Ceratocystis fimbriata Employs a Unique Infection Strategy Targeting Peltate Glandular Trichomes of Sweetpotato (*Ipomoea batatas*) Plants

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ABSTRACT

The infection processes of *Ceratocystis fimbriata* BMPZ13 (BMPZ13) was elucidated on vegetative tissues of sweetpotato plants employing light and scanning electron microscopy. Vegetative tissues infected with *C. fimbriata* BMPZ13 by either wounding or nonwounding inoculation methods developed typical disease symptoms, establishing black rot in stems and necrosis on buds, young leaves, and stems of sprouts, in addition to wilt on leaves and shoot cuttings, typical of vascular associated diseases. The runner hyphae of *C. fimbriata* BMPZ13 formed from germinated conidia were able to directly penetrate the epidermal cuticle for initial infection and invade sweetpotato peltate glandular trichomes, specialized secretory structures to store and secrete metabolites. A two-step biotrophic phase was observed with nonwounding inoculation on leaves and stems, featuring both intercellular and intracellular invasive hyphae, with the latter found within living cells of

the leaf epidermis. Subsequent to the biotrophic phase was a necrotrophic phase displaying cell death in infected leaves and veins. Additionally, this cell death was an iron-associated ferroptosis, supporting the notion that iron is involved in the necrotrophic phase of *C. fimbriata* BMPZ13 infection. Significantly, we establish that *C. fimbriata* employs a unique infection strategy: the targeting of peltate glandular trichomes. Collectively, our findings show that *C. fimbriata* is a plant fungal pathogen with a hemibiotrophic infection style in sweetpotato vegetative tissues.

Keywords: biochemistry and cell biology, *Ceratocystis fimbriata*, etiology, ferroptosis, hemibiotroph, mycology, necrotrophic phase, peltate glandular trichomes, postharvest pathology and mycotoxins, sweetpotato black rot, vegetative infection

Sweetpotato [*Ipomoea batatas* (L.) Lam.] is one of the most widely cultivated storage root crops, especially in tropical and subtropical countries (Lebot 2009; Scott et al. 2000). Key attributes of sweetpotato relative to other major staple food crops include a wide range of production geography, excellent adaptability to marginal soils, climate conditions, and high nutritional values. Sweetpotato is consumed as food, utilized as a reliable feed for livestock and even is employed as a medicinal plant (Panda and Sonkamble 2012). It is an excellent source of nutrients including provitamins, vitamin C, potassium, iron, and calcium minerals (Clark and Moyer 2013). Sweetpotato serves as a staple food in many countries of subtropical regions because the storage roots are rich in starch and the leaves and young shoots are edible (Lim 2016). Sweetpotato has also drawn attention as an emerging alternative to established bioenergy crops (Qiu et al. 2010; Wang et al. 2013).

Black rot disease is one of the most important fungal diseases of sweetpotato, causing significant losses in yield and a drastic decrease in quality (Li et al. 2009). The causative agent of black rot disease is *C. fimbriata*, which has a worldwide distribution in sweetpotato growing regions (Zauza et al. 2004). The *C. fimbriata* complex consists of multiple species which infect a range of host plants. Three host-specific lineages of *C. fimbriata* were identified from cacao (*Theobroma cacao*), sweetpotato (*Ipomoea batatas*), and sycamore (*Platanus* spp.) (Engelbrecht et al. 2004; Harrington et al. 2005). *C. fimbriata* also causes wilt and canker diseases in a wide range of woody hosts including: kiwifruit (*Actinidia* spp.) (Piveta et al. 2016), ficus (*Ficus carica*) (Pasura and Harrington 2004), yerba mate (*Ilex paraguariensis*) (Dos Santos et al. 2018), *Eucalyptus*, mango (*Mangifera indica* L.) (Araujo 2014), plane tree (*Platanus* spp.) (Clerivet et al. 2000), and pomegranate (*Punica granatum*) (Harrington et al. 2015). Besides sweetpotato, other herbaceous nonwoody hosts are also infected by *C. fimbriata* strains causing rot diseases including taro (*Colocasia esculenta*) and Peruvian carrot (*Arracacia xanthorrhiza*) (Harrington et al. 2005; Johnson et al. 2005; Melo et al. 2016). During cultivation and postharvest storage, typical annual yield losses caused by sweetpotato black rot are 5 to 10%, but can be significantly greater in exceptional years (Zhang et al. 2017).

Phytoalexin substances are produced in sweetpotato as a mechanism of defense against *C. fimbriata* infection (Oguni and Uritani 1974). Some of these defense-related furanoterpenoid derivatives, when accumulated heavily in infected sweetpotato storage roots, are toxic to humans and animals. For example, the notorious ipomeamarone is poisonous to both humans and livestock (Akazawa and Uritani 1961; Akazawa and Wada 1961; Imaseki and

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Ikuzo 1964; Wamalwa et al. 2015), with accidental consumption of diseased sweetpotato storage roots often leading to serious health concerns for humans, and the death of livestock (Coxon et al. 1975).

