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PLANT DISEASE FEATURE

Maize Anthracnose Stalk Rot in the Genomic Era

Renata Belisário,[1] Alison E. Robertson,[2] and Lisa J. Vaillancourt[1,†]

[1] Department of Plant Pathology, University of Kentucky, 201F Plant Science Building, 1405 Veterans Drive, Lexington, KY 40546-0312

[2] Department of Plant Pathology and Microbiology, Iowa State University, 1344 Advanced Teaching and Research Building, 2213 Pammel Drive, Ames, IA 50011

Abstract

Anthracnose stalk rot (ASR) of maize results in millions of dollars in losses annually in the United States. ASR, together with anthracnose leaf blight and anthracnose top dieback, is caused by the fungus *Colletotrichum graminicola*. Current ASR management recommendations emphasize host resistance and reduction of plant stressors (e.g., drought, heat, low fertility, or soil acidity). Stress reduction may be more difficult to achieve in the future due to more high-intensity production protocols and climate change. Moreover, cultural and chemical management practices may conflict with other important goals, including environmental sustainability and maximization of yield potential. Thus, future ASR management may rely more heavily on host resistance, for which there are relatively few highly effective sources. The last comprehensive review of *C. graminicola* and maize anthracnose was written over two decades ago. The genomic age has brought important new insights into mechanisms governing the host–pathogen interaction from the application of molecular and cytological technologies. This review provides a summary of our current model of maize anthracnose etiology, including how increased knowledge of molecular and cellular events could contribute to better ASR management. Improved understanding of *C. graminicola* taxonomy has confirmed that the fungus is specific to *Zea mays*, and that it colonizes living maize tissues via a critical biotrophic phase. Successful biotrophic establishment relies on an array of secreted protein effectors and secondary metabolites produced at different stages of infection and dispersed to multiple locations. These molecules could provide therapeutic targets for the next generation of transgenic or gene-edited ASR-resistant hybrids.

Keywords: anthracnose leaf blight, anthracnose top dieback, cereals and grains, *Colletotrichum graminicola*, field crops, fungi

Maize (*Zea mays* L.) is the most valuable crop grown in the United States, worth more than U.S.$52 billion in 2019 (USDA-NASS 2020). One of the most common and serious diseases of maize is stalk rot, which reduces yield by interfering with translocation of carbohydrates to the grain, and by causing premature plant death (Kleczewski 2014; Munkvold and White 2016; Wise et al. 2016). Stalk rot can also cause the collapse of plants below the ear, known as lodging, making harvest difficult or impossible. Moreover, when ears on lodged stalks contact the ground, they are more likely to develop ear mold that lowers grain quality.

Management of maize stalk rot is complicated, in part, because multiple pathogens, including several fungal species as well as oomycetes and bacteria, can cause stalk rot symptoms (Munkvold and White 2016; Wise et al. 2016). Management is also challenging because stalk rot is primarily a postanthesis disease that develops when cellular defenses become compromised by removal of carbohydrates from lower stalk and root tissues to support grain-fill (Dodd 1980). Environmental stressors that negatively impact photosynthetic capacity (e.g., low light intensities or foliar damage due to pathogens or insects) decrease the amount of stored carbohydrate, which can lead to increased stalk rot disease severity and early senescence (Campos et al. 2021; Dodd 1980).

One of the most common and consistently damaging maize stalk rot diseases in North America is anthracnose stalk rot (ASR), caused by the fungus *Colletotrichum graminicola* (Ces.) G. W. Wilson (Bergstrom and Nicholson 1999; Crop Protection Network 2021; Munkvold and White 2016). Estimates of yield reductions from ASR by the Corn Disease Working Group and others range between 1 and 5% in the United States annually; industry estimates are as high as 10 to 20% worldwide (Crop Protection Network 2021; Deleon et al. 2021; Frey et al. 2011; Munkvold and White 2016). If lodging occurs, losses can be close to 100%. Annual costs attributed to ASR in the United States and Canada averaged approximately U.S.$420 million, equivalent to U.S.$4.68 per acre (U.S.$11.56 per hectare), between 2012 and 2019 (Crop Protection Network 2021; Mueller et al. 2016, 2020).

C. graminicola is more aggressive to maize than other stalk rot pathogens. In susceptible genotypes, it has the potential to cause significant damage preanthesis (Dodd 1980; Munkvold and White 2016; White et al. 1979). In addition to ASR, *C. graminicola* also causes a leaf blight disease (anthracnose leaf blight [ALB]) that can result in severe seedling injury or death in susceptible genotypes. ALB generally has less of a negative impact on yield than ASR in modern hybrids (Crop Protection Network 2021). The same *C. graminicola* strains can cause disease in leaves or stalks (Nicoli et al. 2016) but expression of host resistance in these two tissue types is not always correlated (Lim and White 1978).

Resistance to ASR becomes less effective postanthesis and during grain-fill (Dodd 1980). Reduced tillage production methods now in

†Corresponding author: L. J. Vaillancourt; vaillan@uky.edu

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use on much of the maize acreage in the United States allow inoculum to increase to levels that can be damaging on more resistant genotypes (Claassen et al. 2018; Lipps 1985, 1988). Greater losses are associated with higher soil moisture and humidity or rainfall, especially when combined with other environmental host stressors, including nutritional deficits, low light intensity, or abnormally high or low temperatures (da Costa et al. 2019; White et al. 1978, 1979). The strong environmental influence on damage due to ASR means that losses can be difficult to predict from season to season. However, we can anticipate that ASR will continue to be a major problem due to increasing levels of plant stress linked to high-intensity monocultures (e.g., higher seeding densities that can result in lower light intensity in the canopy and nutritional or water deficits) and changing climate (e.g., flooding from more intense precipitation events, as well as hotter temperatures) (Cook 2000; Hunjan and Lore 2020; Prasanna et al. 2021). Milder winters associated with climate change in some locations may result in increased survival of *C. graminicola* primary inoculum and greater disease incidence and severity (Hooda et al. 2016). With so many factors potentially affecting its establishment and impact, ASR represents a serious ongoing threat to maize cultivation.

The last comprehensive review of *C. graminicola* and ASR was written more than 20 years ago (Bergstrom and Nicholson 1999). Our goal here is to summarize our current understanding of ASR disease etiology, particularly important new insights that have been revealed by the application of advanced molecular genomic and cytological technologies. We will discuss how this information could contribute to better ASR disease management for the future, and we will also highlight important gaps in our knowledge that remain to be addressed.

Disease Symptoms

C. graminicola can infect maize roots, stalks, leaves, and ears at all crop developmental stages in highly susceptible genotypes (Nankam and Foley 1988; Sukno et al. 2008; Venard and Vaillancourt 2007a, b; Warren and Nicholson 1975; Williams and Willis 1963). However, because the most susceptible germplasm has been eliminated from elite hybrids, disease symptoms are now observed in the field mainly on seedling leaves (ALB) and mature stalks (ASR) (Munkvold and White 2016; Wise et al. 2016). ALB appears first on the lowest leaves of susceptible seedlings early in the season as oval to elongated necrotic lesions, typically with chlorotic halos (Fig. 1A and B). The appearance of the lesions can vary, depending on the host genotype (Nicholson and Warren 1976). Leaf blight reduces photosynthetic area and can result in early senescence of the leaves (Nicholson et al. 1985). Once the canopy closes, ALB becomes less prevalent.

ASR symptoms develop later in the season, initially as internal water-soaking and discoloration of the stalk pith (Fig. 1C), often associated with dark shiny lesions on the rind (Fig. 1D). Though considered a diagnostic feature, rind lesions are not always present even when substantial pith damage exists. Postanthesis, or earlier in more susceptible genotypes, the discoloration proceeds to rotting and disintegration of pith tissues surrounding the vascular strands, resulting in a shredded appearance (Fig. 1E). The rot can significantly reduce yield because it interferes with translocation and grain-fill, and can also weaken the stem, leading to lodging (Fig. 1F). ASR disease severity and yield loss are related to crop growth stage at the time of infection (Keller and Bergstrom 1988; Matiello et al. 2013).

Anthracnose top dieback (ATD) is another common stalk symptom associated with *C. graminicola* infection. Symptoms of ATD include yellowing and premature death at mid- to late grain-fill of the leaves and stem internodes at the top of the plant (Fig. 1G). Dark lesions on the rind of the upper internodes, like those that occur on the lower stalk, are often visible (Fig. 1H). In the presence of rain or high humidity levels, salmon-pink spore masses may be produced on the senescent upper stalks (Robertson 2013) (Fig. 1I).

Distribution and Importance of ASR

The first severe ASR outbreaks were reported in Germany during the 1930s (Böning and Wallner 1936), and in the south of France in the 1940s (Messiaen et al. 1959). In contrast, *C. graminicola* was a minor issue on maize in North America, described as causing only ALB, through the late 1950s (Sprague 1950; Wilson 1914). Maize stalk rots in North America prior to 1960 were attributed mostly to *Stenocarpella maydis* (Diplodia stalk rot [DSR]) or *Fusarium graminearum* (Gibberella stalk rot [GSR]) (Christensen and Wilcoxson 1966; Koehler 1960; Messiaen et al. 1959; Michaelson 1957).

During the late 1950s and early 1960s, *C. graminicola* was recovered from severe stalk infections of maize in Arkansas, particularly in locations experiencing abnormally high humidity (Dale 1963). Affected stalks were often coinfected with *S. maydis* or *F. graminearum*, suggesting that *C. graminicola* was a secondary pathogen. In controlled inoculations, *C. graminicola* colonized maize stalks but did not cause significant lodging or yield loss (Dale 1963). Meanwhile, a severe stalk rot that had occurred for several years on a research farm of The Ohio State University was also found to be associated with *C. graminicola* infection (Williams and Willis 1963). Controlled inoculations of maize with the pathogen in Ohio produced severe stalk symptoms.

By the early 1970s, ASR epidemics were severely impacting maize production in the United States, with significant yield reductions and lodging reported in multiple states including Maryland, North Carolina, Indiana, and Kentucky (Morgan and Kantzes 1971; Naylor and Leonard 1977; Perkins and Hooker 1979; Smith 1976; Warren and Nicholson 1973; Wheeler et al. 1974; White et al. 1979). Losses of up to 20%, due primarily to reduced kernel weight, were reported in controlled field tests with susceptible hybrids (Perkins and Hooker 1979; Smith 1976). The sweet corn canning industry in Indiana experienced total crop failures due to epidemics of ASR during this period (Warren and Nicholson 1973). ASR continued to be common in the United States throughout the 1980s and 1990s, with recorded losses ranging up to 35% in susceptible genotypes and conducive environments (Callaway et al. 1992; Keller et al. 1986).

