Cuscuta reflexa Roxb. Parasitism: The Development of Haustorium

P. Chitralekha[1]*, Anita Rani[1], Roma Katyal[1], Rashmi Mathur[2] and Saloni Gulati[1]*
[1]*Department of Botany, Dyal Singh College, Lodhi Road, New Delhi-110003, India*
[2]*Department of Botany, Sri Aurobindo College, Malviya Nagar, New Delhi-110017, India*

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ABSTRACT

Cuscuta reflexa Roxb. parasitises by developing and inserting a haustorium into host plant tissues. The development of haustorium in *C. reflexa* was studied in the host plants, *Alstonia scholaris* (L.) R.Br., *Bougainvillea spectabilis* Willd., *Volkameria inermis* L., and *Senna siamea* (Lam.) H.S. Irwin & Barneby. In all the hosts, haustorium development begins with the differentiation of a meristem from a few starch-containing cortical/pericycle cells that are adjacent to the parasite phloem tissue proximal to the host. The meristem cells divide and form files of parenchymatous cells which elongate and give rise to the haustorium. The distal-most file cells of the haustorium grow intrusively and penetrate through the parasite and host tissues. Thus, the haustorium is made up of elongated proximal files of ('axial') cells, and distal intrusive cells. The intrusive cells grow apically through the intercellular spaces and spaces created by the degeneration of cells in the path of the growing haustorium. Near the host vascular bundles, the intrusive cells of the haustorium diverge, grow towards, and make contact with the phloem and xylem tissues.

The growth pattern of the endophytic haustorium was found to vary, based on the type of vasculature present in the four different hosts studied. In the stem of *B. spectabilis*, which possessed medullary, and a peripheral ring of vascular bundles, the haustorium grows deep into the pith between the bundles. The intrusive cells connect with numerous vascular bundles as the haustorium progresses into the pith. In the petiole of *S. siamea* and stems of *V. inermis* and *A. scholaris,* the vasculature consists of a ring of phloem tissue surrounding an inner ring of xylem tissue. Additionally, internal phloem is present inner to the primary xylem in *A. scholaris.* The haustorium in the first two hosts does not breach the cylinder of the xylem but in *A. scholaris,* the intrusive cells grow through the xylem ring and reach the internal phloem tissue. These findings indicate that the intrusive cells are strongly drawn to the host vascular tissues.

Following contact with the host xylem, the intrusive cells differentiate into the xylem elements, and the differentiation continues into the axial cells of the haustorium. However, neither axial cells nor intrusive cells in contact with the host phloem were observed to differentiate into phloem cells or elements. In a mature haustorium, a continuous column of tracheary elements connecting the xylem of the host with that of the parasite is observed. Since only a few of the intrusive cells that are in contact with the host xylem tissue and only the central axial cells of the haustorium are found to differentiate into xylem elements, it is proposed that a two-way interaction between the signal/s derived from the host vascular cells and the cells of the haustorium, probably having procambium-like attribute, is a prerequisite for the differentiation of not only the intrusive cells but also the (procambium-like) cells of the haustorium into conducting cells, to establish a continuous vascular connection between the host and the parasite.

Key words: Conducting cells, endophytic haustorium, intrusive cells, phloic cells, procambium-like cells, two-way host-parasite interaction

***Authors for correspondence:** P. Chitralekha, Saloni Gulati, e-mail: pchitralekha22@gmail.com, salonigulati@dsc.du.ac.in

Introduction

The haustorium (from the Latin, *haurire*, to drink), the defining feature of all parasitic plants (Hibberd & Jeschke, 2001), makes direct contact with the host tissues and obtains water and nutrients required for the survival and growth of the parasite. *Cuscuta reflexa* Roxb. develops lateral haustoria to parasitise aerial parts of host plants (Yoshida et al., 2016). It first attaches tightly to the host plants with the help of adhesive disks (Chitralekha & Rani, 2022). The parasite then forms and inserts the haustoria inside the host where the vascular bundles are targeted. On contact with the host vascular tissues, haustorial cells differentiate into conducting (xylem and phloem-like) cells which connect the host vascular tissues with those of the parasite. The parasite then draws materials required for its survival from the host (Brun et al., 2021; Park et al., 2022).

Though the parasite invades and establishes contact with the host plants, wound or defense responses such as hypersensitive cell death or cell wall appositions are not visible at either macroscopical or microscopical levels (Birschwilks et al., 2007). This suggests that *Cuscuta* has an efficient and strong defense response, including at the host-parasite junction (Ranjan et al., 2014; Birschwilks et al., 2007; Albert et al., 2021) and overcomes tissue incompatibilities (Kokla & Melnyk, 2018). The response mechanism ensures that the haustorium is not recognized as a foreign entity (Liu et al., 2020) enabling it to establish a stable inter-species graft with a wide range of hosts from different families. Increased expression of genes that respond to stress stimuli and plant defenses (Ranjan et al., 2014) and mobile microRNAs (Shahid et al., 2018; Johnson & Axtell, 2019; Johnson et al., 2019) have been implicated in the successful parasitisation of hosts by *Cuscuta*. The extracellular growth of haustorium without penetrating the protoplast of host cells could also be a mechanism adopted by the parasite to avoid detection by the host (Vaughn, 2003).

The nature of the *Cuscuta* haustorium has been debated for a long time. It is often considered to be a highly modified adventitious root (Kuijt & Toth 1976; Lee & Lee, 1989; Dawson et al., 1994; Shimizu et al., 2018). However, according to Truscott (1966), the inability of *Cuscuta* to regenerate roots even under a highly altered environment indicates that the root origin of haustorium "in all probability is not valid". The requirement of cytokinin (Tsivion, 1978) and expression of *SHOOT MERISTEMLESS* (STM) gene involved in the establishment of shoot meristem, during the development of haustorium suggests a shoot-like origin (Alakonya et al., 2012). An analysis of the transcriptome during early developmental stages of haustorium in *C. pentagon* by Ranjan et al. (2014), suggests the recruitment of shoot developmental processes in the evolution of *Cuscuta* haustorium. However, according to Alakonya et al. (2012), neither the anatomy of the haustorium nor the structure of meristem giving rise to the haustorium resemble that of either stem or shoot apical meristem, suggesting "a mixed developmental origin using elements of both the shoot and root developmental programs".

In the induction and development of haustoria, light cues and tactile/contact signals (Tada et al., 1996; Olsen et al., 2016; Kaga et al., 2020), as well as phytohormones such as cytokinins and auxins have been implicated (Paliyath et al., 1978; Ramasubramanian et al., 1988; Löffler et al., 1999). Contact stimulus in the form of coiling of *Cuscuta* stem around the host plant for haustoria formation is well-documented (Haidar et al., 1997; Ihl & Weise, 2000; Lee, 2007; Kaga et al., 2020), and once triggered, haustoria continue to develop even when detached from the host (Tada et al., 1996).

