Infection of Winter Wheat by a β -Glucuronidase-Transformed Isolate of *Cephalosporium gramineum*

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ABSTRACT

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Field-grown winter wheat was inoculated with a β -glucuronidase-transformed isolate of *Cephalosporium gramineum* in two field seasons to elucidate the mode of infection in resistant and susceptible cultivars. Colonization of viable root epidermis and cortical cells occurred as soon as 15 days postinoculation and the pathogen was found in the vascular tissues by 20 days postinoculation, well before freezing soil temperatures

occurred. Penetration occurred directly through the root epidermis and through wounds adjacent to emerging secondary roots. The pathogen also penetrated through root cap cells and colonized meristematic tissues near root tips to gain access to the vascular system. Lower stem base colonization was observed where the pathogen penetrated directly through the epidermis, wounds, or senescent tissues. Appressorium-like structures, which appeared to aid penetration of cell walls, were often found within cells of both roots and stems after initial colonization. The mechanisms of resistance were not apparent, but less colonization occurred in resistant than in susceptible cultivars.

Cephalosporium stripe of winter wheat (*Triticum aestivum* L.) is caused by the fungus *Cephalosporium gramineum* Nisikado and Ikata (sporodochial stage, *Hymenula cerealis* Ellis and Everh). *C. gramineum* is an economically damaging vascular wilt pathogen of small grain cereals in the northwestern United States where yield loss of up to 80% can occur on winter wheat when conditions are favorable for disease development (6,12,14). When cool, wet conditions exist in the autumn, small (5 to 11 $\times$ 1.5 to 3 μ m) hyaline conidia produced on infested plant residues (7,34) are washed into the soil and serve as primary inoculum (13,34). Although infection is thought to take place through wounds in roots created by freeze-thaw cycles that occur during winter and early spring (2,7,19,20) or by damage caused by other means such as root-feeding insects (27), direct evidence for this does not exist. Mathre and Johnston (19) hypothesized that infection occurs when conidia are "vacuumed" into xylem vessels of damaged roots. In greenhouse experiments, however, Anderegg and Murray (1) demonstrated that root breakage is not a prerequisite for disease development and that severe disease can occur without soil freezing under conditions of low pH and high soil moisture.

Some researchers believe that *C. gramineum* is strictly a xylem-inhabiting pathogen incapable of penetrating living cells (33), but Mathre and Johnston (18) suggested the pathogen has a rudimentary ability to penetrate and grow through living root cells. Bailey et al. (2) found that the epidermis and root cortex did not appear to serve as barriers to colonization. They found fungal hyphae could penetrate the root epidermis both intracellularly and intercellularly on tissues that had been frozen to -10°C and thawed overnight, but little colonization was found on nonfrozen roots. The frozen tissues appeared to be intact, as viewed by scanning electron microscopy (SEM), and they concluded that freezing stress may be an important factor in predisposing wheat plants to active penetration and infection by *C. gramineum*. Bonde (5) also observed

C. gramineum colonizing root cortical cells but did not find evidence of vascular infection. No work to date has demonstrated the process of vascular infection under field conditions.

Variability in occurrence of *Cephalosporium* stripe from year to year has made screening for disease resistance difficult (7,16,17). Bruehl et al. (8) found that over a 5-year period susceptible cultivars were consistently susceptible whereas cultivars with some resistance varied widely in the percentage of diseased stems. Variation in resistance screening methodologies has also confused conclusions about resistance. For example, Bruehl (6) categorized four cultivars as resistant to *Cephalosporium* stripe when inoculated with a hypodermic needle, but the same cultivars were susceptible when grown under field conditions (25). There is great variation in the amount of disease between and within cultivars, and highly effective resistance to *C. gramineum* does not exist in wheat (6,8,15,26). Although resistance in winter wheat cultivars is not highly effective, some wheat relatives are resistant (18) and progeny from a cross between the wheat relative *Agropyron elongatum* (Host) Beav. (now called *Thinopyrum ponticum* [Posp.] Barkworth and D. R. Dewey [35]) and *Triticum aestivum* are highly resistant (20,32).