Currently, ASR remains among the most important diseases of maize in the United States and Canada. Between 2012 and 2016, it ranked in the top five most damaging diseases, together with Fusarium stalk rot (FSR), northern corn leaf blight, gray leaf spot (GLS), and Goss's wilt; from 2016 to 2019, only FSR and GLS caused more losses (Mueller et al. 2016, 2020). Because ASR could be misdiagnosed as FSR if the characteristic external dark rind lesions are absent, it might be even more common and damaging than reported.

Although *C. graminicola* is distributed worldwide (McGee 1988; White et al. 1979), the importance of ASR outside North America varies widely if we judge from the frequency with which outbreaks are reported in the literature. Brazil is the world's second-largest maize producer after the United States. ASR was first reported in Brazil in the 1960s, associated with unusually high humidity and resulting in yield reductions of up to 30% in commercial seed production fields (Silveira et al. 1965). Since then, losses from ASR have grown steadily along with increased adoption of no-till practices and expansion of monocultures (Cota et al. 2012; da Costa et al. 2008; Nazareno 1989). Maize is often double cropped during the summer and winter in Brazil; ASR is especially serious in the winter crop, when plants are more likely to be stressed by frost, drought, nutritional deficiencies, insect damage, and other diseases (Arakaki and Minuzzi 2016; Cardoso et al. 2004; da Costa et al. 2019). Curiously, ASR has not been reported as a significant problem elsewhere in South or Central America, to our knowledge.

More recently, outbreaks of ASR have been reported in Europe (Palaversic et al. 2009), where the disease may be growing in importance. ASR was recently recorded for the first time in Portugal, Switzerland, and Bosnia and Herzegovina (Cuevas-Fernández et al. 2019; Sanz-Martín et al. 2016b; Sukno et al. 2014). *C. graminicola* was described from maize leaves and ears in South Africa in the early 1980s (Baxter et al. 1983) but ASR is not a major issue in South Africa or elsewhere on the continent, where other fungal stalk rots

Fig. 1. Symptoms caused by *Colletotrichum graminicola* on maize. **A,** Anthracnose leaf blight (ALB) lesions on maize seedling. **B,** Closer view of ALB lesion. **C,** Early anthracnose stalk rot (ASR) symptom, internal pith discoloration. **D,** External dark raised lesions, typical of ASR. **E,** Internal rotting and shredding, late symptom of ASR. **F,** Collapse of basal stalk tissues due to ASR which can lead to lodging. **G,** Typical symptoms of anthracnose top dieback (ATD). **H,** External dark lesions on upper stalk of a plant with ATD. **I,** Masses of conidia produced on the upper stalk of a plant with ATD. Image in C photographed by C. Venard; all others by A. Robertson.

(DSR, GSR, and charcoal rot, caused by *Macrophomina phaseolina*) are more frequent. In Asia and Oceania, ASR has not been reported, although ALB was found as early as the 1950s in the Philippines (Quebral 1958) and identified for the first time in China in 2019 (Duan et al. 2019). The presence of *C. graminicola* in Australia has not been confirmed (Perrine-Walker and Anderson 2019; Shivas et al. 2016).

The sudden increases in ASR incidence and severity in the United States and Brazil in the 1960s may have been related to introduction of new, more susceptible hybrids, changes in cultural practices, or particularly conducive weather conditions. The focus on breeding for DSR and GSR resistance in the United States prior to 1960 may have allowed ASR to become more dominant, because resistance to *C. graminicola* is not always correlated with resistance to other stalk rot pathogens (Hooker and White 1976; White 1977). Wheeler et al. (1974) suggested that development of more aggressive strains of the pathogen could have been responsible for the emergence of ASR in the United States. Several studies proposed a role for the European corn borer (ECB), because larvae produced wounds that acted as infection courts and directly transmitted *C. graminicola* while feeding on stalk tissues (Bergstrom et al. 1983; Dale 1963; Keller et al. 1986; Messiaen et al. 1959; Muimba-Kankolongo and Bergstrom 1990; Nyhus 1989). Introduction of transgenic maize containing *Bacillus thuringiensis* (*Bt*) genes encoding insecticidal proteins in the 1990s significantly reduced ECB damage but ASR continues to be a significant problem (Gatch et al. 2002; Hutchison et al. 2010). Without a clear understanding of the factors that contributed to the sudden emergence of ASR as a disease problem in the Americas, we cannot predict how rapidly it will expand or how serious it will become in other parts of the world.

Taxonomy and Host Specificity

There are many inaccuracies in the literature regarding the host range and distribution of *C. graminicola*. Wilson (1914) originally combined multiple earlier names and specimens from a variety of hosts and locations to define *C. graminicola* (Ces.) Wils., based primarily on morphological features that included falcate (sickle-shaped) spores produced in setose acervuli. *C. graminicola*, as initially described by Wilson, was a single cosmopolitan species with a very wide host range among grasses, a concept that was reinforced or expanded by subsequent authors (Bruehl and Dickson 1950; Sprague 1950; von Arx 1957). For example, von Arx (1957) combined 35 additional *Colletotrichum* spp. from a variety of grass hosts into *C. graminicola*. However, Sutton (1980) significantly narrowed the species definition of *C. graminicola* and restricted its host range to *Z. mays*. This agrees with most published inoculation studies that demonstrate specificity to maize, and an inability to infect other living grass hosts (Dale 1963; Jamil and Nicholson 1987; Lebeau 1950; Torres et al. 2014; Venard and Vaillancourt 2007a, b; Williams and Willis 1963; Zwillenberg 1959).

During the last 20 years, the genus *Colletotrichum* has been extensively revised by the application of molecular phylogenetics based on multilocus sequence analysis (Cannon et al. 2012; Hyde et al. 2009). *C. graminicola* is now placed within the monophyletic *C. graminicola* species complex (CGSC), which comprises at least 18 closely related species that collectively infect more than 42 grass hosts (Crouch et al. 2006, 2009a, b, 2014; O'Connell et al. 2012). The CGSC includes other important crop pathogens; for example, *C. sublineola* that causes anthracnose leaf blight and stalk rot of sorghum (*Sorghum bicolor* L.) and *C. falcatum* that causes red rot of sugarcane (*Saccharum officinarum* L.). The taxonomic history and biology of the CGSC has been reviewed recently (Crouch and Beirn 2009).

The improved taxonomy of *C. graminicola* and its close relatives has supported Sutton's narrower species definition and made it clear that *C. graminicola* is incapable of infecting living hosts other than *Z. mays*. The few references that suggest an ability to infect other grass species seem to be due either to cases of mistaken identity (Chowdhury 1936), or they were growth chamber studies that included inoculation methods that strongly favored disease development and decreased effectiveness of host defense (e.g., high inoculum pressure, highly susceptible host genotypes, or extended moist or dark periods) (Koehler 1943; Wheeler et al. 1974). Some CGSC members, including *C. graminicola*, are capable of colonizing defensively compromised or senescing nonhost monocot tissues in laboratory and greenhouse settings (Buiate et al. 2017; Jamil and Nicholson 1987; Torres et al. 2014; L. J. Vaillancourt, unpublished data). Moreover, *C. graminicola* was isolated from lesions on senescing older sorghum leaves in a field in Pennsylvania that had been under a maize-sorghum rotation (Gaffoor et al. 2021). The capacity for cross-infection of senescing nonhost tissues deserves further study because it has important implications for the use of crop rotations and weed control in management of ASR and ALB.

Pathogen Center of Origin

The origin of *C. graminicola* as a pathogen of maize is unknown but recent molecular genetic analyses have provided some clues. Genome comparisons among a small number of strains from around the world indicate that they are very similar (>95% identity), suggesting a relatively recent origin or founder event (Buiate et al. 2017; Rech et al. 2014; L. J. Vaillancourt, unpublished data). Polymorphism among strains seems to be concentrated in dispensable and highly variable minichromosomes that include a high percentage of repetitive DNA (O'Connell et al. 2012; Rollins 1996).

The genus *Zea* is native to Central America. A taxon within teosinte, *Z. mays* subsp. *parviglumis*, is thought to have given rise to the progenitor of domestic maize as recently as 9,000 to 10,000 years ago (Bennetzen et al. 2001; Buckler and Stevens 2006; Ross-Ibarra et al. 2009). If *C. graminicola* coevolved with *Zea* spp., then its center of diversity should be in the central Mexican highlands. Unfortunately, little has been published about *C. graminicola* from this region.

Interestingly, molecular phylogenetic analysis indicated that *Colletotrichum* spp. from dallisgrass (*Paspalum dilitatum*) and bahiagrass (*P. notatum*) are close relatives of *C. graminicola* (Crouch et al. 2009a). Both grasses are native to South America and only distantly related to maize. This suggests an alternative hypothesis, that *C. graminicola* arose after the domestication of maize, by either hybridization or a host jump. It was recently reported that South America is a secondary domestication center for maize; a lineage developed there in isolation from other members of genus *Zea* for about 6,000 years (Kistler et al. 2018). Maize hybrids currently grown in North America contain very little germplasm derived from this South American lineage but it is found in landraces that are still grown in Brazil (Kistler et al. 2018). There are at least five races of *C. graminicola* in Brazil based on differential responses of five maize hybrids but there was no evidence for races among nearly 100 representative U.S. isolates when these were used to inoculate the same hybrids (da Costa et al. 2014; D. F. Parreira and L. J. Vaillancourt, unpublished data). Moreover, the U.S. population of *C. graminicola* is genetically less diverse than the population in Brazil (Nicholson and Warren 1981; Parreira et al. 2016; D. F. Parreira and L. J. Vaillancourt, unpublished data). Additional surveys and studies of the population genetics and genomics of the pathogen worldwide are urgently needed to determine whether its center of origin is indeed in South America. If so, South American maize germplasm might include novel sources of resistance to ASR.

Disease Cycle

C. graminicola overwinters on infested crop debris in the field (Figs. 2A and 3A). The fungus can survive on surface debris for at least 20 months (Jirak-Peterson and Esker 2011; Lipps 1983) but it is a relatively poor competitor in the soil; burying the debris significantly reduced primary inoculum and ALB or ASR incidence and severity during the following growing season (Jirak-Peterson and Esker 2011; Lipps 1985; Naylor and Leonard 1977; Williams and Willis 1963). Reduced tillage and continuous cropping of maize

are major factors contributing to increased ASR incidence and severity (Jirak-Peterson and Esker 2011; Munkvold and White 2016).