Upon developing continuity with the host vascular tissue, the parasite becomes a strong sink and competes with the actively growing host for materials required for its survival and growth. Water, carbohydrates, proteins and nitrogenous compounds, macro and micro elements, phytohormones, and other substances are translocated from the host to the parasite via the haustorium (Chang & Lynn, 1986; Dawson et al., 1994; Hibberd & Jeschke, 2001; Birschwilks et al., 2006, 2007; David-Schwartz et al., 2008; Lee , 2009; Smith et al., 2013; Ranjan et al., 2014; Yoshida et al., 2016; Brun et al., 2021; Park et al., 2022). However, *Cuscuta* does not appear to depend solely on host-translocated metabolites but can also synthesize metabolites from the raw materials procured from the host (MacLeod,1963; Vogel et al., 2018; Kumar & Amir, 2021). Besides, secondary metabolites (Smith et al., 2013, 2016; Bais and Kakkar, 2014; Tanruean et al. 2017; Kumar & Amir, 2021) and bioactive molecules such as proteins (Haupt et al., 2001; Birschwilks et al. 2007; Jiang et al., 2013; Smith, et al., 2013; Liu et al., 2020; Shen et al., 2020) and RNA (Roney et al., 2007; David-Schwartz et al, 2008; Kim et al., 2014; Kim and Westwood, 2015; Shahid et al., 2018, Park et al., 2022; Zhang et al., 2021) have been shown to be exchanged extensively between *Cuscuta* and its hosts, influencing the host-parasite relationship.

Thus, *Cuscuta* parasitism offers an excellent system to study not only host-parasite interactions but also the signaling and developmental roles of the myriads of molecules trafficked between host and parasite. A thorough understanding of the basic mechanisms underlying parasitism such as host-detection, penetration, the establishment of infection and the interaction between host and parasite through a functional haustorium, both at the structural and molecular levels will enable development of efficient control measures (Bouwmeester et al., 2021). Although substantial literature already exists on the structure and development of haustorium in *C. reflexa*, comparative haustorium development in hosts with varied vascular structures is not well studied.

This study was undertaken to investigate haustorial development in four unrelated hosts with different types of vasculature. Whereas stem of *Bougainvillea spectabilis* Willd. contains a few large widely separated medullary, and a peripheral ring of small vascular bundles, stems of *Alstonia scholaris* (L.) R.Br. and *Volkameria inermis* L., and the petiole of *Senna siamea* (Lam.) H.S.Irwin & Barneby possess a ring of very closely placed vascular bundles. In addition, internal phloem is present in *A. scholaris* and a many-layered pericycle of lignified fibres in *S. siamea.* Our study revealed new insights into the process of haustorium development in *C. reflexa* such as the directional movement of the intrusive cells towards the vascular tissues, and the variation in growth patterns of the haustorium in hosts with different types of vasculature. The differentiation of conducting tissues in the haustorium is inferred to be a result of interaction between intrusive cells with the host vascular cells and cells of the haustorium with procambium-like attributes.

Materials and Methods

We collected portions of *Cuscuta reflexa* Roxb. (Convolvulaceae) parasitised stems of at least 3 individuals each of *Alstonia scholaris* (L.) R.Br. (Apocynaceae), *Bougainvillea spectabilis* Willd. (Nyctaginaceae), *Volkameria inermis* L. (Lamiaceae), and petioles of *Senna siamea* (Lam.) H.S.Irwin & Barneby (Caesalpiniaceae). Three to four haustorial portions in each host were cut into 5 mm pieces, fixed in FAA and processed for glycol methacrylate (Sigma-Aldrich) embedding (Feder & O'Brien, 1968). Semi-thin sections (2-5 μm) were cut using glass knives, stained either with 0.05% aqueous toluidine blue O (TBO, Sigma-Aldrich) or stained first with periodic acid-Schiff reaction (PAS, periodic acid: Sigma-Aldrich, Schiff reagent: Merck) and counter-stained with TBO (Feder & O'Brien, 1968), and mounted in DPX (SLR Chemical). More than 100 sections of each parasitised host plant were observed and photographed.

Results

In all the four parasitised hosts (*A. scholaris, B. spectabilis, S. siamea, V. inermis*), the adhesive disk and haustorium of *C. reflexa* begin to develop almost at the same time following contact with the host, thus different stages of parasitism were observed.

Initiation of the haustorium

One sign of haustorium development was the presence of starch grains in the cells of cortex/pericycle adjoining the phloem of parasite vascular bundles proximal to the host contact site (Fig. 1A). Groups of cells containing dense cytoplasm and hypertrophied nuclei resembling meristematic cells, were observed outer to the phloem of the parasite (Fig. 1B). These appear to be haustorium meristems differentiated from starch-containing initial cells. The cells of the meristem divide and form files of cuboidal cells (Fig. 1A).

The meristem cells and their derivatives (file cells) enlarge, crushing some cortex cells in the process (Fig 1B). The cuboidal file cells then elongate (Fig. 1A) and push through the outer cortex and epidermis of the parasite, penetrate the host tissues and form the haustorium. The haustorium comprises two parts, the proximal part inside the parasite, the upper haustorium, and the distal part present within the tissues of the host, the endophytic portion (Fig. 1C). The upper part of a young haustorium consists of radial files of vacuolated cuboidal cells and elongated cells (Fig. 1A). Crushed cortical cells of the parasite are generally found around the mature upper haustorium (Fig. 1D) indicating that an enlargement of the upper haustorium occurs during maturation resulting in the crushing of parasite cells. At this stage, cells resembling those of cambium are observed between the xylem and phloem of vascular bundles of the parasite infecting the host, *V. inermis* (Fig. 1D, arrowheads).

Growth of the haustorium

The haustorium consists of a column of long axial cells derived from the elongation of the files of cuboidal cells. The axial cells are thin-walled and parenchymatous with large vacuoles and conspicuous nuclei (Figs. 1C, 2A). The distal-most cells at the tip of the haustorium, also called the intrusive cells, penetrate and grow through the host tissues. The intrusive cells are highly elongated, thin-walled, vacuolated with a dense layer of peripheral cytoplasm and contain large hypertrophied nuclei (Fig. 2A-C). Crushed cells with densely stained material and empty spaces in front of the growing ends of the intrusive cells are observed (Fig. 2A, B), indicating degeneration of host cells in the path of elongation. The intrusive cells appear to penetrate the host tissues by expanding into the intercellular spaces in the host tissues and into spaces created by the degradation of host cells. In all the host species, the endophytic region of haustorium embedded in the host cortex is bound by a layer of dense material (Figs. 1C, 3B, C, 4A-C) seemingly of degraded cell remnants.

