Mycol. Res. **105** (11): 1340–1347 (November 2001). © The British Mycological Society
DOI: 10.1017/S0953756201005111 *Printed in the United Kingdom.*

Hemibiotrophic infection and identity of the fungus, *Colletotrichum destructivum*, causing anthracnose of tobacco

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Received 21 November 2000; accepted 25 June 2001.

The causal agent of tobacco anthracnose was identified as *Colletotrichum destructivum* based on the morphology of the fungus and a comparison of the sequence of the rDNA ITS with those of other *Colletotrichum* species. The infection process on tobacco (*Nicotiana tabacum* and *N. benthamiana*) was examined by light microscopy, which revealed that the pathogen acted as an intracellular hemibiotroph. Penetration occurred preferentially at the anticlinal walls of epidermal cells by an appressorium and penetration peg. An infection vesicle formed in the penetrated host cell by 48 h after inoculation, and out of this, a multi-lobed infection vesicle grew which remained limited to the initially infected cell. The interaction at this point was biotrophic, which was confirmed by plasmolysis and accumulation of a vital stain by the infected host cells. Thin secondary hyphae arose from multi-lobed infection vesicles at 60 h after inoculation, which then penetrated the host cell wall and began the necrotrophic phase of the infection. Acervuli formed on the plant surface by 96 h after inoculation, typically with a single melanized seta. In addition to tobacco, the fungus could infect alfalfa, cowpea, and *Medicago truncatula*, but not soybean. The process of infection of *C. destructivum* in tobacco was very similar to that previously reported in alfalfa and cowpea.

INTRODUCTION

Tobacco suffers from a variety of foliar diseases caused by fungi, including anthracnose (Lucas 1965, Shew & Lucas 1991). Anthracnose of tobacco is primarily a seedling disease that is favoured by low temperature, reduced light and high humidity (Shew & Lucas 1991). The conidia are dispersed by wind or rain to leaves or stems, where they germinate and form melanized appressoria. The appressoria penetrate the epidermis to produce irregularly-shaped primary hyphae from which slender secondary hyphae grow and invade neighbouring cells (Cronin 1958). Small light-green, water-soaked spots appear after several days that can enlarge up to 3 mm and dry to become papery thin (Shew & Lucas 1991). If the lesions are numerous, they can coalesce, and severely infected younger plants can be killed.

The taxonomy of the tobacco anthracnose fungus has been uncertain, and the fungus has been named *Colletotrichum nicotianae*, *C. tabacum*, *C. destructivum* and *C. gloeosporioides* (Cronin 1958, Farr *et al*. 1989, Shew & Lucas 1991). Cronin (1958) noted that the tobacco anthracnose fungus is not restricted to tobacco but has a wide host range, including various legumes, and Cronin (1958) compared a number of morphological features of the tobacco anthracnose pathogen to those of several *Colletotrichum* species and concluded that the tobacco anthracnose pathogen is indistinguishable from the *C. destructivum* that attacks legumes and therefore should be identified as *C. destructivum*. However, more recently, Shew & Lucas (1991) proposed that *C. gloeosporioides* is the appropriate name for the tobacco anthracnose pathogen.

Like many plant pathogenic fungi, the taxonomy of *Colletotrichum* species has been based on its pathogenicity and certain morphological features. However, these criteria are not always reliable since they can be variable, and there can be an overlap of phenotypes. DNA sequences are now available for a number of *Colletotrichum* species, and sequence comparisons of the internal transcribed spacer regions (ITS) of the ribosomal DNA have proven to be particularly useful in the identification of many *Colletotrichum* species, including *C. destructivum* from cowpea and alfalfa (Bailey *et al*. 1996, Latunde-Dada *et al*. 1996, Sherriff *et al*. 1994, Sherriff *et al*. 1995, Sreenivasaprasad *et al*. 1996). Because the tobacco anthracnose fungus has been given several different names, a comparison of DNA sequences may help to resolve its identity.

The host infection process of a number of *Colletotrichum* species has been studied, and can be classified as following either an intracellular hemibiotrophic strategy, a subcuticular intramural strategy, or a combination of both strategies (Bailey *et al*. 1992). The *C. destructivum* isolates that attack cowpea and alfalfa have been shown to have an intracellular hemibiotrophic strategy (Latunde-Dada *et al*. 1996, Latunde-Dada, Bailey & Lucas 1997). In this strategy, the fungus has

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an initial biotrophic phase where it feeds on living host cells and the host is symptomless. This is followed by a destructive necrotrophic phase where the fungus causes extensive degradation of host cells and symptoms become visible.

This study examines the infection process and confirms the identity of the tobacco anthracnose fungus through morphological and ITS sequence comparisons with *C. destructivum* on cowpea and alfalfa, as well as other *Colletotrichum* species.

MATERIALS AND METHODS

Biological materials and inoculation

An isolate of the tobacco anthracnose fungus, *C. destructivum* N150, was kindly provided by M. Maurhofer (Institute of Plant Sciences/Phytopathology, Zurich) and is preserved at the University of Guelph Fungal Culture Collection. Additional tobacco anthracnose isolates, ATCC 10921 and 11995, were obtained from the American Type Culture Collection (Manassas, VA). The fungi were cultured on potato dextrose agar (PDA, Difco, Detroit, MI), SYAS (Manandhar, Hartman & Sinclair 1986) or TSA (tobacco stem agar) under continuous fluorescent lighting at 25 °. TSA was made by autoclaving 500 g fresh weight of *N. tabacum* cv. 'Samsun' stem pieces and 15 g agar (Fisher Scientific) in a total volume of 1 l distilled water.

