The infection process of *Colletotrichum graminicola* and relative aggressiveness on four turfgrass species

A. Khan and T. Hsiang

Abstract: Detached 3-week-old leaves of *Agrostis palustris*, *Lolium perenne*, *Poa annua*, and *Poa pratensis* were inoculated with conidial suspensions of two isolates of *Colletotrichum graminicola* obtained from *A. palustris*. Inoculated leaves were incubated at 23 °C under high relative humidity (>95%). The infection process was investigated by light microscopy from 2 to 168 h after inoculation (AI). Spore germination was observed within 2 h AI, appressoria within 6 h AI, and penetration pores within 8 h AI on all four hosts. Infection hyphae were observed inside epidermal cells within 24 h AI on all four hosts, but significantly greater infection was observed in *A. palustris* and *P. annua* than in *L. perenne* or *P. pratensis* at both 96 and 120 h AI. Acervuli appeared on leaves of *A. palustris* at 72 h AI and on *L. perenne* at 96 h AI but were not found on either *P. annua* or *P. pratensis* during the first 168 h AI. The infection process was similar to that reported for *C. graminicola* from other hosts; however, disease development of the two isolates of *C. graminicola* from *A. palustris* was faster or fungal growth more extensive on detached leaf tissue of *A. palustris* than on other turfgrass species tested.

Key words: annual bluegrass, anthracnose, creeping bentgrass.

Résumé : Des feuilles détachées de *Agrostis palustris*, *Lolium perenne*, *Poa annua* et *Poa pratensis*, vieilles de 3 semaines, ont été inoculées avec des suspensions conidiales de deux isolats de *Colletotrichum graminicola* obtenus à partir de *A. palustris*. Les feuilles inoculées ont été incubées à 23 °C sous humidité relative élevée (>95 %). Le processus d'infection a été examiné sous microscope optique de 2 à 168 h après l'inoculation (AI). La germination des spores fut observée à l'intérieur de 2 h AI, des appressoriums à l'intérieur de 6 h AI, et des pores de pénétration à l'intérieur de 8 h AI, sur les quatre hôtes étudiés. Des hyphes d'infection ont été observés à l'intérieur de cellules épidermiques, à l'intérieur de 24 h AI, chez les quatre hôtes, mais un infection significativement plus importante a été observée chez *A. palustris* et *P. annua* par rapport à *L. perenne* ou à *P. pratensis* à 96 et 120 h AI. Des acervulums sont apparus sur les feuilles de *A. palustris* à 72 h AI et sur celles de *L. perenne* à 96 h AI, mais n'ont pas été retrouvés sur *P. annua* ou *P. pratensis* au cours des premières 168 h AI. Le processus d'infection était semblable à celui décrit pour *C. graminicola* chez d'autres hôtes; toutefois, le développement de la maladie pour deux isolats de *C. graminicola* provenant de *A. palustris* était plus rapide, ou la croissance fongique plus abondante, sur des tissus de feuilles détachées de *A. palustris* que sur d'autres espèces de gazon analysées.

Mots clés : pâturin annuel, anthracnose, agrostide traçante.

[Traduit par la Rédaction]

Introduction

Colletotrichum graminicola was erected by Wilson in 1914 to accommodate most *Colletotrichum* spp. with falcate conidia (Politis 1975). Sutton (1968) found that sclerotia of *C. graminicola* from sorghum (*Sorghum* spp.) or maize (*Zea mays*) differed in size and shape and maintained that *C. graminicola* consisted of at least two dissimilar taxa. Sutton (1980) described *Colletotrichum* isolates from maize and sorghum as two separate species and designated the maize isolates as

C. graminicola and the sorghum isolates as *Colletotrichum sublineolum*. Vaillancourt and Hanau (1992) also concluded that maize isolates of *C. graminicola* were a separate species from sorghum isolates, since they differed morphologically, were not interfertile, and could be distinguished by molecular markers. In addition to sorghum and maize, *C. graminicola* has been found on many cereals and grasses, including wheat (*Triticum aestivum*), oats (*Avena sativa*), barley (*Hordeum vulgare*) (Lapp and Skoropad 1978), and turfgrasses (Smith et al. 1989). *Colletotrichum graminicola* shows physiological specialization, since only isolates originating from the same or closely related host species are generally pathogenic to a given host species (studies cited in Mordue 1967 and Backman et al. 1999). Maize isolates were capable of attacking sorghum but failed to produce symptoms on oats, barley, wheat, tall fescue (*Festuca arundinacea*), or millet (*Panicum miliaceum*) (Wheeler et al. 1974).

There is also evidence of physiological specialization among turfgrass isolates of *C. graminicola*. Generally, only

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one host species is severely affected by anthracnose disease caused by *C. graminicola* when several grasses are grown together (Backman et al. 1999; Browning et al. 1999). For example, anthracnose foliar blight occurs during hot, humid weather and is generally severe only on *Poa annua* (Couch 1995), while anthracnose basal rot occurs under cool or warm, wet conditions and is often found only on *Agrostis palustris*, even in mixed stands with *P. annua* (Hsiang and Goodwin 2000). Isolates from *P. annua* were pathogenic on most lines of *P. annua* tested, but all cultivars of *A. palustris* tested were found to be resistant in seedling tests (Bolton and Cordukes 1981). In pathogenicity tests conducted under controlled environment conditions between turfgrass isolates and turfgrass hosts, Backman et al. (1999) did not find strict host specificity between turfgrass isolates and turfgrass hosts. In similar tests, Browning et al. (1999) found that isolates from *A. palustris* caused disease on both *A. palustris* and *P. annua*, while those from *P. annua* were limited to *P. annua*. Browning et al. (1999) also observed that maize and sorghum isolates were able to cause foliar infection on *P. annua* but not on *A. palustris*.

