



Article

Cell Wall Dynamics in Haustorial Development of *Cuscuta campestris* During Parasitism on Differentially Susceptible Hosts

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Abstract

Dodder (*Cuscuta campestris*) is a parasitic plant that causes severe economic losses to crops such as mung bean (*Vigna radiata*), although some species, including tomato (*Solanum lycopersicum*), exhibit varying degrees of resistance. Dodder parasitism begins with the development of the haustorium, whose endophytic primordium undergoes intrusive growth to penetrate host tissues. While the cell walls of endophytic cells are essential for invasion, the sequential changes occurring in these cell walls are not fully understood. This study aims to characterize cell wall modifications in *Cuscuta campestris* haustoria during parasitism of a susceptible host (*Vigna radiata*) and a resistant host (*Solanum lycopersicum* ‘Minibel’), using histochemical and immunohistochemical approaches focused on homogalacturonan (HG) and arabinogalactan proteins (AGPs). In both hosts, AGPs and HG (predominantly in their demethylesterified form) increased in the host-facing epidermal walls, the aligned file cells of the haustoria, and the boundary layer surrounding the haustorial cone. The boundary layer was enriched in AGPs and initially showed massive HG deposition, later incorporating lignin and callose. In tomato, lignin-based resistance was associated with the outermost cortical cells and did not substantially affect the overall dynamics of the dodder cell walls. These findings highlight the central role of coordinated cell wall remodeling in dodder invasion and reveal broadly similar developmental trajectories of HG and AGPs in haustoria formed on susceptible and resistant hosts.

Keywords: boundary layer; callose; cell wall; dodder; homogalacturonan; immunohistochemistry; mung bean; *Solanum lycopersicum*; tomato; *Vigna radiata*



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1. Introduction

Cuscuta is a genus of parasitic plants (dodders) that causes significant economic losses to crops such as alfalfa, beet, mung bean, cranberry, carrot, potato, sweet pepper, and vetch, among others. Most of these losses are attributed to field dodder *Cuscuta campestris* [1,2]. Due to the biological features of dodder seeds and their broad host range, implementing selective control strategies that do not harm crops remains particularly challenging [3]. However, certain plant species, such as tomato (*Solanum lycopersicum*), exhibit varying degrees of resistance to dodder infestation [1,4–8].

Contact between a parasite and its host can occur in two ways: primary or secondary parasitism [9,10]. In primary parasitism of dodder plants, after seed germination, a seedling

lacking leaves and with minimal chlorophyll content develops. A vestigial root then forms at its base, enabling the plant to survive for a limited time. If it fails to establish a connection with the host plant, it will eventually die [11,12]. Directional growth towards the host is driven by circumnutation movements [9,13] and guided by at least two environmental cues: specific light signals and volatile compounds emitted by the host plant [9,12,14]. In secondary parasitism, invasion is initiated by a lateral branch originating from a dodder plant that has already parasitized a primary host plant.

Once the stem tip comes into contact with the host, mechanosensitive ion channels in dodder tissues perceive these mechanical stimuli, resulting in the dodder stem coiling around the host stem. This phase, referred to in our study as pre-adhesive or F0, triggers the invasion process through the formation of a specialized organ known as the haustorium [15,16]. This organ performs four sequential functions: attachment, penetration, evasion of the host defense system, and establishment of a vascular connection for water and nutrient uptake [11,17]. These functions correspond to three main developmental phases of the haustorium: adhesive, intrusive, and connective [18–20].

The adhesive phase (denoted F1 in our study) initiates with the formation of a disk-shaped structure, the adhesive disk or appressorium, arising from the elongation of epidermal and cortical cells of the dodder stem [15,18]. This is followed by the development of the pre-haustorium, a non-endophytic structure located at the upper part of the haustorium and derived from a disk-shaped meristem [17,21].

Once the pre-haustorium is formed, its cortical cells become active and begin to divide, giving rise to the primary endophytic tissue that constitutes the haustorium proper [22]. This tissue marks the start of the intrusive phase (F2) and is composed of elongated, finger-like cells with large nuclei, together with smaller file cells, both of which grow intrusively into host tissues [17]. These cells form the haustorial cone and penetrate both the cortical tissues of dodder and the epidermal and cortical layers of the host plant [18,23].

During this intrusion, so-called searching hyphae and digitated cells develop from the elongation of epidermal cells at the haustorial apex [18,22,24]. These hyphae grow directionally toward the vascular tissues of the host and establish symplastic connections with host cells through plasmodesmata [24,25].

Upon reaching the vascular system (connective phase, F3), the searching hyphae acquire identity based on the type of host tissue they contact [18]. Hyphae that connect with xylem vessels differentiate into xylem hyphae, forming direct bridges with the host. In contrast, those that contact phloem elements become absorptive hyphae and develop finger-like projections that surround the host's phloem vessels [17]. Once the vascular systems are connected, dodder extracts water, nutrients, and photoassimilates from its hosts; moreover, both dodder and host exchange diverse biomolecules, including systemic signals, mRNAs, small RNAs, and proteins [25,26].

Once a vascular connection has been established, the dodder will grow again, producing new stems and marking the start of an additional fourth stage: the proliferative (F4) phase, defined by extensive lateral branching after functional vascular integration. These stems can reach additional host plants (secondary parasitism), form further haustorial connections, and ultimately develop flowers, fruits, and seeds [10].

In cases where hosts develop a resistance response towards dodder haustoria [1,4–8], two new stages can be identified: the initial disintegration of host cortical cells surrounding the invading haustorium (early resistance, or R1 phase), followed by the loss of turgor and death of the haustorium (late resistance, or R2 phase).

Dodder cell walls play a crucial role during the parasitic invasion process [21,27,28]. The primary cell wall of dicots consists of a cellulose scaffold bound to a matrix of polysaccharides (pectin and hemicelluloses) and glycoproteins [28]. Pectin is composed of galactur-

onans, particularly homogalacturonan (HG), which exhibit varying degrees of methylesterification, rhamnogalacturonan I, which carries arabinan and galactan side chains, and substituted galacturonans, as rhamnogalacturonan II. Hemicelluloses, on the other hand, include mainly xyloglucans, xylans and mannans [29]. Among the glycoproteins, arabinogalactan proteins (AGPs)—characterized by arabinogalactan side chains attached to the core protein and often anchored to plasma membrane via glycosylphosphatidylinositol—play a relevant role in modulating dodder cell wall structure [27,30]. Some cell types produce secondary cell walls after growth ceases; these walls are mainly composed of cellulose and hemicelluloses and incorporate compounds such as lignin, which strengthen the cell wall and make it more impermeable [29].

The plant cell walls of the invaded host act as physical barriers that the dodder haustorium must overcome. To penetrate host tissues, the haustorium releases a suite of cell wall-degrading enzymes targeting specific components of host cell walls. In intrusive dodder tissues, increased expression of genes encoding endo- β -1,4-glucanases, endo- β -1,4-mannanases, polygalacturonases, and pectin methylesterases has been reported [31–34]. Pectin methylesterases reduce the degree of methylesterification of HG, thereby facilitating the activity of polygalacturonases and modifying pectin properties, thus facilitating tissue penetration [28].

As the haustorium of dodder penetrates host tissues, structural modifications arise in the cell walls of both organisms. Notably, a wide and rather irregular boundary layer usually develops at the haustorium–host interface [23]. This layer is rich in lignin, and comparable structures have been described in other parasitic plant–host systems where they have been termed lignolic deposit [35] or interfacial lignin deposit [36]. The nature of this boundary layer is a matter of debate: whether it represents a defensive response of the host [23] or a structure actively produced by the parasitic plant [35,36]. Alternatively, it has been argued that it is the result of contributions from both partners, potentially through selectively stretching or remodeling of the pre-existing host cell wall [24].

Lignin in host tissues acts as a physical barrier against parasite invasion, and certain resistant plants—such as some tomato varieties resistant to dodder—accumulate lignin at infection sites as a part of their defense response [5,7]. However, recent evidence indicates that interfacial lignin deposits in some parasitic plants, including *Striga hermonthica*, *Rhinanthus minor* and *Odontites vernus*, can be primarily produced by the parasite itself [35,37]. These findings suggest that lignin may play additional roles in the parasite–host interactions. To determine the source of these deposits, some studies have compared haustoria attached to the host with non-infective pre-haustoria attached to inert surfaces, such as pot walls. These studies have shown that the latter are capable of producing lignin-rich deposits that are comparable to those found at the haustorium–host interfaces [35].

Further research is needed not only to deepen understanding of the dynamics of lignin interfacial deposit formation, but also to determine whether other cell wall components are involved. Additionally, establishing whether the composition and/or function of these deposits differs between interactions with susceptible and resistant hosts could provide valuable insight into the mechanisms underlying host selectivity and resistance.

