Biology of *Colletotrichum horii*, the causal agent of persimmon anthracnose

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Colletotrichum horii causes serious anthracnose on persimmon (*Diospyros kaki* cv. Wuheshi). The taxon was previously identified as *C. gloeosporioides* and only recently revealed to be *C. horii* based on molecular data and comparisons to type specimens. This fungus provides an important new model for examining plant–fungus interactions in the perennial persimmon crop. In this paper, we review available information on *C. horii*, with special focus on symptoms, morphological characteristics, phylogenetic analysis, host-specificity and pathogenicity testing, infection processes, and the effects of environment factors on anthracnose development, including a discussion on future prospects.

**Keywords:** anthracnose; infection process; morphology; phylogeny; systematics; taxonomy

**Introduction**

*Diospyros kaki*, known as *shizi* in Chinese, is the most widely used species of persimmon (oriental persimmon), having been cultivated in China for over 2500 years. More than 2000 different cultivars exist, with 960 of them being cultivated (Zhang 2008). *Diospyros kaki* cv. Wuheshi has been grown for more than 600 years in the Chunan area of Zhejiang Province (Zhang et al. 2003). However, there have been few records of persimmon anthracnose and local growers are unfamiliar with the disease. In 1992, the local government established 666 ha of persimmon orchard to stimulate the industry with the result that persimmon anthracnose has become increasingly common. In 1996, about half of the planted persimmon trees had died from anthracnose (Zhang et al. 2003). The disease has caused serious economic losses and become a major problem for the persimmon industry (Zhang 2008).

The pathogen causing persimmon anthracnose was previously identified as *C. gloeosporioides* (Zhang et al. 2005). Cytological research of the infection processes and intracellular infection structures have shown that *Colletotrichum* on persimmon is a hemibiotrophic species and, thus, is different from *C. gloeosporioides sensu stricto* (Cannon et al. 2008). During initial colonization of host cells, infection vesicles and primary hyphae are surrounded by an interfacial matrix that separates the fungal cell wall from the invaginated host plasma membrane, closely resembling that of *C. lindemuthianum* on *Phaseolus vulgaris* (Zhang et al. 2003, 2005; Zhang 2008).

Weir and Johnston (2010) described the species causing persimmon anthracnose as *C. horii* Weir and Johnst., and our isolates from Chunan area were found to be conspecific to the ex-epitypes of *C. horii*. Weir and Johnston (2010) considered *C. horii* as part of the *C. gloeosporioides* species complex, but recognized it as a distinct species from phylogenetic analysis based on ITS, EF1\(\alpha\), and GPDH sequences. Although Weir and Johnston (2010) described the morphological characteristics of *C. horii*, other features, such as pathogenicity, host range and characteristics of appressoria and conidia in *viva*, were not detailed. Isolates from China (Chunan, Zhejiang) generally do not produce setae and a teleomorph was not observed on the host in natural or in artificial culture. Field and laboratory observations showed that *C. horii* (as *C. gloeosporioides*) was specific to different species, cultivars and organs of *Diospyros* (Zhang and Xu 2005), although its host range has not been fully determined.

Recent research has elucidated the etiology of anthracnose disease of persimmon (Zhang et al. 2005b), including the primary inoculum source (Zhang and Xu 2003), host-specificity (Zhang and Xu 2005), infection processes and the effects of environment factors on disease and fungal development (Zhang et al. 2005; Zhang 2008). Less attention has been paid to understanding the biological characteristics of this species. In this paper, we describe the morphological characteristics of this species in detail, determine its pathogenicity and host range, and investigate...
its phylogenetic relationships with closely related taxa based on multiple gene sequence analysis.