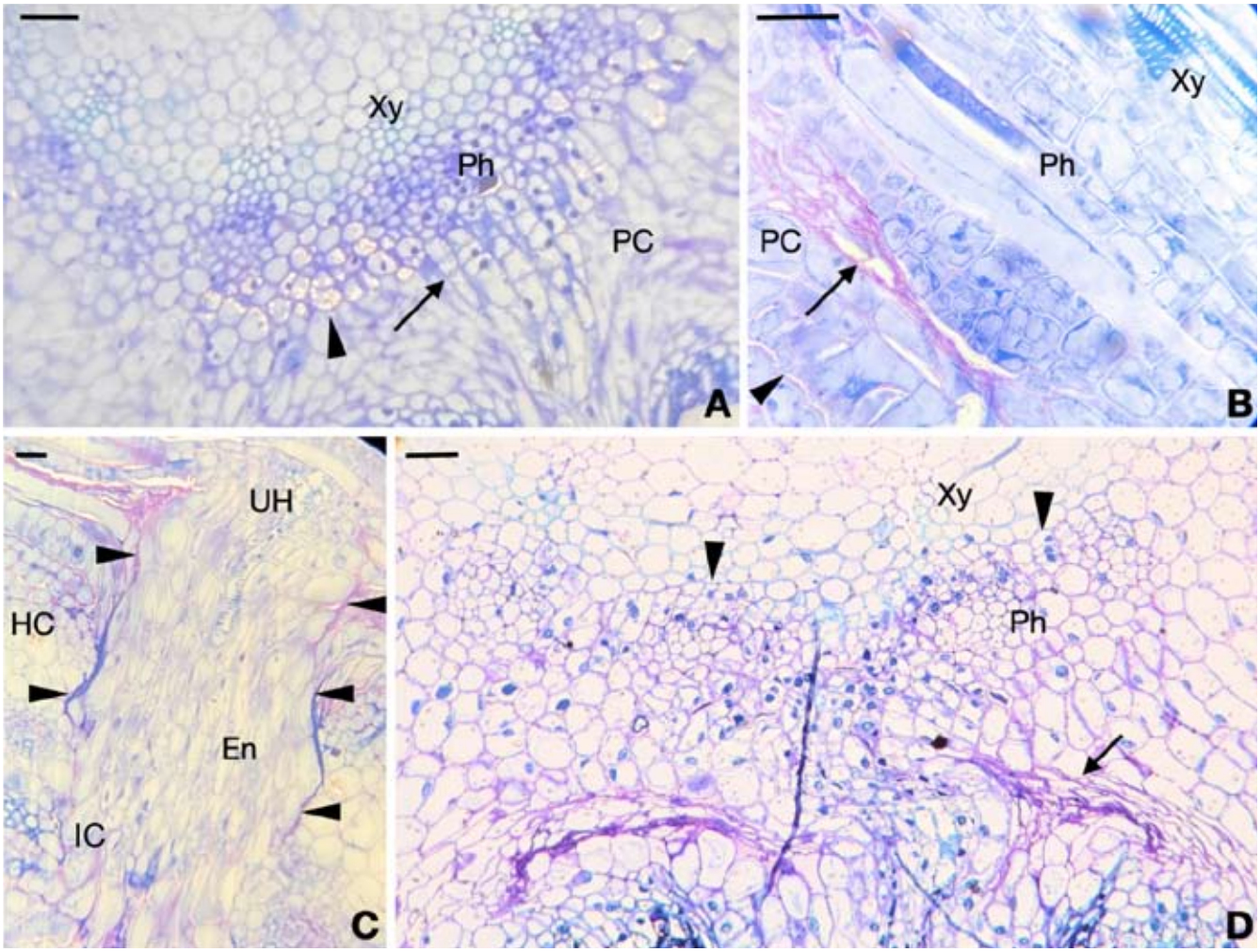

Figure 1. Internal structure of *Cuscuta reflexa* stem (Hosts: A-C, *Bougainvillea spectabilis; D, Volkameria inermis*). (A) T. S. stem showing a young developing haustorium. Cortical cells adjacent to phloem of vascular bundles proximal to host accumulate starch grains (arrowhead). Note the files of cuboidal cells (arrow). (B). L. S. stem showing a group of densely cytoplasmic meristematic cells surrounded by crushed cells of the parasite cortex (arrow); elongated lobed epidermal cells (arrowhead) of the adhesive disk can be seen. (C) L.S. haustorium showing the two parts, upper haustorium present in the parasite, and the endophytic portion inside the host; arrowheads point to the dense boundary layer surrounding the haustorium. (D) The upper part of a mature haustorium showing the crushed cortical cells of the parasite (arrows); arrowheads point to the cambial-like cells. Bar = 50 µm. Staining: A-D,F TBO, B-D – PAS-TBO. Abbreviations: En- endophytic part, HC- host cortex, IC- intrusive cell, PC- parasite cortex, Ph- phloem, UH- upper haustorium, Xy- xylem.

Differentiation of conducting cells in the haustorium

Near the vascular tissue of the host, the intrusive cells appear to diverge, grow towards and make contact with the host phloem and xylem (Figs 2 C-F). At this stage, in *B. spectabilis* stem, some cells in both the xylem and phloem appear to fuse forming larger cells and also fuse with the tip of the intrusive cells establishing continuity with the haustorial cells (Fig. 2D). The intrusive cells in contact with the vascular tissue generally contain a dense layer of cytoplasm with large hypertrophied nuclei (Figs.. 2 D-F) that are sometimes lobed (Fig. 2F). Often, the intrusive cells in contact with the xylem tracheary elements are observed to possess cytoplasm without a discernible central vacuole or nucleus (Figs. 2A, 3A). These cells probably are in the process of losing their cellular contents and differentiating into tracheary elements.

Following contact with host xylem, the intrusive cells of the haustorium differentiate into xylem elements (Figs. 3A, B) by acquiring distinct secondary wall patterns. The axial cells in the center of the haustorium too, differentiate into xylem elements, and a continuous column of xylem connecting the host xylem with