Determining how *C. gramineum* infects and colonizes wheat has been difficult because these events take place in the soil during the winter. In addition, *C. gramineum* has nondescript hyphae and does not produce distinct lesions, which has made understanding the general epidemiology, as well as host resistance difficult. To overcome this problem, *C. gramineum* was transformed with the β -glucuronidase (GUS) reporter gene (24). GUS is normally not produced in fungi (31), which makes it an ideal marker to study host-pathogen interactions. The GUS gene is constitutively expressed and its activity can be visually detected by incubation in the substrate 5-bromo-4-chloro-3-indolyl- β -D-glucuronic acid (X-Gluc) (30). The reaction produces an intensely blue dichloro-dibromindigo (CIBr-indigo) precipitate that is stable throughout normal histological preparations (30). GUS-transformed fungi have been used successfully to study various host-pathogen interactions (9,17,31). No studies, however, have been conducted under field conditions. The objectives of this research were to elucidate the timing and mode of penetration and infection of winter

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wheat by *C. gramineum* and to determine whether differences existed among resistant and susceptible cultivars for these processes.

MATERIALS AND METHODS

Fungal isolate and wheat genotypes. A GUS-transformed isolate of *C. gramineum* (tCg84-3086) and the winter wheat genotypes Stephens, Daws, WA7437, and Agrotriticum 3425 were used in this study. The GUS-transformed isolate of *C. gramineum* behaves normally with respect to pathogenicity, physiological growth, and reproduction (24). The winter wheat genotypes Stephens and Daws are susceptible cultivars, WA7437 is a resistant breeding line, and Agrotriticum 3425 is a highly resistant 56 chromosome amphiploid of wheat and *Thinopyrum ponticum* (35).

Cultural practices. Experiments were conducted in the 1996 to 1997 and 1997 to 1998 growing seasons and hereafter will be referred to as 1997 and 1998 seasons, respectively. Field plots were located at the Plant Pathology Research Farm, Pullman, WA. Seed was sown on 10, 20, and 30 September 1997 at a rate of 33.6 kg ha⁻¹ and on 12 and 22 September and 2 October 1998 at a rate of 40.5 kg ha⁻¹ with a grain drill. Both experiments were arranged as random complete block split-plot designs with planting date as the main plot factor and cultivar as the subplot factor in three replicate blocks. Wheat was sown in four-row plots of approximately 1.2 × 3.6 m in 1997 and 1.2 × 5.2 m in 1998 into Thatuna silt loam (fine-silty, mixed, mesic xeric Agrialboll). Plots in both years were fertilized with a granular mix of 215 N: 22 P: 0 K: 22 S kg ha⁻¹. A single four-row strip of wheat was planted on the north and south sides of the experimental plot and two or three four-row strips were planted on the west and east sides, respectively, to serve as guard rows and to monitor spread of the transformed pathogen outside the plot area. Oat kernels colonized by the GUS-transformed isolate of *C. gramineum* were scattered onto the soil surface in the experimental plot at a rate of 1,614 kg ha⁻¹ on 18 October 1996 and 10 October 1997. Oat kernel inoculum was produced by autoclaving 500 ml of dry oats with 300 ml of distilled water in 2,000-ml flasks for 60 min. A conidial suspension was added and the flasks were incubated at room temperature for 2 to 3 weeks before being spread out and allowed to dry at room temperature.