**Synonyms of Colletotrichum horii**

The fungus causing anthracnose on persimmon was first described by Shotaro Hori (1910a,b) as *Gloeosporium kaki* based on its morphological characteristics, which were similar to *Gloeosporium rufomaculans* and *Gloeosporium fruitigenum* (Hori 1910b). In China, this disease was first recorded in Taiwan and noted as *Gloeosporium kaki* Hori (Sawada 1933). Maffei (1921) had also described a leaf spot disease of persimmon from Italy and the pathogen was described as *Colletotrichum kaki* Maffei. The two taxa were distinguished by the presence of setae and pathogenicity. *G. kaki* was associated with lesions on young twigs and shoots and as spots on unripe fruit, while *Colletotrichum kaki* produced numerous setae and infected only leaves (Maffei 1921). However, there is no evidence that *Gloeosporium kaki* and *Colletotrichum kaki* are synonyms (Weir and Johnston 2010). Von Arx (1957) placed *Gloeosporium kaki* (Maffei 1921). However, there is no evidence that *Gloeosporium kaki* and *Colletotrichum kaki* are synonyms (Weir and Johnston 2010). Von Arx (1957) placed *G. kaki* in synonymy with the conidial state of *Gloeosporium cingulata*, i.e. *Colletotrichum gloeosporioides* and Sutton (1992) did not recognize *G. kaki* as a distinct species. For this reason, the pathogen-causing disease of persimmon in the Chunan area of Zhejiang Province was known as *Colletotrichum gloeosporioides* using morphological and pathogenic characters.

Phylogenetic analysis has increasingly becoming a useful tool for species delimitation in *Colletotrichum* (Shenoy et al. 2007a,b; Than et al. 2008; Cai et al. 2009). However, if type material is lost or in such poor condition that it cannot be used to extract DNA, molecular data cannot be obtained. Epitypification is a good way to resolve this problem and has been applied to several *Colletotrichum* species (Hyde and Zhang 2008; Cannon et al. 2009; Crouch et al. 2009). An isolate causing persimmon anthracnose was designated as the epitype of *Gloeosporium kaki* Hori and the taxon was transferred to *C. horii* (Weir and Johnston 2010).

**Symptoms**

Anthracnose is a destructive disease of persimmon nurseries in the field. The fungus attacks young twigs, leaves (petioles and veins) and fruits, leading to anthracnose lesions, which comprises twig blight, leaf defoliation, fruit drop and fruit rot (Zhang and Xu 2005). If twig blight becomes significant, the growth of a tree may decline and the entire tree can be killed within two or three years (Figure 1E).

Anthracnose symptoms on twigs, leaves and fruits first appear in the spring as darkish, oval or elliptic spots, or as pin-pricks on newly-formed twigs. The minute spots develop into dark purple or dark brown lesions (Figure 1A), with a sharp line of demarcation between diseased and symptomless tissues. Pale orange conidial masses are frequently produced in the lesion centre. Under favorable conditions, adjacent lesions may coalesce, increasing in size until the entire twig is infected (Figure 1B). When a twig is girdled or completely infected, then dieback results (Figure 1C). The lesions may become dormant under unfavorable conditions but, in this situation, the fungus still continues to extend into the xylem, resulting in collapse with longitudinal cracking and finally forming cankers on a twig (Figure 4). Leaf defoliation occurs if lesions develop at the base of petioles.

The pathogen infects petioles and leaf veins to produce the small, round or ovoid, sunken, purple to dark brown spots (Figure 1F), but they form later than those on young twigs. These small spots develop into the larger lesions, but they rarely coalesce on the petioles and leaf veins. If a petiole is infected, the leaf may continue to develop and remain green for an extended period, but may easily defoli ate in the wind.

The persimmon fruits can be infected throughout the entire fruit-growing season. In young fruits, the lesions are often circular or oval, 3–8 mm in diameter, purple to dark purple, and occasionally slightly depressed. As the disease progresses, sometimes fruit lesions reach ~20 mm in diameter (Figure 1G). The centre of the lesions becomes grey-white over time, while the broad margins remain dark purple. Pale orange conidial masses are produced in the lesion centre. Under dry conditions, the diseased lesions are sunken, and a longitudinal crack often occurs through the centre (Zhang 2008). If fruits are badly infected, they may drop in an unripe state (Figure 1H). In pre-mature fruits, the diseased lesions are often dark brown or purple dark, oval, sunken, with small cracks. Larger cracks often form and almost all deep cracks are produced in a longitudinal direction (Figure 1I). Anthracnose of persimmon fruits also occurs in market shelves and storage warehouses, resulting in fruit rot (Zhang 2008).