Results of DNA analyses for specialization among turfgrass isolates of *C. graminicola* have not been in agreement. Comparisons of isolates of *C. graminicola* from *P. annua*, *A. palustris*, and other hosts by random amplified polymorphic DNA (RAPD) analysis have given conflicting results. For example, Backman et al. (1999) grouped *P. annua* isolates with an isolate from sorghum and *A. palustris* isolates with maize isolates; whereas, Browning et al. (1999) grouped maize isolates and one sorghum isolate together, apart from turfgrass isolates. Chen et al. (2002) examined isolates of *C. graminicola* from *A. palustris* and *Poa* spp., using RAPD markers, and found that all *P. annua* isolates tested clustered together, although with low bootstrap values separating *A. palustris* and *P. annua* isolates. Hsiang and Goodwin (2001) sequenced ribosomal DNA of isolates of *C. graminicola* from turfgrasses, including *Poa* spp. and *A. palustris*, and found that while there were distinct differences between the isolates from sorghum and maize, the turfgrass isolates were even more distantly related and showed greater genetic similarity to *Colletotrichum dematium* or *Colletotrichum coccoides* than to sorghum or maize isolates. They found some differences between *P. annua* and *A. palustris* isolates, but bootstrap support in the cluster analysis was low.

Detailed studies on the infection process of *C. graminicola* have been done on oats (Politis 1976), maize (Mims and Vaillancourt 2002; Politis and Wheeler 1973), and sorghum (as *C. sublineolum*) (Wharton and Julian 1996). Although infection studies with *C. graminicola* on turfgrasses have been conducted (Backman et al. 1999; Browning et al. 1999; Herting 1982), detailed infection studies at the cellular level have not been carried out. If physiological specialization is present among turfgrass isolates of *C. graminicola*, then it might be observed in the infection process. The host infection process of a number of *Colletotrichum* spp. has been classified as following either an intracellular hemibiotrophic strategy, a subcuticular intramural strategy, or a combination of both strategies (Bailey et al. 1992; Perfect et al. 1999). Isolates of *C. graminicola* that attack maize are considered to follow an intracellular hemibiotrophic strategy (Bergstrom and Nicholson 1999; Mims and Vaillancourt

2002; Politis and Wheeler 1973). In this strategy, the fungus has an initial biotrophic phase in which it feeds on living host cells and the host is symptomless. This is followed by a destructive necrotrophic phase in which the fungus causes extensive degradation of host cells and symptoms become visible. *Colletotrichum sublineolum* (syn. *C. graminicola*) on sorghum (Wharton and Julian 1996) and *C. graminicola* on maize (Mims and Vaillancourt 2002) also show intracellular hemibiotrophy, and it is likely that isolates of *C. graminicola* from other species also share this strategy of infection.

The purpose of this study was to examine the infection process of *C. graminicola* from *A. palustris* on different turfgrass species to determine (*i*) whether the infection process and structures differ from those found on maize (Mims and Vaillancourt 2002; Politis and Wheeler 1973) or on oats (Politis 1976) and (*ii*) whether the infection process differs among the four turfgrass species.

Materials and methods

Grass culture

Fine sand (Fisher S25-500 washed sea sand, Mississauga, Ont.) was autoclaved twice, and 16 g was added to 3.7-cm-diameter, 16-mL wells in 6-well tissue culture sterile polystyrene plates (Corning 3505, Corning, N.Y.). Approximately 200 seeds per well of *P. annua* (0.09 g), *A. palustris* (0.02 g), *Poa pratensis* (0.09 g), or *Lolium perenne* (0.38 g) were spread out uniformly on the sand. Sterile distilled water (3.5 mL) was added to each well, and another 0.5 g of autoclaved sand was spread over the seeds. Lids were placed on the plates to reduce evaporation, and plates were incubated at 23 °C under continuous low light (50 µE·m^{-2}·s^{-1}). Plate lids were removed after seed germination, and plates were placed into a 40-L clear plastic container (Rubbermaid 2223), which allowed light to reach the plants while maintaining high relative humidity (>95%). Sterile water (2.5 mL) was added to each well at 2- or 3-day intervals, and plants were grown for 3 weeks at 23 °C and continuous low light. No nutrients or fertilizer was added to the plants.

For fungal inoculation, two layers of autoclaved filter paper (Whatman No. 1, 7-cm diameter) were placed in each 9-cm-diameter Petri plate. Sterile water (1.6 mL) was added to each plate to moisten the filter paper. Leaf blades from 3-week-old plants were cut with a sterile scalpel blade, and up to 60 leaf pieces, 1.5 cm long, were placed adaxial surface up on moistened filter paper in each Petri plate.

Fungal isolation and inoculation

Diseased samples showing symptoms of anthracnose basal rot were collected from an *A. palustris* golf course putting green in Guelph, Ont. Two isolates of *C. graminicola* (99359 and 99362) were obtained by surface sterilizing 2-mm leaf segments in 70% ethanol and 0.5% NaOCl for 60 s each and rinsing three times in autoclaved distilled water. Leaf segments were placed on potato dextrose agar (PDA) amended with streptomycin sulfate (100 mg·L^{-1}) and tetracycline (50 mg·L^{-1}), and plates were incubated at room temperature (23 °C) for 10 days. Colonies identified as *C. graminicola* (Sutton 1980) were transferred to fresh PDA plates, and a series of subcultures were made to ensure isolate purity. The

cultures were transferred to PDA slants for long-term storage at 4 °C, and subcultures were made on fresh PDA for spore production. After 2 weeks at 23 °C and continuous low light, 10 mL of sterile distilled water was added to the spore production plates, and spores were dislodged by gentle rubbing with a flamed, bent glass rod. The inoculum suspension was passed through a double layer of cheesecloth to remove mycelial fragments, and the concentration was adjusted to 3×10^6 spores·mL^{-1} of water, following the procedure of Vargas et al. (1993). The inoculum suspension was placed in a small sprayer that produced droplets averaging from 0.5 to 1 mm in diameter, and 0.02 mL was applied uniformly to leaves in each plate. Plates were incubated at 23 °C and continuous low light (50 µE·m^{-2}·s^{-1}). For each isolate, there were four replicate plates per host species.