Plant sampling and specimen preparation. Plants were sampled beginning 26 October 1996 continuing until 15 May 1997 and from 24 October 1997 until 27 April 1998. Plants were sampled at 1-week intervals after inoculation for the first five sample dates and then at 2- to 4-week intervals in 1997. In 1998, plants were sampled every 3 to 4 weeks after inoculation. At each sampling date, approximately 5 to 10 plants in 1997 and 10 to 20 plants in 1998 were taken from each cultivar within each subplot depending on cultivar and seeding date. Plants were carefully dug with a shovel, making sure the root systems were intact. In both years, noninoculated control plants were dug from plots located 50 to 100 m away from the inoculated field plot. Each sample was washed free of soil and the plants were placed in plastic bags and incubated overnight at 10 to 15°C to enhance metabolic activity of β -glucuronidase prior to incubation in X-Gluc reagent (2 mM 5-bromo-4-chloro-3-indolyl- β -D-glucuronic acid [Biosynth Ag, Switzerland], 100 mM NaPO, pH 8.0, 0.5 mM ferricyanide, 0.5 mM ferrocyanide, 10 mM EDTA, 0.1% Triton X-100, and 20% methanol) (38). Whole root systems and the first 3 to 5 cm of stem were vacuum infiltrated at room temperature and incubated in X-Gluc at 37°C in the dark for up to 24 h. Tissues were then fixed in FAA (10% formaldehyde, 5% glacial acetic acid, 70% ETOH) or 0.1 M sodium phosphate buffer (pH 7.0) containing 2.5% glutaraldehyde and 2.5% formaldehyde for at least 48 h before microscopic examination.

Microscopy. Every plant from each subplot sample was observed under a stereo microscope. Observations were recorded on

plant tissues colonized by the transformed pathogen as indicated by the blue precipitate within the hyphae, and then cut into small pieces and placed into vials containing 0.1 M sodium phosphate buffer (pH 7.0), 2.5% glutaraldehyde, and 2.5% formaldehyde. Whole mounts, hand sections, and thin sections (8 to 10 μ m) were examined with a light microscope with a photomicroscopy unit (Olympus BH-2, New York). Thin sections were made by dehydrating tissue samples in a graded series of alcohol and infiltrating the tissues with paraffin. Serial sections (8 to 10 μ m) were made by a standard microtome, floated on water at 45°C, mounted on gelatin-coated slides, and counter-stained with saffranin (1.0% aqueous) for 12 to 24 h before being examined. Samples for SEM were dehydrated in a graded series of alcohol and then critical-point dried (Tousimis Samdre PVT-3B Critical Point Dryer, Rockville, MD). Each sample was placed on a SEM stub mount; regions showing GUS activity were drawn before coating with gold (Technics Hummer V Sputter Coater, Alexandria, VA) in order to differentiate hyphae of the transformant from other fungi. All specimens were examined by a SEM (S570; Hitachi, Tokyo).

Soil sampling. Soil samples were taken every 2 to 4 weeks beginning in October each year and ending 14 May 1997 and 27 April 1998. Soil samples were collected randomly within each replicate block to monitor *C. gramineum* population density and moisture content in the plot without regard to the main or subplot treatments. Eight soil cores (3 × 15 cm) were removed from each replicate block and thoroughly mixed. Care was taken to avoid inoculated oat kernels on the soil surface when sampling. Population density of *C. gramineum* was determined by a modified method of Specht and Murray (36). Two 5-g subsamples of soil per replicate were dilution-plated onto the *C. gramineum* semi-selective media (CGSM) and incubated for 10 to 12 days at 15°C before colonies of *C. gramineum* were counted. The transformant was differentiated from wild-type colonies by spraying the plates with X-Gluc reagent with an atomizer and incubating the plates at 37°C in the dark overnight before counting. Population density of *C. gramineum* was expressed as mean CFUs per gram of dry soil across all three replicates. Soil moisture was determined by taking two subsamples (100 to 200 g) of bulk soil from each replicate and drying them in a forced air oven at 100°C overnight. Soil temperature at a depth of 15 cm was also measured at each sampling date by taking the average of one random measurement in each replicate with an electronic temperature probe (Omega 450 ATH, Stamford, CT).

