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INFECTION PROCESSES OF *COLLETOTRICHUM* ISOLATES FROM FOREST TREES IN THE TROPICS

Maziah Zakaria,

Forest Research Institute Malaysia, Kepong, 52109 Kuala Lumpur, Malaysia

John A. Bailey & Richard J. Pring

Department of Agricultural Sciences, University of Bristol, Long Ashton Research Station, Long Ashton, Bristol BS18 9AF, United Kingdom

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MAZIAH, Z., BAILEY, J. A. & PRING, R. J. 2000. Infection processes of *Colletotrichum* isolates from forest trees in the tropics. Several isolates of *Colletotrichum* obtained from forest trees were found to have wide host ranges. They infected both legumes and other forest trees. All the isolates examined penetrated the cuticle of inoculated plants through production of germ-tubes that arose from appressoria. However, some isolates also penetrated the host through open stomata, sometimes after the production of appressoria but on most occasions appressoria were not formed. Two types of infection process were observed. In the first, shown by isolates 634, 640 and 659, individual infected cells were dead upon infection but growth through adjacent cells and tissues caused no extensive degradation of cell walls. In the second process, shown by isolate 689, infected cells were also killed soon after infection, but as the pathogen began to grow through the tissues, cell walls in advance of the hyphae were extensively degraded. Typically, in both strategies the host cuticle remained unaltered even after complete dissolution of underlying epidermal cell walls.

Key words: *Colletotrichum* - papillae - infection process - tissue colonisation - intracellular hyphae

MAZIAH, Z., BAILEY, J. A. & PRING, R. J. 2000. Proses jangkitan isolat *Colletotrichum* yang diperoleh dari pokok-pokok hutan di kawasan tropik. Kajian terhadap julat perumah beberapa isolat *Colletotrichum* yang diperoleh dari pokok hutan menunjukkan bahawa isolat-isolat tersebut mempunyai julat perumah yang meluas. Isolat-isolat tersebut boleh menjangkiti pokok-pokok hutan dan kekacang. Dalam kajian ini, kesemua isolat boleh menembusi kutikel perumah menerusi tiub germa yang keluar dari apresoria. Terdapat juga isolat yang menembusi perumah melalui stoma yang terbuka, melalui pembentukan apresoria tetapi pada lazimnya apresoria tidak terbentuk. Dua proses infeksi telah dikenal pasti. Proses infeksi yang pertama ditunjukkan oleh isolat-isolat 634, 640 dan 659, sel individu yang diserang mati serta-merta tetapi pertumbuhan melalui sel-sel dan tisu-tisu bersebelahan tidak menyebabkan pendegradan ekstensif dalam dinding sel. Dalam proses kedua yang ditunjukkan oleh isolat 689, sel-sel yang dijangkiti musnah serta-merta selepas jangkitan. Tetapi selepas patogen mula merebak ke dalam tisu, dinding sel mengalami pendegradan yang teruk sebelum hifa sampai kepadanya. Dalam kedua-dua proses, kutikal perumah tidak mengalami apa-apa perubahan walaupun setelah keseluruhan sel epidermal di bawahnya telah musnah.

Introduction

Colletotrichum gloeosporioides is the most dominant and major species of *Colletotrichum* on crops and as currently defined embraces a wide range of variability (Waller 1992).

Many species of *Colletotrichum*, including *C. gloeosporioides*, are known to have an extremely wide host range especially in the tropics, though some forms of *C. gloeosporioides* are very host specific such as *C. gloeosporioides* f. sp. *aeschynomenes*, f. sp. *malvae* and f. sp. *cuscutae* (Templeton 1992).

In Peninsular Malaysia, the most important *Colletotrichum* disease of forest trees was brown needle disease of pines, i.e. in the seventies when there was wide interest in planting pines. The causal organism, *C. gloeosporioides* f. sp. *pinae*, attacked all species of pines grown in nurseries, especially *P. caribaea* (Fielding 1971). More recently, surveys revealed the regular occurrence of *Colletotrichum* species in forest nurseries (Maziah & Bailey 2000). Results from host range studies clearly indicated that isolates of *Colletotrichum* from forest trees have wide host ranges. They are capable of infecting different species of forest trees as well as different legume vegetables.

