The filamentous fungus *Verticillium dahliae* is a soilborne fungal pathogen that causes *Verticillium* wilt, a plant disease in over 400 plant species, and results in big crop losses annually (Bhat & Subbarao, 1999; Fradin & Thomma, 2006). It is extremely difficult to control *Verticillium* wilt, in large part because *V. dahliae* is a vasculature-colonizing pathogen and there are no efficient treatments to cure infected plants. Another reason is that *V. dahliae* produces microsclerotia, melanized dormant structures that can survive in the soil for decades (Chen et al., 2021; Wilhelm, 1955). After stimulation by root exudates from host plants, the microsclerotia germinate and produce hyphae that pass through the root cortex into the host vascular tissue and colonize the xylem of the infected plants (Fradin & Thomma, 2006; Klosterman et al., 2009; Zhang et al., 2013; Zhao et al., 2016). In the vascular tissue, *V. dahliae* produces a large number of conidia that are transported through the xylem transpiration stream to the aerial parts of the plant. Ultimately, the infected host exhibits wilt symptoms, stunting, chlorosis, and necrosis. However, so far, the detailed cellular mechanisms of *V. dahliae* invasion and colonization remain to be explored.

**Abstract**

The soilborne ascomycete fungus *Verticillium dahliae* causes destructive vascular wilt disease in hundreds of dicotyledonous plant species. However, our understanding of the early invasion from the epidermis to the vasculature and the prompt proliferation and colonization in the xylem tissues remains poor. To elaborate the detailed infection strategy of *V. dahliae* in host plants, we traced the whole infection process of *V. dahliae* by live-cell imaging combined with high-resolution scanning electron microscopy. The 4D image series demonstrated that the apex of invading hyphae becomes tapered and directly invades the intercellular space of root epidermal cells at the initial infection. Following successful epidermal invasion, the invading hyphae extend in the intercellular space of the root cortex toward the vascular tissues. Importantly, the high-resolution microscopic and live-cell images demonstrated (a) that conidia are formed via budding at the apex of the hyphae in the xylem vessels to promote systemic propagation vertically, and (b) that the hyphae freely cross adjacent xylem vessels through the intertracheary pits to achieve horizontal colonization. Our findings provide a solid cellular basis for future studies on both intracellular invasion and vascular colonization/proliferation during *V. dahliae* infection and pathogenesis in host plants.

**KEYWORDS**

invasion strategy, live-cell imaging, plant-*Verticillium* interaction, xylem colonization

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In order to demonstrate the detailed colonization strategy, we traced the infection process of *V. dahliae* V592-GFP by live-cell imaging combined with high-resolution scanning electron microscopy (SEM). *V. dahliae* was grown on potato dextrose agar (200 g/L potato, 20 g/L glucose, 15 g/L agar). To collect conidia, mycelial plugs were cultured in liquid Czapek-Dox medium (2 g/L NaNO₃, 1 g/L K₂HPO₄, 1 g/L MgSO₄·7H₂O, 1 g/L KCl, 0.02 g/L FeSO₄, 30 g/L sucrose) with shaking at 160 rpm at 25°C for 3–5 days. The roots of infected *Arabidopsis* and cotton seedlings were observed in a time-lapse series using spinning-disc confocal microscopy (UltraView VOX; Perkin Elmer) (Feng et al., 2018). During microscopy, 488-nm and 561-nm lasers were used to excite GFP and mCherry, respectively. All images were analysed with Volocity (Perkin Elmer) and ImageJ (http://rsbweb.nih.gov/ij).

Due to the lack of fluorescent marker-tagged lines of cotton and the large diameter of cotton roots, we observed the initial infection process (16–24 h postinoculation [hpi]) in *Arabidopsis thaliana* ecotype Columbia expressing the plasma membrane marker PIP2A-mCherry, and then observed colonization and proliferation (2–13 days postinoculation [dpi]) in cotton by propidium iodide staining. *A. thaliana* Columbia was used in these experiments as it is the most susceptible ecotype to *V. dahliae* (Veronese et al., 2003). *A. thaliana* was planted on 1/2× Murashige & Skoog plates with 15 g/L sucrose, and then 7-day-old seedlings were incubated in conidial spore suspensions (10⁷ cfu/ml) for 15 min. The roots were then rinsed three times with sterile water and cultured at 22°C in a greenhouse. At 16–24 hpi, we observed that more than 90% (n = 699, four independently repeated experiments) of *V. dahliae* conidia on the root surface germinated.

**FIGURE 1** 4D illustration of early *Verticillium dahliae* invasion into host plants. (a) Scanning electron microscopy images showing invasion of *V. dahliae* hyphae (left) and germ tubes (right) into the intercellular space of *Arabidopsis thaliana* root epidermal cells. The scale bar represents 10 μm. The yellow arrows indicate the invasion sites. Representative images are shown from four independent experiments. (b) 4D image series of *V. dahliae* expressing cytoplasmic green fluorescent protein (green, V592-GFP) and *A. thaliana* expressing PIP2A-mCherry (magenta), showing that the apex of invading hyphae becomes tapered and directly invades the junctions of root epidermal cells. The yellow arrows indicate the invasion sites. A time-lapse image is the maximum z-projection of image stacks at 1.5-μm intervals (18 z-slices). See also Movie S1.
and the elongating hyphae attached to the Arabidopsis root surface. High-resolution SEM observation revealed that the germ tubes and elongated hyphae invaded the intercellular space between epidermal cells (Figure 1a). The statistical data showed that fewer than 10% \((n = 748, \text{ four independently repeated experiments})\) of germ tubes and elongated hyphae could successfully invade the root epidermal space. The 4D image series showed that the invading hyphae first extended close to root epidermal cells; at suitable penetration sites, the apex of invading hyphae became tapered and directly invaded the junctions of root epidermal cells (Figure 1b; Movie S1). V. dahliae did not form any conspicuous infection structures like appressoria; only slight hyphal swellings were formed before epidermal invasion (Figure 1b; Movie S1).

