

Anthracnose: The sophisticated rot

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Corn anthracnose disease

The mold fungus *Colletotrichum graminicola* causes anthracnose, one of the most economically damaging corn diseases worldwide. Anthracnose can occur either as a stalk rot (ASR), or a leaf blight (ALB) (4; 27). The leaf blight phase is generally insignificant in North America as a cause of yield loss, although in the tropics and subtropics it is much more important. Resistance to ASR is usually not correlated with resistance to ALB, complicating efforts to breed resistant corn varieties (2; 4). Resistance to ASR and ALB is mostly quantitative, although sources of major gene resistance have been described (10; 29). Hybrids containing some of these major-gene resistance sources are likely to become available for management of ASR in the near future.



Figure 1. A cornfield in Daviess County, Kentucky, in August of 2000. This field was about 95% lodged due to stalk rot. Photograph taken by Paul Vincelli

In the corn belt of the United States (U.S.), stalk rot is estimated to cause losses of between 5 and 10% of the potential corn yield annually (10; 24). Corn is the most valuable crop in the U.S., worth more than 52 billion dollars in 2014 (USDA/NASS). That means losses from stalk rot in 2014 were probably more than 2.5 billion dollars. When it is very prevalent, stalk rot can cause lodging, resulting in up to 100% loss (4; 24; 29). Fungal stalk rot was particularly widespread in the U.S. during the summer of 2000, when many fields experienced severe lodging (Figure 1).

In addition to *C. graminicola*, several other fungi can cause corn stalk rot, including *Gibberella zeae* and *Diplodia* (aka. *Stenocarpella*) *maydis*. Resistance to ASR is not usually correlated with resistance to *Gibberella* stalk rot (GSR) or *Diplodia* stalk rot (DSR) (2; 7). Incorporation of resistance to one pathogen species often results only in the increased incidence of another. Currently, ASR is the most common and the most damaging stalk rot in the U.S.

Much has been published about the effects of various agronomic practices on stalk rots. In general, anything that stresses the plants and/or decreases photosynthetic capacity can increase stalk rot incidence (7; 24; 29). Some aspects are less clear, however. For example, nitrogen levels are important, but have an unpredictable effect on stalk rot severities (28). This suggests a complex relationship not just related to plant stress but perhaps also to the ability of the fungus to sense and utilize nutrients *in planta*. A dominant theory holds that drawing of carbohydrate reserves from stalks during grain fill causes increased susceptibility to stalk rot (9). This may be due to an associated reduction in natural host defenses, and/or to fungal sensing of changing nutrient status, since fungi are known to be very responsive to carbon quality and quantity. Resistance to stalk rot fungi is not expressed efficiently once the plant reaches physiological maturity, and breeding for increased stalk rot resistance eventually becomes self-defeating since producing corn that keeps carbohydrate in the stalk, and thus maintains resistance, leads to a depression of yield potential (29). Does this mean that we are stuck with stalk rots, as a byproduct of high yields? One thing suggesting this is not the case is that corn stalks are not susceptible to all fungi. For example, they do not succumb to the closely-related sorghum anthracnose pathogen, even though that fungus has the capacity to cause a very similar stalk rot disease in sorghum (26). Clearly corn stalks do have an ability to defend themselves, even post-anthesis, and stalk rot fungi must have specific mechanisms that they use to circumvent those defenses. If we had a better understanding of these mechanisms, we might be able to develop more targeted tools for disease management. In the Vaillancourt laboratory at the University of Kentucky, we have spent more than 15 years attempting to address this question. Our progress so far is summarized below.

How does C. graminicola enter and grow in corn tissues?

Fungal pathogens of plants can be classified as biotrophs, which feed only on living plant tissues, or necrotrophs, which live primarily on dead or dying cells (17). Stalk rot fungi are traditionally considered to be necrotrophic, like all rot fungi. However, a majority of *Colletotrichum* fungi are intracellular hemibiotrophs, an interesting third category that are biotroph-like or necrotroph-like during successive phases of their disease cycles. There was previously some doubt about whether *C. graminicola* was truly hemibiotrophic, but we and others have been able to confirm this by cytological means, by using plasmolysis assays and vital staining to show that the fungus typically enters cells that are still alive in both the leaves and the stalks (20; 23).