Seeds of *Nicotiana tabacum*, *N. benthamiana* and *N. pauciflora* were provided by J. Brandle (Agriculture and Agri-Food Canada, London, ON), S. Kamoun (Ohio State University, Wooster, OH), and V. Sisson (North Carolina State University, Oxford, NC), respectively. Seeds of *Medicago truncatula*, *Vigna unguiculata* and *Glycine max* were provided by M. Harrison (Samuel Roberts Noble Foundation, Ardmore, OK), M. Heath (Dept. of Botany, University of Toronto, Toronto, ON), and I. Rajcan (Dept. of Plant Agriculture, University of Guelph, Guelph, ON), respectively.

Plants were grown at 25 ° with a 16 h photoperiod at an intensity of 200 μmol s^{-1} m^{-2} photosynthetically active radiation. At the 8th leaf stage, entire plants were inoculated by spraying until runoff with a conidial suspension of 2×10^6 conidia per sml in sterile distilled water. Conidia of N150, ATCC 10921 and ATCC 11995 were obtained from 7 d-old cultures grown on SYAS as described above. The plants were then immediately enclosed in a plastic bag and incubated at 25 ° at low or medium light (25 or 200 μmol s^{-1} m^{-2} , respectively) for 9 d. All tests were carried out with three replicates.

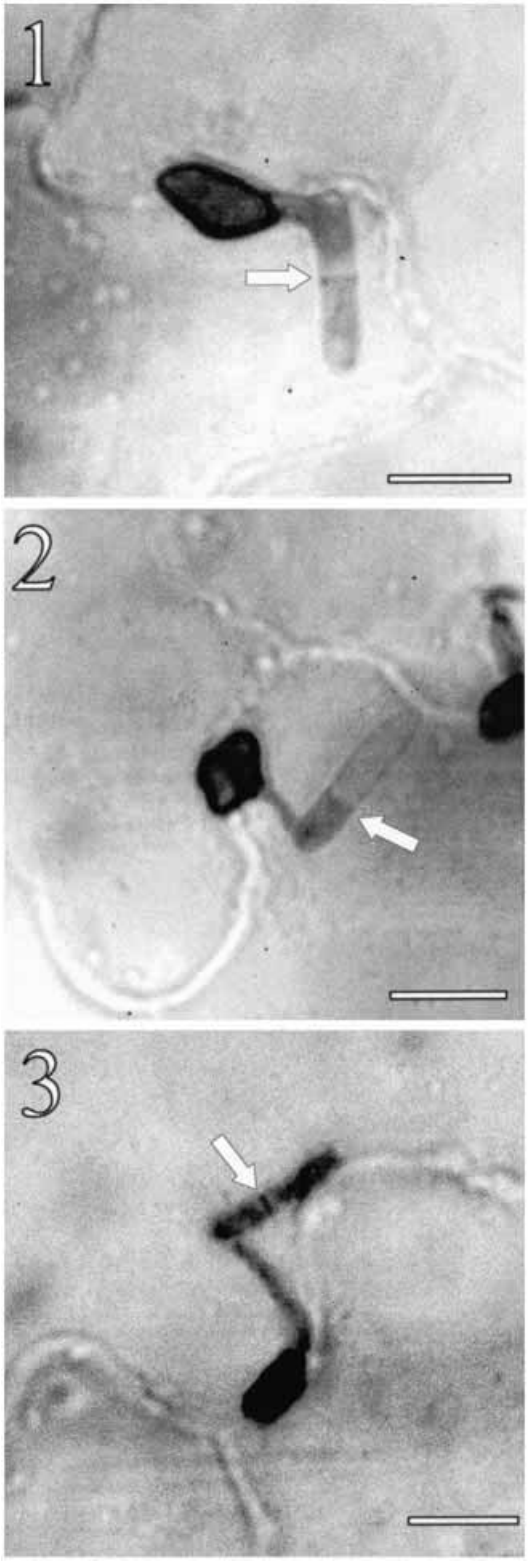
Microscopy

Pieces of mature fourth leaves of *Nicotiana tabacum* cv. 'Xanthi' and *N. benthamiana* containing visible infection sites

Table 1. List of *Colletotrichum* isolates used for sequence comparisons in this study.

