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Colletotrichum destructivum from cowpea infecting *Arabidopsis thaliana* and its identity to *C. higginsianum*

Hu Sun · Jing-Ze Zhang

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Abstract *Colletotrichum* isolates isolated from cowpea in the Hangzhou area of China were identified as *C. destructivum* based on morphological characteristics, pathogenicity tests, sequence analysis of the internal transcribed spacer (ITS)1, 5.8S RNA gene and ITS2 regions of ribosomal DNA and the infection process. The ability of the *C. destructivum* isolates to infect *Arabidopsis thaliana* was investigated under laboratory conditions and showed a two-phase hemibiotrophic infection process. In addition, the sequences of the rDNA ITS region of *C. destructivum* isolates from cowpea were identical with 100% similarity to that of isolates of *C. higginsianum* originating from cruciferous plants. This article presents new evidence in support of *C. higginsianum* as a synonym of *C. destructivum.*

Keywords Cowpea anthracnose · Biotrophy · rDNA-ITS · Infection process

Introduction

The large ascomycete genus *Colletotrichum* is one of the most economically important groups of plant pathogens, causing anthracnose disease on a wide range of crops. *Colletotrichum* species develop a series of specialised infection structures including germ tubes, appressoria, intracellular hyphae, and secondary necrotrophic hyphae (Bailey and Jeger 1992). Many species have evolved a hemibiotrophic strategies for colonisation of plants (Perfect et al. 1999). These fungi therefore provide excellent models for studying the molecular and cellular bases of fungal pathogenicity (Dufresne et al. 2000; Perfect et al. 2000; Yang and Dickman 1999; Shimada et al. 2006). In addition, *Colletotrichum* species have the advantage of being haploid organisms, which can be cultured axenically and transformed. This greatly facilitates mutational analysis and the critical assessment of gene function by targeted gene disruption.

More recently, it has been reported that isolates of *C. higginsianum*, which originated from cruciferous plants, infected *Arabidopsis thaliana* (Narusaka et al. 2004; O'Connell et al. 2004). These findings provide a new *Arabidopsis* pathosystem. *Colletotrichum higginsianum* was reported to cause typical anthracnose lesions on a wide range of cruciferous plants (Higgins 1917; Scheffer 1950; Sutton 1980). It was regarded as a distinct species on the basis of conidial morphology and consistent association with cruciferous hosts (Sutton 1980; 1992). Differences between *C. higginsianum* and *C. destructivum* are hardly distinguished by the morphology of their conidia and appressoria, and the infection process of the former closely resembles that of *C. destructivum* on other hosts

H. Sun · J.-Z. Zhang (✉)
Institute of Biotechnology, College of Agric. & Biotechnology, Zhejiang University,
Kaixuan road 258,
Hangzhou 310029, P.R. China
e-mail: jzzhang@zju.edu.cn

(e.g. cowpea, alfalfa, and tobacco). Likewise, the rDNA sequences of *C. higginsianum* isolates are closely related to *C. destructivum*. O'Connell et al. (2004) suggested that *C. higginsianum* should be accommodated within the species concept of *C. destructivum*. However, no evidence showed that the sequence differences between *C. higginsianum* isolates and *C. destructivum* isolates on other hosts were within levels of intraspecific variability of *C. destructivum*. In addition, there have been no reports of any *C. destructivum* isolates, from the family Leguminosae, causing infection of *A. thaliana*.

In this report, we demonstrate that isolates of *C. destructivum* from cowpea (*V. unguiculata*) in the Hangzhou area of China are able to infect *A. thaliana* in laboratory experiments. In addition, the sequences of internal transcribed spacer (ITS)1, 5.8S RNA gene and ITS2 regions of *C. destructivum* isolates from cowpea and *C. higginsianum* isolates from cruciferous hosts were analysed. The phylogenetic relationship between *C. destructivum* and *C. higginsianum* are discussed.

Materials and methods

Collection of *Colletotrichum* isolates

The isolates of *Colletotrichum* spp. used in this study were obtained from anthracnose lesions on the leaves of *Vigna unguiculata* cv. Xinyou 1 grown in the grounds of Zhejiang University, Hangzhou, China. Conidia removed directly from sporulating acervuli, or small pieces of diseased tissues taken from the margin of diseased leaves, were sterilised for up to 5 min in 0.5% hypochlorite before being placed onto PDA (potato dextrose agar) containing streptomycin. Isolates of interest were transferred to PDA, and single-conidial isolates were prepared from each isolate. They were cultured on PDA at 25°C. Plugs (5 mm) were cut from the margin of fresh colonies and stored in 20% glycerol in a freezer at −70°C.