In this context, the aim of this study was to characterize cell wall modifications in the haustorial cells of *Cuscuta campestris* throughout the parasitic process, during parasitism of both susceptible (mung bean, *Vigna radiata*) and resistant (‘Minibel’ tomato) hosts, with a focus on the boundary layer. As pectin and AGPs were previously identified as key cell wall constituents accumulating in the haustoria of *Cuscuta campestris* and *Cuscuta japonica*, we focused on these components [27]. To this end, we employed a combination of histochemical and immunohistochemical approaches to examine changes in cell wall composition across the stages of haustorium development.

2. Results

2.1. Invasion of Dodder on Mung Bean and ‘Minibel’ Tomato Plants

The distribution of *Cuscuta campestris* developmental stages at 13 days post-infection differed markedly between the susceptible host (mung bean) and the resistant host (tomato ‘Minibel’), as well as between primary and secondary parasitism (Figure 1).

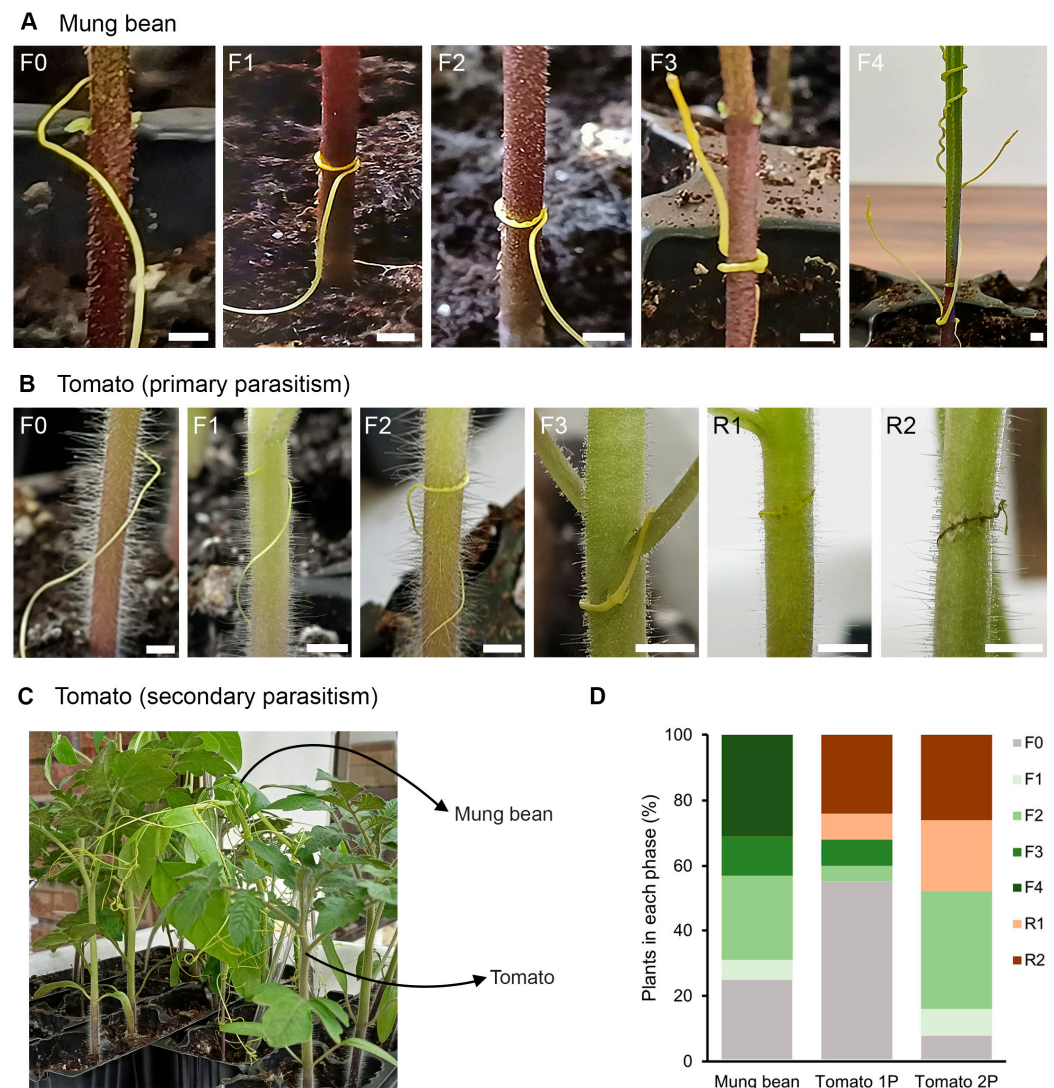


Figure 1. Stages of *Cuscuta campestris* parasitism on (A) mung bean and (B) ‘Minibel’ tomato during primary parasitism (dodder stems emerging from seeds), and on (C) ‘Minibel’ tomato during secondary parasitism (dodder stems arising from previously parasitized mung bean). Parasitism stages are classified as follows: F0, pre-adhesive; F1, adhesive; F2, intrusive; F3, connective; and F4, proliferative. Two resistance stages are also identified in the interaction between dodder and tomato stems: R1 (early resistance) and R2 (late resistance). Detailed descriptions of each stage are provided in the Introduction section. (D) Proportion of plants at each developmental stage of dodder parasitism after 13 days on mung bean, tomato during primary parasitism (tomato 1P) (N = 25), and tomato during secondary parasitism (tomato 2P) (N = 14). Scale bars: (A) 3 mm; (B) 5 mm.

In our experiment, mung bean proved to be a susceptible host for *Cuscuta campestris*. During primary parasitism on mung bean (Figure 1A), most (75%) dodder plants had progressed to advanced stages of parasitism. The majority reached the connective (F3) and proliferative (F4) phases, indicating successful penetration and establishment of functional

haustorial connections. Only a small proportion of plants remained at early stages (F0–F1), reflecting efficient transition through the pre-adhesive and adhesive phases (Figure 1D).

In contrast, during primary parasitism on ‘Minibel’ tomato (Figure 1B), haustorial development was strongly restricted. Most plants remained in the pre-adhesive (F0) or adhesive (F1) phases, and only a minority reached the intrusive (F2) stage. Notably, none advanced to the connective (F3) or proliferative (F4) phases, demonstrating an early and effective blockage of haustorial progression in the resistant host. About 55% of dodder plants failed to attach to tomato plants. As dodder survival depends on establishing a parasitic connection, the unattached seedlings died within 13 days. Even among the 45% of seedlings that were able to form haustoria, around 30% triggered a defensive response in the tomato plants. This was initially evidenced by slight brown discoloration of the tomato epidermis at the site of contact with the dodder (early resistance, R1), followed by loss of turgor and dark browning of the dodder stem, ultimately resulting in its death (late resistance, R2) (Figure 1B,D).

However, when tomato plants were challenged through secondary parasitism using dodder stems originating from previously infected mung bean plants (Figure 1C), invasion rates increased substantially: 90% of dodder stems had established interactions with tomato plants; a proportion of haustoria successfully reached F1 and F2 stages, indicating that prior establishment on a compatible host facilitates subsequent invasion of a resistant host. Nevertheless, the overall distribution remained shifted towards earlier stages (F1 and F2) compared to the mung bean plants (greater abundance of F3 and F4 than F1 and F2), confirming that the tomato plants still impose significant constraints on dodder development. In fact, 40% of these interactions resulted in the tomato plants exhibiting resistance (R1 and R2 stages), whereas no resistance stages were observed in the interactions between mung bean and dodder plants (Figure 1D).

2.2. Histological and Immunohistochemical Characterization of the Early Stages of Parasitism of *Cuscuta Campestris* on Tomato Stems

Focusing on dodder parasitism in tomato, during the initial steps of the pre-adhesive phase (F0) dodder stems displayed the differentiation of a group of dividing cells emerging in a region between the cortex and the vascular tissue and located adjacent to the surface facing the host (Figure 2A). In the early adhesive phase (F1), consecutive divisions of these cells produced rows of parenchymatous, isodiametric cells, which subsequently elongated markedly in a direction perpendicular to the stem surface to form file cells (Figure 2B,C).

During the early intrusive phase, these file cells formed the inner axial domain and were arranged in a columnar configuration (Figure 2D–F). As the columns of file cells elongated, they extended toward the apical epidermal cells of the dodder stem. Concurrently, the parasite stem swelled, pushing the apical epidermal cells against the host surface and exerting increasing mechanical pressure (Figure 2D–I).

Immunohistochemical analyses revealed distinct modifications in the cell walls of both file cells and apical epidermal cells throughout the adhesive and early intrusive phases. File cell walls differed markedly from those of cortical cells in the pre-haustorium, showing a clear asymmetry in their composition along the basal to apical axis. In the basal zone (opposite to the host), file cell walls were strongly immunolabeled with LM19, which recognizes low methylesterified or unesterified HG (Figure 2B). By contrast, apical regions exhibited little to no LM19 labeling. Notably, in the last basal file cells showing LM19 signal, labeling was particularly intense at the cell corners (Figures 2B and S2A).