Understanding the modes of infection shown by *Colletotrichum* may be very important in preventing and controlling disease development and spread. The mechanisms by which species of *Colletotrichum* penetrate their hosts have been discussed for many years. Several modes are possible: by direct penetration of the cuticle, through natural openings, e.g. stomata, and through wounds. Of these, the first appears to be the most common means of penetrating plant tissues (Bailey *et al.* 1992). Penetration through stomata is rare in species of *Colletotrichum.* However, it has been observed to occur in infection of *Hevea brasiliensis* by *C. gloeosporioides* (Radziah 1984, Senechal *et al.* 1987). Like penetration through stomata, penetration through wounds is also not common. However, it is essential for some diseases such as crown and finger stalk rot of banana (Krantz *et al.* 1978, Agrios 1988). For other species, e.g. *C. capsici*, although wounding is not essential for infection it does increase the plant's susceptibility to disease (Ramakrishnan 1940, Maziah 1990, Pring *et al.* 1995).

The aim of this study was to examine the infection strategies of several pathogens isolated from tropical forests that lead to the production of destructive water-soaked lesions. Studies were performed using hypocotyls of bean (*Phaseolus vulgaris*) and leaves of rubber (*Hevea brasiliensis*).

Materials and methods

In the preliminary studies, host susceptibility, initial host reactions and infection strategies were examined using 13 isolates from forest trees. Details of these isolates are described in Table 1. These were tested on hypocotyls of *Phaseolus vulgaris* cv. La Victoire, a host that has been used extensively for assessing the presence of infection hyphae (O'Connell *et al.* 1985). A more detailed study on the infection processes was conducted on four isolates (634, 640, 659 and 689) using leaves of *H. brasiliensis.*

Inoculation of legume hypocotyls and Hevea brasiliensis leaves

Hypocotyls and leaves were excised, placed in plastic boxes lined with moist tissue paper and inoculated with 5-7 µl drops of conidia suspended in sterilised distilled water (5×10^5 conidia per ml). For the leaves, inoculation was performed on the lower side. The plant tissues were incubated in an illuminated cabinet at 25 °C. The inoculation droplet was maintained on the plant surface for at least 24 h.

Table 1. The origins of the isolates of *Colletotrichum*

Isolate No.	Host	Disease symptoms	Collection site
630	*Acacia mangium*	Leaf spots and lesion	FRIM, Malaysia
634	*Hevea brasiliensis*	Leaf spots	Dengkil, Malaysia
635	*Chrysalidocarpus lutescens*	Leaf spots	FRIM, Malaysia
640	*Schizostachym branchycladium*	Leaf spots	FRIM, Malaysia
645	*Magnolia malayana*	Leaf lesions	FRIM, Malaysia
657	*Calamus manan*	Leaf spots and lesions	FRIM, Malaysia
659	*C. manan*	Leaf spots and lesions	FRIM, Malaysia
660	*Pterocarpus indicus*	Leaf spots and lesions	FRIM, Malaysia
662	*P. indicus*	Leaf spots and lesions	FRIM, Malaysia
664	*P. indicus*	Leaf spots and lesions	FRIM, Malaysia
665	*P. indicus*	Leaf spots and lesions	FRIM, Malaysia
674	*Schoutenia accrescens*	Leaf spots	FRIM, Malaysia
689	*Gliricidia sepium*	Leaf spots	Gualan, Guatemala

Assessment of cell viability

Legume hypocotyls have been used extensively to study the initial infection process of *Colletotrichum* spp. especially to assess whether these pathogens can establish initial biotrophic infections typical of hemibiotrophic fungi (Bailey *et al.* 1992). The ability of bean hypocotyl cells to plasmolyse and to accumulate neutral red was tested using a method described by O'Connell *et al.* (1985). Stained tissues were mounted on slides, and the presence of plasmolysed cells was observed immediately by bright field microscopy. Successful plasmolysis is evidence that the cells are alive.

Light microscopy

Infection processes were studied by examining thin longitudinal strips of tissue beneath inoculation droplets, mounted in distilled water. Examination of leaf tissues was done using the method of O'Connell *et al.* (1993). Where browning and granulation in cells appeared to obscure hyphae, the tissues were mounted in 0.5 N sodium hydroxide for 1 to 2 min, in an attempt to dissolve the pigmentation. All specimens were viewed using either bright field or Nomarski interference contrast optics.