Sequencing and analysis of rDNA-ITS regions

Fungal mycelium was produced by shaking (120 r.p.m. for 3 days at 25°C) in liquid growth media (PD broth, PDA devoid of agar) and collected on filter papers, ground in liquid N_2 and freeze-dried. Established methods were used for DNA extraction and amplification, and sequencing of rDNA-ITS regions (Zhang et al. 2005). Sequence files were assembled and edited and consensus sequences were constructed using DNAMAN 4.0 (Lynnon bioSoft). The ITS sequences generated in this study were submitted to GenBank database, and compared to those already deposited in GenBank using the Basic Local Alignment Search Tool (BLAST). The ITS sequences for reference isolates were downloaded from GenBank. All sequences were aligned using CLUSTAL X (Thompson et al. 1994). Phylogenetic analyses were performed using test Version 4.0b10 (PPC) of PAUP (David Swofford, Smithsonian Institution, Washington DC.). Phylogenetic trees were inferred from the ITS sequence data set using parsimony analysis and all characters weighted equally and unordered.

Plant cultivation

Seeds of *V. unguiculata* (cvs XinYou 1, TeZao 30, CunqiuHong and ZhiJiang 884) and *Lens culinaris* were purchased from the Hangzhou Seed Company, Zhejiang province, China. The cv. Xinyou 1 has been shown to be especially susceptible to the anthracnose pathogen in the field. Seeds of *A. thaliana* and tobacco were obtained from the Institute of Horticulture, Zhejiang University.

In addition to *A. thaliana*, all seeds were surface-disinfected with 10% hypochlorite for 5–8 min, rinsed exhaustively with distilled water, soaked in distilled water for 10 min, sown in pasteurised potting medium (vermiculite: perlite = 3:1) and maintained at 25°C in a growth chamber using a 12-h photoperiod.

Seeds of *A. thaliana* were soaked overnight, sterilised for 12 min by 10% hypochlorite and washed 4–5 times. These seeds were then planted in 0.8% agar medium containing 1/2 MS inorganic salt and transferred to a growth chamber with a 16-h photoperiod at an intensity of ca. 150 μmol $s^{-1}m^{-2}$ photosynthetically active radiation, and day and night temperatures of 23°C and 18°C, respectively, after incubation in the dark at 4°C for two days. After 7–10 days, young seedlings were transplanted into the pasteurised potting medium and grown in a 12-h photoperiod at an intensity of ca. 400 μmol $s^{-1}m^{-2}$ photosynthetically active radiation and night temperatures of 23°C and 18°C, respectively.

Fungal culture and inoculation of plants

Plugs were removed from cryotubes and transferred to Petri dishes containing PDA. Colonies were grown at 25°C under a mixture of white and near-UV light with a 12 h photoperiod. Conidia and setae taken from 7 day-old cultures were examined for size and shape. To produce appressoria *in vitro*, drops (50 µl) of conidial suspension (10^{-5} spores ml^{-1}) were placed on polystyrene Petri dishes and incubated in a humid chamber at 25°C.

Arabidopsis thaliana plants were inoculated 6 weeks after sowing before the start of flowering. All other plant species were grown to the four- to six-leaf stage before being used for inoculation. All plants were inoculated by spraying with a conidial suspension (1×10^6 spores ml^{-1}) using an atomiser. After covering the plants with plastic bags, they were incubated under the above conditions. Control plants were treated with sterile deionised water.

Microscopy

Pieces were cut from the inoculated leaves and decolourised in a 0.15% (*w/v*) solution of trichloroacetic acid in a 3 : 1 (*v/v*) mixture of ethanol and chloroform for 14 h. They were then stained in a 0.025% (*w/v*) solution of Aniline Blue in lactophenol for 3–4 h. Light microscopic examinations were made with a Zeiss Axiophot 2 microscope with Axiocam CCD camera and Axiovision digital imaging software (AxioVision Software Release 3.1., ver. 3-2002; Carl Zeiss Vision Imaging Systems).

