Interaction Between *Fusarium oxysporum* f. sp. *medicaginis* and *Corynebacterium insidiosum* in Alfalfa

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ABSTRACT

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The development of *Corynebacterium insidiosum* was adversely affected by the presence of *Fusarium oxysporum* f. sp. *medicaginis* in the field, the glasshouse, and in vitro. In the field, bacterial wilt symptoms were less severe in roots of alfalfa plants inoculated with a mixture of *C. insidiosum* and *F. oxysporum* than in plants inoculated with *C. insidiosum* alone. This effect was greater in cultivars susceptible to bacterial wilt than in resistant cultivars. In the glasshouse, *C. insidiosum* developed more slowly in alfalfa roots inoculated with a mixture of both pathogens than in roots inoculated with *C. insidiosum* alone. Only occasional bacteria were found in various cells of vascular bundles when vessel elements contained *F. oxysporum*. Eighteen of 19 *Fusarium* isolates including *F. oxysporum* f. sp. *medicaginis*, killed before being placed on agar media seeded with *C. insidiosum* or *Rhizobium meliloti*, inhibited the development of both bacterial species. The sterile filtrates of four isolates of *F. oxysporum* f. sp. *medicaginis* grown in a nutrient broth in still and in shake culture were added to agar medium seeded with *C. insidiosum*. Filtrates from the still cultures (mostly mycelium) inhibited *C. insidiosum*, but filtrates from the shake cultures (mostly conidia) had no effect on the bacterial growth. *F. oxysporum* apparently produces a substance that inhibits *C. insidiosum* and reduces bacterial wilt severity in alfalfa plants.

Additional key words: *Medicago sativa*.

Fusarium wilt (caused by *Fusarium oxysporum* Schl. f. sp. *medicaginis* [10]) and bacterial wilt (caused by *Corynebacterium insidiosum* (McCull.) Jens. [4,5]) are important alfalfa diseases that can cause stand losses. These diseases and their causal agents have long been studied individually (6,11), but little is known about their relationship when both are present in the same plant. Frosheiser and Barnes (3) studied field-grown alfalfa inoculated with a mixture of these two pathogens and found that symptoms of bacterial wilt were readily distinguishable from those of Fusarium wilt. The bacterial wilt symptoms were less severe when plants were inoculated with a mixture of *F. oxysporum* and *C. insidiosum* than when inoculated with *C. insidiosum* alone. Resistance to one of the pathogens was not correlated with resistance to the other. The study also suggested that *F. oxysporum* might inhibit *C. insidiosum* or have some type of competitive advantage. In in vitro studies, *Fusarium* inhibited the development of many *Rhizobium* spp. (1,2). The inhibition was ascribed to an antibiotic substance. An antibiotic, enniaten, described as a mycobactericide, was isolated from several *Fusarium* spp. (7); however, the effect of this antibiotic on *C. insidiosum* is unknown.

The objectives of this study were to investigate the interaction between *F. oxysporum* and *C. insidiosum* during disease development in alfalfa in the field, in the greenhouse, and in vitro and to determine if *F. oxysporum* produces a substance in vitro that is inhibitory (antibiotic) to *C. insidiosum*.

MATERIALS AND METHODS

Disease development in the field. Three isolates of *F. oxysporum* f. sp. *medicaginis* (BW7, 7F1, and 31F3), obtained from diseased alfalfa roots collected in Minnesota and maintained in autoclaved sandy loam soil, were increased by adding a few infested soil particles to a sterile nutrient broth and incubating it on a shaker for 4 days at approximately 23 C (3). Abundant microconidia and few hyphal fragments were produced. The isolates were mixed and diluted to approximately 1.5×10^6 microconidia per milliliter, as estimated with a hemacytometer.

Alfalfa root tissue infected with *C. insidiosum* was used to prepare the bacterial wilt inoculum. The infected roots were washed, ground, and stored in plastic bags at −18 C. Frozen roots were soaked in tap water (50 g/L) for about 30 min with frequent

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agitation, then the inoculum was strained through cheesecloth to remove the plant material.