C. graminicola enters corn epidermal cells by producing a dome-shaped melanized appressorial structure. The appressorium builds up a very high internal osmotic pressure, and this is translated into turgor to drive mechanical penetration via the penetration peg (3). The process of hemibiotrophic colonization of corn by *C. graminicola* has been described in detail in several of our research papers (15; 23; 25; 26). During biotrophic development, initial penetration of the host cell is followed by the production of a swollen primary infection hypha (Figure 2). Within a few hours, branches emerge and begin to infect other host cells via narrow connections through intact host cell walls. Newly infected cells remain alive, while the host plasma membrane expands and envelops these primary hyphae. Approximately 24 hours after infection, the plasma membrane of an invaded cell loses its functional integrity, and the cell begins to die. Tissue damage is confined to cells that have been infected by hyphae, and there is no extensive dissolution of the host cell walls at this point. Primary infection hyphae continue to colonize new cells, expanding the infection front until the more destructive necrotrophic phase begins, 24-48 hours after initial infection. This phase is characterized by production of numerous narrow secondary hyphae that ramify through the host tissues both inter- and intracellularly. Host cells are killed rapidly, and host cell walls become degraded and rotted. It is only at this stage that visible anthracnose lesions begin to appear. The dimorphism of primary versus secondary hyphae can be observed both in stalk and leaf tissues (Figure 2). Hemibiotrophic development in *C. graminicola* is asynchronous and spatially defined (23; 25; 26). Thus, colony centers exhibit necrotrophy, while colony margins continue to advance by biotrophic

invasion of adjacent living cells.

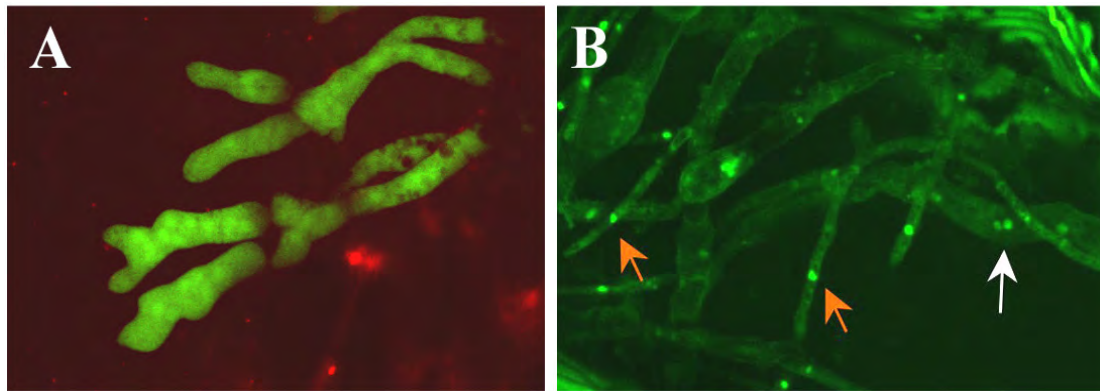


Figure 2. A. Primary infection hyphae in a corn leaf. The dark areas are where the hyphae have passed through a very narrow opening from one cell to the next. B. Thick, irregularly shaped primary infection hyphae (white arrow) with narrower secondary hyphae arising from them (gray arrows) in corn stalk cells.

Very little is known about how other stalk rot fungi colonize corn cells, but our data suggest that they do not routinely enter living cells as *C. graminicola* does. *D. maydis* appears to be a true necrotroph that kills cells prior to penetration, presumably by the secretion of a phytotoxin. There is evidence in the literature that a secondary metabolite produced by *D. maydis* called diplodiatoxin functions as a phytotoxin (30). *G. zeae* only rarely enters corn cells directly, and when it does, it remains confined there, seeming to have a difficult time moving out again. Colonization of wheat by *G. zeae* has been more extensively investigated (5; 6; 13). In wheat, *G. zeae* grows initially in intercellular spaces between cells and along vascular bundles, and enters the cells only after they have been killed, presumably due to activity of the phytotoxin deoxynivalenol (DON).