**Morphological characteristics**

*Conidiophores* (11–) 11.5–25–35 (~50)×3–3.5–4.5. μm (*n = 30*), congregated, produced in acervuli, fasciculate, straight or occasionally geniculate (Figure 2B), 1-3-septate, dark grey at the base, reduced to a single hyaline conidiogenous cell on the natural host. *Conidiogenous cells (7.5–) 9.5–13–15 (~16) × 3–3.5–4.5. μm, produced at the apex of conidiophores, cylindrical, rarely ampulliform, smooth, with a dark collarette (Figure 2B) or occasionally annellidic at the conidigenous sites. *Conidia* (16.5–) 17–19.5–20.5 (~22.5) × (4.5–) 5–5.5–6 (~6.5) μm (*n = 30*), formed in yellowish-orange masses, holoblastic, cylindrical, straight or slightly curved, non-septate, smooth, apex obtuse, obtuse at both ends, with a hilum-like low protuberance at the base (Figure 2A). Under scanning electron microscopy,
apex of conidia obtuse, base truncate (Figure 2C), with a truncate hilum that is hollow (Figure 2D).

Colonies on PDA velvety, floccose, grey to dark grey, with the large numbers of yellowish-orange conidial masses, edge regular (Figure 3A), reverse dark grey to dark brown, with concentric zonation (Figure 3B). Margin of colony regular and the mean daily growth rate at 25°C was 12.8 ± 0.8 mm per day. Conidia produced across the whole colony, forming slimy, pale orange conidial masses amongst the aerial mycelium. Conidiophores 3.5–5 μm diam., short-cylindrical. Conidiogenous cells 10–15 × 3–5 μm, cylindrical. Conidia (17–) 18.5–20–21.5 (–22.5) × (4.5–) 5–5.4–5.7 (–5.9) μm (n = 30), cylindrical (Figure 3C). Appressoria were 8–9 × 7.8–8.7 μm, smooth, globose and dark brown (Figure 3D) on polystyrene Petri dishes, as described by O’Connell et al. (2004) and Sun and Zhang (2009). On coverslips, appressoria were dark, globose to subglobose, smooth, and similar to those on plastic Petri dishes (Figure 3E), as described by Cei et al. (2009).

Material examined: CHINA: Zhejiang Province, Chunan County, Weiping Town (118° 20′ E, 118º 32′ N), on Diospyros kaki cv. Wuheshi (Ebenaceae), 30 Apr. 2009, J.Z. Zhang and L. Xie (HMAS 197044). The living cultures (TSG001, TSG002, TSG003, TSG004 and TSG005) are deposited in the collection of Biotechnology Institute, Zhejiang University, Zhejiang Province, China. Sources of isolates used in this study are given in Table 1.
Notes: Numerous conidia aggregate on the acervuli in cone-shaped masses (Zhang et al., 2005B) and the conidia are embedded within and surrounded by mucilage (Figure 2E). In the process of conidial formation, transmission electron microscopy clearly showed that the outer wall of an apical conidiogenous locus breaks and forms the collar-ette, but its inner wall takes part in the formation of the outer wall of a new conidium (Figure 2E, F).

Most conidia on PDA are straight and less curved than that on the natural host, but they were similar in size with mean dimension of $19.5 \times 5.5 \mu m$ on the natural host and $20 \times 5.4 \mu m$ on PDA. Similarly, conidia size of isolates from Japan on PDA were $(13–21) \times 4.5–5.5 \mu m$ (mean = $17.5 \times 4.8 \mu m$) and those from New Zealand were $16–29.5 \times (4–) 4.5–6 \mu m$ (mean = $22 \times 5 \mu m$) (Weir and Johnston 2010). There is a large variation in conidial size in isolates from different locations. Colletotrichum horii is morphologically similar to C. gloeosporioides, but can be differentiated as the latter epitype by conidial size of $17–20.5 \times 5–6 \mu m$ for the epitype of C. horii compared to $12–17 \times 4.5–6 \mu m$ for the epitype of C. gloeosporioides (Cannon et al. 2008).

Host specificity and results of pathogenicity testing

Colletotrichum horii isolates from the Chunan area of Zhejiang Province have been tested for host specificity in the field and laboratory (Zhang and Xu 2005). They found that a cultivar of Diospyros glaucifolia from the Chunan area was completely resistant; D. kaki cv. Wuweshi, was very susceptible; D. kaki cv. Dongshi was susceptible and the fruit is infected; while D. kaki var. sylvestris (wild persimmon), whose twigs were infected, was only slightly susceptible.