The combined inoculum was prepared by mixing the undiluted microconidial suspension of *F. oxysporum* with an undiluted bacterial suspension. Each pathogen in the combined inoculation was at the same dilution as in single inoculation preparation.

Three alfalfa cultivars and one experimental line were chosen for this experiment: Agate, resistant to both pathogens; Glacier, susceptible to both; U.C. Salton, resistant to *Fusarium* but susceptible to the bacterial pathogen; and experimental line MnPL-4, which is susceptible to *Fusarium* but resistant to the bacterial pathogen.

The alfalfa seedlings were grown in glasshouse benches filled with steamed sand. In mid-June the seedlings (~10 wk old) were dug and their roots were washed in tap water and immersed in the desired inoculum for 20–30 min. Plants receiving the same inoculum treatment were grouped together, their tops and roots were clipped, wrapped in paper towels, and refrigerated overnight until transplanted into the field at St. Paul and Rosemount, MN. The plants were transplanted about 20 cm apart in rows spaced 1 m apart. This study was organized as a split-plot design. Main plots were three inoculation treatments (*F. oxysporum* alone, *C. insidiosum* alone, and a mixture of both pathogens). Subplots were the four alfalfa entries. The study was replicated four times at each location with 50–70 plants per plot.

The number of living plants was recorded 2 wk after transplanting and about every 3 wk thereafter during the summer. Surviving plants were dug in mid-September. The taproot of each plant was sectioned and rated for severity of both Fusarium wilt and bacterial wilt on the basis of stele discoloration. Fusarium wilt was characterized by a reddish-brown discoloration, usually confined to vascular bundles within the stele. Bacterial wilt was characterized by a tan discoloration that was not limited to any particular tissue within the stele. The disease severity ratings were based on a scale of 0–5: 0 = no discoloration, 1 = small dark strands in the stele, 2 = small discolored areas on the cut surface, 3 = partial ring of discolored tissue under cortex, 4 = complete ring of discolored tissue under the cortex, and 5 = stele completely discolored or plant dead. Disease severity indexes (DSI) (average of disease severity scores) were calculated for each entry. The statistical significance of the data was determined by analysis of variance.

Early disease development in the glasshouse. *F. oxysporum* f. sp. *medicaginis* isolate BW7 was isolated, cultured, and inoculum prepared as described above except that the inoculum was diluted to 10^4 microconidia per milliliter. *C. insidiosum* inocula were prepared from infected root tissue (as described above) and from a pure culture of the bacterium maintained on beef lactose agar. Each bacterial inoculum suspension was diluted to about 10^5 cells per milliliter. Each pathogen in the combined inoculum was at the same dilution as in single inoculum preparations.

Seedlings of cultivar Glacier were grown in 15-cm-diameter pots (five plants per pot) containing 3 cm of sand on sandy loam soil. Approximately 12 wk after planting, the plants were clipped leaving a 3–4 cm stubble. The crowns and upper taproots of the plants were exposed by removing the sand from the soil surface, washed with 70% ethanol and sterile water, and the roots were wounded by scratching them once with a sterile needle. Either 0.25 ml of sterile water, or about 2.5×10^4 cells of *C. insidiosum*, 2.5×10^3 microconidia of *F. oxysporum*, or the two combined were placed onto the wound and the sand was replaced in the pot. One hundred fifty plants received each treatment.

At 4, 7, 14, 21, and 30 days after inoculation, roots of 30 plants from each inoculation treatment were rated for disease severity according to the amount of each type of stele discoloration. Statistical significance of the data was confirmed by analysis of variance. Twenty of the roots were prepared for examination by light microscopy to determine the location of the pathogen(s). These roots were fixed in formalin-acetic acid-alcohol (FAA), rinsed with water, and hand-sectioned at 4- to 5-mm intervals beginning at the site of the inoculation and extending to the root apices. Sections were stained with 0.05% toluidine blue O in 0.1 M phosphate buffer (pH 7.0) (8). Additional sections were stained with Gram stain (9). Sections stained by either method were examined immediately with a light microscope at ×900. Five of the remaining 10 roots were prepared for scanning electron microscopy to examine the appearance of the pathogen(s). These roots were fixed with FAA, rinsed with water, dehydrated in a graded acetone series, dried in a critical-point drier, 5-mm segments were mounted on stubs, coated with gold-palladium in a vacuum evaporator, and examined with a Philips 500 scanning electron microscope (Philips, Eindhoven, The Netherlands). The five remaining roots were surface sterilized and segments were placed on either beef lactose agar (BLA) or acidified potato-dextrose agar (APDA) to culture the microorganisms present in the stele.