C. graminicola, *G. zeae*, and *D. maydis* are all believed to invade corn stalk tissues via the roots and leaf sheaths (7; 29). All three pathogens also readily enter stalks through insect wounds, although the relationship of the pathogens to the insects (e.g. whether they act as vectors) is not well understood (29). *G. zeae* and *C. graminicola* were the two most frequent causes of stalk rot occurring in Bt corn, suggesting that they may depend less than on insect wounding to gain entry to plants than *D. maydis* (12).

It has been suggested that *C. graminicola* behaves as a vascular wilt fungus, colonizing the xylem and producing dieback of the upper parts of the stem (4; 19). We have not been able to find any evidence that *C. graminicola* is a wilt, although it does efficiently colonize and move through the fiber cells that surround the vascular bundles, and that also underlie the epidermal cells in the stalk rind (Figure 3) (25). Our work suggested for the first time that fiber colonization is an important pathogenicity factor for this fungus. Movement through the mostly non-living fibers may allow the fungus to avoid host defenses, and could provide a base from which it can invade adjacent parenchyma cells. We don't know anything about how other stalk rot fungi grow inside corn stalks, or whether fibers are also important for them. Investigating this question is an important goal, since fiber colonization may provide a unifying theme and a universal target for management of stalk rot fungi. *G. zeae* is known to enter fibers routinely in the wheat stalk, where the hyphae adopt a characteristic appearance and become filled with lipid droplets (13). We have observed *G. zeae* occupying fibers in corn stalks, but we have not followed up on these observations nor have we done any cytological work with *D. maydis* in corn stalks yet.

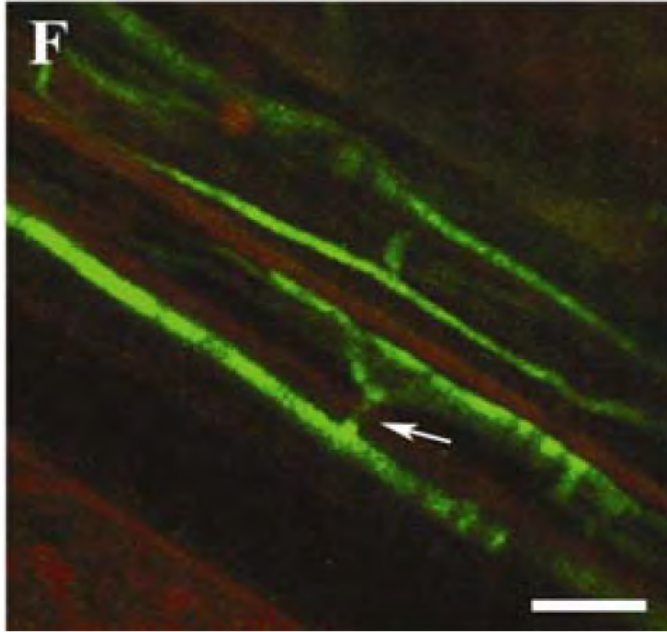


Figure 3. Hyphae colonizing fiber cells and moving from one fiber to another via narrow extensions (arrow) that may be passing through pits. These hyphae have been labeled with GFP to facilitate visualization.

How does C. graminicola enter living cells?