Visual disease index. Visual disease ratings were determined by removing 0.5-m sections of a row from each subplot after anthesis (18 to 22 June 1997 and 16 to 18 June 1998). In 1997, plants were taken from more than one row in subplots where uninterrupted 0.5-m sections could not be found due to the previous removal of samples. Individual stems were rated for disease based on a 0 to 5 scale, where 5 = dead stems (whitehead), 4 = stems with stripes and or necrotic vascular bundles in the flag leaf, or extending to the head, 3 = stems with stripes and or necrotic vascular bundles in the leaf subtending the flag leaf, 2 = stems with stripes and or necrotic vascular bundles in the second leaf down, 1 = stems with stripes and or necrotic vascular bundles in the third leaf down, and 0 = stems with no visual symptoms in the uppermost four leaves (37). Disease incidence was calculated as the percentage of stems with symptoms and disease severity was calculated as the mean rating of stems with symptoms. Disease index was calculated by multiplying disease incidence by disease severity and dividing by five (37). Disease index was subjected to analysis of variance using a random complete-block split plot model with planting date as the main plot factor and cultivar as the subplot factor using the SAS general linear models procedure (version 8.0, SAS Institute, Cary, NC). Fisher's protected least significant differences test was used to differentiate cultivar and seeding date effects.

Fig. 1. Light micrographs of a β -glucuronidase-transformed isolate of *Cephalosporium gramineum*, which turns blue in response to incubation in X-Gluc on soil-dilution plates and colonizing winter wheat plants. **A**, Soil dilution plate differentiating the wild-type (w) *C. gramineum* from the transformant (t). **B**, Colonization of a root tip of Stephens 20 days postinoculation (DPI) in 1997 (bar = 21 μ m). **C**, Cross section (10 μ m) of a root of *Agrotriticum* 3425 49 DPI in 1998 showing colonization of root cortex cells (bar = 89 μ m). **D**, Colonization of the coleoptile and vascular bundles of the stem from WA7437 26 DPI in 1997. **E**, Direct penetration of a hypha (arrowhead) through the epidermis of the lower stem base of Daws 108 DPI in 1997 (bar = 37 μ m). **F**, Penetration of hyphae into the vascular system of a vascular bundle from the lower stem base of Stephens 132 DPI in 1998 (bar = 22 μ m). **G**, Colonization of the lower stem base (s), adventitious roots (r), and emerging secondary roots (arrowheads) of the crown region of *Agrotriticum* 3425 108 DPI in 1997. **H**, Longitudinal section through the crown region of Daws 108 DPI in 1997 with evidence of extensive colonization of the crown tissues and vascular bundles (arrowheads).

Isolation of *C. gramineum*. *C. gramineum* was isolated from diseased stems and leaves following visual disease ratings to determine the proportion of stems colonized by the transformant and wild type. Up to 25 leaves or stems with symptoms of Cephalosporium stripe from individual tillers were collected from each subplot. In subplots where there were fewer than 25 diseased tillers, leaves or stems with symptoms were collected from all possible tillers making a total of 803 samples in 1997 and 317 in 1998. In both years, leaves or stems were cut into 1- to 3-cm-long pieces, surface disinfected with 1.0% NaOCl for 2 to 3 min, and placed on CGSM (37). The plates were incubated at 15 to 20°C for 2 weeks then sprayed with X-Gluc to differentiate the transformant from wild-type pathogen.

RESULTS

Soil population density, moisture, and temperature. Wild-type colonies of *C. gramineum* and those of the GUS transformant were differentiated by their reaction with the X-Gluc reagent (Fig. 1A). In 1997, soil population density of both wild-type *C. gramineum* and the GUS transformant were approximately 1×10^6 CFU/g of soil 8 days after inoculation and ranged up to 1×10^7 CFU/g of soil for the GUS transformant in November (Fig. 2). *C. gramineum* was no longer detectable by dilution plating in mid-May. In 1998, soil population density of both wild-type *C. gramineum* and the GUS transformant were around 1×10^2 CFU/g of soil 14 days after inoculation and never rose above 1×10^4 CFU/g of soil and 1×10^5 CFU/g of soil, respectively (Fig. 2).

In 1997, relatively high soil moisture (30 to 45%) predominated from October through December (Fig. 3). Soil moisture during the same period in 1998 was much lower (12 to 30%). Soil moisture from December through May did not vary greatly between years but was higher in 1997 than 1998 (Fig. 3). Soil temperatures were similar from October to January in both years, but were much higher in 1998 from January to April than in 1997 (Fig. 3).

