Hyperparasitism in *Cuscuta reflexa* Roxb.: A structural study

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Abstract

Obligate stem parasites, *Cuscuta reflexa* Roxb. and *Cuscuta campestris* Yunck. parasitise many plant species, and they also exhibit the phenomenon of facultative hyperparasitism. Both the species have been observed to parasitise plants belonging to their own species (autoparasitism/intra-specific hyperparasitism). When growing together, *C. campestris* is also found to parasitise *C. reflexa* (intra-generic hyperparasitism) but not vice versa. Some of the autoparasitized *C. reflexa* branches were observed to wither and dry. Anatomical investigations of *C. reflexa* stems parasitised by *C. reflexa* (intra-specific hyperparasitism) and by *C. campestris* (intra-generic hyperparasitism) were carried to find out if structurally normal haustoria develop and vascular continuity is established between the parasitic host and the hyperparasite. In both the types of hyperparasitism, at the point of contact with the parasitic host, the hyperparasite forms adhesive disks composed of enlongated and lobed epidermal cells and enlarged cortical cells. Haustoria with elongated parenchyma cells develop and emerge from the centre of the disks and penetrate the parasitic host tissues. In the case of intra-specific hyperparasitism, generally, the haustoria grow through the vascular tissues and reach the pith of the parasitic host. The haustoria, however, are surrounded by a densely stained boundary layer preventing direct cellular contact with the parasitic host tissues. Often the phloem tissue of the parasitic host near the penetrating hyperparasite haustorium appears necrotised. Some haustoria are found to be growing towards other haustoria in the parasitic host, suggesting that the haustoria, in general, are attracted towards nutrient-rich regions in the plant. In intra-generic hyperparasitism, the densely stained boundary layer is absent around the elongated intrusive cells at the distal end of hyperparasite (*C. campestris*) haustoria. The intrusive cells grow and connect with the phloem cells of the parasitic host, *C. reflexa,* but later, the two cell types in contact degenerate. Xylem differentiation is lacking in the haustoria of both the types of hyperparasites in the majority of the samples studied. These findings indicate that the haustoria of hyperparasites are not structurally entirely similar to those of the parasites infecting non-parasitic hosts, and may also be functionally compromised. The manifested structural differences appear to be a post-penetration

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hypersensitive / resistance reaction elicited as a result of incompatibility between the closely related partners in intra-specific and intra-generic hyperparasitism, restricting the haustoria from becoming fully functional in such parasitic relationships. The probable significance of intra-specific and intra-generic hyperparasitism is discussed. In addition, the information on the responses exhibited by resistant nonparasitic host plants during parasitisation by *Cuscuta* is briefly summarised to present our understanding of the mechanism of resistance to *Cuscuta* infection.

Introduction

Many parasitic plants, both holo- and hemi-parasites, often infect other parasitic plants. This phenomenon is frequently referred to as "hyperparasitism" or "epiparasitism". Calvin and Wilson (2009), Piwowarczyk et al. (2018), Szczesniak et al. (2019) use the terms synonymously to define parasitic relationship between two parasitic plants belonging to different species. However, others have made a distinction between the two terms. According to Mathiasen et al. (2008), hyperparasitism and epiparasitism pertain to, respectively, a facultative and an obligatory parasitic relationship between two parasitic plants. Krasylenko et al. (2021) consider hyperparasitism to be a parasitic relationship between any two parasitic plants, and designate epiparasitism specifically to such a relationship between two aerial parasitic plants. In this study, the term hyperparasitism is used to describe a parasitic relationship between any two parasitic plants. Whereas obligate hyperparasites such as *Agelanthus dichrous, Loranthus kaio, Dendrophthora epiviscum,* etc., survive only by parasitising other parasitic plants,

facultative hyperparasites including *Cuscuta,* which generally parasitise nonparasitic autotrophic plants, are not dependent on parasitic plants for their survival. Hyperparasitism in which both the partners involved in the parasitic relationship belong to the same species or are conspecific is known as intra-specific hyperparasitism (Musselman & Dickison, 1975), mutual parasitism (Furuhashi et al., 1995) or autoparasitism (see Krasylenko et al., 2021). Self-parasitism is a type of autoparasitism/intra-specific hyperparasitism wherein haustoria from one part of a parasitic plant infect a different part of the same plant (Fineran, 1965). In intra-generic and inter-generic hyperparasitism, a parasitic plant parasitises another parasitic plant belonging to a different species of the same genus or a different genus, respectively (Krasylenko et al., 2021). Autoparasitism is well documented in root and shoot hemiparasites, and shoot holoparasites with wide host ranges (Krasylenko et al., 2021) but not in root holoparasites which are more host specific, enabling them to recognise and not parasitise self or closely related species (Jhu & Sinha, 2022 a).

Cuscuta is a shoot holoparasite which infects a large number of angiosperms.

