Infection process of *Colletotrichum dematium* on cowpea stems

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Colletotrichum dematium is the cause of a new fungal stem disease on cowpea in South Africa. The pre-penetration and infection process of this fungus on cowpea stems was studied by light microscopy and SEM. Conidia began germinating at 6 h post inoculation (hpi), forming appressoria directly or at the ends of germ-tubes. By 14 hpi appressoria had melanized and direct penetration of host tissue had begun. At 20 hpi infection vesicles formed in epidermal cells. Thick, knotted primary hyphae formed from these vesicles and entered adjacent cells. At $\pm$40 hpi *C. dematium* produced secondary hyphae which were highly branched, and grew extensively inter- and intracellularly. After approximately 48 hpi light brown lesions appeared on the stem, associated with the invasion of secondary hyphae into cells and cell necrosis. Acervuli with one or two melanized setae were visible on lesions by 70 hpi.

Colletotrichum dematium (Pers.) Grove is the causal agent of a new anthracnose disease of cowpea (*Vigna unguiculata* (L.) Walp.) in South Africa (Smith & Aveling, 1997). It was previously recorded on cowpea in India and Malaysia (Lenné, 1992) and on numerous other hosts (temperate and tropical), many of them legumes (Sutton, 1962; Holliday, 1980; Lenné, 1992). Stem symptoms on cowpea begin as tan brown discolourations that enlarge, becoming dark to purplish-brown. Sunken, necrotic lesions and small black acervuli are visible on the stems (Smith & Aveling, 1997). Three other *Colletotrichum* species are important pathogens on cowpea, namely *Colletotrichum lindemuthianum* (Sacc. & Magn.) Briosi & Cavara, *Colletotrichum capsici* (Syd.) E. J. Butler & Bisby and *Colletotrichum truncatum* (Schwein.) Andrus & W. D. Moore (Lenné, 1992). *Colletotrichum* spp. penetrate through natural openings (e.g. stomata), through wounds and/or by direct penetration of the plant cuticle (Bailey *et al.* 1992). *Colletotrichum* spp. exhibit three main initial infection strategies, according to which species are loosely categorized (Bailey *et al.*, 1992). The first group of species are known as intracellular hemibiotrophs, e.g. *C. lindemuthianum* on bean. These species exhibit a two-phase infection process. During the first phase, the pathogen grows biotrophically and no symptoms are produced on the plant. The second phase is a visibly destructive phase during which the pathogen grows necrotrophically. Other *Colletotrichum* spp. are known as 'subcuticular intramural' pathogens as they grow beneath the cuticle and cause extensive dissolution of the pectic matrix of the epidermal cell walls. The third group of species exhibits both intracellular hemibiotrophic and subcuticular intramural infections.

As yet no studies have been conducted on the infection process and structures of *C. dematium* on cowpea. The main aim of this study, therefore, was to investigate the nature of infection processes of *C. dematium* on cowpea.

