European Journal of Plant Pathology **103:** 35–41, 1997.

Infection process of *Colletotrichum destructivum* O'Gara from lucerne (*Medicago sativa* L.)

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Accepted 8 July 1996

Key words: alfalfa, anthracnose, cowpea, intracellular infection structures, legumes, *Vigna*

Abstract

The infection process of *Colletotrichum destructivum*, a cause of anthracnose in lucerne (= alfalfa, *Medicago sativa*) was studied by light microscopy. At the onset of the host-pathogen interaction, the fungus produced large, multilobed, multiseptate infection vesicles with elongated neck regions. Each infection structure packed the lumen of the initially-infected epidermal cell and remained confined within its walls for 48 h. Subsequently, narrow, invasive secondary hyphae radiated from the multilobed vesicles, grew through the walls of host cells and rapidly colonized the surrounding tissues. Acervuli emerged on the surface of colonized leaves 96 h after inoculation. These observations are discussed in relation to the infection process and specificity of a genetically closely-related isolate of *Colletotrichum destructivum* causing anthracnose in cowpea (*Vigna unguiculata*).

Introduction

Colletotrichum destructivum O'Gara is one of several fungal species which are capable of causing anthracnose disease in lucerne (= alfalfa, *Medicago sativa* L.) [Boland and Brochu, 1989]. It is regarded, along with *C. coccodes* (Wallr.) Hughes, *C. dematium* (Pers. ex Fr.) Grove f. *truncata* (Schw.) v. Arx, and *C. truncatum* (Schw.) Andrus and Moore, as a secondary pathogen in comparison to the more virulent *C. trifolii* Bain and Essary (Graham et al., 1976; Koch et al., 1989). Nevertheless, sole infections of lucerne by *C. destructivum* have caused considerable yield losses in North America (Boland and Brochu, 1989), Europe (Pauly, 1974; Raynal, 1977; Robotic and Klokocar-Smit, 1983), northern Africa (Troeung and Gosset, 1987) and South Africa (Thompson and van der Westhuizen, 1985; Koch et al., 1989). The host range of *C. destructivum* is wide and includes such legumes as *Phaseolus lathyroides* L., *Glycine max* (L.) Merr., *Trifolium* spp., *Lotus* spp., *Leucaena leucocephala* (Lam.) De Wit., *Melilotus albus* Desr., *Vigna unguiculata* (L.) Walp., *Coronilla varia* L., as well as tobacco (*Nicotiana tabacum* L.) and dodder (*Cuscuta* spp.) (Leach, 1958; Forer et al., 1973; Massenot and Raynal, 1973; Baxter et al., 1983; Manandhar et al., 1986; Wolcan and Dal Bello, 1988; Koch et al., 1989; Latunde-Dada et al., 1996). Host specificity and differences in pathogenicity have also been observed among *C. destructivum* isolates (Wolcan and Dal Bello, 1988).

The infection processes of a number of *Colletotrichum* species have been elucidated (see Skipp et al., 1995), including *C. trifolii* in lucerne (Porto et al., 1988; Mould et al., 1991a, b). However, the nature of pathogenesis of lucerne anthracnose caused by *C. destructivum* remains unknown. The infection process and nature of the intracellular fungal structures produced by a cowpea (*V. unguiculata*) isolate of *C. destructivum* have been described (Bailey et al., 1990; Latunde-Dada et al., 1996). In the latter study, the nucleotide sequences of the amplified ITS-2 and D2 regions of rDNA obtained from a cowpea isolate of *C. destructivum* were found to be highly homologous (97–99%) to those of some lucerne isolates of the pathogen, indicating that these isolates were closely related and suggesting that they might have similar

modes of infection in their hosts. The current paper presents the results of studies on the infection process of three isolates of *C. destructivum* from lucerne and compares them with the behaviour of the isolate of *C. destructivum* from cowpea.

