

Orobanche cumana-Sunflower Interaction: Histological Dissection of Sunflower Parasitic Resistance Mechanisms

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Method Article

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Abstract

Background

Orobanche cumana (*O. cumana*), a root parasitic plant, causes significant losses to crop like sunflowers. While breeding resistant varieties is a key solution, a standardized method for analyzing resistance mechanisms has been lacking due to the complex infection process. In this study, we established a method to analyze *O. cumana* resistance phenotypes using *O. cumana* and two sunflower (*Helianthus annuus*) lines with differing resistance levels, a susceptible cultivar LD5009 and a resistant cultivar JK103.

Results

Specifically, we combined statistical analysis and tissue sectioning to track the parasite infection process, through which seven distinct infection stages were clearly defined. Using this established method, we analyzed the *O. cumana* resistant line JK103. Results revealed that chemical signaling communication before physical infection remained unaffected. However, the parasite infection process was prolonged, with high mortality observed in the tubers. Furthermore, additional investigations showed that in JK103, parasite invasion induced lignin accumulation adjacent to parasite invading cells; the vascular bridge failed to develop properly. These observations most likely explain the prolonged infection duration and programmed cell death in the terminal haustorium of *O. cumana* infecting JK103.

Conclusions

This research provides a standardized screening strategy for breeding sunflower germplasm resistant to *O. cumana*, facilitates the exploration of parasite-host molecular interactions, and serves as a reference for similar systems (e.g. *Striga* parasitism on cereals).

Introduction

The parasitic weed *O. cumana* has a broad host range and is a devastating root parasitic plant that poses a serious threat to various important economic crops, such as sunflower, tomato, tobacco, chickpea, faba bean, and carrot [1, 2, 3]. As a destructive parasitic weed, it severely competes with hosts for water and nutrients, causing significant yield loss [4]. The parasitic plant *Striga* produces strigol, a highly efficient germination stimulant, which induces its seeds to germinate near the roots of host plants [5]. The parasitic plant *O. cumana* systemically hijacks host physiology by producing phytohormones like cytokinins and auxins, ultimately leading to severe growth weakening, yield loss, or plant death [6, 7]. Long dormancy period and high reproductive capacity of *O. cumana* seeds make the eradication of this weed extremely difficult once it established [8]. Additionally, as a root parasitic plant, *O. cumana* infects its hosts underground by attaching to their roots, rendering all conventional weed control methods

ineffective. Taking all these factors into account, breeding crops resistant to *O. cumana* is the most sustainable and efficient approach to reducing losses. To achieve this goal, we must understand the molecular mechanism underlying the interaction between crops and this parasitic plant.

Sunflower, the crop most severely affected by *O. cumana*, suffers annual losses exceeding €2 billion and field yield reductions of 10–60%, with total loss in heavily infested areas, while the emergence of more virulent races continues to threaten global production [9, 10]. This damage adversely affects both yield and seed quality, resulting in reduced oil content and unfavorable alterations in fatty acid composition. Therefore, *O. cumana* is considered the most destructive parasitic weed in sunflower production. This makes the breeding of *O. cumana* resistant cultivars a core strategy for the sustainable development of the global sunflower industry. Currently the selection of *O. cumana* resistant phenotypes mainly relies on field selection, a method that is time-consuming and labor intensive. Therefore, a standardized laboratory screening protocol is urgently needed.

Based on morphological observations, the basic process of *O. cumana* infesting sunflower can be divided into germination, infection, haustorium initiation, tubercle formation, shoot emergence, and flowering. Notably, failed infections, where the parasite is unable to complete subsequent developmental steps and the infection process is terminated prematurely, may occur at any stage (Fig. 1). However, resistance mechanisms vary significantly among different parasitic plant-host combinations. This heterogeneity makes it difficult to pinpoint which specific stage or what exact biological process underpins host resistance. Therefore, it is essential to shift from broad morphological observations to systematic, tissue-level investigations of the infection process.

A successful infection of a host by a parasitic plant involves many steps: parasitic seed germination, host recognition, radicle attachment, invasion, vascular bundle connection and nutrient uptake [11]. Firstly, host-secreted chemicals play multiple roles throughout the infection process. For example, strigolactones, hydroquinones, and volatiles (e.g., β -myrcene) act as stimulants for parasitic seed germination, pre-haustoria formation and host recognition [12, 13, 14]. Once the parasitic plant cells reach the host root, they secrete carbohydrate rich molecules that help form a physical and chemical bond between the parasite and host during attachment. Meanwhile, cell wall-degrading enzymes such as cellulases and pectinases are secreted to soften the host cell wall during penetration [15, 16]. After the parasitic cells enter the host plant tissue, a vascular connection is established to facilitate nutrient uptake by the parasite from the host plant. This relies on the synergistic regulation of local cell proliferation and differentiation by cytokinin (CK) and auxin [17]. Then a fully functional haustorium is established, which acts as a metabolic hub redistributing host-derived nutrients (e.g., sugars, amino acids) to the developing parasite shoots. During these processes, host plants can defeat parasitic invaders via diverse pathways, such as: cell wall thickening, lignin deposition, and callose accumulation [11]; reactive oxygen species burst, the phytoalexin synthesis, and activation of pathogenesis related proteins [18]; hypersensitive response [19]; and signal interference [6].

This study used the susceptible sunflower line LD5009 and the resistant line JK103 as experimental materials. Previous studies have confirmed that JK103, when infected by *O. cumana*, rapidly activates defense responses including callose deposition, a reactive oxygen species (ROS) burst, and increased activity of related scavenging enzymes. It also significantly upregulates key genes in the salicylic acid (SA) and jasmonic acid/ethylene (JA/ET) signaling pathways [20]. Based on these, here we use these plant materials to analyze the stage-specific cytological responses of the parasitic infection. *O. cumana* was used as the parasitic agent, together with the susceptible sunflower cultivar LD5009 and the resistant line JK103, to establish and evaluate a screening protocol for *O. cumana* resistance in sunflower germplasm.

By combining statistical analyses of parasitic seed germination, tubercle formation, and stem emergence with semi-thin sectioning and confocal microscopy. This approach allowed us to track the infection process from the initial parasitism (radicle attachment) to the establishment of a vascular connection (xylem bridge formation) and established a standardized evaluation protocol for *O. cumana* resistance in sunflowers. This protocol was then successfully applied to analyze the resistant line JK103, thereby establishing a standardized methodology for screening parasitic plant-resistant germplasm in sunflower breeding. This work also provides a theoretical foundation for elucidating the molecular mechanisms underlying host-parasite interactions.

Materials and methods

Sunflower Seed Germination and Growth Conditions

Plant Material: Two sunflower cultivars were used: the susceptible cultivar LD5009 and the resistant cultivar JK103. LD5009 was purchased from Beijing Kaifengrui Seed Co., Ltd. JK103 was kindly provided by the Sunflower Breeding Team, Baicheng Academy of Agricultural Sciences, Jilin Province. **Seed Treatment and Seedling Culture:** Sunflower seeds were surface-sterilized with 70% (v/v) ethanol for 2 min, germinated on moist filter paper in Petri dishes, and cultured at 25°C under a 16/8-h light/dark photoperiod for 1 week.

Germination and Growth Conditions for Sunflower Broomrape

O. cumana seeds were collected from Dadoupu Village, Wuchuan County, Hohhot City, China N 41.17, E 111.61. This population was identified as having moderate pathogenicity, and its physiological race was characterized as type G [21]. One-week-old sunflower seedlings were placed in a Petri dish filter paper system (Fig S1A, B). Then, 0.04 g of seeds were evenly spread over the roots in approximately two-thirds of the dish for germination. Co-culture was maintained at 25°C under a 16/8-hour light/dark cycle for one week. Broomrape germ tubes emerged by the seventh day of co-culture, and the first day of tubercle attachment to the sunflower roots was designated as the start of parasitism.

