INFECTION PROCESSES OF *CIBORINIA CAMELLIAE* ON CAMELLIA FLOWER TISSUE

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ABSTRACT

The infection process of *Ciborinia camelliae*, the cause of camellia flower blight, was studied using light and confocal microscopy. Germination and infection structures of the pathogen on petals were stained with trypan blue in lactophenol and with glutaraldehyde for light and confocal microscopy respectively. Within 6 h of inoculation, the ascospores germinated and formed short germ tubes. The host cuticle was penetrated and within a further 6 to 18 h, a large swollen structure formed underneath each penetration. From here, a hypha grew under the cuticle to the junction between two cells and then developed as intercellular hyphae. The mycelium continues to develop intercellularly for the next 72 h but without causing extensive destruction of the host cell walls.

Keywords: infection process, *Ciborinia camelliae*, confocal microscopy, glutaraldehyde.

INTRODUCTION

Ciborinia camelliae Kohn causes flower blight of camellia, which is the only host for this pathogen. The ascospores infect flower petal tissue and cause extensive damage to flowers (Kohn and Nagasawa 1984).

Details of the infection process for *C. camelliae* are not known. The infection processes of *Venturia inaequalis* and *Colletotrichum* spp. have been well studied (Bailey *et al.* 1992; Skipp *et al.* 1995) and the different strategies of infection defined. Some *Colletotrichum* spp. achieve infection through wounds caused by physical damage or insects (Boher *et al.* 1983). Others penetrate cuticles and establish a benign but often extensive sub-cuticular infection (sub-cuticular intramural pathogens) prior to development of necrotrophic hyphae which grow through and destroy tissue (Walker 1921). A third group, typified by *C. lindemuthianum* on bean has "intracellular biotrophy" development (O'Connell *et al.* 1985). This process involves the establishment of a biotrophic relationship sequentially in each successive cell colonized by primary hyphae. The fourth group of *Colletotrichum* spp. are known as "intracellular hemibiotrophic" pathogens and are typified by *C. destructivum* in infected cowpea. Hemibiotrophic infections form in viable epidermal cells; hyphae are large and become highly branched, but remain within the initially infected epidermal cells (Bailey *et al.* 1990). This intracellular phase lasts only for 72 h (Latunde-Dada *et al.* 1996). *Ciborinia camelliae* could follow one of the above strategies of the *Colletotrichum* infection process.

The objectives of the present study were to understand the strategy of the infection process for *C. camelliae*, and to visualize and measure the time sequence of important events after inoculation of host tissue with this pathogen.

MATERIALS AND METHODS

Inoculum

Apothecia of *C. camelliae* were collected from the camellia section of the Wellington Botanic garden during the 1999 blight season (August – October), placed *New Zealand Plant Protection* 53:151-156 (2000)

in a humid chamber and stored at 5°C until required. Ascospore suspensions were prepared by suspending a sclerotium with an apothecium upside down in a sterilised universal bottle with moistened paper tissue around the sclerotium. After 8 - 16 h (overnight) ascospores had been released into the bottom of the bottle and sufficient sterile distilled water was added to obtain an inoculum concentration of 5×10^4 ascospores/ml. For direct dry inoculation, airflow from an aquarium pump was passed over apothecia in a plastic chamber and it carried fresh ascospores directly from the apothecia to a settling chamber where they were deposited by gravitation onto the test petals (Tyler 1999).

Host

Flowers were collected from camellia plants (*Camellia japonica* cv. Brian) located in the Massey University arboretum, and undamaged, healthy petals were removed for inoculation.

Inoculation and incubation

For wet inoculation, 2 µl of ascospore suspension was placed at each of 5-6 sites per flower petal using a micro-pipette. Inoculated petals were allowed to dry for about 1 h until the inoculum suspension became a thin layer of water. They were then placed in a plastic tray with moist paper towels in the bottom and covered with a clear polyethylene bag to ensure high humidity. The tray was incubated at 21°C for 72 h. During incubation, petals were taken at random and about 25 mm² was cut from each inoculated site of the tissue at 6 h intervals for microscopic examination.

Light microscopy

For examination by light microscopy, two or three pieces of inoculated petal tissue were placed on glass slides in a few drops of 0.05% trypan blue in lactophenol (Wei *et al.* 1997) and heated over a flame until boiling. Excess stain was removed from the specimens by washing with distilled water and stained pieces were mounted with Citifluor (glycerol/PBS) mounting medium before examination.

Confocal microscopy

For examination by confocal laser scanning microscopy (CLSM), samples were incubated with PBS-GA 8 solution (8 ml of 25% electron microscopy standard glutaraldehyde mixed with 92 ml of phosphate buffered saline solution) for 6 h or overnight to induce fluorescence of fungal structures. Specimens were cleared overnight with chloral hydrate (5 g in 2 ml of water). The treated specimens were mounted on a slide with Citifluor mounting medium and observed under CLSM using exciter filters of 488 and 568 nm and barrier filters of 530 and 590 nm.

RESULTS

Images of the stages of ascospore germination and hyphal development, from germ tube initiation to primary hyphae or secondary hyphae, were obtained by both light microscopy and CLSM. Most ascospores germinated from one side at the sharp end of the spore (Fig. 1) and a short germ tube formed within 6 h. Each germ tube directly penetrated through the cuticle of petal tissue (Fig. 2) and swelled underneath.

