Colonization of Wheat Seedlings by Cephalosporium gramineum in Relation to Symptom Development

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ABSTRACT

Wheat seedlings (*Triticum aestivum* L., 'Genesee') inoculated with a conidial suspension of *Cephalosporium gramineum* were systemically invaded by the fungus as early as 3 days after inoculation. The development of chlorotic leaf stripe symptoms followed fungal invasion of leaf blades and sheaths by 5 to 7 days. The fungus, predominantly in conidial form, invaded only the proto- and metaxylem vessels of occasional vascular bundles. In such vessels, conidial germination and blastogenous reproduction were common, and acropetal movement of the fungus occurred. Leaf striping was initiated around the fungus-containing xylem vessels, and laterally encompassed numerous layers of fungus-free cells including several adjacent uninfected vascular bundles.

Prior to and in the early stages of leaf striping, the causal fungus was sparsely present in xylem vessels, and vascular occlusion was not apparent. During this time, healthy and diseased leaves showed equivalent capacities to conduct and accumulate dye solutions in their vascular bundles, but the lateral and intervenous transport of dye in diseased leaves was markedly impaired. The infected xylem vessels of prominently striped leaves frequently were occluded by conidial masses, and such leaves showed impaired conduction and accumulation of dye solutions. Thus, in *Cephalosporium*-infected wheat seedlings an initial interruption of lateral transport about infected vessels may contribute to leaf stripe formation. This dysfunction is distinct from the occlusion of the invaded xylem vessels which occurs later apparently as the result of fungus proliferation.

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The Cephalosporium stripe disease frequently affects winter wheat in the Great Lakes Region (12) and in the Pacific Northwest (1, 2, 8). The causal agent, *Cephalosporium gramineum* Nisikado & Ikata, is soil-borne and enters plant roots at sites of injury (2, 7, 8, 11). In cereals, the fungus becomes established as a vascular pathogen and causes a characteristic chlorotic and necrotic striping of leaf blades and sheaths (1, 2, 7).

Studies of *C. gramineum* as a wheat pathogen have been concerned with inoculation techniques (2, 10), survival in soil (3, 4, 5, 6), performance in culture (2, 7), and control through cultural practices (7, 8). Less attention has been given to pathogenesis and to the establishment of the fungus in wheat. In a single report, Spalding et al. (13) contend that vascular occlusion is a major factor in pathogenesis. These workers implied that fungal cells, a polysaccharide, and pectin plugs reduced or prevented flow in vascular streams. Pool & Sharp (9) partially characterized a polysaccharide from *C. gramineum* which when administered to wheat leaves reduced the intervenous transport of dye solutions. Hence, cellular impermeability (13) and impaired transport (9) may be involved in the development of leaf stripe symptoms.

While these earlier observations provided some initial insights into disease mechanisms in *Cephalosporium*-infected wheat, they largely reflect comparisons of healthy and visibly striped tissues

often collected from the field. The present study was primarily concerned with the establishment and effects of *Cephalosporium* in wheat at points in time prior to and during leaf stripe development. In order to clarify the movement, location, and prevalence of *Cephalosporium* in wheat in relation to symptom development, this study employed wheat seedlings in which stripe symptoms were induced with regularity following controlled inoculation with *C. gramineum*.

Cephalosporium has already been reported as a vascular pathogen based on its presence in conidial and mycelial form in the xylem of affected plants (2, 7). However, the rate and extent of host colonization by *Cephalosporium* prior to and during the production of visible symptoms was not elucidated. A knowledge of the distribution of *Cephalosporium* in wheat in relation to symptom development is essential to assess the involvement of diffusates in pathogenesis; i.e., to distinguish between proximal and distal effects of the pathogen. Likewise, precise explanations for the leaf striping phenomenon require a knowledge of the time during disease development when disease-associated events, like vascular occlusion, and/or impaired transport are first evident and significant.