Materials and methods

Fungal material

Three isolates of *C. destructivum* obtained originally from diseased lucerne were used. They were LARS 202, isolated by B.M. Troeung, Universite Mohammed – 1er, Morocco (Troeung and Gosset, 1987); LARS 319, isolated by G.J. Boland, University of Guelph, Ontaria, Canada (Boland and Brochu, 1989); and LARS 709, isolated by A.A. Al-Rokaibah, College of Agriculture, Burraydah Gassim, Saudi Arabia. In addition, LARS 056, a cowpea isolate of *C. destructivum* (Latunde-Dada et al., 1996) was used in comparative studies. The fungi were cultured in the dark on CM medium (Mathur et al., 1950). Inoculum was obtained from 7-day-old Petri dish cultures by irrigation with sterile deionised H_20. The conidial concentrations were adjusted by dilution after counting with a haemocytometer.

Plant material

Seeds of lucerne, cv. Europe (Sinclair McGill Ltd, Lincolnshire, U.K.) were sown in trays filled with Fisons Levington M3 soil-less compost (Levington Horticulture Ltd, Bramford, UK) and maintained at 25 °C in a growth cabinet using a 12 h photoperiod (50μ mole PAR m^{-2} s^{-1}). Cowpea seeds (cv. IT82E-60) were obtained from the International Institute of Tropical Agriculture, Ibadan, Nigeria. They were sown in trays filled with vermiculite and maintained under the same conditions used for lucerne.

Inoculation

Leaves were excised from 4-week-old lucerne seedlings and inoculated on the abaxial surface with four 3μl droplets of a 1×10^5 ml^{-1} conidial suspension of the fungi. They were incubated in moist plastic boxes at 25 °C in the dark (Latunde-Dada et al., 1996). Intact unifoliate primary leaves of 10-day-old cowpea seedlings were inoculated with ten 3μl droplets of inoculum. Seedlings were maintained at 25 °C under moist conditions in a propagator (Humex Greenhouse System, New Milton, UK). Control leaves were treated with droplets of sterile deionized H_20.

Light microscopy

Leaves were decolourized in a 0.15% (w/v) solution of trichloroacetic acid in a 3:1 (v/v) mixture of ethanol and chloroform for 12 h. They were then stained in a 0.025% (w/v) solution of Aniline Blue in lactophenol for 2 h. Light microscopic examinations were done with a Zeiss Axiophot microscope using Nomarski differential interference contrast. Photomicrographs were recorded on Kodak T-Pan film rated at 80ASA and developed in Diafine (Acufine, Chicago, USA).

Results

The morphological characteristics of the conidia obtained from CM cultures of the isolates of *C. destructivum* used in this study are given in Table 1. Isolates LARS 202, 319 and 709 developed similarly on lucerne leaves. Representative micrographs of stages in the infection process are provided in Figures 1–9. Conidia of *C. destructivum* became septate upon germination on the surface of lucerne leaves and formed melanized appressoria within 12 h of inoculation (Figure 1). The appressoria were sub-globose, with irregular and highly variable margins. By 24 h after inoculation, epidermal cells of lucerne leaves had been penetrated and contained intracellular fungal structures comprising swollen, saccate infection vesicles with elongated neck regions (Figure 2). The infection vesicles enlarged by developing bulbous lateral lobes (Figure 3) and assumed, by 48 h after inoculation, a multiseptate, multilobed structure (Figure 4), which was variable in shape (Figure 5). At this stage of the host-pathogen interaction, infected lucerne leaves were symptomless and the multilobed vesicles always remained confined within the initially-infected epidermal cells, often packing the cell lumen with bulbous fungal lobes.

Beyond 48 h after inoculation, bud-like outgrowths developed around the periphery of the multilobed vesicle (Figure 6) and these extended rapidly to produce invasive filamentous secondary hyphae (Figure 7). These hyphae radiated from the multilobed vesicles and grew through the walls of the infected cells to invade surrounding tissues, producing water-soaked lesions on the surfaces of infected leaves. Knotted hyphal aggregates developed from the multilobed

Table 1. Morphological characteristics of isolates of *Colletotrichum destructivum*

Characteristics	Isolates			
	LARS 056[1]	LARS 202[2]	LARS 319[2]	LARS 709[2]
Conidial size[3]	16.31 ± 1.97 by 4μm	17.84 ± 1.64 by 4μm	17.23 ± 1.86 by 4.4μm	16.39 ± 2.09 by 4μm
Conidial shape	Cylindrical, predominantly straight, but some slightly curved with a truncate base and a slightly-pointed apex			
Septation of conidium upon germination	Yes	Yes	Yes	Yes
Appressorial shape	Sub-globose with irregular and variable margins			

[1] Isolated from cowpea.
[2] Isolated from lucerne.
[3] Mean of 30 measurements ±SD.