Disease index visual ratings. In 1997 and 1998 differences among seeding dates were not significant, nor was the interaction between seeding date and genotype ($P = 0.05$). Therefore, the means of disease index for genotype effects across all seeding dates were compared. Disease index in 1997 was higher than in 1998 for all genotypes (Table 1). In 1997, all four genotypes were significantly ($P = 0.05$) different from one another with disease index being the highest in Stephens followed by Daws, WA7437, and Agrotriticum 3425, respectively (Table 1). In 1998, Stephens also had the highest disease index followed by Daws, WA7437, and Agrotriticum 3425, respectively (Table 1). The only significant difference ($P = 0.05$) in disease index, however, was between the susceptible genotype Stephens and the resistant genotype Agrotriticum 3425 (Table 1).

Isolation of *C. gramineum* at anthesis. *C. gramineum* was isolated from 798 of 803 plant pieces with symptoms of Cephalosporium stripe in 1997. Of these, 72% were the GUS transformant and 28% were wild-type isolates of *C. gramineum*. In 1998, *C. gramineum* was isolated from 306 of 324 plant pieces with symptoms of Cephalosporium stripe. Of these, 93% were the GUS transformant and 7% were wild-type isolates of *C. gramineum*.

Seeding date, year, and cultivar effects on colonization by *C. gramineum*. In both years, *C. gramineum* colonized root and lower stem tissues throughout the study. In 1997, colonization of root epidermis and cortex cells, as well as epidermal and mesophyll cells of lower stem bases was observed as early as 15 days postinoculation (DPI) (3 November) on all genotypes from seeding date 1. By contrast, almost no colonization was observed on plants from seeding dates 2 and 3 at this time. By 20 DPI (8 November), however, *C. gramineum* was readily observed colonizing plants from all seeding dates. In general, plants from seeding date 1 had more extensive colonization than plants from seeding dates

2 or 3 at all sample dates throughout the study and the susceptible genotypes Stephens and Daws were usually more extensively colonized than the resistant genotypes WA7437 and Agrotriticum 3425. Similar results were observed in 1998, but colonization by the GUS transformant was not readily observed until 49 DPI (28 November) and the amount of colonization was not as extensive as in 1997. Likewise, differences in the extent of colonization among genotypes and seeding dates were not as evident in 1998 as in 1997, although susceptible genotypes were usually colonized more extensively than resistant genotypes.

Root colonization by *C. gramineum*. *C. gramineum* infected root tips by penetrating through root-cap cells and colonizing the surrounding tissues (Figs. 1B and 4A). Following root cap penetration, *C. gramineum* colonized meristematic tissues where vascular infection through differentiating cells of the stele occurred (Fig. 4B). Small root tips of primary seminal and secondary adventitious roots were colonized more often than root tips of large primary adventitious roots and ranged from none to a few root tips colonized per plant.

C. gramineum was observed colonizing root cortex and vascular tissues of resistant and susceptible genotypes as early as 15 DPI (3 November) in 1997 and 49 DPI (28 November) in 1998 (Fig. 1C). Appressorium-like structures were observed in both years within colonized cells of the root cortex, endodermis, and vascular tissues (Fig. 4C and D). These structures were only found in colonized root tissues and not on the root epidermis. Hyphae appeared to adhere to cell walls and become enlarged at the tip. In some samples a small structure was detected that emerged from the bulbous tip and appeared to penetrate through the cell wall, similar to a penetration peg emerging from an appressorium (Fig. 4D).

Fig. 2. Soil population density of wild-type and β -glucuronidase-transformed *Cephalosporium gramineum* in the 0- to 15-cm zone of soil at the Washington State University Plant Pathology Farm, Pullman. Population density was determined by dilution-plating soil onto the *C. gramineum* semiselective medium. The transformant was differentiated from wild type by the blue precipitate in response to over-spraying the plates with X-Gluc reagent and incubating them at 37°C in the dark for up to 24 h. Bars represent $\pm$ the standard error of the mean.