Plant fungal pathogens infect and interact with their host plants through a number of distinct mechanisms including biotrophic, necrotrophic, and hemibiotrophic lifestyles (Presti et al. 2015). Biotrophs invade and propagate in living cells, while necrotrophs kill host cells and feed off the nutrients released from dead tissues. Hemibiotrophic pathogens initiate a biotrophic phase after entering plant tissues and then switch to necrotrophy (Kabbage et al. 2015). Recently, necrosis associated with plant pathogenic fungal infection has been linked to ferroptosis, an iron-dependent ferroptotic cell death, which plays a key role in the interaction of rice with the hemibiotrophic fungal pathogen *Magnaporthe oryzae* (Dangol et al. 2019). Hemibiotrophic pathogens typically enter host cells by mechanical penetration of the cuticle by employing dome-shaped and melanized appressoria, or enter via natural openings such as stomata (De Wit 1977; Tucker and Talbot 2001). Whether alternative strategies are used by plant fungal pathogens to enter host tissues remain to be established.

Black rot of sweetpotato is generally considered to be a seedborne and soilborne postharvest disease because *C. fimbriata* infects sweetpotato storage roots through wounds during postharvest storage, however, severe diseases occur in seed beds and during vegetative growth in the field, including plant stunting, wilting, yellowing, leaf drop, and death (Clark and Moyer 2013; Hildebrand and Cook 1959). *C. fimbriata* is believed to be a necrotrophic pathogen, at least on storage roots (Johnson et al. 2005). Much attention was paid to black rot disease of storage roots, while relatively little is known about the infection style of *C. fimbriata* on sweetpotato vegetative tissues either in the field or under laboratory conditions. Thus, the *C. fimbriata*–sweetpotato pathosystem warrants detailed characterization of *C. fimbriata* infection on vegetative tissues of sweetpotato plants. In this context, it is important to determine the possible role of any vegetative infection in disease cycles of black rot in the field, and the resulting impact on disease occurrence at the postharvest storage stage.

Our study aimed to determine whether *C. fimbriata* possessed the capability to infect sweetpotato vegetative tissues, and if so, whether the pathogenic lifestyle on vegetative tissues is different from that on storage roots and of other plant fungal pathogens. This information may help guide future control strategies for *C. fimbriata* infection of sweetpotato.

MATERIALS AND METHODS

The fungal strain and culture conditions. *C. fimbriata* BMPZ13 was provided by the Sweet Potato Research Center at Xuzhou Academy of Agricultural Sciences, Jiangsu, China. This particular strain was isolated from a sweetpotato exhibiting typical symptoms of black rot in the field in Xuzhou. *C. fimbriata* BMPZ13 is a representative strain that is virulent in many varieties including Guishu 07-98 (data not shown). The mycelia of *C. fimbriata* BMPZ13 was cultured on potato dextrose agar (PDA) in an incubator at 25°C in the dark or potato dextrose broth at 25°C in a shaker at 150 rpm.

Fungal genomic DNA extraction. Acetyl trimethyl ammonium bromide (CTAB)-based DNA extraction protocol was adapted with minor modification (Murray and Thompson 1980). The colonies were grown on PDA plates for 3 to 4 days to around 1.2 to 1.5 cm in diameter. A mycelium mass of 1 cm (about 3/4 of the colony) was mixed with quartz sand in an Eppendorf tube and frozen at –20°C followed by an incubation with 200 µl of 2% CTAB at 65°C for 1 h. An equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) was added and vigorously shaken for 1 min followed by centrifugation at 12,000 rpm for 10 min. The aqueous phase was mixed with two volumes of 100% ethanol and incubated at –20°C for 2 h. Fungal genomic DNA was then pelleted by

centrifuging at 12,000 rpm for 10 min and washed with 75% ethanol. Air dried fungal genomic DNA pellets were dissolved in ddH₂O and stored at –20°C for later use.

Identification of ITS and LSU rDNA. The conserved sequences among fungal pathogens of two genomic regions, internal transcribed spacer (ITS) and large subunit ribosomal DNA (LSU rDNA), were determined using a procedure previously reported (Baker et al. 2003). The primers for PCR amplification are listed in Supplementary Table S1. The ITS region including 5.8S rDNA operon was amplified by PCR with primers ITS4 and ITS5 (Supplementary Table S1). LSU rDNA region was amplified with primers LROR and LR5 (Supplementary Table S1) (Stielow et al. 2015). *Taq* DNA polymerase-based PCR amplification was conducted in a BIOER LifePro Thermal cycler PCR System (Hangzhou Bioer Technology Co. Ltd., China) with the following thermocycling conditions: initial denaturation at 94°C for 5 min, followed by 35 cycles of 94°C for 30 s, and 55°C for 30 s, and 72°C for 2 min, and then a final extension at 72°C for 10 min. The amplified PCR products were purified from agarose gels and sequenced in both directions by a commercial sequencing service provider (Sangon Biotech, Shanghai, China).

Sequence analysis. The resulting sequences were compared with ITS or LSU rDNA sequences in GenBank database at the National Center for Biotechnology Information (NCBI) (<https://www.ncbi.nlm.nih.gov>) using basic local alignment search tool (BLAST) (Altschul et al. 1990). Phylogenetic analysis was performed using MEGA 6 and a maximum likelihood phylogenetic tree was constructed with combined datasets of the ITS and LSU rDNA sequences (Tamura et al. 2013). Sequences were deposited in GenBank and assigned accession numbers MN396458 and MN396459.

Plant material. Sweetpotato variety Guishu 07-98 was provided by Jinfeng Hua at Guangxi Academy of Agricultural Sciences, Guangxi, China. Sweetpotato storage roots were kept in pots filled with sandy soil within a greenhouse of 75% relative humidity and 16 h of lighting at 25°C. Sprout development usually started in 10 to 18 days. One-month-old shoots (12 to 15 cm long) were detached and then transplanted individually into pots for rooting and maintained to grow to 30 to 45 cm tall in greenhouse. Different types of sweetpotato vegetative tissues were used in pathogenicity assays and for the purposes of microscopic experiments.