Pathogenicity testing was conducted in the laboratory on both healthy young twigs of D. kaki cv. Wuweshi and the fruits of several other plant species, including Musaceae (banana, Musa acuminata), Solanaceae (tomato, Lycopersicon esculentum; green pepper, Capsicum annum), Cucurbitaceae (pumpkin, Cucurbita pepo;
marrow, Cucurbita pepo, Rutaceae (orange, Citrus sinensis), Anacardiaceae (mango, Mangifera indica), and Leguminosae (common bean, Phaseolus vulgaris; cowpea, Vigna unguiculata). An aqueous suspension $1 \times 10^5$ conidia/ml was prepared from 8–10-day cultures. The inoculated fruits were washed three times with sterile water, and 50% were pricked with insect needles prior to inoculation. The wounded/unwounded plants materials were then inoculated with a 100 $\mu$l conidial suspension as described by Zhang and Xu (2005).

Five isolates showed strong virulence, with anthracnose symptoms occurring on unwounded/wounded persimmon twigs and leaf veins within 3 days following inoculation (Figure 4A). Serious symptoms resulted within 5 days. Lesion size varied greatly on inoculated twigs and leaf veins, depending on the age of the persimmon twigs. Average lesion size on the unwounded/wounded persimmon twigs was 3.75 ($\pm$0.94) cm and 3.68 ($\pm$1.73) cm, respectively; and 2.07 $\pm$ 0.67 ($\pm$0.67) cm and 2.26 ($\pm$0.72) cm on the unwounded/wounded leaf veins. Large numbers of yellow-pink conidial masses were produced on the central parts of lesions (Figure 4B). Seven days after inoculation, the lesions extended to almost entire twigs or main leaf veins (Figure 4C). In contrast, smaller lesions were found on unwounded/wounded banana (0.68 $\pm$ 0.08/0.8 $\pm$ 0.07 cm) (Figure 4D) and marrow (0.76 $\pm$ 0.09/0.92 $\pm$ 0.08 cm) fruits (Figure 4E), as well as on unwounded green pepper fruits (confined to inoculation sites), 5 days after inoculation. No conidial masses were produced on the lesions until 7 days and no infected symptoms were observed on the other plants inoculated.

Pathogenicity testing indicated that D. horii infected unwounded banana and marrow fruits. Interestingly, marrow plants are grown in the region but no infection has been reported under field conditions. Banana plants do not grow in this location. Artificial host inoculation is usually not reliable enough for determining host-specificity (usually being determined by natural infection), but may indicate the potential for infection (Freeman et al. 1998), cross-infection potential and cytological characteristics of the infection process.

**Infection process**

**On persimmon**

The infection process of C. horii on susceptible Diospyros kaki cv. Wuheshi has been well documented (Zhang et al.
Table 1. Sources of isolates used in this study.

| Colletotrichum species | Isolates | ACT       | TUB-2     | CAL       | GS        | ITS        |
|------------------------|----------|-----------|-----------|-----------|-----------|------------|
| C. horii               | TSG001   | GU133374  | GU133375  | GU133376  | GU133377  | AY787483   |
| C. horii               | TSG002   | GU133379  | GU133380  | GU133381  | GU133382  | AY791890   |
| C. horii               | TSG003   | GU133384  | GU133385  | GU133386  | GU133387  | AY791894   |
| C. horii               | TSG004   | GU133388  | GU133389  | GU133390  | GU133391  | AY791893   |
| C. horii               | TSG005   | GU133393  | GU133394  | GU133395  | GU133396  | AY791892   |
| C. asiamum             | BML-13   | FJ 903188 | FJ 907434 | FJ 917501 | FJ 972586 | FJ 972605  |
| C. asiamum             | BPD-I4   | FJ 907424 | FJ 907439 | FJ 917506 | FJ 972595 | FJ 972612  |
| C. asiamum             | BML-114  | FJ 907421 | FJ 907436 | FJ 917503 | FJ 972598 | FJ 972615  |
| C. siamense            | BML-16   | FJ 907420 | FJ 907435 | FJ 917502 | FJ 972599 | FJ 972604  |
| C. siamense            | BPD-I2   | FJ 907423 | FJ 907438 | FJ 917505 | FJ 972596 | FJ 972613  |
| C. siamense            | BML-15   | FJ 907422 | FJ 907437 | FJ 917504 | FJ 972597 | FJ 972614  |
| C. fruticola           | BPD-I8   | FJ 907427 | FJ 907442 | FJ 917509 | FJ 972592 | FJ 972602  |
| C. fruticola           | BPD-I12  | FJ 907425 | FJ 907440 | FJ 917507 | FJ 972594 | FJ 972611  |
| C. fruticola           | BPD-I16  | FJ 907426 | FJ 907441 | FJ 917508 | FJ 972593 | FJ 972603  |
| C. acutatum            | BRIP 28519 | FJ 907428 | FJ 907443 | FJ 917510 | FJ 972591 | FJ 972601  |
| C. acutatum            | CBS 294.67 | FJ 907429 | FJ 907444 | FJ 917511 | FJ 972590 | FJ 972610  |
| C. falcum Epitype      | FAL      | FJ 907431 | GQ 289454 | FJ 917513 | FJ 972600 | FJ 972606  |
| C. gloeosporioides     | CBS 953.97 | FJ 907430 | FJ 907445 | FJ 917512 | FJ 972589 | FJ 972609  |
| C. kahawae             | IMI 319418 | FJ 907432 | FJ 907446 | FJ 917514 | FJ 972588 | FJ 972608  |
| C. kahawae             | IMI 363578 | FJ 907433 | FJ 907447 | FJ 917515 | FJ 972587 | FJ 972607  |