Hypae, however, were observed apparently penetrating cell walls where no appressorium-like structures were found.

Stem and leaf colonization by *C. gramineum*. Colonization of developing stems occurred from the soil line to the crown throughout the study. The leaf tips of the coleoptiles and the prophylls of developing shoots were commonly colonized by *C. gramineum* (Fig. 1D). Based on colonization patterns, the pathogen seems to have entered the vascular system through the hydathodes of these leaves, although penetration of hydathodes was not observed directly. The pathogen penetrated leaf epidermal cells directly and colonized vascular tissues of leaf sheaths as early as 20 DPI (8 November) in 1997 and 49 DPI (28 November) in 1998 (Fig. 1E and F). Colonization of the leaf sheaths ranged from a few small areas to almost the entire leaf sheath. It was common to find the pathogen sporulating within and on cells of the leaf sheaths. Most of the colonized areas were around vascular bundles and the surrounding mesophyll and epidermal cells (Fig. 1D). Appressorium-like structures, similar to those observed in the roots, were observed in cells of leaf tissues (data not shown).

Crown colonization by *C. gramineum*. *C. gramineum* was able to colonize the inner tissues of the crown from infections that occurred through the roots and the lower stem base. Stem-base infections were commonly observed around wounds apparently created by the emergence of tillers and stem elongation (Fig. 1G). *C. gramineum* was observed colonizing the inner crown tissues of Stephens and Daws from seeding date 1 at 129 DPI (25 February) (Fig. 1H). By 157 DPI (25 March), however, colonization of the inner crown tissues of plants from all genotypes and seeding dates was observed. Mycelium and conidia were abundant within the inner crown tissues and the pathogen colonized adjacent vascular elements by penetrating through the cell walls. More extensive colonization of the inner crown tissues occurred in Stephens and

Daws than WA7437 and Agrotriticum 3425. In 1998, colonization of the inner crown tissues was first observed in Stephens from seeding date 1 at 132 DPI (19 February). Very limited colonization of the inner crown tissues of Stephens and Daws was observed from subsequent sampling dates and almost no colonization was observed within the inner crown tissues of WA7437 and Agrotriticum 3425 at these later sample dates.

DISCUSSION

C. gramineum colonized vascular and nonvascular stem and root tissues of field-grown plants well before soil freezing occurred. This observation is consistent with the work of Stiles and Murray (37) who were able to isolate *C. gramineum* before soil freezing occurred and provides additional evidence that soil freeze-thaw cycles are not required for infection. This study revealed that *C. gramineum* can penetrate and colonize stems directly through the epidermis of leaf sheaths or wounds where tillers emerge. Evidence for stem-base infection was also found by Stiles and Murray (37) and Bonde (6), who were able to isolate the pathogen from stems but not roots of the same plants, indicating that the fungus may have colonized the stems directly. An important aspect of stem-base infections is that *C. gramineum* can spread from stem to stem by colonizing the crown tissues without having to transgress from the roots to the stems. Mathre and Johnston (23) found evidence for this when they injected a conidial suspension into primary tillers and observed disease symptoms on subsequent tillers, indicating that the pathogen had moved down the stem into the crown tissues and colonized other stems.

Stiles and Murray (37) isolated *C. gramineum* from crown roots and seminal roots but rarely from the subcrown internode and concluded that under Pacific Northwest conditions, crown roots appear to be the most important infection sites. In the current study, colonization of the subcrown internode was rare in both years. If the seminal roots are a major source of vascular colonization and movement, the subcrown internodes should have been colonized extensively because the vascular tissues of the seminal root system have to pass through the subcrown internode. These observations support Stiles and Murray's conclusions (37) that adventitious roots are the most important infection courts of the root system.

