Etiology

Interaction of *Fusarium avenaceum* and *Pseudomonas viridiflava* in Root Rot of Red Clover

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ABSTRACT

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Fusarium avenaceum and *Pseudomonas viridiflava* were inoculated separately or together into roots of red clover to determine if these pathogens interacted synergistically to cause more severe disease symptoms. When these pathogens were inoculated together into wounded roots of 4-wk-old plants grown gnotobiotically, they caused significantly longer rot lesions and more severe disease symptoms than when inoculated separately. Samples for microscopic examination were produced by the inoculation of 8-wk-old plants grown hydroponically on slant boards. Inoculations were made by introducing inoculum suspensions into severed lateral roots about 1.5 cm below the crowns. Examination of cross sections, made through the taproot at the juncture with the inoculated lateral root, showed that only minor tissue damage was caused by either organism inoculated alone, and that damage was confined mainly to the inoculated lateral root. Inoculation with both pathogens together resulted in the destruction of periderm, secondary phloem, vascular cambium, xylem parenchyma, xylem fibers, and some xylem vessels in the taproot. The organisms were intercellular and intracellular in the areas of severe tissue disruption. Histological evidence and in vitro tests indicated that the combined organisms degraded cellulose more extensively than could be expected from the individual organisms.

Additional keywords: root rot complex, synergism, *Trifolium pratense*.

The factor most limiting the continued productivity of forage legume stands is the destruction of the taproot by a complex of organisms. Fungal pathogens most frequently associated with this complex are *Fusarium* spp. (5,7,16); *F. avenaceum* (Fr.) Sacc. is the most common in red clover (*Trifolium pratense* L.) (5,16). These fungi colonize wound sites (16) on the roots, vary in virulence (14), and usually are found in close association with other pathogenic and saprophytic organisms in diseased roots.

In 1977, a survey of bacteria and fungi associated with root rots of alfalfa was completed in Pennsylvania. Many green, fluorescent pseudomonads were isolated, including a strain that produced a blue, extracellular pigment on yeast extract carbonate agar. In 1978, a similar bacterium was isolated from alfalfa (*Medicago sativa* L.) near the original sampling site, and in 1980, it was isolated from birdsfoot trefoil (*Lotus corniculatus* L.) at another site almost 200 km distant in Pennsylvania.

The bacteria isolated from alfalfa and trefoil were morphologically, biochemically, and physiologically identical to authenticated strains of *Pseudomonas viridiflava* (Burkholder 1930) Dowson 1939, 177AL (1,17,21). *P. viridiflava* is an opportunistic pathogen, causing leaf and fruit spots on a wide variety of plants (26,27) and root diseases on birdsfoot trefoil (18) and parsnip (9). Strains of *P. viridiflava* are known to degrade vascular tissues of *Vicia faba* L. and *Phaseolus vulgaris* L. (3,4).

The objective of our research was to define more clearly the role of *P. viridiflava* in the root rot complex and to evaluate whether this bacterium interacts synergistically with *F. avenaceum* to destroy plant tissue.

MATERIALS AND METHODS

General. Red clover was used as the host species because a strain of *F. avenaceum* with low virulence in red clover was available. Plants grown from breeder seed of Kenland red clover were used in all experiments. All plants were randomly distributed in a growth chamber, with 15 ± 1 C night and 25 ± 1 C day temperatures and a 15-hr daily photoperiod of incandescent and fluorescent light of 300 μE m^{-2} sec^{-1} intensity at plant height. All plants were watered with half-strength Hoagland solution (8). *F. avenaceum*, isolate 814 from red clover, and *P. viridiflava*, isolate 531 from alfalfa, were used in all experiments.

Macroscopic evaluation. All evaluations were made on plants grown in gnotobiotic conditions in slant-board units (12,25). Seed were surface disinfested with 2.5% sodium hypochlorite for 10 min, rinsed with sterile distilled water, and incubated for 48 hr on vegetable juice agar (20). Uncontaminated seeds were aseptically placed on 1% agar prepared with half-strength Hoagland solution (8) in large, cotton-stoppered glass tubes. When the plants were 2 wk old, they were aseptically transferred to sterile slant-board units (11), which then were placed in a growth chamber. The four plants in each unit were allowed to grow for 2 wk before inoculation.