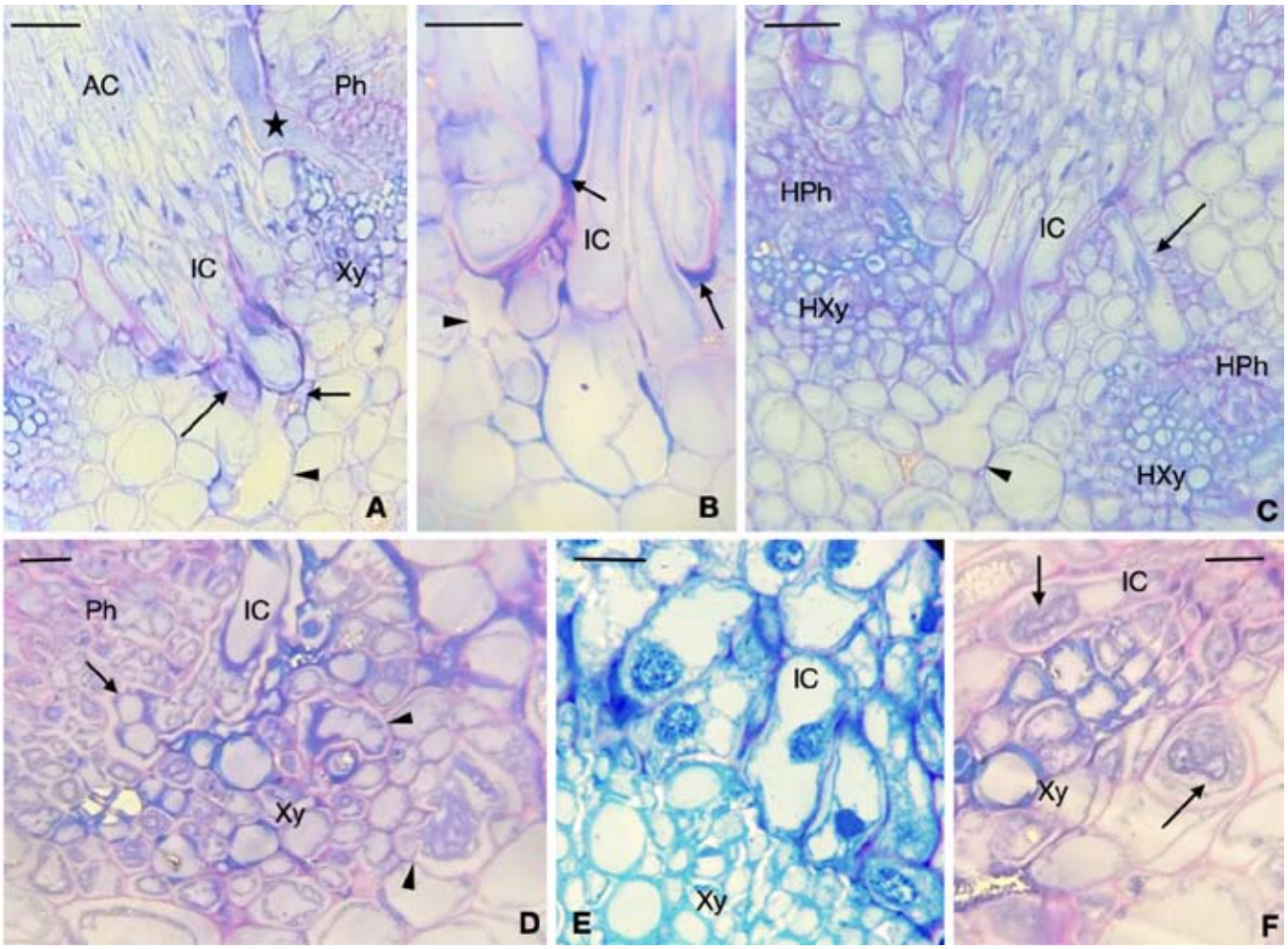

Figure 2. Intrusive cells of *C. reflexa* inside host stem (A-D, F: *B. spectabilis*; E: *V. inermis*). (A, B) Intrusive cells in ground tissue surrounded by remnants of degenerated cells (arrows) and empty spaces (arrowheads); an intrusive cell (star in A) in contact with xylem has evacuolated cytoplasm; (C) Intrusive cells in contact with host phloem (arrow) and xylem; arrowhead points to empty space devoid of cellular contents in front of the intrusive cell; (D) Fused cells of host phloem (arrow) and xylem (arrowheads) following contact with intrusive cells; (E) Intrusive cells with dense cytoplasm and hypertrophied nuclei in contact with host xylem; (F) Intrusive cells with dense cytoplasm and lobed nuclei (arrows) near vascular tissue. Bar in A-C = 25 µm, in D-F = 10 µm. Host: Staining: A-D, F, PAS-TBO; E, TBO. Abbreviations: AC, axial cells, HPh, host phloem, HXy, host xylem, IC, intrusive cell, Ph, phloem, Xy, xylem.

that of the parasite is observed (Figs. 3C, D). But differentiation of intrusive cells in contact with host phloem tissue (Fig. 2C) into phloem elements could not be discerned.

The stem of *B. spectabilis* possesses numerous large medullary vascular bundles in the ground tissue surrounded by a ring of smaller vascular bundles (Fig. 4A). The intrusive cells of a haustorium are seen to establish contact with more than one vascular bundle (Figs. 2C, 3B, C). A single intrusive cell that has differentiated into a xylem element is also seen to branch at the tip and connect with adjacent tracheary elements (Fig. 3C). Often two or more haustoria penetrating a host stem come in contact and merge in the ground tissue (Fig. 4A). Whether there is any exchange of metabolites between them needs to be investigated.

Secondary growth was found to have occurred in all the hosts studied. Vasculature in the parasitised petiole of *S. siamea* and stems of *A. scholaris* and *V. inermis*, consisted of an inner ring of xylem surrounded on the outer side by a ring of phloem (Figs. 4B-D). In *A. scholaris,* internal phloem tissue is also present inner to the primary xylem at the periphery of the pith (Fig. 4D). The haustorium, in *S. siamea* petiole, penetrates through pericyclic fibres and reaches the vascular tissue. The intrusive cells at the periphery of the haustorium in these three hosts connect with the