Pathogenicity assay. *C. fimbriata* BMPZ13 was cultured for 7 to 10 days on PDA. Conidia were flushed with 1 ml of distilled water and collected in Eppendorf tubes. Mycelium mass was removed by filtering conidia spore suspension through a cheese cloth. The spores were pelleted by centrifugation at 3,000 rpm for 10 min and washed twice with 1 ml of distilled water. The conidia were resuspended with 1 ml of distilled water, and the numbers were counted with a hemocytometer under 10× magnification. A final spore resuspension for inoculation was adjusted to 1×10^5 spores/ml for inoculations.

Pathogenicity assays involved nonwounding and wounding inoculation methods. For nonwounding inoculation, 8-day-old sprouts (usually 4 to 6 cm in length) with unfolded young leaves were spray-inoculated with 1 ml of *C. fimbriata* BMPZ13 spore suspension using a small atomizer (10 ml volume) for nonwounding inoculations. In each independent assay, six detached sweetpotato sprouts were spray-inoculated with *C. fimbriata* BMPZ13 on young stems and leaves and H₂O as mock treatment. For nonwounding inoculation of detached leaves, six fully expanded sweetpotato leaves were drop-inoculated by spotting a 2-µl spore suspension on one half of the leaves. A mock inoculation was distilled H₂O on the other half. The inoculated sweetpotato sprouts or leaves were placed in petri dishes with a layer of wet filter paper for maintaining moisture for 9 days to monitor disease development.

Wounding inoculation was used for pathogenicity assays on sweetpotato storage roots, fully expanded leaves and shoot cuttings.

Six storage roots were pierced 0.5 cm deep with a needle and injected with 20 µl of spore suspension. The inoculated storage roots were maintained in a growth chamber at 25°C with 16 h of lighting and 75% relative humidity. Six fully expanded leaves were wounded with needle and each wounding site was drop-inoculated by spotting with 2 µl of spore suspension. The inoculated tissues were maintained in a growth chamber under the same conditions as for storage roots, and disease development was monitored for 14 days for disease progression and symptom development. A mock inoculation with distilled H₂O was made as the control. Six shoot cuttings of sweetpotato plants were inoculated at the base with 300 µl of *C. fimbriata* BMPZ13 spore suspension at 1×10^5 spores/ml and distilled H₂O employed as a mock treatment. The inoculated cuttings were kept in a growth chamber with the same conditions above and monitored for disease progression and symptom development for up to 21 days.

All the pathogenicity assays were repeated three times for both symptom development and microscopic observation. For pathogenicity assays involving in nonwounding drop inoculation, half of the inoculated tissues were kept for disease symptom development while the other half were collected as samples for light microscope and scanning electron microscope (SEM) observation.

Leaf tissue clearing. For clear visualization of infected tissues under microscope, the leaf tissues were cleared by removing chlorophyll. A leaf tissue clearing protocol was adapted from the previously reported method with modification (Xie et al. 2018). Leaves were drop-inoculated without wounding and tissue samples of inoculated area were taken at time points 0, 4, 6, and 8 h, and 1, 2, 3, 5, 7, and 10 days after inoculation. The tissue samples were fixed in formaldehyde acetic acid (FAA) solution at 4°C overnight followed with washes of phosphate buffered saline (PBS), pH 7.4, twice. The fixed tissues were subjected to a dehydration procedure with a serial ethanol solutions: 50% ethanol for 1 h, 70% ethanol for 1 h, 90% ethanol for 1 h, and finally 100% ethanol for 3 h. The dehydrated samples were then immersed in 10% potassium hydroxide solution for 2 h and gently mounted on slides for light microscopic visualization with a Leica DM5000 B light microscope.

Histochemical Prussian blue staining of Fe³⁺ accumulation. A protocol for Prussian blue Fe³⁺ staining was followed as previously described for other fungal infections with slight modification (Dangol et al. 2019; Liu et al. 2007). Young leaves or stems of 8-day-old sweetpotato sprouts were spray-inoculated with a *C. fimbriata* BMPZ13 spore suspension. The epidermal peels of leaves or stems were sampled at time 0 and 4 days postinoculation and incubated in 7% (wt/vol) potassium ferrocyanide and 2% (vol/vol) hydrochloric acid (HCl) (1:1, vol/vol) for 15 h at room temperature. Bright Prussian blue pigments, or ferric ferrocyanides resulted from reaction of Fe³⁺ with ferrocyanides inside leaf or stem epidermal cells. Experiments were repeated three times to observe and document Prussian blue pigment accumulation with a Leica DM5000 B light microscope.

Trypan blue staining. To monitor the *C. fimbriata* BMPZ13 hyphal progression and visualize cell death of infected cells, a Trypan blue staining previously described was adapted with slight modifications (Koch and Slusarenko 1990; Takemoto et al. 2003). Sweetpotato leaves were infected with either wounding or nonwounding drop inoculation methods. Four days postinoculation, six disc (1 cm in diameter) of the infected or mock-treated leaf tissues were cleared in ethanol for 24 h and stained by boiling for 3 min in lactophenol trypan blue solution (10 ml of H₂O, 10 ml of lactic acid, 10 ml of glycerol, 10 g of phenol, and 10 mg of trypan blue) followed with cooling to a room temperature. The stained tissues were destained with chloral hydrate solution at 1 g/ml by boiling for 20 min and visualized under a Leica DM5000B microscope. Experiments were repeated three times.