Results

Pathogen morphology

Three isolates of *Colletotrichum* spp. (CD-hz 01, CD-hz 02 and CD-hz 03) were obtained from diseased leaves of cowpea. The colony morphology of three isolates was similar on PDA, with grey-white to grey aerial hyphae. They produced orange conidia in mass and sporulated profusely. All the isolates had straight, cylindrical conidia with obtuse apices. Conidia of CD-hz 01, CD-hz 02 and CD-hz 03 were 18.36± 1.11 µm long (mean ± standard deviation of 100 spores) by 3.96±0.28 µm wide, 18.11±1.35 by 4.01± 0.28 µm and 18.85±0.96 by 3.91±0.24 µm, respectively. Three isolates were not significantly different ($P<0.005$) in length and width. Mature appressoria of all three isolates generally were subglobose (approximately 8 by 6 µm) with an irregular, lobed margin on hydrophobic polystyrene (Fig. 1).

Sequencing and analysis of rDNA-ITS regions

Nucleotide sequences of the ITS regions of ribosomal (r)DNA (ITS1 and ITS2) and the 5.8S RNA gene were determined and submitted to GenBank for the three test isolates (Table 1). A blast search of GenBank nucleotide database showed that three isolates were 100% identical to the isolates of *C. higginsianum* and very similar to that of *C. destructivum*. In order to illustrate their level of similarity, one reference isolate of *C. destructivum* (LARS 056) and two *C. higginsianum* isolates (MAFF 305635 and MAFF 238563) were downloaded from GenBank. In addition, three *C. higginsianum* isolates (IMI 349061, IMI 349063A and IMI 349063B) also were downloaded and included in the comparative analysis (Table 1).

The three test isolates had an identical sequence and were 100% identical to that of two *C. higginsianum* and three *C. destructivum* isolates (originated as *C. higginsianum*), which all derived from cruciferous plants. However, the sequences of three test isolates were 98.7% identical to that of the *C. destructivum* isolate (LARS 056) also from cowpea (Fig. 2). All reference isolates from cruciferous plants were iden-

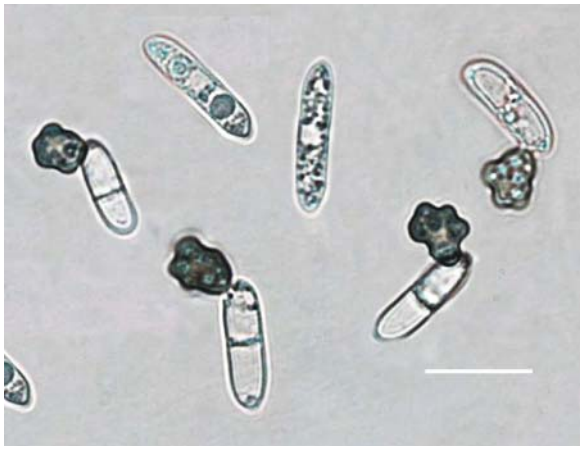

Fig. 1 Conidia (C) of *Colletotrichum destructivum* isolate CD-hz 01 germinating on polystyrene to form irregularly-shaped, melanised appressoria. Bar=10 µm

Table 1 Origins of the *Colletotrichum* isolates used in this study and the reference isolates used for ITS ribosomal DNA sequence comparisons

Culture collection numbers	*Colletotrichum* spp.	Host plant	Country of origin	GenBank accession no.
Isolates used				
CD-hz 01	*C. destructivum*	*Vigna unguiculata*	China	EU070911
CD-hz 02	*C. destructivum*	*V. unguiculata*	China	EU070912
CD-hz 03	*C. destructivum*	*V. unguiculata*	China	EU070913
Reference isolates				
LARS 056	*C. destructivum*	*V. unguiculata*	Nigeria	AJ558108
N150	*C. destructivum*[a]	*Nicotiana tabacum*	France	AF320564
ATCC10921	*C. destructivum*[a]	*Nicotiana tabacum*	America	AF320562
LARS 202	*C. destructivum*[b]	*Medicago sativa*	Morocco	AJ558106
LARS 319	*C. destructivum*[b]	*Medicago sativa*	Canada	AJ558107
UQ343	*C. destructivum*	*Lens culinaris*	Australia	AF451908
MAFF 238563	*C. higginsianum*	*Matthiola incana*	Japan	AB042303
MAFF 305635	*C. higginsianum*	*Brassica rapa*	Japan	AB042302
IMI 349061	*C. destructivum*(orig. as *C. higginsianum*)[c]	*B. campestris*	Trinidad	AJ558109
IMI 349063A	*C. destructivum*(orig. as *C. higginsianum*)[c]	*B. campestris*	Trinidad	AJ558110
IMI 349063B	*C. destructivum*(orig. as *C. higginsianum*)[c]	*B. campestris*	Trinidad	AJ558111
BBA 70709	*C. trifolii*	*Trifolium*	USA	AJ301941
TSG001	*C. gloeosporioides*[d]	*Diospyros kaki* cv. Wuheshi	China	AY787483
BBA 67866	*C. acutatum*	*Fragaria* sp.	Denmark	AJ301950
MAFF 511343	*C. graminicola*	Unknown	Japan	AB057436