Note: ACT: actin; TUB-2: partial ß-tubulin (tub2); CAL: calmodulin; GS: glutamine synthetase; GDPH: glyceraldehydes-3-phosphate dehydrogenase; ITS: complete rDNA-ITS region; MFU: Mae Fah Luang University, Thailand; IFRD: International Fungal Research and Development Centre, China; CBS: Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; BRIP: Plant Pathology Herbarium, Department of Primary Industries, Queensland, Australia; IMI: CABI Europe UK, Bakeham Lane, Egham, Surrey TW209TY, UK; *: holotype.

2004; Zhang 2008). C. horii exhibits an infection strategy of intracellular colonization and fungal hyphae growth within the cell lumen without penetrating host protoplasts. This is similar to that of C. lindemuthianum on beans and C. sublineolium on sorghum (O’Connell et al. 1985; Wharton et al. 2001). This research also revealed that cytological and ultrastructural characters are similar in the process of infection of twigs and petioles, closely resembling that of C. lindemuthianum on bean (Zhang et al. 2003, 2005). In contrast, conidia of C. horii germinate on twigs of the resistant Zhejing persimmon (D. glaucifolia) and produce germ tubes that form dark appressorium which, in turn, produce infection pegs and penetrate the cuticle within 24 h of inoculation. The fungus then became quiescent and did not develop further and no visible symptoms of disease were observed (Zhang and Xu 2005). This phenomenon may be similar to that of quiescent infection in unripe avocado fruits attacked by Colletotrichum gloeosporioides (Prusky 1996).

**Infection process on banana and marrow**

The infection process of C. horii on banana (Musa acuminata) and marrow (Cucurbita pepo) was observed with fruits inoculated with a conidial suspension, as described above. Pieces were cut from the inoculated banana and marrow skins and decolorized 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, as described by Sun and Zhang (2009). Light microscopic examination was made with a Zeiss Axiopt 2 microscope with Axiochrom CCD camera and Axioscience digital imaging software (AxioVision Software Release 3.1, ver. 3-2002; Carl Zeiss Vision Imaging Systems). The preinfection stages of C. horii isolates on banana are very similar to those on Diospyros kaki (Zhang et al. 2003, 2005), in which conidia adhere to and germinate on the plant surface, producing germ tubes that form appressoria 12 h after inoculation, which in turn produced infection pegs 24 h after inoculation and penetrate the cuticle directly. About 48 h after inoculation, the inoculated tissue sites became brown, but no infection vesicle were seen; these appeared 60 h after inoculation. Ninety six hours after inoculation, the primary hypha formed in the initial infection cell (Figure 5A) and was surrounded by an invaginated plasma membrane. The primary hyphae did make contact with the plasma membrane and were separated by an interfacial matrix (Figure 5B), closely resembling that on Diospyros kaki (Zhang et al. 2005; Zhang 2008). The host plasma membrane surrounding the primary hypha became thick and was probably deposited by the opaque material, as previously described on Diospyros kaki (Figure 5B) (Zhang et al. 2003). However, in contrast to Diospyros kaki (Zhang 2008), the disease development was limited and the primary hyphae developed very slowly. Even 120 h after inoculation, the lesions were only slightly larger than the inoculated sites, and dark brown. The primary...
hyphae was confined to the initial infection cell but did not produce branches and septa (Figure 5C), and an interfacial matrix separated the cell wall of the primary hyphae from the host plasma membrane (Figure 5C). Then, 144 h after inoculation, the primary hypha produced septa, but was still restricted to the invading cell and no secondary hyphae were produced from it. At the same time, the interfacial matrix disappeared (Figure 5D) and the host cell was killed.