Fungal inoculum consisted of minicultures grown on autoclaved, 1-cm-long pieces of braided nylon fishing line placed on the surface of potato-dextrose agar (PDA). Cultures were maintained at 21 ± 1 C in the dark for 1 wk to enhance vegetative growth and minimize sporulation. Bacterial inoculum was cultured on King's medium B agar (13) in ambient laboratory conditions (about 22 C) for 24 hr. Bacterial culture slime was used as inoculum because previous use of washed bacterial cells yielded inconsistent results.

Primary roots were inoculated, about 1 cm below the crown, by placing a piece of fishing line beneath the root and stabbing through the root with a dissecting needle into the fishing line. Treatments consisted of fishing line carrying no organisms, fungal mycelium, bacterial slime, or both organisms, and controls consisted of pieces of fishing line that had been autoclaved and maintained on PDA before use. Bacterial slime was coated onto sterile pieces of fishing line just before inoculation. Four plants on each slant board received the same treatment and constituted one replication. Two replications were used in each of three trials. An analysis of variance was done on data from each trial. Homogeneous error variances permitted data to be pooled for an overall analysis of variance.

Plants were evaluated for severity of root rot and foliar symptoms 7 days after inoculation. The length of individual, external root rots was measured, and foliar symptoms were rated on a scale of 1 to 5, with 1 = no symptoms, 2 = cotyledons wilted, 3 = cotyledons dead and unifoliolate leaf chlorotic, 4 = plant wilted and unifoliolate leaf dead, and 5 = plant dead.

Microscopic evaluation. Tissue samples for histological examination were obtained from plants grown in conventional plant boards. Plants were started from seed in moist vermiculite, transplanted to slant boards when 2 wk old, and grown an additional 4 wk before inoculation. Four plants were grown in each slant-board unit.

Fungal inoculum was produced on vegetable juice agar (20) at 21 ± 1 C with 50 μE m^{-2} sec^{-1} of continuous fluorescent light to enhance conidial production. Bacterial culture was as described in the macroscopic evaluation section.

Roots were inoculated by the uptake of conidia, bacterial cells, or combined pathogen suspension into a severed lateral root. A lateral root located about 1 cm below the crown was severed with a flamed scalpel about 2 cm from the taproot. The attached portion of the severed root was immersed for the duration of the test in a glass vial (28 × 2 mm ID) containing about 40 μl of liquid. Conidia were rinsed from culture plates and used in an aqueous suspension containing 6×10^5 spores/ml as estimated with a hemacytometer. Bacterial suspensions were prepared by washing cells twice with sterile tap water, with a final suspension containing 1×10^9 cells/ml, as determined by light transmission in a spectrophotometer. Combined inoculum was prepared by adjusting the suspension to 3×10^5 fungal spores plus 0.5×10^9 bacterial cells. Controls consisted of sterile tap water.

Sampling involved harvesting four replications, consisting of one plant per treatment, at 3, 5, 7, 10, 13, and 15 days after inoculation for a total of 96 plants. Plants were sampled by excising the crown and the adjacent 20 mm of taproot, which included the inoculation site. The tissue was surface disinfested in 0.5% sodium hypochlorite for 5 min, drained on paper towel, and aseptically divided into four sections. The apical 2-mm section was placed on Nash medium (22) for the isolation of *F. avenaceum*, and the basal 2-mm section was placed in 0.5% phosphate buffer at room temperature until buffer samples were streaked on King's medium B and yeast dextrose carbonate agar (24) and assayed for oxidase activity (19) to isolate and identify *P. viridiflava*. The remaining two 5-mm sections were fixed in Formalin-acetic acid-alcohol (10). After fixation, histological specimens were dehydrated in a standard tertiary butyl alcohol series (10) and embedded in Paraplast-Plus (Monoject Scientific, St. Louis, MO). Radial and longitudinal, serial, 10-μm-thick sections were cut on a rotary microtome, affixed to chemically cleaned slides with Haupt's adhesive, and stained with either Johansen's quadruple stain or Harris hematoxylin and orange G stain (10). Specimens were examined under a light microscope. Tests were performed to detect pectin by the iron absorption method, cellulose by the zinc-chlor-iodide test, and birefringence in polarized light (23).