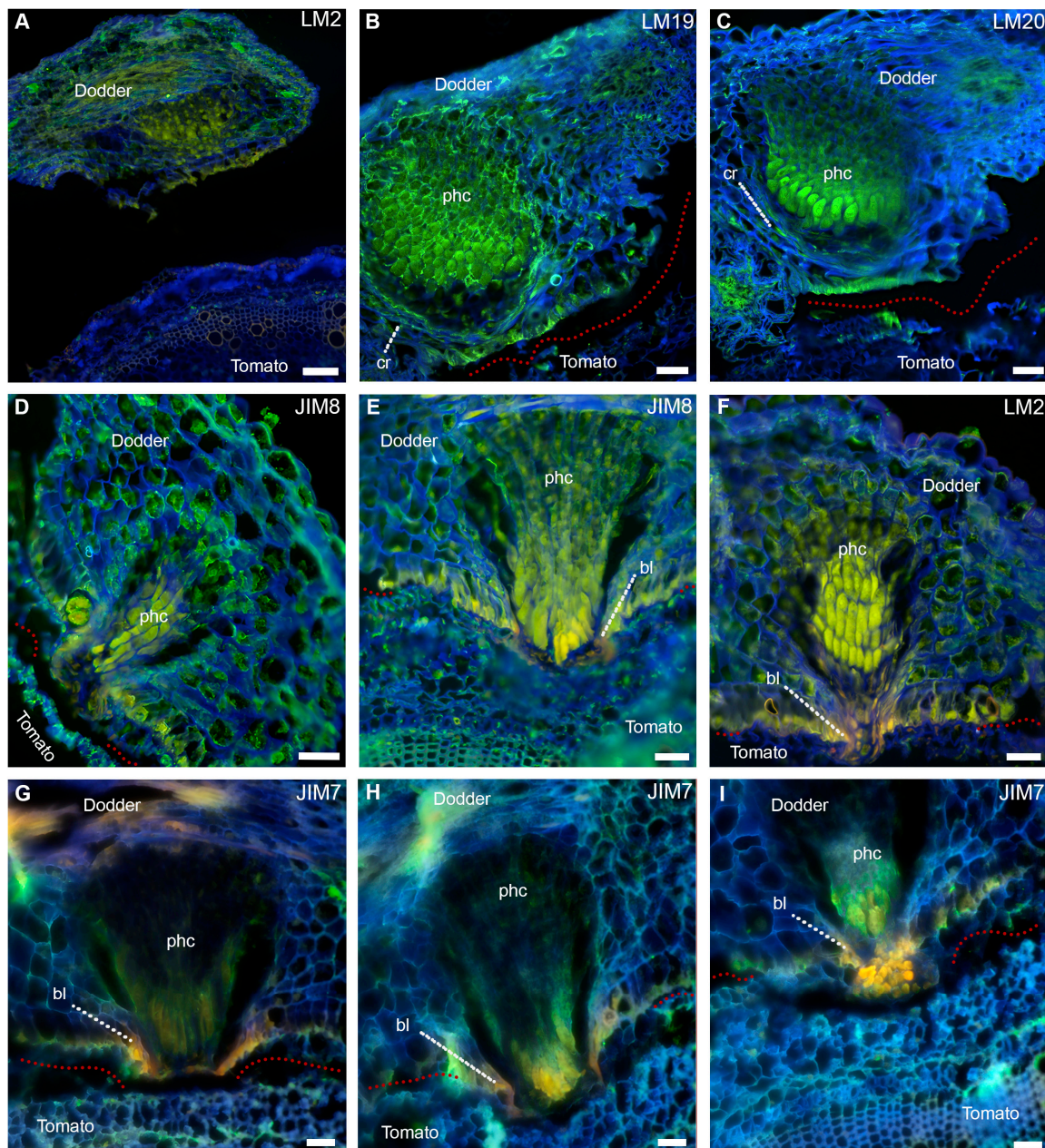


Figure 2. Role of cell walls during the initial phases of *Cuscuta campestris* parasitism of ‘Minibel’ tomato, revealed by immunolocalization of different cell wall components (green signal) and Calcofluor counterstaining (blue signal) in cross-sections. In each image, the upper part corresponds to the dodder stem and the lower part to the host stem. (A–C) Pre-adhesive and adhesive phases. (A) A dodder stem is approaching the tomato stem during the pre-adhesive phase, with arabinogalactan proteins (AGPs) immunolabeled using LM2. Note the stronger signal in the dodder tissues and the virtual absence of labeling in the tomato stem. (B) Interaction in the adhesive phase showing low methylesterified homogalacturonan (HG) immunolabeled with LM19. LM19 marks the cells of the pre-haustorial cone (phc), but not as many at the top. It also labels the epidermal cells facing the host. Crushing (cr) of some dodder cortical cells is also evident. (C) Pre-haustorium showing highly methylesterified HG immunolabeled with LM20. A more defined signal is forming a gradient towards the apex of the haustorial cone, and is particularly evident in epidermal cells facing the host. (D–I) Early intrusive phase. (D) Haustorium showing AGPs immunolabeled using JIM8. The advance of three aligned cell files is visible. (E) Haustorium in a more advanced state, with AGP immunolabeled using JIM8. Eight aligned cell files are observed, along with the onset of the boundary layer (bl). (F) Haustorium showing AGPs immunolabeled using LM2. The labeling is mainly associated with the dodder stem and is only weakly associated with the tomato stem. Eight aligned

cell files and a more defined boundary layer are visible. (G–I) Progressive aspect of haustoria immunolabeled with JIM7 to locate partially methylesterified HG. The boundary layer with phenolic impregnation is clearly visible. The JIM7 signal remains associated with the apical region of the haustorial cone. The analysis of samples was repeated independently at least twice, and the results were consistent. Abbreviations: bl, boundary layer line; cr, crushing of cells; phc, pre-haustorial cone. The dotted red line delineates the interface between dodder and host stems. Scale bar: (A), 100 μm ; (B–I), 50 μm .

In contrast, immunolabeling with LM20 (Figure 2C) and JIM7 (Figure 2G–I) antibodies, which recognise methylesterified HG epitopes, was more intense in the apical region of the file cell columns. Taken together, these results indicate that both the abundance of pectin and the degree of HG methylesterification are higher in the apical than in the basal regions of the file cell columns, suggesting differential pectin remodeling along the developing haustorium axis.

In the epidermal cell walls, HG labeling was detected specifically in the apical region, but not in the remaining epidermal cells. This was seen using LM19 (Figure 2B), LM20 (Figure 2C) and JIM7 (Figure 2G–I). The fluorescence signal was particularly intense on the outer (apical) face of the walls. These results indicate the presence of localized enrichment of both low- and highly methylesterified forms of HG in the apical epidermal cell walls.

A decrease in Calcofluor labeling in the central cells of the haustoria was observed, indicating a lower cellulose content (Figure S3B). The cell walls of these cells are particularly thin, making them flexible and dynamic, which supports their penetrating and transport functions.

Differences were also detected in the distribution of AGPs. Two AGP-directed antibodies, LM2 and JIM8, were used in this study. Both LM2 and JIM8 showed positive labeling in isodiametric cells during the pre-adhesive phase and later in the elongated file cells (Figure 2A,D–F). Numerous JIM8- and LM2-labeled vesicles were found in cortical cells adjacent to the file cells, and AGP labeling was also observed in the inner cell wall (Figure S2B,C). These findings suggest an active accumulation of AGPs in transit toward the cell wall, where they are eventually deposited during the early stages of haustorial differentiation.

AGP labeling was also intense in dodder apical epidermal cells, particularly along the cell surface facing the host. This was observed using LM2 (Figure 2A,F) and JIM8 (Figure 2D,E). These dodder epidermal cells were found to be tightly attached to the host epidermis, which exhibited a low level of JIM8 labeling (Figure 2D–F). This asymmetric distribution suggests that AGPs accumulate at the parasite–host interface, potentially contributing to adhesion and/or early recognition processes during the initial stages of contact.

Finally, Figure 2D–I shows the formation of a boundary layer in sequence, with the boundary layer progressively occupying an increasing surface area and displaying an intensifying brown-orange signal, which could indicate phenolic impregnation.

2.3. Modifications of the Dodder Cell Walls in the Haustorial Stages of Parasitism in Tomato Hosts

During the intrusive phase of haustorial development in tomato stems, resistance responses in the host tissues became evident (Figure 3). These responses were characterized by the disintegration of cortical cells surrounding the invading haustorium. In more advanced stages, phenolic materials are deposited in cortical cells, which is consistent with a defensive response involving lignin (Figure 3H). Within the endophytic haustorium, the distribution of low-methylesterified pectin showed intense LM19 labeling at the haustorial apex forming a basal-to-apical gradient, and in epidermal cells at the host–parasite

boundary (Figure 3A,D). Notably, the cortical and epidermal cell layers of the tomato stem appear disrupted, while the epidermal layers of dodder in close contact with the tomato stem remain intact.