Pathogen interaction in vitro. Fourteen isolates of *F. oxysporum*, one isolate each of *F. moniliforme* Sheld. emend. Snyd. and Hans., *F. solani* (Mart.) App. and Wr. emend. Snyd. and Hans., *F. roseum* (Lk.) emend. Snyd. and Hans., and two isolates of *F. tricinctum* (Cda.) emend. Snyd. and Hans. were grown over pieces of sterile filter paper laid on the surface of nutrient broth-yeast agar (NBYA), potato-dextrose agar (PDA), beef lactose agar (BLA), or yeast extract agar (YEA). After 4 days, the fungus on the filter paper was killed with ether. Half of the filter paper pieces bearing fungus (hereafter called "filter paper"), chosen at random, were autoclaved for 20 min. Autoclaved or nonautoclaved filter paper pieces were placed on NBYA, PDA, or BLA that contained 10^6 cells of *C. insidiosum* or on YEA that contained 10^6 cells of *Rhizobium meliloti* Dang. The filter paper was always placed on the same kind of medium that it was on when the fungus grew onto it. The experiment was done four times with three plates per treatment each time. Sterile filter paper, untreated or treated with ether, placed on the inoculated media, served as the control. After 10 days, the agar surface in the petri plates was examined for the development of zones of inhibition around the filter paper.

Four isolates of *F. oxysporum* (LJ, BW7, 7F1, and 31F3) from alfalfa, previously tested for ability to inhibit *C. insidiosum* and *R. meliloti* in vitro, were incubated individually for 4 days in sterile nutrient broth (3) in still and shake cultures. The cultures were then centrifuged at 2,310 *g* for 15 min to remove the fungus. The supernatant was decanted and filtered through a 0.1-μm Millipore filter to remove any suspended mycelium and conidia. Part of the filtered supernatant was autoclaved for 20 min. One milliliter of autoclaved or nonautoclaved sterile supernatant from each *F. oxysporum* culture was added to petri dishes containing 10 ml of BLA with 10^6 cells of *C. insidiosum*. This experiment was replicated four times. As a control, 1 ml of sterile nutrient broth was added to petri dishes containing 10 ml of BLA inoculated with 10^6 cells of *C. insidiosum*.

RESULTS

Disease development in the field. Data from the two locations were pooled after an analysis of variance indicated that differences due to locations were not significant.

Within 3 mo, 20–62% of plants inoculated with *F. oxysporum* were dead (Table 1). More than 90% of these died within 5 wk after transplanting. In contrast, only 2–4% of plants inoculated with *C. insidiosum* were dead within 3 mo and these began dying 9 wk after transplanting (Table 1). Reacting like plants inoculated with *F. oxysporum*, 22–67% of plants inoculated with the combined inoculum died within 3 mo after transplanting and most of these died within 5 wk. Generally, their roots had the dark-brown localized vascular discoloration typical of Fusarium wilt (Table 1).

Since symptoms of Fusarium wilt and of bacterial wilt were easily distinguishable, each root was rated individually for each disease. The DSI for Fusarium wilt symptoms was similar for plants inoculated either with the combined inoculum or with *F. oxysporum* alone. However, the DSI for bacterial wilt symptoms was much lower in plants inoculated with the combined inoculum than with *C. insidiosum* alone (Table 1). Roots inoculated with a single pathogen usually showed only the symptoms of the associated disease indicating that cross-contamination between diseases was negligible.