Primary inoculum is produced in acervuli on the surface of the infested debris during periods of high humidity or rain (at least 10 to 12 h at 100% relative humidity) (Figs. 2A and 3A) (Bergstrom and Nicholson 1999). Acervuli can be recognized with a hand lens by the presence of darkly pigmented whisker-like setae among the masses of spores, which are produced in a salmon-pink, mucilaginous matrix (Fig. 3B). The uninucleate aseptate falcate spores are formed on short phialides in response to light (Fig. 3C) (Panaccione et al. 1989; Yang et al. 1991). The setae capture water droplets that dissolve the mucilaginous matrix, facilitating dispersal (Ramadoss et al. 1985). The matrix is chemically complex, composed largely of polysaccharide, and protects the spores from desiccation and other stresses (Epstein and Nicholson 1997; Nicholson et al. 1989; Ramadoss et al. 1985). It contains the germination self-inhibitor mycosporine-alanine (Leite and Nicholson 1992) as well as proline-rich proteins that can bind toxic plant phenolic esters and glycosides (Nicholson et al. 1986, 1989).

Falcate spores are spread primarily by splashing or wind-blown water droplets. Spores that land on foliar tissues of nearby seedlings (Fig. 2B and C) adhere to them by a combination of a preformed

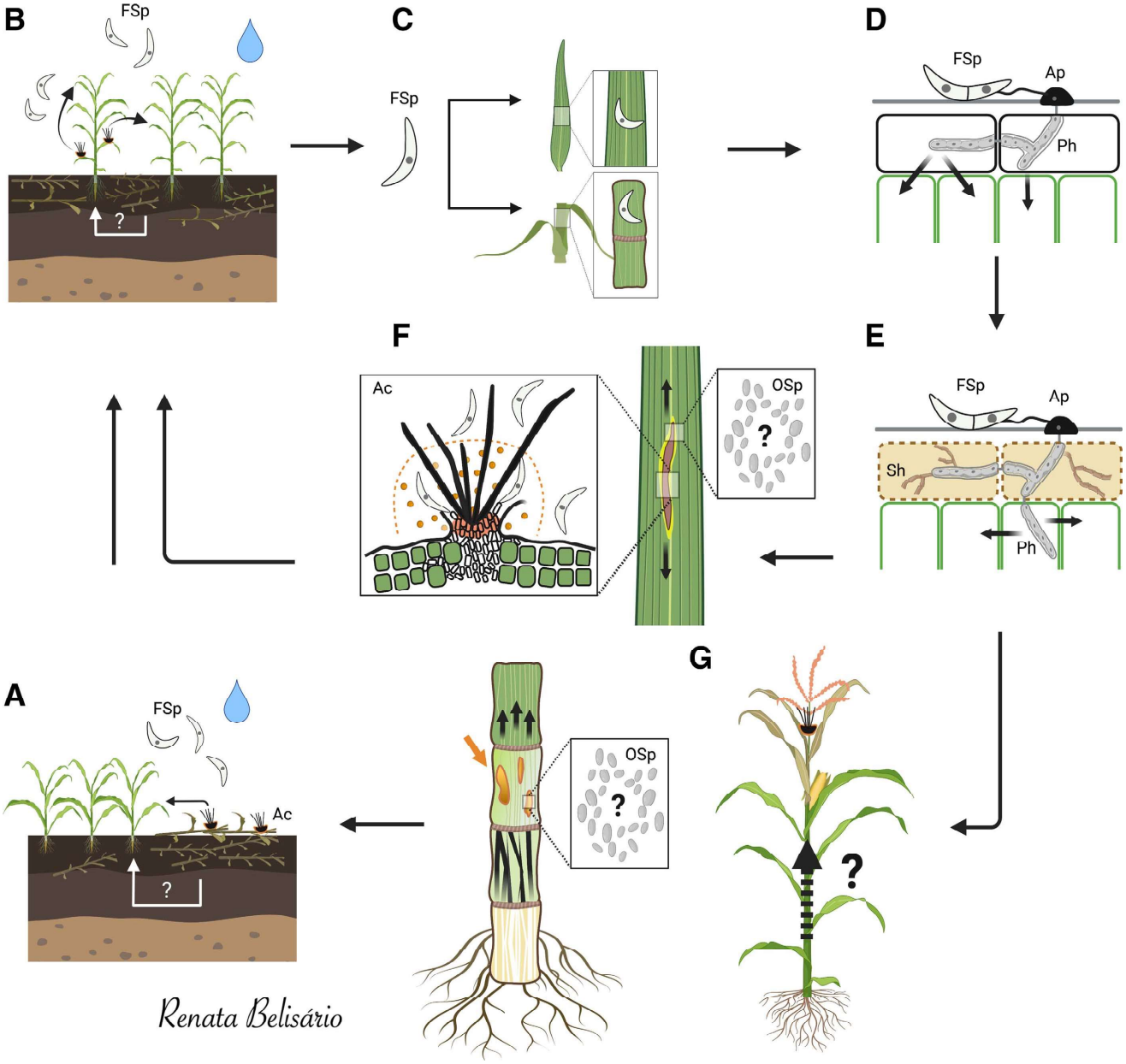

Fig. 2. Disease cycle of *Colletotrichum graminicola* on maize. **A,** The pathogen overwinters on crop debris on the soil surface; in the spring, acervuli are produced, and falcate spores are splashed by rain onto emerging seedlings. Buried inoculum may infect roots. **B,** Inoculum produced on the foliage of infected plants is spread by splashing rain within and between plants in a secondary cycle. **C,** Falcate spores (FSp) adhere to foliar surfaces (leaf or stalk) and **D,** germinate to produce a melanized, dome-shaped appressorium (Ap). The pathogen invades the host cells from the appressorium via a narrow infection peg. Once inside the cells, the fungus grows from cell to cell biotrophically as bulbous primary hyphae (Ph). **E,** About 48 to 72 h after infection, narrower secondary necrotrophic hyphae (Sh) emerge from the primary hyphae in the dead cells behind the advancing edge of the colony. At this point, **F,** necrotic lesions are produced and acervuli (Ac) containing falcate spores form. **G,** Lesions on leaves and in stalks expand longitudinally via the vascular bundles, or stalk rind. Oval spores (OSp) are produced in parenchyma cells in both leaf and stem lesions but their role is not clear. The fungus can emerge from the bundles or rind to produce discontinuous secondary lesions in the stalk (arrows). Stalk lesions ultimately progress to tissue degradation and rotted cavities that weaken the stem and interrupt the flow of water and nutrients to the upper plant, possibly leading to anthracnose top dieback symptoms. Secondary sporulation can occur on senescent stalks. Infested stalk and leaf debris left on the soil surface gives rise to primary inoculum in the spring, completing the disease cycle. Created with BioRender.com.

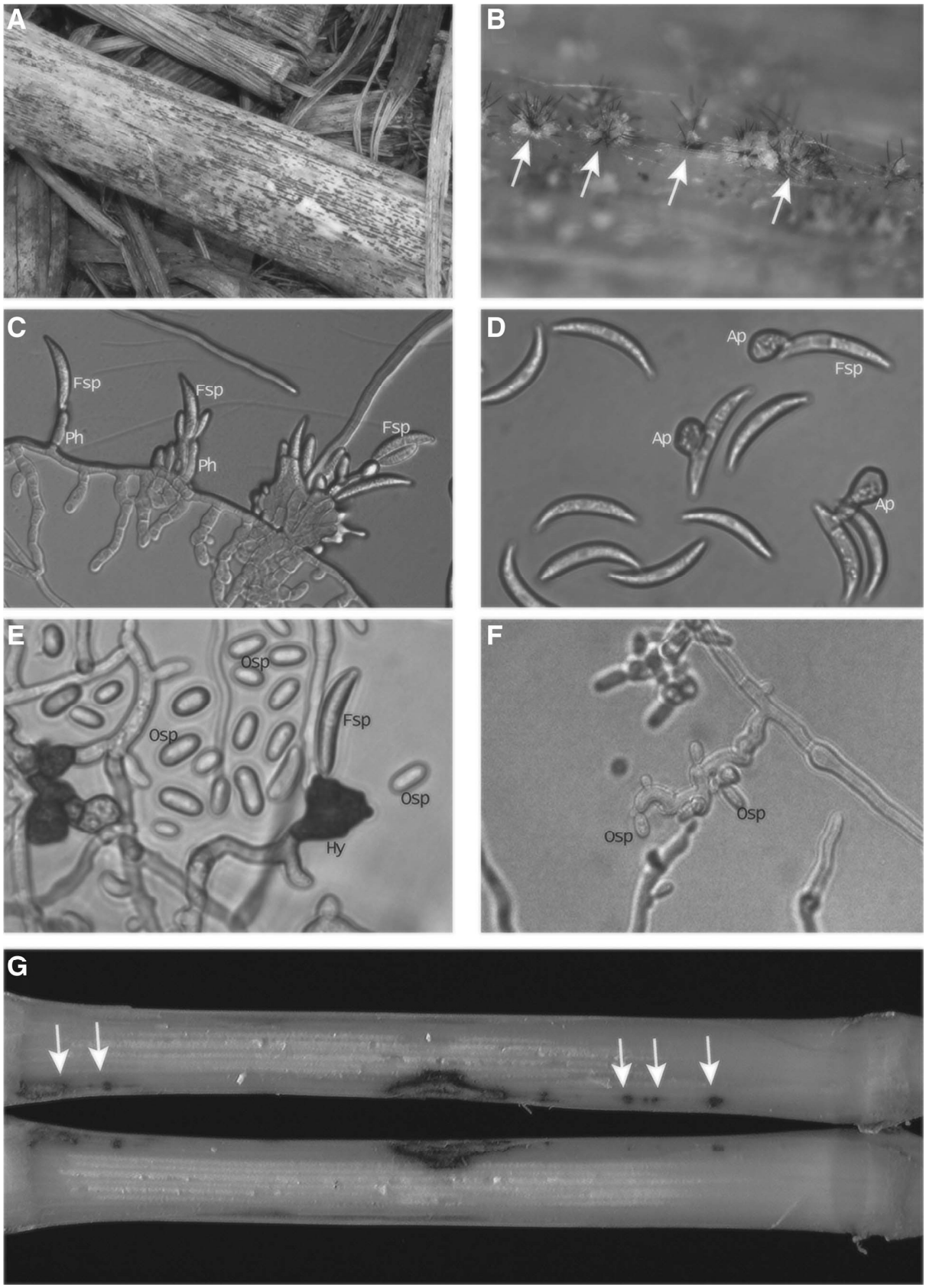

Fig. 3. Disease cycle of *Colletotrichum graminicola*. **A,** Fungus overwinters on surface debris and **B,** produces setose acervuli on the surface of the tissue (arrows). **C,** Falcate spores (FSp) embedded in a mucilagenous matrix are produced in the acervuli from phialides (Ph). **D,** Falcate spores germinate and produce melanized appressoria (Ap) on the plant epidermal surface. **E,** Oval spores (OSp) are produced in submerged cultures. They germinate and produce vegetative hyphal networks that can produce hyphopodia (Hy) capable of infection. **F,** Oval spores are produced from small pegs on specialized conidiogenous mycelia and not in an acervulus. **G,** Discontinuous secondary lesions (arrows) can be produced in stalks at a distance from the primary lesion. Images A and B are by A. Robertson; other images are by C. Venard.

adhesive matrix and active mechanisms in response to hydrophobicity and chemical signals (Chaky et al. 2001; Epstein and Nicholson 1997, 2006; Mercure et al. 1994; Nicholson and Epstein 1991; Sugui et al. 1998). Adhesion to a hydrophobic surface in the presence of free water is essential for germination of the falcate spores in the absence of external nutrients (Chaky et al. 2001; Mercure et al. 1994; Nicholson and Moraes 1980).