[a] Shen et al. (2001)

[b] Latunde-Dada et al. (1996, 1997)

[c] O'Connell et al. (2004)

[d] Zhang et al. (2005)

tical. A phylogenetic analysis including other *Colletotrichum* spp. showed that the three test isolates formed a single group together with the reference isolates of *C. destructivum* and *C. higginsianum* supported by 99% bootstrap (Fig. 3). Previous studies have shown the high level of similarity between *C. destructivum* and *C. higginsianum* isolates in rDNA regions and these were suggested to be a single species (O'Connell et al. 2004). For this reason, isolates CD-hz 01, CD-hz 01, and CD-hz 01 will be referred to hereafter as *C. destructivum*.

Pathogenicity test

The pathogenicity of the three isolates of *C. destructivum* was tested by inoculating the original host and some of the potential hosts from the families Leguminosae, Cruciferae, and Solanaceae. These plant species were selected from the reported hosts of *C. destructivum* and *C. higginsianum* and their responses to the three isolates were identical. In the families Leguminosae, the *V. unguiculata* cvs Xinyou 1 and Tezao 30 were susceptible to *C. destructivum*, cvs Cunqiuhong and Zhejiang 884 were resistant and the only lentil (*Lens culinaris*) was completely resistant (Table 2). In the cultivars of cowpea, seedlings of the cvs Xinyou 1 and Tezao 30 showed the visible symptom of water-soaked anthracnose lesions at 5 days after inoculation, and no symptoms on seedlings of the cvs Cunqiuhong and Zhijiang 884 were visible until 6 days after inoculation. When lesions developed on the susceptible cultivars, a small lesion enlarged or several neighbouring lesions coalesced into a large lesion by 8 days (Fig. 4a). In contrast, many small, restricted, necrotic flecks were observed on inoculated surfaces of the resistant

Fig. 2 Aligned sequences of the ITS 1, 5.8S RNA gene and ITS2 of *Colletotrichum* isolates. A dash indicates an introduced gap. The alignment was generated using CLUSTAL W (Thompson et al. 1994)

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CD-hz-01      ACATACCTCAACTGTTGCTTCGGCGGGCAGGAGGACAACCCCCCTCGGGGGGCGGTCCCCCTCCCGGCCG
CD-hz-02      ********************************************************************************
CD-hz-03      ********************************************************************************
IMI-349063A   ********************************************************************************
IMI-349063B   ********************************************************************************
IMI-349061    ********************************************************************************
MAFF-305635   ********************************************************************************
MAFF-238563   ********************************************************************************
LARS-056      ******************************************TC--**********************************

CD-hz-01      CGCCCTCACGGGCGTGGCGCCCGCCGGAGGATACCAAACTCTATTTTAACGACGTTTCTTCTGAGTGGCA
CD-hz-02      ********************************************************************************
CD-hz-03      ********************************************************************************
IMI-349063A   ********************************************************************************
IMI-349063B   ********************************************************************************
IMI-349061    ********************************************************************************
MAFF-305635   ********************************************************************************
MAFF-238563   ********************************************************************************
LARS-056      ******************************************************C*************************

CD-hz-01      CAAGCAAATAATTAAAACTTTTAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATG
CD-hz-02      ********************************************************************************
CD-hz-03      ********************************************************************************
IMI-349063A   ********************************************************************************
IMI-349063B   ********************************************************************************
IMI-349061    ********************************************************************************
MAFF-305635   ********************************************************************************
MAFF-238563   ********************************************************************************
LARS-056      ********************************************************************************

CD-hz-01      CGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGCA
CD-hz-02      ********************************************************************************
CD-hz-03      ********************************************************************************
IMI-349063A   ********************************************************************************
IMI-349063B   ********************************************************************************
IMI-349061    ********************************************************************************
MAFF-305635   ********************************************************************************
MAFF-238563   ********************************************************************************
LARS-056      ********************************************************************************

CD-hz-01      TTCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCCCAGCTTGGTGTTGGGGCCCTACGGT
CD-hz-02      ********************************************************************************
CD-hz-03      ********************************************************************************
IMI-349063A   ********************************************************************************
IMI-349063B   ********************************************************************************
IMI-349061    ********************************************************************************
MAFF-305635   ********************************************************************************
MAFF-238563   ********************************************************************************
LARS-056      ******************************************************T*************************