Many species of *Cuscuta* are known to exhibit hyperparasitic behaviour, including intra-specific, intra-generic and inter-generic hyperparasitism (Szczesniak et al., 2019; Krasylenko et al., 2021). The phenomenon of self-parasitism or autoparasitism in *Cuscuta* has been recorded since long (Uloth, 1860; Dixon, 1901; Audus, 1939; Lackey, 1946; Pazourek, 1958; Pizzolongo, 1963, 1964; Wellman, 1964; Singh, 1965). More recently, reports have been published on autoparasitism or intra-specific hyperparsitism in *C. reflexa* (Madhavan & Gupta, 1982; Jacob et al., 1986; Furuhashi et al., 1995, 2011; Dey & Mukherjee, 2013), *C. pedicillata* (Lyshede, 1985), *C. australis* (Lee, 1993), *C. epilinum* (Toma et al., 2004-2005), *C. chinensis* and *C. campestris* (Fathoulla & Duhoky, 2008; Hong et al., 2011) and *C. lupiliformis* (Szczesniak et al., 2019), on intra-generic hyperparasitism between *C. monogyna* and *C. campestris* (Fathoulla & Duhoky, 2008), and between *C. euproaea* and *C. lupiliformis* (Szczesniak et al., 2019), and on inter-generic hyperparsititsm in *Cuscuta* species infecting many hemiparasites belonging to Orobanchaceae and Santalaceae (Toma et al., 2004-2005; Piwowarczyk et al., 2018; Krasylenko et al., 2021). Though a large amount of information is available on the structure of haustorium and the mechanism of solute transfer between *Cuscuta* and its autotrophic hosts, the phenomenon of hyperparasitism in *Cuscuta* has received scarce attention as is evident from the very small number of reports available (Krasylenko et al., 2021). So far, little information exists on the structure and function of haustoria formed by the hyperparasites. According to prevailing literature, autoparasitic and intra-generic hyperparasitic infection in *Cuscuta* is initiated with the development of adhesive disks at the point of attachment on the parasitic host stem, followed by the development and penetration of the hyperparasite haustoria into the parasitic host tissue (Uloth, 1860; Audus, 1939; Pazourek, 1958; Pizzolongo, 1963; Madhavan & Gupta, 1982; Lyshede, 1985; Jacob et al., 1986; Lee, 1993; Furuhashi et al., 1995; Toma et al., 2004-2005; Fathoulla & Duhoky, 2008; Hong et al., 2011; Szczesniak et al., 2019). However, conflicting evidence is presented on the differentiation of vascular tissue in the haustoria. Vascular tissue, especially xylem, appears to be completely lacking (Uloth, 1860, Lyshede, 1985) or well-developed (Pazourek, 1958; Toma et al., 2004-2005; Hong et al., 2011; Szczesniak et al., 2019). Moreover, information regarding the function of the *Cuscuta* haustoria formed in a hyperparasitic relationship is extremely meagre. In the only investigation directly examining the conduction function of hyperparasite haustorium, a complete absence of transport of labeled sucrose from a host filament of *C. reflexa* to a parasitising shoot of the same species was reported (Jacob et al., 1986). These findings question the functionality of the hyperparasitic haustoria, and the benefit to a hyperparasite in these relationships. The paucity of information signals an urgent need to investigate further, at the structural, physiological and molecular levels, the phenomenon of hyperparasitism.

A prerequisite for an effective hyperparasitic relationship is the presence of a functional haustorium, and one way to verify the functionality of a haustorium is through anatomical observations (Krasylenko et al., 2021). This anatomical study investigating the structure of haustoria formed in two types of hyperparasitism, the intra-specific/

autoparasitism in *C. reflexa* and the intra-generic between *C. reflexa* and *C. campestris*, was undertaken to augment the information on hyperparasitism in *Cuscuta*. It was found that in both intra-specific and intra-generic hyperparasitism, the hyperparasite attaches to the parasitic host plant by developing adhesive disks. Haustoria with elongated parenchymatous cells form and emerge from the centre of the disks and penetrate the parasitic host tissues. In intra-specific hyperparasitism, a boundary layer, probably, of lysed cells, surrounds the penetrating haustorium preventing direct contact with the parasitic host tissues, especially the vascular tissue. Phloem tissue near the haustoria appears degenerated. In intra-generic parasitism, no boundary layer is found around the penetrating distal region of the haustoria. The intrusive cells at the distal region make contact with the parasitic host phloem tissue. However, the intrusive cells as well as the host phloem cells in contact appear to degenerate subsequently. Xylem was not evident in the hyperparasite haustoria in both the types of hyperparasitism. These finding suggest that the hyperparasite haustoria in intra-specific and intra-generic hyperparasitism possess a structure which is different from that of *C. reflexa* haustoria formed during parasitisation of nonparasitic hosts (Chitralekha et al, 2022), and hence, they may not be fully functional. The structural abnormalities observed could be due to a hypersensitive response elicited at the post-penetration stage as a result of incompatibility between closely related partners in these relationships. *Cuscuta,* which is known to parasitise a large number of plants, possibly infects self and other parasites (hyperparasitism) by default. Further investigations are required to understand

the phenomenon of hyperparasitism in *Cuscuta*, and to determine the functionality of the haustoria formed by the hyperparasites.

Materials and methods

Field observations
Roadside trees, shrubs and herbs in the city of Delhi, India, infected with *Cuscuta reflexa* Roxb. and *Cuscuta campestris* Yunck. (Convolvulaceae) were observed. Occurrence of intra-specific and intra-generic hyperparasitism in the two species was recorded and photographed.

Anatomical study
Portions of stem of *Cuscuta reflexa* with haustoria of parasitising *C. reflexa* (intra-specific hyperparasitism/autoparasitism) and haustoria of parasitising *Cuscuta campestris* (intra-generic hyperparasitism), growing on four different hosts, *Alstonia scholaris* (L.) R.Br. (Apocynaceae), *Bougainvillea spectabilis* Willd. (Nyctaginaceae), *Volkameria inermis* L. (Lamiaceae) and *Senna siamea* (Lam.) H.S. Irwin & Barneby, were collected. Some material was sectioned using razor blade. The hand sections were stained with 1% aqueous safranin O (Merck) solution and mounted in 15% glycerin. Rest of the collected material was cut into 5 mm pieces. The pieces were 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, and were 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). The stained dry sections were mounted in DPX (SLR

Chemical). Mounted sections were observed under microscope (Olympus) and photographed.

Results

Morphological observations

The occurrence of hyperparasitism, intra-specific as well as intra-generic, in *C. reflexa* and *C. campestris* is observed to be very common (Fig. 1A-K). In intra-specific hyperparsitism, the parasite and the hyperparasite belong to the same species. Three types of intra-specific hyperparasitism are possible in *C. reflexa*: (i) the parasitising and hyperparasitising strands belong to different plants (autoparasitism), (ii) the parasitising and hyperparasitising strands are different branches of the same plant (self-parasitism), (iii) a single strand folds back and parasitise itself (self-parasitism), and hence, acts both as parasitic host and hyperparasite (Fig. 1E,F). However, while collecting the material, it was difficult to ascertain whether the strands belonged to the same *Cuscuta* plant or different plants in the first two types, and therefore, no distinction was made. All the material collected, where the host and parasite strands belonged to *C. reflexa,* were considered to represent autoparasitism or intra-specific hyperparasitism. In general, the hyperparasite is observed to parasitise an autotrophic host plant as well (Fig. 1H). More than two strands may associate and infect each other (Fig. 1A-D, G). A single strand can act both as a host and a parasite (Fig. 1I). *C. campestris* too, was observed to exhibit similar intra-specific parasitism (Fig. 1A, B). Stem of *C. reflexa* is greener and thicker with a much larger diameter as compared to that of *C. campestris* (Fig. 1A, B, J). When present together, strands of *C. campestris* are