The infection pegs produced from appressoria penetrated the cuticle to invade the host cell and also entered the intercellular tissues (Figure 6A) or the host cell walls (Figure 6B,C). Similarly, development of the primary hyphae was evidently restrained and no secondary hyphae were produced. In contrast to hyphal length, the primary hyphae (7.12 ± 0.22 μm) in the intercellular tissues and host cell walls were shorter than in the invading cells (13.8.12 ± 0.20 μm) and no septum was produced until 132 h after inoculation (Figure 6C). The walls of primary hyphae made contact with the cell walls of the host and occupied the space between the cell walls (Figure 6A–C). After invading the host cell wall, swollen primary hypha may produce cell wall-degrading enzymes and exert mechanical pressure with its development, causing the host cell wall to rupture (Figure 6C). Such observations clearly indicate that the hyphal growth of C. horrii isolates were inhibited at ∼3 days after inoculation, compared with the extensive hyphal development on Diospyros kaki (Zhang et al. 2003, 2005), and no secondary hyphae differentiation was produced after primary hyphae differentiation at the transition between the biotrophic and necrotrophic phases. We have demonstrated that an interfacial matrix also was formed in the interaction between C. horri isolates and banana plant.
Similarly, the initial stages of *C. horii* isolates on marrow are very similar to that on banana, in which the appressoria produce infection pegs 24 h after inoculation, penetrating the cuticle directly. However, the infection pegs seemed to cease development until 144 h after inoculation, as described on twigs of the resistant Zhejing persimmon (*D. glaucifolia*) (Zhang and Xu 2005).

Species delineation in *Colletotrichum* is confused, being based on few morphological characters and host relationships. Cytological studies clearly show that the infection process and intracellular infection structures of *C. horii* are different from that of *C. gloeosporioides* (Sutton 1992) and similar to hemibiotrophic species of *Colletotrichum* (Perfect et al. 1999), but more closely related to that of *C. lindemuthianum* on bean (O’Connell et al. 1985; Zhang et al. 2003, 2005; Zhang 2008) and banana. However, morphologically, *C. horii* is different from *C. lindemuthianum* when conidial size 9.5–11.5 × 3.5–4.5 μm is compared (Sutton 1992).

**Effects of environment factors on growth and development of *C. horii***

*Colletotrichum horii* overwinters mainly in lesions of living twigs (Zhang and Xu 2003). Although it was believed that
pathogen could overwinter on dead leaves and fruits, no experimental data has been provided (Jia et al. 1997). In the field, the disease first appears in the vicinity of previously diseased twigs and no symptoms are visible on newly-formed twigs near the ground. Consequently it is speculated that the pathogen may disappear when leaves and fruits rot on the ground. Detection of pathogen from various plant parts showed that the survival rate of C. horii was 16.67% in lesions of living twigs, and 1.56% in the segments of dead diseased twigs (Zhang and Xu 2003). Mycelia in diseased tissues are, therefore, considered to be an important source of inocula-producing primary conidia in field. In spring, mycelia in diseased tissues produce conidia and are dispersed by rain splash and wind to newly formed twigs. The pathogen can also be dispersed in symptomless seedlings over long distances (Zhang and Xu 2003). Symptomless persimmon seedlings from diseased areas produced anthracnose lesions within 1 year on newly formed twigs. The pathogen can also be dispersed by rain splash and wind to newly formed twigs. According to the position of the lesions, it was believed that the infection source was related to the bud scales that carried the pathogen. In the spring, when favorable weather conditions occur, conidia that develop from the overwintering mycelium act as the primary source of inocula. Successful infection involves various environment factors, such as temperature, humidity, pH and nutrition on the surface of the host, which determines disease occurrence.