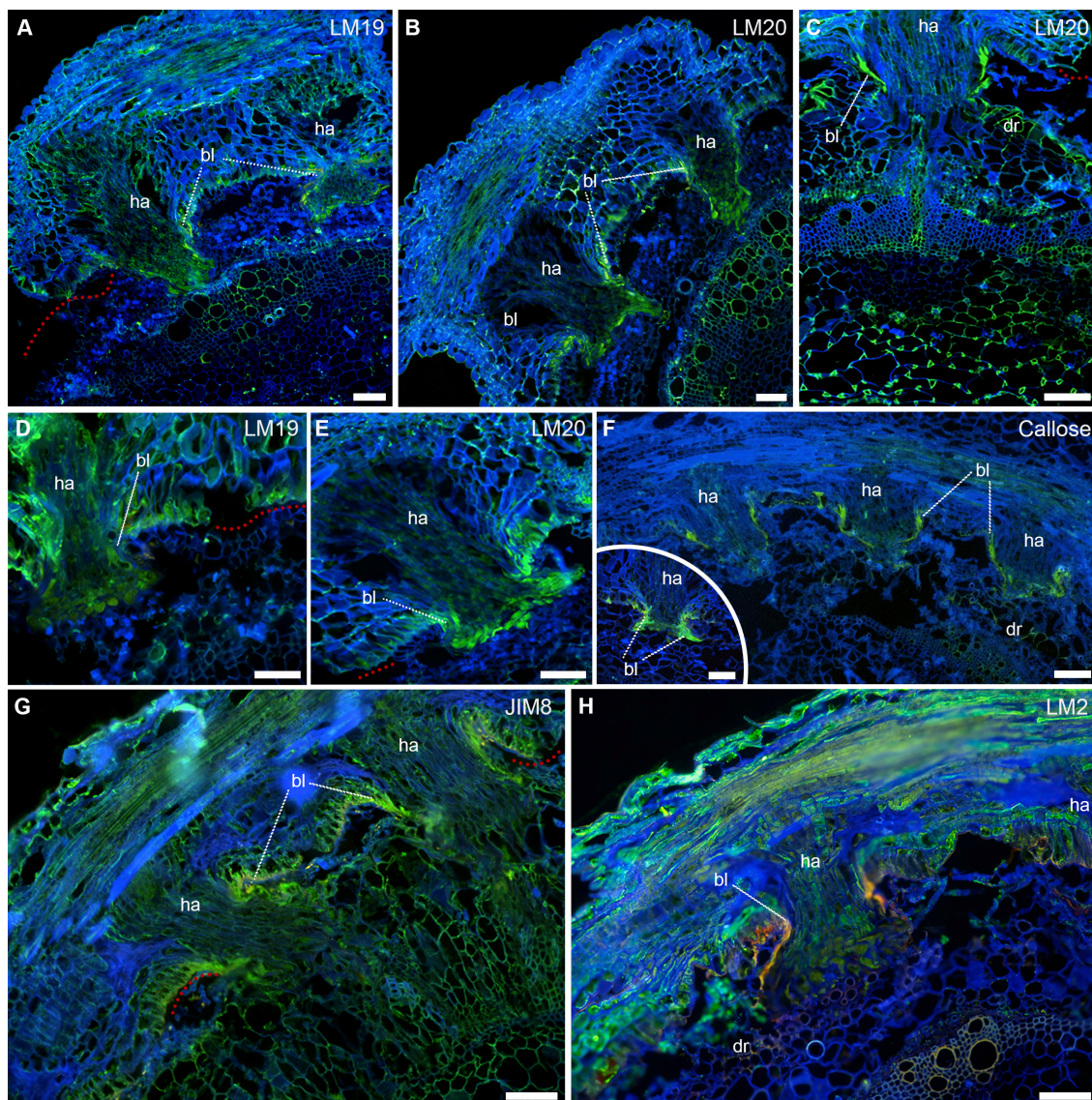


Figure 3. Immunohistochemical analysis of cell wall AGPs, HG and callose in cross sections during advanced stages of *Cuscuta campestris* endophyte invasion in ‘Minibel’ tomato stems. In each image, the upper part corresponds to the dodder stem and the lower part to the host stem. (A) LM19 immunolabeling of a cross section of dodder and tomato stems. Two haustoria (ha) are visible. Low methylesterified HG is mainly associated with the apical zone of the file cells and the epidermal cells of dodder. LM19 immunolabeling in the tomato stem was weaker. (B) LM20 immunolabeling of two dodder haustoria penetrating the tomato stem. Methylesterified HG is not immunolocalized in the file cells at this stage, although strong labeling persists in epidermal cells. Tomato cortex cells display the typical labeling at the outer region of their cell wall corners. (C) LM20 immunolabeling at a more advanced developmental stage of the haustorium. Strong signal is detected associated with the development of a xylem vessel and in the boundary layer (bl), as well as in tomato cortex cells undergoing deformation due to the hypersensitive response associated with the defense reaction (dr) near the site of penetration. Tomato cortex cells display the typical labeling at the outer region of their cell wall corners. (D) A closer view of a haustorium labeled with LM19, showing a basal-to-apical gradient of labeling. (E) A closer view of a haustorium labeled with LM20, showing a strong signal associated with the boundary layer. (F) Callose immunolocalization. Three haustoria can be observed.

In the haustoria on the left, callose accumulates in the epidermal cells and the boundary layer; in those on the right, it is mainly restricted to the boundary layer. Callose deposition is also detected in the tomato cortex during the early steps of interaction with the haustorium (rightmost haustorium). A resistance response is also visible in the cortex cells of the tomato. The inset shows a callose immunolocalization in a single haustorium with a close-up view. (G) AGP immunolocalization with JIM8. Two haustoria are visible. AGPs are mainly localised in the epidermal cells and the boundary layer. (H) AGP immunolocalization with LM2. Two haustoria are shown; the one on the left exhibits a well-defined boundary layer. AGPs are detected in dodder, predominantly in the protoplasts of digitated cells, with minimal labeling in the tomato stem. Defense-related changes, including phenolic deposition and cell disorganization, are visible in the tomato cortex. (A,D): LM19; (B,C,E): LM20; (F): anti-callose; (G): JIM8; (D): LM2. All sections were counterstained with Calcofluor. The analysis of samples was repeated independently at least twice, and the results were consistent. Abbreviations: ha, haustorium; bl, boundary layer; dr, defense reaction. The dotted red line delineates the interface between dodder and host stems. Scale bars: 100 μ m.

Concomitantly, in the connective phase, highly methylesterified pectin detected with LM20 accumulated in the apical region of the endophyte, particularly in the inner axial aligned file cells, coinciding with the differentiation of developing xylem vessels, as well as in the epidermal cells at the contact zone (Figure 3B,C,E).

The anti-callose antibody revealed strong callose accumulation in the dodder epidermal cells at the contact zone with the tomato stem, particularly within the boundary layer and in epidermal cells penetrating into the endophyte (Figure 3F). At this stage, virtually no callose deposition was detected in cortical cells of the tomato stem at the host–parasite interface, except during the early steps of interaction with the haustorium, observed in the rightmost haustorium.

Finally, with respect to AGPs, JIM8 labeling appeared diffuse but was more intense in the epidermal cells at the adhesion zone and in the boundary layer (Figure 3G). LM2 labeling showed an asymmetric distribution, being more intense in the dodder tissues, and localized mainly in the apical region of the endophyte (Figure 3H).

2.4. Modifications of the Dodder Cell Walls During the Parasitism of Dodder on Mung Bean Stems

In the early stages of dodder parasitism—pre-adhesive (F0) and adhesive (F1)—on mung bean stems, no noticeable structural differences were observed compared to tomato host stems (Figure S1). This was evidenced by the differentiation of the same cell types, and by the absence of a boundary layer between parasite and host tissues at these stages (compare Figure S1 and Figure 2, respectively).

During the intrusive phase (F2), the aligned file cells progressively penetrate the host tissues until they reach the host vascular bundles, marking the transition to the connective phase (F3) (Figure S1).

Histological analyses of mung bean hosts show that the endophytic haustorium first breaches the epidermal layer and then advances through the cortical tissues before finally reaching the host vascular cylinder, with no evidence of resistance responses in the stem tissues. Figure 4A illustrates two consecutive stages: the haustorium on the right has penetrated into the inner cortical layers but has not yet reached the vascular bundle (intrusive phase), whereas the haustorium on the left has already contacted it (connective phase).

In the haustorium, axial cells are characterized by conspicuous nuclei, large vacuoles, and thin primary cell walls (Figures 4A–C,G–I and S2D). At higher magnification, the previously isodiametric cells arranged in orthogonal files during the pre-haustorial stage adopt more irregular shapes upon invading the host tissues, giving rise to the searching hyphae or digitated cells. These cells stain intensely green with safranin–fast green (Figure 4B,C,G–I).