TABLE 1. Disease severity index (DSI) and percentage of dead plants in alfalfa entries in the field 3 mo after inoculation with *Fusarium oxysporum*, *Corynebacterium insidiosum*, or the two pathogens combined

Entry	DSI[a] after inoculation with:				Dead plants per treatment		
	F. oxysporum	*C. insidiosum*	Combined inoculum				
	Fusarium wilt symptoms[b]	Bacterial wilt symptoms[b]	Fusarium wilt symptoms[b]	Bacterial wilt symptoms[c]	*F. oxysporum* (%)	*C. insidiosum* (%)	Combined (%)
Agate	2.18	1.46	2.18	0.63	20	3	31
Glacier	3.17	3.53	3.35	1.32	62	4	67
MnPL-4	3.82	1.53	3.63	0.75	54	3	60
Salton	2.09	3.16	1.97	1.64	23	2	22
Average	2.82	2.42	2.78	1.09	40	3	45
LSD $P = 0.05$	0.85	1.11	0.86	0.49	21	1	22

[a] Average disease severity rating based on a 0–5 scale. 0 = No internal root discoloration, 5 = stele entirely discolored or plant dead. Each value is an average of 50–70 plants in each of eight replications.
[b] Based on total plants established.
[c] Based on plant surviving 3 mo.

Early disease development in the glasshouse. Bacterial wilt progressed at the same rate in roots of Glacier alfalfa inoculated with either *C. insidiosum* prepared from infected roots or from a pure culture, indicating that contaminants present in the inoculum prepared from roots did not change symptom expression. Data from these two treatments were therefore combined. Bacterial wilt symptoms, in plants inoculated with *C. insidiosum* alone, developed slower than did symptoms of Fusarium wilt. The DSI for bacterial wilt 30 days after inoculation was approximately half of that observed after 3 mo in the field study (Tables 1 and 2). None of the plants wilted or died during the 30-day study.

Fusarium wilt symptoms, in plants inoculated with *F. oxysporum* alone in the glasshouse, developed at rates comparable to those observed in the field (Tables 1 and 2). Some plants wilted within 21 days after inoculation and 40% had died within 30 days after inoculation.

Symptoms of Fusarium wilt in plants inoculated with the combined inoculum developed at the same rate as in those inoculated with *F. oxysporum* alone. Thirty-eight percent died within 30 days after inoculation. However, bacterial wilt symptoms in plants inoculated with the combined inoculum developed at less than half the rate for plants inoculated with *C. insidiosum* alone (Table 2).

When healthy alfalfa roots were examined by light microscopy after staining with toluidine blue O, walls of vessel elements were turquoise blue, vascular parenchyma cells were violet, and fibers were white to pale blue. In roots examined by electron microscopy, vessel elements appeared to be empty except for a few starch grains, which may have been introduced during section preparation (Fig. 1).

Roots inoculated with *F. oxysporum* alone, examined by light microscopy after staining with toluidine blue O, had infected dark-brown vascular bundles containing fungal mycelium stained blue violet. Upon examination by scanning electron microscopy, mycelium was found in the vessel elements, sometimes filling them. Microconidia were seen in these mycelium-filled elements (Fig. 2). Mycelium was found inside vessel elements only until the plants were moribund, then it was observed in other cell types within the stele.

Roots inoculated with *C. insidiosum* alone, examined by light microscopy after staining with toluidine blue O, had infected tan to light-yellow vascular bundles containing bacteria staining blue to dark blue. The identity of the bacteria was confirmed by staining an adjacent section with Gram stain (9); only Gram-positive bacteria were seen in these sections. In roots examined by scanning electron microscopy, bacteria were found in vessel elements (Fig. 3), vascular parenchyma, and fibers throughout diseased tissue.

Roots inoculated with the combined inoculum, examined by either light- or scanning-electron microscopy 4 days after inoculation, sometimes contained both pathogens within the same vessel element (Fig. 4). However, at later dates, the two organisms were not closely associated and 30 days after inoculation bacteria were usually found only in vascular bundles devoid of mycelium.