Preparation of Root Extracts

Root systems from 1-week-old seedlings (approximately 1 g fresh weight per cultivar) were harvested, immediately frozen in liquid nitrogen, and ground to a fine powder in pre-chilled mortars. The powder was transferred to centrifuge tubes, mixed with 20 mL of ice-cold sterile deionized water, and extracted at 4°C for 1–2 hours. The extracts were centrifuged at 10,000×g for 10 minutes at 4°C. The supernatant (root aqueous extract) was aliquoted for immediate use or stored at -80°C. The extract was used to determine the germination rate of *O. cumana* seeds.

Statistical Methods

Data collection commenced on the first day following the sowing of 0.04g of *O. cumana* seeds over the sunflower root systems. The number of tubercles was determined by counting the tubercles on resistant or susceptible sunflowers. The stem emergence rate was calculated as the ratio of the number of emerged stems on resistant or susceptible sunflowers to the total number of tubercles on the respective sunflower type at the same time point. The mortality rate was calculated as the ratio of the number of dead tubercles on resistant or susceptible sunflowers to the total number of tubercles on the respective sunflower type at the same time point. All figures were generated using GraphPad Prism 9 software (GraphPad Software, USA).

Histological Analysis of Host-Parasite Interface Using Semi-Thin Sectioning

Sample Preparation: Root tissues of sunflower parasitized by *O. cumana* were collected at the host-parasite interface. Samples were thoroughly rinsed with sterile water and fixed in a 4% paraformaldehyde (PFA) solution. Fixation was performed under vacuum pump assistance (vacuum: 0.09 MPa) for 1 hour to ensure complete infiltration.

Sectioning and Staining: Post-fixation samples were dehydrated in a graded ethanol series, immersed in Solution A for 3 days, and embedded in polymerized resin prepared by mixing Solution A and Hardener II at a 15:1 (v/v) ratio. Embedded tissues were sectioned (5–10 µm thickness) using a plastic microtome (e.g., Leica RM2265) following established methodology [22]. Sections were dried on a slide warmer at 55°C to enhance adhesion, stained with 0.05% (w/v) Toluidine Blue O aqueous solution for 30 seconds, rinsed thoroughly with deionized water, and mounted with Canada Balsam. **Microscopy:** Stained sections were imaged under an upright light microscope (Olympus BX53, Japan).

Vibratome Sectioning of Host-Orobanche Interaction Tissues

Sample Preparation and Sectioning: Root tissue segments (1–5 cm length) containing the host-parasite interface from parasitized sunflowers were collected. Samples were placed in a 1.5 ml centrifuge tube containing 1 ml of PFA solution. Infiltration was performed under vacuum pump assistance (vacuum: 0.09 MPa) for 30 minutes at 4°C to ensure complete penetration. The tissue segments were then embedded in 0.6% (w/v) low-melting-point ag-rose. Embedded tissues were sectioned (80–100 µm thickness) using a vibrating microtome (Leica VT1200 S) to preserve structural integrity, as previously described [23].

Dual-color Fluorescence Staining with Confocal Microscopy

Direct Yellow Staining: Samples were stained with 0.1% (w/v) Direct Yellow 96 for 6 h. Stained samples were imaged using an inverted confocal laser scanning microscope (Leica DMI8 or Leica STELLARIS). Imaging parameters: Excitation 488 nm, laser power 9%, PMT gain 600, pinhole diameter 1 Airy Unit (AU), emission detection range 500–550 nm.

Basic Fuchsin Staining: Sections were treated and stained with 0.2% Basic Fuchsin for 3 hours. Imaging was performed using the same confocal system. Parameters: Excitation 561 nm, laser power 2%, PMT gain 700, pinhole diameter 1 AU, emission detection range 600–650 nm.

Results

JK103 Resistance to *O. cumana* Occurs During Parasite Invasion

The sunflower cultivar JK103 is resistant to *O. cumana*, whereas LD5009 is a susceptible cultivar [20]. Using these two resistant and susceptible cultivars, we evaluated resistance-associated morphological traits by monitoring the infection process of *O. cumana* on sunflower roots grown in the Petri dish-filter paper system (Fig S1A).

O. cumana seeds were spread onto sunflower roots cultured in the Petri dish-filter paper system (Fig S1B), and the germination rates were recorded respectively. No significant difference in the germination rates of *O. cumana* seeds was observed between JK103 and LD5009 (Fig. 2A). Small tubercle structures appeared 15 days after cocultivation. The tubercle formation rate was lower in JK103 compared to LD5009 (Fig. 2B). Initially, there was no significant difference in shoot emergence rate between JK103 and LD5009; but later, the rate for JK103 became significantly lower. (Fig. 2C), which was consistent with the significantly higher tubercle mortality rate observed on JK103 (Fig. 2D). These results suggest that the resistance of JK103 to *O. cumana* is mainly exerted during the parasitic invasion stage, rather than during the early stage of chemical signaling.

Figure 2 Statistic Evaluation of the *O. cumana* Resistance trait in Sunflower Varieties. (A) Germination rates of *O. cumana* on resistant sunflower JK103 and susceptible sunflower LD5009. (B) Tubercle formation numbers of *O. cumana* on JK103 and LD5009. (C) Stem induction rate in JK103 and LD5009.

(D) Tubercle mortality rates on JK103 and LD5009. Figure caption: Data are presented as the mean value ($n = 6$). Symbols (ns, no significant difference; *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$) indicate significant differences between the resistant cultivar JK103 and susceptible cultivar LD5009 under the same conditions.

Hypersensitive Necrosis and Disrupted Xylem Connections during *O. cumana* Infection of JK103

As previously observed, *O. cumana* infecting resistant sunflowers JK103 exhibited higher tubercle mortality than that infecting the susceptible cultivar LD5009. To further investigate this phenomenon, we examined the host-parasite interface of *O. cumana* at the tubercles and shoot emergence stages, in both resistant and susceptible lines at the tissue level using semi-thin plastic sectioning technology.

At the tubercle stage, a well-developed haustorium was observed in *O. cumana* infecting the susceptible host LD5009, accompanied by successfully established xylem bridges (Fig. 3A, A') and intact tubercles structures. During shoot elongation, a fully developed xylem connection was established between the host and parasite via a vascular bridge (Fig. 3B). In contrast, the *O. cumana* infecting the resistant hosts JK103 exhibited numerous shriveled dead cells in its tubercle cells (red arrows, Fig. 3C'). Additionally, haustorial xylem cells adjacent to the host-parasite interface showed irregular alignment and cell size, indicating an impaired xylem structure (red arrows, Fig. 3D'). These findings demonstrate that resistance mechanisms in JK103 persist throughout both the tubercle and shoot development stages, effectively preventing the establishment of parasitic connections.

Infection Process of *O. cumana* in Susceptible Sunflower

Based on statistical observations, we concluded that the resistant cultivar JK103 delays *O. cumana* infection and is associated with a high tubercle mortality rate. Cytological analysis indicates that the latter phenomenon may result from the disruption of host vascular connections. To elucidate the defense mechanism of JK103, we first employed semithin plastic sectioning to analyze the *O. cumana* infection process in the susceptible cultivar LD5009[22]. Samples were prepared using an *in vitro* co-culture system for parasitic-host interaction studies [6].