Transmission electron microscopy (TEM)

Inoculated rubber leaf tissue pieces (5 mm × 5 mm) were fixed in a mixture of formaldehyde(4%):glutaraldehyde(5%) in 0.1 M cacodylate buffer (pH 7). After rinsing in distilled water and post-fixing with 4% osmium tetroxide in cacodylate buffer, the tissues were rinsed with distilled water and left overnight in fresh distilled water. After fixation, the samples were dehydrated in graded concentrations of ethanol. Following dehydration, the tissues were infiltrated with Spurr's low viscosity epoxy resin. The tissues were placed in silicone rubber flat embedding moulds and the resin was left to polymerise at 70 °C. Polymerised blocks were sectioned, stained with lead citrate and the sections were examined in a Hitachi H7000 TEM.

Scanning electron microscopy (SEM)

For cryo-SEM, tissues bearing symptoms were cut and attached to the specimen carrier using a mixture of colloidal graphite and "Tissue Tek" (Agar Scientific, UK). The tissues were frozen in the pre-chamber block at -150 °C. Tissue samples were examined using a Hexland CT 1000 cryo preparation unit (Oxford Instrument, UK) interfaced with a Philips 505 SEM. Photographs were taken on Kodak T-Max 100 film.

Results

Infection and colonisation of legume hypocotyls

Results of the preliminary study showed, with the exception of isolate LARS 630, which did not infect bean hypocotyls, all the isolates produced water-soaked lesions within 3 to 5 days after inoculation. Isolate 689 appeared to be the most pathogenic, followed by isolates 634 and 665 respectively. Analysis of tissues by light microscopy showed that isolate 630 produced extensive mycelial growth on the host surface but no hyphae penetrated the tissues. All the other isolates successfully penetrated the host, developed intracellular hyphae and subsequently caused extensive damage to the host tissues. The results are summarised in Table 2.

Microscopic examination of the infection process revealed that conidia of most isolates germinated within 2–4 h after inoculation, in a manner similar to that observed *in vitro* with a few forming hyaline appressoria. After 12 h, abundant melanised appressoria had formed on the surface of the tissue. All isolates were observed to penetrate the host directly through the cuticle (Figure 1). However, on a few occasions, isolates 634, 640 and 659 were also observed to penetrate through stomata. In addition, penetration through trichomes via very fine penetration hyphae was also observed in a few isolates.

In response to initial penetration by the pathogen, *Phaseolus vulgaris* responded by producing wall appositions (papillae) of varying sizes at the penetration site

Table 2. Infection strategies of the *Colletotrichum* isolates on *Phaseolus vulgaris*

Isolate	Initial host reaction	Initial infection process	Host reaction
630	Haloes, HR	Surface hyphae only	R
634	HR	Intracellular	S
635	Haloes, HR	Intracellular	S^l
640	Haloes, HR	Intracellular	S^l
645	Haloes, papillae, HR	Intracellular	S^l
657	Papillae, HR	Intracellular	S^l
659	Papillae, HR	Intracellular	S^l
660	Haloes, HR	Intracellular	S^l
662	Haloes, HR	Intracellular	S^l
664	HR	Intracellular	S^l
665	Haloes, HR	Intracellular	S^l
674	Haloes, HR	Intracellular	S^l
689	HR	Intracellular/Intramural	S

Observations were made on superficial sections of inoculated hypocotyls (O'Connell *et al.* 1985). HR = hypersensitive reaction; R = resistant; S = susceptible; S^l = susceptible, though involving extensive initial host reaction.

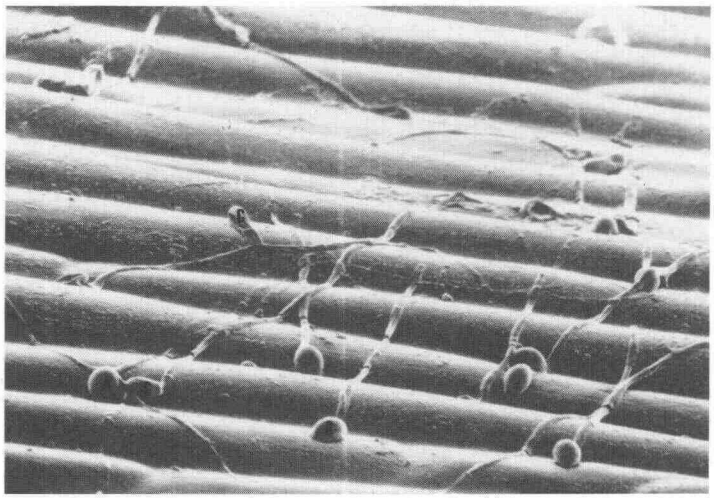

Figure 1. Hyphal growth and appressorial formation of isolate 634 on *Phaseolus vulgaris* hypocotyl. Germinated conidium (C) and appressorium (A). (x 742)