CD-hz-01      CGACGTAGGCCCTTAAAGGTAGTGGCGGACCCTCCCGGAGCCTCCTTTGCGTAGTAACTTAACGTCTCGC
CD-hz-02      ********************************************************************************
CD-hz-03      ********************************************************************************
IMI-349063A   ********************************************************************************
IMI-349063B   ********************************************************************************
IMI-349061    ********************************************************************************
MAFF-305635   ********************************************************************************
MAFF-238563   ********************************************************************************
LARS-056      ********************************************************************************

CD-hz-01      ACTGGGATCCGGAGGGACTCTTGCCGTAAAACCCCCAAAACTTTTAC
CD-hz-02      ************************************************
CD-hz-03      ************************************************
IMI-349063A   ************************************************
IMI-349063B   ************************************************
IMI-349061    ************************************************
MAFF-305635   ************************************************
MAFF-238563   ************************************************
LARS-056      ************************************************
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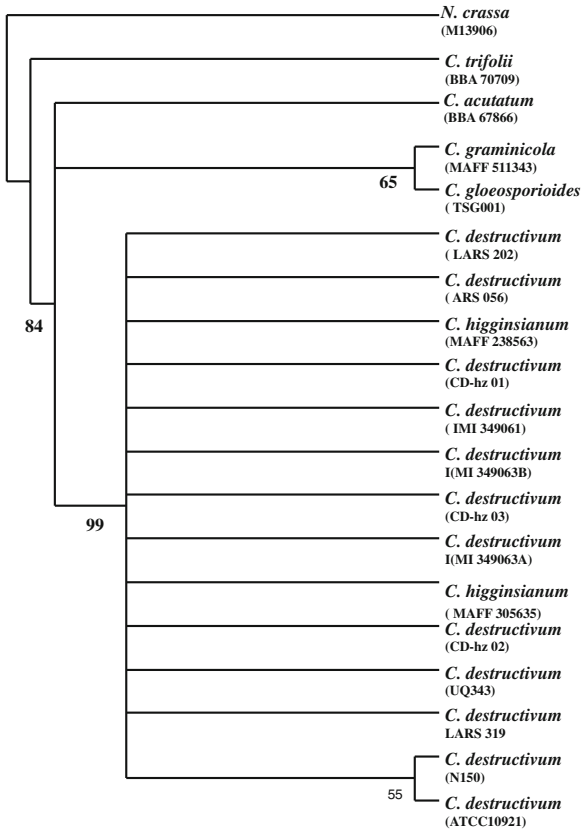

Fig. 3 Parsimony consensus tree prepared using Paup software, based on ITS sequences, depicting the relationship of the three *Colletotrichum destructivum* isolates used in this study (CD-hz 01, CD-hz 01, and CD-hz 01) with reference isolates of *C. higginsianum*, *C. destructivum,* and other *Colletotrichum* spp. representing the diversity of the genus. Numbers above branches represent percentages out of 1000 bootstrap replications. *Neurospora crassa* was included as an outgroup

cultivars at that time (Fig. 4b). The symptomatic characters of host resistance were similar to those described previously (Latunde-Dada et al. 1999).

In the family Cruciferae, two *Arabidopsis* accessions tested in this study were highly susceptible to three isolates of *C. destructivum* from cowpea while Chinese cabbage (*Brassica pekinense*) was completely resistant (Table 2). In seedlings of *A. thaliana* accessions, the leaf lesions appeared 96 h after inoculation. When small water-soaked lesions expanded, the leaves became chlorotic and the infected tissues were macerated and water-soaked (Fig. 4c). The lesions coalesced, resulting in the death of individual leaves or the entire plant at 8 days (Fig. 4d). Two species of *Nicotiana* were completely resistant.

Infection process in susceptible cowpea

The infection process of three isolates was similar in leaves of two susceptible cultivars of cowpea. About 12 h after inoculation, the conidia became septate and formed melanised subglobose appressoria at the ends of germ-tubes on the surface of the susceptible cv. Xinyou 1. By 36 h after inoculation, epidermal cells of leaves had been invaded. At this stage of infection, the intracellular structures of the fungus, emanating from the appressorium, consisted of a long, narrow neck, which gave rise to a swollen, saccate infection vesicle (not shown). The infection vesicle developed and produced the multilobed infection vesicles or primary hypha within an epidermal cell, 48 h after