TABLE 2. Disease severity index (DSI) for Glacier alfalfa roots in a glasshouse 4–30 days after inoculation with *Fusarium oxysporum*, *Corynebacterium insidiosum*, or the two pathogens combined

Days after inoculation	DSI[a] inoculation treatment			
	Fusarium wilt symptoms		Bacterial wilt symptoms	
	F. oxysporum[b]	Combined[c,d] inoculum	*C. insidiosum*[c]	Combined[c,e] inoculum
4	1.00	0.90 NS	0.30	0.31 NS
7	1.00	1.00 NS	0.59	0.35 *
14	1.60	1.65 NS	0.87	0.49 *
21	2.80	2.61 NS	1.58	0.56 *
30	3.65	3.55 NS	1.60	0.80 *

[a] Average disease severity based on a 0–5 scale, 0 = No internal root discoloration, 5 = stele entirely discolored or plant dead. DSI in plants treated with sterile water ranged from 0.20–0.30.
[b] Each value is the average of 30 plants.
[c] Each value is the average of 60 plants.
[d] Data compared for significance with roots inoculated with *F. oxysporum*. NS indicates no significant difference.
[e] Data compared for significance with roots inoculated with *C. insidiosum*. NS indicates no significant difference, * indicates a significant difference, $P = 0.05$.

The difference in color of the plant tissues due to both the plant reaction to infection and to staining aided in determining the location of the two organisms. Vascular bundle discoloration by *F. oxysporum* was found 2–3 mm behind the furthest advance of the fungus, whereas vascular bundle discoloration by *C. insidiosum* was found 7–10 mm behind the furthest advance of the bacteria.

The location of both *F. oxysporum* and *C. insidiosum* in the vascular bundles beyond the inoculation site was determined at 4, 7, 14, 21, and 30 days after inoculation (Fig. 5). Hypha of *F. oxysporum* grew rapidly throughout the vascular system of the root and did not seem to be adversely affected by the presence of *C. insidiosum*. Hyphae of *F. oxysporum* were seen 20–40 mm beyond the site of inoculation four days after inoculation, and at 30 days were 55–75 mm beyond it. Cells of *C. insidiosum* spread more slowly throughout the vascular system of the root and their distribution was limited when introduced with *F. oxysporum*. Four days after inoculation of roots with *C. insidiosum* alone, the bacteria were observed 10–20 mm beyond the site of inoculation and at 30 days were 35–50 mm beyond it. Four days after inoculation with both pathogens, the bacteria were observed 5–10 mm beyond the inoculation site and at 30 days were 5–25 mm beyond it (Fig. 5).