MATERIALS AND METHODS

Fungal cultures

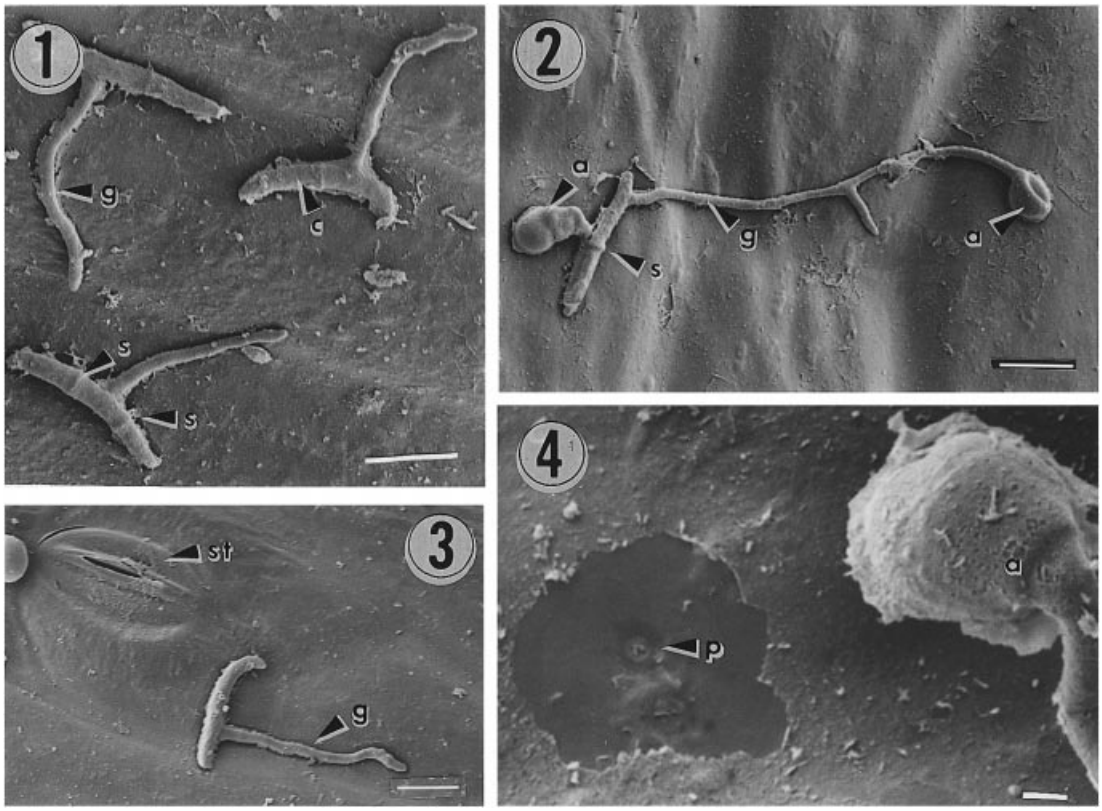
Colletotrichum dematium was consistently isolated from diseased cowpea stems. Stems were surface sterilized in 1% (w/v) sodium hypochlorite for 1 min before lesions were excised and plated on potato dextrose agar (PDA) amended with 0·025% w/v chloramphenicol. An isolate of *C. dematium* was deposited with the National Collection of Fungi, Pretoria (designated PPRI 6121). *C. dematium* was cultured on PDA, incubated at 24 °C in the dark and stored on agar plugs in liquid nitrogen.

Plant material

Cowpea seeds, cv. Rhino, were planted in plastic pots containing pasteurized soil and maintained in a greenhouse at 22°. The stems of 3 wk old seedlings were used for infection studies.

Inoculation

Inoculum was prepared aseptically by pouring sterile distilled water over *C. dematium* cultures and agitating. In order to determine the effect of wounding on the infection process of *C. dematium*, the stems of half of the cowpea seedlings were wounded with fine sandpaper. All stems were painted to run-off with a spore suspension (1×10^5 conidia ml^{-1}). Inoculated plants were covered with plastic bags to maintain a high humidity and kept at 22° in a glasshouse.



Figs 1–4. Scanning electron micrographs of pre-penetration structures formed by *Colletotrichum dematium* on the stem of cowpea. **Fig. 1.** Germ-tube (g) formation 6 hpi. Conidia (c) have formed septa (s). Bar = 10 μm. **Fig. 2.** Formation of a sessile appressorium (a) and an appressorium (a) subtended from a germ-tube (g) s, septum. Bar = 10 μm. **Fig. 3.** Micrograph showing lack of orientation of germ-tube (g) to stoma (st). Bar = 10 μm. **Fig. 4.** Penetration point (p) in the centre of the area of adhesion of a mechanically dislodged appressorium (a). Bar = 1 μm.

Light microscopy

To study the infection process and quantify pre-penetration structures, stems were cut into 20 mm long segments at various time intervals after inoculation. The epidermis of these segments was stripped using a scalpel and forceps, mounted on microscope slides and stained with lactophenol cotton blue. The preparations were studied and photographs were taken with a Nikon Optiphot photomicroscope.