Results showed that when the radical tip of *O. cumana* contacted the host root epidermis, the parasite induced minimal lignin deposition specifically at the physical interaction site (1 dpi, Attachment phase, Fig. 4A–A"). Subsequently, *O. cumana* penetrated the host epidermis and cortical tissues, extending to the endodermis; here, the radicle tip cells polarized

Figure 4 Histological analysis of parasite-host interaction between *O. cumana* and sunflower cultivar LD5009. (A–G) Stereomicroscope micrographs showing *O. cumana* infecting the host (sunflower) at different time points. (A'–G') Sections of parasite-host interaction sites at different time points. (A"–G") Magnified views of red-boxed regions in A'–G'. Developmental timeline: (A–A') attachment phase. (B–B") vascular bundle contact phase. (C–C") vascular bundle penetration phase. (D–D") nutrient absorption initiation phase. (E–E") haustorium formation phase. (F–F") tubercle phase. (G–G") stem emergence

phase. Staining: 0.05% toluidine blue. Labels: H, Host plant (sunflower); P, Parasitic plant (*O. cumana*); X, Xylem; XB, Xylem; bridge. All scale bars: 50 μ m.

towards the xylem pole and differentiated into a wedge-shaped infection structure (2 dpi, Vascular bundle contact phase, Fig. 4B'–B"). Next, the radicle tip cells successfully penetrated the endodermis and further differentiated into an arrowhead-shaped structure, which then connected to the host vessels (3 dpi, Vascular bundle penetration phase, Fig. 4C'–C"). *O. cumana* cells proliferated rapidly and occupied most of the space within the host vascular bundle (4 dpi, Nutrient absorption initiation phase, Fig. 4D'–D"), leading to the expansion of parasitic tissue and its connection to the host phloem pole (7 dpi, Haustorium formation phase, Fig. 4E'–E"). parasitic cells adjacent to parasitic-host interface differentiate into xylem, indicating the successful establishment of xylem bridges. With the formation of xylem bridges, *O. cumana* developed a tubercle (Fig S2I'–K", 18 dpi; Tubercle phase, Fig. 4F'–F"). Subsequently, the tubercle volume increased and the shoot primordia emerged. Mature xylem bridges exhibited increased vessel diameter and wall thickness (25 dpi, Stem emergence phase, Fig. 4G'–G"). In summary, the successful parasitism of sunflowers by *O. cumana* was fully characterized at both the cellular and histological levels.

To further track the differentiation of xylem bridge and the potential defense responses of the host plant, we assessed lignin accumulation at different parasite infection stages using the Basic Fuchsin Staining method [23]. The xylem bridge is a specialized structure through which parasitic plants connect to their hosts for nutrients and water uptake [25, 26], which is a hallmark of successful parasitic relationship establishment. Notably, lignin **Fig. 5** Lignin dynamics during *O. cumana* infection of the susceptible sunflower cultivar LD5009. (A–G') Direct Yellow was used for cell wall staining (yellow). (A–G") Basic Fuchsin was used for lignin staining (red). Image panels: (A–G) 4 $\times$ confocal overlay micrographs at different developmental stages. (A'–G') 40 $\times$ magnified confocal overlays of white-boxed areas in (A–G). 40 $\times$ Basic Fuchsin channel of white-boxed areas (A"–G"). Developmental stages: (A–A") attachment phase. (B–B") vascular bundle contact phase. (C–C") vascular bundle penetration phase. (D–D") nutrient absorption initiation phase. (E–E") haustorium formation phase. (F–F") tubercle phase. (G–G") stem emergence phase; Abbreviations: X, Xylem; XB, Xylem bridge; LA, Lignin accumulation; H, Host plant; P, Parasitic plant. All scale bars: 50 μ m.

accumulation is well-documented to play critical roles in xylem maturation and host plant resistance against parasitic plant.

We observed that on day 1 of parasitism, the physical contact between *O. cumana* and sunflower roots resulted in minimal lignin deposition in the parasite. (Attachment phase, Fig. 5A–A"). At the vascular bundle contact and penetration phases, as parasitic cells penetrated the host vascular tissue, lignin accumulated in the epidermal cells of the parasite at the host-parasite interface. However, no lignin accumulation was detected in the host plants, and xylem bridge formation had not yet been initiated (Fig. 5BC–B"C"). Four days after attachment, lignin began to appear in the infective cells of *O. cumana*, indicating the formation of xylem bridge structures that connected the parasite to host vessels (Nutrient

absorption initiation phase, indicated by white arrows xylem bridge, Fig. 5D"). Furthermore, as shoot apical primordia emerged within the tubercle tissue, the xylem bridges further connected to the host protoxylem (Haustorium formation phase, indicated by white arrows shoot apical primordia, Fig. 5EF–E' 'F" and Fig S3E–K"), a key event marking the initiation of the parasite autonomous vascular system development (Tubercle phase, indicated by white arrows xylem bridge, Fig. 5F–F"). Mature xylem bridges become fully integrated with the xylem of the parasite stem (Stem emergence phase; indicated by white arrows xylem bridge, Fig. 5G–G"), forming a continuous vascular network that supported sustained resource acquisition. Following the formation of xylem bridges, *O. cumana* efficiently absorbed host nutrients and water, enabling its continuous growth and expansion.

Using tissue sectioning and staining, we characterized the successful infection process of *O. cumana* in sunflower plants at different time points and across cellular and histological levels. Additionally, we observed that no specific lignin accumulation occurred in host cells; in contrast, in parasite cells, lignin accumulation was not limited to xylem differentiation, it also appeared to function as a protective mechanism for the invading parasite cells.

Infection Process of *O. cumana* in Resistant Sunflower

To clarify the successful infection process and reveal the precise resistance mechanism of JK103 to *O. cumana*, we compared the infection process of *O. cumana* between the resistance cultivar JK103 and the susceptible cultivar LD5009 using tissue sectioning and staining. Cytological analysis revealed that compare with the susceptible cultivar LD5009, *O. cumana* infection process is significantly delayed in resistance cultivar JK103. Specifically, while the parasite had already reached the host xylem in LD5009, it was still blocked by the endodermis in JK103 (vascular bundle contact phase to vascular bundle penetration phase, Fig

6A–A'). Furthermore, investigation of the lignification pattern in the resistant cultivar JK103 following *O. cumana* infection revealed significant lignin deposition in the epidermal tissue of the host plant during the attachment phase (Fig. 6B–B'). Notably, while the parasite had progressed to the nutrient absorption initiation phase in the susceptible cultivar LD5009, it remained in the vascular bundle penetration phase in the resistant JK103. During this phase, invasive cells of *O. cumana* in JK103 exhibited markedly stronger lignification compared to those in LD5009. In addition, substantial lignin accumulation was also detected within the host vascular tissues. Even in most case the xylem connections between the parasite and host were eventually established in JK103, the xylem bridge structures were discontinuous, accompanied by prominent lignin accumulation (Fig. 5D", Fig S4E" and Fig. 6C–C'). Compared with in susceptible cultivar, lignin accumulated in the vasculature of the resistant cultivar during the haustorium formation phase (Fig. 6D–D'). The resistant cultivar exhibited structurally compromised vascular bridges, characterized by sparse and discontinuous xylem bridges (Tubercle phase, Fig. 6E red arrows indicate vascular bridges, Fig. 6E–F'). During the stem emergence phase, the vascular bridges in the resistant cultivar were disrupted and collapsed, with substantial lignin deposition, particularly at the host-parasite interface. (white boxes indicate the parasitic interface, Fig. 6G–G'), indicating impaired connectivity

between the parasite and host. Cross-sectional views of the vascular bridges exhibited markedly greater lignin accumulation and subsequent structural failure in the resistant versus the susceptible cultivar (Fig. 6I–I').

Overall, our data suggests that the resistant sunflower cultivar JK103 can restrict the *O. cumana* infection across multiple phases. The physical contact of *O. cumana* invasive cells likely induces lignin accumulation in the host epidermis and vascular tissue, which may result in the prolonged infection period. Meanwhile, lignin deposition in *O. cumana* invasive cells reflects the defensive reactions of the parasite caused by the resistance host. After the parasite reaches the host vascular tissues, the resistant host can further inhibit the differentiation of the xylem bridge in the parasite haustorium.