Staining

At intervals of 2 h up to 10 h after inoculation (AI), then at 24-h intervals up to 168 h AI, one inoculated leaf blade was removed from each of the four replicate plates for each isolate by host combination. The leaves were placed in 1.5 mL of acetic alcohol (1 part glacial acetic acid and 3 parts 95% alcohol) for 48 h to remove the chlorophyll (Busch and Walker 1958; Lubani and Linn 1962). After 24 h, the acetic alcohol was removed with a pipette and replaced with a fresh solution for another 24 h. The cleared leaves were removed and placed on a glass slide with the adaxial surface up in drops of 0.05% trypan blue (w/v) in lactophenol (20% phenol, 20% lactic acid, 40% glycerine, and 20% water) for 24–48 h. After 48 h, leaves from *A. palustris* and *P. annua* were placed in 1.5 mL of lactophenol for 12–24 h to remove excess stain and mounted on glass slides in drops of lactophenol. Tissues of *P. pratensis* or *L. perenne* usually required additional staining, and the glass slides with tissues and stain were placed on a hot plate and gently heated for 15–45 minutes at 40 °C to enhance staining.

Microscopy and photography

Prepared slides were observed under brightfield light microscopy with a Nikon Labophot. Images were taken with a Nikon Coolpix 800, with the camera lens positioned directly over the top ocular lens of the microscope. Focal distance was set to "landscape mode" to prevent constant automatic refocusing by the digital camera and the flash from firing. Optical zoom was used to fill up the view frame to prevent a silhouette effect.

Infection structures

Spore germination was measured on slides prepared 2–48 h AI. Each leaf blade was considered a separate replicate, with four replicates per isolate by host combination. Starting at the basal end of a leaf, a total of 25 spores were observed for germination on each leaf blade at × 1000 magnification. A spore was considered germinated if the germ tube was at least half the width of the spore. Clusters of spores less than half their width apart were not assessed.

Appressorial and penetration pore formation were observed on leaf blades collected 4–48 h AI. Each leaf blade was considered a separate replication. Starting at the basal end of a leaf, the first 25 spores, germinated or not, were observed for the presence of appressoria on each leaf blade at × 1000 magnification. A germ tube tip cell that was 4 µm or wider was considered an appressorium, irrespective of color. Appressoria originating from mycelial fragments were not counted. Each appressorium was observed for penetration pore formation where a bright-white, circular spot was observed through the appressorial wall by light microscopy.

The occurrence of infection hyphae was assessed in leaf blades mounted at 10, 24, 48, 72, 96, and 120 h AI. Each leaf blade was considered a separate replication. Starting at the basal end of a leaf, a total of 25 spores per leaf were observed for the presence of infection hyphae at × 1000 magnification. Penetration was considered successful if the appressorium produced hyphae inside the epidermal cell. Leaf blades mounted at 48, 72, 96, 120, 144, and 168 h AI were observed for the presence of acervuli and spore production. At 2 weeks AI, the occurrence of acervuli on leaf segments was also assessed.

Results and discussion

Isolates and conidial morphology

The two isolates of *C. graminicola* used in this study were both from an *A. palustris* golf course putting green in Guelph, Ont. A previous study with multiple isolates from this green found >95% similarity in RAPD banding patterns (Chen et al. 2002), indicating a high level of genetic relatedness among the isolates. Since the two isolates studied did not differ significantly in cultural morphology, growth rates, or infection on the four host species in this study, all data for the two isolates were pooled. All images presented in the figures are of isolate 99359.

Conidia of the two isolates were mostly allantoid (slightly curved with rounded ends, as described by Browning et al. 1999) when produced on PDA (Figs. 1A and 1B)[2]; however, acervuli formed on dead host tissues were found to produce falcate spores (crescent shaped with pointed ends; Fig. 1C). The spore sizes did not differ significantly between the two isolates, averaging 12–25 µm × 3–5 µm. These descriptions of spore size and shape are similar to those reported by Browning et al. (1999) for isolates of *C. graminicola* from *P. annua* or *A. palustris*, with allantoid to falcate spores averaging 16–29 µm × 4–6 µm (produced in culture); by Backman et al. (1999) for isolates from *P. annua* and *A. palustris* with average lengths of 17–27 µm (in culture); and by Smith (1954) with fusoid spores for isolates from *P. annua* in planta (19–32 µm × 3–6 µm) or in culture (22–35 µm × 4–7 µm). However, these descriptions are different from those of maize isolates in which the spores were falcate (in planta, Fig. 2 in Politis 1975) and the sizes were larger, measuring 28–43 µm × 5–6 µm (in culture, Mims et al. 1995).

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[2] All figures for this article are available in colour as supplementary data and are available on the Web site or may be purchased from the Depository of Unpublished Data, Document Delivery, CISTI, National Research Council Canada, Ottawa, ON K1A 0S2, Canada. DUD 3530. For more information on obtaining material refer to <http://www.nrc.ca/cisti/irm/unpub_e.shtml>.

Fig. 1. Spore morphology of *Colletotrichum graminicola* from *Agrostis palustri*s. (A) Two fusoid spores. (B) Two allantoid spores. (C) One falcate spore and the pointed ends of two other spores. Bar = 10 μm.