Two hypotheses have been proposed to explain root infections by fungal vascular wilt pathogens: first, the pathogen colonizes root cortex cells, both intercellularly and intracellularly, before moving through the endodermis to reach the xylem; and second, the pathogen infects root tips and colonizes differentiating meristematic cells before reaching the xylem (39). Under field conditions in this study, *C. gramineum* infected the vascular tissues of roots by both processes. *C. gramineum* was observed colonizing

Fig. 3. Average soil temperature and gravimetric soil moisture at 0 to 15 cm depth during the 1997 and 1998 growing seasons at Washington State University Plant Pathology Research Farm, Pullman.

TABLE 1. Cephalosporium stripe visual disease index for four winter wheat genotypes sown on three dates during the 1997 and 1998 growing seasons at the Washington State University Plant Pathology Farm, Pullman

Cultivar	Disease index ^a	
	1997	1998
Stephens	46.0	18.5
Daws	39.2	12.1
WA 7437	16.9	2.2
Agrotriticum 3425	3.9	0.3
LSD (5%)	5.1	11.8

^a Values within columns are the mean of three replicates and three seeding dates. Individual stems were rated for disease severity based on a 0 to 5 scale. Disease incidence was calculated as the percentage of stems with symptoms and disease severity was calculated as the mean rating of stems with symptoms. Disease index was calculated by multiplying disease incidence by disease severity and dividing by five to give a scale ranging from 0 to 100.

root cortex cells and growing through the endodermis and was also observed colonizing root tips and meristematic tissues before colonizing the vascular tissues. Previous work on the infection process of *C. gramineum* under controlled environmental conditions found partial evidence for the first hypothesis. Bailey et al. (2) found the root epidermis and root cortex were colonized by *C. gramineum*, but did not observe hyphae penetrating through the endodermis. Bonde (6) also observed *C. gramineum* colonizing root cortical cells but never found evidence of vascular infection.

Previous studies found no evidence for root-tip colonization by *C. gramineum*. Stiles and Murray (37) rarely isolated *C. gramineum* from roots with intact root tips and Bonde (6) did not find root-tip colonization in plants inoculated aseptically in growth pouches. Colonization of root tips in this study was rare compared with colonization of the root cortex but is probably significant. For example, Mathre and Johnston (23) demonstrated disease development in plants inoculated in single roots with a conidial suspension using capillary tubes.

Fig. 4. Scanning electron micrographs (SEM) of field-grown winter wheat colonized by a β -glucuronidase (GUS)-transformed isolate of *Cephalosporium gramineum* in 1997. Each sample was placed on a SEM stub mount, and regions exhibiting GUS activity were drawn before coating with gold in order to differentiate hyphae of the transformant from other fungi. **A**, Hyphae penetrating directly between root cap cells (arrowhead) of Daws 26 days postinoculation (DPI) (bar = 36 μ m). **B**, Longitudinal section of root meristematic cells of Daws showing extensive colonization 108 DPI (bar = 36 μ m). **C**, Cross section of an adventitious root from Stephens showing extensive colonization of the cortex (c), endodermis (e), and vascular tissues (vt). Hyphal swellings can be seen within the endodermis (arrowheads) as well as other cells of the stele where direct penetration has occurred (bar = 54 μ m). **D**, Cross section of an adventitious root from Agrotriticum 3425 showing what appears to be a penetration peg (arrowhead) at the end of an appressorium-like hyphal swelling. Other hyphal swellings can also be seen appressed to the cell walls of the endodermis (e) as well as colonizing other cells of the stele (bar = 9 μ m).

Pool and Sharp (31) suggested that early planted winter wheat is more susceptible to *Cephalosporium* stripe than later planted winter wheat because the root biomass is greater, and therefore, more susceptible to root wounds caused by freeze-thaw heaving. This aspect of the disease was investigated by examining plants seeded at different times. In 1997 when extensive colonization of root and lower-stem tissues was observed soon after inoculation, plants from the earliest seeding date were more extensively colonized than plants from later seeding dates. By contrast, little colonization was observed after inoculation in 1998 and differences among seeding date were not as apparent. These observations correlated with visual disease ratings in the respective years but the overall seeding date effects on disease index were not significant at the 95% confidence level in either year. Plants seeded earlier, however, had a higher disease index than plants seeded later.