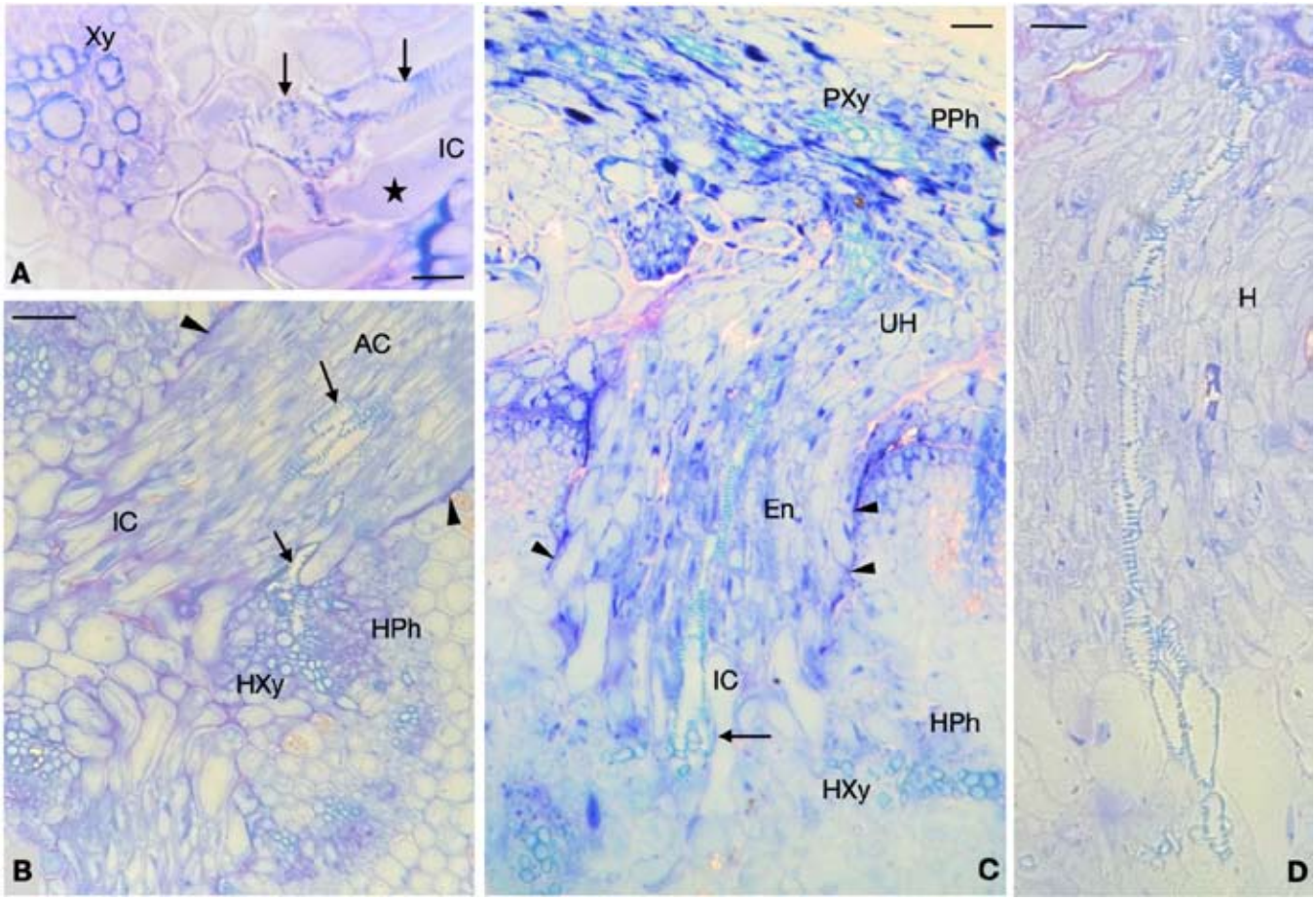

Figure 3. Intrusive cells of *C. reflexa* haustorium in *B. spectabilis*. (A) Intrusive cells (arrows) forming secondary thickenings and differentiating into xylem elements; a nearby intrusive cell (star) has evacuolate cytoplasm; (B) Haustorium axial cells and intrusive cells in contact with host xylem elements differentiate into tracheary elements (arrows); (C, D) Haustorium with a central xylem strand connecting the host xylem with parasite xylem. Arrow in C points to a branched intrusive cell differentiated into a xylem element. Bar in A= 10 µm, B-D = 25 µm. Staining: A, B, D, PAS-TBO; C, TBO. Abbreviations: AC, axial cells; En, endophytic part; H, haustorium; HPh, host phloem; HXy, host xylem; IC, intrusive cell, PPh, parasite phloem; PXy, parasite xylem; UH, upper haustorium; arrowheads point to the boundary layer around the endophytic part.

phloem tissue while the cells in the center penetrate through the phloem tissue to connect with the xylem elements (Figs. 4B-D). The haustorium, however, does not breach the xylem ring in *S. siamea* and *V. inermis* (Figs. 4B, C). In contrast, intrusive cells of haustoria in *A. scholaris* stem invade and destroy a part of the xylem tissue to reach the internal phloem at the periphery of the pith (Fig. 4D). Interestingly, all the intrusive cells in contact with the xylem elements of the host do not seem to differentiate into tracheary elements (Figs. 3A-C, 4A-D). Haustorium in *B. spectabilis* possesses a single bundle of centrally located xylem tissue connecting the host xylem with that of the parasite (Fig. 3C, D). In the other three hosts too, a similar haustorial structure was observed (images not included).

Discussion

The formation of haustorium in *C. reflexa* is discussed under three sections, (1) differentiation of the haustorium, (2) penetration of the haustorium through the host tissues, and (3) contact with the host vascular tissue and differentiation of conducting tissues in the haustorium.

Differentiation of the haustorium

In the present study, the first signs of change observed as the parasite perceives a host/support nearby are the elongation of epidermal cells and accumulation of starch grains in the parenchyma cells of the parasite cortex/pericycle immediately external to the vascular bundles proximal to the host. Similar deposition of

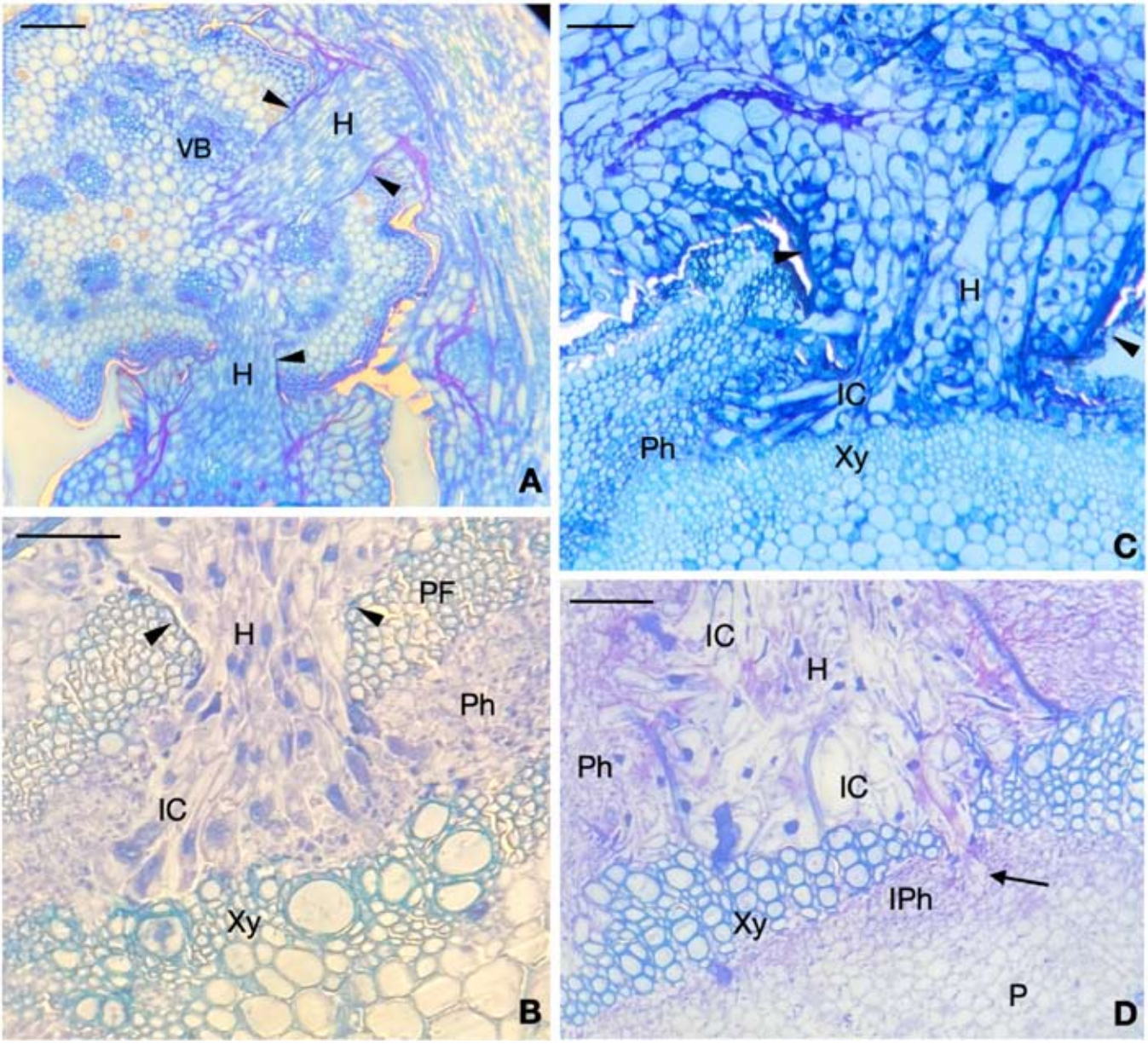

Figure 4. A-D: *C. reflexa* haustoria in host stem tissues. (A) Two haustoria deep inside the stem of *B. spectabilis* have come in contact with each other in the pith; (B) Haustorium in petiole of *Senna siamea* penetrates through the host pericyclic fibres but not through the xylem ring; (C) Haustorium in *V. inermis* stem; intrusive cells do not breach the host xylem ring; (D) Haustorium in *Alstonia scholaris* stem; intrusive cells are in contact with the host xylem and phloem tissue external to xylem, and have intruded through the ring of xylem tissue to reach the internal phloem (arrow). Arrowheads point to boundary layer around the endophytic part. Bar in A&D = 100 µm, B&C = 50 µm. Staining: A, D, PAS-TBO; B, C, TBO. Abbreviations: H, haustorium; IC, intrusive cells; P, pith; IPh, internal phloem; PF, pericyclic fibers; Ph, phloem; Xy, xylem; VB, vascular bundle.

starch grains in the mid-cortical as well as epidermal cells of the parasite stem at the host contact site has also been observed in *C. australis* (Lee & Lee, 1989) and in *C. japonica* (Lee, 2007; 2008). According to Lee (2008), the deposited starch may be used as an energy source for subsequent cellular activities, including cell division and elongation during the development of the upper haustorium. These starch-containing cells are considered to be the haustorial initials (Lee, 2007).