The first observable sign of germination is nuclear division and septation, followed by the production of one or occasionally two germ tubes (Fig. 2D). On a hydrophobic surface such as the maize epidermis, germ tubes produce melanized, dome-shaped infection structures called appressoria (Figs. 2D to E and 3D) (Chaky et al. 2001; Mims and Vaillancourt 2002; Politis and Wheeler 1973). Vegetative hyphae can form similar structures known as hyphopodia; thus, mycelia can also be infectious on intact plant surfaces (Fig. 3E) (Du et al. 2005; Sukno et al. 2008; Werner et al. 2007). Appressoria accumulate significant turgor pressures, up to 5 MPa, facilitating the physical breach of the host cell wall via narrow penetration pegs (Bechinger et al. 1999), which is followed by colonization of host cells by infection hyphae (Fig. 2D to E). Although spore germination occurs across a wide range of temperatures (15 to 35°C), efficient penetration is limited to between 25 and 30°C (Skoropad 1967). *C. graminicola* can also invade wounded tissues without forming an appressorium. In this case, it appears that germination occurs in response to chemical or nutritional cues produced by the damaged cells; falcate spores germinate in vitro in the absence of an inductive surface if they are exposed to glucose (Chaky et al. 2001).

Mutations in class I, III, and V chitin synthase genes, or in genes encoding melanin biosynthetic enzymes, prevent the production of functional appressoria, indicating that wall rigidity and structure are important for direct invasion (Ludwig et al. 2014; Rasmussen and Hanau 1989; Werner et al. 2007). In the rice blast pathogen *Magnaporthe oryzae*, which also produces melanized appressoria, melanin plays a critical role in accumulation of glycerol to generate turgor pressure (Foster et al. 2017). However, in *C. graminicola*, melanin is not essential for turgor; osmotic potentials were similar in wild-type appressoria and in those produced by melanin-deficient mutants, and mutant appressoria were still capable of supporting penetration of artificial membranes, although they failed to produce successful infections in planta (Ludwig et al. 2014). Melanin may have other important functions in *C. graminicola*, including protection of the appressoria and directing penetration by guiding secretory activity to the infection site (Ludwig et al. 2014).

Foliar lesions become visible a few days after fungal infection and expand longitudinally, especially along the midrib (Fig. 2F). They are larger in more susceptible host genotypes, on senescing leaves, or under low light (Hammerschmidt and Nicholson 1977; Jenns and Leonard 1985; Schall et al. 1980). Acervuli and falcate spores are produced in necrotic lesion centers during periods of high humidity or rain (at least 10 to 12 h at 100% relative humidity) (Bergstrom and Nicholson 1999) (Fig. 2F). Spores are subsequently dispersed to the leaves, leaf sheaths, and stalks of neighboring plants within the same growing season, initiating a secondary infection cycle.

C. graminicola produces a second type of asexual spore that is oval and smaller than the falcate spores (Panaccione et al. 1989; Nishihara 1975; Nordzieke et al. 2019; Venard et al. 2008). Oval spores are produced in abundance in submerged cultures (Fig. 3E) and have also been observed inside foliar and stalk tissues (Fig. 2F and G), as well as in root epidermal cells and root hairs (Nordzieke et al. 2019; Panaccione et al. 1989; Sukno et al. 2008; Venard and Vaillancourt 2007b; Venard et al. 2008). Unlike falcate spores, oval spores do not require light for induction. Moreover, they are not produced from phialides in acervuli, instead originating from short pegs on individual hyphae that appear to be developmentally committed for their production (Fig. 3F) (Panaccione et al. 1989; Venard et al. 2008; C. M. Venard and L. J. Vaillancourt, unpublished data). Oval spores are infectious when applied to aerial tissues; however, their germination does not depend on surface exposure or nutrient content, and they do not form mycosporine-amino acids (Chaky et al. 2001; Nordzieke et al. 2019).

Germinated oval spores readily form fused germling networks that appear to be important for production of hyphopodia and for infection and colonization. The absence of network formation results in less severe symptoms (Nordzieke et al. 2019). A mutation in *Strl*, an ortholog of the calmodulin-binding protein striatin, produced defects in hyphal fusion and delayed infection, suggesting a function for calcium signaling in this process (Wang et al. 2016). The precise role of oval spores in the disease cycle is not clear. However, because they remain inside the host tissues, they probably facilitate pathogenic colonization within plants, as opposed to secondary spread between plants.

The perithecial sexual form of *C. graminicola* has been produced in the laboratory where it can be used for genetic analysis (Politis and Wheeler 1972; Vaillancourt and Hanau 1991; Vaillancourt et al. 2000) but it appears to be rare or absent in the field and, consequently, is not thought to play a significant role in the ASR or ALB disease cycles.

Host Colonization: Hemibiotrophy

Like other *Colletotrichum* spp., *C. graminicola* is a hemibiotroph that initially invades living host cells and then shifts to necrotrophic colonization of dead plant tissues. Bergstrom and Nicholson (1999) described hemibiotrophic development of *C. graminicola* as similar to the better studied *C. lindemuthianum*, in which the fungus makes a complete switch to necrotrophy after several days of biotrophic development and begins to kill host cells at the colony margins in advance of its growth. However, more recent work, including live-cell imaging and transgenic strains expressing fluorescent proteins (Behr et al. 2010; Mims and Vaillancourt 2002; Münch et al. 2008; O'Connell et al. 2012; Torres et al. 2014; Venard and Vaillancourt 2007a, b), indicate that *C. graminicola* is more like its close relative *C. sublineola* (Wharton and Julian 1996; Wharton et al. 2001). These two species undergo a hemibiotrophic process that has been called sequential biotrophy (SB) (Crouch et al. 2014; O'Connell et al. 2012).

In SB, living cells at colony margins are invaded biotrophically by primary hyphae, then die after approximately 12 to 24 h (Fig. 4). Necrotrophic secondary hyphae develop in the dead and dying cells, at the same time as primary hyphae continue to invade living cells at the colony margins. Thus, biotrophic and necrotrophic lifestyles coexist in different parts of the fungal colony and the fungus is truly biotrophic only in the newly invaded cells at the colony edges (Behr et al. 2010; Mims and Vaillancourt 2002; Münch et al. 2008; O'Connell et al. 2012; Torres et al. 2014).

The biotrophic primary hyphae of *C. graminicola* are thick, multinucleate, irregular in shape, and surrounded by a membrane that

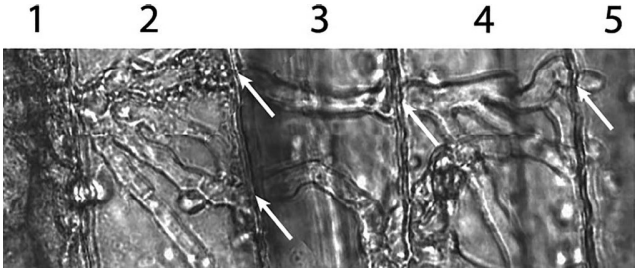

Fig. 4. *Colletotrichum graminicola* primary hyphae colonizing five maize epidermal cells over the course of 36 h. The fungus initially entered the cell on the far left (1) and progressed from cell to cell via narrow hyphal connections (arrows), finally entering the cell on the far right (5), which is the only cell that is still alive. The rest of the cells (1 to 4) are already dead or dying, based on evidence from plasmolysis assays (Torres et al. 2014). Granulation can be seen in the first two cells that were invaded (1 and 2). In the first (1), the entire cell has been filled and, in the second (2), the granules are mostly associated with the hyphae. These granules may be a defensive response of the plant, akin to hypersensitive resistance. If so, the response was apparently too slow to stop pathogen growth. Micrograph by E. Buiate.

separates them from the cytoplasm of the living host cells (Figs. 4 and 5A) (Behr et al. 2010; Mims and Vaillancourt 2002; Torres et al. 2014; Venard and Vaillancourt 2007a, b). They do not extensively degrade host cell walls; instead, they pass from one cell to another via narrow connections (Figs. 4 and 5A) like those that have been observed in *M. oryzae* traversing plasmodesmata (Kankanala et al. 2007).

Secondary hyphae are thinner than the primary hyphae, mononucleate, and lack a surrounding membrane (Fig. 5B) (Mims and Vaillancourt 2002; Torres et al. 2014; Venard and Vaillancourt 2007a, b). Plant tissue destruction follows the emergence of the secondary hyphae that secrete large quantities of lytic enzymes in the dead cells behind the advancing colony margin (Crouch et al. 2014; Mims and Vaillancourt 2002; Münch et al. 2008; O'Connell et al. 2012; Torres et al. 2014; Venard and Vaillancourt 2007a, b). Cells in the centers of the lesions collapse even if they do not contain hyphae, typical of necrotrophic development (Behr et al. 2010; Mims and Vaillancourt 2002).