Among the three major corn stalk rot species, the ability to enter living host cells seems to be unique to *C. graminicola*. Mutagenesis can be a useful approach to understanding the nature of hemibiotrophic development in *Colletotrichum*. We created a mutation in a gene called *cpr1* (Colletotrichum Pathogenicity Related 1), and the resulting mutant strain (MT) was nonpathogenic to both leaves and stalks of corn, although it grew normally in culture (21; 22). Closer inspection revealed that the MT germinated and formed appressoria normally on the plant surface, but was delayed by about 24 hours in penetration of the host (15; 23). Aside from this delay, penetration efficiency was normal, and primary hyphae encased with a membrane were formed (15; 23). However, once it penetrated the host epidermal cell, the MT was significantly debilitated in comparison to the WT, as more than 95% of the infection sites remained confined to the first cell at 72 hours after inoculation (hpi), whereas more than 95% percent of the WT infection sites had progressed several cells beyond the first penetrated cell at only 48 hpi (Table 1) (23). Interestingly, if the host tissue was killed by localized injury with dry ice, the MT penetrated normally, grew beyond the first cell quickly, and proceeded all the way to sporulation, in a manner that was indistinguishable from the WT (23). Thus, the MT does not have a deficiency in its ability to utilize corn tissue for growth: instead, it appears to have a problem colonizing corn tissue that is actively defending itself, and is unable to establish a normal biotrophic interaction in the living host. The nonpathogenic *cpr1* mutant reveals an “Achilles heel”, by demonstrating that the capacity for biotrophic invasion is critical for pathogenicity of *C. graminicola*.

Table 1. Maximum number of cells colonized by the WT (48 hpi) or the MT (72 hpi) on corn leaf sheaths.

	Maximum number of colonized cells (%)				
	1 cell	2 cells	3 cells	4 cells	5 cells
WT	3.8 ± 4.1	30.1 ± 7.1	45.7 ± 7.4	15.4 ± 5.4	4.7 ± 4.6
Cpr1	95.9 ± 3.7	3.5 ± 3.2	0.95 ± 2.0	0	0

The *C. graminicola cpr1* gene encodes a protein predicted to belong to a highly conserved family of signal peptidase proteins, called SPC22/23 in mammals (8). The protein is part of the signal peptidase complex (SPC), a multi-subunit protein complex that is located on the endoplasmic reticulum (ER) membrane, and serves to cleave signal peptides from nascent proteins as they are being passed from the cytoplasm to the ER for further processing and secretion. The identification of the MT as potentially affected in protein processing and secretion suggests that it may be altered in its ability to secrete proteins that are important for successful pathogenicity.

Biotrophic plant pathogens are known to reprogram living host cells by secreting an array of small proteins (SSPs) that suppress host defense responses and cell death (18). In contrast, necrotrophs (e.g. blights, wilts, and rots) take advantage of plant defense responses to enhance pathogenicity, and induce host cell death by secreting necrosis-inducing proteins or phytotoxic secondary metabolites (SMs) (1). Evidence in the literature suggests that hemibiotrophic *Colletotrichum* fungi do both, first suppressing, and then later inducing, host cell death (11; 14; 16). Both processes are associated with the expression of genes encoding large and diverse suites of SSPs, and genes involved in production of SMs, (aka. effectors), production of which must be tightly regulated to provide the correct function at the appropriate time and place.

We considered two hypotheses to explain why the *C. graminicola* MT is nonpathogenic. First, it may fail to secrete proteins or SMs that allow the establishment of biotrophy. Second, it may inappropriately secrete elicitors or toxins, which are not released in the wild type (WT) until the necrotrophic phase, that activate host defenses and kill the host tissues prematurely. To test these alternate hypotheses we conducted co-inoculation studies (23). We reasoned that, if the MT was inappropriately secreting toxic proteins or elicitors of the defense response, co-inoculation of the MT with WT should prevent the WT from infecting. Alternatively, if the MT was failing to secrete effectors allowing establishment of biotrophy, co-inoculation of the MT with WT should allow the MT to colonize normally. Results of the co-inoculation experiments revealed that inoculation of MT and WT spores together in very close proximity allowed the MT to establish biotrophy and grow normally (23). The distance over which the effect operated was limited to approximately 2 mm, corresponding to about 8 maize epidermal cells (Table 2) (23). This suggested that the WT produces a diffusible substance/signal, most likely to be one or more protein or SM effectors, that cause the neighboring cells to become receptive for biotrophic invasion, whereas the MT was unable to produce these substances.