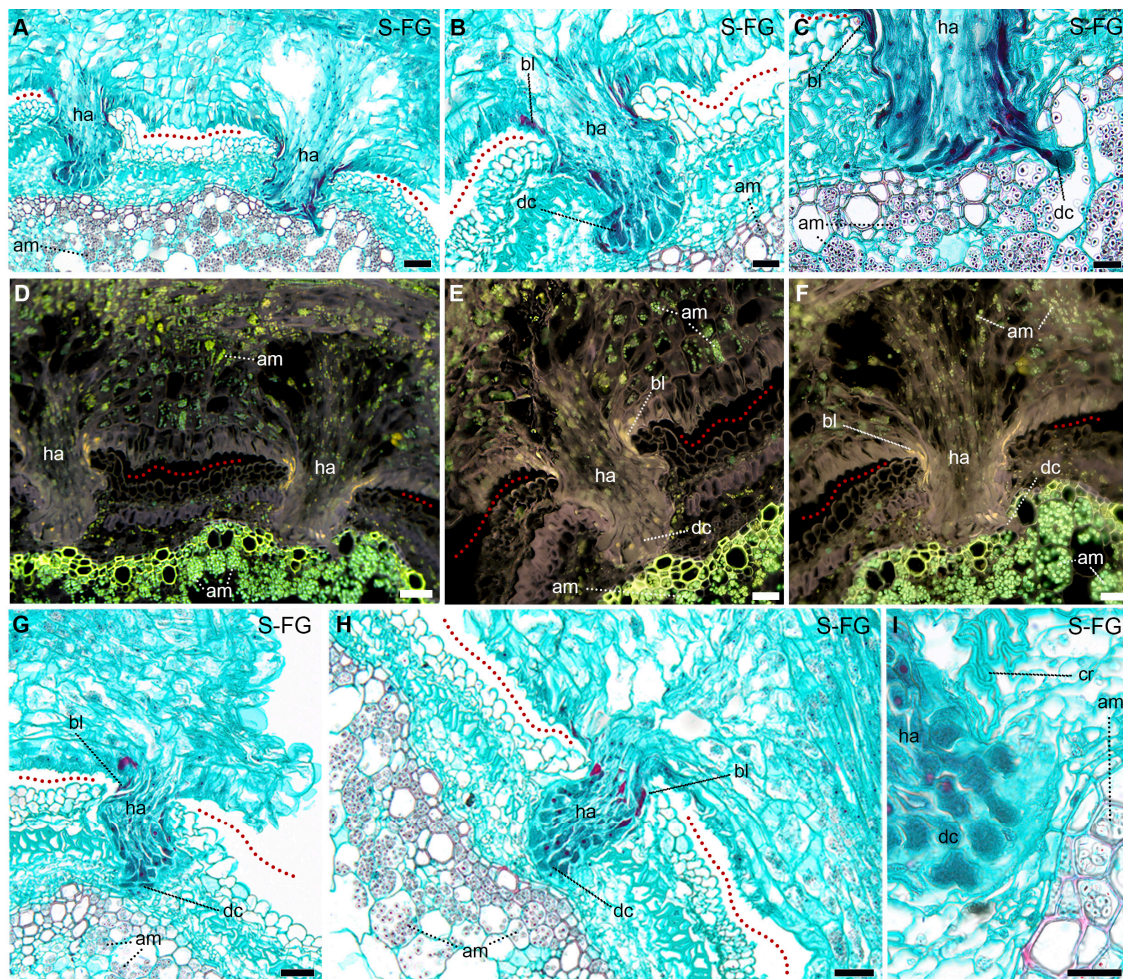


Figure 4. Histological analysis of the development of the *Cuscuta campestris* endophyte in mung bean stem. In each image, the upper part corresponds to the dodder stem and the lower part to the host stem. (A) Two haustoria (ha) are visible: the right haustorium is at an earlier intrusive stage, while the left has already established contact with the host vascular system (connective phase). (B) Close-up of the right haustorium. Note the morphology of the digitated cells (dc) and the formation of the boundary layer (bl). (C) Close-up of the left haustorium, showing digitated cells reaching the vascular bundles and the boundary layer. (D–F) Fluorescence images corresponding to images (A–C), respectively. Note the different shape of the amyloplast (am) between the two species and the characteristics of the boundary layer. (G) Haustorium at the end of the intrusive stage and at the early connective phase. (H) Another view of a haustorium in the initial connective phase. (I) Close-up of the haustorium in image H; note the presence of crushed host cells (cr). (A–C,G–I): safranin–fast green (S-FG) staining; (D–F): fluorescence (images merged) of samples shown in (A–C) as a result of UV (UV-2A), blue (B-2A) and green (G-2A) excitation. The analysis of samples was repeated independently at least twice, and the results were consistent. Abbreviations: am, amyloplasts; bl, boundary layer; cr, crushed cells; dc, digitated cells; ha, haustorium. The dotted red line delineates the interface between dodder and host stems. Scale bars: (A,D,G,H) 100 μ m; (B,C,E,F,I) 50 μ m.

Autofluorescence microscopy of these samples revealed the presence of amyloplasts within these cells (Figure 4D–F). Although amyloplasts were spherical in both species, those in mung bean were generally larger and more abundant. Similar differences in amyloplast morphology were also visible with other staining approaches (e.g., safranin-fast green, Figure 4C,H).

As the haustorium advances, the surrounding host epidermal cells appear to undergo strong mechanical compression, resulting in conspicuous protoplast shrinkage and significant changes to the composition of the cell wall. These changes are evident in the

distinct appearance of the cells, as well as their staining and fluorescence properties. The compressed epidermal cells eventually die, contributing to the formation of the boundary layer (Figure 4B,H).

As penetration proceeds, the host cortical cells become increasingly compressed, leaving, in some cases, only remnants of their cell walls (Figures 4I and S2E). Eventually, the haustorium penetrates through the cortex and establishes a connection with the host vascular cylinder (Figure 4C,F; enlarged view of the left haustorium in Figure 4A,D).

During the connective phase, the cell walls of the endophytic haustorium continued to undergo the modifications initiated during the intrusive phase. A clear enrichment in pectin was observed, particularly in the epidermal cells in contact with the host (the boundary layer) and in the apical regions of the digitated cells (Figure 5A,B).

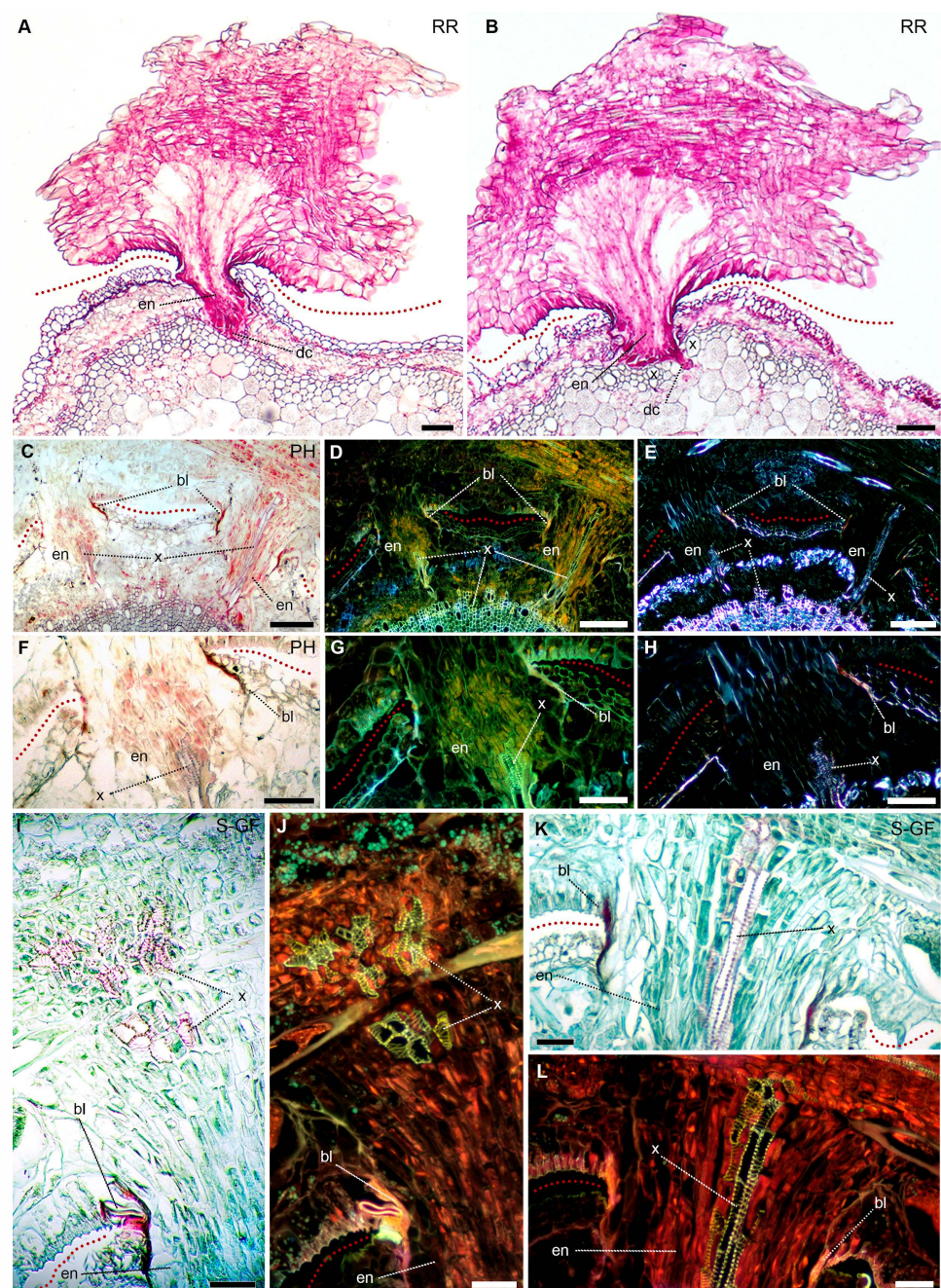


Figure 5. Cell wall modifications during endophyte development of *Cuscuta campestris* in mung bean (intrusive and connective phases), as revealed by histological analyses of cross section. In each image,