FIGURE 1:

Confocal micrograph. *Ciborinia camelliae* ascospore germination (closed diamond arrow head) and formation of multilobed structure (open arrow head) 30 h after inoculation. A germinated spore from which a hypha grew towards an epidermal inter-cellular junction is shown by a closed arrow head. Bar=20 µm.

FIGURE 2:
Confocal micrograph. *Ciborinia camelliae* ascospore germination and penetration of the cuticle layer of camellia flower petal tissue at 6 h after inoculation. Bar=40 μ m.

FIGURE 3:
Light microscopy. *Ciborinia camelliae* ascospore germination and hyphal growth underneath the cuticle layer towards the epidermal intercellular junction of camellia flower petal tissue at 18 h after inoculation. Bar=40 μ m.

FIGURE 4:
Confocal micrograph. *Ciborinia camelliae* ascospore germination and intercellular hyphal growth in camellia flower petal tissue at 18 h after inoculation. Bar=20 μ m.

FIGURE 5:
Confocal micrograph. *Ciborinia camelliae* intercellular hyphal growth in camellia flower petal tissue at 48 h after inoculation. Bar=200 μ m.

FIGURE 6: Confocal micrograph. Intracellular hyphae of *C. camelliae* in a well established disease lesion. Bar=20 μ m.

In a few cases, relatively large multilobed structures developed and showed restricted growth for 30 h (Fig. 1). A narrow tube then emerged from the penetrated enlarged germ tube and immediately formed a relatively large, primary hypha that grew underneath the cuticle towards the junction of the epidermal cells over the next 18 h (Fig. 3). Upon reaching a junction of the epidermal cell, the primary hypha branched and grew intercellularly (Figs 3 and 4). The growth of the fungal hyphae continued intercellularly for at least the next 48-72 h (Fig. 5). During this period, the cell walls became brown and after 72 h a visible brown lesion had developed. Intracellular hyphae could be found in the cells at this stage (Fig.6).

DISCUSSION

Light and confocal microscopy of the infection process of *C. camelliae* have revealed differences from other pathogens. In all cases, conidia or ascospores germinate and the host barrier is penetrated, but the subsequent growth and behavior of infection hyphae varies. Smart (1991) stated that the plant cell wall represents the next physical barrier to a fungal pathogen after breaching the cuticle. In the present study, the ascospores produced a short germ tube, which immediately penetrated the cuticle and there was no evidence that the hyphae penetrated through the epidermal cell walls into cells in the first 72 h of the infection process. This contrast with the case of *Colletotrichum lindemuthianum* where the fungus first penetrates an epidermal cell via the cuticle and epidermal cell wall then continues as an “intracellular biotrophic” pathogen in several successive cells after it becomes necrotrophic (O’Connell *et al.* 1985). Similarly, *C. destructivum* penetrates epidermal cells but remains as an “intracellular hemibiotrophic” pathogen for 72 h in each initially penetrated cell (Latunde-Dada *et al.* 1996). The initial infection process of *C. camelliae* thus differs from that of these two species of *Colletotrichum*.

McKeen (1974) observed that *B. cinerea* infections frequently started from short germ tubes without appressorium formation. Cole *et al.* (1996) also observed penetration from short germ tubes of *B. cinerea* under conditions of dry inoculation but high humidity. However, they also found that long germ tubes were produced when water with glucose was present. In the present study, *C. camelliae* produced short germ tubes under both wet or dry but humid conditions and readily penetrated petal cuticles. Germ tubes of *Venturia inaequalis*, commonly contact the cuticle and appresoria are formed, but sometimes germ tubes may penetrate the cuticle directly without forming an appressoria (MacHardy 1996). Appresoria of *C. camelliae* were not as obvious as those of *B. cinerea* (McKeen 1974). Other fungi such as *Sclerotinia sclerotiorum*, *Rhizoctonia solani*, *Verticillium albo-atrum*, *Fusarium* species and *Cochliobolus carbonum* have been recorded as invading plant tissue without formation of appresoria (Mendgen and Deising 1993). Although no obvious appressorium was observed in our study soon after cuticle penetration, the penetration hyphae produced sub-cuticular swellings before development of primary infection hyphae.

During the early stages of infection some pathogens (e.g. *Colletotrichum lindemuthianum*) act as intracellular hemibiotrophic pathogen and infect the host cells without killing them. Others (e.g. *C. capsici*, *C. circinans* and *Venturia inaequalis*) act as subcuticular intramural pathogens that grow exclusively beneath the cuticle and within the walls of host epidermal cells without entering them (Bailey *et al.* 1992). *Ciborinia camelliae* clearly belongs to the second group. Bailey *et al.* (1992) have speculated that *Colletotrichum* spp. that establish hemibiotrophic relationships involving intimate cytoplasmic interaction with their hosts appear to be highly host-specific, while those that grow only in host cell walls (e.g. *C. capsici*) have a wide host range. *Ciborinia camelliae* does not fit this categorisation since it grows hemibiotrophically in the cell walls for the first 24-48 h but has a very restricted host range. Whether this relationship is dependent on the host tissue infected (petals versus leaves and fruit) or whether it is characteristic of *C. camelliae* requires further elucidation. The appearance of disease symptoms within 24-48 h even though intracellular inversion does not appear to occur within this time span also requires further investigation.

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