In vitro cellulase assay. The ability of the fungus and the bacterium to degrade cellulose, when grown as separate or combined cultures, was determined with Fernley's (6) cellulose-azure method. Organisms were grown on carnation extract medium (2), with alfalfa leaves and stems in place of carnation. The medium was prepared by comminuting 100 g of fresh alfalfa leaves and stems with 400 ml of distilled water in a blender at high speed for 10 min. The suspension was filtered through four layers of cheesecloth, and the filtrate was diluted to 1 L with distilled water. This was adjusted to pH 7.0 with 1 N NaOH and dispensed into 250-ml baffled Erlenmeyer flasks in 50-ml amounts. The flasks and medium were autoclaved for 20 min at 121 C.

Treatments included control, bacterium, fungus, and bacterium plus fungus, and there were three replicate flasks per treatment. Eight of the flasks were infested with several mycelial fragments of

TABLE 1. Root rot and foliar disease ratings of red clover plants grown under gnotobiotic conditions after inoculation with *Pseudomonas viridiflava* and *Fusarium avenaceum*

Inoculation treatment	Root rot length (mm)	Foliar symptom disease rating[a]
Control	1.0 A[b]	1.0 A
P. viridiflava	1.0 A	1.0 A
F. avenaceum	1.2 A	1.1 A
P. viridiflava + *F. avenaceum*	10.0 B	3.6 B

[a] Rating system: 1 = no symptoms; 2 = cotyledons wilting; 3 = cotyledons dead, unifoliolate leaf chlorotic; 4 = plant wilting, unifoliolate leaf dead; 5 = plant dead.

[b] Means in columns followed by the same letter are not significantly different based on an *F*-test of orthogonal contrast effects at $P = 0.05$.

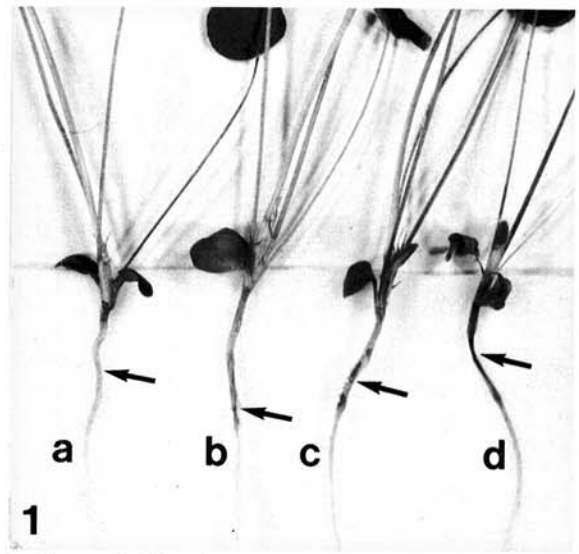
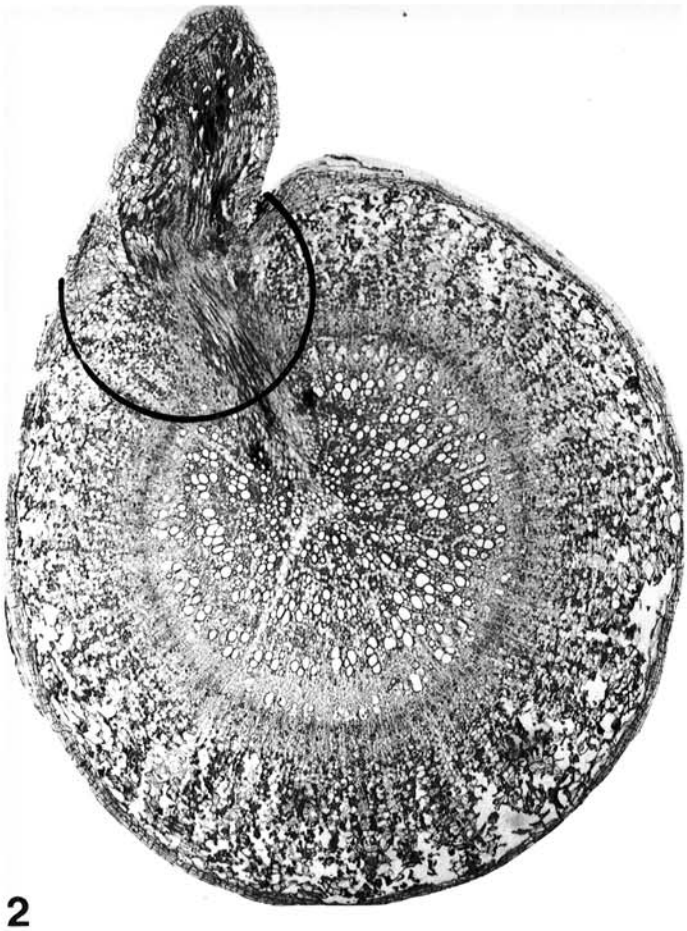