Species	Isolate	Host	Origin	GenBank accession ITS1	GenBank accession ITS2	Source
C. acutatum	794	*Prunus* sp.	Israel	AF207794	AF207794	Freeman *et al.* (2000)
C. acutatum	915	*Fragaria* × *ananassa*	USA	Z32915	Z32914	Sreenivasaprasad *et al.* (1996)
C. acutatum	928	*Fragaria* × *ananassa*	UK	Z32928	Z32927	Sreenivasaprasad *et al.* (1996)
C. coccodes	930	*Lycopersicon esculentum*	Bulgaria	Z32930	Z32929	Sreenivasaprasad *et al.* (1996)
C. destructivum	N150	*Nicotiana tabacum*	France	AF320564	AF320564	this study
C. dematium	607	—	—	AB046607	AB046607	Moriwaki & Tsukiboshi, unpub.
C. destructivum	56	*Vigna unguiculata*	Nigeria		Z18977	Latunde-Dada *et al.* (1996)
C. destructivum	709	*Medicago sativa*	Saudi Arabia		NA	Latunde-Dada *et al.* (1996)
C. destructivum	921	*Nicotiana tabacum*	ATCC10921	AF320562	AF320562	this study
C. destructivum	940	*Medicago sativa*	Canada	Z32940		Sreenivasaprasad *et al.* (1996)
C. destructivum	995	*Nicotiana tabacum*	ATCC11995	AF320563	AF320563	this study
C. fragariae	854	*Fragaria vesca*	—	AF090854	AF090854	Moriwaki & Tsukiboshi, unpub.
C. gloeosporioides	855	*Olea europaea*	Spain	AF090855	AF090855	Martin & Garcia-Figueres, (1999)
C. graminicola	310	*Sorghum halpense*	USA		NA	Sherriff *et al.* (1995)
C. graminicola	334	*Zea mays*	USA		NA	Sherriff *et al.* (1995)
C. graminicola	337	*Zea mays*	USA	Z33377		Sreenivasaprasad *et al.* (1996)
C. graminicola	975	*Sorghum bicolor*	USA	Z32975		Sreenivasaprasad *et al.* (1996)
C. linicola	609	—	—	AB046609	AB046609	Moriwaki & Tsukiboshi, unpub.
C. linicola	900	*Digitalis lanata*	—	Z32900		Sreenivasaprasad *et al.* (1996)
C. linicola	901	*Trifolium* sp.	Netherlands	Z32901		Sreenivasaprasad *et al.* (1996)
C. linicola	908	*Medicago sativa*	New Zealand	Z32908		Sreenivasaprasad *et al.* (1996)
C. lindemuthianum	975	*Phaeolus vulgaris*	Europe		Z18975	Sherriff *et al.* (1994)
C. lindemuthianum	989	*Phaeolus vulgaris*	Brazil	Z32989		Sreenivasaprasad *et al.* (1996)
C. orbiculare	379	*Cucumis sativus*	Netherlands	Z33379		Sreenivasaprasad *et al.* (1996)
C. orbiculare	997	*Cucumis sativus*	Japan		Z18997	Sherriff *et al.* (1994)
C. trifolii	4	*Medicago* sp.	USA	Z33004		Sreenivasaprasad *et al.* (1996)
C. trifolii	990	*Medicago* sp.	USA		Z18990	Sherriff *et al.* (1994)
C. truncatum	941	*Arachis hypogaea*	Gambia	Z32941		Sreenivasaprasad *et al.* (1996)
C. truncatum	978	*Pisum sativum*	USA		Z18978	Sherriff *et al.* (1994)

GenBank accession number obtained from the website 'http://www.ncbi.nlm.nih.gov/'.
NA, the sequence is not available in GenBank but is in the reference cited.
—, not stated.



Figs 1–3. Germination of conidia of *Colletotrichum destructivum* isolate N150 on *Nicotiana tabacum* cv. 'Xanthi' leaf surface at 24 h post inoculation. Figures 1–3 show septation of the conidium with a septum (arrow), varying germ tube lengths and shapes of the appressorium, and the preference for appressorium formation at the host anticlinal cell wall. Bar = 10 μm.

were placed in lactophenol with 0.05% (w/v) trypan blue, and heated over a flame for approx. 10 s until boiling. The heated lactophenol caused destaining of plant pigments, and the trypan blue stained the fungal structures. Leaf pieces were examined by light microscopy and photographed (Nikon Labophot). Randomly selected portions of the stained tissues were observed to determine the percentage conidial germination and percentage of germ tubes producing appressoria. Four replicate observations of at least 25 spores or 25 appressoria were taken. Proportions calculated from these observations were analysed in Student's *t* tests for differences between host species after angular transformation calculated as the arcsine of the square root of the proportion.

To determine host cell viability at time of staining, infected leaf pieces were infiltrated with a vital stain of 0.85 M $NaNO_3$ containing 0.01% (w/v) Neutral red, and examined for host cell plasmolysis as described by O'Connell *et al.* (1993).

The average size of spores and appressoria of isolate N150 were measured by microscopy from 100 randomly selected conidia taken from 7 d-old PDA cultures and 100 randomly selected appressoria on infected *N. tabacum* cv. 'Xanthi' leaves at 24 h after inoculation.

Extraction of DNA, PCR and sequence analysis

Isolates were cultured for 2 wk at 25 ° on PDA that was overlain with a cellophane membrane sheet (Flexel, Atlanta, GA). Mycelium was harvested and DNA extracted by the method of Edwards, Johnstone & Thompson (1991). The ITS region of genomic rDNA was amplified by the polymerase chain reaction (PCR) with the primer pair ITS1 (5′-TCCGTAGGTGAACCTGCGG) and ITS4 (5′-TCCTCCGC-TTATTGATATGC), described by White *et al.* (1991). The 30 μl reaction mixture for PCR amplification contained the following: 10 ng DNA, 1 × DNA polymerase buffer (50 mM Tris–HCl at pH 8.5), 1.5 mM $MgCl_2$, 0.5 μM of each primer, and 1 unit *Tsg* DNA polymerase (Biobasic, Scarborough, ON). Amplifications were performed in a Perkin–Elmer GeneAmp 2400 (PE Applied Biosystems, Mississauga, ON), with an initial denaturation step of 94 ° for 2 min, followed by 35 cycles of 94 ° for 30 s, 55 ° for 1 min, and 72 ° for 1 min, and a final extension at 72 ° for 10 min. Aliquots (3 μl) of amplification products were electrophoresed through 1.4% agarose gels along with a 100 bp ladder size marker (Pharmacia, Mississauga, ON). The gels were stained with ethidium bromide and bands visualized on a UV trans-illuminator.

When single bands of the appropriate size (approx. 700 bp) for an ITS region were observed, the remaining 27 μl PCR product was electrophoresed through 1.0% agarose gels in a modified TAE buffer (40 mM Tris-acetate, pH 8.0, 0.1 mM Na_2EDTA), and the target bands excised. DNA from excised bands was purified using an Ultrafree®-DA DNA extraction kit (Millipore, Bedford, MA) and sequenced on an Applied Biosystems 377A automated DNA sequencer (Perkin–Elmer, Mississauga, ON) using the forward and reversed primers, ITS1 and ITS4, separately. Consensus sequences were then constructed from the forward and reverse sequences.