MATERIALS AND METHODS.—Winter wheat seedlings, *Triticum aestivum* 'Genesee', were grown in a growth chamber in a steamed, compost-amended field soil. The chamber was operated continuously at 17-18 C. A mixture of incandescent bulbs and cool-white fluorescent tubes provided ca. 1,500 ft-c of light at plant level. Light and dark periods alternated and were of 12-hr duration.

Nine to 12 days after planting, the seedlings had developed two fully expanded leaves and were inoculated with conidial suspensions of an isolate of *Cephalosporium gramineum* Nisikado & Ikata collected from Michigan wheat. Since vernalized and nonvernalized seedlings of the Genesee cultivar show the same initial responses to infection by *C. gramineum*, the seedlings were not vernalized prior to inoculation. The nonvernalized seedlings, furthermore, offered the advantage of being more uniform in terms of morphology and physiological age than vernalized seedlings. *Cephalosporium* conidia to be used as inoculum were rinsed with distilled water from 7-day-old cultures of the fungus grown on potato-dextrose agar (PDA). Conidial concentration was adjusted to 10^6 or 10^7 conidia/ml based on hemocytometer counts and turbidity measurements at 400 nm.

The inoculum was either injected into plants at soil level or applied to roots. For injection, a syringe equipped with a No. 24 needle was used to puncture the seedlings at the soil line and deposit a droplet of inoculum in the wound created. This procedure introduced the fungus directly into the sheaths of the first and second leaves and into the third leaf approximately at midblade. The puncture injury itself induced no appreciable adverse effects on subsequent seedling growth and development. Plants to be root-inoculated were started in sterile sand and transplanted to soil when they had developed two fully expanded leaves. During the transplantation, approximately one-half of the root mass of each seedling was removed and the plants were placed momentarily in the conidial suspension. For both methods of inoculation, control plants were similarly treated with distilled water or with an autoclaved suspension of conidia. All data presented herein were obtained using root-inoculated seedlings unless specifically stated otherwise.

At intervals during a 4-week period following inoculation, the first, second, and third seedling leaves were harvested separately, examined for disease symptoms, and cut into 4- or 5-mm segments. *Cephalosporium* in the segments was detected by a culture plating method and by light and electron microscopy. Leaf segments to be plated were surface-sterilized in 0.15% sodium hypochlorite and placed on PDA. Counts of sections producing colonies of *C. gramineum* were made after incubating the cultures 10-14 days at room temperature.

Fresh leaf segments were sectioned using a Labline-Hooker plant microtome and stained with aceto-carmine or toluidine blue. Segments to be fixed were placed in 3% glutaraldehyde or 3% acrolein followed by 2% osmium tetroxide at pH 7.0. Fixed tissues were transferred to a graded ethanol series, to propylene oxide, and finally to ERL epoxy resin (14). Thin-sections of the embedded tissues were stained with toluidine blue and mounted in balsam for examination with a light microscope or stained with 0.5% uranyl acetate and 0.4% lead citrate for examination in a Zeiss EM 9A electron microscope.

Leaves to be used for measurements of dye uptake were excised below the blade with a wet scissors, and their cut ends placed immediately in a solution of 0.5% aqueous acid fuchsin in the growth chamber. The blades of such leaves were allowed to accumulate the dye during intervals up to 4 hr. After dye treatment the blades were harvested, cut into segments, dried overnight at 80-90 C, and extracted with water (50 mg leaves dry wt/20 ml) in a 1-min grind in a high speed blender. The ground mixture was filtered through Whatman No. 2 paper and the absorption of the filtrate measured at 542 nm, the absorption maximum, for acid fuchsin.

RESULTS.—Microscopic examinations and the development of colonies of *C. gramineum* from segments of inoculated seedlings showed the fungus to be widely distributed in wheat prior to symptom development. This preliminary dispersal of the fungus occurred in seedlings inoculated by either the stem puncture or root cut techniques. In root-inoculated plants the fungus traversed the rudimentary crown of the seedlings and was dispersed in leaf sheaths and blades beginning 4-6 days after inoculation. Visible leaf stripe symptoms on such plants were first apparent 10-12 days after inoculation (Fig. 1). During the 3rd week after inoculation, chlorotic leaf stripes were prominent and necrosis was initiated. Necrosis developed within the chlorotic stripes and progressed from leaf tip to base. Occasional leaf veins within the stripes became visibly darkened.