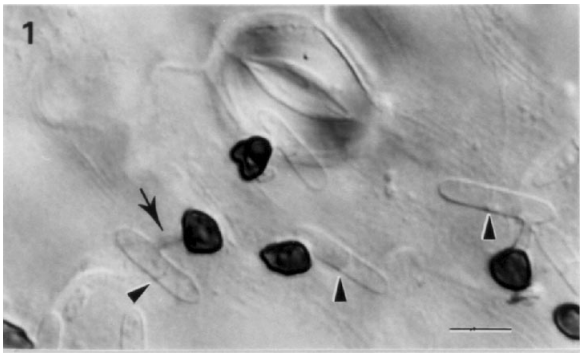


Figure 1. Germination of conidia of isolate LARS 202 on lucerne leaf surface 12 h after inoculation. Arrow heads indicate septa formed at the equatorial zone of the conidia upon germination; arrow indicates a germ tube terminating in a melanized appressorium. Bar = 10μm.

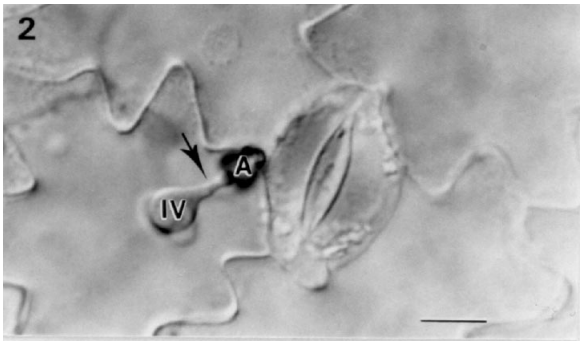


Figure 2. Formation of an intracellular saccate infection vesicle (IV) of LARS 202, with an elongated neck (arrow) emanating from a melanized appressorium (A), in an epidermal cell of lucerne leaf 24 h after inoculation. Bar = 10μm.

vesicles by 72 h after inoculation (Figure 8). These aggregates represented the initials of acervuli, which were observed in abundance by 96 h after inoculation.

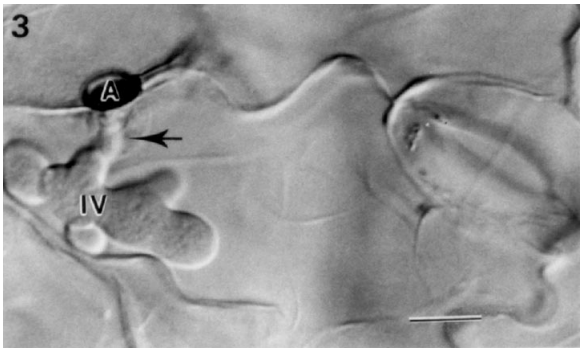
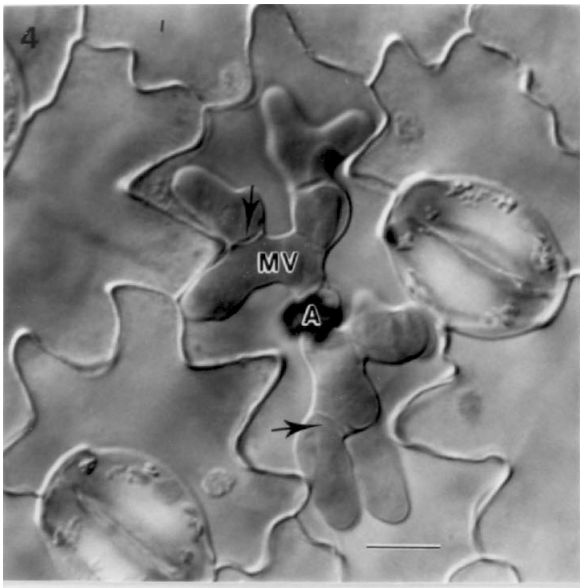
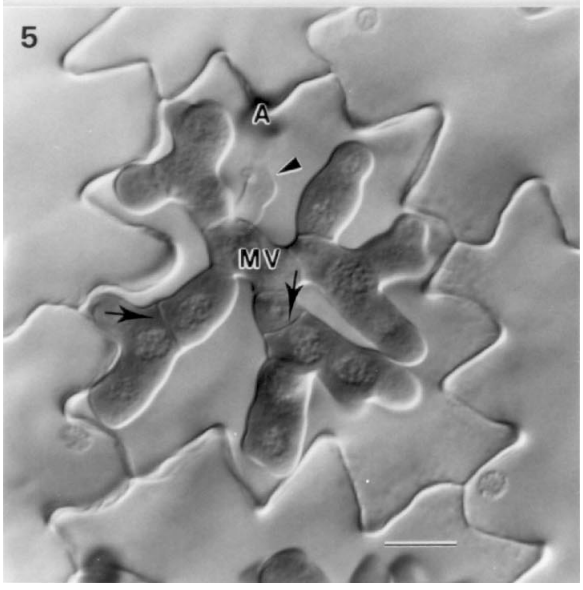