Fig. 2. Conidial germination of *Colletotrichum graminicola* from *Agrostis palustris* photographed at 2 or 6 h after inoculation. Conidial germ tubes produced (A and B) from single end, (C) laterally, and (D) in multiples. Bar = 15 μm.
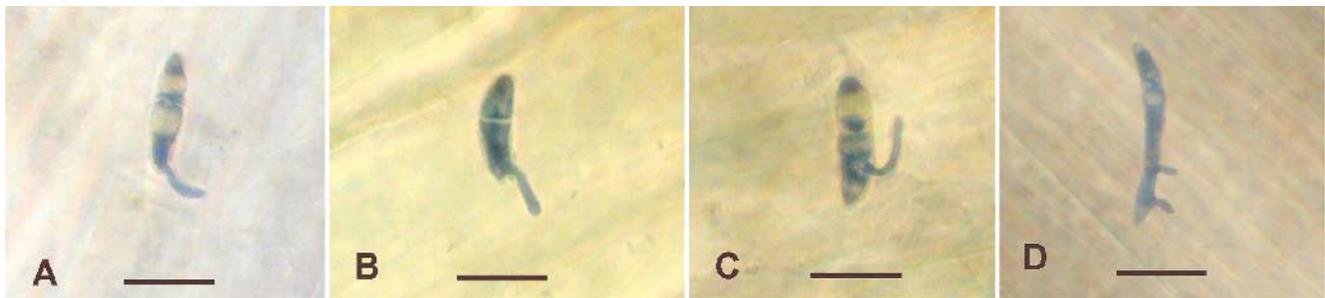

Table 1. Conidial germination of *Colletotrichum graminicola* spores on 3-week-old detached leaves of four turfgrass species at defined intervals after inoculation.

Host	Conidial germination on leaf surfaces (%)						
	2 h	4 h	6 h	8 h	10 h	24 h	48 h
Agrostis palustris	10	41	69	71	83	96	96
Poa annua	13	30	45	61	71	88	93
Poa pratensis	12	31	47	55	60	86	93
Lolium perenne	12	33	49	50	61	87	94
LSD	ns	ns	14	14	12	ns	ns

Note: Each mean is based on 8 replications (4 per isolate) of 25 spores each. LSD, least significant difference; ns, not significant in an ANOVA test; both at *P* = 0.05.

Table 2. Percentage of conidia of *Colletotrichum graminicola* that germinated and formed appressoria on 3-week-old detached leaves of four turfgrass species at defined intervals after inoculation.

Host	Appressorial formation on leaf surface (%)					
	4 h	6 h	8 h	10 h	24 h	48 h
Agrostis palustris	0	9	19	47	87	95
Poa annua	0	6	12	45	81	95
Poa pratensis	0	16	20	29	77	92
Lolium perenne	0	11	23	29	56	92
LSD	ns	ns	ns	10.1	ns	ns

Note: Each mean is based on 8 replications (4 per isolate) of 25 spores each. LSD, least significant difference; ns, not significant in an ANOVA test; both at *P* = 0.05.

Spore germination

Conidial germination (Fig. 2) started within 2 h AI (Table 1) on all four host species and increased with time. There was no significant difference in the percentage of germination of conidia on different hosts at 2 or 4 h AI. At 6 h AI, conidial germination of the two *A. palustris* isolates was significantly higher on *A. palustris* than on *P. annua*, *P. pratensis*, or *L. perenne* by up to 50%. Conidial germination remained higher on *A. palustris* than on the other species at 8 and 10 h, but by 24 h AI, there were no longer significant differences in total conidial germination among the four species, with all germination rates over 93% (Table 1). Conidial germ tubes were produced at the spore tips or laterally in almost equal number. Most conidia produced a single germ tube (Figs. 2A–2C), but a few produced two tubes and in rare cases three or more (Fig. 2D). In a previous study, Politis and

Wheeler (1973) did not find multiple germination for any of the maize isolates, while Skoropad (1967) found this for a wheat isolate. At the time of germination, a septum (Fig. 2B) formed in the center of the germinating spore.

Appressorial morphology and formation

Appressoria were first observed at 6 h AI on all four turfgrasses (Table 2, Fig. 3). There were no significant differences in the percentages of appressorial formation among the four hosts at each time interval of 6, 8, 24, or 48 h AI. Appressoria were produced either directly from conidia (Figs. 3A and 3B) or from the tips of germ tubes (Figs. 3C and 3D). The frequency of these forms of appressorial formation depended on the location of the appressoria: most penetration occurred at the anticlinal wall position between epidermal cells on the plant surface (>50%), and some germ

Fig. 3. Spore germination and appressorial formation of *Colletotrichum graminicola* photographed between 6 and 10 h after inoculation on *Agrostis palustris*. (A) Appressorium forming below conidium without spore germ tube. (B) Two appressoria forming at opposite ends of a spore with limited spore germ tube development. (C and D) Spore germ tube growth and appressorial formation on anticlinal wall. Bar = 15 μm.
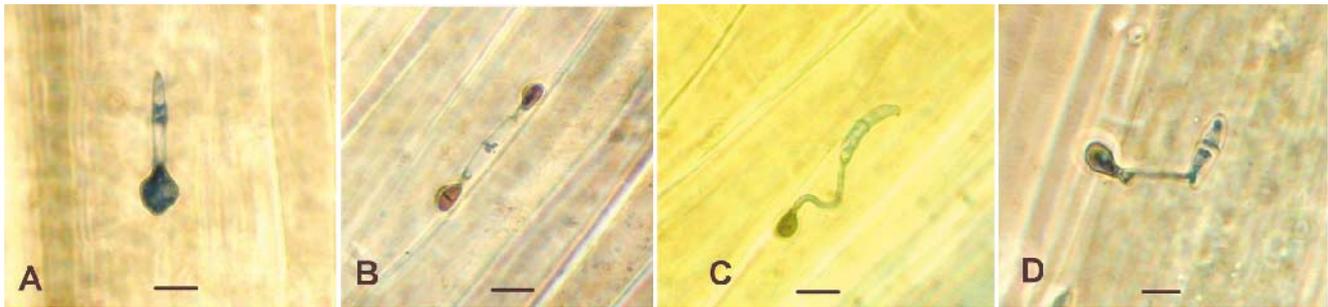

Table 3. Percentage of conidia of *Colletotrichum graminicola* with penetration pores on 3-week-old detached leaves of four turfgrass species at defined intervals after inoculation.