Table 2. Growth of the MT in co-inoculations with the WT is affected by distance. Different treatments indicate numbers of cells and total distance separating MT and WT fungal colonies at the time of inoculation, and percentage of MT infection sites in which hyphae colonized at least two cells. The control was the MT co-inoculated with water. Different letters indicate a significant difference.

Treatment	No. cells	Distance	Infection sites beyond one cell	Class
D1	8.5 ± 1.5	2.6 ± 0.46 mm	33.5 ± 15	a
D2	13.3 ± 2	4.1 ± 0.62 mm	8.2 ± 14	b
D3	22.8 ± 4.9	7 ± 0.12 mm	9.9 ± 13	b
Control	N/A	N/A	4.6 ± 4.2	b

What are these substances/signals?

With our collaborators, we sequenced the genome of *C. graminicola* a few years ago (16). Our analysis revealed that this pathogen encodes diverse collections of SSPs, and more cell wall degrading enzymes (CWDE) than other sequenced plant-pathogenic fungus, including even the well-known white rot and gray rot fungi *Sclerotinia sclerotiorum* and *Botrytis cinerea*, respectively. We also found that *C. graminicola* encodes a larger number of putative SM genes and gene clusters than any other sequenced fungal pathogen.

We collaborated with scientists at Agrilife Genomics and Bioinformatics Services at Texas A&M University to conduct an analysis of fungal gene expression in *C. graminicola*-infected corn sheath tissues. Samples represented pre-penetration melanized appressoria; late biotrophic development (when the fungus had colonized 3-4 cells beyond the infection site); and necrotrophy (when the fungus had begun to produce secondary hyphae). We subjected the WT fungal data to statistical analysis and compared it with a parallel set of data that was generated for the nonpathogenic MT fungus during two stages of infection: pre-penetration melanized appressoria; and biotrophic development, in which approximately 95% of the successful penetration sites consisted of primary hyphae that were limited to the initially infected cell.

We found that more than 2000 fungal genes were differentially transcribed in “waves” during infection of corn by *C. graminicola*. Secreted proteins, SSPs, SM genes, and membrane receptors were over-represented among the differentially expressed genes, suggesting that the fungus engages in an intimate and dynamic conversation with the host, beginning prior to penetration. This communication process probably involves reception of plant signals triggering subsequent developmental progress in the fungus, as well as production of signals that induce responses in the host. During the late appressorial phase and early biotrophy, numerous SSPs and SM genes were induced. There was a bias in favor of genes that were unique to *C. graminicola* during these phases of development. During the necrotrophic phase, pectate lyases (which are involved in degradation of pectin and production of “rot” symptoms) and other CWDE were heavily over-represented. The late phases of biotrophy were more similar to necrotrophy, and featured increased production of secreted proteases, inducers of plant cell necrosis, hydrolases, and membrane bound transporters for the uptake and egress of potential toxins, signals, and nutrients. This work has revealed the identities of fungal genes that are specifically expressed during critical phases of host penetration and biotrophic establishment. The products of these genes may provide targets for chemical or biological controls to manage this important disease.

Summary

Work in the Vaillancourt laboratory over the years has revealed much about how *C. graminicola* establishes itself in living corn cells. We have demonstrated that biotrophic colonization is critical for subsequent disease development. We have shown that the pathogen is able to colonize fiber cells, which may serve as a refuge until the host defenses weaken sufficiently to be overcome. We have exposed the intimate conversation between the pathogen and its host, which induces the living cells at the edges of advancing colonies to become receptive to fungal invasion. We have even identified some of the potential “words” in this conversation, in the form of SSPs, receptors, SM enzymes, and other proteins that are expressed during critical phases of disease development. Our work with other stalk rot pathogens is still in its very early phases, but preliminary results suggest that these other pathogens have very different mechanisms of infection compared with *C. graminicola*. However, we have hope that further study of these pathogens may reveal a common theme in the ability of stalk rot fungi to circumvent corn resistance pathways, and thus reveal a universal target that could be used for efficient simultaneous management for all stalk rots. This is our ultimate goal for the future.

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