Discussions

This study addresses the lack of a standardized cytological evaluation system for resistance to *O. cumana* in sunflower breeding. We successfully established an analytical methodology combining in vitro co-culture, time-course statistics, and tissue sectioning techniques. Using this method, we clearly defined, at the cellular and histological levels, seven consecutive, distinct developmental stages of the successful infection process of sunflower roots by *O. cumana* (Fig. 4, 5, 7). A schematic diagram of these seven parasitic phases is provided (Fig. 7), while their corresponding functional characteristics are summarized in Table 1.

Our data provide a detailed analysis of the entire infection process of sunflower by *O. cumana*, from the attachment of the parasite radicle to maturation of a functional xylem bridge, thereby established a phenotyping method for the precise identification of parasitic plant resistance mechanisms. And as the convergent evolutionary characteristics of infection processes in parasitic plants [27], this methodology also potentially applicable to other systems, especially those originating from the Orobanchaceae, such as the *Striga*-cereal interaction. First, determine whether resistance is induced by modified chemical signals that regulate seed germination or haustorium induction, such as altered strigolactone composition in sorghum [28], or changed flavonoids that regulate haustorium induction [29]. Then we can further investigate the cytological features of the parasitic infection process in resistance hosts, following the framework of this study.

Using the *O. cumana* resistant cultivar JK103 as an example, our statistical analysis revealed that its root exudates' ability to stimulate *O. cumana* seed germination did not differ significantly from that of the susceptible cultivar LD5009 (Fig. 2A), indicating that early chemical signaling remains unaffected. Impeding parasitic progression via cortical cell wall thickening or lignification is a conserved host defense strategy. Analogous physical barrier mechanisms have also been reported in *Oryza sativa*, *Pennisetum glaucum*, *Sorghum bicolor* or *Vigna unguiculata* [30]. Here, we show that a similar mechanism also exists in sunflowers against *O. cumana*. Our cytological analysis, revealed that upon *O. cumanae* contact and attempt penetrate the JK103 roots, the host plant activates a strong lignification response at the infection site during attachment phase and vascular bundle penetration phase (Fig. 6),

by which lead to delayed parasitic infection process. However, how host plant sense parasite invasion and induce lignin accumulation at specific locations during parasite-host plant interactions remains to be elucidated.

In addition, when *O. cumana* invading cells successfully penetrate the epidermal tissue of JK103, significant lignin accumulation is observed within these parasitic invading cells (before vascular contact phase). This indicates either robust defensive activity or senescence in the parasitic plant cell, which may be caused by either an unsuccessful vascular connection or the toxic compounds production by host plants [31]. Furthermore, the subsequent development of the xylem bridge is severely compromised in JK103, resulting in xylem bridges that are discontinuous, malformed, and ultimately collapse (Fig. 3, 6). This directly disrupts the water and nutrient flow between the parasite and the host, consequently leading to the death

Table 1
Cytological Definition and Characterization of the Haustorial Development Stages of *O. cumana* in Sunflower.

Developmental phase	Parasitic Timeline	Cytological Events	Structural Characteristics	Developmental Function
I. Attachment phase	1 dpi	The radicle tip of <i>O. cumana</i> contacts the host epidermis, causing deformation of the epidermal cells at the contact site.	Radicle attachment to the epidermis.	Establishing initial physical contact and host recognition.
II. Vascular bundle contact phase	2 dpi	The parasite penetrates the epidermis and cortical tissues, with its radicle tip cells polarizing toward the xylem pole and differentiating into a wedge-shaped infection structure.	Wedge-shaped infection structure oriented toward the endodermis/vascular bundle.	Orienting growth toward the host vascular system.
III. Vascular bundle penetration phase	3 dpi	The radicle tip successfully penetrates the endodermis and further differentiates into an arrowhead-shaped structure that establishes connection with host vessels.	Arrowhead-shaped structure establishing initial host-parasite vascular contact.	Breaking through the endodermal barrier and achieving preliminary xylem connection.
IV. Nutrient absorption initiation phase	4 dpi	Parasitic cells proliferate rapidly and occupy most of the space within the host vascular bundle.	Parasitic cell mass occupying the vascular bundle.	Establishing and expanding foothold within the host vascular system.
V. Haustorium formation phase	7 dpi	Parasitic tissue expands and connects to the host phloem pole, accompanied by haustorium formation.	Mature haustorium with established connections to host xylem and phloem.	Forming a complete interface for bidirectional nutrient transport (xylem/phloem).
VI. Tubercle phase	≥ 13dpi	Parasitic cells adjacent to the host-parasite interface differentiate into xylem, establishing	Discernible tubercle with functional xylem bridges.	Consolidating nutrient acquisition to support subterranean

Developmental phase	Parasitic Timeline	Cytological Events	Structural Characteristics	Developmental Function
		xylem bridges, while tubercle development occurs.		growth of the parasite.
VII. Stem emergence phase	≥ 18dpi	The tubercle increases in volume, shoot primordia emerge, and xylem bridges mature with thickened walls and enlarged vessel diameter.	Shoot emergence with mature xylem bridges featuring thickened walls and enlarged vessel diameter	Enabling photosynthesis, completing the life cycle, and producing seeds.

of *O. cumana* tubercles (Fig. 2D). Thus, the host can inhibit the differentiation of *O. cumana* haustorial cells and formation of xylem bridges, although the exact mechanisms underlying this inhibition require further investigation.

Haustorium formation, which involves reprogramming of multiple phytohormones, is a critical phase for parasitic establishment. *Striga* parasitism of *Santalum album* is associated with an elevated auxin/cytokinin ratio during haustorium development [32], a process driven by local auxin biosynthesis and transport [18]. Additionally, salicylic acid (SA) and jasmonic acid (JA) play roles in parasitic interactions, as demonstrated in the *Cuscuta*-tomato system [23, 33]. Beyond these hormones, a hypersensitive response, which ultimately blocks establishment of functional xylem bridges, has been observed in tomato during *Cuscuta* invasion [34]. JK103 resistance may be mediated by the host interfering with haustorial tissue differentiation either by disrupting the hormone balance or inducing specific R genes that recognize parasitic invasion and trigger the host defense signaling.

In summary, we have established a standardized cytological evaluation system for studying *O. cumana* resistance in sunflowers and revealed the resistance mechanism in JK103 involving phase-specific lignification and disrupted xylem bridge function. Additionally, we also provided a clear, replicable research paradigm for broader studies on parasitic-host plant interactions, which will significantly advance resistance breeding efforts.

Declarations

Supplementary Information

Additional Supporting Information may be found in the online version of this article.

Figure S1. Petri dish-filter paper system.

Figure S2. Cytological Observation of Susceptible Host-Parasite Plant Interactions During Parasitization.

Figure S3. Formation of Xylem Bridge During Parasitization of Susceptible Host-Nonhost.

Figure S4. Xylem Bridge Formation During Parasitization of Resistant Host-Nonhost Plants.

Figure S5. Cytological and histological changes in resistant cultivar JK103 and susceptible cultivar LD5009 following *O. cumana* infection.

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Data availability

The raw data supporting the conclusions of this article will be made available by the authors without undue reservation.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Consent and approval for publication obtained from all the authors.

Competing interests

The authors declare no competing interest.

Author contributions

RongL drafted the manuscript and also undertook data collection and analysis, semi-thin plastic sectioning, vibratome sectioning, image acquisition, and schematic diagram preparation. MW provided guidance on semi-thin plastic sectioning techniques. NY, TL, RL, JY, and JZ participated in the collection of *Orobancha cumana* seeds. JZ, TX, and QC supervised the research process and provided overall guidance. RongL, JZ, TX, and QC contributed to the final version of the manuscript. All authors reviewed the manuscript.