Host	Penetration pore formation on leaf surface (%)					
	4 h	6 h	8 h	10 h	24 h	48 h
Agrostis palustris	0	1	6	15	58	90
Poa annua	0	1	3	12	39	85
Poa pratensis	0	0	4	10	41	60
Lolium perenne	0	0	3	5	18	53
LSD	ns	ns	ns	ns	13.2	20.3

Note: Each mean is based on 8 replications (4 per isolate) of 25 spores each. LSD, least significant difference; ns, not significant in an ANOVA test; both at *P* = 0.05.

Table 4. Percentage of conidia of *Colletotrichum graminicola* that formed infection hyphae onto 3-week-old detached leaves of four turfgrass species at defined intervals after inoculation.

Host	Infection hyphae observed in epidermal cells (%)					
	10 h	24 h	48 h	72 h	96 h	120 h
Agrostis palustris	0	15	37	51	60	82
Poa annua	0	10	34	47	62	76
Poa pratensis	0	13	27	40	24	34
Lolium perenne	0	6	10	48	47	67
LSD	ns	8.3	16.7	ns	19.3	13.1

Note: Each mean is based on 8 replications (4 per isolate) of 25 spores each. LSD, least significant difference; ns, not significant in an ANOVA test; both at *P* = 0.05.

tubes were seen to grow toward these positions to produce appressoria (Figs. 3C and 3D). As appressoria matured, septa formed between appressoria and germ tubes, and the conidia became empty and hyaline (Fig. 3B). Over 93% of spores on all four hosts were seen to be germinated by 48 h AI (Table 1), and over 92% of spores successfully germinated and formed appressoria by 48 h AI (Table 2), indicating that nearly all spores that germinated produced appressoria.

Appressoria of the isolates of *C. graminicola* from *A. palustris* varied in shape, from ovate to lobed. Most appressoria were round and smooth (Fig. 4A) with fewer exhibiting dentations (Fig. 4B) or lobes (Figs. 4C and 4D), and this type of variation was consistent across the different hosts and both isolates. Browning et al. (1999) described the appressoria of *C. graminicola* from *A. palustris* and *P. annua* as varying in size and shape from round to lobate, with some irregularly shaped. As noted by Browning et al. (1999), the appressoria from turfgrass isolates were more similar to appressoria of sorghum isolates, which were described as pyriform, elliptical, obovate to broadly clavate (Sutton 1968), than they were to isolates from maize, which were described as more variable in shape, ranging from obovate, pyriform to elliptical in small appressoria to completely irregular in large appressoria with one to five distinct lobes (Sutton 1968). The size of the appressoria of *A. palustris* isolates produced in planta in this study averaged 10.0 μm × 6.5 μm, and they were smaller than those of isolates grown in culture originally from sorghum

(12.8 μm × 8.8 μm), maize (18.4 μm × 13.6 μm), *A. palustris* (14.6 μm × 9.7 μm), or *P. annua* (13.4 μm × 9.6 μm) (Browning et al. 1999; Sutton 1968).

Penetration pores, infection hyphae, and acervuli

Under light microscopy, the penetration pore appeared as a small, circular bright spot in the middle of an appressorium (Fig. 5A). The penetration pore is an area of the appressorium through which the penetration peg emerges, but the peg is not easily visible by light microscopy, since it is small and embedded in the cell wall. Penetration pores were first seen at 6 h AI in appressoria on *A. palustris* and *P. annua*, and at 8 h AI on *P. pratensis* and *L. perenne* (Table 3). Penetration pore formation at 24 h AI was significantly higher on *A. palustris* than on *P. annua* or *P. pratensis* and was the lowest on *L. perenne*. By 48 h AI, 85%–90% of the spores on *A. palustris* or *P. annua* had formed penetration pores compared with only 53%–60% on *P. pratensis* or *L. perenne* (Table 3). Stomatal penetration or direct penetration by germ tubes or hyphal tips without appressoria was not observed.

Single hypha (Fig. 5B) or two hyphal strands (Fig. 5C) grew from the infection point, but the latter was observed with slightly greater frequency. Infection hyphae inside the epidermal cells were first observed at 24 h AI in all four species (Table 4). Hyphae were significantly more commonly observed in *A. palustris* and less frequently observed in *L. perenne*, at either 24 or 48 h AI. There was no signifi-

Fig. 4. Appressorial morphology of *Colletotrichum graminicola* from *Agrostis palustris* photographed at 24 h after inoculation. (A) Round, smooth appressoria. (B) Slightly irregular appressoria. (C) Lobed appressoria. (D) Multilobed appressorium. Bar = 10 μm.
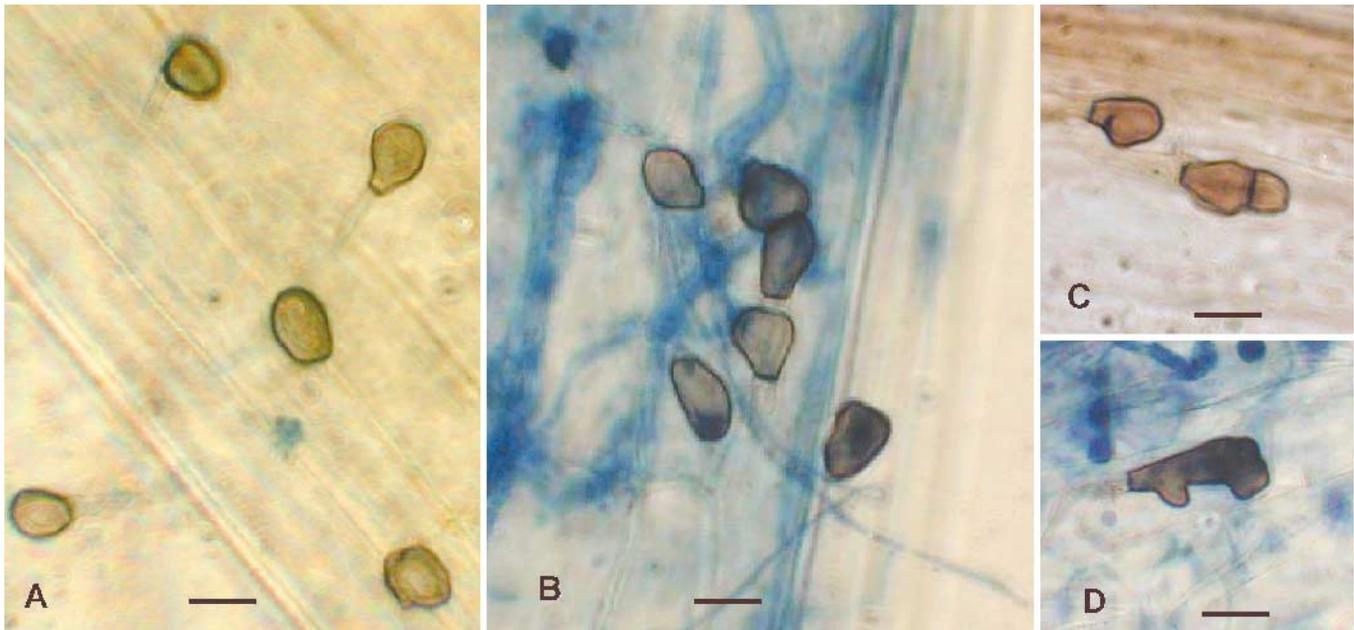