Light microscope. A Leica DM5000 B light microscope (Leica, Germany) was used for visualization and imaging of

samples. Culture samples were directly mounted for microscopic observation of microstructures and the sizes of microstructures were measured with an internal ruler and Leica imaging software. Measurements of 60 microstructures were recorded of each type. Other samples include peels of live epidermis, cleared or stained sweetpotato tissues either inoculated or mock treated at the indicated time points.

SEM. A procedure of fixation previously reported was modified for SEM sample preparation (Cai et al. 2008). Leaves and sprouts were infected with *C. fimbriata* BMPZ13 by nonwounding inoculation method. After infection, 10 epidermal peels of inoculated leaves or stems of sprouts were collected at 6, 24, 48, 72, and 96 h after inoculation. Samples were washed with 0.1 M phosphoric acid buffer (pH 7.2) for 15 min three times following a fixation in 1% osmium acid liquid for 2 h. The fixed tissues were dehydrated with ethanol at a concentration gradient of 30, 50, 60, 70, 80, 90, 95, and 100%, for 30 min at each concentration. The ethanol dehydrated tissues were then subjected to critical point drying with Leica EM CPD030 critical point dryer. The dried tissues were coated with gold and mounted for observation by SEM S3400.

RESULTS

Identification and microstructures of *C. fimbriata* BMPZ13. Species identification and pathogenicity of *C. fimbriata* BMPZ13 was determined on a number of sweetpotato varieties displaying typical black rot disease symptoms (data not shown). To confirm the species identification of *C. fimbriata* BMPZ13, the ITS and LSU regions were PCR-amplified and sequenced. Sequence comparison showed that ITS of *C. fimbriata* BMPZ13 was identical to those of four isolates in China from *Ipomoea batatas* and shared a 99.84% sequence identity to those of Korean sweetpotato isolates (SPL17100 and SPL17101). Similarly, the LSU sequence of *C. fimbriata* BMPZ13 was identical to those of other sweetpotato strains. A phylogenetic analysis determined that BMPZ13 was present in the same clade with reference strains (SPL17100, SPL17101, CMW15049, CBS103.40, and CBS114723) and Chinese strains (Li et al. 2016a, b) (Supplementary Fig. S1).

The *C. fimbriata* BMPZ13 culture became yellowish brown with a smell of fruity odor after incubation on PDA at 25°C for 7 days in the dark. *C. fimbriata* BMPZ13 developed all types of microstructures that were commonly seen with other *C. fimbriata* strains from sweetpotato, albeit with minor differences (Paul et al. 2018). The main characteristics and sizes of microstructures are listed in Table 1.

Flask-shaped conidiophore started to produce cylindrical single-celled conidia after incubation for 5 days at 25°C under a 12 h light photoperiod (Fig. 1A to C). Aleuroconidiophores developed on PDA medium (Fig. 1D) producing rounded aleuroconidia (Fig. 1E and F). The globular perithecia were dark brown with slim necks and usually found near the center of the colony after incubation for 10 days at 25°C under a 12 h light photoperiod, of which the ostiole forming hyphae attenuated from the base to the tip (Fig. 1G). The ascospores produced were helmet-shaped (Fig. 1H and I).

***C. fimbriata* BMPZ13 infects vegetative tissues of sweetpotato.** To determine how *C. fimbriata* BMPZ13 might cause disease through wounding, we assayed the pathogenicity of this fungus on storage roots and vegetative tissues, including shoot cuttings and leaves of the sweetpotato variety Guishu 07-98. When storage roots were inoculated at wound sites, Guishu 07-98 was susceptible to *C. fimbriata* BMPZ13 infection and developed typical black rot disease symptoms after about 7 days, similar to other strains on susceptible sweetpotato varieties (Fig. 2A, Supplementary Table S2) (Paul et al. 2018). Shoot cuttings were drop-inoculated at the base with a *C. fimbriata* BMPZ13 spore suspension. Symptoms of black rot or canker of stems near the inoculation sites appeared 9 days postinoculation. A more severe

chlorosis of upper leaves started to appear about 14 days after inoculation. The wilt symptoms of the shoot developed 9 to 14 days after the appearance of stem cankers, typically followed by eventual death (Fig. 2C on the left, Supplementary Table S2).

TABLE 1. Characteristics of microstructures of *Ceratocystis fimbriata* BMPZ13

Microstructure	Size (μm)	Characteristics
Conidiophores	6.5–8.9 × 38.2–59.3	Flask-shaped and produce conidia (Fig. 1A)
Conidia	3.3–7.6 × 6.8–40	Cylindrical (Fig. 1B and C)
Aleuroconidiophores	4.8–9.2 × 10.4–48.7	Produce aleuroconidia (Fig. 1D)
Aleuroconidia	8.3–13.2 × 11.9–20.4	Round, thick walls, slightly brown (Fig. 1E and F)
Perithecia	Base: 161.3–343.7 Neck: 446.3–627.6 Ostiole forming hyphae: 22.3–35.2 Ostiole diameter: 14.9–24.7	Globular, dark brown (Fig. 1G)
Ascospores	4.3–6.6 × 5.9–8.4	Helmet-shaped (Fig. 1H and I)

All leaves inoculated through wound sites developed typical symptoms of necrosis around the inoculation site and fine veins turned necrotic beyond the inoculation site (Fig. 2D, left), while no symptoms were seen following mock inoculation (Fig. 2D, right). Moreover, necrotic lesions were developed on all wound-inoculated leaves at locations distal from inoculation sites and *C. fimbriata* BMPZ13 could be recovered from these distal lesions (Supplementary Fig. S2).