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Figures

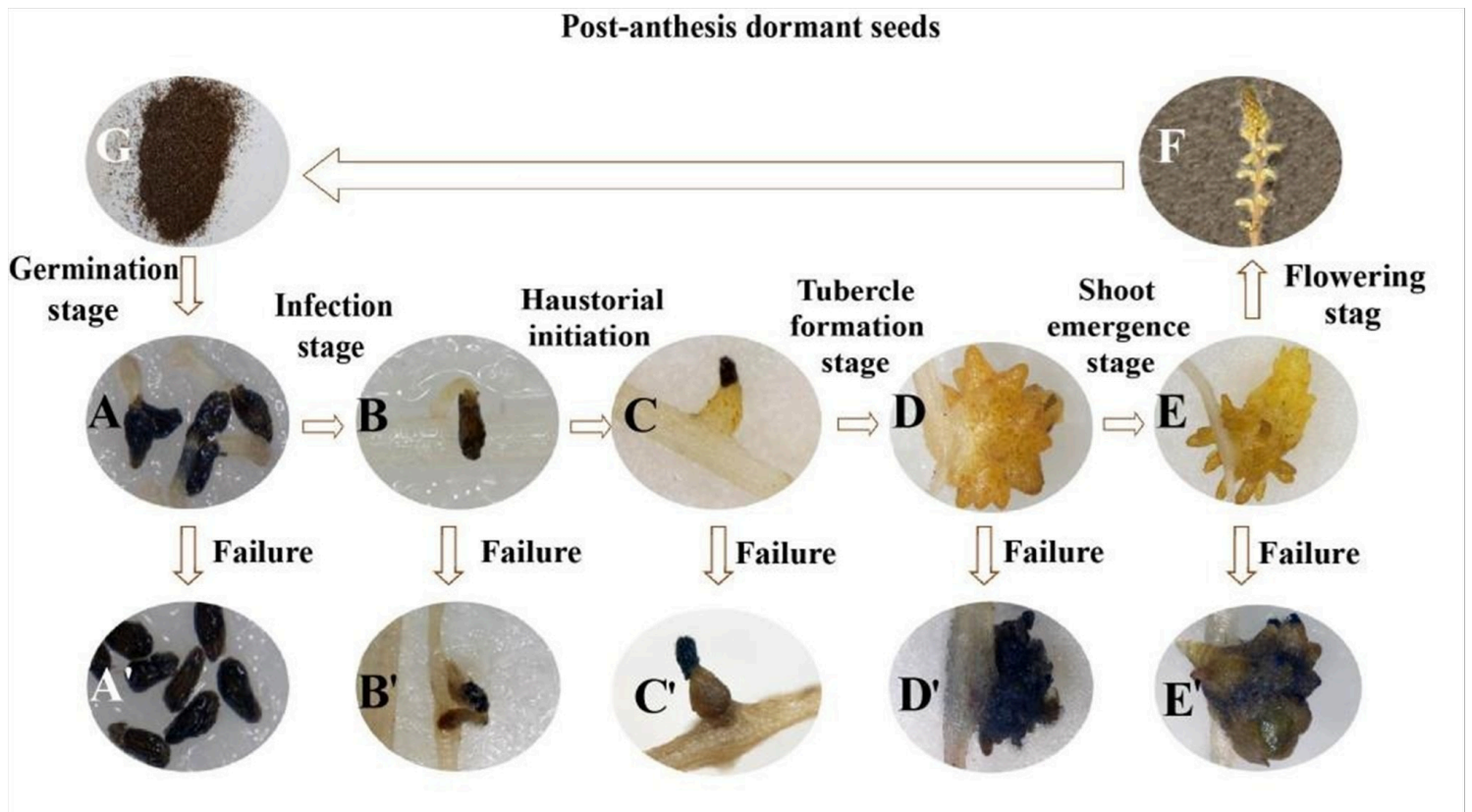


Figure 1

The process of successful *O. cumana* infection in sunflowers and potential failure cases caused by host resistance. (A) Successful germination. (A') failed germination. (B) successful infection. (B') failed infection. (C) successful haustorium formation. (C') failed haustorium formation. (D) successful tubercle formation. (D') failed tubercle formation. (E) successful shoot emergence. (E') failed shoot emergence. (F) flowering. (G) seed setting.

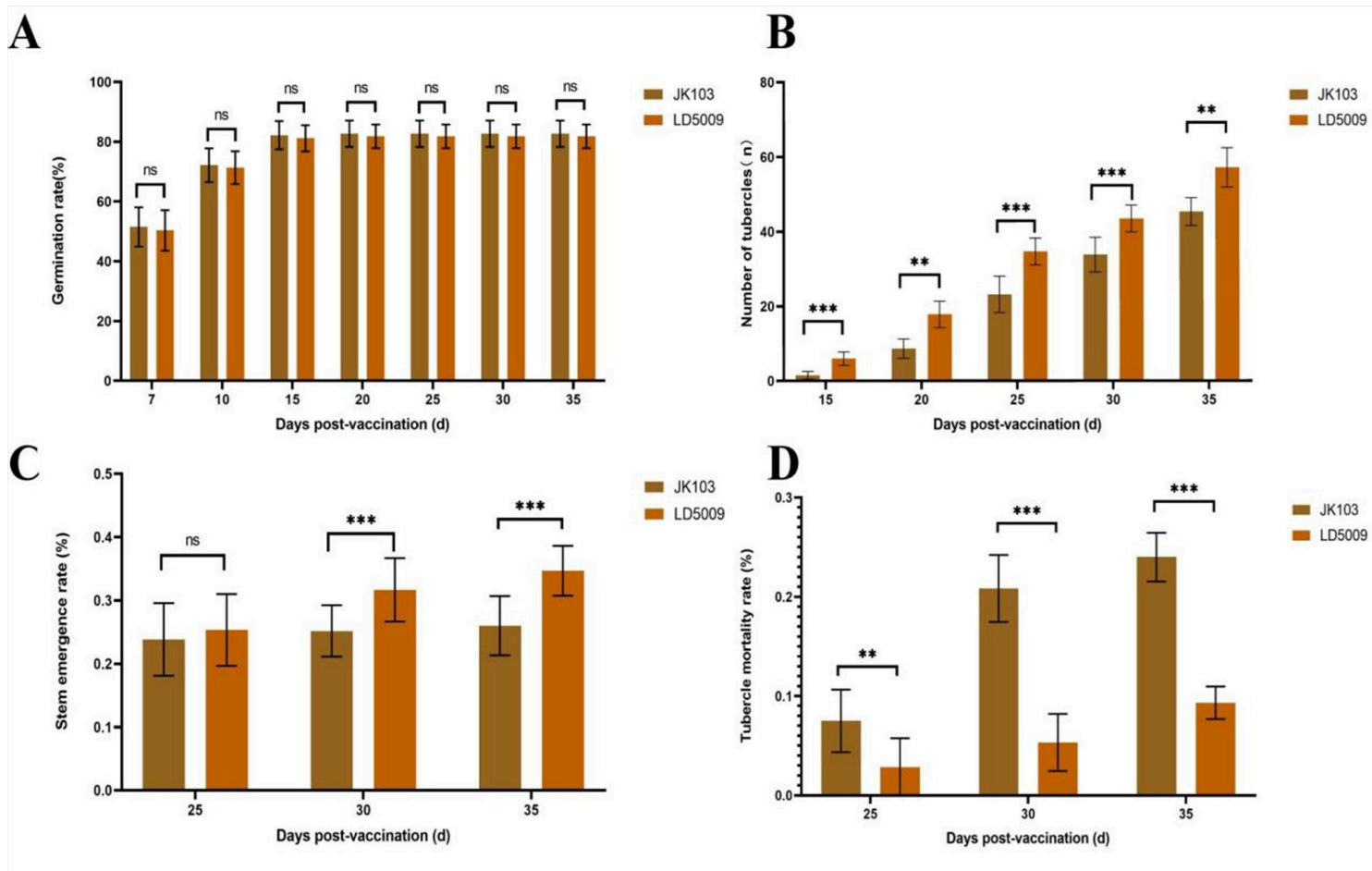


Figure 2

Statistic Evaluation of the *O. cumana* Resistance trait in Sunflower Varieties. (A) Germination rates of *O. cumana* on resistant sunflower JK103 and susceptible sunflower LD5009. (B) Tubercle formation numbers of *O. cumana* on JK103 and LD5009. (C) Stem induction rate in JK103 and LD5009. (D) Tubercle mortality rates on JK103 and LD5009. Figure caption: Data are presented as the mean value (n = 6). Symbols (ns, no significant difference; *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$) indicate significant differences between the resistant cultivar JK103 and susceptible cultivar LD5009 under the same conditions.