Fig. 5. Formation of penetration pores and infection hyphae of *Colletotrichum graminicola* on *Agrostis palustri*s. (A) Penetration pores (p) of *C. graminicola* in the middle of mature melanized appressoria, appearing as a bright spot at 24 h after inoculation (AI). (B) A swollen vesicle (v) and infection hyphae (h) at 24 h AI. (C) Infection hyphae growing in different directions at 48 h AI. (D) Hyphal strand passing from cell to cell, exhibiting constriction (c) at host cell walls at 48 h AI. Bar = 15 μm.
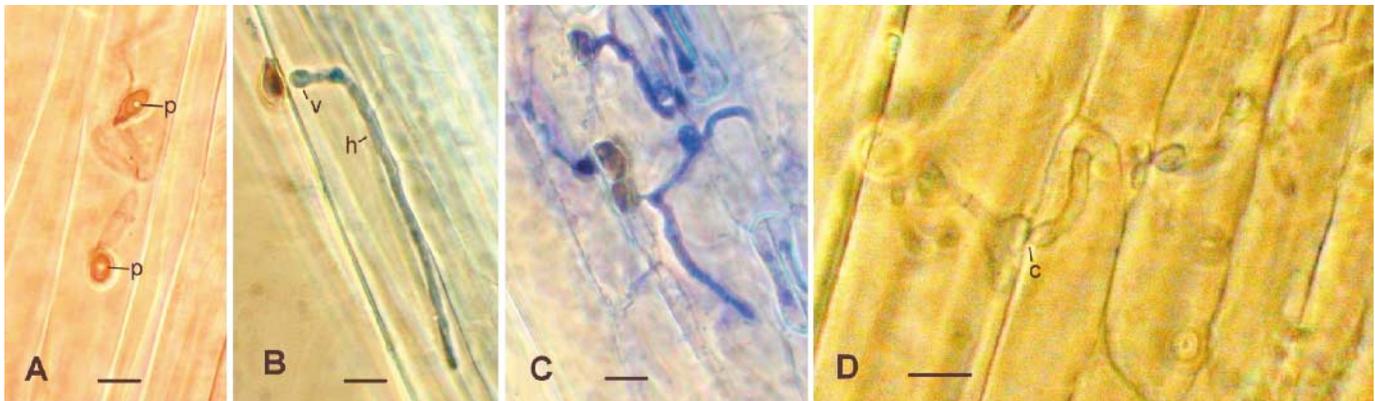
cant difference in the frequency of infection hyphae in the different hosts at 72 h AI, but at 96 and 120 h AI, the incidence of infection hyphae was significantly higher in *A. palustris* than in *P. pratensis* (Table 4).

Among certain *Colletotrichum* spp., the end of the biotrophic phase is signaled by the appearance of thinner secondary infection hyphae, widespread intercellular growth, and lack of hyphal constrictions as they penetrate host cell walls (Bailey et al. 1992). In our study, up to 168 h AI, hyphae were not observed to differentiate into thinner secondary infection hyphae. Initially, the hyphae appeared constricted at penetration points through epidermal cell walls (Fig. 5D), but with increased hyphal colonization, constrictions were no longer visible. For *A. palustris* and *L. perenne*, widespread ramification of hyphae occurred after 72 h AI, while

for *P. annua* and *P. pratensis*, it was still not observed by 168 h AI.

Because we did not use vital staining techniques in our study, we could not prove the occurrence of biotrophy. However, for *C. graminicola* on *Z. mays*, Mims and Vaillancourt (2002) confirmed the occurrence of a biotrophic phase in which epidermal cells deposited wall appositions around hyphae. They stated that this phase was short, lasting less than 12 h within a cell, and that a switch to necrotrophy occurred between 48 and 72 h AI. For *C. sublineolum* on *Sorghum bicolor*, Wharton and Julian (1996) observed the development of narrow secondary infection hyphae at 66 h AI. The primary infection hyphae, which were found only in epidermal cells, were twice the width and showed constrictions where they penetrated host cell walls. The development