found to parasitise *C. reflexa* (intra-generic hyperparasitism) (Fig. 1A, B, J). Surprisingly, parasitisation of *C. campestris* by *C. reflexa* was not observed. In both intra-generic and intra-specific hyperparasitism, the parasitised host strand remains more or less straight; it is the parasitising strand which coils anticlockwise around the host strand (Fig. 1A-C, E, G-J). At the point of contact with the host strand, the parasitising strand develops swollen adhesive disks (Fig. 1C, D). Single haustorium emerges from the centre of each disk. Rarely, two haustorial scars are seen within a single adhesive disk (Fig. 1D) indicating development of two haustoria from one disk. In *C. campestris,* prehaustoria are prominently developed even when not in contact with a host (Fig. 1K). Some of the parasitised stems of *C. reflexa*, especially in intra-specific parasitism, wither and turn brown (Fig. 1G).

Anatomical observations

In this study, the structure of stem of parasitic host and of hyperparasite with haustoria in the two instances of hyperparasitism, (i) intra-specific hyperparasitism in *C. reflexa* and (ii) intra-generic hyperparasitism (*C. campestris* parasitising *C. reflexa)*, were examined. The stem of *C. reflexa* and *C. campestris* appears to be similar anatomically, except for a fewer number of vascular bundles in the latter. In both the species, the stem consists of an outer epidermal layer, followed by a few-layered cortex, a ring of widely spaced collateral vascular bundles and a central pith (Figs 2A-I, 3A). Large parenchyma cells make up the cortex, the interfascicular regions or medullary rays and the pith. Vascular bundles consist of phloem and xylem tissues.

Fig. 1. Intra- and inter-specific hyperparasitism in *Cuscuta*. (A, B) Intra-specific hyperparasitism in *C. reflexa* (white arrow) and *C. campestris* (cyan arrow), and intra-generic hyperparasitism of *C. campestris* on host *C. reflexa* (arrowheads), (C-I) Intra-specific hyperparasitism in *C. reflexa*. Arrows in (C & D) point to adhesive disks; note scars of two haustoria in a single adhesive disk in (D). The strand has coiled back on itself and parasitises its own self in (E & F). One of the parasitised strand in (G) appears brown and shrivelled. In (H), a strand of *C. reflexa* is parasitising the autotrophic host as well as another strand of *C. reflexa*. A single strand of *C. reflexa* acts as a host at one region (red arrow) and parasite at another (black arrow) in (I). (J) *C. campestris* parasitising a strand of *C. reflexa* (intra-generic hyperparasitism). (K) Prehaustorial projections (arrow) in a strand of *C. campestris*.