Table 2 Pathogenicity of *Colletotrichum destructivum* isolates

Family, genus, species	Common name or cultivar	response to the isolates
Leguminosae		
Vigna unguiculata	Cowpea (cv. Xinou 1)	Susceptible[c]
V. unguiculata	Cowpea (cv. Tezao 30)	Susceptible
V. unguiculata	Cowpea (cv. Cunqiuong)	Resistant[b]
V. unguiculata	Cowpea (cv. Zhijiang 884)	Resistant
Lens culinaris	Lentil	Completely resistant
Cruciferae		
Arabidopsis thaliana	ecotype Columbia (Col)	Susceptible
A. thaliana	ecotype Landsberg erecta (Ler)	Susceptible
Brassica pekinenses	Chinese cabbage (cv. Chengqin 2)	Completely resistant[a]
Solonaceae		
Nicotiana glutinosa	Tobacco	Completely resistant
N. tabacum	Tobacco	Completely resistant

Disease reaction scales: a represents no symptoms; b represents restricted, necrotic flecks; c represents spreading, water-soaked lesions

Fig. 4 Infection of cowpea and *Arabidopsis thaliana* by *Colletotrichum destructivum*. **a** Symptoms on seedlings of *Vigna unguiculata* cv. XinYou 1 incubated for 8 days. Leaves show large and small brown lesions (*arrows*). **b** Symptoms on leaf of *V. unguiculata* cv. CunqiuHong incubated for 8 days. The leaves show a hypersusceptible phenotype, with many small, brown to dark brown, necrotic flecks (*arrows*). **c** Symptoms on 6 week-old plants incubated for 120 h. The leaves of *A. thaliana* ecotype Columbia (Col) show small, dark, water-soaked lesions and a few leaves were collapsed and macerated. **d** Symptoms on 6 week-old plants incubated for 8 days. The leaves of *A. thaliana* ecotype Columbia (Col) show completely collapsed and dead leaves

inoculation (Fig. 5a). The primary hyphae grew progressively longer, and septa were visible. By 72 h after inoculation, this fully developed multiseptate primary hypha remained inside the initially penetrated epidermal cell (Fig. 5b). Subsequently, narrower secondary hyphae began to emerge from the end of the primary hypha and grew through the host cell wall to invade the surrounding tissues (Fig. 5c). The development of the secondary hyphae was followed by the appearance of water-soaked lesions on the surface of infected tissues, 120 h after inoculation. By 144 h after inoculation, acervuli, each with a single melanised seta, were observed on the leaf surface (Fig. 5d). A similar pattern of infection was also observed on the cv. TeZao 30.

Infection process in *A. thaliana*

The infection process of three isolates on ecotype Columbia (Col) was identical. Infection proceeded more rapidly than in cowpea and a small saccate infection vesicle became visible within leaf epidermal cells at 24 h; this became branched and expanded to form the primary hypha with the septum by 48 h (Fig. 6a). Primary hyphae subsequently enlarged and became multiseptate and multilobed (Fig. 6b). However, primary hyphae eventually filled the first infected epidermal cell but never expanded beyond it. Narrower secondary hyphae began to emerge from the primary hyphae at 72 h, and penetrated the host cell wall to invade neighbouring cells (Fig. 6c). By 96 h, host tissues were extensively colonised by a dense mycelium of intra- and intercellular hyphae, which extended from the upper to the lower epidermis of leaves. At this time, the appearance of water-soaked lesions on the surface of infected tissues was observed clearly (not shown). By 108 h, hyphal aggregates, which represented the initial of an acervulus, produced a cluster of short conidiophores around a long, melanised seta which erupted through the cuticle onto the surface of the infected host. The asexual cycle was completed with the production of