The puncture inoculations introduced the fungus

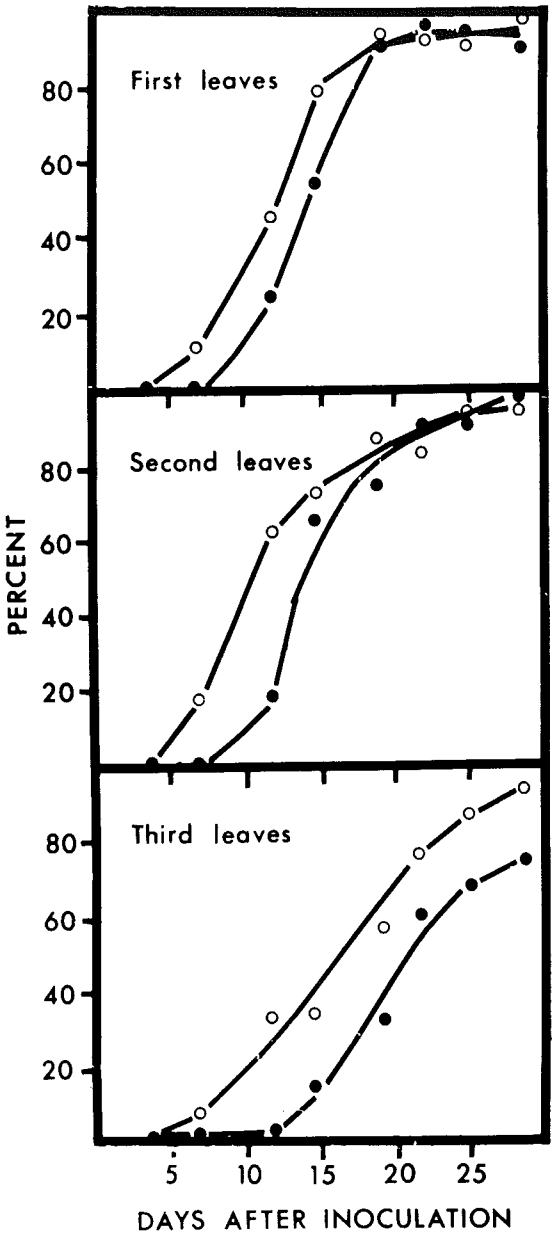

Fig. 1. Per cent of seedling wheat leaf blades containing *Cephalosporium gramineum* (-o-) and showing leaf stripe symptoms (-•-) after root inoculation. Percentages calculated from a minimum of 30 leaves.

directly into leaf sheaths, and hastened its detection in blades to as early as 2 days after inoculation. The development of visible symptoms in puncture-inoculated plants was likewise hastened to within 7-10 days. The progression and appearance of the fungus in leaves of puncture-inoculated plants was somewhat more uniform than in leaves of root-inoculated seedlings, but both methods of inoculation led to the production of equivalent and typical leaf stripe symptoms.

Leaf segments submitted to microscopic examination showed the fungus to be primarily in conidial form and located usually in one or two vascular bundles/leaf, where it inhabited only the proto- and metaxylem vessels (Fig. 2, 3). All other host cells, including those immediately adjacent to the infected vessels, were free of the fungus and remained so until leaf striping was well advanced.

Hyphal fragments were present in these vessels (Fig. 3, 4), but mycelial masses were nonexistent. The conidia in the host often appeared in stages of germination or blastogenous multiplication either from conidiophores (Fig. 5) or more often directly from existing conidia (Fig. 5, 6). Observations with the electron microscope made it possible to follow the accumulation of an electron-dense material on the outer walls of the fungus. This material eventually was liberated, and lined the walls of the infected xylem vessels (Fig. 7). In leaves bearing prominent symptoms, the material was readily detected in or on all cells comprising the vascular bundle.