(Figure 2). In some instances, these papillae were observed to completely encase the hyphae, thus preventing further development. However, in most others, the hyphae managed to advance despite the barrier. Another type of response that was observed was an early hypersensitive reaction, visible as localised death and browning of cells around the infection point (Figure 3). The affected cells quickly became darkly pigmented and this could be seen as superficial flecking on the host surface. Clearing of tissue with NaOH showed that most infections at this point consisted of short hyphae within single epidermal cells. The cytoplasm of the infected cells was completely disorganised and in some cases adjacent uninfected cells also appeared to be dead. Staining of early infections with KNO_3/neutral red failed to show any evidence that initially infected cells remained alive. In most cases, these hypersensitive reactions did not stop the advancement of infection hyphae and within a few days the hyphae grew into adjacent cells. During the next 72 to 96 h hyphae began to advance, accompanied by progressive death of infected cells and subsequently death of uninfected adjacent cells. This was seen as loss of the ability of the hypocotyls' cells to plasmolyse when tested with KNO_3/neutral red stain. Five days after inoculation, intracellular, intramural and intercellular hyphae had extensively colonised host tissues, causing the lesions to become water-soaked.

Detailed infection and colonisation processes on Hevea brasiliensis leaves

Detailed examination of the infection process on leaves of *H. brasiliensis* was conducted using four isolates (634, 640, 659 and 689). Inoculation was performed on the lower side of the leaves, which had earlier proved to be very susceptible. The development of these pathogens on the host surface and their initial penetration processes were observed to be very similar. Most conidia germinated within 2–4 h and penetration was observed as early as 12 h after inoculation. All isolates penetrated the host directly through cuticle, normally after appressoria had been produced,

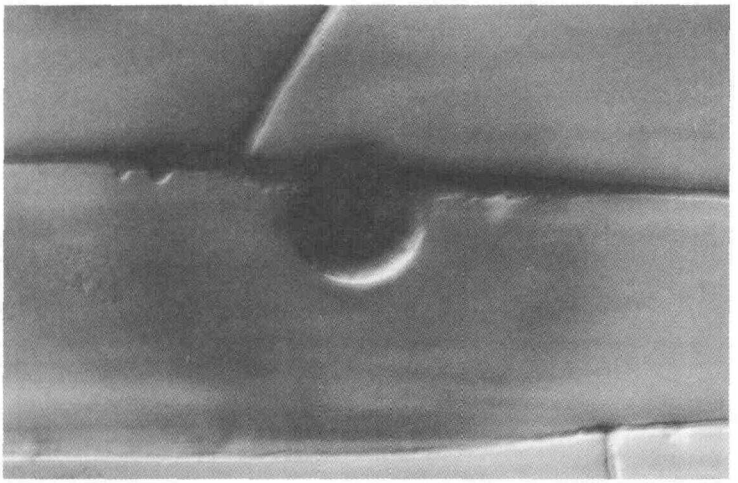

Figure 2. Papilla (wall apposition) formed at the site of fungal penetration of hypocotyls of *P. vulgaris* in response to infection by isolate 635 (4 days after inoculation)