Lesion formation and production of acervuli occur only after tissue collapse during necrotrophy but the biotrophic phase is a

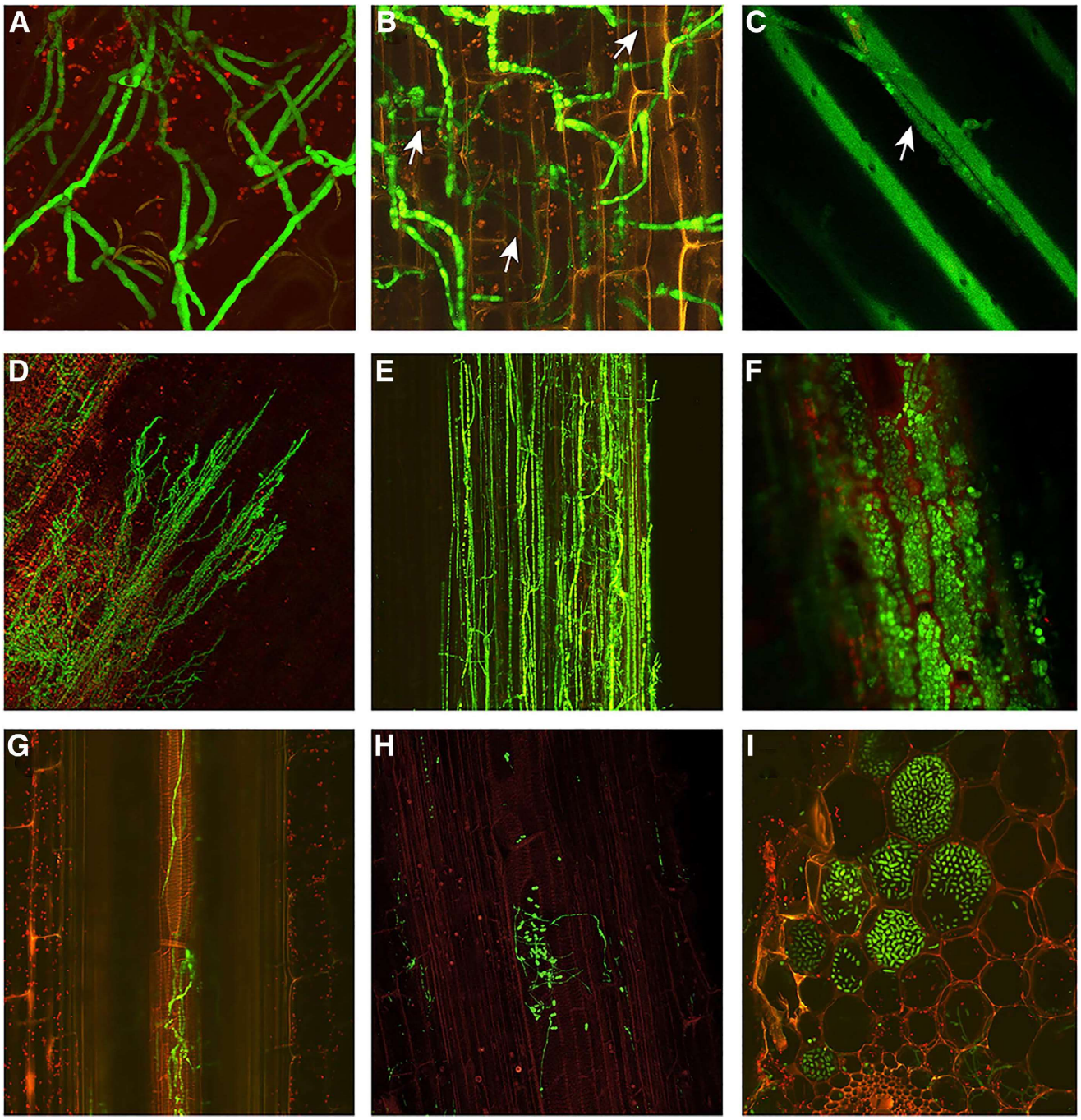

Fig. 5. Infection of maize stalks and leaves by *Colletotrichum graminicola* expressing green fluorescent protein. **A,** Growth of primary hyphae from cell to cell in stalk pith parenchyma. Note intact cell walls and passage through walls via very narrow connections. **B,** Production of thinner secondary hyphae (arrows) from primary hyphae in a pith lesion behind the advancing margin of the lesion. **C,** Hypha (arrow) inside a stalk bundle fiber cell. **D,** Colonization of bundle fibers and bundle sheath of leaf midrib, longitudinal lesion expansion. **E,** Heavy colonization of rind fibers. **F,** Dark lesions in epidermal cells are due to fungal stromata. **G,** Hyphae in xylem vessel of stalk bundle. **H,** Oval spores inside a xylem vessel of stalk bundle. **I,** Dormant oval spores accumulating in pith parenchyma cells. All micrographs courtesy of Corteva.

prerequisite for *Colletotrichum* spp. to establish infection in living host tissues (Münch et al. 2008; Pellier et al. 2003; Stephenson et al. 2000; Torres et al. 2014). Cytological evidence has established that intact stalk pith parenchyma cells at colony margins are also invaded biotrophically, even if the fungus initially enters the stalks via wounds (Tang et al. 2006; Venard and Vaillancourt 2007a, b). The pattern of biotrophic and necrotrophic growth of *C. graminicola* in stalks is like that in foliar tissues (Venard and Vaillancourt 2007a, b). Thus, studies of SB in the experimentally more tractable ALB pathosystem are relevant to understanding of ASR.

Host Colonization: Systemic Infection

Studies in which spores were placed behind leaf sheaths suggested that the intact rind is an effective barrier to penetration because the levels of ASR that resulted were low (Bergstrom et al. 1983; White and Humy 1976). Wounding the rind significantly increases the speed and efficiency of infection (Keller et al. 1986) but the fungus is also capable of penetrating unwounded stalks by passing through the pits in the intact rind fiber cells (Venard and Vaillancourt 2007b). Discolored lesions that are correlated in size with fungal biomass are produced in the pith parenchyma and extend longitudinally from the point of stalk infection (Fig. 2G) (Muimba-Kankolongo and Bergstrom 2011). New secondary lesions can form at a distance from the primary infection in the same or different internodes (Figs. 2G and 3G) (Venard and Vaillancourt 2007a). Pathogen spread into the upper stem from the basal internodes, together with interruption of water supply due to degradation of the lower stem, may result in ATD; earlier ATD symptoms are typically associated with the earlier onset and presence of more severe basal ASR symptoms (A. E. Robertson, unpublished data).

Bergstrom and Nicholson (1999) proposed that *C. graminicola* hyphae colonized maize tissues systemically via the xylem, and some have called *C. graminicola* a wilt pathogen, especially in relation to ATD (Smith and White 1988; White et al. 1979). True wilt fungi target and occupy the xylem, blocking the flow of water and nutrients. *C. graminicola* can be readily recovered from individual vascular bundles isolated from infected maize stalks (Bergstrom and Nicholson 1999; Sukno et al. 2008). However, recent cytological studies reveal that the primary route of colonization for *C. graminicola* in stalk bundles is via thick, straight hyphae through the mostly nonliving fiber cells surrounding the xylem and phloem (Figs. 5C and 6) (Venard and Vaillancourt 2007a). In leaves, the fungus enters the bundle sheath and fibers associated with veins and grows from cell to cell longitudinally along the leaf blade (Fig. 5D) (Behr et al. 2010; Mims and Vaillancourt 2002; Venard and Vaillancourt 2007b). *C. graminicola* extensively colonizes the rind fibers (Fig. 5E) (Venard and Vaillancourt 2007a). The black rind lesions often associated with ASR consist of stromata that eventually form from hyphae accumulating within the rind epidermis (Fig. 5F). Fungal hyphae have been observed emerging from the fiber cells in the rind or the vascular bundles to form new biotrophic infection foci (Tang et al. 2006; Venard and Vaillancourt 2007a, b; Venard et al. 2008). Hyphae can be seen in xylem and phloem but usually relatively late in the process of colonization, after the associated pith tissues have rotted and bundles have become disrupted and broken (Fig. 5G) (Venard and Vaillancourt 2007a).

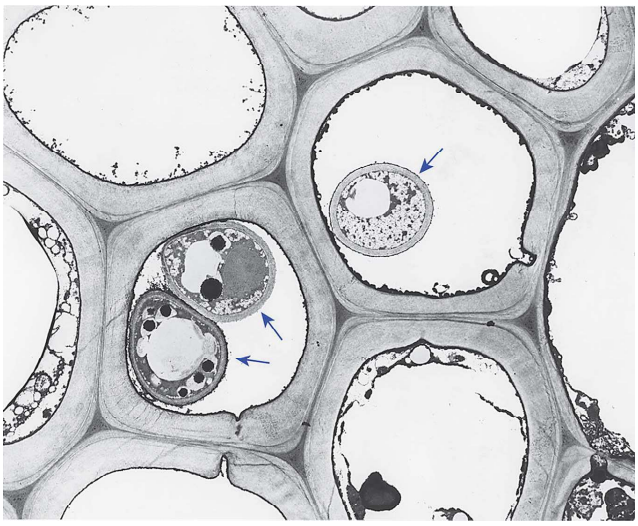

Fig. 6. Transmission electron micrography image cross-section, showing *Colletotrichum graminicola* hyphae (arrows) inside stalk bundle fiber cells. Micrograph courtesy of Corteva.

Due in part to their resemblance to microconidia of some wilt fungi (e.g., *Ophiostoma ulmi*) (D'Arcy 2000), oval spores produced by *C. graminicola* were proposed to facilitate systemic spread via the xylem (Bergstrom and Nicholson 1999; Nordzieke et al. 2019; Panaccione et al. 1989). Oval spores can be observed in xylem vessels (Fig. 5H) but are seen much more often accumulating in large numbers within the parenchyma cells in stalks and leaves (Fig. 5I) (Venard et al. 2008). They appear to be dormant in these cells, because they were observed to germinate only very rarely, and then only in tissues that were already degraded and rotted (C. M. Venard and L. J. Vaillancourt, unpublished data). Because oval spores are highly efficient in producing mycelial networks (Nordzieke et al. 2019), they may utilize the substrate more effectively during necrotrophy, or facilitate rapid colonization of the debris after overwintering.

Evidence supporting *C. graminicola* as a true wilt pathogen that specifically targets the xylem is not strong, although the fungus clearly uses the vascular bundles for lesion expansion in stalks and leaves. Preliminary research suggests that ATD symptoms are associated with internal stalk rot and are likely to result from destruction of the transpiration stream due to damage to vascular bundles (Munkvold 2002; A. E. Robertson, unpublished data). However, more research on the etiology and economic importance of ATD is needed. It has been reported that *C. graminicola* can infect maize seedlings and then remain dormant until becoming active later in the season to cause symptoms of ASR and ATD (Munkvold 2002). We speculate that this dormancy may take the form of asymptomatic colonization of the rind and the bundle fibers. The details of colonization and movement by *C. graminicola* in maize vascular bundles remain unclear; given its potential importance in the disease cycle, this topic deserves further study.