Intra-specific hyperparasitism (autoparasitism) in C. reflexa

Intra-specific parasitism in *C. reflexa* was found in all parasitised host plants observed. Haustoria (Fig. 2A-I) with adhesive disks (Fig. 2C, D, F, I) develop on the hyperparasitising strand at the region of contact with the parasitic host strand. The adhesive disks are made up of radially elongated epidermal cells and underlying enlarged cortical cells (Fig. 2C, D, I). The surface of the epidermal cells in contact with the parasitic host strand is invaginated or infolded giving a lobed appearance (Fig. 2C). A layer of cementing material present between the lobed
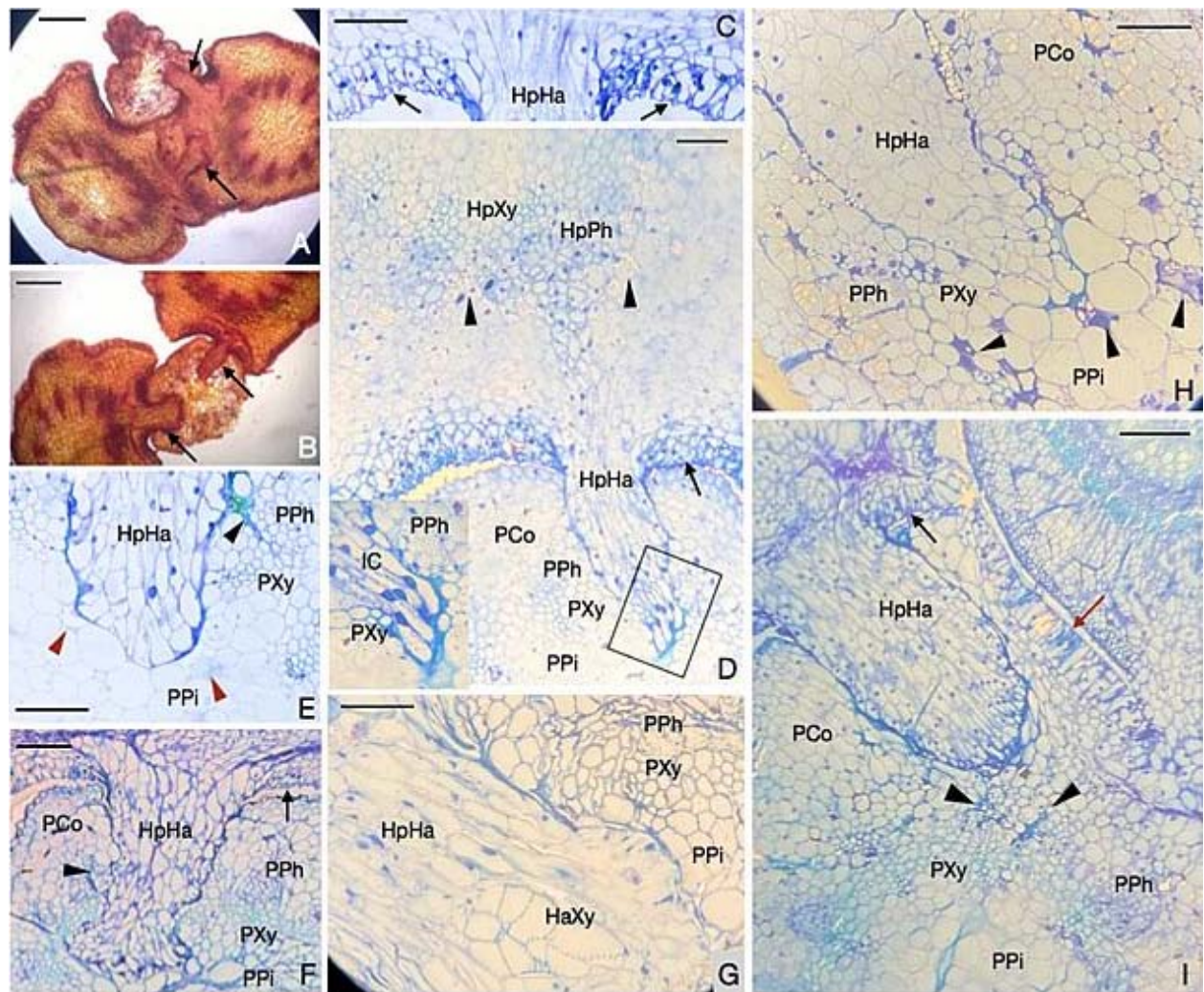
Fig. 2. Intra-specific hyperparasitism in *C. reflexa*. A-I: Transverse sections of *C. reflexa* stem with haustoria parasitising stem of another *C. reflexa*. (A, B) Hand sections of three parasitising strands; arrows point to the haustoria. In (A), the lower haustorium appears to be growing in the cortex towards the upper haustorium. (C) Adhesive disk of hyperparasite showing the lobed epidermal cells (arrows). (D-H) The hyperparasite haustorium has grown through the parasitic host stem epidermis, cortex, vascular ring and reached the pith. The haustorium is made up of elongated parenchyma cells surrounded by a densely stained boundary layer. Arrows point to the adhesive disks. In (D), the young haustorium possesses elongated axial cells in the proximal region and intrusive cells at its distal end. Boxed region is enlarged in the inset to show the degenerated parasitic host cells surrounding the intrusive cells. The black arrowheads point to the starch-containing cortical cells around the vascular bundles of the hyperparasite. In (E), intrusive cells appear to be hemmed in by the densely stained layer; red arrowheads indicate the disorganised cells of the parasitic host near the intrusive cells. Black arrowheads in (E) & (F) point to degenerated phloem cells near the haustorium. The rare presence of xylem elements in the hyperparasite haustorium is evident in (G). In (H), many disorganised cells (black arrowheads) are seen in the withered parasitic host stem. (I) The hyperparasite haustorium appears to be growing in the cortex towards the parasite haustorium infecting the stem of a nonparasitic host, *Senna siamea*, visible in the upper right hand corner. The black and red arrows point to the adhesive disks of the hyperparasite and parasite haustorium, respectively. The parasitic host phloem (arrowheads) near the hyperparasite haustorium appears degenerated. Bar in A, B = 250 µm, C-F, H, I = 100 µm, G = 50 µm. Staining: A, B, safranin; C-E,TBO; F-I, PAS-TBO. HaXy, haustorium xylem; HpHa, hyperparasite haustorium; HpPh, hyperparasite phloem; HpXy, hyperparasite xylem; IC, intrusive cells; PCo, parasite cortex; PPh, parasite phloem; PPi, parasite pith; PXy, parasite xylem.
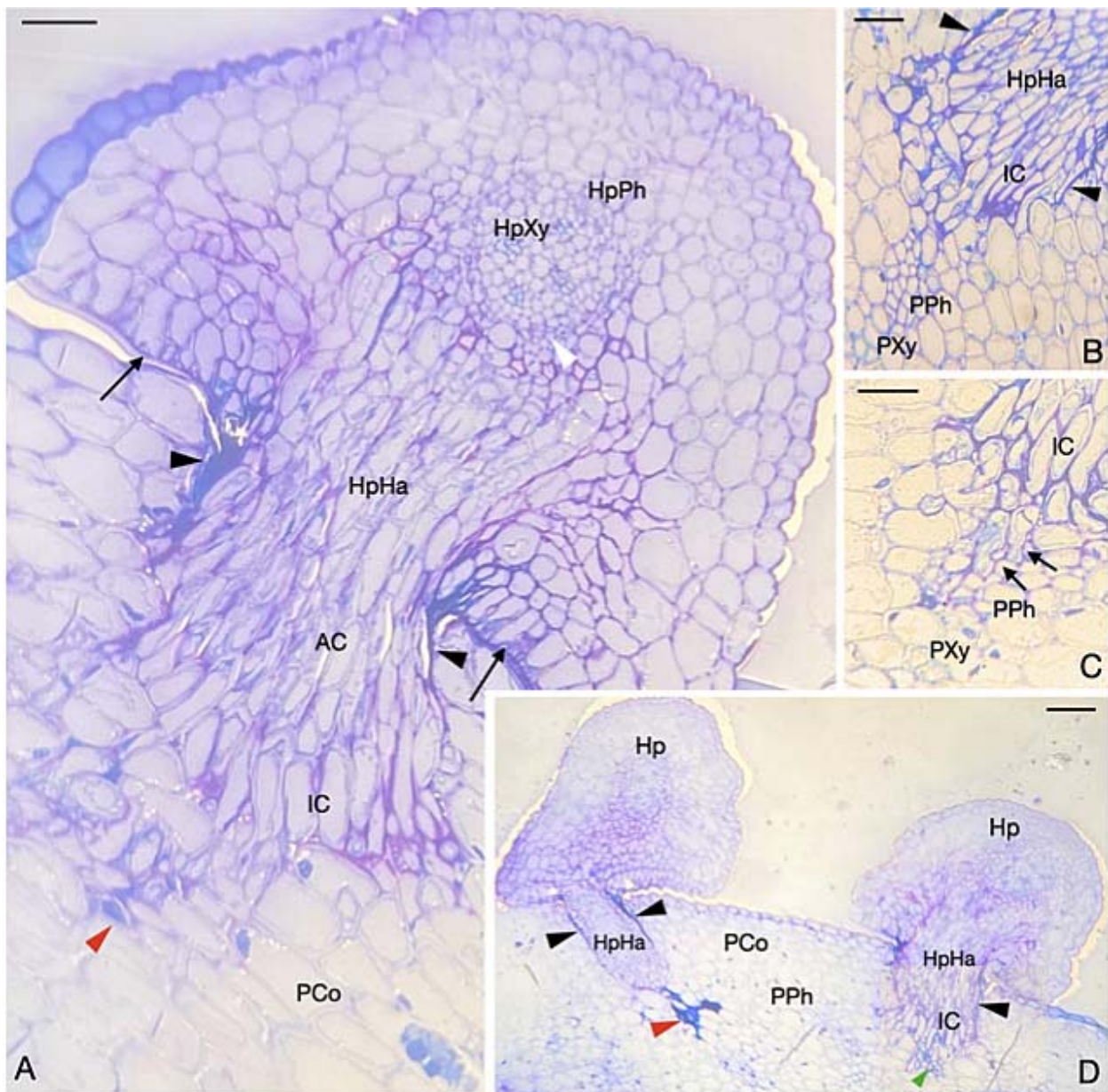
Fig. 3. Intra-generic hyperparasitism between *C. reflexa* and *C. campestris*. A-D: Transverse sections of stem of *C. campestris* (hyperparasite) with haustoria parasitising stem of *C. reflexa* (parasitic host). (A) Haustorium with adhesive disc. Arrows point to the infolded surface of the elongated epidermal cells of adhesive disk. Haustorium, consisting of proximal axial cells and distal intrusive cells, has penetrated the parasitic host cortex. The white, black and red arrowheads point to the radial files of cells near the hyperparasite vascular bundles facing the parasitic host, the densely stained boundary layer in the proximal region of the haustorium, and the degenerating cortical cells of the parasitic host, respectively. (B) Intrusive cells of hyperparasite haustorium have reached the phloem tissue of parasitic host. (C) Some intrusive cells appear to have fused with the phloem cells (arrows). (D) Two haustoria of the hyperparasite have penetrated the parasite stem. Black and Red arrowheads point to the densely stained layer around the proximal part of haustoria and the degenerated cells of parasitic host cortex, respectively. Host phloem tissue and the intrusive cells of the haustorium in contact with the host phloem appear disorganised (green arrowhead). Bar in A, B, C = 50 µm, in D = 100 µm. Staining: A-D, PAS-TBO. AC, axial cells; Hp, hyperparasite; HpHa, hyperparasite haustorium; HpPh, hyperparasite phloem; HpXy, hyperparasite xylem; IC, intrusive cells; PCo, parasite cortex; PPh, parasite phloem; PXy, parasite xylem.