After incubation of tissues inoculated through a nonwounding procedure in a growth chamber at 25°C with 16 h light and 8 h darkness, necrotic lesions developed on tender leaves and stems of sprouts at about 3 days after spray inoculation (Fig. 2E and F, Supplementary Table S2). Small microscopic necrosis developed on leaves 3 to 5 days after drop inoculation with *C. fimbriata* BMPZ13 (Fig. 2G), with typical necrotic veins and chlorosis appearing 7 to 10 day postinoculation (Fig. 2H), while the mock treated leaves remained normal (Fig. 2I and 2J). When sweetpotato storage roots were spray- or drop-inoculated with spore suspensions in a nonwounding manner, only minor necrotic lesions were observed on adventitious buds, while no obvious symptoms developed on storage roots (data not shown). Time for disease

Fig. 1. Microstructures of *Ceratocystis fimbriata* BMPZ13. **A**, Conidiophore. **B** and **C**, Cylindrical conidia. **D**, Aleuroconidiophores. **E**, Aleuroconidia. **F**, Aleuroconidia and an endoconidium. **G**, Long neck perithecium. **H** and **I**, Ascospores. Microstructures of *C. fimbriata* BMPZ13 observed with light microscopy are shown in **A**, **B**, **D**, **E**, **G**, and **H** and scanning electron microscopy in **C**, **F**, and **I**.

development on different tissues varied slightly with different inoculation methods (Supplementary Table S2).

***C. fimbriata* BMPZ13 infection involves a two-step biotrophic phase.** To elucidate the entry strategy and subsequent progression

of *C. fimbriata* BMPZ13 infection in the absence of host wounding, a spray inoculation procedure was deployed, which more closely mimics natural infection. Young sprouts and leaves of sweetpotato variety Guishu 07-98 were spray-inoculated with a *C. fimbriata*

Fig. 2. *Ceratocystis fimbriata* BMPZ13 infects vegetative tissues of sweetpotato. **A**, Symptoms of storage root infected with *C. fimbriata* BMPZ13 8 days after wounding drop inoculation. **B**, Mock (H_2O) inoculation control for **A**. **C**, Cutting infected (left) and mock inoculation (right). Shown are symptoms of wilt and rot on stem base 15 days after inoculation. **D**, Leaf infected by wounding drop inoculation (left) and mock (right) 5 days after inoculation. **E**, Symptoms of sprouts infected with nonwounding spray inoculation. Shown are the symptoms of young leaf and stems 5 days after inoculation. **F**, Mock (H_2O) inoculation control for **E**. **G and H**, Symptoms of leaves 3 days (**G**) and 9 days after nonwounding drop inoculation (**H**). **I and J**, Mock (H_2O) inoculation control for **G** and **H** at the same time points after inoculation, respectively. Pathogenicity assays were repeated three times with consistent results and disease symptoms.

BMPZ13 spore suspension. The rod-shaped conidia asymmetrically germinated from one side of the middle cell, forming a T-shaped germinating tube structure within 6 h (Fig. 3A and Supplementary Fig. S3A). The germinated tube developed a runner hypha and grew on the epidermal surface. Runner hyphae extended to several hundred micrometers in length and entered epidermal cells within 24 h or continued growing until reaching a suitable entry site. The majority of runner hyphae did not form special structures for penetration of the cuticle, typically penetrating directly ~2 days postinoculation (Fig. 3C, Supplementary Fig. S3A). Veins and stem epidermis were infected in a similar fashion (Supplementary Fig. S3C and D). However, hyphae with enlarged tips were observed occasionally (<2% of total hyphal entry events) at penetration sites on the epidermis (Fig. 3B). Runner hyphae were also found to penetrate peltate glandular trichomes in 100% of observed samples infected 2 to 3 days after conidia germination (Fig. 3D, Supplementary Fig. S3E). Interestingly, peltate glandular trichomes were found to be more preferable sites for hyphal entry, accounting for 67.60 and 87.70% of total observed entry events on infected sprout stems and leaves, respectively, compared with 32.40 and 12.30% entry events that were direct cuticle penetration (Table 2). In contrast, no hyphal penetration of stoma was observed. Typically, *C. fimbriata* BMPZ13 runner hyphae bypassed stomata on the epidermis (Supplementary Fig. S3F).

After penetration of the epidermal layer, the runner hyphae differentiated into two types of invasive hyphae (IH): intercellular

IH and intracellular IH. Intercellular IH grew between epidermal and mesophyll cells before entering epidermal cells or leaf veins (Fig. 4A and B). Intercellular IH were less segmented and thicker than normal runner hyphae, with long tubular structures possessing a brown cell wall. Intercellular IH were the dominant IH forms during continuous infection between cells, especially around vascular bundles (Fig. 4A to E). Intracellular IH and intercellular IH could be transformed reciprocally. Intercellular IH entered living epidermal cells and transformed into irregular swollen oval-shaped and nodose intracellular IH (Fig. 4B and Supplementary Fig. S4A). Both intercellular IH and intracellular IH were observed in cells adjacent to peltate glandular trichomes (Fig. 4C).