the upper part corresponds to the dodder stem and the lower part to the host stem. (A) Ruthenium red staining of the dodder endophyte (en) penetrating the mung bean stem and reaching the vascular bundles. Note the intense red signal associated with pectin in the digitated cells (dc) of the endophyte, in the epidermis initially in contact with the mung bean stem, and in the aligned file cells of the (pre)haustoria. (B) A more advanced stage of endophyte penetration. Note a digitated cell that has clearly advanced to a xylem vessel (x). (C) Phloroglucinol staining of two dodder haustoria penetrating the mung bean stem. The left haustorium has initiated the differentiation of xylem elements, with xylem vessels visible in pink. The right haustorium is at a more advanced differentiation stage, showing the formation of a xylem vessel. A distinctive boundary layer, stained pink with phloroglucinol, is visible at the contact zones between both haustoria and the mung bean stem. (D) Fluorescence image of panel (C). Xylem elements are identifiable by their intense green fluorescence. Note also the fluorescence emitted by the boundary layer (bl). (E) The same view as in (C), observed under polarized light, showing birefringence associated with the differentiation of xylem elements, and also with the boundary layer. (F–H) Close-ups of the right haustorium from panel (C), using the same techniques as in (C–E), respectively. Note the presence of epidermal remnants in the boundary layer. (I) Early differentiation of xylem elements from the aligned cells of the (pre)haustorium, stained with phloroglucinol. (J) Same sample as in (I) under autofluorescence; note the slightly positive signal in the boundary layer. (K) Haustorium in the connective phase stained with phloroglucinol, showing a pink xylem vessel and the characteristic coloration of the boundary layer. (L) Autofluorescence image of (K). The xylem vessel displays strong fluorescence, while the boundary layer shows a weaker signal. (A,B): ruthenium red; (C,F): phloroglucinol; (I,K): safranin-fast green; (D,G): fluorescence of samples shown in (C–F) as a result of UV (UV-2A) excitation. (J,L): fluorescence (images merged) of samples shown in (I–K) as a result of UV (UV-2A), blue (B-2A) and green (G-2A) excitation; (E,H): birefringence under polarised light of the samples (C,F), respectively. The analysis of samples was repeated independently at least twice, and the results were consistent. Abbreviations: bl, boundary layer; dc, digitated cells; en, endophyte; x, xylem. The dotted red line delineates the interface between dodder and host stems. Scale bars: (A–E), 100 μm ; (F–H), 50 μm ; (I–L), 25 μm .

Toward the initial stage of the connective phase, some of the digitated cells—particularly those located in the most apical region—begin to differentiate into developing xylem elements (Figure 5C–E, haustorium of the left, and Figure 5I–J), and later into mature vessel elements (Figure 5C–E, haustorium of the right, and Figure 5K–L). Lignin deposition in these cells was readily detected by both safranin–fast green staining and autofluorescence. Birefringence denoted a high enrichment in crystalline cellulose that is characteristic of the vessel elements (Figure 5E,H).

Simultaneously, the dodder epidermal cells that were compressed in the pre-haustorial stage become flattened within the boundary layer and start accumulating phenolic compounds, as evidenced by autofluorescence, safranin–fast green staining, and polarized light microscopy (Figure 5C–H). In advanced stages, host epidermal cell integrity was lost, leaving only their thickened cell walls, which showed strong phenolic deposition within the boundary layer (Figure 5C–H).

Immunohistochemical analyses showed that the cell walls of digitated haustorial cells were enriched in low methylesterified pectin, as indicated by strong LM19 labeling (Figure 6A,B). In contrast, labeling with LM20—recognizing highly methylesterified pectin—was weak or absent in these cells (Figure 6C,D).

With respect to AGPs, JIM8 labeling (Figure 6E) was predominantly observed in the aligned file cells and in the epidermal cells at the host–parasite interface, but occurred more sporadically in the digitated cells. Labeling intensity varied considerably among individual cells, ranging from strong to nearly undetectable. In contrast, LM2 labeling showed a comparatively uniform pattern, although the signal intensity was somewhat higher in the inner axial file cells relative to the outer cells (Figure 6F). In all of these immunohistological images, a brown-orange boundary layer was consistently observed.

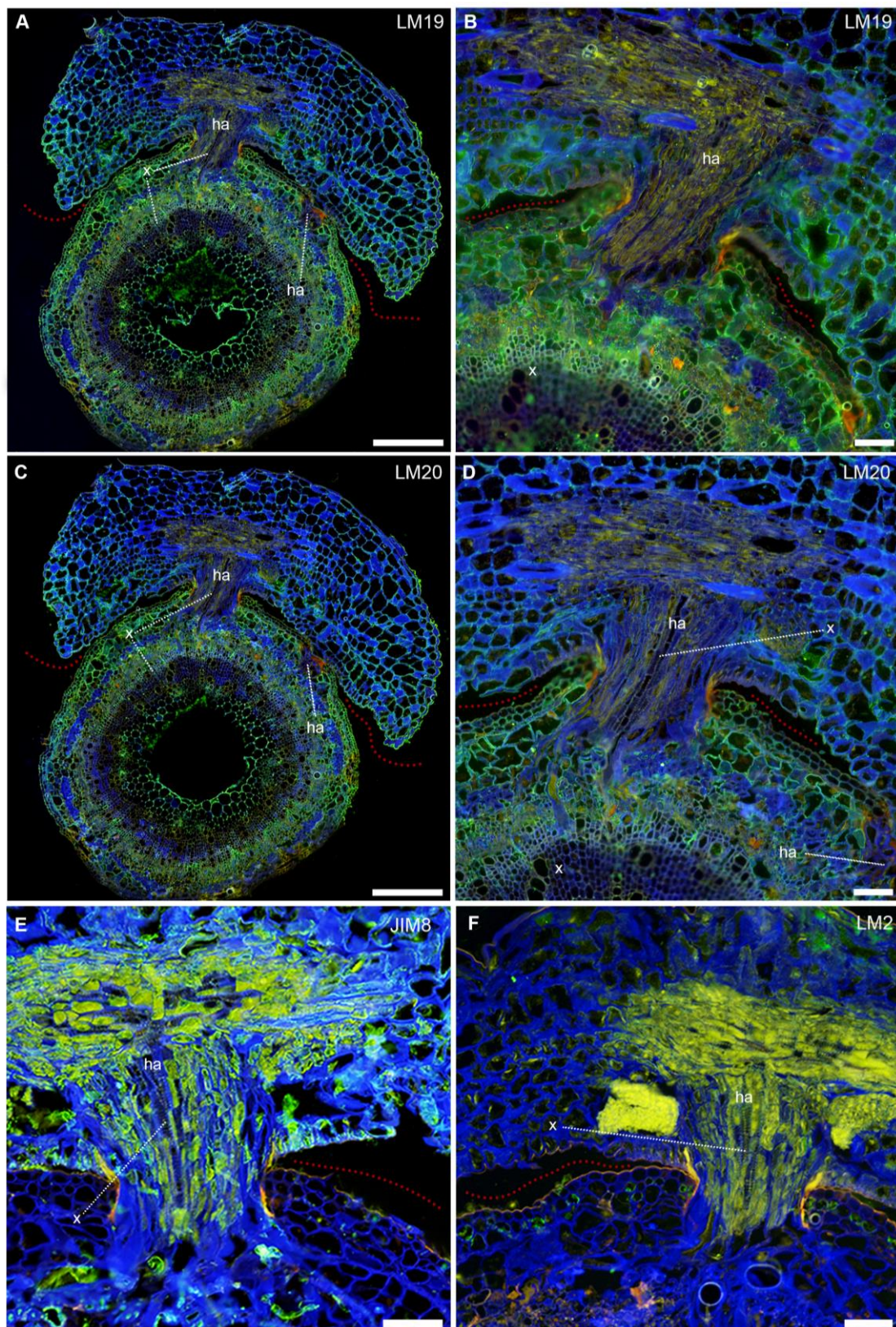


Figure 6. Immunohistochemical analysis of cell wall AGPs and HG during advanced stages of *Cuscuta campestris* endophyte invasion in mung bean in cross section. In each image, the upper part corresponds to the dodder stem and the lower part to the host stem. (A) LM19 immunolabeling of dodder and mung bean stems at the connective stage. Two haustoria (ha) are visible: the left haustorium is well developed, showing a newly formed xylem vessel (x), whereas the right one is incipient. See detailed view of LM19 labeling in Figure S2F. (B) Close-up of the right haustorium from panel (A). Weakly methylesterified HG is mainly localized in the inner, axially aligned file cells