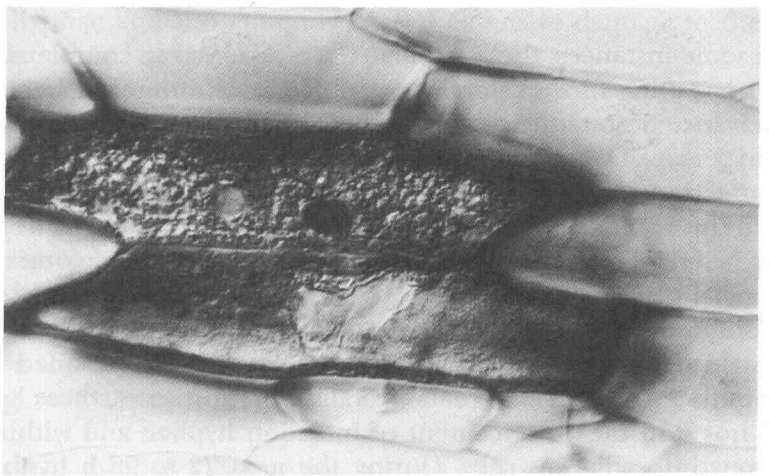

Figure 3. Hypersensitive reaction of epidermal cells of *P. vulgaris* following infection by isolate 635

and/or through stomata, normally in the absence of an appressorium (Figure 4). Penetration through stomata depended greatly on the availability of open stomata, as they were never observed to penetrate closed stomata.

Subsequent developments of isolates 634, 640 and 659 were very similar. All these isolates produced a very extensive development of intracellular hyphae of variable diameter in many epidermal leaf cells. There were no signs of any special infection structures such as a vesicles or large diameter primary hyphae. After 48 h, extensive intracellular hyphae and a few intramural hyphae had grown extensively, colonising the entire depth of the host tissues (Figure 5). This growth was accompanied by progressive death of infected host cells as well as surrounding cells. The walls of these cells remained intact with no evidence of wall dissolution away from the sites of hyphal penetration. The hyphae grew through the leaf tissues and became subcuticular before

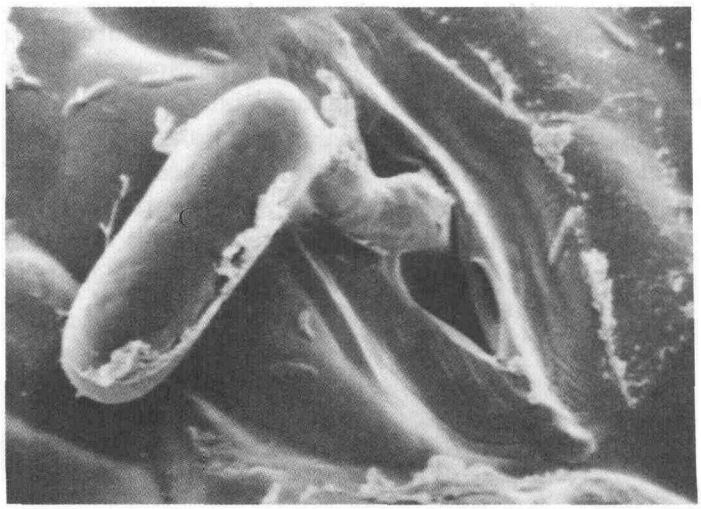

Figure 4. Penetration through stomata. Germinating conidium (C) of isolate 640 penetrating open stomata of *Hevea brasiliensis*, photograph taken 24 h after inoculation. (x 4900)

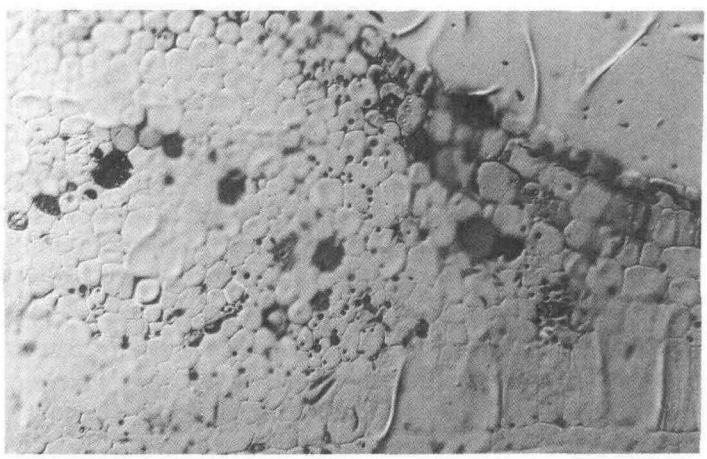

Figure 5. Colonisation of *H. brasiliensis* by isolate 634, showing intracellular hyphae in the entire cross-section of the leaf

emerging on the upper surface of the leaf. Soon afterwards, usually within three days after inoculation, the entire leaf became water-soaked. At this time, the cell walls were extensively degraded.