Genetics and Physiology of the Host–Pathogen Interaction

Penetration and the establishment of biotrophy, and the switch from biotrophy to necrotrophy, are critical steps in determining success of *C. graminicola* as a pathogen. The availability of a high-quality genome sequence (O'Connell et al. 2012) has led to significant recent advances in our understanding of these processes in *C. graminicola*. The fungal genome is predicted to encode more than 12,000 genes (O'Connell et al. 2012; Schliebner et al. 2014). At least 20% of the genes are differentially expressed (DE), transcribed in "waves" across the different phases of development in planta, including prepenetration and mature appressoria, initial biotrophic establishment in epidermal cells and cell-to-cell movement during SB, the switch to necrotrophic growth and production of secondary hyphae, and the lytic phase characterized by host-cell degradation and lesion formation (O'Connell et al. 2012; Torres et al. 2016). Genes predicted to encode secreted and membrane-localized proteins are overrepresented among the DE group and include many that are unique to *C. graminicola*, suggesting distinct roles in specific aspects of host–pathogen signaling and recognition (Buiate et al. 2017; O'Connell et al. 2012; Torres et al. 2016). There are at least 15 DE transcription factors that may regulate the transition between developmental phases (Torres et al. 2016).

Prior to host penetration and during infection and initial biotrophic establishment in the epidermal cells, *C. graminicola* relies on stored nutrients from the spore. Genes encoding glyoxylate cycle enzymes

are upregulated during these early phases, indicating utilization of lipids as a primary source of carbon (Torres et al. 2016). Purine catabolism pathway genes *URE1* and *ALA1* were expressed during appressorial development and were required for infection in the absence of an exogenous nitrogen source, suggesting that conversion of purines to ammonium via uric acid provides an essential source of nitrogen during early infection (Perino et al. 2020). Expression of stress response genes indicates that the fungus is experiencing oxidative and other stresses at these early time points (Torres et al. 2016).

During subsequent colony expansion and cell-to-cell growth of the primary infection hyphae, expression of genes encoding hydrolase enzymes and nutrient transporters increases significantly, indicating that the pathogen switches to utilization of amino acids and sugars obtained from killed plant tissues for its nutritional needs. Expression of genes encoding acid phosphatases and phytases, siderophores, and aryl sulfatase suggests that phosphorus, iron, and sulfur are also acquired from the host during these stages of infection (Albarouki et al. 2014; Tang et al. 2006; Torres et al. 2016). The uptake and sequestering of these important minerals may provide essential nutrients for the pathogen or subvert normal immune function in the host (Albarouki et al. 2014; Tang et al. 2006).

An increase in expression of secreted proteases, including homologs of some implicated in the inhibition of plant defense proteins by other fungi, indicates that *C. graminicola* is actively targeting maize defenses during SB (Torres et al. 2016). The biotrophic hyphae evade recognition by host pathogen-associated molecular pattern (PAMP) receptors by manipulating wall polysaccharides, including converting chitin to chitosan via activity of chitin deacetylases, and downregulating β-1,3-glucan synthase, thus reducing the content of defense-triggering β-1,3-glucan (Oliveira-Garcia and Deising 2013; Torres et al. 2016). An array of lytic enzymes targeting the plant cell walls are strongly upregulated once secondary hyphae develop, leading to host tissue collapse and lesion formation (O'Connell et al. 2012; Torres et al. 2016).

It was previously suggested that the biotrophic primary infection hyphae of *C. graminicola* function primarily in nutrient uptake (Bergstrom and Nicholson 1999). We now know that a major role of these hyphae is to produce an array of small, secreted protein effectors and secondary metabolites (SM) that suppress plant defenses and cell death, and foster host compatibility necessary for the establishment of biotrophy (Fig. 7) (Kleemann et al. 2012; O'Connell et al. 2012; Torres et al. 2016). Cytological studies utilizing a signal peptidase mutant (Thon et al. 2002) expressing green fluorescent protein (GFP) coinoculated with the wild-type fungus expressing red fluorescent protein suggested that unknown secreted fungal factors promote compatibility in the uninvaded host cells beyond the edge of the

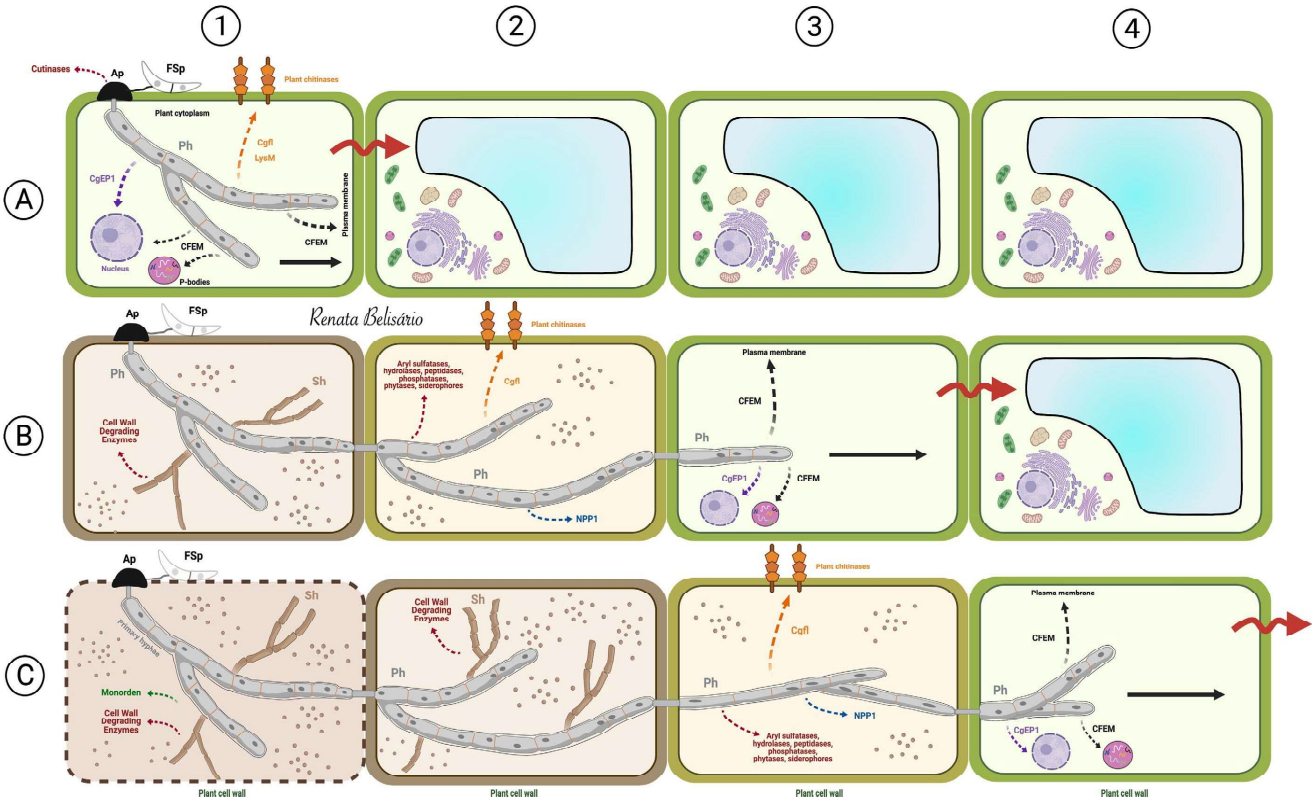

Fig. 7. Hemibiotrophic infection of maize by *Colletotrichum graminicola*. **A,** In the first stage of infection, a falcate spore (FSp) has attached to the host surface and germinated to produce a dome-shaped melanized appressorium (Ap). Prior to penetration, the fungus expresses genes for cutinases and a subset of unique effectors and secondary metabolites that may play roles in preparing the host for invasion. Penetration occurs via a narrow penetration hypha that emerges from the base of the appressorium. Once inside the living plant cell, the fungus produces bulbous primary hyphae (Ph) that are separated from the host cytoplasm by a membrane. Biotrophic primary hyphae produce effectors that include common in-fungal extracellular membrane (CFEM) classes that target various cellular compartments, as well as the characterized nuclear localization signal effector *CgEP1*, and the metallopeptidase *Cgfl* that may target host chitinases. These molecules probably function to facilitate biotrophic invasion and to prepare adjacent cells for invasion (red arrow) by targeting host defenses and engineering the host cell to accommodate the primary hyphae. **B,** In the next stage of sequential biotrophy, the primary hyphae grow cell to cell via narrow connections that may traverse plasmodesmata. At the edge of the colony (cell 3), the hyphae continue to invade living cells biotrophically but, behind that (cell 2), the intercalary primary hyphae begin to produce molecules that indicate that they are utilizing host nutrients and minerals (sulfur, iron, and phosphorus). They also produce classes of effectors (e.g., the NPP1 family) that are known to induce host cell death. Cytological analysis indicates that these cells are already dead or dying. In the dead cells (cell 1), narrower secondary hyphae (Sh) are produced as branches from the primary hyphae, and these begin to produce various classes of cell-wall degrading enzymes (CWDE). **C,** In this final stage of infection, the earliest invaded cells (cell 1) begin to collapse, and the walls disintegrate due to the activity of the CWDE produced by the secondary hyphae. The fungus is completely necrotrophic in this region of the colony, and adjacent cells are destroyed even if not occupied by hyphae. At this stage, visible necrotic lesions develop. Specialized effectors and secondary metabolites are also produced, including monorden, which may function in induction of cell death in the lesion or, alternatively, may protect the dead tissue from competitors. Meanwhile, the fungus continues to invade living host cells biotrophically at the edge of the colony (cell 4). Created with BioRender.com.

lesion, enabling cell-to-cell progression of the primary hyphae during SB (Torres et al. 2014).

C. graminicola encodes at least 40 predicted SM clusters (Buiate et al. 2017; O'Connell et al. 2012; Torres et al. 2016). Mutants lacking the key SM regulator 4′phosphopantetheinyl-transferase were nonpathogenic, establishing the general importance of SM for compatibility (Horbach et al. 2009). *C. graminicola* also encodes hundreds of small secreted proteins (SSPs) with potential effector activity (Buiate et al. 2017; O'Connell et al. 2012; Torres et al. 2016). These include homologs of many known fungal effectors and effector classes, including BAS2 and BAS3 from *M. oryzae* (Mosquera et al. 2009), and LysM-domain chitin-binding proteins that have been functionally characterized in other *Colletotrichum* fungi (Takahara et al. 2016). Many other SSP and SM genes seem to be unique to *C. graminicola* (Buiate et al. 2017).

Subsets of SM and SSP genes are transcribed at different stages of hemibiotrophic development, with some appearing only early before or during penetration, some produced only in the appressoria or in biotrophic hyphae in newly invaded living epidermal cells, and others not expressed until late in the infection during or just after the switch to necrotrophy (O'Connell et al. 2012; Torres et al. 2016). Effectors unique to *C. graminicola* are enriched during early infection and include classes that are likely to target host defense responses—for example, cysteine-rich or common in fungal extracellular membrane (CFEM) domain proteins—while late effectors include members of the *NPP1* family and other proteins that are likely to induce programmed cell death (Fellbrich et al. 2002; Torres et al. 2016). This suggests that *C. graminicola* first suppresses and then induces host cell death during hemibiotrophic infection. In older leaves that have already initiated senescence, the fungus can manipulate host cytokinins to delay cell death and maintain photosynthetic activity to produce green islands. Host invertase levels are significantly upregulated in these green islands to produce carbon sinks that deliver more sugars to the fungus at the expense of the plant (Behr et al. 2010, 2012).