epidermis of the hyperparasite adhesive disk and the outer surface of host epidermal cells at the site of contact attaches the hyperparasite to the parasitic host.

The cortical cells of the hyperparasite adjacent to the vascular bundles near the parasitic host possess starch grains (Fig. 2D). The haustoria appear to originate from these starch-containing cells. In most cases, the haustoria penetrated the parasitic host stem epidermis and cortex (Fig. 2A-I) as well as the vasculature to reach the pith (Fig. 2A-H). Interestingly, some haustoria are observed to grow within the cortex, not towards the vascular bundles of the parasitic host but towards another haustorium of the hyperparasite (Fig. 2A) or parasite (Fig. 2I). The young growing haustoria consist of elongated proximal axial and distal intrusive cells (Fig. 2D). These elongated parenchymtous cells are thin-walled, vacuolated and contain hypertrophied nuclei. The apical end of the intrusive cells are surrounded by degenerating parasitic host cells (Fig. 2D, E). In all the mature haustoria observed, a densely stained boundary layer, probably made of the degenerated host cells, encloses the endophytic part from all sides (Fig. 2E-H). No direct contact between cells of hyperparasite haustorium and vascular tissues of the parasitic host is evident (Fig. 2D-I). The parasitic host phloem bundles near the haustorium appear disorganised (Fig. 2E, F, G-I). Xylem tracheidal elements are not found in the hyperparasite haustoria (Fig 2D-F, H, I) except for a small group in one haustorium (Fig. 2G). The brown and withered parasitic host stem contains many degenerated cells (Fig. 2H).

Intra-generic hyperparasitism between C. reflexa and C. campestris

C. campestris (hyperparasite) is often seen to parasitise *C. reflexa* (parasitic host) when they are growing together, and the haustoria of the hyperparasite invade the parasitic host stem (Fig. 2A-D). At the site of contact with parasite strand, normal adhesive disks consisting of radially elongated epidermal cells and enlarged cortical cells inner to the epidermis develop on the hyperparasite strand (Fig. 3A, D). The surface of the epidermal cells of the disk in contact with the parasite is infolded (Fig. 3A). The haustorium of the hyperparasite appears to be derived from the meristematic activity of cells around the vascular bundles nearest to the parasite strand (Fig. 3A). The haustoria penetrate the epidermis and cortex of the parasitic host stem (Fig. 3A,D). They consist of elongated and vacuolated proximal axial and distal intrusive cells (Fig. 3A-D). A densely stained boundary layer surrounds the proximal part of the haustoria (Fig. 3A, B, D). Sometimes cortical cells of the parasitic host stem near the haustoria are found degraded (Fig. 3A, D). The intrusive cells connect with the phloem of the parasitic host vascular bundles (Fig. 3B-D). A few intrusive cells seem to fuse with the phloem cells (Fig. 3C). However, in most cases, the phloem tissue as well as the intrusive cells in contact appear to degenerate subsequently (Fig. 3D). The haustoria were not observed to grow beyond the phloem tissue of the parasitic host or differentiate xylem tracheary elements (Fig. 3A-D).