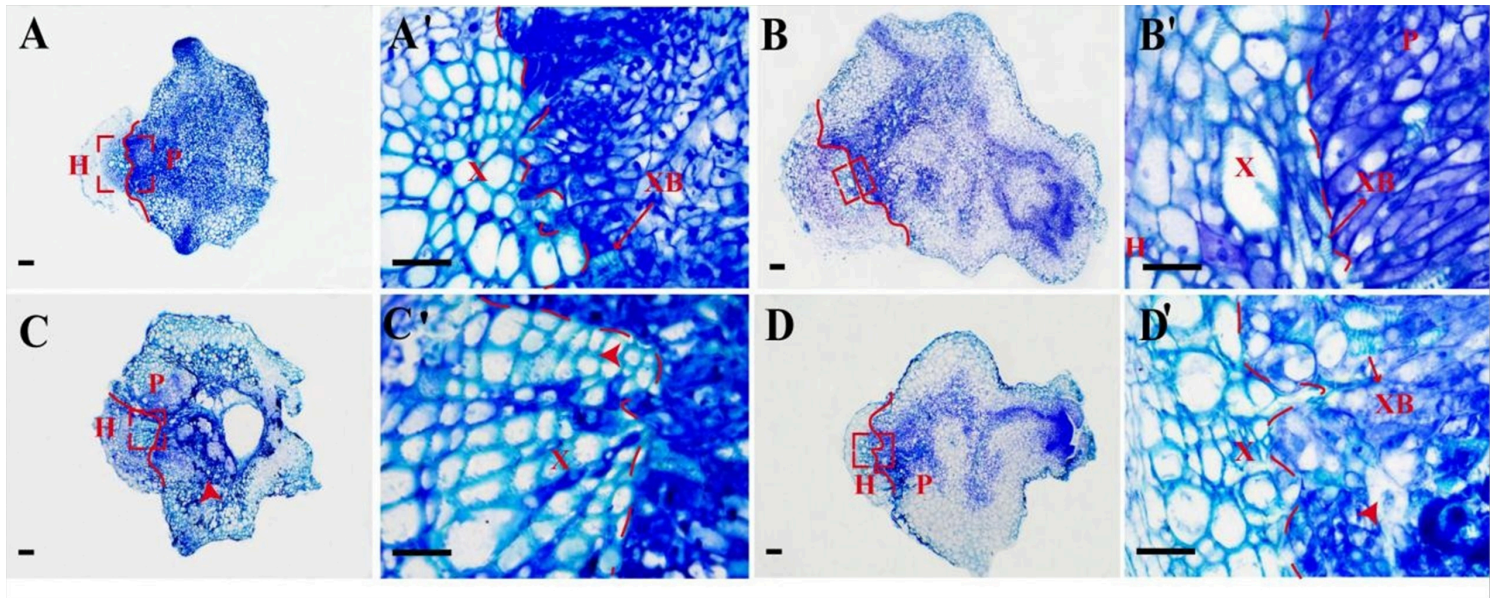


Figure 3

Histological analysis of *O. cumana* infection sites in susceptible and resistant sunflower cultivars. (A–A') tubercle formation on the susceptible cultivar LD5009 (15 dpi). (B–B') stem emergence stage in susceptible cultivar LD5009 (25 dpi). (C–C') tubercle formation on the resistance cultivar JK103 (15 dpi). (D–D') stem emergence stage in the resistance cultivar JK103 (25 dpi). (A'–D') magnified views of red-boxed areas in (A–D). Staining: 0.05% toluidine blue (semi-thin sections). Labels: H, Host plant; P, Parasitic plant; X, Xylem; XB, Xylem bridge. All scale bars: 50 μm.

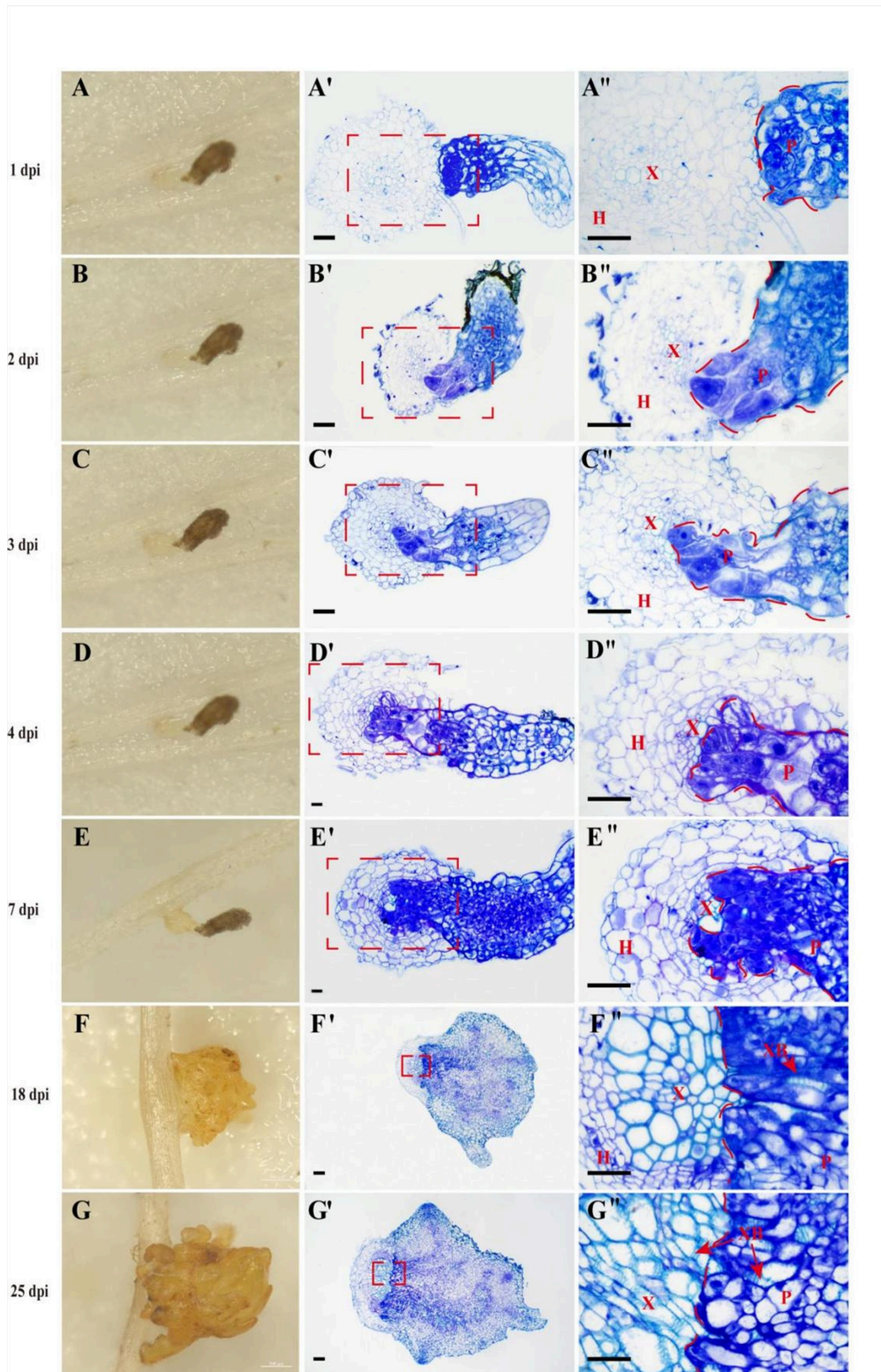


Figure 4

Histological analysis of parasite-host interaction between *O. cumana* and sunflower cultivar LD5009. (A–G) Stereomicroscope micrographs showing *O. cumana* infecting the host (sunflower) at different time points. (A'–G') Sections of parasite-host interaction sites at different time points. (A''–G'') Magnified views of red-boxed regions in A'–G'. Developmental timeline: (A–A') attachment phase. (B–B'') vascular bundle contact phase. (C–C'') vascular bundle penetration phase. (D–D'') nutrient absorption initiation