Figure 3. Development of an intracellular infection vesicle (IV) of LARS 319, showing bulbous lateral lobes and an elongated neck (arrow), in an epidermal cell of lucerne leaf 30 h after inoculation. Note appressorium (A). Bar = 10μm.

Each acervulus possessed a single septate melanized seta which was surrounded by copious amounts of conidia (Figure 9).

The three lucerne isolates of *C. destructivum* infected and colonized the leaves of lucerne cv. Europe at comparable rates in a disease cycle lasting for 72–96 h as described above. When cowpea leaves were inoculated with the same isolates, however, epidermal cells were not penetrated until after 96 h, even though conidial germination and appressorial formation occurred within 24 h after inoculation. Within 24 h after penetration, intracellular multilobed vesicles were produced in the epidermal cells of cowpea leaves by each of the lucerne isolates used in this study (Figures 10–12). Acervuli were produced sparsely on small non-spreading lesions, 168 h after inoculation, i.e. 72 h after penetration. Conidia of the cowpea isolate of *C. destructivum* germinated and produced melanized appressoria on the leaves of lucerne cv. Europe within





Figures 4 and 5. Fully developed multiseptate, multilobed vesicles (MV) of LARS 709, produced in epidermal cells of lucerne leaf, 48 h after inoculation. Arrows indicate septa, arrow head an elongated neck emanating from a melanized appressorium (A). Bar = 10μm.

24 h after inoculation. Penetration of host cells was not observed on detached leaves but occurred on intact leaves by 48 h after inoculation. Although multilobed vesicles were produced at low frequencies in epidermal cells, tissue colonization was rapidly inhibited at these penetration sites. Anthracnose lesions did not develop on lucerne leaves inoculated with the cowpea isolate.

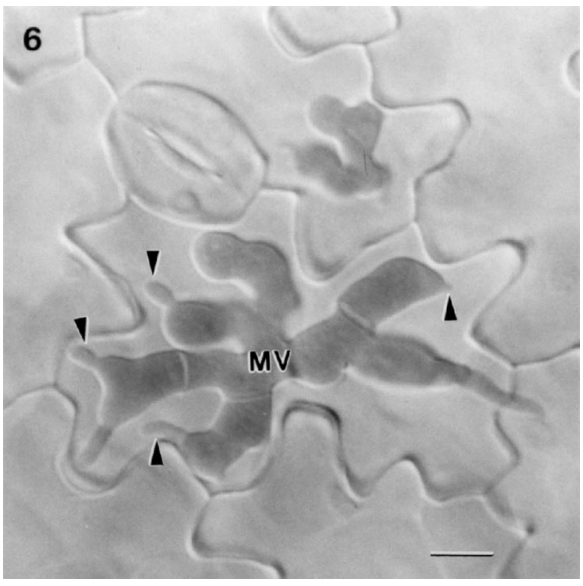


Figure 6. Development of bud-like outgrowths (arrow heads) from the distal ends of a multilobed vesicle (MV) of LARS 709 in an epidermal cell of lucerne leaf, 60 h after inoculation. Bar = 10μm.