Discussion

Intraspecific and intrageneric hyperparasitism in *Cuscuta,* though known since a long time (Uloth, 1860; Dixon, 1901; Audus, 1939), have not been well studied and there is a dearth of information on the subject, especially on intra-generic hyperparsitism. *C. reflexa* and *C. campestris* stems infect their own stems (intra-specific hyperparasitism). Unlike the observation of Lyshede (1985) that self-parasitism in *Cuscuta pedicillata* occurs only in stem parts distant to the weakened host plants, in both *C. reflexa* and *C. campestris,* autoparasitism was observed between strands growing vigorously on healthy as well as weakened host plants. Multiple strands can associate and infect each other as also reported by Madhavan & Gupta (1982). *C. campestris* also infects *C. reflexa*. However, this intra-generic hyperparasitism appears to be nonreciprocal as *C. reflexa* has not been found to parasitise *C. campestris*. It could be because either *C. reflexa* is unable to coil around and form adhesive disks on the comparatively very thin stem of *C. campestris* or the latter species has developed resistance to infection by the former. A similar unidirectional hyperparsitism has been reported in inter-generic hyperparasitism in root hemiparasites, between *Olax phyllanthi* (Olacaceae) and parasitic hosts *Exocarpos odoratus, E. sparteus* and *Leptomeria squarrulosa* (Santalaceae) (Pate et al., 1990a, b, 1994) and between *Rhinanthus minor* and parasitic host *Melampyrum nemorosum* (Krasylenko et al., 2021). A single stem may act both as a host and a parasite. Interestingly, it is always the hyperparasite which coils round the parasite, as also observed by Jacob et al. (1986). Autoparasitism in *Cuscuta* has

been shown to be induced by mechanical contact and light quality and not by host-derived chemical signals (Furuhashi et al., 1995; Heide-Jørgensen, 2008). The inability of *Cuscuta* spp. to differentiate between living and non-living supports (*Cuscuta* has been shown to move towards and coil around any vertical object) suggests that host-derived chemical cues are often not involved in host attachment and haustoria formation in *Cuscuta* (Dawson et al., 1994; Furuhashi et al., 2011; Kaiser et al., 2015). This and the fact that haustoria can be induced by mechanical contact and light (Furuhashi et al., 2011) could be the reason why *Cuscuta* is able to parasitise any plant it comes in contact with, leading to its wide host range. Hence, *Cuscuta* probably parasitises its own self as well as other parasites (intra-specific, intra-generic and inter-generic) by default. According to Krasylenko et al. (2021), the generalist hyperparasites, such as *Cuscuta,* tend to infect other parasites opportunistically.

In the present study, in both intra-generic and intra-specific hyperparasitism, the process of parasitism begins with the anticlockwise twining of the hyperparasite stem and the development of adhesive disks, prerequisites to initiate infection, at the point of attachment on the parasitic host stem by the hyperparasite stem, as also reported by Pazourek (1958), Madhavan & Gupta (1982), Furuhashi et al. (1995). The adhesive disks are made up of enlarged cortical cells and radially elongated lobed epidermal cells. A cementing material attaches the disk to the parasitic host. This structure of adhesive disk is similar to that formed during parasitisation of nonparasitic hosts by *C. reflexa* (Chitralekha & Rani, 2022). The development of normal adhesive disks during hyperparasitic infection is not

surprising as such disks are reported to form on contact with any type of support including non-living and artificial ones (Kaiser et al., 2015; Bernal-Galeano et al., 2022). Haustoria emerge from the centre of the disks. The meristematic activity of the cells around the phloem of vascular bundles of hyperparasite nearer to the parasitic host strand appears to give rise to the haustoria as also observed during parasitisation of non-parasitic hosts by *Cuscuta* (Chitralekha et al., 2022). The haustoria are made up of elongated parenchymatous axial and distal intrusive cells. These haustoria penetrate the host epidermis and cortex. A similar development and penetration of hyperparasite haustoria into tissues of the parasitic hosts has been found in almost all cases of hyperparasitism involving *Cuscuta.* It has been reported in intra-specific and intra-generic hyperparasitism by Uloth (1860), Audus (1939), Pazourek (1958), Pizzolongo (1963), Madhavan & Gupta (1982), Jacob et al. (1986), Lee (1993), Furuhashi et al. (1995), Toma et al. (2004-2005), Fathoulla & Duhoky (2008), Hong et al. (2011), Szczesniak et al. (2019), and in inter-generic hyperparasitism with *Cuscuta* as the hyperparasite by Toma et al. (2004-2005), Piwowarczyk et al. (2018) and Krasylenko et al. (2021).

The present investigation has revealed a difference in the subsequent growth of the haustoria of hyperparasites, *C. reflexa* in intra-specific hyperparasitism, and *C. campestris* in intra-generic hyperparasitism, within the tissues of parasitic host *C. reflexa.* In intra-specific hyperparasitism, though the haustorium penetrates the parasitic host tissues, often reaching the pith, the endophyte is enclosed in a densely stained boundary layer of probably degraded cells of the parasitic host. Lysis of host cells and the

subsequent absorption of the lysed cellular contents is reportedly a common activity undertaken by penetrating haustoria to make space for their invasion (Chitralekha et al., 2022). However, in intra-specific hyperparasitism, the degraded cellular contents apparently are not absorbed and persist as a boundary layer around the haustoria, preventing the intrusive cells from diverging and making contact with the cells of the parasitic host. Thus, any kind of symplastic association with the host tissues is completely inhibited, and the hyperparasite is restricted from directly accessing water and nutrients of the parasitic host. An earlier report by Jacob et al. (1986) also mentions presence of such a layer around self haustoria of *C. reflexa.* The isolating layer prevented the haustorial cells from reaching the vascular bundles of the parasitic host stem. Lyshede (1985) found a similar isolating layer of degenerated cells enclosing the self haustoria of *C. pedicellata.* However, such an isolating boundary layer was not present around the autoparasitic haustoria of *Cuscuta austalis* (Lee, 1993). The self haustoria in the present study lacked differentiated xylem. Uloth (1860) and Lyshede (1985), too, did not find any vascular tissue in the autoparasitic haustoria of *Cuscuta,* whereas Madhavan & Gupta (1982) reported feebly developed xylem in the self-haustoria of *C. reflexa.* But well-developed xylem tissue in the haustoria connecting the vascular bundles of the two partners in autoparasitism has been observed in other *Cuscuta* spp.*,* such as in *C. epithymum* (Pazourek, 1958)*, C. epilinum* (Toma et al., 2004-2005) and *C. campestris* (Hong et al., 2011) suggesting the probable presence of functional haustoria capable of transferring solutes from parasite to hyperparasite.