phase. (E–E'') haustorium formation phase. (F–F'') tubercle phase. (G–G'') stem emergence phase. Staining: 0.05% toluidine blue. Labels: H, Host plant (sunflower); P, Parasitic plant (*O. cumana*); X, Xylem; XB, Xylem; bridge. All scale bars: 50 μ m.

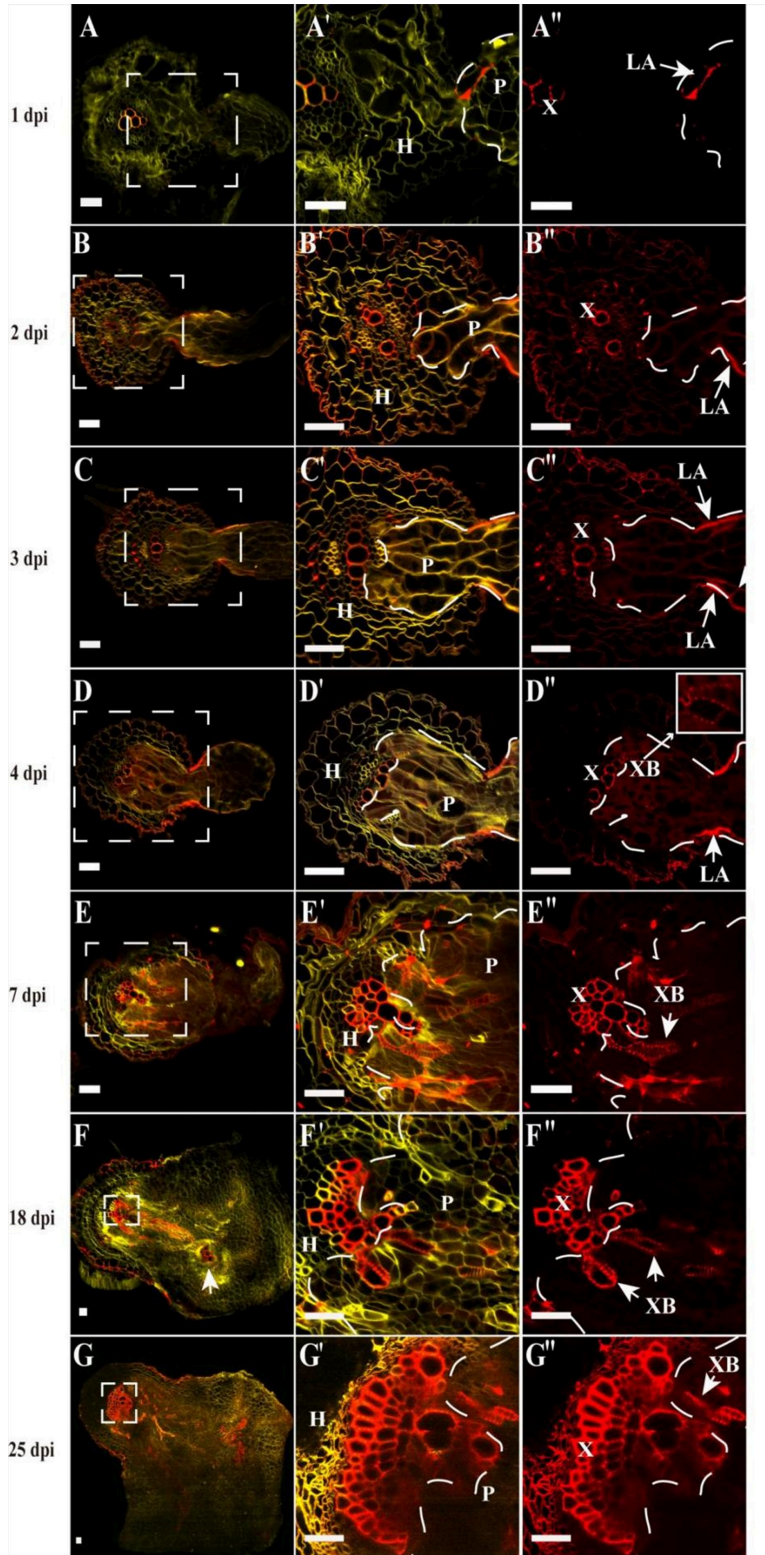


Figure 5

Lignin dynamics during *O. cumana* infection of the susceptible sunflower cultivar LD5009. (A–G') Direct Yellow was used for cell wall staining (yellow). (A–G'') Basic Fuchsin was used for lignin staining (red).

Image panels: (A–G) 4× confocal overlay micrographs at different developmental stages. (A'–G') 40× magnified confocal overlays of white-boxed areas in (A–G). 40× Basic Fuchsin channel of white-boxed areas (A''–G''). Developmental stages: (A–A'') attachment phase. (B–B'') vascular bundle contact phase. (C–C'') vascular bundle penetration phase. (D–D'') nutrient absorption initiation phase. (E–E'') haustorium formation phase. (F–F'') tubercle phase. (G–G'') stem emergence phase; Abbreviations: X, Xylem; XB, Xylem bridge; LA, Lignin accumulation; H, Host plant; P, Parasitic plant. All scale bars: 50 µm.