Fig. 6. Formation of acervuli. (A) Aggregation of *Colletotrichum graminicola* hyphae and production of mycelial knots to form proto-acervular stroma below the cuticle 72 h after inoculation (AI) on *Agrostis palustris*. (B) Stroma at 72 h AI on *A. palustris*. (C) The stroma ruptures the cuticle, and spores of *C. graminicola* appear on the surface of inoculated leaves of *A. palustris* at 72 h AI. (D) Conidiophore growing out of a stomate at 96 h AI on *A. palustris*. (E) Falcate conidia produced by young acervuli at 96 h AI on *A. palustris*. (F) The base of the acervulus begins to darken (melanize) as it matures at 96 h AI on *A. palustris*. (G) An acervulus exhibiting setae and conidiophores at 96 h AI on *A. palustris*. (H) Spore matrix with setae seen on *Poa annua* at 2 weeks AI. Bar = 20 µm.
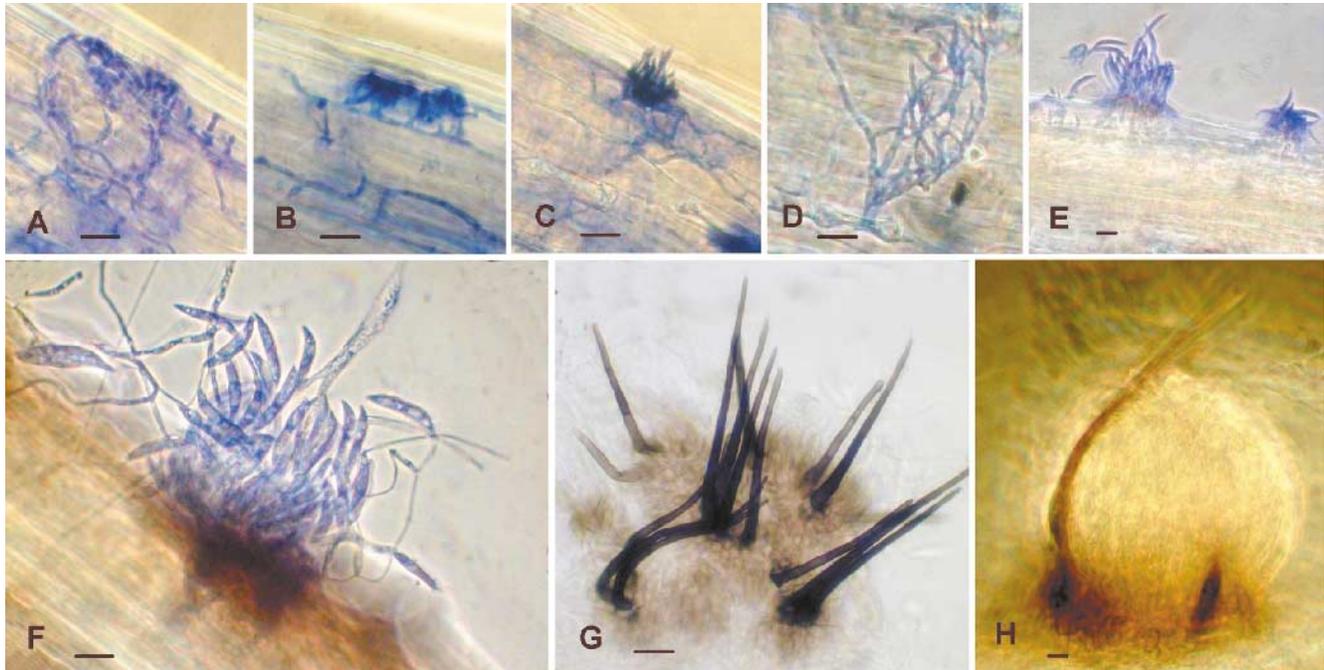

Table 5. Number of acervuli per leaf segment at defined intervals after inoculation of spores of *Colletotrichum graminicola* onto 3-week-old detached leaves of four turfgrass species.

	No. of acervuli						
Host	48 h	72 h	96 h	120 h	144 h	168 h	2 weeks[a]
Agrostis palustris	0	2.6	4.0	16.5	12.6	16.3	100%
Poa annua	0	0	0	0	0	0	20%
Poa pratensis	0	0	0	0	0	0	43%
Lolium perenne	0	0	2.6	1.8	2.5	5.5	100%

Note: Leaf segments (1.5 cm long) inoculated with *C. graminicola* were observed for the presence of acervuli, and each value is the average over 4–8 leaf segments.

[a]At 2 weeks after inoculation, the number of leaves bearing acervuli out of the total number of leaves (ranging from 31 to 89) was assessed. The leaves were senescent at this time.

of narrower secondary infection hyphae has not been reported for *C. graminicola* on other hosts, such as maize and oats, and we did not observe it for the turfgrass hosts.

At 48 h AI, hyphae were observed in the mesophyll tissue in *A. palustris* and *P. annua* but not in *P. pratensis* and rarely in *L. perenne*. By 72 h AI, the mycelium colonized both epidermal cells and underlying mesophyll cells in *A. palustris* and *L. perenne* but not in *P. annua* or *P. pratensis*. In *A. palustris*, by 120 h AI, the hyphae proliferated extensively in epidermal cells and upper mesophyll but were not as frequent in the lower mesophyll. The greater frequency in the upper mesophyll could have been related to the inoculation of the upper (adaxial) surface. In *L. perenne*, colonization of adjacent cells occurred with penetration into the mesophyll, but colonization of the whole leaf segments was

not seen by 120 h AI. In *P. annua*, the infection hyphae spread from cell to cell in the epidermal layer and later into the mesophyll, but branching of the mycelium was not frequently observed in mesophyll cells by 168 h AI. In *P. pratensis*, the infection hyphae were restricted to the infected epidermal cells and rarely colonized neighboring cells up to 120 h AI.

By 72 h AI in *A. palustris*, hyphae were observed under the cuticle (Fig. 6A), which formed proto-acervular stroma (Fig. 6B). The stroma erupted through the cuticle (Fig. 6C) and began to produce conidiophores and then conidia. In some cases, hyphae grew out of stomata (Fig. 6D), and the conidiophores produced unicellular spores singly at their tips. On *A. palustris*, conidia were first observed at 72 h AI but without setae (Fig. 6E). Setae formed between and

Fig. 7. Encased penetration pegs. (A–C) Appressoria of *Colletotrichum graminicola* that were unsuccessful in penetrating epidermal cells. The penetration pegs became encased at 24–48 h after inoculation on *Agrostis palustri*s. Bar = 15 µm.

Table 6. Comparison of the timing of infection events among different hosts of *Colletotrichum graminicola* from time of inoculation.