Scanning electron microscopy (SEM)

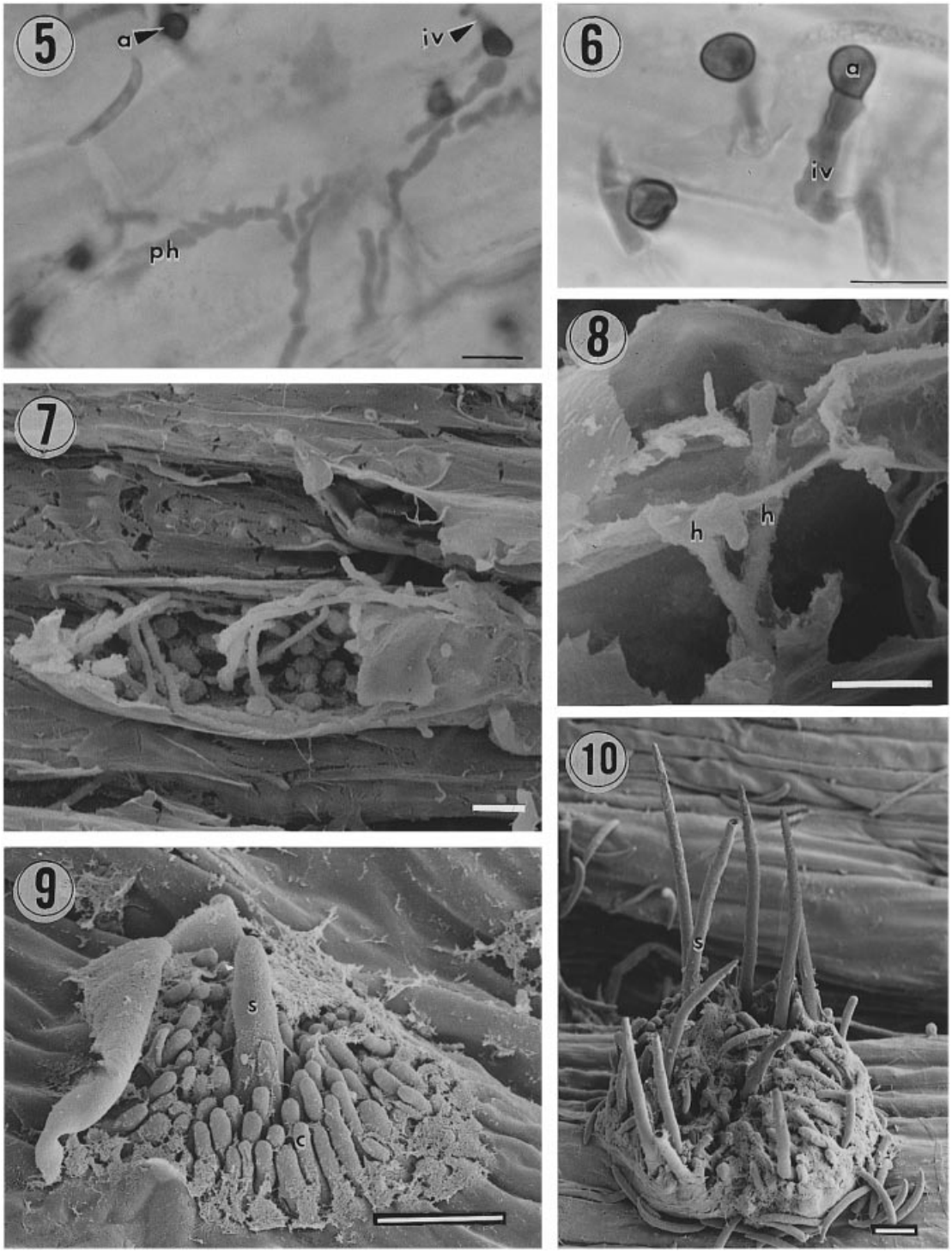
To study pre-penetration behaviour, 5 mm stem segments were cut from the inoculated plants at various time intervals after inoculation. Material was fixed in 2·5% v/v glutaraldehyde in 0·075 M phosphate buffer (pH 7·4–7·6) for 2 h. Material was then rinsed in the same buffer and post-fixed in 0·25% w/v aqueous osmium tetroxide for 2 h, followed by three successive washing steps in distilled water. Material was dehydrated in an ascending acetone series, critical point dried in a Bio-rad critical point dryer and mounted on SEM stubs. The stem epidermis of some of the specimens was removed using the stub method of Hughes & Rijkenberg (1985) in order to study the infection process beneath the epidermis and within the stem. All specimens were coated with gold in a Polaron sputter coater and examined with a JEOL JSM 840 scanning electron microscope operating at 5 kV.