Infection by isolates 640 and 659 was similar to that of 634, penetration being either direct through the cuticle with or without the presence of appressoria, or through stomata (Figure 6). Within 48 h extensive growth of surface mycelium of isolate 640 was accompanied by growth of intracellular hyphae but intramural hyphae were rarely observed. Cells around the initially-infected area remained alive. Again, during the initial colonisation of the leaf tissues by these isolates, there was no evidence of extensive degradation of the host cell wall (Figure 6).

In contrast, development of isolate 689 was more rapid and subsequently quite distinct. As with the other isolates, penetration was either direct through the cuticle following the production of appressoria or through stomata without the presence of appressoria. Initial hyphal penetration triggered immediate death of infected cells, in which the pathogen developed extensive intracellular hyphae. Within 12 h, a few isolated intracellular hyphae had grown out of these cells and penetrated through the entire depth of the leaf. These intra- and intercellular hyphae caused death of both the infected cells and the adjacent uninfected cells and there was extensive degradation of their cell walls (Figure 7). Within 24 h the infection had usually caused visible water-soaked symptoms through the entire leaf.

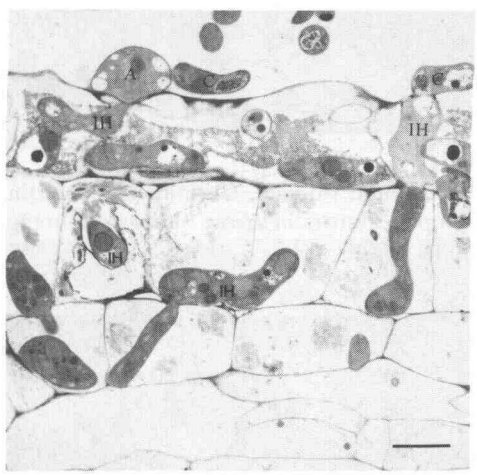

Figure 6. Penetration and infection of *H. brasiliensis* leaves by isolate 659, 48 h after inoculation. Note the penetration with the presence and absence of appressorium (A), followed by development of intracellular mycelium (IH) in the absence of extensive cell wall degradation. C = germinating conidium. Bar = 5 μm.

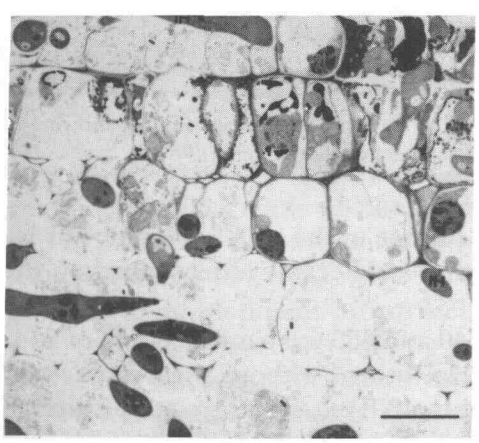

Figure 7. Infection of *H. brasiliensis* leaves by isolate 689, 48 h after inoculation. Note the development of the intracellular hyphae (IH) and extensive degradation of cell walls as well as death of cells in advance of the hyphae, especially in the palisade layer. Bar = 10 μm.

Discussion

Many isolates of *C. gloeosporioides* are known to have an extremely wide host range, though some others such as *C. lindemuthianum* from *Phaseolus* bean, *C. malvarum* from Malvaceae, *C. orbiculare* from cucurbits, *C. trifolii* from alfalfa and *C. destructivum* from cowpea have very narrow host ranges (von Arx 1957, Vaillancourt & Hanau 1992, Bailey *et al.* 1995). For these latter species, it has been proposed that their restricted host range is a direct consequence of their initial, albeit brief, biotrophic phase of growth (Bailey *et al.* 1995).

In the present study, four isolates of *Colletotrichum* obtained from different hosts but able to attack the same tropical forest species and several legume vegetables were studied. Two modes of host penetration were observed. Direct penetration of the host cuticle via appressoria was seen most often, but penetration through stomata with or without the presence of appressoria also occurred. Penetration through natural openings such as stomata has rarely been reported in species of *Colletotrichum*, although penetration of isolates of *C. gloeosporioides* through vein stomata of rubber was reported by Senechal *et al.* (1987).