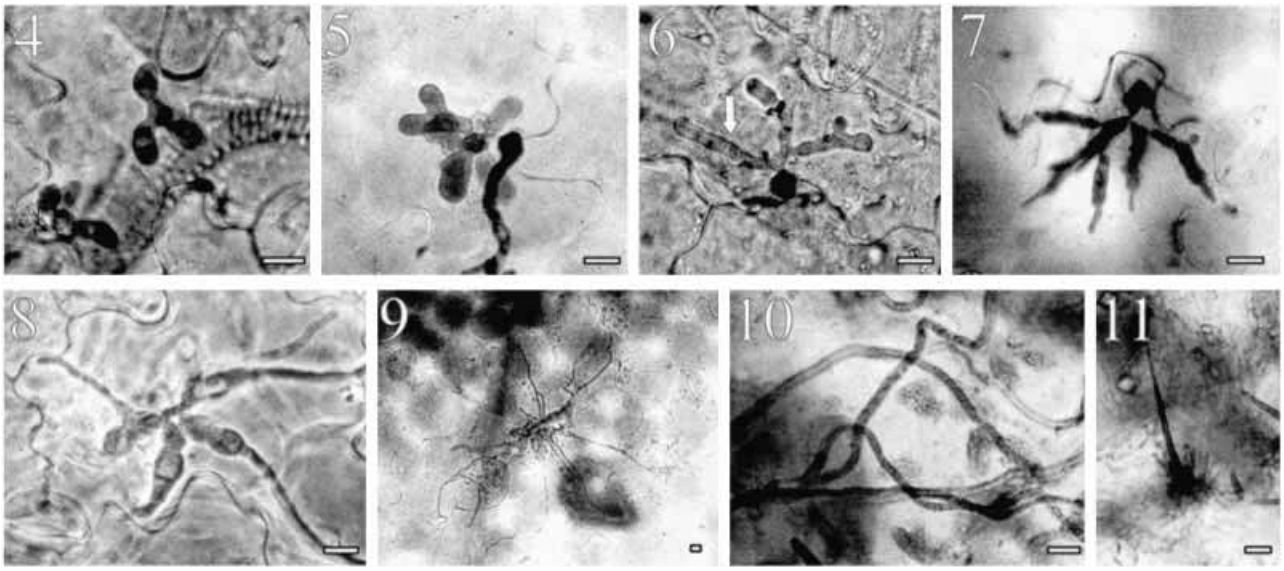
Sequences were aligned with the program CLUSTAL-W (Thompson, Higgins & Gibson 1994) using default parameters. In addition to the *Colletotrichum destructivum* isolates sequenced

Table 2. Rates of conidial germination and appressorial formation of *Colletotrichum destructivum* isolate N150 on *Nicotiana* leaves.

	12 h		24 h		96 h
Host	Germination %	Appressoria %	Germination %	Appressoria %	Lesions cm^{-2}
N. benthamiana	50.0[a]	56	81.7	68.3	7.3
N. tabacum cv. 'Xanthi'	71.5	89.3	95.3	97.3	2
t test[b]	$P < 0.01$	$P < 0.01$	$P < 0.05$	$P < 0.01$	$P < 0.01$

[a] Mean percentages based on a minimum of 100 observations (four replications of at least 25 spores or appressoria).

[b] Significance of Student's *t* tests performed between cultivars (percentage germinated or percentage with appressorial formation) after angular transformation (arcsine of the square root of the percentage/100).



Figs 4–11. Infection of *Nicotiana tabacum* and *N. benthamiana* by *Colletotrichum destructivum* isolate N150. Bar = 10 μm. **Fig. 4.** Early development of multi-lobed infection at 48 h post inoculation on *N. benthamiana*. **Fig. 5.** Expansion and development of additional lateral lobed vesicles at 48 h post inoculation on *N. benthamiana*. **Fig. 6.** Multi-lobed vesicles at 48 h post inoculation on *N. tabacum* cv. 'Xanthi'. Note the formation of a septum (arrow) in one of the lobes. **Fig. 7.** Appearance of narrow secondary hyphae from the distal ends of the multi-lobed vesicle at 60 h post inoculation on *N. tabacum* cv. 'Xanthi'. **Fig. 8.** Growth of thin secondary hyphae in the initially infected epidermal cell by at 72 h post inoculation on *N. tabacum* cv. 'Petit Havana'. **Fig. 9.** Invasion of adjacent epidermal host cells by thin secondary hyphae from multi-lobed vesicle at 72 h post inoculation on *N. tabacum* cv. 'Xanthi'. **Fig. 10.** Intercellular growth of secondary hyphae in necrotic leaf tissue at 96 h post inoculation on *N. tabacum* cv. 'Xanthi'. **Fig. 11.** An acervulus with a single setae on the surface of leaf tissue at 96 h post inoculation on *N. tabacum* cv. 'Xanthi'.

in this study, GenBank sequences of other *Colletotrichum* isolates were used in alignment of either the ITS1 (21 sequences) or the ITS2 (19 sequences) (Table 1). ITS1 and ITS2 sequences were compared separately because both types of sequences are not available for many *Colletotrichum* isolates on GenBank. Minor adjustments were made to improve alignments following visual inspection.

Dendrograms were generated with distance methods, and bootstrap analysis was performed to provide confidence limits on tree topology. The alignments were analyzed with the software package PHYLIP (Felsenstein 1989) using the programs SEQBOOT (100 bootstrap replications), DNADIST (Jukes–Cantor genetic distance), NEIGHBOR (UPGMA algorithm), and CONSENSE. Finally, dendrograms were produced with the PHYLIP program DRAWGRAM and modified using Corel® PRESENTATIONS.