The striping symptom, unlike the fungus itself, was not confined to vascular tissues but radiated from infected vessels to the upper and lower epidermis and to and beyond adjacent uninfected vascular bundles. The host cells comprising the stripe showed marked chlorosis, and later became necrotic and distorted. At no time did cells other than xylem harbor the fungus inter- or intracellularly.

The puncture inoculations at midblade or midsheath frequently resulted in the production of stripe symptoms above and below the point of inoculation. Although the fungus could be recovered from areas of the leaves several centimeters below the site of inoculation, it was not possible to demonstrate a redistribution of the fungus at the rudimentary seedling crown and its subsequent presence in noninoculated leaves.

Before stripe symptoms developed, it was possible to discern only occasional conidia in xylem vessels. While the fungus could be readily isolated from symptomless, inoculated leaves (Fig. 1), it was not microscopically in abundance in such tissues until stripe symptoms were evident. Once leaf striping was prominent, xylem vessels often appeared densely packed with conidia (Fig. 2, 3, 6). Vascular occlusion by materials other than fungal cells, like plugs of polysaccharide or pectin as previously reported (13), were not in evidence during the 4-week period of observation after inoculation.

In an attempt to further assess the extent of vascular occlusion in *Cephalosporium*-infected seedlings, the leaves of healthy and diseased plants were compared in tests of dye uptake. Conceivably, the quantity of dye accumulated and the pattern of its distribution in leaves might reveal vascular occlusions and possible transport dysfunctions not detected by microscopy.

When striped leaves were submitted to dye treatments, they distributed the dye irregularly (Fig. 8). Microscopic examinations of the dye-treated leaves revealed two distinct dysfunctions in dye transport in diseased leaves. Whereas healthy leaves