The effect of temperature on growth of mycelium is significant. The optimal temperature for growth in C. horii is ~25°C; higher temperature inhibits mycelium growth (Zhang and Hu 2004). While conidia germinate and form appressoria over a wide range of pH 2.0–9.0, the optimal pH for conidial germination and appressorial formation was between pH 5.0 and 6.0. When combining temperature and pH, pathogenicity testing showed interesting results. Visible symptoms of anthracnose occurred on new twigs at 23°C within pH 4.0–8.0 with conidial masses on lesions after 80–90 h; at 17°C and pH 6.0 without spore masses after 7 days, but were absent at temperatures lower than 17°C and pH 5.0–6.0 (Zhang and Hu 2004).

Glucose also influences conidial germination and appressoria formation (Zhang and Hu 2004). The percentage of conidial germination increases with increasing concentrations of glucose and time (Table 2). The percentage of conidial germination increased between 21 and 59% with the concentration of glucose (0.1–5%) after 12 h and, subsequently, the percentage in all treatments increased with time. After 48 h, the percentage of conidia germination went from 57 to 87% with increasing glucose concentrations.

Glucose inhibited appressorial formation (Table 3), which decreased from 32 to 0% after 12 h with glucose concentrations (0.1–5%); similarly, with increasing time, the percentage also increased correspondingly. After 48 h, the percentage climbed from 31 to 87% with increasing concentrations of glucose.

### PCR, sequencing and phylogenetic analysis

Partial actin (ACT), β-tubulin (TUB2), calmodulin (CAL), glutamine synthetase (GS), glyceraldehyde-3-phosphate dehydrogenase (GPDH) genes and the complete rDNA-ITS (ITS) region from five Colletotrichum strains were amplified by PCR, as described by Prihatsuti et al. (2009). The amplified DNA fragments were purified by an

### Table 2. Rate of conidial germination in different concentration of a glucose solution.

| Concentration of glucose (%) | 0.1 | 0.5 | 1.0 | 2.0 | 3.0 | 4.0 | 5.0 |
|-----------------------------|-----|-----|-----|-----|-----|-----|-----|
| Time (h)                    |     |     |     |     |     |     |     |
| 12                          | 21.0 ± 3.6a | 25.7 ± 2.1ab | 33.7 ± 5.8b | 39.3 ± 4.9c | 41.3 ± 4.9cd | 47.3 ± 4.1d | 59.3 ± 5.0d |
| 18                          | 27.3 ± 2.1a | 30 ± 1.0ab   | 41.7 ± 3.8b | 45.0 ± 2.0c | 47.7 ± 2.1cd | 55.7 ± 3.1d | 63.0 ± 5.3d |
| 24                          | 30.7 ± 5.1a | 39 ± 4.3a    | 49.3 ± 4.9b | 51.7 ± 4.5c | 56.3 ± 2.5d  | 64.0 ± 1.0e | 77.7 ± 4.7e |
| 36                          | 45.7 ± 2.9a | 50.7 ± 3.1a  | 57.0 ± 2.6b | 64.3 ± 3.1c | 69.0 ± 5.2d  | 74.7 ± 3.1d | 82.7 ± 2.8d |
| 48                          | 57.3 ± 3.8a | 61 ± 3.5ab   | 65.0 ± 4.5b | 71.3 ± 3.8c | 75.7 ± 4.5d  | 82.7 ± 7.6de| 85.7 ± 6.1e |

Note: Different letters following the data in the same row show significant difference at 0.5%.

### Table 3. Percentage of appressorial formation in different concentrations of a glucose solution.

| Concentration of glucose (%) | 0.1 | 0.5 | 1.0 | 2.0 | 3.0 | 4.0 | 5.0 |
|-----------------------------|-----|-----|-----|-----|-----|-----|-----|
| Time (h)                    |     |     |     |     |     |     |     |
| 12                          | 31.9 ± 6.9a | 27.0 ± 4.4ab | 21.7 ± 4.8b | 11 ± 0.2c  | 4.9 ± 0.6cd | 2.9 ± 1.4cd | 0 ± 0d  |
| 18                          | 56.0 ± 6.0a | 49.9% ± 9.2a | 43.4 ± 9.4a | 19.43 ± 5.5b| 10.4 ± 4.6b | 7.7 ± 1.2b | 6.1 ± 1.8b |
| 24                          | 65.9 ± 7.9a | 60.6% ± 4.7ab| 50.6 ± 4.8b | 35.47 ± 0.7c| 21.4 ± 3.9d | 17.2 ± 2.9d | 12.6 ± 3.8d|
| 36                          | 75.1 ± 8.2a | 73.1% ± 8.7a | 61.9 ± 2.3b | 41.1 ± 6.6bc| 29.3 ± 2.2a | 26.3 ± 1.9c | 20.2 ± 3.9c|
| 48                          | 87 ± 3.5a  | 82.9% ± 4.4ab| 77.4 ± 3.7b | 58.33 ± 4.1c| 40.4 ± 6.8d | 37.1 ± 0.5de| 31.3 ± 5.1e|