In the intra-generic hyperparasitism

between *C. reflexa* (parasitic host) and *C. campestris* (hyperparasite), the host-penetrated hyperparasite haustoria possess the densely stained boundary layer in the upper part of the haustoria but not around the distal intrusive cells. In most cases, the intrusive cells reach the host phloem tissue. The tip of some of the intrusive cells appear to fuse with the phloem cells of host as reported to happen when *C. reflexa* haustorium penetrates a autotrophic host (Chitralekha et al., 2022). However, there is a subsequent degeneration of host phloem tissue as well as the intrusive cells in contact. Hence the intrusive cells of *C. campestris* haustoria associate with cortical cells but not with the vascular tissue of *C. reflexa*. It is tempting to assume that, before their degeneration, the intrusive cells form symplastic connections with cortical cells of parasitic host through plasmodesmata, and facilitate transfer of nutrients and water to some extent. The occurrence of plasmodesmata between non-parasitic host parenchyma cells and *Cuscuta* intrusive cells/searching hyphae is well documented (Fischer et al., 2021). No xylem differentiated in the hyperparasite haustoria unlike in the other intra-generic (*C. euproaea* parasitising *C. lupiliformis*) hyperparasite haustoria (Szczêœniak et al., 2019).

Most hyperparasite haustoria grow towards the parasitic host vascular bundles in both intra-specific and intra-generic hyperparasitism (present study). Interestingly, some autoparasitic haustoria, however, were found growing within the parasite cortex, not towards the vascular bundles but towards other nearby haustoria invading a parasitic or nonparasitic host. This is unlike the findings of an earlier study where the *C. reflexa* haustoria in nonparasitic hosts are found to grow directly toward the vascular bundles (Chitralekha et al., 2022). It was proposed that the directed growth of the haustoria is due to the perception of signal/s emanating from the host vascular tissues, by the intrusive cells. Autoparasitic haustoria seem to either lack such a perception, may be because of the presence of the boundary layer around them, or the signal/s from the close by metabolites-absorbing haustoria is stronger. Degenerated phloem bundles near the haustoria imply that the latter possibility is more likely, and haustoria, in general, are attracted to the nutrient-rich regions, such as the phloem of host vascular bundles or the nearby nutrient-absorbing haustoria. The earlier study by Chitralekha et al. (2022) seems to support this supposition since the intrusive cells of *C. reflexa* haustoria were observed to breach the xylem ring to reach the internal phloem in stem of host, *Alstonia scholaris*.

In this study, in both, intra-specific and intra-generic hyperparasitism, certain abnormal structures were observed. The haustoria lacked xylem, and may be also phloem-like cells involved in absorption of nutrients. There was an apparent degeneration of parasitic host phloem in both the cases. Moreover, the haustoria in intra-specific hyperparasitism were almost completely enclosed by a layer of dead cells, while those in intra-generic hyperparasitism possessed intrusive cells which degenerated on contact with parasitic host phloem. These structural features are unlike those observed during *C. reflexa* parasitism of nonparasitic hosts (Chitralekha et al., 2022) and imply that the hyperparasitic haustoria in *C. reflexa* may not be involved much in the withdrawal of nutrients and water from the parasitic host vascular tissues. Pazourek (1958) and Jacob et al. (1986)

observed that the starch reserves and growth of the hyperparasite decreased markedly if the hyperparasite is detached from its autotrophic host despite the fact that the hyperparasite is still strongly attached to the parasite through its haustoria. This indicates that the hyperparasite was obtaining most of its resources from the nonparasitic autotrophic host and very little from the parasitic host, and that the hyperparasite haustoria lack the functional ability of parasite haustoria. Jacob et al. (1986) directly assessed the absorption and conduction function of self haustoria of *C. reflexa* using radiolabelled sucrose and found the transfer of a negligible amount of sucrose from the parasite to hyperparasite. The very small amount transferred was not sufficient to sustain the survival of the parasite, and may have taken place apoplastically. However, Lee (1993) reports a lack of symplastic continuity through plasmodesmata between the parasitic host and hyperparasite haustorial cells despite the absence of an isolating layer enclosing the self haustoria of *C. australis*. On the contrary, the withering and extensive degradation of tissues found in some autoparasitised strands in the present study imply that autoparasitism can cause substantial damage to the parasitic host plant. Szczesniak et al. (2019) report that the stem of *C. lupiliformis* dries out when parasitised by *C. europaea* in intra-generic hyperparasitism, and the hyperparasitic association seems to be unprofitable to both the partners. According to Dey & Mukherjee (2013), the strands wither for want of nutrition as nutrition is drawn away by the parasitising stands. Further investigations are required to ascertain to what extent, if at all, the hyperparasite haustoria function in absorption of resources from the parasitic host and whether the death of parasitic host strands are because of starvation from such a withdrawal of nutrients by the hyperparasite.

The abnormal structural features observed during the present investigation on hyperparasitism involving *C. reflexa,* may have developed in response to a hypersensitive reaction due to incompatibility between the hyperparasite and parasite host. The incompatibility reaction sets in at the post-penetration stage, i.e., after the attachment and development of haustoria. It would seem that, the more closely related the host and parasite are, the more incompatible they are, triggering an aggravated immune reaction and manifesting greater hypersensitive response. When the host and parasite belong to same species, *C. reflexa* (intra-specific hyperparasitism), the hypersensitive reaction causes formation of a dense boundary layer around the haustoria obstructing direct physical contact with host cells and degeneration of host phloem, and prevents vascular differentiation in the haustoria. If the host and parasite belong to different species of the same genus (intra-generic hyperparasitism), *C. campestris* parasitising *C. reflexa,* the hypersensitive reaction appears to be less severe; haustoria grow and connect with (parasitic) host phloem cells but later, the intrusive cells of the haustoria and the phloem degenerate before vascular differentiation can occur in the haustoria. Details of anatomical investigations in intra-generic and inter-generic hyperparasitism with *C. reflexa* as the hyperparasite were not found. However, such investigations with other species of *Cuscuta* as hyperparasites have been carried out (Toma et al., 2004-2005;