Hyphae extended and reached the proximity of leaf veins 4 days after inoculation, and a large number of oval-shaped intracellular IH accumulated in the mesophyll cells surrounding vascular bundles (Fig. 4D and Supplementary Fig. S4B). Intercellular IH also abundantly grew along veins promoting continuous infection after 5 to 6 days in vascular tissues, eventually leading to whole leaf chlorosis (Fig. 4D and E, Supplementary Fig. S4B).

Intercellular hyphae were often seen to extend out of stoma (Fig. 4F, Supplementary Fig. S4C) or the leaf epidermal septum, at a late stage (7 to 10 days) of infection (Supplementary Fig. S4D). Conidia and aleuroconidia were produced on infected tissues generally at 7 days after inoculation (Supplementary Fig. S5A to D).

Fig. 3. Invasion of *Ceratocystis fimbriata* BMPZ13 into leaf tissues of sweetpotato. **A**, A germinating conidium on the leaf epidermis of sweetpotato. **B**, Enlarged tip of a *C. fimbriata* BMPZ13 hypha at the site of invasion 2 days postinoculation. **C**, An invasive hypha penetrating the epidermis of a sweetpotato leaf. **D**, Hyphae invading peltate glandular trichomes of sweetpotato. The leaf tissues (A to D) were infected with *C. fimbriata* BMPZ13 with the nonwounding drop inoculation method. RH, runner hyphae; P, peltate glandular trichome.

Peltate glandular trichomes are targeted by *C. fimbriata* BMPZ13. As peltate glandular trichomes were preferred penetration sites (Fig. 3D and 4C, Supplementary Table S2), the ability of *C. fimbriata* BMPZ13 to target peltate glandular trichomes was monitored. Directional growth of runner hyphae toward peltate glandular cells on the leaf epidermis was often observed (Fig. 5A). Subsequently, penetration typically ensued, followed by hyphae passing through the eight-cell-structured peltate glandular trichomes 2 to 3 days after spray inoculation (Fig. 5B). Growth of runner hyphae was routinely extended to other peltate glandular trichomes to form a hyphal network associated with these structures 5 days postinoculation (Fig. 5C). Runner hyphae developed lateral secondary hyphae, which also targeted peltate glandular trichomes 4 to 5 days after inoculation (Fig. 5D). Hyphae accumulated over time, eventually resulting in the destruction of peltate glandular trichomes 5 days after inoculation (Fig. 5E and F).

Necrotrophic phase of *C. fimbriata* BMPZ13 infection. Necrotrophic growth of *C. fimbriata* BMPZ13 was assessed with trypan blue staining to visualize cell death associated with infection (Joo et al. 2005; Koch and Slusarenko 1990; Takemoto et al. 2003). *C. fimbriata* BMPZ13 infection led to microscopic lesions (Fig. 6B), while mock inoculation on stems of sprouts resulted in no visible lesions (Fig. 6A). Necrotic lesions occurred as early as

4 days post-spray inoculation. Necrotic veins were commonly observed, which may have resulted from aggressive growth of IH (Figs. 6B and 4D and E). In contrast, wound inoculation led to more rapid cell death in the proximity of the inoculation site within 2 days, and similarly, necrotic veins extended to distal areas routinely at 4 days postinoculation (Fig. 6C).

Recently, iron-dependent ferroptotic cell death was reported in rice-*Magnaporthe oryzae* interactions (Dangol et al. 2019). To determine whether *C. fimbriata* BMPZ13 employs a similar mechanism in its necrotrophic growth, Prussian blue staining was applied to examine ferric accumulation at infection sites (Dangol et al. 2019; Liu et al. 2007). Blue pigment sedimentation was observed in cells of the stem of sprouts infected with *C. fimbriata* BMPZ13 2 days after spray inoculation, indicating iron accumulation is indeed involved in the interaction between sweetpotato and *C. fimbriata* (Fig. 6D and F). More interestingly, iron accumulation was also seen in cells adjacent to peltate glandular cells (Fig. 6E and F), consistent with *C. fimbriata* infection being linked to peltate glandular trichomes (Figs. 4C and 5).

DISCUSSION

ITS and LSU sequences of *C. fimbriata* BMPZ13 are identical to those of all Chinese isolates or share high similarity (99.84%) to Korean isolates in the comparison (Supplementary Fig. S1). The measurements of microstructures were also similar to those reported (Paul et al. 2018). This indicates that *C. fimbriata* BMPZ13 is most likely the same lineage originating from the clonal haplotypes isolated from sweetpotato in China, supporting the hypothesis that *C. fimbriata* was introduced to Japan on sweetpotato storage roots and then introduced to China in the 1930s (Li et al. 2016a, b).

TABLE 2. Frequency of hyphal penetration events into cuticle and peltate glandular trichomes

	Cuticle	Peltate glandular trichome	Total penetration events	<i>t</i> test
Stem	32.40%	67.60%	68	<i>P</i> = 0.00018
Leaf	12.30%	87.70%	140	<i>P</i> = 0.00417

Fig. 4. *Ceratocystis fimbriata* BMPZ13 develops invasive hyphae (IH). **A**, An intercellular IH formed after penetration of the sweetpotato leaf cuticle. **B**, A tubular intercellular IH entering into a leaf epidermal cell of sweetpotato and subsequent formation of nodose intracellular IH in a sweetpotato leaf cell observed with scanning electron microscopy (SEM). **C**, IH in leaf cells near peltate glandular trichomes and **D**, in cells adjacent to leaf veins. **B to D**, Labels and arrows in black color denote intracellular IH and the ones in white indicate intercellular IH. **E**, IH grow along adjacent leaf veins. **F**, Intercellular hyphae under epidermis grow out of stoma on leaf. Images shown in A, B, E, and F were taken with SEM and images in C and D were taken with light microscope. A to C show the inner side of leaf epidermal peel. All images were captured from leaf tissues infected with *C. fimbriata* BMPZ13 by nonwounding drop inoculation method. P, peltate glandular trichome; RH, runner hyphae; and S, stoma.