Only a few of the putative secreted protein effectors or SM of *C. graminicola* have been characterized in detail (Fig. 7). An analysis of 10 predicted secreted effectors with CFEM domains revealed that they targeted multiple locations in the plant cell, including the plasma membrane, nucleus, and cytoplasmic bodies (Gong et al. 2020). Five of the 10 also suppressed plant cell death in a *Nicotiana benthamiana* heterologous assay (Gong et al. 2020).

Eisermann et al. (2019) deleted 26 individual candidate secreted effector genes and seven gene clusters (for a total of 32 genes) and found that only two of these genes (GLRG_04686, also known as *CLU5a*, and GLRG_04689, also known as *CLU5d*), both residing in the same gene cluster, were essential for pathogenicity. Deletion mutants did not produce functional appressoria. These two proteins are highly conserved in fungi but otherwise have no recognizable functional motifs.

Twenty-seven putative *C. graminicola* effector genes encoding nuclear localization signals were identified and one of these (GLRG_04079, also known as *CgEP1*) was functionally characterized (Vargas et al. 2016). The effector was produced early during infection and was essential for pathogenicity. It targeted the host nucleus and bound to DNA, where it presumably manipulates host gene expression to promote compatibility.

Another functionally characterized effector is a metalloprotease (GLRG_06543, also known as *Cgfl*) that is highly expressed during early infection and may target host chitinase defense proteins (Sanz-Martín et al. 2016a). Deletion mutants were significantly reduced in aggressiveness compared with wild-type strains.

Other than the melanin cluster, the product of only one other SM cluster has been identified and characterized in *C. graminicola*. This cluster (encompassing the genes GLRG_11836 to GLRG_11340), is identical to the radicicol (also known as monorden) cluster in *Pochonia chlamydosporia* (Reeves et al. 2008; Torres et al. 2016). Monorden is an antibiotic that targets the Hsp90 chaperone (Schulte et al. 1998). Monorden was isolated from *C. graminicola*-infected stalks and the compound was proposed to suppress basal defense responses during early infection and biotrophic colonization of maize (Wicklow et al. 2009). However, transcriptome analysis indicated that genes in the *C. graminicola* monorden cluster are most highly expressed during necrotrophy (Torres et al. 2016). Monorden has been associated with the generation of reactive oxygen species and induction of programmed cell death in animal systems (Ryhänen et al. 2008; Soga et al. 2003) and, thus, it may play a role in the induction of host cell death during the switch to necrotrophy in maize. It may also serve an antibiotic function, protecting the dead tissue from competitors.

Given the tractability of *C. graminicola* for reverse genetic studies, there is more work that can be done to characterize the functions and targets of other secreted effectors and SM clusters. Those essential for pathogenicity will be of special interest, because they may be therapeutic targets for disease management (e.g., by an RNA interference approach or by manipulating the interacting host factors with gene editing techniques) (Goulin et al. 2019; Rosa et al. 2018).

Management of Maize Anthracnose

Despite significant progress in the past 20 years in elucidating the etiology of ASR, recommendations for managing the disease in the field have not changed very much. This is due mainly to a lack of new field-based information. There is a critical need for additional research on the potential impact of a changing climate (e.g., extremes of temperature and rainfall, higher summer temperatures, and milder winters), and also on the effects of newer maize production protocols (e.g., shifts in irrigation or nutrient management practices, higher seeding rates and earlier planting dates, availability of new genetically modified hybrids with multiple pest resistance and drought tolerance traits, and expansion in the use of foliar fungicides) (Cook 2000; Saavoss et al. 2021).

The most effective tool for management of ASR is the use of hybrids that express genetic resistance to *C. graminicola*. Numerous studies over the years have investigated sources and patterns of inheritance of resistance to ASR (Callaway 1989; Carson and Hooker 1981, 1982; Lim and White 1978; Matiello et al. 2012, 2013; Nicoli et al. 2016; Rezende et al. 2004; Toman and White 1993). Resistance can be conferred by multiple major and minor genes, with both additive and dominance effects. Significant heterosis was detected at some loci, indicating the presence of partial dominance. Levels of resistance were increased in some cases through the recombination of transgressive resistant individuals (Matiello et al. 2013).

Producing ASR symptoms in the field for screening maize germplasm is very labor intensive: the most reliable method involves injecting inoculum directly into individual stalks (White and Humy 1976; White et al. 1979). Use of molecular markers (also known as marker-assisted selection) has significantly reduced the time needed to develop improved hybrids (Balint-Kurti and Johal 2009; Deleon et al. 2021; Poland et al. 2009) but it is nonetheless a lengthy process to incorporate multiple genes to achieve effective levels of resistance through traditional breeding.

A few major genes that provide high levels of resistance against ASR have been identified, and these offer an advantage for traditional breeding as well as for transgenic or genome editing approaches (Badu-Apraku et al. 1987; Cook 2000; Goulin et al. 2019; Toman and White 1993). One of these major genes was localized to a specific interval on chromosome 4 of the inbred MP305 by using molecular markers (Jung et al. 1994). Additional fine mapping and sequencing resulted in the identification of the *Rcg1* locus that contains the first fully characterized *C. graminicola* resistance (*R*) genes, two tightly linked analogs of the nucleotide-binding site–leucine-rich repeat (NBS-LRR) class (*Rcg1* and *Rcg1b*) (Broglie et al. 2006, 2009). Both are necessary to confer full resistance to ASR (Broglie et al. 2006, 2009). The mechanism of resistance is likely to be via recognition of specific pathogen avirulence factors (McHale et al. 2006). There appeared to be no fitness costs for near-isogenic hybrids carrying the *Rcg1* locus in the absence of the pathogen, and there were significant benefits in the presence of disease (Frey et al. 2011).

The *Rcg1* locus occurs in only 5% of inbreds that represent more than 90% of the genetic diversity in public sources of maize germplasm; all of these *Rcg1* inbreds are tropical (Frey 2006). The germplasm enhancement of maize (GEM) project was developed to increase the genetic base of hybrid maize in temperate regions by incorporating exotic sources, including tropical germplasm (Pollak 2003). Four inbred lines with improved resistance to ASR have been developed from 75% exotic populations through the GEM project (Smith et al. 2015). Moreover, another NB-LRR *R* gene (NLR02) was recently characterized on chromosome 6 in the tropical inbred Tzi8 (Deleon et al. 2021). This gene appears to be the result of an unusual fusion event between two *R* genes and performed well when introduced as a transgene in greenhouse assays (Deleon et al. 2021). It is likely that tropical germplasm will be a good source of novel resistance genes to ASR and, with the application of new, faster approaches for marker-assisted selection and next-generation methods for gene editing, we can expect more of these genes to be identified and deployed in the future.

Resistance conferred by major genes may be less durable than multiple genes with partial effects because there is a possibility for rapid emergence of new, virulent pathogen genotypes that can overcome the resistance. The *Rcg1* locus has been deployed in some commercial hybrids in the United States since 2011. Because it is a compound locus that includes two *R* genes, it might be harder for the pathogen to overcome. Furthermore, durability relates to the diversity of the population and potential for genetic shifts and, in the United States, *C. graminicola* appears to lack race structure, and putative effectors appear to be relatively low in genetic diversity (Du et al. 2005; Nicholson and Warren 1981; Vaillancourt and Hanau 1992; D. F. Parreira and L. J. Vaillancourt, unpublished data). *C. graminicola* also does not seem to undergo sexual recombination in the field. However, studies of population diversity in *C. graminicola* are still very limited. Furthermore, a lack of overall sequence diversity in effectors may not translate to a lack of pathogenic diversity because strains may also differ in the expression of the effectors (Rech et al. 2014).

Major gene resistance to ASR does not produce a hypersensitive response even against incompatible races (D. F. Parreira and L. J. Vaillancourt, unpublished data). Instead, it results in reduced and slower fungal germination, infection, and biotrophic and necrotrophic colonization of pith cells (Muimba-Kankolongo and Bergstrom 2011; M. F. Torres and L. J. Vaillancourt, unpublished data). Resistance genes may function to activate maize basal defense pathways more quickly and more intensely in response to pathogen attack. Host defenses are active and detectable within ALB lesions before and after the switch to necrotrophic growth (Vargas et al. 2012). Quantitative variation in aggressiveness to maize among isolates of *C. graminicola* has been reported (White et al. 1987), and more aggressive isolates may be more successful than less aggressive isolates in infecting or colonizing resistant germplasm.

Physiological mechanisms that have been associated with ASR resistance include the expression of various defensive metabolites and the presence of physical barriers. Several groups of defense-associated compounds are produced by maize in association with ASR, sometimes inhibiting and sometimes promoting disease. These include benzoxazinoid hydroxamic acids (e.g., 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one) (Ahmad et al. 2011), terpenoid phytoalexins (i.e., kauralexins and zealexins) (Christensen et al. 2018; Huffaker et al. 2011; Schmelz et al. 2011), flavonoids (i.e., maysin, apimaysin, and methoxymaysin; and lignin precursors 4-coumarate, caffeic acid, and ferulic acid) (Balmer et al. 2013; Bergstrom and Nicholson 1999; Lee et al. 1998), anthocyanins (Doehlemann et al. 2008), oxylipins (Borrego and Kolomiets 2016; Gao et al. 2007), and jasmonates (Balmer et al. 2013; Gorman et al. 2020). Maize also expresses multiple salicylic-acid- and jasmonic-acid-associated pathogenesis-related proteins in response to fungal infection (Torres et al. 2016; Vargas et al. 2012). Many of these compounds and proteins also accumulate in maize in response to a wide range of other biotic challenges as well as to wounding in some cases, and some have been shown to have antifungal activity, at least in vitro.

Physical barriers involve lignification and callose deposition, including production of papillae in response to attempted penetrations, and localized thickening of walls and apoplast occlusion that also occur in response to wounding (Muimba-Kankolongo and Bergstrom 1990; Venard and Vaillancourt 2007a). Wounding induced increased resistance to subsequent infection by *C. graminicola* (Muimba-Kankolongo and Bergstrom 1990, 1992, 2011).