RESULTS

Wounding of the stems had no effect on the time of formation or orientation of pre-penetration structures, as conidial germination on unwounded and wounded stems occurred simultaneously and the structures formed showed no specific orientation to wounded tissue. Identical symptoms were produced on both wounded and unwounded stems.

Both germinated and ungerminated conidia adhered strongly to the stem surface since they were not dislodged during SEM preparation. Conidia of *C. dematium* were falcate, 2–4 × 16–34 μm and aseptate, but one to two septa became visible upon germination (Figs 1, 2). Germination of conidia had begun by 6 h post inoculation (hpi), and by 9 hpi up to 80% had germinated. Germ-tubes developed from random points on the conidia. The majority of conidia formed only one germ-tube (Figs 1, 3), but occasionally more than one germ-tube (branched or unbranched) was formed (Fig. 2). The appressoria (4–6 × 6–10 μm) were bulbous and either sessile, developing from any point on the conidium, or were subtended from the tips of short (2–6 μm) or longer (15–20 μm) germ-tubes (Fig. 2). Germ-tubes and appressoria formed directly above epidermal cells or stomata, with no specific orientation to stomata, whether open or closed (Fig. 3).

Fig. 4 illustrates the area of contact on the host tissue representing the site of adhesion beneath a mechanically dislodged appressorium. A penetration point on the stem, in



Figs 5–10. Light and scanning electron micrographs of post-penetration events of the infection of cowpea stems by *Colletotrichum dematium*. Bars = 10 μm. **Fig. 5.** Light micrograph showing melanized appressoria (a) and knotty primary hyphae (ph) from infection vesicles (iv) penetrating adjacent epidermal cells. **Fig. 6.** Light micrograph showing formation of infection vesicles (iv) in epidermal cells below melanized appressoria (a). **Fig. 7.** Infected epidermal cell packed with hyphae, 40 hpi. **Fig. 8.** Inter- and intracellular growth in cortical cells and branching of secondary hyphae (h). **Fig. 9.** Immature acervulus and developing conidia (c) emerging from the surface of the stem, 65 hpi. s, seta. **Fig. 10.** Mature acervulus and conidia on the surface of the stem, 80 hpi. s, seta.

the centre of the area of adhesion, is visible (Fig. 4). By 10 hpi, the appressoria had begun to melanize and by 14 hpi 90–95 % of appressoria had become melanized and penetration of host tissue had begun (Figs 5, 6). Penetration by infection pegs occurred directly through the plant cuticle and cell wall. Direct penetration by germ-tubes was not observed. At 20 hpi, appressoria had formed infection vesicles within epidermal cells (Fig. 6). Thick ($\pm$3–4 μm diam.), knotty primary hyphae formed from these infection vesicles and entered adjacent epidermal cells (Fig. 5). The primary hyphae branched and grew rapidly for 20–30 h until the initially infected epidermal cells were packed with hyphae (Fig. 7).

Having established an infection in epidermal cells, *C. dematium* produced secondary hyphae (±40 hpi). The secondary hyphae were branched, thinner than primary hyphae and grew extensively inter- and intracellularly, passing through cell walls from one cortical cell to the next (Fig. 8). After approx. 48 hpi, light brown lesions appeared on the stems, due to the invasion of secondary hyphae into cells and necrosis of epidermal cells. By 65 hpi the eruption of acervuli through the cuticle to the stem surface was visible (Fig. 9). These acervuli were characterized by a single, melanized seta surrounded by numerous developing conidia (Fig. 9). After 70 hpi small black acervuli with one or two setae were visible on lesions under the dissecting microscope. Mature, sporulating acervuli became visible at approx. 80 hpi (Fig. 10). By 100 hpi brown lesions also began to appear on leaves of inoculated plants.

DISCUSSION

The infection process of *C. dematium* on cowpea stems is generally consistent with that of other *Colletotrichum* spp. on various hosts, including cowpea. Conidial germination and appressorial formation occurred within 6 hpi. This is earlier than that of *C. lindemuthianum* on French bean (18 hpi) (O'Connell, Bailey & Richmond, 1985), *C. capsici* on cowpea hypocotyls (16 hpi) (Pring *et al*., 1995), *C. lindemuthianum* on cowpea (12 hpi) (Bailey *et al*., 1990) and *C. destructivum* on cowpea (12 hpi) (Latunde-Dada *et al*., 1996), but slightly later than the germination of *C. truncatum* on pea (4 hpi) (O'Connell *et al*., 1993). The rate of spore germination and appressorium formation can be influenced by the presence of nutrients, the chemical nature of host surfaces and the concentration of epicuticular wax (Mercer, Wood & Greenwood, 1971; Prusky & Plumbley, 1992).