Note: Different letters following the data in the same row show significant difference at 0.5%.
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Axygen PCR purification kit. Purified PCR products for partial actin, beta-tubulin, calmodulin and glutamine synthetase were ligated into a pGEM-T vector (TaKaRa Co., Japan), and the ligated products were transformed into DH5α. The positive clone was propagated, the recombinant plasmids were extracted according to the manufacturer’s instructions (Axygen Bioscience), and identified by PCR and restriction endonuclease enzyme digestion. The sequence determination of PCR products and recombinant plasmid was carried out by Hangzhou Genomics Institute for sequencing in both directions. DNA sequencings were performed at the SinoGenoMax Company Limited. The accession numbers of all sequences are listed in Table 3. Phylogenetic analyses were performed using PAUP* 4.0b10 (Swofford 2002) and MrBayes 3.0b4 (Huelsenbeck and Ronquist 2001), with details outlined by Cai et al. (2006).

The combined dataset included 20 sequences with 2291 characters after alignment. Parsimony analysis resulted in six equally parsimonious trees. The KH test showed that these trees were not significantly different. One of these trees is shown in Figure 7. Phylogenetic analysis showed that the five tested isolates of *C. horii* clustered together with the reference isolates of *C. kahawae* (IMI 363578 and IMI 319418), with 98% bootstrap

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Figure 7. Phylogram generated from parsimony analysis based on combined actin, Bt2, calmodulin, GS and ITS sequences. Data were analysed with random addition sequence, unweighted parsimony and treating gaps as missing data. Bootstrap values ≥50% are shown above or below branches. Thickened branches indicate Bayesian posterior probabilities ≥95%. The tree is rooted with *Colletotrichum falcum*. 
support. Two species could be well differentiated by the conidia size, as *C. horii* has much larger conidia than *C. kahawae* (12.5–19.0 × 4.0 μm) (Waller et al. 1993).

The phylogenetic relationship between *C. horii* and its closely related species in *Colletotrichum* is analyzed based on combined partial actin, beta-tubulin, calmodulin, glutamine synthetase and ITS/5.8S gene sequence data. The results of phylogenetic analysis clearly indicated that *C. horii* is a distinct species, closely related to *C. kahawae*.

**Future prospects**

In this paper, we review the available information on *Colletotrichum horii*, which will facilitate its correct and accurate identification. Improved agricultural production requires accurate identification of pathogens to enable more effective disease control and management (Than et al. 2008). Previously, we had found that symptomless seedlings of persimmon carried *C. horii*, which lead to its dispersal over long distances (Zhang and Xu 2003). As this pathogen was previously identified as *Colletotrichum gloeosporioides*, which has a wide range of hosts, it has not been added to list of quarantine pests in China. In addition, the percentage of bud scales that carried the pathogen is very low, ranging from 3.3 to 4% (Zhang and Xu 2003). Although latent infection by *C. horii* in persimmon seedlings is undergoing extensive research (http://www.cab.zju.edu.cn/instswjs/people/zhang-jz/c3.htm), methods that enable its rapid and accurate detection from a large numbers of persimmon seedlings need to be improved.

The infection process in *C. horii* is well understood. *C. horii* provides an excellent pathosystem for studying the molecular basis for infection and fungal–plant interactions. To understand the molecular mechanisms of interactions between the pathogen and host, a genomic library of *C. horii* has been constructed and cloning of pathogenesis-related genes have also been performed by *Agrobacterium tumefaciens* - mediated transformation technique (Sun et al. 2008). A few mutants have been shown to be related to pathogenicity, and corresponding gene fragments had have been cloned (Sun et al. 2008). Identification and functional analysis of pathogenesis-related genes are a major undertaking for future studies but will provide new insights into the molecular mechanism of infection structure differentiation and fungal–plant interactions.

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