Haustorium development, in all the four hosts studied, begins with the differentiation of a meristem, most probably from the starch-containing haustorial initials. The meristem cells then divide and give rise to radial files of cells which elongate to form

the haustorium. Earlier investigations have revealed the differentiation of haustorial initials or meristems from cortical cells (Heide-Jørgensen, 1991; Dawson et al., 1994; Lee & Lee, 1989; Lee, 2007; Hong et al., 2011; Alakonya et al., 2012). López-Curto et al. (2006) report that the haustorium originates from the fascicular and interfascicular regions of the parasite stem. According to Lee (2007), contact with a live host triggers division of meristem cells. The derivatives of the meristem form the haustorium primordium which reportedly consists of two types of cells, the file cells and the distal-most file cells called the tip cells (Hong et al., 2011) or digitate cells (Lee & Lee, 1989; Lee, 2007; Alakonya et al., 2012).

Cuscuta does not exhibit secondary growth (Carlquist & Hanson, 1991) but other members of Convolvulaceae show a normal secondary growth by activation of intra- and inter-fascicular cambia, and/or anomalous secondary growth by formation of successive rings of cambia from pericycle or parenchyma located outside the phloem (Rajput et al. 2014). It is possible that in *Cuscuta,* the parenchyma cells outside phloem tissue have retained the inherent ability to divide. Stimulus from light and contact with a support (living or nonliving) (Tada et al., 1996; Haidar et al., 1997; Lee & Lee, 1989) probably initiates the synthesis of phytohormones required for the differentiation of the cells into initials and meristems, and the division of the meristem cells to form the haustorium (Ramasubramanian et al., 1988; Haidar et al., 1997, 1998; Ihl & Weise, 2000). In the host *V. inermis, C. reflexa* stem with mature haustoria exhibited active intrafascicular cambium (Fig. 1D) indicating that the vascular cambium of *C. reflexa* can be activated under certain conditions.

On contact with the host, the haustorium penetrates the host tissues by the elongation of its cells (present study). The haustorium thus consists of two parts, the upper part that arises inside the parasite and the endophytic part present inside the host. The upper part and the proximal region of the endophytic part are made up of elongated axial cells derived from file cells. The distal end of the endophytic haustorium is made up of long, filamentous hypha-like intrusively growing cells. Inside the host, the hypha-like intrusive cells establish contact with the host phloem and xylem tissues. Most of the earlier investigators have reported similar findings though different terms were used for the haustorial structures. The upper part of the haustorium has been variously referred to as the upper haustorium (Kuijt, 1977; Lee & Lee, 1989; Lee 2007) or pre-haustorium (Tsivion, 1978; Hozumi et al., 2017; Albert et al., 2006; Alakonya et al., 2012; Kaiser et al., 2015) which included the "adhesive disk" along with the haustorium meristem (Dawson et al., 1994; Dörr, 1968; Heide-Jørgensen, 1991), the endophytic portion inside the host as the inner haustorium (Kuijt, 1977; Lee, 2009; Hong et al., 2011), and the hypha-like cells as either the intrusive cells (López-Curto, et al., 2006) or the search/searching hyphae (Dörr, 1972; Costea & Tardif, 2006; Lee & Lee, 1989; Dawson et al., 1994; Vaughn, 2003, 2006; Birschwilks et al., 2006, 2007; Lee, 2009; Hong et al., 2011; Alakonya et al., 2012) (Lee & Lee, 1991; Lee 2009). The upper haustorium may differentiate on contact with any type of support (Kuijt, 1977; Lee & Lee, 1989; Lee 2007). For the development and elongation of searching hyphae, however, a live host is required (Heide-Jørgensen, 2008; Hong et al., 2011; Olsen et al., 2016). On the other hand, Kaga et al. (2020) have reported the growth of search hyphae in the absence of a host

The axial cells of the haustorium not only elongate but also enlarge in width. The dense boundary layer, apparently of cell remnants, found around the haustorium in the present study, indicates that the host and parasite tissues surrounding the developing haustorium are crushed and degraded. A similar observation was made by Heide-Jørgensen (1991) in *C. reflexa* infecting *Pelargonium zonale.* Fathoulla & Duhoky (2008), however, believe that the boundary layer is composed of cementing material deposited by the cells of the haustorium to secure a tighter adhesion to the host.

Penetration of haustorium through the host tissues

The host cells form a physical barrier to penetration of the haustorium. In this study, the axial as well as the intrusive cells of the haustorium of *C. reflexa* are highly elongated in all the host species studied. The apical end or tip of intrusive cells is surrounded by either empty spaces or dense material resembling degraded cell remnants. These observations suggest that the mechanical pressure exerted by the elongating cells of the haustorium probably results in the degeneration of host cells and facilitates penetration through the host tissues. The intrusive cells of the haustorium appear to grow mostly by extension at the tip. The tip growth is extracellular, through the intercellular spaces in the host tissues as well as through the spaces created by crushing, degradation and absorption of the host tissues. Vaughn (2003), too, reported the presence of crushed,

cytoplasm-less cells at the site of formation of the endophytic part. In *C. australis*, the tip cells appeared to engulf the debris of broken host cells (Lee & Lee, 1991). Similar extracellular penetration of haustorium into host tissue has been reported by Dörr (1967), Nagar et al. (1984), Dawson et al, (1994), Vaughn (2003), Lee and Lee. (1991), Birschwilks et al. (2007), Lee (2009), and Shimizu and Aoki (2019). A combination of physical pressure exerted by the elongating cells and enzymatic degradation are employed by the haustoria of *Cuscuta* to penetrate host tissues (Kuijt, 1969; Nagar et al. 1984; Dawson et al., 1994; Srivastava et al., 1994; Lee & Lee, 1989; López-Curto et al., 2006; Lee, 2007; Fathoulla & Duhoky 2008; Johnsen et al., 2015; Kaiser et al., 2015). Alternatively, the intrusive cells/hyphae of the haustorium may intrude into the host tissues without crushing and lysing the cells. They grow by pushing into the host cells covered by a layer of stretched (Dörr, 1968; Dawson et al. 1994; Vaughn, 2003) or newly synthesised (Vaughn, 2003) host cell wall. This mechanism gives rise to a chimeric wall of the penetrating hyphae derived from both the host and the parasite. The intrusive cells have also been found to penetrate between the host cells by degrading the middle lamella and separating the cells (Vaughn, 2003; Lee & Lee, 1991; Lee, 2009; Shimizu & Aoki, 2019). These extracellular growth pathways are expected to minimize stress to the host (Vaughn, 2003). A few reports also mention intracellular growth of the unicellular searching hyphae within the host parenchyma (Birschwilks et al. 2007; Lee, 2009). Several investigations have revealed that parasitic plants directly secrete or induce secretion by hosts, substances that remodel cell walls of the host (Johnsen et al. 2015; Sarić-Krsmanović, 2019; Shimizu & Aoki, 2019) as well as parasite (Johnsen et al., 2015; Olsen & Krause, 2017) to enable penetration of the haustorium.