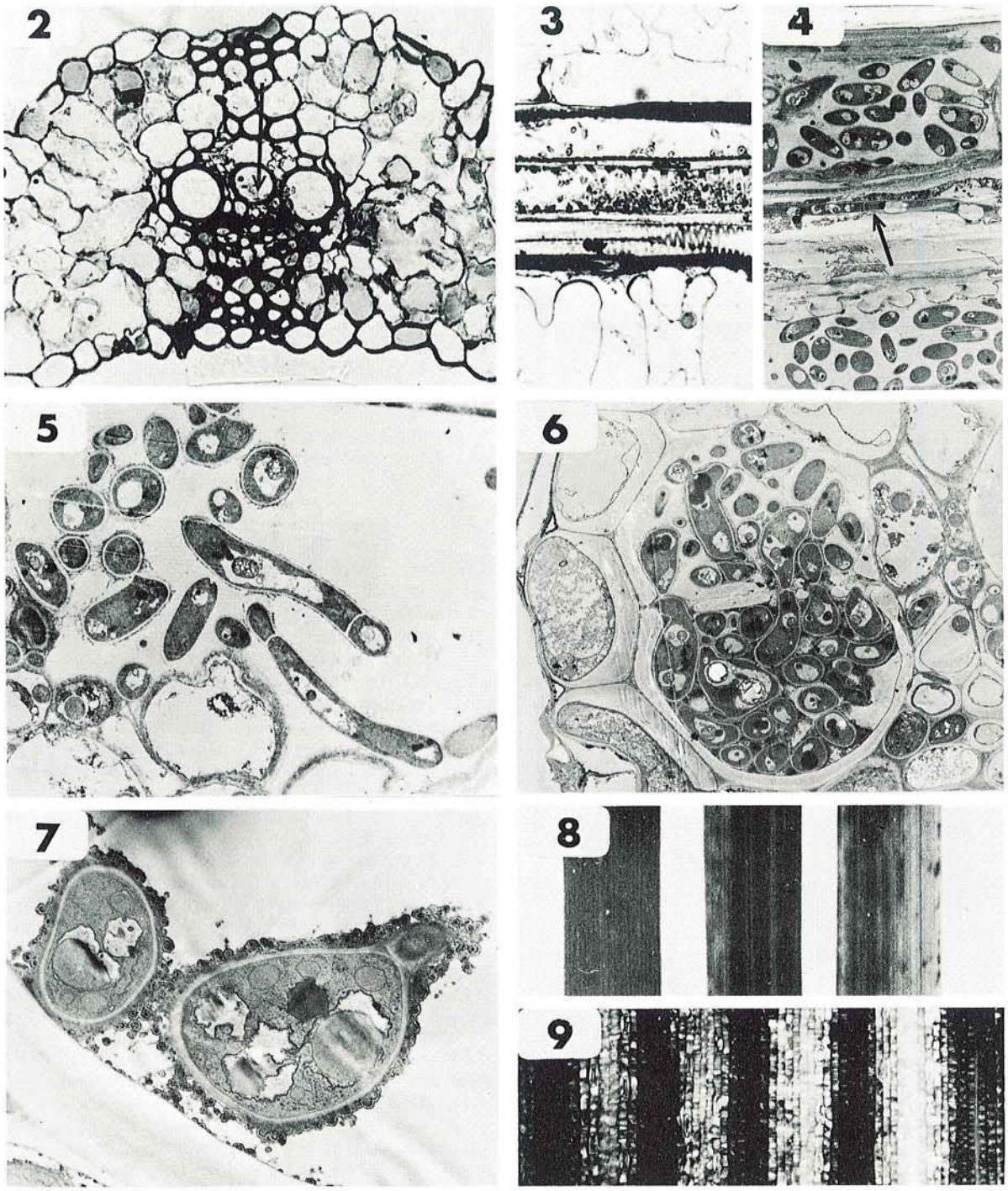

Fig. 2-9. 2-7) *Cephalosporium gramineum* in xylem vessels of wheat. 2) Cross section of an incipiently chlorotic portion of leaf blade showing fungus confined to a vascular bundle where it occupies proto- and metaxylem vessels (arrow). 3, 4) Light and electron micrographs, respectively, of xylem vessels in longisection revealing predominant conidial form of *Cephalosporium* in its seedling host. Arrow marks hyphal strand. 5) Conidial development from conidiophores and directly from existing conidia. 6) Conidial mass occluding the xylem in prominently striped leaf blade. Note budding conidia in top vessel. 7) Accumulation of electron-dense material on outer walls of *Cephalosporium* conidia and its deposition on host cell walls. 8, 9) Distribution of dye in healthy and *Cephalosporium*-infected leaf blades. 8) Uniform dye distribution in healthy leaf blade (left) and patterned dye distribution in symptomless (center) and incipiently chlorotic (right) diseased leaf blades. 9) Micrograph of dye-treated symptomless diseased leaf blade showing dark-colored dye accumulated in vascular bundles but not distributed intervenously.

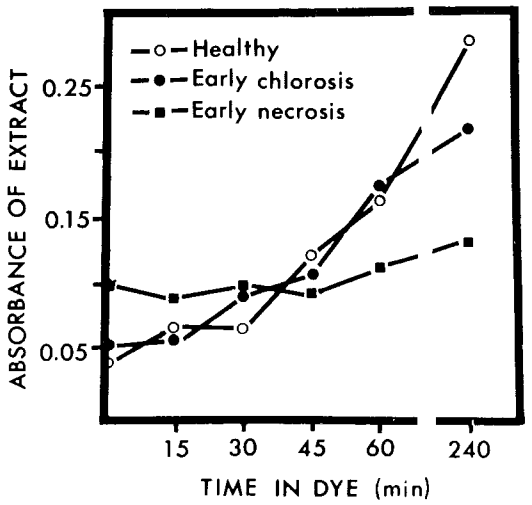

Fig. 10. Rate of dye accumulation in wheat leaf blades in different stages of symptom development.