RESULTS

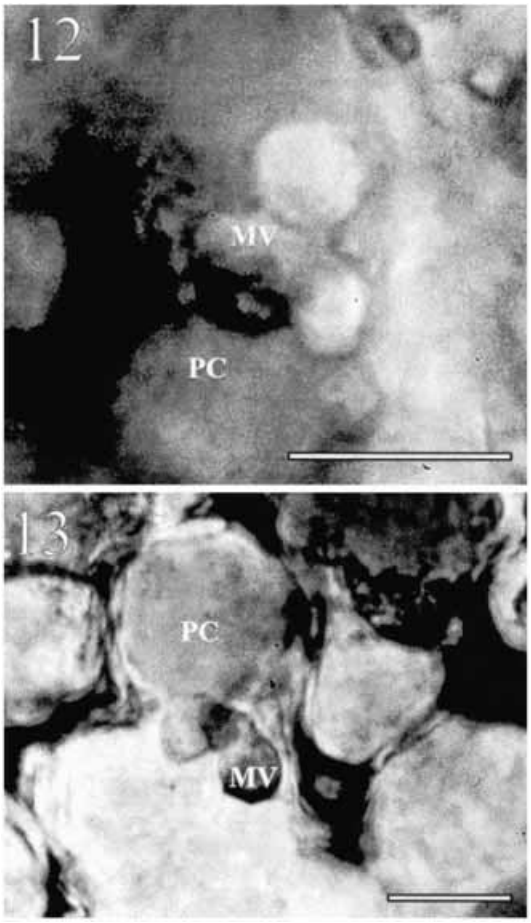
The cultures of *Colletotrichum destructivum* isolates N150, ATCC 10921 and ATCC 11995 produced grayish aerial hyphae with orange conidia in mass when grown under continuous light on PDA or SYAS media for 7 d. However, on TSA, the colonies were sodden, appressed, moderate orange colour with small sclerotia near the centre of the colonies. The conidia of isolate N150 produced on PDA measured 16.5 ± 1.4 μm by 4.8 ± 0.2 μm and were aseptate and straight to slightly curved with obtuse ends. This was similar for isolates ATCC 10921 and ATCC 11995.

Seedlings of *N. tabacum* cvs. 'Burley 21', 'Delgold', 'Petit Havana', 'Samsun NN' and 'Xanthi' inoculated with N150 all showed small (2 mm) water-soaked lesions at 3 d after inoculation, and these enlarged to 3 mm. Seedlings of *N.*

benthamiana and *N. pauciflora* PI 555546 also showed water-soaked lesions by that time. The severity of infection was greater on *N. pauciflora* than on *N. benthamiana*, which were both greater than on any of the *N. tabacum* cultivars. Primarily this was due to a much greater number of small coalescent lesions, but there were also a number of larger (4 mm) lesions on *N. pauciflora* and *N. benthamiana*, which were not observed with *N. tabacum*. The entire inoculated area on *N. pauciflora* leaves was dead by 3 d. Large (10 mm) water-soaked lesions appeared on *V. unguiculata* cv. 'California Blackeye' leaves at 5–6 d after inoculation. By 5 or 6 d, *M. truncatula* cv. 'Jemalong' showed 2 mm water-soaked lesions similar to that on *N. tabacum*, but *M. sativa* cv. 'Saranac' showed irregular-shaped lesions at the edge of the leaves. No symptoms were ever observed with inoculated *G. max* cv. 'Shire' plants. Acervuli were observed on all the infected plant tissues after necrosis appeared. Symptoms were identical at low or medium light (25 or 200 μmol s^{-1} m^{-2} PAR, respectively), but the disease was more severe at lower light intensities as previously described (Shew & Lucas 1991). Isolates ATCC 10921 and 11995, which are described as tobacco anthracnose fungi, also produced the same symptoms on *N. tabacum* and *N. benthamiana*.

By 12 h to 24 h post-inoculation on tobacco, the conidia had germinated, and appressoria had formed (Figs 1–3). During the germination process, a septum was produced in the conidium, and the germ tube emerged from only one of the cells (Figs 1–3). In some cases, the staining showed that there was no cytoplasm in the conidial cell that had not germinated (Fig. 2). The melanized appressorium that had formed ranged from clavate (Fig. 3) to clavate with one (Fig. 1) or two lobes (Fig. 2). The appressoria measured 10.4 ± 1.6 μm in length and 6.1 ± 0.9 μm in width. There was a preference for penetration sites at the epidermal cross walls (i.e. the anticlinal epidermal walls) (Figs 1–3). The percentage of appressoria forming at the epidermal cross walls on leaves was 59% on *N. benthamiana* and 66% on *N. tabacum* cv. 'Xanthi', but the difference between the two species was not significant ($P = 0.72$ after angular transformation of the data). Both germination and appressorial formation occurred more rapidly on cv. 'Xanthi' than on *N. benthamiana* (Table 2). Despite this, *N. benthamiana* was more susceptible than cv. 'Xanthi' based on the number of lesions which eventually appeared (Table 2), which agreed with previous observations of greater anthracnose severity on *N. benthamiana* (Sievert 1972).