of the haustorium; in contrast, it is absent from the outer tissues. (C) LM20 immunolabeling of a comparable stem region shown in (A), revealing the abundance of the epitope in protoplasmic vesicles (see detailed view in Figure S2G). (D) Close-up of the right haustorium from panel (C). Methyl-esterified HG signal is detected in dodder, in contrast with the stronger labeling observed in the mung bean stem. (E,F) Immunolocalization of AGPs using JIM8. (E) JIM8 labeling was observed at an advanced endophyte developmental stage, with a stronger signal in the endophyte, in the epidermal cells of dodder, and in the cortex and vascular tissue of the host. Several newly formed xylem vessels are observed. (F) AGP immunolocalization with LM2, showing signal in the inner axial aligned pre-haustorial file cells. A well-developed brownish-pink boundary layer is evident in all samples. (A,B): LM19; (C,D): LM20; (E): JIM8; (F): LM2. All sections were counterstained with Calcofluor. The analysis of samples was repeated independently at least twice, and the results were consistent. Abbreviations: ha, haustorium; x, xylem. The dotted red line delineates the interface between dodder and host stems. Scale bars: (A,C) 500 µm; (B,D–F) 100 µm.

3. Discussion

The parasitism of several *Cuscuta* species (e.g., *C. campestris*, *C. reflexa*, *C. japonica*, *C. pentagona*) on diverse hosts follows a conserved developmental sequence of events that can be broadly divided into three phases: an adhesive, pre-haustorial phase, followed by an intrusive phase, which leads to a connective phase, during which both functional xylem and phloem connections are established [2,22].

In our study, the interaction of *C. campestris* stems with their host (regardless of whether these were compatible, such as mung bean, or partially resistant, such as ‘Minibel’ tomato) followed this dynamic process, in which cell walls were demonstrated to play a crucial role.

During the pre-adhesive and adhesive phases, which involve coiling of the dodder stem around the host and the subsequent adhesion of epidermal cells to the host surface, independently of the host, both AGPs and HG (predominantly in their demethylesterified form) accumulated in the outer walls of the dodder epidermal cells destined to contact host epidermal cells. Although HG is secreted in a highly methylesterified form, it becomes largely demethylesterified within the adhesion layer by pectin methylesterases (PME) [29]. This demethylesterification enables the formation of calcium-mediated crosslinks and pectic gels.

Together with AGPs, this HG gelation appears to be essential for establishing intimate physical contact between dodder and their hosts, facilitating stable attachment [28,38]. Similar enrichment of HG and AGPs during adhesion has been reported in facultative root parasites such as *Phtheirospermum japonicum* and *Rhinanthus minor* [35,39], as well as in the adhesive secretions of climbing plants such as *Hedera helix* and *Parthenocissus quinquefolia* [38].

Previous immunolabeling studies have shown that AGPs accumulate in the epidermal cell walls of dodder stems following host contact [27,40]. Significantly higher levels of AGPs were observed in *Cuscuta reflexa*, particularly in its haustoria, than in its host (*Pelargonium zonale*) [21]. Similarly, AGPs labeled with the JIM8 antibody were found in *Cuscuta pentagona* cells, but were virtually absent in the host plant, *Impatiens sultanii* [24]. Consistently, in our study, AGPs detected with JIM8 and LM2 antibodies localized within the adhesion zone of dodder epidermal cells and remained concentrated in the boundary layer. Our observations further indicate that these antibodies recognize distinct AGP epitopes, since JIM8 was predominantly associated with the cell walls, whereas LM2 labeling was mainly protoplasmic, consistent with earlier findings [40].

AGPs are known to be more abundant in elongated haustoria than in pre-haustorial structures, supporting the notion that their accumulation is developmentally regulated [40],

a conclusion that is reinforced by the fact that some AGP genes are specifically upregulated during the adhesion phase [40].

Pectin distribution also displays clear spatial organization during haustorium development. Demethylesterified HG was abundant in the outer haustorial cells, whereas highly methylesterified HG accumulated at the apex, within the aligned file cells, and in the inner vascular tissues, including the xylem bridge that ultimately secures the parasite to the host. These spatial patterns are consistent with previous observations of *Cuscuta reflexa* parasitizing *Pelargonium zonale* or susceptible tomato lines. In those interactions, HG with a low degree of methylesterification was present in both the dodder and the hosts, and infection was accompanied by HG demethylesterification, or was facilitated by a pre-existing scarcity of highly methylesterified HG [21]. In that study, rhamnogalacturonan I galactan and arabinan side chains showed stronger signals in the dodder than in the host and increased upon infection, paralleling the modifications observed for low methylesterified HG [21]. Altogether, these spatial patterns underscore the dynamic and tissue-specific modulation of HG distribution, as well as the broader pectin remodeling processes that accompany haustorium formation and differentiation [31,41].

Changes in cellulose content were also apparent throughout haustorium development. Digitated cells of dodder showed reduced Calcofluor labeling, suggesting a lower cellulose content, consistent with thin, flexible cell walls adapted for intrusive growth [15,18]. In contrast, developing xylem vessels exhibited increased birefringence, indicative of a higher proportion of crystalline cellulose, associated with secondary wall formation and vascular differentiation [42].

In tomato stems, host resistance responses became evident during the intrusive phase of haustorial development. These responses included localized lignification and the degeneration of cortical cells surrounding the early invading haustorium, consistent with previous descriptions of tomato defense against dodder [1,7]. Notably, however, the composition of the endophytic dodder cell walls remained comparable to that observed in haustoria formed on mung bean, for which a defense response was not observed. This suggests that the architecture of the parasite's cell walls is largely maintained regardless of the host.

In some cases, elongation of the aligned file cells of dodder results in the crushing of some adjacent cortical cells of the parasite itself. Such crushed cortical cells were frequently observed surrounding differentiating pre-haustoria, suggesting that pre-haustorial enlargement during differentiation exerts mechanical pressure leading to collapse of neighboring parasite cells. Epidermal cells in the boundary layer also undergo marked morphological modifications during this process.

Once the haustoria of *Cuscuta campestris* begin to penetrate the host epidermis and cortex, crushed host cells can be observed directly ahead of the advancing intrusive cells. These cells exhibit densely stained remnants and empty spaces, which are indicative of the cellular degeneration of host cells along the penetration path. Similar observations have been reported for *Cuscuta reflexa*, where intrusive cells expand into host intercellular spaces and into cavities generated by the degradation of host cells, ultimately producing the boundary layer, consisting primarily of degraded host cell remnants [23].

During the early stages of parasitism (pre-adhesive and adhesive phases), a distinct boundary layer is not yet detectable on the outer region of the pre-haustorial cone. Nevertheless, the epidermal cell walls of dodder adjacent to the host already exhibit alterations, particularly on the face oriented toward the host epidermis, corresponding to the adhesion zone. Regarding the composition of this emerging layer, intense deposition of pectin—mainly low-methylesterified HG—was observed, along with the concomitant accumulation of AGPs, consistent with their proposed role in adhesion to the host surface [27,41].

During the intrusive phase, as the endophyte penetrates deeper, the dodder epidermal cells in contact with the host become flattened, lose volume, and undergo pronounced cell wall remodeling. In addition to the accumulation of pectin (as shown by ruthenium red staining), these cells also retain considerable amounts of AGPs. Frequently, they accumulate lignin-like polymers, evidenced by birefringence, autofluorescence, and staining with safranin–fast green and, more specifically, phloroglucinol.

Although lignin deposits have been traditionally attributed to a host defense response, either to encapsulate the haustorium or impede penetration [3,7], or as a consequence of the cell degradation around the invading endophyte [23], recent findings have shown that at least part of these deposits originate from the parasite itself [35]. Moreover, recent investigations in various species, including *Cuscuta campestris*, have proposed that lignification and the upregulation of lignin-related genes contribute to haustorium development, thereby supporting and preserving the structure of the (pre)haustorium, maintaining mechanical continuity during invasion, and insulating intrusive tissues from unpredictable host responses, thus facilitating host invasion [37,42].