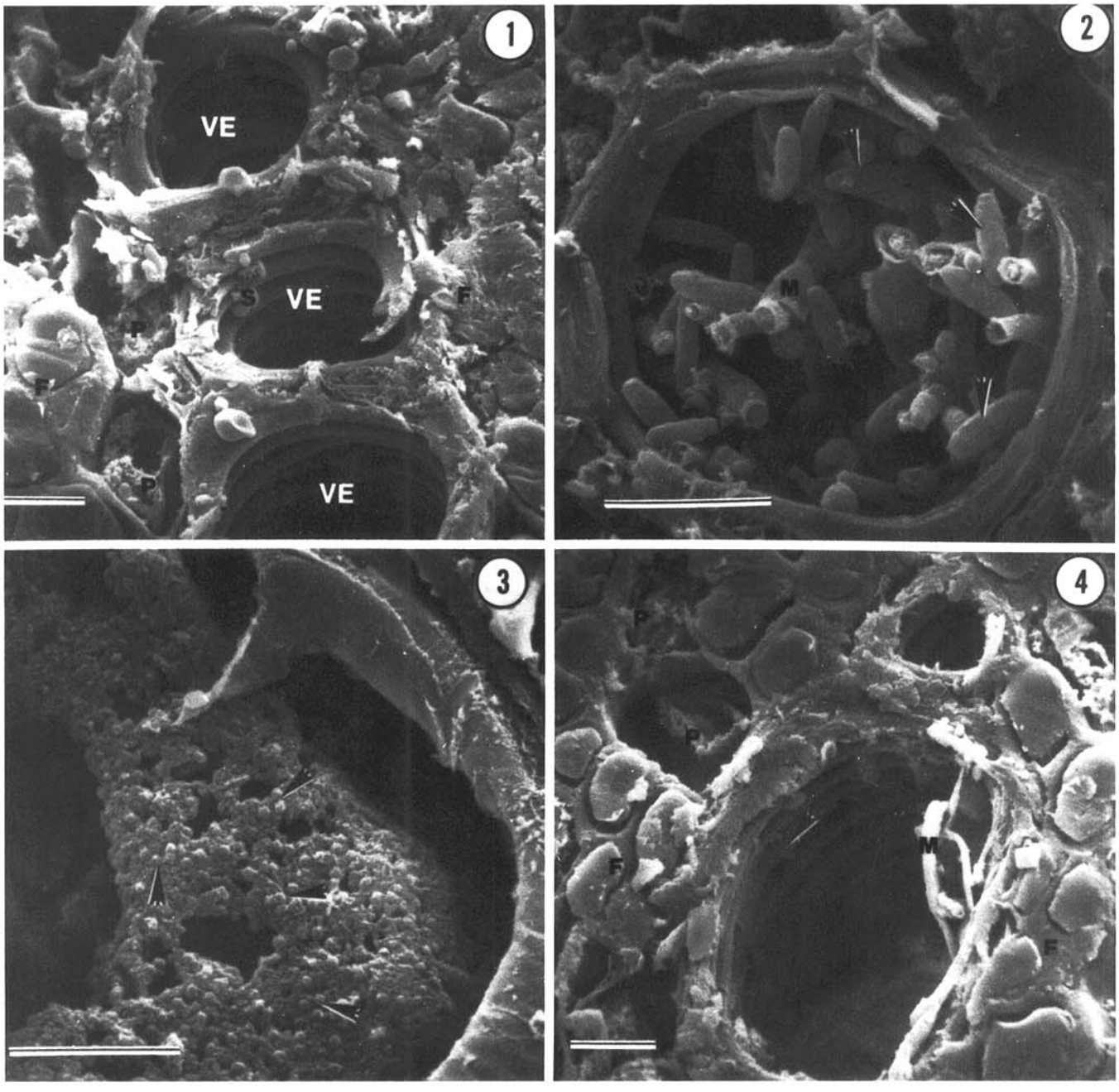
When surface-disinfested root segments were plated on the appropriate agar media, *F. oxysporum* was the only fungus cultured from the roots inoculated with *F. oxysporum* alone or with combined inoculum. *C. insidiosum* was isolated from roots inoculated with *C. insidiosum* alone. Rod-shaped Gram-negative bacteria were isolated from roots inoculated with either sterile

water or *C. insidiosum*. Isolating bacteria from roots inoculated with combined inoculum was difficult because *F. oxysporum* overgrew the plates very rapidly. However, *C. insidiosum* was isolated twice from roots inoculated with combined inoculum, once from roots collected 4 days after inoculation and once from roots collected 7 days after inoculation.

Interaction of the pathogens in vitro. Eighteen of 19 *Fusarium* isolates, killed on filter paper pieces, inhibited the growth of both *C. insidiosum* (Fig. 6) and *R. meliloti* in vitro. The *Fusarium* that did not inhibit bacterial growth was a variant with atypical colony growth on agar selected from another *F. oxysporum* isolate.

The media on which the Fusaria were grown had no effect on either the development or size of the inhibition zone around the filter paper carrying the dead fungi. Inhibition zones were 5–15 mm wide. No inhibition of *C. insidiosum* or *R. meliloti* was observed around sterile filter paper, sterile filter paper treated with ether or around autoclaved fungus-bearing filter paper (Fig. 7).

Isolates of *F. oxysporum* f. sp. *medicaginis* (LJ, BW7, 7F1, and 31F3), grown in liquid shake culture, formed abundant microconidia (10^8–10^{10}/ml) and few hyphal fragments. These isolates, grown in the same medium in still culture, formed mycelium on the surface with few microconidia. The supernatant from the cultures of *F. oxysporum* grown without agitation prevented development of *C. insidiosum*. Bacterial development was not inhibited by the supernatant from the cultures grown with agitation, by autoclaved supernatant from cultures grown either with or without agitation, or by liquid medium without fungal growth.