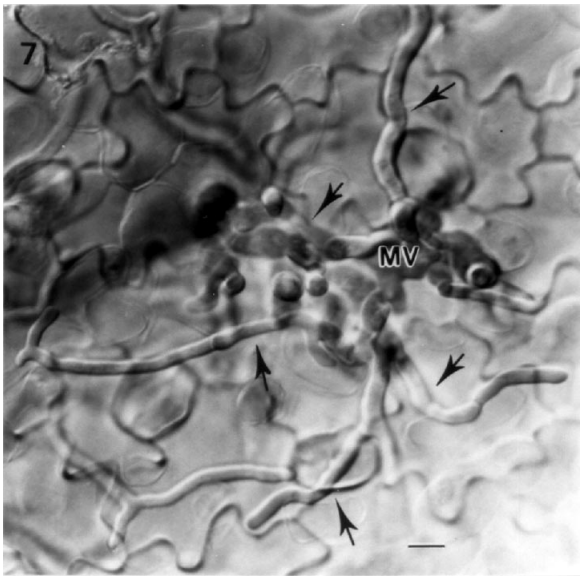


Figure 7. Invasion of host tissue by secondary hyphae (arrows) radiating from a multilobed vesicle (MV) of LARS 202 and growing through the walls of surrounding lucerne cells 72 h after inoculation. Bar = 10μm.

Discussion

The results obtained in the present work indicate that *C. destructivum* can act, under warm humid conditions, as a sole and primary pathogen of lucerne. Although

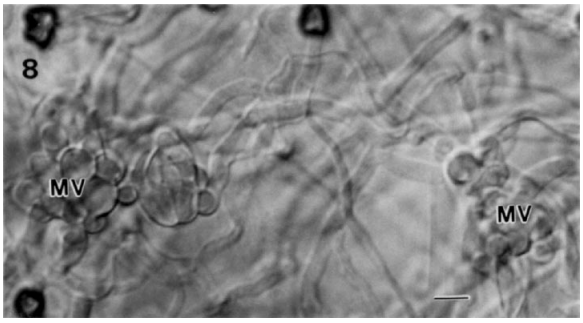


Figure 8. Knotted hyphal aggregates formed in lucerne cells around multilobed vesicles (MV) of LARS 709, 72 h after inoculation. Bar = 10μm.

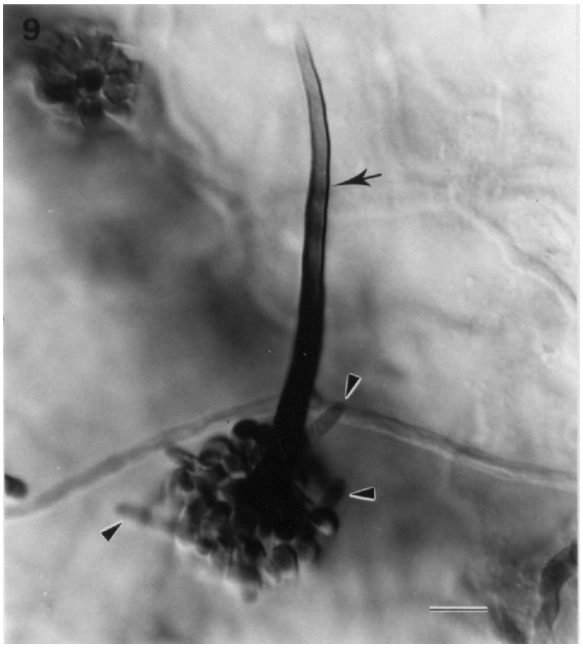
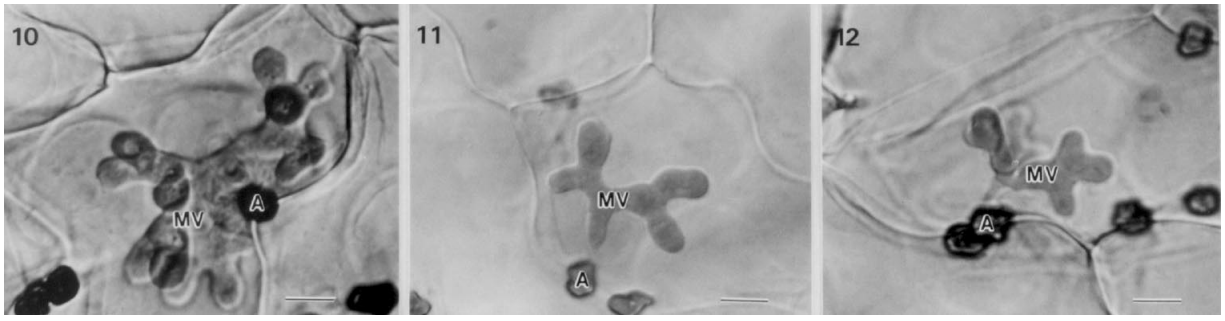