The difference in the degree of colonization early in the growing season and final disease index between 1997 and 1998 may be related to pathogen soil population density, which was greater and peaked sooner in 1997 than in 1998. Soil population density of *C. gramineum* was approximately fourfold greater 2 weeks after inoculation in 1997 than in 1998. These differences in soil population density between years are likely due to the difference in soil moisture, which was approximately three times greater in 1997 than 1998. This speculation is supported by previous studies that have shown sporulation by *C. gramineum* is favored by high soil moisture (28). Observations based on GUS activity and visual disease ratings suggest that early seeding results in more disease as a result of early and extensive root and crown colonization by *C. gramineum*.

Although *C. gramineum* colonized root cortex cells of all genotypes, vascular invasion is not related to the ability of a pathogen to colonize root cortex, regardless of whether the plant is susceptible or resistant (14) and it is possible that resistance to *Cephalosporium* stripe occurs at the endodermis. Bishop and Cooper (5) found little difference in the reaction of root epidermal cells and cortical cells between resistant and susceptible tomato and pea cultivars to *Fusarium oxysporum* f. sp. *lycopersici* and *F. oxysporum* f. sp. *pisi*, respectively, but found varietal differences in both pea and tomato at the endodermis. No detectable differences were observed between *Cephalosporium* stripe-resistant and -susceptible genotypes with respect to host cell reactions. There were host responses in the cortex and around the stele, as indicated by necrotic cells, but further research at the histochemical and transmission electron microscopic level is needed to determine whether such subtle chemical differences exist between susceptible and resistant genotypes.

Resistance to vascular wilt pathogens can occur during one or both stages in the disease cycle known as the determinative phase I and II (3,4). Determinative phase I describes the initial vascular invasion, either directly through the endodermis, through wounds, or through the apical meristems. Determinative phase II describes the subsequent colonization and upward movement of the pathogen within the vascular tissues. Evidence for both types of resistance were found in the genotype Agrotriticum 3425 and to a lesser extent in WA7437. Roots and stems of these genotypes were usually colonized less extensively than those of the susceptible genotypes. *C. gramineum* was found in the vascular system of Agrotriticum 3425 early in the season but little disease developed. Gold and Robb (13) investigated the rate of vascular colonization of *Verticillium dahliae* race 1 on near isogenic lines of tomato and found that plants with the *Ve* resistance gene were able to rapidly suberize xylem vessel walls, thus not allowing the pathogen to pass easily from vessel to vessel. Whether a similar response exists in Agrotriticum 3425 is unknown, but seems likely because colonization of the vascular system by *C. gramineum* was limited.

The crown region of Agrotriticum 3425 was previously identified as a possible site of resistance to *C. gramineum* (22). In 1997, colonization of the crowns was observed first in susceptible culti-

vars seeded early. Only later was colonization of resistant genotypes and plants from later seeding dates observed. Agrotriticum 3425 was usually colonized the least regardless of seeding date. Similar observations were made in 1998 even though overall disease severity was lower than in 1997. These observations combined with visual disease ratings suggest that resistance to *Cephalosporium* stripe may be a function of the ability of the host to retard colonization of the crown. It is unclear, however, if the resistance is a function of excluding the pathogen from the crown region through the root system or from the inability of the pathogen to successfully colonize the crown region from stem-base infections.

The timing and events involved in infection of field-grown winter wheat by *C. gramineum* have not been described previously. Use of the β -glucuronidase-transformed isolate of *C. gramineum* allowed specific detection of this pathogen within plant tissues making it possible to study the infection process under field conditions. Although specific events involved in resistance were not apparent, it appears that resistance in Agrotriticum 3425 is largely associated with limiting colonization. Further studies are needed to understand where the most important sites for resistance to *C. gramineum* occur. Such information would be useful both in understanding the biology of the disease and in identifying resistant genotypes more efficiently.

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