Figs. 1–4. 1, Scanning electron micrograph of healthy vessel elements of alfalfa roots (VE) surrounded by fibers (F) and xylem parenchyma (P). A few starch grains (S) are present in the vessel elements. Bar equals 10 μm. **2,** Scanning electron micrograph of a vessel element of alfalfa roots filled with *Fusarium oxysporum* mycelium (M) and microconidia (arrow) 21 days after inoculation. Bar equals 10 μm. **3,** Scanning electron micrograph of a vessel element of alfalfa root containing *Corynebacterium insidiosum* bacteria (arrows) in a matrix 14 days after inoculation. Bar equals 10 μm. **4,** Scanning electron micrograph of a vessel element of alfalfa root containing both *Fusarium oxysporum* mycelium (M) and a few cells of *Corynebacterium insidiosum* (arrow) 4 days after inoculation with both pathogens. Bar equals 10 μm.

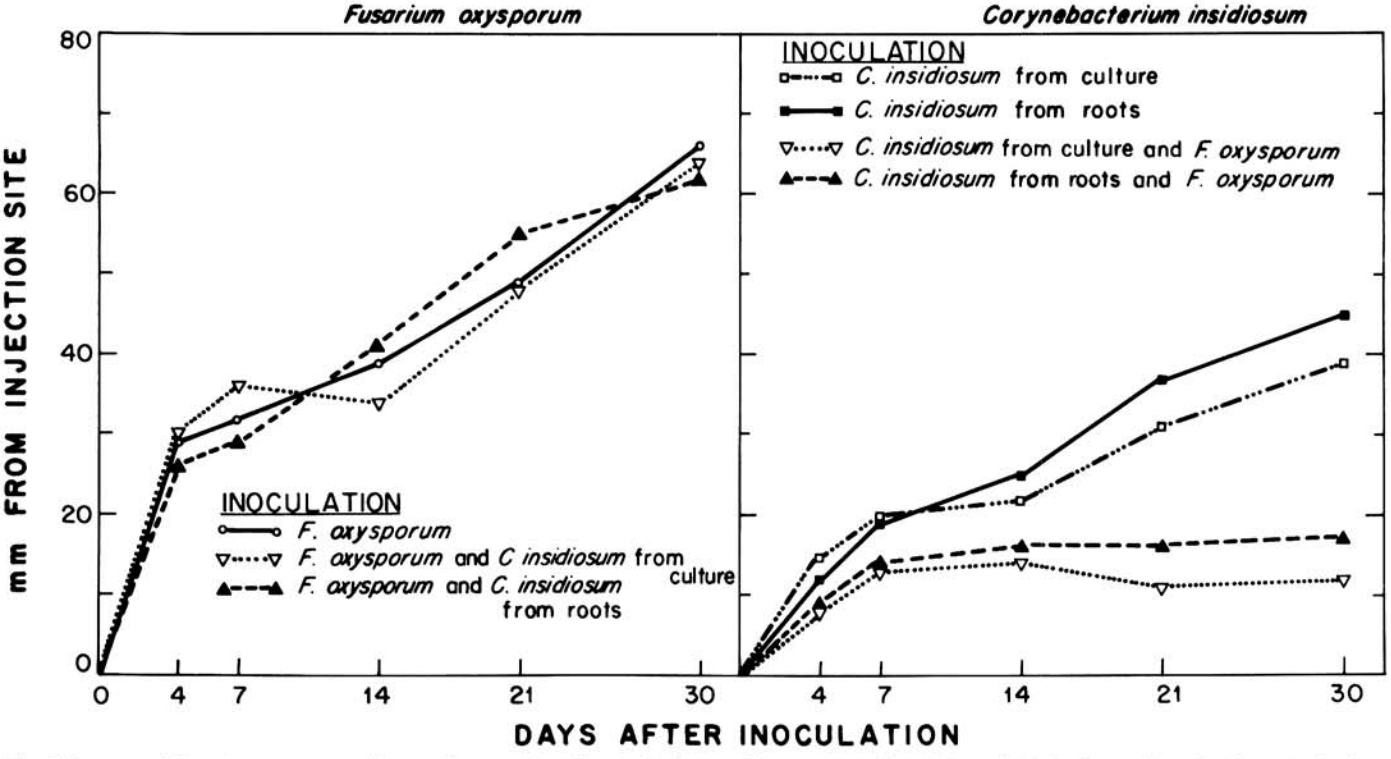
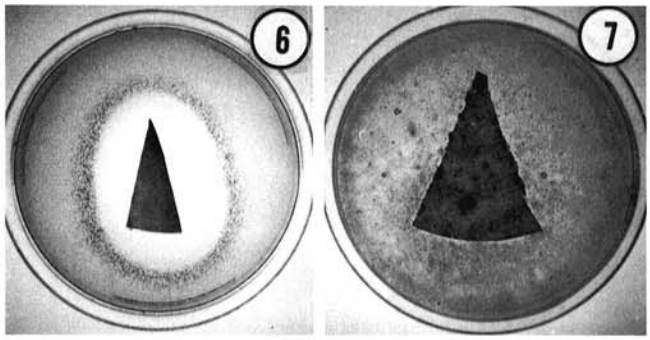