In the present study, the intrusive cells are found to be vacuolated with a thin dense layer of peripheral cytoplasm and hypertrophied nuclei. Birschwilks et al. (2007) and Lee (2007, 2009) report that though the intrusive cells/search hyphae are mostly vacuolate, their apical ends possess dense cytoplasm rich in organelles and large conspicuous nuclei. Consequently, the apical end of these cells appears to be metabolically active and well-equipped to synthesise and secrete the wall modifying and degrading substances required for host invasion (Lee, 2007).

Contact with the host vascular tissue and differentiation of conducting tissues in the haustorium

The intrusive cells of the haustorium grow towards and connect with the host vascular tissues. In all the host plants studied in this report, the haustorium of *C. reflexa* grows more or less straight through the host tissues and does not diverge in the host cortex until it nears the vascular tissues of the host where the intrusive cells separate and grow towards the host xylem or phloem tissues. The growth pattern of the haustorium is observed to vary with the type of host vascular tissue encountered. In host plants with a continuous cylinder of vascular tissue (e.g. *S. siamea*, *V. inermis*), some intrusive cells on contact with the phloem ring cease further growth and connect with phloem elements while others continue through the phloem tissue and stop at the xylem ring. Surprisingly, in *A. scholaris,* the intrusive cells appear to sense the presence of phloem tissue inner to the host xylem ring, and part of the ring is degraded to access the internal phloem tissue. The stem of *B. spectabilis* contains a ring of small vascular bundles along with widely spaced large medullary vascular bundles. The haustorium penetrates deep inside the pith, possibly attracted by the vascular bundles on the other side of the pith. On the way, the intrusive cells connect with the xylem and the phloem tissues of the nearby bundles. These findings suggest that the intrusive cells of the haustorium are strongly drawn to the host vascular tissues. The directed growth of the intrusive cells toward host vascular tissues is possibly due to signal/s emanating from the latter tissues (Fig. 5A). The signal/s appears to be strong enough to impel the intrusive cells to degrade xylem elements in *A. scholaris* stem and connect with the internal phloem. It would be interesting to find out the nature of the signal/s. Birschwilks et al. (2007), however, report that the growth of the searching hyphae towards the vascular bundles occurs by chance and is not actively directed. In host species, *Duranta plumieri* Jacq., *Catharanthus roseus* Don., *Hamelia erecta* Jacq. and *Ixora coccinea* L., Nikam et al. (2014) found that *C. reflexa* haustorium reaches up to the secondary xylem ring and does not grow beyond. Toma et al. (2004-2005) too, showed the growth of the haustorium till the outer layers of xylem in hosts parasitised by three species of *Cuscuta, C. epilinum, C. epithymum* ssp. *trifolii and C. europaea.*

The intrusive cells of *C. reflexa* (present study) near the host vascular tissues possess dense cytoplasm

and lobed hypertrophied nuclei, as also reported in the searching hyphae of *C. australis* (Lee & Lee, 1989) and *C. japonica* (Lee, 2009). The hyphae may almost grow to a length of 100-200 µm (Vaughn, 2003) to 800-2000 µm (Dawson et al., 1994) to reach the host vascular tissues. Upon contact with host vascular tissues, the intrusive cells differentiate into conducting elements (Dörr, 1972; Dawson et al., 1994; Vaughn, 2006; Lee & Lee, 1989; Lee, 2009; Birschwilks et al., 2007; Hong et al., 2011, present study). Interestingly, contact of intrusive cells with the vascular tissue of *B. spectabilis* stem (present study) appears to trigger fusion among some host xylem and phloem cells, apparently by the dissolution of walls between them. This results in the fusion of a single intrusive cell with a group of phloem/xylem cells. The significance of this could not be ascertained and needs further investigation.

In the present study, contact with the host xylem initiates the differentiation of intrusive cells into xylem elements by development of secondary thickenings and fusion with host tracheary elements. The tip of the intrusive cell may branch and connect with adjacent host xylem elements, as also observed in *C.pentagona* by Vaughn (2006). According to Heide-Jørgensen (2008), the searching hyphae connect with the host xylem vessels through the pits in the cell wall. Subsequently, the hyphal wall at the point of contact becomes thin, perforated and finally disappears forming an open connection with the host xylem vessels (Shimizu & Aoki, 2019). Similar lumen-to-lumen connections between the host and haustorium xylem tracheary elements have also been reported by Dawson et al. (1994) and Vaughn (2006). Numerous osmiophilic particles containing expansin at the tip of the hyphal wall are suggested to aid in the loosening of the host xylem wall and penetration of hyphae (Vaughn, 2006). However, Lee (2009) failed to detect any differentiation of xylic hyphae or hyphal penetration into the host vessels or direct lumen-lumen contact between the host and parasite xylem. Vaughn (2006) reports that the secondary wall formed in the xylic hyphae is not like that of typical xylem elements. Crushing of xylem tissue by some intrusive cells of *C. monogyna* was observed by Fathoulla and Duhoky (2008).

The differentiation of intrusive cells into xylem elements continues into the haustorium, and the axial parenchyma cells in the center of the haustorium develop into xylem elements. A continuous column of xylem tracheary elements connects the host xylem to the parasite xylem (present study). Such a differentiation of an unbroken xylem strand in the haustorium from host to parasite appears to be a common feature, as is evident from the reports of Dörr (1972), Lee and Lee (1989), Haupt et al, 2001, Toma et al. (2004-2005), Birschwilks et al. (2006, 2007); Lee (2009); Alakonya et al. (2012) and Shimizu et al. (2018).

In contrast to a clear xylem development, the present study could not identify differentiation of the intrusive cells in contact with host phloem into any component of phloem tissue. Dörr (1969, 1972, 1990), Lee and Lee (1989), Vaughn (2006), Lee (2009), and Hong et al. (2011) report the differentiation of search hyphae/intrusive cells into absorbing or phloic hyphae but not into sieve tube elements. The searching hyphae grow around the host sieve elements like the fingers of a hand (Dörr, 1972, Dawson et al.,1994, Vaughn, 2006) and develop wall infoldings (Dörr 1972, 1990) increasing the transfer surface area many times. They appear to function both as conducting and transfer cells. Plasmodesmata connecting the phloic hyphae of *C. japonica* with the sieve pores of host sieve elements are reported by Lee (2009). Thus, structural studies carried out so far have not documented either the presence of characteristic phloem sieve elements or sieve pore continuity with the host elements in *Cuscuta* haustorium phloic cells (Westwood & Kim, 2017) except for the reports of MacLeod (1962) and Schlenzka (1992) where the presence of haustorium hyphae with well-defined sieve-like areas resembling the sieve plates of sieve tubes have been mentioned. However, expression of marker genes for phloem companion cells and sieve elements could be demonstrated in the haustoria of *C. japonica* indicating differentiation of cells with companion cell- and sieve element-like attributes in the haustoria (Shimizu et al., 2018).