Fig. 1. Five-week-old red clover plants grown gnotobiotically and inoculated with: **A,** no pathogen; **B,** *Pseudomonas viridiflava*; **C,** *Fusarium avenaceum*; **D,** *P. viridiflava* and *F. avenaceum*. Note discoloration around wound/inoculation site (arrows) and condition of cotyledons.



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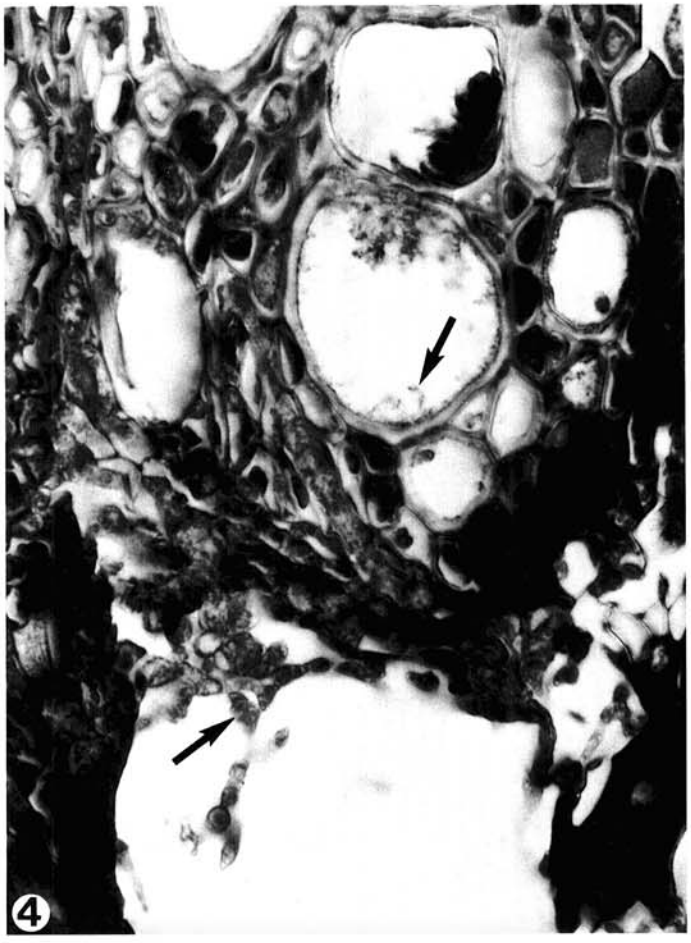