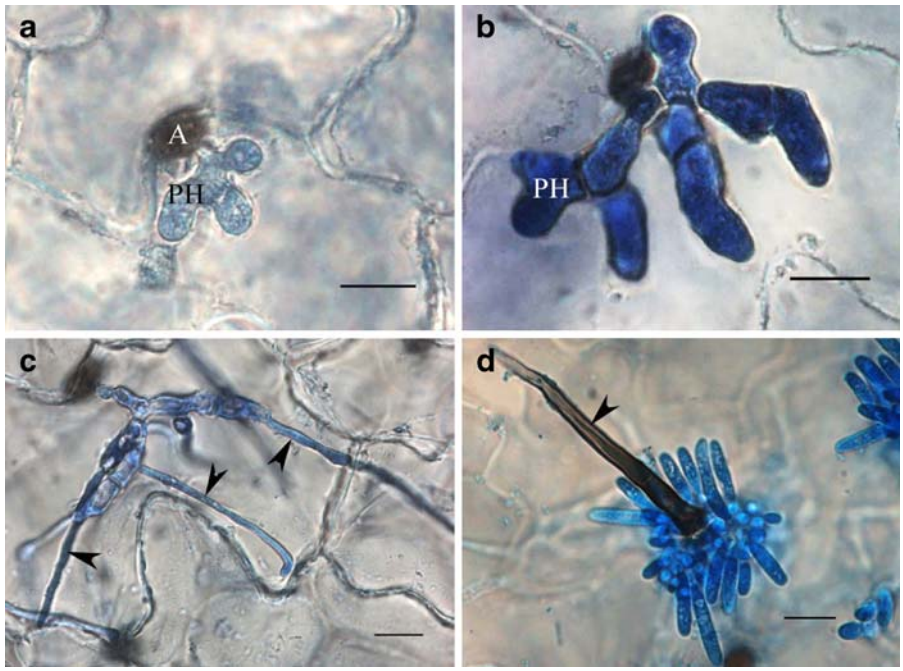

Fig. 5 Infection of cowpea by *Colletotrichum destructivum*. **a** Appressorium (A) penetrating an epidermal cell of cowpea leaf to form a small primary hypha (PH), 48 h after inoculation. **b** Fully developed multiseptate PH, produced in an epidermal cell, 72 h after inoculation. **c** Appearance of narrow secondary hyphae (*arrows*) from the PH, 96 h after inoculation. Note the PH eventually filled the first infected epidermal cell but never developed beyond it. **d** An acervulus with a melanised single seta (*arrow*) surrounded by conidia on cowpea leaf surface, 144 h after inoculation. Bar=10 μm

abundant acervuli on the surface of the dead host tissue. The acervuli formed large numbers of conidia produced from the short conidiophores (Fig. 6d). The infection process of three isolates on the ecotype Landsberg erecta (Ler) were also similarly observed and there were no significant cytological differences between the ecotype Columbia (Col) and Ler.

Discussion

Pathogenicity and infection process of *C. destructivum*

Colletotrichum destructivum is considered to be a major cowpea pathogen worldwide and causes important economic losses. In the field, the symptoms caused by the pathogen on *V. unguiculata* cv. Xinyou 1 leaves were typical anthracnose lesions (Fig. 7). The pathogenicity test demonstrated that the *V. unguiculata* cvs Xinyou 1 and Tezao 30 were susceptible to the pathogen and the cvs Cunqiuhong and Zhijiang 884 were resistant. The infection process of the isolates from cowpea in this study appeared virtually the same as that of *C. destructivum* on infected alfalfa and tobacco (Latunde-Dada et al. 1996, 1997; Shen et al. 2001).

Colletotrichum destructivum has a wide host range, including many legumes (e.g., cowpea, soybean, and alfalfa), solanaceous plants (e.g., tobacco), and cruciferous plants (species of *Arabidopsis*) (Latunde-Dada et al. 1997; Shen et al. 2001; O'Connell et al. 2004). However, different isolates of *C. destructivum* showed host specificity. Previous studies have shown *Brassica* isolates could infect cowpea but not tobacco or alfalfa, while cowpea and alfalfa isolates were unable to infect *A. thaliana* (O'Connell et al. 2004). *Colletotrichum higginsianum* was considered as a distinct species on the basis of conidial morphology and a consistent association with cruciferous hosts (Sutton 1980, 1992). Interestingly, our results displayed, for the first time, that cowpea isolates also could infect and complete the asexual cycle on *A. thaliana*. The infection process showed that in the initial biotrophic phase, intracellular primary hyphae were confined to one epidermal cell, whereas in the subsequent necrotrophic phase, second-