The formation of septa in germinated conidia is consistent with the results presented for *C. lindemuthianum* (Bailey *et al*., 1990), *C. capsici* (Pring *et al*., 1995) and *C. destructivum* (Latunde-Dada *et al*., 1996) on cowpea and *C. truncatum* on hemp sesbania (van Dyke & Mims, 1991; O'Connell *et al*., 1993). Conidia of these *Colletotrichum* spp. also germinated to produce either sessile appressoria, or appressoria subtended on the ends of germ-tubes, as was found in this study.

Bailey *et al*. (1990) reported that appressoria of *C. lindemuthianum* on cowpea were formed indiscriminately over the surface, except over stomata. Our results show that germ-tubes of *C. dematium* on cowpea stems form appressoria on the host epidermis with no specific orientation to stomata. Russo & Pappelis (1981) in their studies of *C. dematium* on onion, found that although the fungus is capable of penetrating through the cell wall, it was often observed to produce appressoria over stomata. Hyphae were observed below the stomata indicating that *C. dematium* can use stomata as an alternative method to enter the host. In contrast to the results of Lapp & Skoropad (1978) for *Colletotrichum graminicola* (Ces.) G. W. Wilson on barley leaves, we found that production of appressoria of *C. dematium* is unaffected by grooves above the anticlinal walls of epidermal cells.

C. dematium produces melanized appressoria and this is consistent with previous observations (van Dyke & Mims, 1991; Bailey *et al*., 1992; O'Connell *et al*., 1993, 1996; Latunde-Dada *et al*., 1996). As was reported for *C. truncatum* on stems, leaves and petioles of pea (O'Connell *et al*., 1993) and *C. capsici* on cowpea hypocotyls (Pring *et al*., 1995), *C. dematium* enters the host directly through the cuticle and epidermal cell wall, and is not reliant on wounds or natural openings for penetration. Penetration of cowpea by *C. dematium* at 14 hpi is earlier than Russo & Pappelis (1981) observed for the same fungus on onion. Penetration by *C. dematium* f. sp. *circinans* on white, yellow and red onion epidermis was observed to have occurred 24 hpi.

This study suggests that *C. dematium* could be a hemibiotrophic pathogen, like *C. lindemuthianum* on bean (O'Connell *et al*., 1985) and cowpea (Bailey *et al*., 1992). However, *C. dematium* on onion was shown by Russo & Pappelis (1981) to exhibit a sub-cuticular infection strategy. On cowpea, *C. dematium* produces globose infection vesicles in epidermal cells. These vesicles give rise to large primary hyphae, which also branch and grow rapidly for 20–30 h until the initially infected cells are packed with convoluted mycelium. As was found with *C. truncatum* (O'Connell *et al*., 1993) and *C. lindemuthianum* (Bailey *et al*., 1990), this symptomless phase lasts for approximately two days, after which a second phase begins, associated with the invasion of secondary hyphae into cells. An extensive primary mycelium is needed to support this phase, during which uninfected cells die leading to lesion formation, acervuli production and sporulation (O'Connell *et al*., 1985). Wijesundera *et al*. (1989) suggested that the switch is associated with changes in the synthesis and/or activity of cell wall degrading enzymes. Secondary hyphae of *C. truncatum* (O'Connell *et al*., 1993) and *C. lindemuthianum* (Bailey *et al*., 1990) began to radiate from primary hyphae after 3–4 d, fill rapidly colonising adjacent cells, as was also found in this study. O'Connell *et al*. (1993) believe that the ability of certain *Colletotrichum* species to invade cells without killing them, thereby avoiding host defences such as hypersensitive reactions, is the key to their success. The appearance of acervuli at ±65 hpi is much earlier than in *C. destructivum* on cowpea, where acervuli are produced in abundance only at 120 hpi (Latunde-Dada *et al*., 1996).

Much is known about the cytology of infection of several *Colletotrichum* spp., even on cowpea, but this is the first study on the infection processes of *C. dematium* on cowpea.

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