Host resistance is typically combined with cultural management practices in an integrated approach for ASR disease management. Tillage to bury infested crop residue and crop rotation both reduce *C. graminicola* primary inoculum and can also result in reduction of ALB and ASR (Jirak-Peterson and Esker 2011; Lipps 1985). Although rotation away from maize for at least a growing season is generally recommended to manage anthracnose, leaving crop residues on the surface has numerous environmental advantages including reduced erosion, improved water infiltration, cooler soil temperatures, and increased soil organic matter (Blanco-Canqui and Lal 2009; Wilhelm et al. 1986, 2007). These considerations may outweigh concerns about increased ASR inoculum. Two-thirds of maize acres in the United States were in conservation tillage in 2016 (Claassen et al. 2018).

Duncan and Dominy (1989) reported that use of a cover crop resulted in slower development and reduced severity of ALB caused by *C. sublineola* in grain sorghum. They attributed this to obstruction of infested surface crop debris by cover crop residues, resulting in reduced splash dispersal of inoculum. Preliminary data indicated that severity of ASR in maize due to *C. graminicola* was also reduced following a winter cereal rye (*Secale cereale*) cover crop (A. E. Robertson, unpublished data). However, the cover crop also reduced maize plant vigor (perhaps as a result of competition), and this resulted in smaller ears that removed less stalk carbohydrate. Thus, it is not clear whether the increase in stalk carbohydrate could explain the reduced ASR severity versus reduced inoculum dispersal due to physical obstruction by the cover crop.

High ASR incidence can occur even in the absence of surface residue or ALB, suggesting a role for long-distance dispersal of inoculum (Lipps 1988). Systemic invasion from buried inoculum in the soil via the roots has also been proposed to explain these cases, although very few studies have addressed this hypothesis directly (Bergstrom and Bergstrom 1987; Bergstrom and Nicholson 1999; Munkvold and White 2016; Sukno et al. 2008).

Sukno et al. (2008) inoculated roots of transplanted susceptible seedings in a growth chamber with a transgenic *C. graminicola* strain expressing GFP and antibiotic resistance and recovered the strain from upper plant parts. In addition, melanized runner hyphae, hyphopodia, and microsclerotia were observed on roots of inoculated susceptible maize seedlings in Petri dishes (Sukno et al. 2008). The fungus also produced acervuli and falcate spores but this is likely to be an artifact of the experimental design, in which the roots were exposed to light. The fungus penetrated and colonized individual epidermal and cortical cells of inoculated roots (Sukno et al. 2008; Venard and Vaillancourt 2007b). Hyphae entered the vascular cylinder only after the root system had become extensively colonized (Sukno et al. 2008).

In a field study, *C. graminicola* was recovered from the roots and subcrown mesocotyl of most seedlings 3 weeks after infested oat kernels were incorporated into the planting furrows with the seeds (Lipps 1985). However, by the end of the season, very few mature plants still harbored the pathogen, and there was no significant increase in ASR resulting from the furrow inoculation, apart from a single replication involving the most susceptible hybrid. Although seedlings grown from *C. graminicola*-inoculated kernels can develop root decay in highly susceptible genotypes (Warren and Nicholson 1975), *C. graminicola* is rarely reported now as a root or kernel pathogen. Thus, the mechanism and importance of root infection as a major route for ASR development in the field remain unclear.

A frequent management recommendation for ASR, and for stalk rots in general, is to reduce crop stress, which will theoretically increase photosynthetic activity and the amount of stored carbohydrates in stalks, increasing their resistance to ASR (Dodd 1980).

High nitrogen fertility levels combined with low potassium levels can increase the risk for stalk rots. Low soil nitrogen can also result in increased stalk rot and lodging (Jardine 2006; Nielsen and Colville 1986). Scouting for ASR and other stalk rots before maize reaches physiological maturity to assess the risk of lodging is important to reduce yield loss (Cota et al. 2012; Freije and Wise 2016). Standard recommendations are to schedule an earlier harvest when stalk rot incidence is above 10%. Although irrigation can prevent drought stress, it may also increase the risk of disease development because higher moisture levels favor spore production and dispersal (Bergstrom and Nicholson 1999; Nielsen and Colville 1986). Unfortunately, there is not a lot of research to directly investigate the effect of specific production practices on ASR severity; more work is needed if improved cultural management practices are to be developed.

The use of foliar fungicides on hybrid field maize for both disease management and physiological benefits has increased considerably since 2006 (Wise and Mueller 2011). Foliar fungicides applied during reproductive stages reduced stalk rot severity in some studies (Byamukama et al. 2013; Harbour and Jackson-Zeims 2016; Mueller and Smith 2019; Shriver and Robertson 2009) but not in others (Mallowa et al. 2015; Paul and Wallhead 2011; Peltier et al. 2015, 2019; Price et al. 2018). However, it was not clear in these studies whether stalk rot was due to *C. graminicola* or to other stalk rot pathogens. Applications at V5 and V6 stages may not reduce ASR, because the pathogen is likely to be already established in the crop (Robertson 2013). Two studies specifically reported the effect of fungicides on ATD. Robertson et al. (2008) found that an application of foliar fungicide at tasseling (VT stage) reduced ATD incidence. Similarly, Adee and Duncan (2017) showed that application of fungicides at VT, or 7 or 14 days after VT, reduced ATD, whereas an application of a fungicide at V5 to V8 (five to eight leaf collars visible) had no effect on ATD. Because ATD may indicate the presence of ASR (A. E. Robertson, unpublished data), these two studies suggest that foliar applications of fungicides during maize early reproductive stages may reduce ASR severity. However, there are concerns with applying fungicides for the management of stalk rots, because the practice poses an elevated risk for development of fungicide resistance in *C. graminicola* and other pathogens (FRAC 2019; Robertson et al. 2020). Moreover, applying fungicides can also delay senescence, resulting in high-moisture maize at harvest, and can also reduce crop yields (Byamukama et al. 2013; Mueller and Smith 2019; Wise and Mueller 2011).

Future Prospects

Further improvements in management of ASR will depend on a better understanding of the disease cycle, including the role of the environment in pathogen survival and infection and in the expression of host resistance. Addressing important questions that remain regarding sources of infection and mechanisms of colonization of plants in the field, and the influence of environmental factors on these processes, could improve management of ASR by cultural means. However, some practices that mitigate ASR may also have negative impacts on the environment; for example, tillage (Uri 1999) or application of foliar fungicides (Wise and Mueller 2011; Zubrod et al. 2019). Consequently, managing ASR in the 21st century will continue to rely most heavily on the development of more effective and durable sources of host resistance which, in turn, will benefit from a more complete understanding of the molecular basis for fungal pathogenicity.

Advances in our understanding of the cytology and molecular biology of the disease interaction have revealed potential targets for intervention via transgenic manipulation. We now know that biotrophic invasion is necessary for ASR to spread through stalks, even if the pathogen is initially introduced via wounds. We have learned that biotrophic establishment is dependent on the production of a suite of secreted protein effectors and SM that target various cellular compartments and host pathways to promote compatibility. These effectors and SM and their regulation must be a focus for further investigation because interference with their production and function would be expected to decrease ASR incidence and severity. Host targets of these compounds also will be important to identify because those could be manipulated by transgenic or gene editing methods.

We still lack critical information about the natural diversity of *C. graminicola* effectors and SM, and of plant receptors and *R* genes. More research in this area is needed if we are to identify new sources for novel *R* genes and manage them for durability. Uncovering the evolutionary history of *C. graminicola* and its center of diversity, whether in Central or South America or elsewhere, will be an important part of this work.

Most of the cytological and molecular studies to date relate to highly susceptible maize hybrids or inbreds, and a single fungal genetic background, strain M2 (also known as M1.001) that was isolated in the 1970s (Forgey et al. 1978). More work with resistant maize hybrids and inbreds and diverse fungal genotypes is needed to better understand the nature and expression of resistance. Further studies that explore the mechanisms of nonhost resistance of maize to closely related members of the CGSC could also be useful. For example, the *C. graminicola* and *C. sublineola* genome sequences differ mostly in genes that code for potential molecular disease determinants, including signaling proteins, transcription factors, transporters, carbohydrate-active enzymes, and SM (Buiate et al. 2017). This suggests that host specificity of *C. graminicola* to maize (and of *C. sublineola* to sorghum) involves specific molecular interactions that include suppression of basal resistance pathways and induction of host susceptibility. Transcriptomic and proteomic studies are also needed to characterize the role of differential expression in host versus nonhost interactions. A comprehensive understanding of the nature of nonhost resistance could ultimately lead us to the holy grail for maize stalk rot disease management, which is universal and durable resistance against all stalk rot pathogens.

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Renata Belisário

Renata Belisário is a plant pathology Ph.D. candidate at the University of Kentucky. Her dissertation research relates to understanding the mechanisms regulating the production and secretion of effector molecules and other secreted proteins by *Colletotrichum graminicola* during infection of maize. The goal is to target highly conserved secretory processes to develop a broadly effective method for management of biotrophic and hemibiotrophic fungal pathogens of crop plants. She has an M.S. in Plant Pathology (2018) from the Universidade Federal de Viçosa, Brazil, and BSc (2015) and BEd (2016) degrees in Biological Sciences from Pontifícia Universidade Católica de Minas Gerais, Brazil. She is the co-founder and president (2021–2023) of the Women in Plant Pathology Collective (Mulheres na Fitopatologia).

Alison Robertson

Alison Robertson is a professor and extension plant pathologist at Iowa State University. Her research and extension programs are tightly integrated. Through her extension program, she strives to increase the plant pathology expertise of her clientele to facilitate more effective disease management and increased net returns for farmers. Research in her lab focuses on current issues in the management of corn diseases, including anthracnose stalk rot. She has been at Iowa State University since 2004. She received her Ph.D. degree from Clemson University (2003), her M.Phil. degree from the University of Zimbabwe (1999), and her B.Sc. from the University of Kwazulu-Natal (Pietermatritzburg) in 1991, all in Plant Pathology.

Lisa Vaillancourt

Lisa Vaillancourt is a professor of plant pathology at the University of Kentucky. Her research program focuses on the genetics of fungal pathogenicity in Colletotrichum and Fusarium diseases, including rots of tree and soft fruit, and stalk and ear rots of grain crops, including wheat, maize, and sorghum. She has been studying *Colletotrichum graminicola* and maize anthracnose for more than 30 years and was part of the team that published the pathogen genome in 2012. She has been at the University of Kentucky since 1996. Before that, she was a Postdoctoral Fellow at the University of Vermont, where she helped to elucidate the molecular structure of the mating type loci of *Schizophyllum commune*. She received her Ph.D. and M.S. degrees in Plant Pathology from Purdue University and the University of Illinois, respectively, and her B.S. in Biology from the University of Connecticut.

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