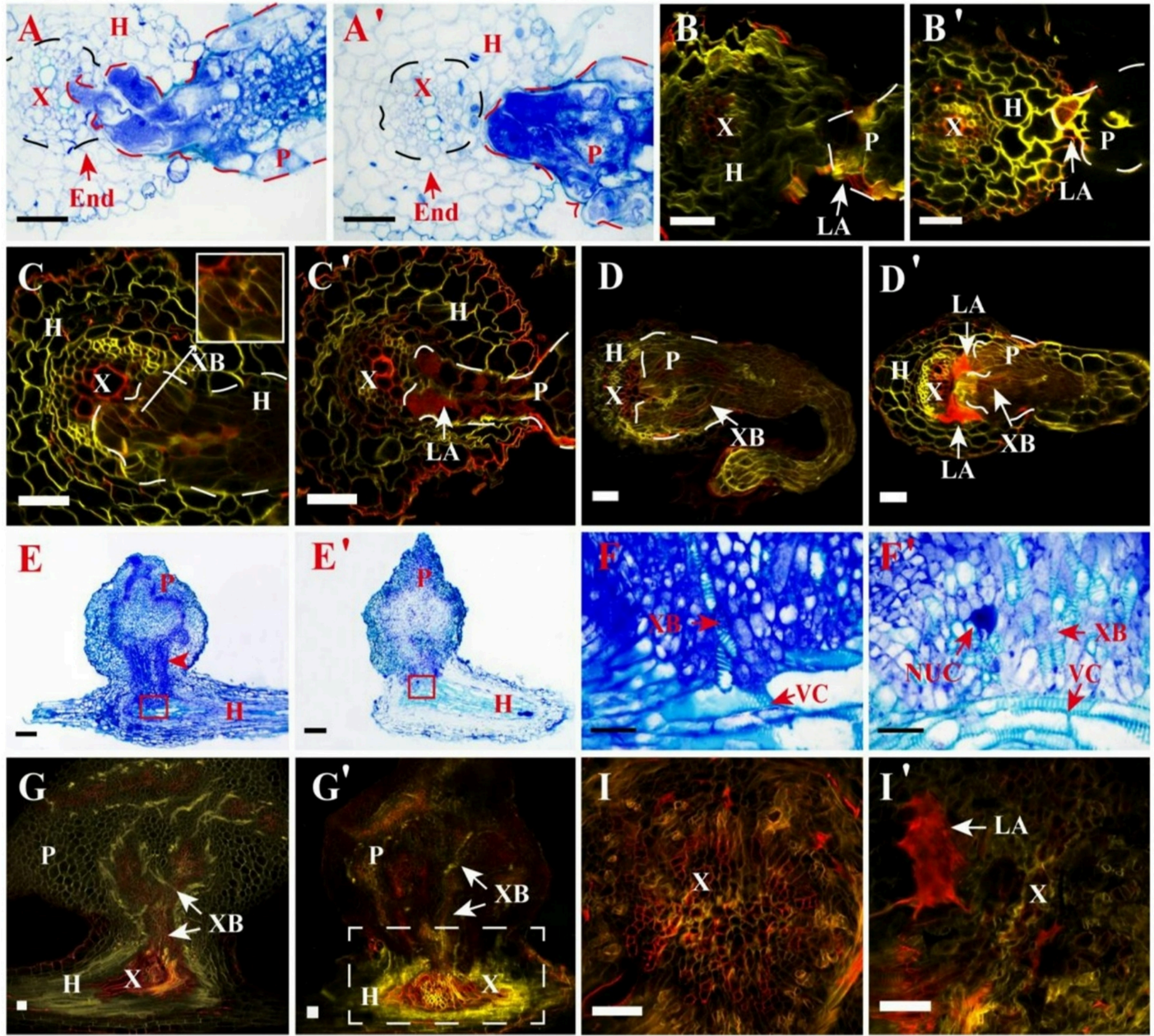


Figure 6

Analysis of the *O. cumana* resistance phenotype of cultivar JK103 via tissue sectioning and staining. (A–A') *O. cumana* infection in susceptible cultivar LD5009 (A) and resistant cultivar JK103 (A') at day 3. (B–B') *O. cumana* infection in susceptible cultivar (B) and resistant (B') sunflower at day 1. (C–C') *O.*

cumana infection in susceptible cultivar (C) and resistant (C') sunflower at day 4. (D–D') *O. cumana* infection in susceptible cultivar (D) and resistant (D') sunflower at day 8. (E–E') 4× *O. cumana* infection in susceptible cultivar (E) and resistant (E') sunflower at day 18. (F–F') 40× magnification of the boxed areas in (E) and (E'), showing *O. cumana* infection in the susceptible cultivar (F), from boxed region in (E) and resistant cultivar (F'), from boxed region in (E') at 18 days post-inoculation. (G–G') *O. cumana* infection in susceptible (G) and resistant (G') sunflower at day 20. (I–I') *O. cumana* infection in susceptible (D) and resistant (D') sunflower at day 20 (another view). Abbreviations: End, Endodermis; X, Xylem; XB, Xylem bridge; LA, Lignin accumulation; Nuc: Nucleus; VC: Vascular Connection (containing the xylem bridge and phloem bridge); H, Host plant; P, Parasitic plant. Staining: 0.05% toluidine blue. All scale bars: 50 µm.

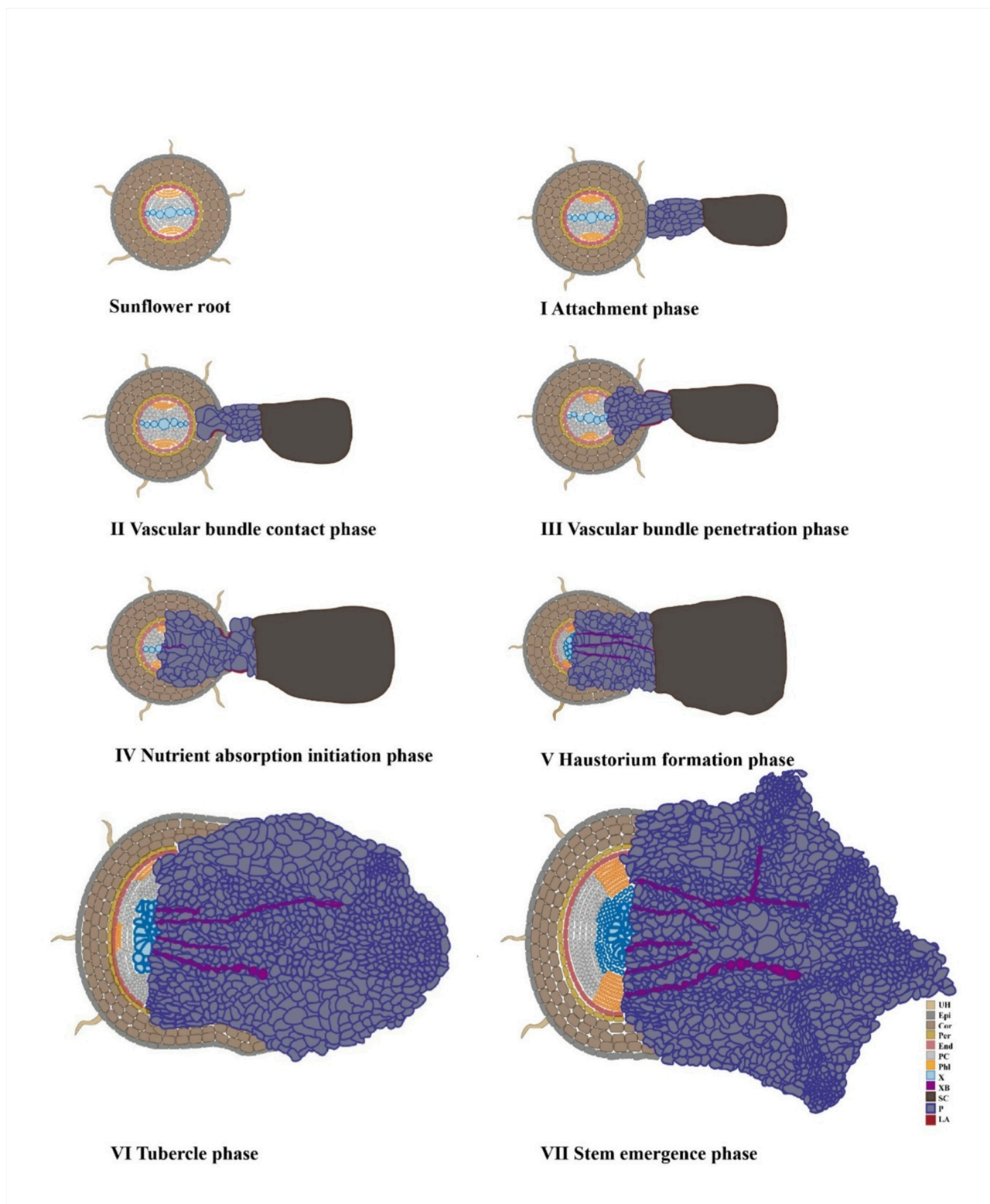


Figure 7

Schematic diagrams of parasitic growth process at different Stages. Abbreviations: UH, Root hair; Epi, Epidermis; Cor, Cortex; Per, Pericycle; End, Endodermis; PC, Parenchyma cell; Phl, Phloem; X, Xylem; XB, Xylem bridge; LA, Lignin accumulation; SC, *O. cumana* seed coat; P, Parasitic plant.

Supplementary Files

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