Fathoulla & Duhoky, 2008; Piwowarczyk et al., 2018; Szczesniak et al., 2019; Krasylenko et al., 2021). In these hyperparasitic relationships too, the haustoria develop and invade the tissues of parasitic hosts. In intra-generic hyperparasitism, xylem differentiated in the haustoria connecting the vascular bundles of hyperparasite, *C. euproaea,* and the parasitic host, *C. lupiliformis* (Szczesniak et al., 2019). In inter-generic hyperparasitism, species of *Cuscuta* have been reported to parasitise other parasitic genera belonging, especially, to the hemiparasites of Orobanchaceae and Santalaceae. Whereas in some of these hyperparasites, the intrusive cells of haustoria die before making contact with the parasitic host vascular bundles (Krasylenko et al., 2021), in the others, xylem differentiates in the hyperparasite haustoria bridging the vascular bundles of the hyperparasite and the parasitic host (Toma et al., 2004-2005). Where *C. reflexa* infects plants belonging to nonparasitic genera (intergeneric parasitism), except in some resistant species and varieties, fully functional haustoria are formed establishing vascular continuity between the host and parasite (Jhu & Sinha, 2022a, Chitralekha et al., 2022). Hence, it appears that the phylogenetic closeness and compatibility between the host and parasite play a critical role in development of structurally well-formed haustoria in *C. reflexa.*

In such a scenario, the significance of hyperparasitism in *Cuscuta* needs to be understood. According to Jhu & Sinha (2022a), non-conductive self haustoria may be beneficial to *Cuscuta* spp. as anchoring devices enabling them to occupy larger area and find new hosts. But where the hyperparasite haustoria have well-developed vasculature and function in conduction, they may provide shortcut to sap flow, shortening distances that assimilates must travel. Auto- or Self-parasitism has been suggested to facilitate long-distance (McLuckie, 1924) and optimum and shorter pathways of (Lyshede, 1985; Dey & Mukherjee, 2013) water and nutrient transport, ensuring their sharing among and within the parasitic plants (Krasylenko et al., 2021). If future investigations reveal that substantial amounts of nutrients are withdrawn by hyperparasites, then the possibility of elimination of certain competing individuals, populations, or even species through hyperparasitism can be thought of. Heide-Jørgensen (2008), Szczesniak et al. (2019) have reported that, often, the hyperparasites harm their parasitic hosts substantially, reducing the damage caused by the latter to their nonparasitic hosts. Thus, hyperparasitism appears to control the survival of the parasites. In fact, obligate epiparasites have been suggested as efficient and targeted biocontrol agents (Pundir, 1981; Lian et al., 2006). Such hyperparasitic behaviour may affect the population ecology of the parasitic plants, which in turn can impact the communities and ecosystems of the region (Krasylenko et al., 2021) underscoring the need to study and understand the importance of hyperparasitic relationships.

Infection by *Cuscuta* spp. is obstructed by certain resistant hosts as well, which has been investigated in some detail and comparatively more information is available on the process of resistance. As in hyperparasitism, the defense/resistance mechanism operates at the post attachment stage, i.e., after the formation of adhesive disks or pre-haustoria and the attachment of parasite to the host (Jhu and Sinha, 2022b). The resistant hosts

prevent the development of functional haustoria by the parasite. The prevention may be at the pre-penetration stage where the haustoria are blocked from penetrating the host tissues as in some *Euphorbia* spp. (Forstreuter & Weber, 1984), resistant tomato varieties (Ihl et al., 1988; Singh & Singh, 1997; Goldwasser et al.,2001; Jhu et al., 2022; Bawin et al., 2024), *Ipomoea batatas* (Singh & Singh, 1997), resistant genotypes of chickpea (Goldwasser et al., 2012), non-host, *Mangifera indica* (Guo et al., 2022), or at the post-penetration stage where the haustorium penetrates the host tissues but is prevented from being functional as in *Gossypium hirsutum* (Capderon et al., 1985), *Hibiscus rosa-sinensis* (Schlenzka, 1992), *Ancistrocladus heyneanus* (Bringmann et al. 1999) and *Impatiens balsamina* (Lee & Jernstedt, 2013). In response to *Cuscuta* attack, an initial localised hypersensitive reaction in the resistant host is brought about by the release of reactive oxygen species and stress-related phytohormone ethylene (Fürst et al., 2016), and, in some cases, cytotoxic compounds such as phytoalexins and acetogenic secondary metabolites (Bringmann et al., 1999) and growth inhibiting soluble phenolic compounds (Sahm et al., 1995). The hypersensitive reaction activates programmed host cell death around the haustorium. The layer with dead cells forms a barrier to haustorial ingression (Ihl et al., 1988). The hypersensitive reaction and cell death also trigger a characteristic wound response resulting in the formation of an impenetrable physical barrier tissue consisting of cells with lignified/suberised or modified walls, usually by the host, at the host-parasite interface (Capderon et al., 1985; Sahm et al., 1995; Kaiser et al., 2015; Jhu et al., 2022) restricting the growth of the haustorium. The

composition of host cell walls is also suggested to play a role in non-penetration of haustoria (Johnsen et al., 2015). Guo et al. (2022), who studied the transcriptomic and metabolomic differences in *C. japonica* during parasitisation of host and non-host plants, infer that the latter resist infection by interfering with the parasite metabolism during development of haustoria. Ihl et al. (1988) found that removal of a few surface layers of the resistant tomato host resulted in the penetration of the haustoria which subsequently became functional indicating that the ability to resist the parasite resides in a few outer layers of the host stem. On the contrary, Singh & Singh (1997) demonstrated that the defense response is distributed throughout the resistant host since haustoria which were able to penetrate the host stem stripped of the outer cell layers, burst without making contact with host vasculature. In a resistant host-parasite relationship, parasite-released effector/s are believed to interact with specific host receptors and trigger elevated host immune response resulting in the non development of functional haustoria (Albert et al., 2008; Clarke et al., 2019; Brun et al., 2021). Hegenauer et al. (2016, 2020), Krause et al. (2018) and Jhu et al. (2022) have identified several molecular factors responsible for the resistance reaction exhibited during the parasitisation of resistant tomato plants by *Cuscuta*. There is a need to identify the active molecules and understand the mechanism/s eliciting the incompatible/ resistance reactions during hyperparasitism as well. The knowledge may aid in engineering crop plants resistant to *Cuscuta* infection (Hegenauer et al. 2020; Jhu et al., 2022; Hartenstein et al., 2023).

Acknowledgments

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