Infection vesicles were first observed at 48 h post inoculation, beginning as small lobed vesicles within an epidermal cell (Fig. 4). These lobed vesicles, or primary hyphae, grew progressively longer, and septa were visible (Figs 5–6). These hyphae always remained inside the initially penetrated epidermal cell. At 60 h, narrower secondary hyphae began to emerge from the ends of the lobes (Fig. 7). The secondary hyphae then grew towards the edge of the originally infected epidermal cell (Fig. 8), penetrated the host cell walls, and then radiated outwards at 72 h (Fig. 9). The secondary hyphae showed extensive intercellular growth (Fig. 10), and their appearance corresponded with the development of lesions. At 96 h, acervuli, each with a single melanized seta,



Figs 12–13. Viability of infected epidermal cells at 48 h post inoculation during the biotrophic phase on *N. tabacum* cv. 'Xanthi'. Viability of infected cells was determined by plasmolysis of the host cytoplasm in 0.85 M $NaNO_3$ coupled with the accumulation of 0.01% Neutral red in the host cell vacuole. Bar = 10 μm. **Fig. 12.** An unstained multi-lobed vesicle (MV) is observed adjacent to the stained plamolysed host cytoplasm (PC). **Fig. 13.** A multi-lobed vesicle (MV) is seen within the same cell adjacent to the stained plamolysed host cytoplasm (PC).

were observed on the leaf surface (Fig. 11). There were, however, a few examples where two setae were observed within an acervulus. The infected epidermal cells during the growth of the multi-lobed primary vesicles appeared to be alive suggesting that the fungus was undergoing biotrophic nutrition. The viability of infected epidermal cells was demonstrated by the presence of the multi-lobed vesicle adjacent to a plant cell with a plasmolysed plasma membrane and tonoplast that had Neutral red accumulated in the plant cell vacuole (Figs 12–13). The viability of infected host cells continued until around the time that the secondary hyphae began to develop. A similar pattern of infection was also observed with isolates ATCC 10921 and 11995. A diagrammatic view of the early stages of the infection process of tobacco anthracnose is shown in Fig. 14.

A comparison of the ITS1 (Fig. 15) and ITS2 (Fig. 16) sequences was made between several *Colletotrichum* species and isolates N150, ATCC 10921 and ATCC 11995. ITS1 and ITS2 sequences were compared separately because both types of sequences are not available for many *Colletotrichum* isolates.

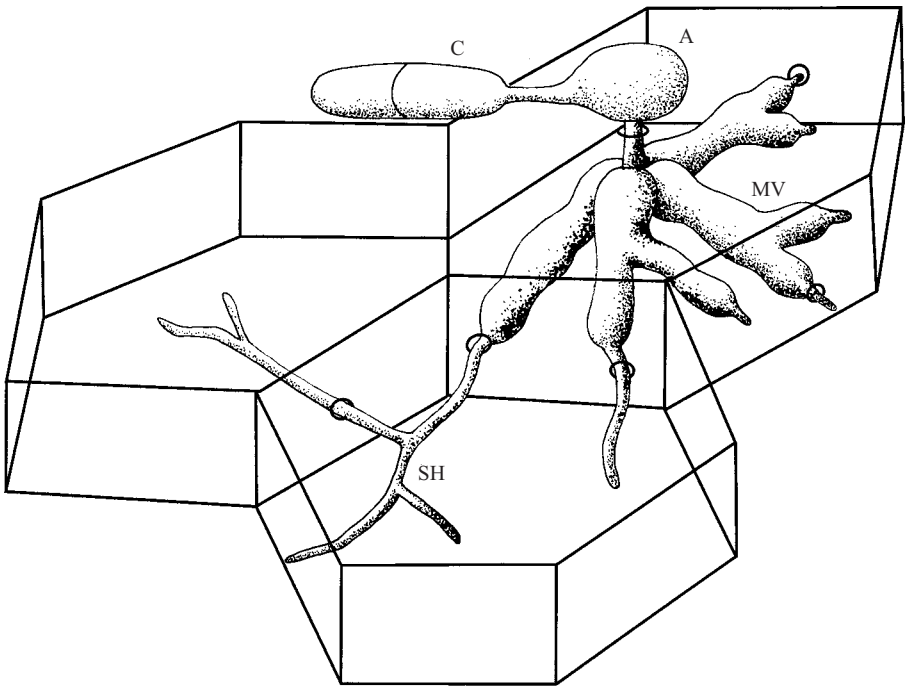


Fig. 14. Diagrammatic view of infected tobacco epidermal cells showing the septate conidium (C), appressorium (A), penetration peg below the appressorium, multi-lobed vesicles (MV) and thin secondary hyphae (SH) growing from the vesicles.

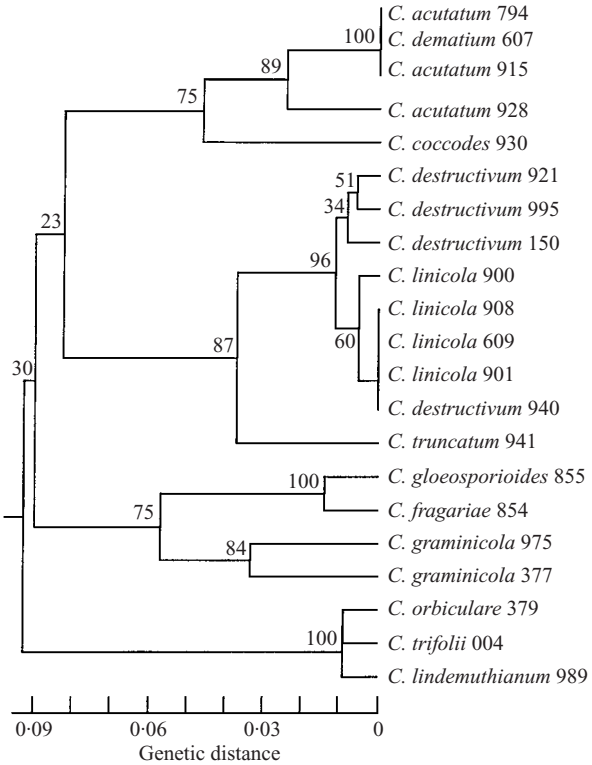


Fig. 15. Dendrogram of 21 isolates of *Colletotrichum* spp. inferred by genetic distance analysis of sequence data from rDNA of the ITS1 region. Designations of the isolates are provided in Table 1. Sequences were aligned with CLUSTAL-W (Thompson *et al.* 1994), and then analyzed with PHYLIP (Felsenstein 1989) programs DNADIST (Jukes–Cantor genetic distance) followed by NEIGHBOR (UPGMA algorithm) or DNAPARS. Percentage from 100 bootstrap replications with the PHYLIP programs SEQBOOT and CONSENSE are shown near the corresponding branches.