Figure 9. An acervulus of LARS 709 with a characteristic single seta (arrow) surrounded by conidia (arrow heads) on lucerne leaf surface 96 h after inoculation. Bar = 10μm.

in the mid-Atlantic region of the United States of America this species is considered as a secondary invader of anthracnose lesions caused by *C. trifolii* (Graham et al., 1976), all three lucerne isolates of *C. destructivum* successfully infected leaves by directly penetrating the host cuticle, in the absence of other *Colletotrichum* species. All the lucerne isolates initiated an intracellular relationship which lasted for 48 h. During this phase of the host-pathogen interaction, large, intracellular fungal infection structures comprising multilobed, multiseptate vesicles with elongated neck regions were elaborated and these remained restricted within the initially-infected epidermal cells. These structures are very similar to the biotrophic multilobed vesicles produced in infected epidermal cells of cowpea by LARS 056, the isolate of *C. destructivum* causing anthracnose in cowpea (Bailey et al., 1990; Latunde-Dada et al., 1996). In both hosts, the anthracnose disease cycle begins with localized intracellular infections (lasting for 48 h in lucerne and 72 h in cowpea). The infection progresses into an invasive necrotrophic phase and culminates in the erumpent emergence of acervuli onto the host surface, with each conidioma possessing a single, septate melanized seta surrounded by copious amounts of aseptate conidia. The uniqueness of the infection strategy of *C. destructivum* in colonizing its host resides in the nature of the intracellular fungal structures produced during the early phase of pathogenesis. An isolate, designated as *C. truncatum*, which shares a high degree of rDNA sequence homology with the cowpea isolate of *C. destructivum* (Sherriff et al., 1994) produces, in pea (*Pisum sativum* L.), similar biotrophic intracellular infection structures which are also restricted within initially infected epidermal cells (O'Connell et al., 1993).

The intracellular hemibiotrophic infection strategy of *C. destructivum* differs markedly from the intracellular necrotrophy (sensu Skipp et al., 1995) described for the interaction between lucerne and *C. trifolii*, in which a recognizable biotrophic phase has not been observed (Mould et al., 1991a, b). It differs, also, from the intracellular hemibiotrophy observed in bean (*Phaseolus vulgaris* L.) tissues infected by *C. lindemuthianum* [Sacc. & Magn.] Bri & Cav., in which biotrophy is not restricted to the initially penetrated cell, but spreads as filamentous primary hyphae emanating from small spherical infection vesicles colonize new cells (O'Connell et al., 1985).

In the current study, lucerne isolates of *C. destructivum* colonized cowpea less aggressively. The cowpea isolate, on the other hand, did not appear to be pathogenic to the lucerne cultivar (Europe); the few multilobed vesicles formed were inhibited at the penetration sites and the fungus failed to invade host tissue. These results, although based on a single cultivar, indicate an adaptation of *C. destrutivum* isolates to their host species. They confirm earlier observations on the host specialization of individual isolates of this pathogen, in spite of the wide host range of the species as a whole (Wolcan and Dal Bello, 1988).



Figures 10–12. Multilobed vesicles (MV) of *Colletotrichum* isolates from lucerne [LARS 319 (Figure 10), LARS 709 (Figure 11) and LARS 202 (Figure 12)] in epidermal cells of cowpea leaves 120h after inoculation. A = appressorium. Bar = 10μm.

Acknowledgements

IACR receives grant-aided support from the Biotechnology and Biological Research Council of the United Kingdom. One of us (A.O. L-D) thanks the Royal Society for the award of a Third World Fellowship. The secretarial assistance of E.M. Cooke and the photographic expertise of K.J.C. Williams are acknowledged.

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