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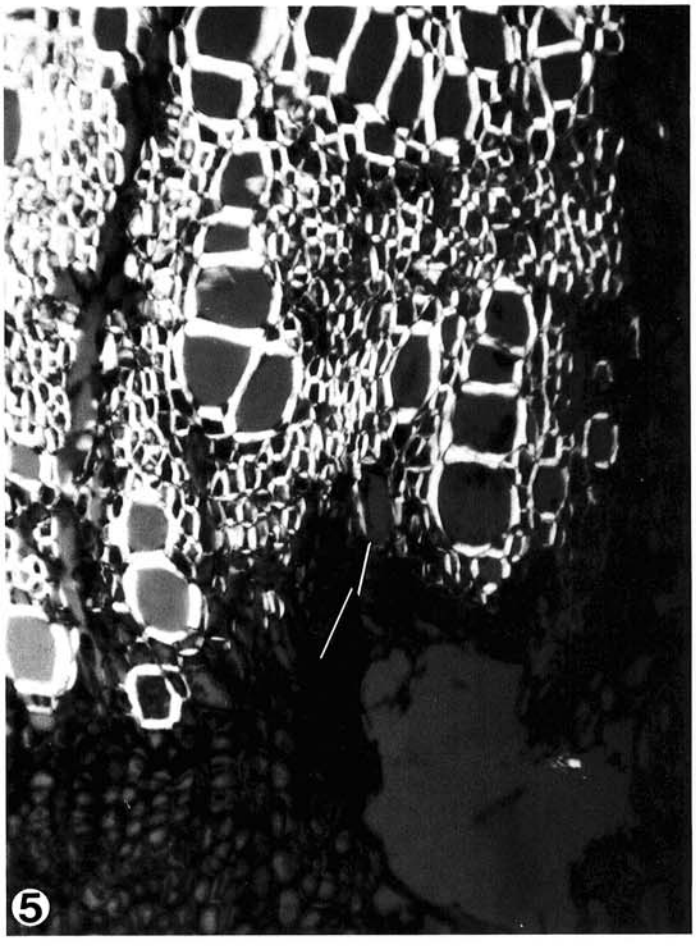

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F. avenaceum from a 5-day-old culture and incubated on an orbital shaker at room temperature (about 20 C). After 4 days, four flasks containing the fungus and four sterile flasks were infested with a suspension of washed bacterial cells from a 24-hr-old culture of *P. viridiflava* (the cells were washed twice with a phosphate buffer, pH 7.0, before use). Flasks with the added bacteria were incubated for 2 days, and then all cells and mycelia were harvested. The liquid was filtered through a milk-filter pad and centrifuged at 20,000 *g* for 15 min. Eighteen milliliters from each of the pooled supernatants was mixed with 2 ml of a 0.2 M citric acid/0.4 M Na_2HPO_4 buffer (pH 6.1). Any precipitate was removed by centrifugation at 20,000 *g* for 30 min. The resultant solutions were stored in a freezer and thawed before use. The cellulose-azure suspension (#C8647, Sigma Chemical Co., St Louis, MO) was made up in a 0.02 M citric acid/0.04 M Na_2HPO_4 (pH 6.1) buffer (240 mg/100 ml). Culture filtrates and cellulose-azure solutions were mixed 1:1 and shaken together at 27 C for 60 min.

Color yields from the dyed cellulose were measured by stopping the reactions with 5 ml of 2.4 M K_2HPO_4 adjusted to pH 9.0 with HCl and centrifuging at 1,500 *g* for 20 min. Color densities were measured with a spectrophotometer at 550 nm. The experiment was repeated once.

RESULTS

Macroscopic evaluation. Results of three trials were similar, and pooled data are presented in Table 1. Typical disease symptoms produced in this system are shown in Figure 1. Only slight discoloration of the roots and almost no foliar disease symptoms were caused by the pathogens inoculated independently. However, the combined pathogens caused significant increases in length of root rots and foliar disease ratings, and the degree of increase indicated a synergistic interaction. In all cases, only the expected organisms were isolated from inoculated tissues and none were isolated from the controls.

Microscopic evaluation. Histological observations supported the gnotobiotic experiment results. The general root location in which observations were made is shown in Figure 2. *P. viridiflava*, when inoculated without *F. avenaceum*, was unable to cause extensive cell breakdown in the taproot. The bacterium colonized the xylem vessel elements of the taproot in the region from which the inoculated lateral root emerged but caused no cell dissolution. Xylem vessel elements were frequently plugged with material that tested positive for pectin. Similarly, *F. avenaceum* inoculated alone was incapable of causing cell dissolution in the taproot. The fungus was confined to the xylem vessels, which were frequently occluded with material staining positive for pectin. In both cases, there was only a very slight reduction in cell-wall birefringence in the xylem vessels colonized by either pathogen, an indication that, separately, the two organisms have very limited abilities to degrade cellulose.