The *C. fimbriata* species complex specialized for infection of sweetpotato is an economically important pathogen and a major factor in postharvest infection leading to both significant yield losses and a marked decrease in storage root quality (Baker et al. 2003). The toxic phytoalexin, ipomeamarone, produced in sweetpotato storage roots in response to *C. fimbriata* infection is a serious health concern for humans as well as livestock (Akazawa and Wada 1961; Imaseki and Ikuzo 1964). To date, it has remained largely unclear how this specialized pathogen might infect host tissues during the vegetative growth of sweetpotato, which would potentially constitute an important component of disease cycle. Here we determined that besides storage roots, *C. fimbriata* can also effectively infect vegetative tissues of sweetpotato through wounds and in the absence of wounds. Previously, only necrotic lesion symptoms of stems of sweetpotato vines were reported in greenhouse trials (Stahr and Quesada-Ocampo 2019). Our pathogenicity assays demonstrated *C. fimbriata* BMPZ13 caused necrotic diseases on sprouts, leaves, and stems when inoculated through wounds and in the absence of wounds. We show *C. fimbriata* is a versatile pathogen deploying a unique strategy to infect live sweetpotato vegetative tissues in the absence of wounding. *C. fimbriata* BMPZ13 shares similar characteristics with hemibiotrophic pathogenesis, but its infection strategy also has distinctive features (Presti et al. 2015; van Kan 2006). Hemibiotrophs such as *M. oryzae* or *Colletotrichum* spp., produce dome-shaped and melanized appressoria responsible for mechanical entry into the host, utilizing the accumulation of turgor pressure to penetrate the cuticle (Tucker and Talbot 2001). Similar to phytopathogenic necrotrophs, *C. fimbriata* BMPZ13 does not form appressorium-like structures for penetration, even though in rare cases inconspicuous enlarged tips were seen (Fig. 3B), but possess the ability to promote direct cell entry through the sweetpotato cuticle (Fig. 3) (Presti et al. 2015).

Our observations support a two-step biotrophic infection for *C. fimbriata* that is strikingly different from those of classical

necrotrophs, which develop only intercellular IH after cell entry through the cuticle. Initial intercellular IH of *C. fimbriata* BMPZ13 form after penetration and grow among epidermal cells, subsequently entering living cells to form intracellular IH (Fig. 4), which is characteristic of pathogenic fungal biotrophic growth (Presti et al. 2015). The ability to form both intercellular and intracellular IH among or within live cells indicates that *C. fimbriata* BMPZ13 has a typical biotrophic phase during the early stage (3 to 5 days after inoculation) of vegetative tissue infection (Fig. 4 and Supplementary Fig. S4). In contrast, pathogenic necrotrophs continue with the necrotrophic phase, killing host cells after a short biotrophic phase of intercellular IH (Oliver and Ipcho 2004).

C. fimbriata appears to have evolved a lifestyle more similar to that of hemibiotrophs, with a two-step biotrophic phase coupled with a necrotrophic phase of infected tissues, where host cell death is mainly associated with leaf veins. This two-step biotrophic phase is distinct from that of typical hemibiotrophs, which involves rapid development of intracellular IH structures following appressorium-mediated penetration (Presti et al. 2015).

For initial infection, *C. fimbriata* does not penetrate stoma openings. This is in contrast to facultative biotrophs, including *Passalora fulva* (*Cladosporium fulvum*), which favors stomatal entry for primary infection (De Wit 1977), together with many other fungal pathogens (Presti et al. 2015). In addition to direct entry through the cuticle, our results demonstrated that *C. fimbriata* is also capable of entering sweetpotato tissues through peltate glandular trichomes (Figs. 3, 4, and 5; Table 2), indicating *C. fimbriata* targets peltate glandular trichomes for infection, a unique strategy for a fungal pathogen that has not been reported previously.

This suggests peltate glandular trichomes are a novel target for infection and *C. fimbriata* utilizes a specialized strategy for pathogenesis of sweetpotato vegetative tissues, which is radically different from any known mechanisms employed by other fungal

Fig. 5. Peltate glandular cells are targets of *Ceratocystis fimbriata* BMPZ13 hyphae during the infection process. **A**, Hyphae grow toward peltate glandular trichomes. **B**, Hyphae penetrate a peltate glandular trichome. **C**, Hyphae form a network associated with peltate glandular trichomes. **D to F**, Runner hyphae accumulate near peltate glandular trichomes on the leaf epidermis of sweetpotato leading to destruction of these structures. All images were captured from leaf tissues infected with *C. fimbriata* BMPZ13 by a nonwounding drop inoculation method. H, hyphae; P, peltate glandular trichomes; RH, runner hyphae; and S, stoma.

pathogens (Presti et al. 2015). Unlike nonglandular trichomes, peltate glandular trichomes are specialized secretory structures that produce, store, and secrete metabolites, including volatile as well as nonvolatile chemicals and essential oils (Glas et al. 2012; Li et al. 2009, 2014). Glandular trichomes are common structures found in approximately 30% of all vascular plants (Duncan 1973; Glas et al. 2012; McDonald and Austin 1990). Trichomes are thought to be involved in plant defense (Levin 1973): nonglandular trichomes serve as physical barriers to thwart insects, while glandular trichomes provide a complementary chemical barrier against attempted pathogen ingress (Glas et al. 2012; Levin 1973; Weinhold and Baldwin 2011). A particularly prominent class of secondary metabolites secreted by glandular trichomes include terpenoids, which typically have defensive functions against herbivorous insects in addition to fungal pathogens (Carter et al. 1989; Langenheim 1994; Mayer 2006; Weinhold and Baldwin 2011). In this context, ipomeamarone is a furanoterpenoid and thus may also be produced in sweetpotato glandular trichomes.