invariably became totally and uniformly reddened by the acid fuchsin, diseased leaves showed intermittent lateral dye transport and limited or no conduction of dye in xylem vessels harboring masses of fungus. Leaves in different stages of symptom development, when submitted to dye treatment, showed these two transport dysfunctions well separated in time of occurrence. The interruption of lateral dye movement (Fig. 9) was apparent prior to stripe formation, as early as 3 days after inoculation, whereas occlusion of the main vascular bundles was not apparent until striping was prominent, and included only those vessels which harbored the fungus. The numerous fungus-free vascular bundles in diseased leaves appeared to conduct dye as readily as those in healthy leaves.

Striped leaves submitted to 4-hr dye treatments accumulated lesser quantities of dye than did healthy leaves (Fig. 10). However, there was little difference in the amounts of dye accumulated by healthy and nonnecrotic diseased leaves during the 1st hour of dye treatment when dye occupied the main veins. Times of dye treatment greater than 1 hr were necessary to fully visualize the interrupted lateral transport of dye in diseased leaves and to measure its effect on decreased dye accumulation (Fig. 10). All vessels of diseased leaves, whether invaded by the fungus or not, failed to conduct dye once surrounding tissues became necrotic.

DISCUSSION.—The dispersal of *Cephalosporium gramineum* in wheat seedlings can best be explained by its movement in conidial form in transpirational streams. While the fungus has been reported to produce abundant mycelium in wheat (2, 7), abundant conidia and only infrequent hyphal fragments were apparent in the seedling host. Mycelium, therefore, apparently is not necessary for systemic extension of the pathogen and production of leaf stripe symptoms, but may be formed in greater quantity in longer term infections such as occur in the field (2, 7).

As in an earlier study (2), *Cephalosporium* was most readily isolated from aboveground plant parts, but preferential distribution of the fungus to different leaves was not in evidence (Fig. 1). Apparently in traversing the rudimentary seedling crown, the fungus is afforded continuous and perhaps equivalent paths to both fully developed and expanding leaves. The predominant upward flow of transpirational streams may account for the depletion of *Cephalosporium* from roots and its accumulation in leaves. However, some bidirectional dispersal of the fungus did occur in this study as indicated by the development of symptoms and the presence of the causal fungus above and below the site of puncture inoculation. Since the basipetal movement observed did not account for dispersal of the fungus to remote plant parts, the phenomenon might be explained in terms of conidia being drawn bidirectionally by water columns broken at the time the leaves were punctured.

The localized presence of *Cephalosporium* in wheat leaves was a prerequisite for leaf stripe formation. Leaf striping cannot be considered, therefore, as a *Cephalosporium*-induced dysfunction originating in roots or in other plant parts. Rather, striping appears to result from a laterally induced dysfunction about infected xylem vessels. While the complete nature of this dysfunction requires further characterization, it involves, at least in part, an interruption of lateral transport between the main vascular bundles in wheat leaves (Fig. 8, 9).

The lateral extension of the leaf stripe reaction up to and beyond fungus-free vessels suggests further that pathogenesis involves a diffusible product from infected vessels whose diffusible range determines the limits of the leaf stripe symptom. It is conceivable, therefore, that a product emitted from *Cephalosporium*-invaded vessels capable of limiting the transport or utilization of nutrients and/or water by intervenous parenchyma cells would eventually bring about their chlorosis and death and, hence, leaf striping. In this light, Fig. 7 may represent visual evidence of such a diffusible product, and in essence supports the contention that fungal byproducts may be involved in pathogenesis (9, 13).

This study further suggests that the occlusion of the main vascular bundles in diseased leaves is of secondary importance in pathogenesis because of its delayed appearance. In this study, the occlusion of xylem appeared after stripe formation as a result of fungal proliferation. Like the occurrence of conidial masses, the occlusion of xylem by plugs of polysaccharide and pectic substances reported previously (13) may represent a product of prolonged infections.

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