When the two pathogens were simultaneously inoculated into the lateral root, extensive cell destruction occurred in the taproot in the area from which the lateral root emerged (Fig. 3). The pathogens were not restricted to the xylem vessels, as was the case when inoculated separately, but caused dissolution of the periderm, secondary phloem, vascular cambium, xylem parenchyma, xylem fibers, and some vessel elements (Fig. 3). *F. avenaceum* was both intercellular and intracellular in the area of massive destruction (Fig. 4) and infrequently was observed in xylem vessel elements, but *P. viridiflava* commonly was observed in the xylem vessel lumens as well as extracellularly in the areas of cell dissolution. Both the zinc-chlor-iodide test and polarized light birefringence indicated that cellulose was eroded in the xylem vessel elements and completely degraded in the xylem parenchyma cells (Fig. 5). Pectic materials also were reduced in the area of massive cell destruction.

TABLE 2. Changes in absorbance at 550 nm due to enzymatic degradation of azure cellulose by culture filtrates of *Pseudomonas viridiflava* and *Fusarium avenaceum* grown alone and in combination at 27 C

Filtrate	Absorbance units (× 100) Run 1	Run 2
Control (no organism)	0.0[a]	0.0[a]
Bacterium	−2.0	2.2
Fungus	−1.0	3.5
Bacterium + fungus	4.5	10.0

[a] Means of determinations for three flasks.

In vitro cellulase assay. The observation of a greater amount of cellulose degradation in vivo when the two pathogens were coinoculated was supported in the experiments with culture extracts and dyed cellulose to detect and quantify the production of cellulase (Table 2). When filtrates from the mixed cultures were tested, they contained more than double the amount of cellulase activity than the filtrate from either pathogen grown alone for the same period of time.

DISCUSSION

In red clover, *P. viridiflava* and *F. avenaceum* together caused more severe root rot and foliar disease symptoms and destroyed more tissues than was expected on the basis of either organism's individual activity. In addition, the results of an in vitro cellulase assay indicated that, in combination, these organisms possessed an enhanced capability to degrade cellulose. The disease macrosymptoms and microsymptoms and the cellulase assay all indicate a synergistic relationship between *P. viridiflava* and *F. avenaceum*.

Other strains of *P. viridiflava* have induced necrosis and destroyed vascular tissue and adjacent cells of other legumes (3,4). Failure to obtain similar results with our strain #531 could have resulted from differences in enzymic capabilities among strains, inoculation techniques, the number of bacterial cells present in the tissues, or the host tissue vulnerability.

The destruction of roots of forage legumes traditionally has been attributed to fungal activity, and the pathogenicity of several fungi has been demonstrated (7,14,15,16). Seldom are surveys designed to identify organisms other than fungi, and usually bacteria that are detected are treated as contaminants. We believe that *P. viridiflava* can play an active role in the root rot complex of forage legumes, and it is likely that, with more selective isolation and more controlled pathogenicity testing, a greater involvement of bacteria in the root rot complex of forage legumes will be demonstrated.

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Figs. 2–5. 2, Cross section through the taproot of a red clover plant in the zone of lateral root juncture. Circled area is that in which micrographs for Figures 3, 4, and 5 were taken (28.8 ×). **3,** Cross section through a taproot of red clover simultaneously inoculated with *Pseudomonas viridiflava* and *Fusarium avenaceum* via a lateral root. Note extensive dissolution of tissues extending beyond limits of vascular elements (69 ×). **4,** Close-up of portion of Figure 5, showing *P. viridiflava* and *F. avenaceum* (arrows). Note the extensive cell breakdown and the reduced thickness of some xylem parenchyma cell walls (617 ×). **5,** Portion of cross section through a taproot of red clover simultaneously inoculated with *P. viridiflava* and *F. avenaceum* viewed with polarized light. Loss of birefringence (arrow) indicates cellulose degradation (240 ×).

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