Host	Spore germination (h)	Appressorial formation (h)	Percentage peg formation (h)	Infection hyphae (h)
Agrostis palustris[a]	4–6	10–24	10–24	48–72
Avena sativa[b]	12–14	14–18	24–48	72
Echinochloa crus-galli[c]	—	<24	—	—
Lolium perenne[a]	6–8	10–24	24–48	48–72
Poa annua[a]	6–8	10–24	24–48	48–72
Poa pratensis[a]	6–8	10–24	24–48	48–72
Sorghum bicolor[d]	<9	<18	—	42
Zea mays[b]	3–8	5–10	—	—
Zea mays[e]	6–8	15–18	18–24	>36
Zea mays[f]	<24	<24	24–36	<36

Note: —, data not given or not available.

[a]Current study. Timing defined by the period when 50% of the events had occurred. Penetration peg formation in this study was represented by penetration pore formation viewed as a small circular bright spot in the middle of an appressorium.

[b]Politis (1976).

[c]Yang et al. (2000).

[d]Wharton and Julian (1996). Pathogen listed as *Colletotrichum sublineolum*.

[e]Bergstrom and Nicholson (1999).

[f]Mims and Vaillancourt (2002).

around conidiophores often 2–3 days later, and spores became detached by the time the setae appeared. At 72 h, conidiophores were not found on *L. perenne*, *P. annua*, or *P. pratensis*. By 96 h, acervuli were observed on *A. palustris* and *L. perenne*, with increased melanization at the base (Fig. 6F). The mature setae were melanized and contained 1–6 septa (Fig. 6G). No acervuli or spores were found on *P. annua* or *P. pratensis* by 168 h AI (Table 5). However, when inoculated leaves were observed under a dissecting microscope 2 weeks AI, acervuli and spores, some in a gelatinous matrix (Fig. 6H), were observed in *P. annua* and *P. pratensis*, indicating that the two *A. palustris* isolates could complete the infection process in these hosts but at a much slower rate, with the production of acervuli only on dead host tissues.

Not all appressoria caused successful infections. By 120 h AI, only 34% of the spores on *P. pratensis* had produced observable hyphae in the epidermal cells, while 67%, 76%, and 82% were seen, respectively, for *L. perenne*, *P. annua*, and

A. palustris (Table 4). In some cases, appressoria produced a swelling at the point of entry into the tissue, appearing as a bright bulbous structure attached to the appressoria, encasing the penetration peg (Figs. 7A–7C). This was probably a lignified papilla, preventing entry of the penetration peg into the cell, as described by Bergstrom and Nicholson (1999), and no hyphae were seen growing from these structures.

Infection process

The general infection process of *C. graminicola* was similar on all turfgrass hosts tested and consistent with that of *C. graminicola* on other hosts, although the pace seemed to differ in some cases (Table 6). These differences in pace of development, however, are confounded by the conditions used in each study. Important factors, such as the level of senescence (Skoropad 1967) and host tissue age (Jamil and Nicholson 1987), are thought to influence the infection processes of *C. graminicola*. In our tests with turfgrass hosts, we used detached leaves, which may have hastened the pace

of infection, since senescence is associated with high levels of disease on turfgrass hosts (Couch 1995; Vargas 1995). To assess whether the pace of infection was affected by using detached leaves, we inoculated intact plants with *C. graminicola*, but the results were variable, with a lack of symptoms in most cases.

On turfgrass hosts, spore germination, appressorial formation, production of infection hyphae, and formation of acervuli by two *A. palustris* isolates were similar, but these events occurred more quickly or more extensively on or in *A. palustris* tissue. In addition to the possible physiological specialization of *A. palustris* isolates on *A. palustris*, another reason for the more aggressive growth of *C. graminicola* in *A. palustris* and the failure to quickly colonize and produce acervuli on *P. annua* and *P. pratensis* could be the incubation temperature (23 °C) used in this study. Couch (1995) found that infection and colonization of *P. annua* did not occur unless the leaves were exposed to temperatures ranging from 30 to 35 °C. In pathogenicity tests on whole seedlings, Herting (1982) found that isolates of *C. graminicola* from *A. palustris* were not able to cause disease symptoms on seedlings of *A. palustris* or *P. annua* when incubated at 10 or 25 °C. Anthracnose foliar blight is known to seriously damage *P. annua* during periods of high temperature and high humidity (Vargas 1995), and in inoculation tests, the optimum temperature for infection was between 30 and 33 °C (Bolton and Cordukes 1981).

Conclusions

Spore morphology of the two isolates of *C. graminicola* from *A. palustris* in this study was similar to those previously reported for turfgrass isolates (Backman et al. 1999; Browning et al. 1999; Smith 1954), but the spores were smaller than those of isolates from maize (Backman et al. 1999; Mims et al. 1995; Politis 1975). This morphological evidence combined with molecular evidence (Browning et al. 1999; Hsiang and Goodwin 2001) supports the distinction of turfgrass isolates of *C. graminicola* from those of other hosts. The observation that spores of *C. graminicola* from *A. palustris* produced on agar were commonly allantoid in contrast to the falcate spores produced in acervuli on host tissue demonstrates the importance of assessing spore morphology on the same substrates. Similarly, the appressoria produced on host tissue in this study were generally smaller than appressoria produced on agar (Browning et al. 1999; Sutton 1968).

The general infection process of *C. graminicola* was similar on all turfgrass hosts tested and similar to previous reports of *C. graminicola* on other hosts. However, under our test conditions, detached leaf tissue of *A. palustris* appeared to be relatively more susceptible to *C. graminicola* than leaf tissue of the other turfgrass species tested, with significantly faster spore germination (6 h), faster penetration pore formation (24 h), more hyphal growth in epidermal cells (120 h), and greater levels of acervular formation. Although *C. graminicola* from *A. palustris* was able to complete the disease cycle on other hosts, the rate was slower or fungal growth was less extensive. This provides evidence for weak physiological specialization among isolates of *C. graminicola* from *A. palustris*.

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