Interestingly, the clavicipitaceous endophytic fungus, *Periglandula ipomoeae*, establishes a symbiotic relationship with *Ipomoea asarifolia*, a plant from the family *Convolvulaceae*, and the mycelium of *P. ipomoeae* localizes to peltate glandular trichomes that may strengthen its association with host tissues (Leistner and Steiner 2009, 2018; Steiner et al. 2006, 2011). In addition, it has been postulated that peltate glandular trichome-secreted metabolites might constitute a source of nutrients for these fungi (Steiner et al. 2008). Thus, *C. fimbriata* may exploit sweetpotato glandular trichomes in a similar manner to promote successful pathogenesis. The unique behavior of *C. fimbriata* entering peltate glandular trichomes dramatically contrasts to the infection strategies of other pathogenic or symbiotic fungi that enter the host plant by either direct cell penetration or via stomatal openings (Presti et al. 2015), suggesting *C. fimbriata* may have evolved a distinct mechanism

of infection that targets the specialized secretory structures of its host.

Compared with the rot of storage roots, vegetative tissues of sweetpotato spray-infected with *C. fimbriata* BMPZ13 produce fewer macroscopic lesions than typical necrotrophs. Infection by *C. fimbriata* BMPZ13 lead to gradual chlorosis of the whole leaf, while infected seedling cuttings developed black rot on the stem base, followed by chlorosis and eventual wilt of entire branches 11 to 22 days after inoculation (Fig. 2B), similar to those caused by *C. fimbriata* on the Peruvian carrot (*Arracacia xanthorrhiza*), a nonwoody herbaceous host (Melo et al. 2016). For woody plants, wilt and canker caused by the *C. fimbriata* complex produces similar symptoms in kiwifruit (*Actinidia* spp.) (Piveta et al. 2016), mango (*Mangifera indica* L.) (Oliveira et al. 2015), pomegranate (Huang et al. 2003; Somasekhara 1999), plane tree (*Platanus* spp.) (Clerivet et al. 2000), and *Caryocar brasiliense* trees (da Silva et al. 2017). Wilt and subsequent plant death appears to be associated with colonization of the xylem, which may result in blockage of the vascular tissue (Araujo et al. 2014, 2016). Our results show that inoculation at wound sites enabled *C. fimbriata* BMPZ13 to form necrotrophic hyphae along veins, which likely progressed through the vascular system. We posit that *C. fimbriata* is a specialized vascular-associated pathogen, as evidenced by necrotic lesions that extended to surrounding leaf veins, damaging the vascular system (Fig. 4). Cytohistological observations showed intercellular IH grew rapidly within veins, facilitating necrotrophic growth marked by extensive necrosis in veins. This is consistent with *C. fimbriata* being a vascular pathogen on sweetpotato vegetative tissue (Fig. 4).

Our data also indicate a role for iron in *C. fimbriata* pathogenesis, although the underlying mechanism remains to be established (Verbon et al. 2017). In this context, iron-associated ferroptosis was observed, which has also recently been reported during an incompatible interaction of *Magnaporthe oryzae* with rice (Dangol

Fig. 6. *Ceratocystis fimbriata* BMPZ13 infection leads to necrosis and ferric accumulation in infected leaf tissues. **A**, Mock inoculated leaf. **B**, Necrotic lesion on leaf following nonwound drop inoculation of *C. fimbriata* BMPZ13. **C**, Lesion at wound inoculation site and vein necrosis on leaf tissues. **D**, Mock inoculation does not result in pigment formation in uninfected cells near peltate glandular trichomes. **E**, Infected peltate glandular trichomes and cells around these structures of leaf tissues form blue pigments derived from Prussian blue staining reporting ferric iron accumulation. **F**, An enlarged area showing infected leaf cells with blue pigment accumulation resulting from Prussian blue staining. Shown in **A**, **B**, **D**, **E**, and **F** are infected leaf tissues by nonwounding drop inoculation method with either H₂O (**A** and **D**) or *C. fimbriata* BMPZ13 (**B**, **E**, and **F**). Shown in **C** is infected leaf tissue by wounding drop inoculation method. Arrows indicate ferric iron accumulation marked by Prussian blue staining. In **A** to **C**, bar = 40 μm.

et al. 2019). In contrast, our data show a role for iron-associated ferroptosis during a compatible interaction of *C. fimbriata* with sweetpotato, suggesting the underlying mechanism of iron involvement in cell death associated with necrotrophic growth of *C. fimbriata* is distinct from that of *M. oryzae* triggered during the immune response. Collectively, our findings establish a novel mechanism of fungal pathogenesis: infection of peltate glandular trichomes. This data may help guide future control strategies for *C. fimbriata* infection of sweetpotato.

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