On legume hypocotyls host resistance responses were observed. The two common types of response were browning of host cells and formation of papillae. The death and browning of host cells (a hypersensitive reaction) is usually followed by accumulation of phytoalexins and other defensive agents and cessation of pathogen development (Bailey 1982). However, in the present study, neither the papillae nor the hypersensitive response was effective. Most of the hyphae managed to grow through the papillae, or out of the dead cells, into adjacent cells allowing subsequent development to produce extensive water-soaked lesions.

Many *Colletotrichum* species exhibit a two-phase infection process involving an initial symptomless phase followed by a final destructive phase. Modes of infection by *Colletotrichum* species have been classified into three main strategies: (i) intracellular hemibiotrophic, (ii) subcuticular intramural, and (iii) combining both intracellular, subcuticular and intramural growth. The final phase for all three infection strategies is always highly necrotrophic, a process that explains the very destructive nature of these pathogens (Bailey *et al.* 1992).

The results from the present study demonstrated two different infection strategies. All pathogens were totally necrotrophic, there being no evidence of any initial biotrophic infections, nor of the presence of any specialised infection structure, such as vesicles, which may be required to establish a functional biotrophic relationship with host cells (Marks *et al.* 1965, Stumm & Gessler 1984, O'Connell & Bailey 1986, Trevorrow *et al.* 1988, Ogle *et al.* 1990, Bailey *et al.* 1992, O'Connell *et al.* 1993). In the first process, demonstrated by isolates 634, 640 and 659, the initial formation of intracellular hyphae killed the individual infected cells, while adjacent uninfected cells remained alive. There was no degradation of the cell walls of the infected cells or the associated uninfected cells. Only during the later phase of infection did the subsequent growth of intracellular hyphae cause cell wall degradation to occur. At this late stage of infection, when lesions became visible, hyphae also grew intramurally and intercellularly causing massive destruction of the host tissues.

The second infection process, demonstrated by isolate 689, was extremely aggressive, causing tissues to rot completely within three days. Penetration was either direct or through stomata, but in contrast to the other isolates, the initial penetration by isolate 689 caused immediate death of infected cells which was also immediately associated with massive degradation of the walls of both the infected and the adjacent uninfected cells. This fungus very soon became highly aggressive, producing a few rapidly growing hyphae that quickly grew across the entire leaf. Again growth of these hyphae was associated with extensive degradation of cell walls and death of cells, often well in advance of the hyphae, especially in the cells in the palisade layer. In both these processes, it appears that the rapid necrotrophic growth of the pathogen is so quick that it prevents the accumulation of the hosts' defence responses. Finally, the destructive phase was accompanied by the development of water-soaked symptoms on the entire leaf. Despite the destruction of most of the tissues, it is notable that both the upper and lower cuticular layers remain intact. Bailey *et al.* (1992) has suggested that intact cuticles play an important role in maintaining the fungal growth required for the development of the reproductive structures like acervuli and perithecia.

Wijesundera *et al.* (1989) presented evidence that pectin lyase activity was expressed at certain stages of *C. lindemuthianum* and that formation of enzyme was correlated with the destructive necrotrophic growth, during which enormous increases in pectin lyase activity were found. Several workers have suggested that cell death could be due to toxicity of the lyase, or in response to pectin or polygalacturonate substrates produced by the enzymes (Cervone *et al.* 1987, 1989, Wijesundera *et al.* 1989, Benhamou *et al.* 1991, Mathieu *et al.* 1991). The different patterns of necrotrophic infection shown by these isolates suggest that their regulation of pectin lyase synthesis will not be the same.

All the pathogens studied were isolated from leaves of tropical forest trees. Assessments of the pathogenicity revealed that they were highly pathogenic and able to attack a wide range of different hosts in addition to the trees from which they were isolated. All isolates infect plants in a totally necrotrophic manner causing rapid destruction of tissues. Isolates 634, 640 and 659 had similar infection processes which differed from that of isolate 689. Whether this indicates that these isolates may be different species requires more detailed taxonomic work. Traditional analysis of conidia failed to show significant differences. Their necrotrophic nature is totally consistent with their abilities to attack different plants. Since these pathogens were isolated from trees, it is likely that spores from these pathogens could be distributed widely and represent a serious threat to adjacent or underlying crops.

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