For observation of colonization and proliferation in the host xylem, 2-week-old cotton seedlings (line TM-1) were inoculated with conidial spore suspensions \((10^7 \text{ cfu/ml})\) by the unimpaired root-dip inoculation method. Following successful epidermal invasion, the invading hyphae extended into the intercellular space of the root cortex in cotton. The 3D images showed that most of the invading hyphae elongated along the longitudinal axis of the root at 2–3 dpi (Figure 2; Movie S2). We observed the V592-GFP signals in vascular tissues of cotton roots until 3–5 dpi (Figure 3a); in the protoxylem and metaxylem, the V592-GFP signal increased as the invasion continued (Figure 3a,b). Longitudinal sections showed that conidia were produced from the tip of hyphae in xylem vessels (Figure 3c).

We tracked the whole process of conidium production in Czapek-Dox liquid medium by live-cell imaging because it is highly challenging to operate in plant vascular tissues. Conidia \((10^5 \text{ cfu/ml})\) were incubated in coverslip-bottomed chambers (Thermo Fisher Scientific, catalogue number 155380) at 26°C for 24 h. The results showed that V. dahliae formed simple conidiophores and produced a large number of conidia in liquid medium (Figure 3d). It took about an hour to produce a conidium at the apex of the hyphae, where multiple conidia are continuously produced (Figure 3d; Movie S3). There were no typical verticillate conidiophores in plant vascular tissues and in Czapek-Dox liquid medium.

In order to further understand the details of V. dahliae colonization and proliferation in xylem vessels, we used SEM to observe longitudinal sections of infected cotton roots. The samples were fixed in 2.5% glutaraldehyde, buffered with phosphate-buffered saline (PBS) (pH 7.2) at 4°C overnight, washed four times with PBS, and dehydrated in an ethanol series (50%–100%) for 15 min per concentration. The samples were critical-point dried before platinum spraying and observation with an S-3000N scanning electron microscope.
microscope (Hitachi). The results showed that the hyphae undergo spore-budding growth at the apex (Figure 4a). In xylem vessels, a large number of conidia accumulated on the inner wall of vessels. The conidia were unevenly distributed in the vessels, with 3–10 conidia per 100 μm² in the enrichment areas (an area of 8.9 mm² was analysed in three independent experiments). The hyphae freely passed from one vessel to another through the intertracheary pits (Figure 4b,c).

The above microscopic observations showed that invasive hyphae of *V. dahliae* grow into the intercellular space of root epidermal cells, and after reaching the vascular tissue, invasive hyphae differentiate into simple conidiophores to produce a large number of conidia for further invasive proliferation. Our findings provide elaborate and novel information about *V. dahliae* infection and colonization in host plants. In particular, the 4D image series demonstrated that the apex of invading hyphae becomes tapered and directly invades the intercellular space of root epidermal cells. Our data support the notion that *V. dahliae* does not form a conspicuous infection structure at the penetration site (Eynck et al., 2007; Zhang et al., 2013; Zhao et al., 2014). In addition, it is generally accepted that *V. dahliae* produces a large number of conidia to rapidly proliferate in the vascular tissue (Fradin & Thomma, 2006; Klimes et al., 2015; Klosterman et al., 2009). However, few reports have shown the details of the conidial production and diffusion patterns of *V. dahliae* in xylem vessels. Importantly, our high-resolution microscopic and live-cell images demonstrated that conidia are formed via budding at the apex of the hyphae. Our findings shed light on the cellular mechanism of *V. dahliae* pathogenicity.

*V. dahliae* colonizes xylem tissues of host plants (Pegg, 1985; Yadeta & Thomma, 2013). Until now the sexual life cycle of *Verticillium* pathogens has not been characterized, and conidia are the only known reproductive form of *Verticillium* pathogens (de Jonge et al., 2013). Thus, the conidial production of *V. dahliae* in the xylem tissue represents a key strategy for adaptation to the ecological niche, and is required for its pathogenicity (El-Bebany et al., 2010; Luo et al., 2016; Sarmiento-Villamil et al., 2018; Tri-Thuc et al., 2019; Xiong et al., 2016). Therefore, it will be intriguing to further explore the regulatory mechanism of *V. dahliae* conidial production in vascular tissues in future studies.
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AUTHOR CONTRIBUTIONS

J.T. performed most of the experiments, analysed the data, and drafted the manuscript. Z.K. conceived the project, interpreted the data, and revised the article.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author.

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FIGURE 4 The details of Verticillium dahliae hyphal expansion in xylem vessels. Scanning electron microscopy analysis was conducted to reveal the details of hyphal expansion in xylem vessels. (a) Spore budding at the apex of the hyphae. The scale bar represents 5 μm. (b) A large number of conidia on the inner wall of vessels. The scale bar represents 10 μm. (c) The hyphae pass through the intertracheary pits. The scale bar represents 10 μm. The red arrows indicate intertracheary pits through which the hyphae pass.
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