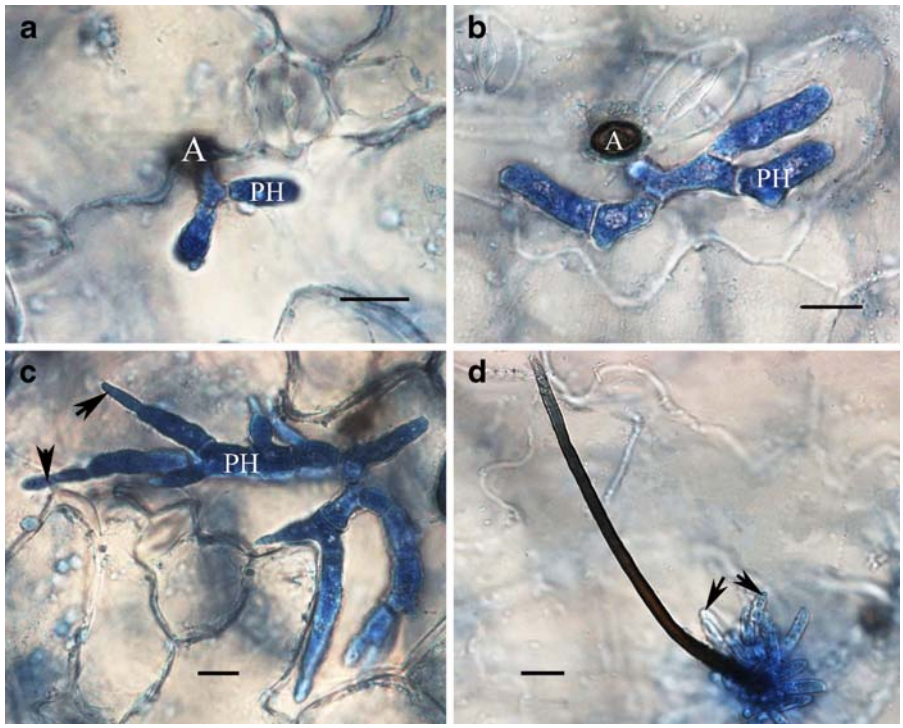

Fig. 6 Infection of *Arabidopsis thaliana* Columbia (Col) by *Colletotrichum destructivum*. **a** Appressorium (A) penetrating an epidermal cell of the Col leaf to form a small, branched primary hypha (PH), 36 h after inoculation. **b** Fully developed multiseptate PH, produced in an epidermal cell of the Col leaf, 60 h after inoculation. **c** Appearance of narrow secondary hyphae (*arrows*) from the PH, 72 h after inoculation. Note that the PH eventually filled the first infected epidermal cell but never developed beyond it. **d** An acervulus with a melanised single seta surrounded by conidia (*arrows*) on leaf surface, 108 h after inoculation. Bar=10 μm

Fig. 7 Typical symptoms of cowpea anthracnose in field

ary hyphae invaded the neighbouring cells, in the same way as *C. higginsianum* originating from cruciferous plants (O'Connell et al. 2004). The results implied that the host range or the cruciferous hosts would not be a reliable criterion to delimit the two species. Compared with the infection processes and intracellular infection structures on cowpea and *Arabidopsis* plants, *C. destructivum* was characterised by the production of multilobed primary hyphae restricted to the first penetrated cell. These characteristics held true for *C. truncatum* and *C. linicola*, and correlate consistently with ITS sequence data (Latunde-Dada and Lucas 2007; Liu et al. 2007).

Variability of rDNA ITS sequences among *C. destructivum* isolates

Analysis of the rDNA sequences showed the test isolates were identical to *C. higginsianum*. The sequence comparison of the cowpea isolates of *C.*

destructivum exhibited levels of intraspecific variability in rDNA ITS regions (Fig. 2). Similarly, analogous results had also been observed among some *Colletotrichum* species (Sherriff et al. 1994; Sreenivasaprasad et al. 1996). Due to the high level of similarity within the ITS region and entirely consistent morphological characteristics, O'Connell et al. (2004) ascribed *C. higginsianum* to *C. destructivum* by high levels of overall similarity (97.9 to 100% identity). Our results demonstrated that the rDNA ITS sequences of *C. destructivum* isolates from cowpea were exactly the same as those of *C. higginsianum* isolates from cruciferous plants. Apparently, the variation of rDNA ITS sequences of *C. higginsianum* isolates were within the levels of intraspecific variability of *C. destructivum*. These provide new evidence to support the conclusion, proposed by O'Connell et al. (2004), that *C. higginsianum* is only a synonym of *C. destructivum*. More recently, Liu et al. (2007) showed that the ITS region of the *C. linicola* isolate clustered with >99% identity to *C. higginsianum* and *C. destructivum*. Latunde-Dada and Lucas (2007) showed that the nucleotide sequences of the D2 and ITS-2 regions of rDNA amongst *C. truncatum*, *C. destructivum* and *C. linicola* had very high similarities (97–99%), and proposed, by a combination the phylogenetic relationships, as well as morphology, infection processes and intracellular infection structures, that *C. destructivum* was a species aggregate, which also includes two other species, namely *C. linicola* and *C. truncatum*. Therefore, three isolates from cowpea in our study belong to a member of this group.

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