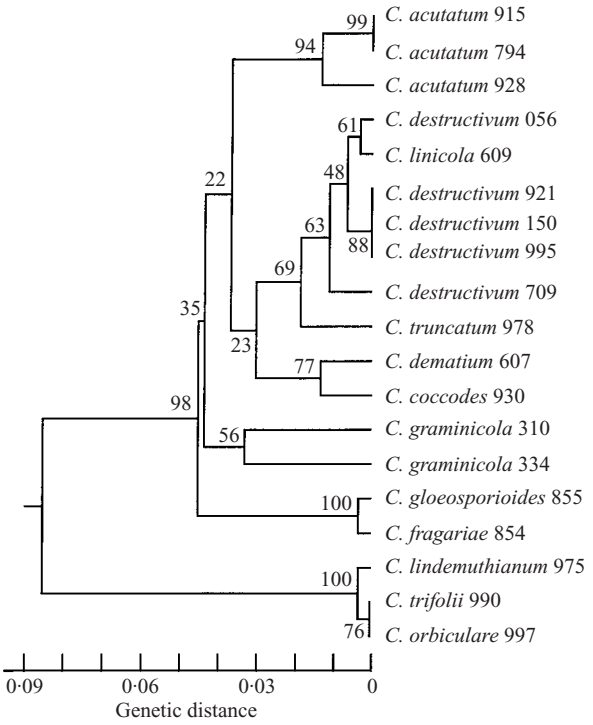


Fig. 16. Dendrogram of 19 isolates of *Colletotrichum* spp. inferred by genetic distance analysis of sequence data from rDNA of the ITS2 region. See Fig. 15 legend for methods.

There was a greater number of polymorphic sites in the ITS1 compared to the ITS2 sequence alignments (TreeBASE accession S633). For both ITS1 and ITS2 sequences, the three tobacco isolates were most similar to each other, but they were part of a group that included isolates of *C. destructivum*

and *C. linicola* from several hosts. There was no clear separation between *C. linicola* and *C. destructivum* isolates on either the ITS1 or ITS2-based dendrogram. The bootstrap value for this clade was 96% for ITS1 and 63% for ITS2. The next most similar species was *C. truncatum* (syn. *C. dematium* f. *truncatum*). The other *Colletotrichum* species were in several groups, which was consistent between the ITS1 and ITS2 analysis, with the notable exception of *C. dematium*, which was either more similar to *C. acutatum* based on ITS1 or *C. coccodes* based on ITS2. Bailey *et al.* (1996) previously observed a cluster containing *C. orbiculare*, *C. lindemuthianum* and *C. trifolii*, and grouped them together as the *C. orbiculare* aggregate species, which is in agreement with our results.

DISCUSSION

The tobacco anthracnose isolate, N150, used in this study matched the description given by Sutton (1992), who described *Colletotrichum destructivum* cultures as sodden with apricot to salmon-coloured centres. Sutton (1992) also stated that the appressoria were irregularly clavate to ovate, 6–16 × 6–10 μm, and the conidia were orange, 10–22 × 4–6 μm and straight to slightly curved with abruptly tapered, obtuse ends. The morphological features of the tobacco anthracnose fungus also closely match those described for *C. destructivum* attacking cowpea and alfalfa (Latunde-Dada *et al.* 1996, Latunde-Dada *et al.* 1997), including conidial shape and size, septation of the conidium upon germination, appressorial shape, and the infection process. Cronin (1958) examined the conidial shape and size, and setal length and septation of *Colletotrichum* isolates from tobacco and concluded that their morphological features most closely matched those of *C. destructivum* isolated from legumes.

The infection process of isolate N150 on tobacco appears to be virtually the same as that of *C. destructivum* attacking alfalfa and cowpea (Latunde-Dada *et al.* 1996, Latunde-Dada *et al.* 1997). The only notable difference was that the fungi on cowpea and alfalfa always formed mono-setate acervuli, and while this was predominantly the case in tobacco, there were a few examples of acervuli with two setae. In the case of cowpea, the biotrophic phase of *C. destructivum* infection persisted until 60–72 h after inoculation, the same observed here on tobacco (Latunde-Dada *et al.* 1996). Development of necrotrophy appears to be faster in alfalfa where secondary hyphae and necrotrophy was visible at 48 h after inoculation (Latunde-Dada *et al.* 1997). The only other species of *Colletotrichum* that has a similar infection process is *C. truncatum* on pea (O'Connell *et al.* 1993). Although the primary hyphae of *C. truncatum* are limited to the initially infected epidermal cell, they are highly branched and densely convoluted. However, the ITS2 sequence from a *C. truncatum* isolate on pea was most similar to those of *C. destructivum* and *C. linicola*, indicating that they may be closely related.