Our findings support the conclusion that the boundary layer is derived predominantly from parasitic cell wall material. First, this layer begins to be detectable in pre-haustorial structures before physical host contact. Second, during the intrusive phase, crushing of dodder epidermal cells is sometimes observed at the interface with the host, contributing directly to boundary layer formation. Third, lignification of the boundary layer occurs independently of the type of host (i.e., the response is not bound to the lignification response of resistance in tomato stems), indicating that its development is not merely a by-product of the host defensive lignification.

Interestingly, our immunohistochemical analyses using an anti-callose antibody revealed callose deposition within the boundary layer, adding another component to the lignified matrix. The co-occurrence of callose and lignin is notable, as these polymers often act synergistically to reinforce mechanical barriers [43]. In some samples, additional staining patterns typical of suberin-like deposition were also detected (Figure S3A). Such combined deposition of callose, lignin, and suberin has been frequently associated with the formation of protective or sealing barriers in plant tissues [44,45].

Thickening of cell walls at the host–parasite interfaces has also been observed in other parasitic systems. In dodder–host interactions as well as in root parasites such as *Rhinanthus minor* and *Odontites vernus*, lignin- and phenolic-rich interfacial deposits accumulate at the contact region, and have been termed lignin-rich interfacial deposits (LIDs) [35]. These LIDs contain AGPs but lack pectin, further illustrating lineage-specific strategies of cell-wall modification [35].

Taken together, our findings reveal that the earliest stages of *Cuscuta campestris* parasitism, whether on a susceptible host such as mung bean or a resistant one such as ‘Minibel’ tomato, are underpinned by the orchestrated remodeling of the parasite’s cell walls. This process involves the targeted accumulation of AGPs and the dynamic regulation of pectin methylesterification. This coordinated restructuring of cell walls generates an adhesive and mechanically resilient interface that enables stable attachment and prepares the epidermal and aligned file cells for the subsequent intrusive haustorial growth. In addition, our characterization of the *Cuscuta campestris* boundary layer reveals that it is primarily formed and controlled by the parasite itself, involving the deposition of AGPs, pectin, lignin-like polymers and callose, thereby contributing to haustorial reinforcement and anchoring and, therefore, to the stabilization of the haustorium inside the host stem. Together, these findings provide new insight into the dynamic remodeling of cell wall components during parasitic attachment and penetration, highlighting the boundary layer as a developmental structure behind the success of dodder parasitism.

4. Materials and Methods

4.1. Plant Material

Commercial seeds of cherry tomato (*Solanum lycopersicum* 'Minibel') (Mascarell Semillas S.L., Benissoda, Valencia, Spain) and mung bean (*Vigna radiata*) ("Legumbres La Asturiana", Vidanes, León, Spain) were germinated and grown in 50-well plastic trays (each well with a volume of 100 mL), filled with pre-hydrated, fertilized universal substrate (Compo Sana[®], Barcelona, Spain). Germination took place in a growth chamber at 23 ± 1 °C under light ($\approx 41 \mu\text{mol m}^{-2} \text{s}^{-1}$) with 16 h light/8 h dark photoperiodic conditions and 50–60% of relative humidity [46]. Twice a week, the trays were watered to reach $\approx 90\%$ of field capacity and fertilized with a universal liquid fertilizer (COMPO NPK 7-5-6).

Approximately 3–4 weeks after sowing the hosts, *Cuscuta campestris* seeds (originally collected from alfalfa (*Medicago sativa*) infestations in León province, Spain, and stored under cold conditions) were placed on a P60 Petri dish lined with two moistened filter paper discs. Germination was carried out in a growth chamber under darkness at 23 ± 1 °C. Once germinated, the dodder seedlings were used to infest a batch of tomato plants ($N = 25$) and a batch of mung bean plants ($N = 14$) that were five weeks old, when they had reached a height of approximately 20 cm (primary parasitism). The dodder seedlings were placed directly on the substrate in contact with the stem of host plants.

Once mung bean plants had been successfully parasitized by dodder, secondary infestation of tomato plants was initiated in some cases. Tomato plants were placed in contact with the parasitized mung bean plants, allowing the lateral branches of the dodder to reach and parasitize the tomato stems. For this setup, one row of mung bean plants ($N = 12$) was positioned between two rows of tomato plants ($N = 24$). The sowing and infestation procedure was repeated three times.

4.2. Histological and Immunohistochemical Techniques

Stem segments of approximately 15 mm in length were collected using a scalpel from plants parasitized by dodder, representing different stages of infestation and resistance, and fixed in formalin–acetic acid–alcohol (FAA) (24–48 h) [47]. After fixation, samples were dehydrated in ascending grades of ethanol (30 min in 70%, 60 min in 96%, 75 min in 100%) and placed in isoamyl acetate (75 min in 100%). Afterward, isoamyl acetate was gradually replaced with Paraplast[®] paraffin, and the segments were embedded in this paraffin for 6 h at 60 °C, and then paraffin blocks were cast using Leuckart molds, which were cooled on ice. Paraffin blocks were sectioned into 12 μm slices by using a rotatory microtome (Leitz-Wetzlar 1512, Leitz, Milton Keynes, Bucks, UK) [45]. The 12 μm tissue sections were deparaffinized with xylene and rehydrated for use with bright-field microscopy techniques, observation under polarized light, and epifluorescence microscopy. For bright-field microscopy, the sections were stained using four methods: Safranin-fast green, ruthenium red, phloroglucinol and Sudan III. Safranin–fast green staining was used as a general histological stain to visualize tissue organization and cell wall structure. Ruthenium red staining was used to detect acidic pectic polysaccharides. Phloroglucinol–HCl staining was used for the histochemical detection of lignin, whereas Sudan III staining was applied to reveal suberin and suberin-like compounds. For safranin-fast green staining, the sections were stained with safranin solution, rinsed in distilled water, differentiated in ethanol and counterstained with fast green solution. For ruthenium red staining, the sections were incubated with 0.02% (w/v) ruthenium red in distilled water. For phloroglucinol staining, the sections were covered with a 1% (w/v) phloroglucinol solution and subsequently treated with 18% hydrochloric acid to develop the reaction. For Sudan III staining, the sections were incubated in a 0.3% Sudan III solution and then rinsed in 70% ethanol to remove the excess dye.

After staining, the tissue sections were dehydrated in ascending grades of ethanol and dewaxed in xylene, except for the phloroglucinol- and Sudan III-stained samples, which were mounted with Entellan synthetic resin (Merck, Madrid, Spain) [45,48]. For epifluorescence microscopy, the sections were mounted as previously described, without staining, to observe the autofluorescence of the tissues. For immunohistochemistry specifically, after rehydration, the sections were placed on slides coated with Vectabond™ reagent (Vector Laboratories®, Burlingame, CA, USA) and then incubated with M-PBS (Milk-Phosphate-Buffered Saline) containing the primary antibody (Table 1, Plant Probes, Leeds, UK) at a dilution of 1:10 for 2 h [46,49]. After washing with PBS, the sections were incubated for 2 h with a 1:100 dilution of anti-rat immunoglobulin G linked to fluorescein isothiocyanate (Merck) in M-PBS. Negative controls were performed by omitting either the primary or the secondary antibody (Figure S4). The antibody incubations were performed in darkness and at room temperature. Finally, contrast staining was performed using 0.005% Calcofluor White (Fluorescent Brightener 28, Merck).

A Nikon E600 microscope (Nikon, Tokyo, Japan) with bright-field and epifluorescence modes (equipped with UV-2 and B-H2 filters) was used for the microscopic study. The UV-2 (Ex. 330–380 nm; DM 400 nm; BA 420 nm) filter was used for autofluorescence and Calcofluor White imaging, while the B-H2 (Ex. 450–490 nm; DM 505 nm; BA 520 nm) filter was used for fluorescein isothiocyanate detection in immunohistochemistry. Stained sections were often observed under the UV-2, B-H2 and/or G-2A (Ex. 510–560 nm; DM 575 nm; BA 590 nm) to increase dye contrast. Some sections were examined under polarized light to check for the presence of birefringent structures. The image acquisition software used was NIS-Elements F v3.2 [46,49,50].

For each histological technique, antibody and developmental phase, a minimum of 30 sections derived from at least two independent biological samples were analyzed.

Table 1. Monoclonal antibodies used as primary antibodies for immunohistochemistry.

Antibody	Epitope	References
	Pectin: homogalacturonan (HG)	
LM19	Range of HG samples, preference to unesterified HG	[51]
LM20	Does not bind to unesterified HG (requires methylesters for recognition)	[51]
JIM7	Partially methylesterified HG but not unesterified HG	[52]
	Arabinogalactan proteins (AGPs)	
LM2	AGP (carbohydrate epitope containing β -linked glucuronic acid)	[53]
JIM8	AGP (carbohydrate epitope containing β -linked glucuronic acid)	[54]
	Callose	
Anti-callose	Recognises linear (1→3)- β -linked oligosaccharide segments in (1→3)- β -glucans	[55]

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/ijms27041914/s1>.

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