Consistent with the results of the present study, Heide-Jørgensen (2008), Hong et al. (2011), Alakonya et al. (2012) and Kaga et al. (2020) report that the search hyphae inside the host tissue differentiate into vascular elements only after contact with the respective host vascular tissues and suggest that some kind of signal from the host tissue triggers the differentiation. Interestingly, after contact with host vascular tissues, the xylic and phloic hyphae reportedly differentiate in opposite directions (Kuijt, 1969; Kuijt & Toth, 1976); xylic hyphae differentiate basipetally from the tip to the base of hypha in contact with the haustorium, while phloic hyphae differentiate acropetally from the

base of the hypha near the haustorium to its tip (Dörr, 1990; Dawson et al.,1994; Vaughn 2006).

An intriguing fact revealed in almost all investigations till date, including the present one, is that the xylem tissue is always found in the center of the *Cuscuta* haustorium, while the nutrient conducting phloem-like cells are probably present surrounding the xylem (Fig. 1, Haupt et al, 2001, Fig. 1,; Toma et al., 2004-2005 Figs 5, 10, 15; Fig. 3C, Birschwilks et al., 2006, Fig. 3C,; Fig. 3a, Birschwilks et al., 2007, Fig. 3a; Fig. 1G, Alakonya et al., 2012, Fig. 1G; Fig. 1D, Shimizu et al., 2018, Fig. 1D). The stem of *Cuscuta* has a ring of widely spaced vascular bundles but such an arrangement of vascular tissue is not found in the haustorium. In addition, only a few intrusive cells in contact with host xylem, and the axial cells in the center of the haustorium differentiate into tracheary elements. If only host-derived signal/s is involved in differentiation of vascular tissues, then all the intrusive cells in contact with the host xylem should have differentiated into tracheary elements which clearly is not the case. These observations suggest the involvement of signal/s derived from both the host vascular tissues and the haustorium of *C. reflexa,* in the differentiation of conducting cells in the haustorium. In general, primary xylem and phloem tissues differentiate from procambial cells derived from apical meristems and embryos. Recently, the presence of (pro)cambial-like domains within the haustorium of *C. japonica* has been revealed by the use of marker genes (Shimizu et al., 2018), and it appears that the haustoria possess, even before contact with host vascular elements, cells with procambium-attribute (Shimizu & Aoki, 2019). Within the developing haustorium, the xylem differentiating region appears to be present in the center surrounded by procambium or phloem differentiating domains (Shimizu et al., 2018). According to Brun et al. (2021), these observations imply that the default vascular development from procambium (-like) cells also operates in *Cuscuta* haustorium. Hence, it is proposed that only those intrusive cells which are in contact with both the host vascular tissues (xylem and phloem) and the cells of the haustorium with procambium-attribute differentiate into the respective conducting elements, implying the involvement of not just the host-derived signal/s alone but signal/s from parasite as well (Figs. 5B, C). A

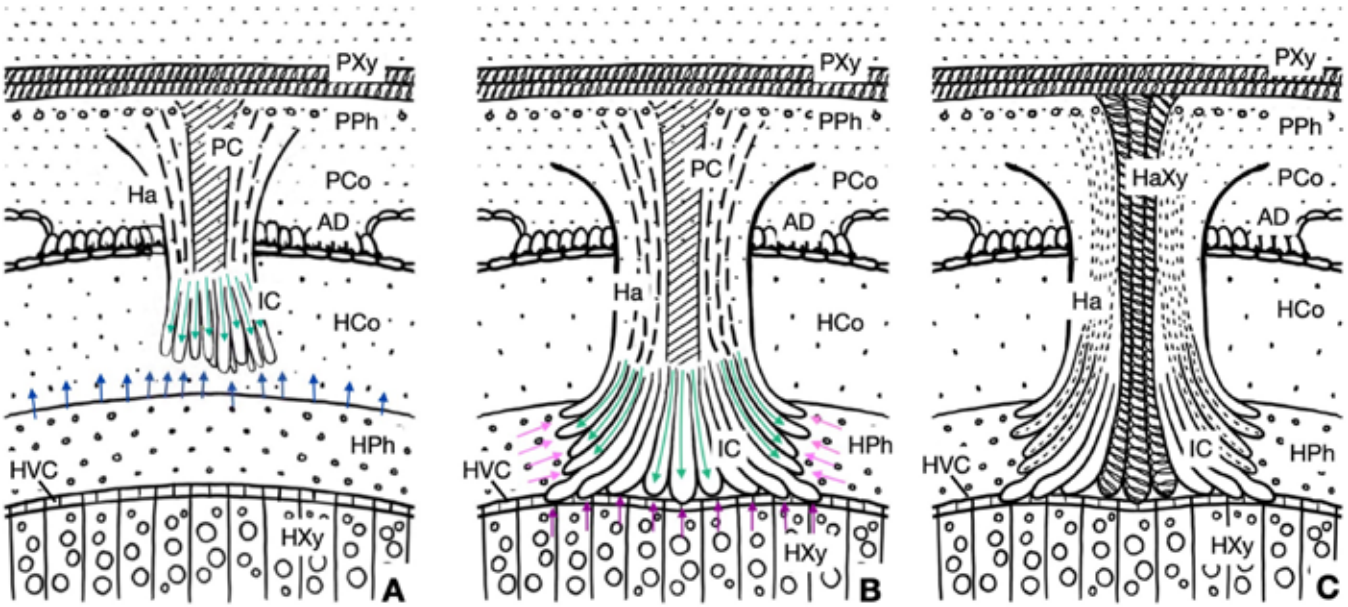

Figure 5. Diagrammatic representation of proposed interaction between the haustorium of *C. reflexa* and the host vascular tissues (not to scale). (A) Haustorium possessing axial cells with procambium-like attributes in the center. Signal/s (blue arrows) from the host vascular tissues attract the haustorium intrusive cells. Green arrows represent the signal/s from cells with procambium-like attributes; (B) Intrusive cells in contact with host vascular tissues. A two-way interaction between signal/s from haustorium cells with procambium-like attribute (green arrows) and host vascular tissues (pink and magenta arrows) occurs to initiate differentiation of vascular elements in haustorium; (C) Intrusive cells in contact with the host xylem on one end and central axial cells of haustorium with xylem procambium-like attribute at the other end along with the central axial cells differentiate into xylem elements. Abbreviations: AD, adhesive disk; Ha, Haustorium; HCo, host cortex, HVC, host vascular cambium; HPh, host phloem; HXy, host xylem; HaXy, haustorial xylem; IC, intrusive cells; PC, cells with procambium-like attribute; PCo, parasite cortex, PPh, parasite phloem; PXy, parasite xylem.

two-way interaction between the signal/s from both the host and parasite is essential for differentiation of conducting tissues in the haustorium and establishing a continuous vascular connection from host to parasite. Coincidentally, parasitised stem and petiole parts of the host plants studied in this investigation showed (though not very clearly evident in the attached micrographs) vascular cambium activity and formation of secondary vascular tissue. We, therefore, believe that the derivatives of vascular cambium are most likely involved in the differentiation of vascular tissues of the haustorium. Investigations are underway to ascertain this hypothesis.

Further studies are required both at the structural and molecular level, involving a variety of host plants to shed more light on the process of parasitism and help in the identification of the primary receptors participating in the induction of haustoria. Such investigations may also reveal the signals/molecular mechanisms involved in preventing the elicitation of defense response of the host against the penetrating parasite, attraction of the intrusive cells towards the host vascular tissues, differentiation of the intrusive cells into conducting elements and the growth of the haustorium inside the host. The information can be used in the control of parasitism by *C. reflexa*.

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