Our results concur with those of Cronin (1958) that there is no justification for regarding the tobacco anthracnose pathogen as being limited to tobacco. Isolate N150 caused symptoms on alfalfa, *M. truncatula* and cowpea as well as tobacco. *C. destructivum* has been described as a pathogen of a number of legumes (Lenné 1992). Isolates of the tobacco anthracnose fungus have been found to have a wide host range including various legumes, solanaceous plants and others (Abdul Wajid & Elias 1988, Morgan 1956). A tobacco anthracnose isolate had a similar host range to that of an isolate from a legume, and the tobacco isolate could attack legumes and vice versa (Cronin 1958). Further evidence that *C. destructivum* from legumes can cause anthracnose of tobacco is the use of a *C. destructivum* strain, originally isolated from alfalfa (ATCC 42492) to test transgenic tobacco for anthracnose disease resistance (Cary *et al.* 2000).

Although *C. destructivum* has a wide host range, there may be some host specificity among *C. destructivum* isolates (Latunde-Dada *et al.* 1997). Alfalfa isolates of *C. destructivum* could infect cowpea, whereas a cowpea isolate could not infect alfalfa (Latunde-Dada *et al.* 1997). However, the alfalfa isolates did not produce lesions on cowpea leaves until 7 d after inoculation compared to 3 d on alfalfa leaves (Latunde-Dada *et al.* 1997). Morgan (1956) also found some host specificity as the tobacco anthracnose fungus could infect alfalfa but not cowpea. Although isolate N150 could infect cowpea, *Medicago truncatula* and alfalfa, there was a delay in the appearance of symptoms on these hosts compared to tobacco. In addition, isolate N150 could not attack soybean, which is reported as a host of *C. destructivum* (Farr *et al.* 1989).

Isolate N150 infected all five *Nicotiana tabacum* cultivars that were tested. Sievert (1972) tested nearly 1000 lines, and Reddy, Chandwani & Nagarajan (1975) tested nearly 500 lines of *N. tabacum*, and all were found to be susceptible to anthracnose. *N. benthamiana* and *N. pauciflora* were reported to be more susceptible than *N. tabacum* cv. 'Burley 21', and the entire *N. pauciflora* plant was killed by anthracnose (Sievert 1972). *N. pauciflora* was also highly susceptible to anthracnose in our tests, and the lesions were so numerous and coalesced so quickly that the entire inoculated areas were killed by the fungus. Cronin (1958) believed that *C. destructivum* was limited to small lesions on living *N. tabacum* tissue due to an inhibition of the fungus by the living plant cells surrounding the lesions. In our study, small lesions were also observed with *N. tabacum*, but slightly larger lesions were observed with *N. benthamiana* and *N. pauciflora*, which may be due to these plants having less ability to inhibit the spread of the fungus.

Comparisons of the ITS regions of the rDNA have greatly improved our understanding of the taxonomy of *Colletotrichum* (Bailey *et al.* 1996, Latunde-Dada *et al.* 1996, Sherriff *et al.* 1994, Sherriff *et al.* 1995, Sreenivasaprasad *et al.* 1996). A comparison of the ITS2 and D2 regions of the rDNA from *C. destructivum* from cowpea and alfalfa with that of several *Colletotrichum* species showed that *C. destructivum* isolates were in a separate cluster from *C. lindemuthianum*, *C. orbiculare* and *C. gloeosporoioides* (Latunde-Dada *et al.* 1996). A comparison of the ITS1 and ITS2 sequences from isolates N150, ATCC 10921 and ATCC 11995, which all originated from tobacco, showed that all were very similar to each other, and were clearly in a group that contained only *C. destructivum* and *C. linicola* isolates. Sreenivasaprasad *et al.* (1996) had noted that *C. destructivum* and *C. linicola* clustered together with a 100% bootstrap value. These fungi had the fewest (1.1%) differences in ITS1 sequences between any *Colletotrichum* species, and these differences were lower than the

threshold values for divergence among other *Colletotrichum* species. Sreenivasaprasad *et al*. (1996) suggested that *C. linicola* and *C. destructivum* should be combined into a single species. Our results with additional isolates and both ITS1 and ITS2 sequences support this conclusion and now includes tobacco anthracnose isolates in this group.

The descriptions by Sutton (1980, 1992) of *C. linicola* and *C. destructivum* show that both fungi have a number of similarities, such as similar sizes and shapes of appressoria and conidia. One notable difference is that the appressoria of *C. linicola* can be crenate, which is not observed with *C. destructivum*. However, Schwinghammer (1954) noted that appressoria of *C. linicola* were typically globose to pear-shaped, and crenate appressoria were described as abnormal types found only in weakly pathogenic isolates. Additional features should also be considered in classifying *Colletotrichum* species. In recognizing the *C. orbiculare* as an aggregate species, Bailey *et al*. (1996) concluded that the production of a globular infection vesicle and primary hyphae in host epidermal cells was characteristic. For *C. destructivum*, the production of a multi-lobed vesicle in infected alfalfa and cowpea epidermal cells appears to be a unique characteristic (Latunde-Dada *et al*. 1996, 1997). Virtually identical vesicles were observed in infected tobacco epidermal cells, and multi-lobed vesicles have also been described in flax epidermal cells infected with *C. linicola* (Schwinghamer 1954). Therefore, it appears that this infection structure is at least one characteristic feature of these fungi, and we believe that *C. destructivum*, like *C. orbiculare*, is also an aggregate species, which should include isolates of *C. linicola*. Based on ITS sequences, cultural characteristics and the infection process, the appropriate identification of the tobacco anthracnose fungus is *C. destructivum*.

ACKNOWLEDGEMENTS

Funding for this study was provided by the Natural Sciences and Engineering Research Council of Canada.

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Corresponding Editor: D. T. Mitchell