Fig. 5. Progress of *Fusarium oxysporum* f. sp. *medicaginis* mycelium and of *Corynebacterium insidiosum* bacteria (cells) beyond the site of inoculation into roots of Glacier alfalfa on different dates after inoculation based on histological examination.

DISCUSSION

The results of the field disease development study confirmed the report by Frosheiser and Barnes (3) that bacterial wilt was less severe in plants inoculated with *C. insidiosum* mixed with *F. oxysporum* than in plants inoculated with *C. insidiosum* alone. This effect was more pronounced in cultivars Glacier and Salton, which are susceptible to bacterial wilt, than in the resistant cultivars, Agate and MnPL-4.

Several possible mechanisms could account for the reduced development of bacterial wilt in plants inoculated with both *F. oxysporum* and *C. insidiosum*: symptoms due to *F. oxysporum* might develop more rapidly than those due to *C. insidiosum* and thus mask the symptoms of bacterial wilt; *F. oxysporum* might have a competitive advantage over *C. insidiosum* due to more rapid development within the plant; or *F. oxysporum* might inhibit the development of *C. insidiosum*. Both the field and glasshouse studies indicated that symptoms of Fusarium wilt in plants inoculated with *F. oxysporum* alone and with the mixture, developed more rapidly than did symptoms of bacterial wilt in plants inoculated with *C. insidiosum* alone. In the glasshouse study, the relatively mild bacterial wilt symptoms in susceptible plants inoculated with both pathogens were accompanied by fewer bacteria beyond the inoculation site. This suggested that the milder bacterial wilt symptoms were due to fewer bacteria rather than the masking of symptoms due to the more rapid Fusarium wilt symptom development. This reduced development of *C. insidiosum* did not seem to be due to a competitive advantage possessed by *F. oxysporum*. Although the fungus developed more rapidly within the root than did the bacterium, it was found only in vessel elements in the living plant, whereas *C. insidiosum* was found in vessel elements and in vascular parenchyma and fibers. Even though the small numbers of *C. insidiosum* cells in vascular bundles infected with *F. oxysporum* were not associated with the invasion of parenchyma or fibers by the fungus, by the time the study was terminated, few bacteria were found in the cells of vascular bundles of vessel elements that contained *F. oxysporum*. These findings favor the hypothesis that *C. insidiosum* was inhibited by *F. oxysporum* and therefore symptoms of bacterial wilt did not develop readily in the presence of the fungus.



Figs. 6 and 7. 6, Zone of inhibition around a piece of filter paper bearing mycelium of *Fusarium oxysporum* isolate BW7 killed by ether before being placed on agar seeded with *Corynebacterium insidiosum*. **7,** Lack of a zone of inhibition around filter paper bearing mycelium of *Fusarium oxysporum* isolate BW7, autoclaved after fungus was present, placed on agar seeded with *Corynebacterium insidiosum*.

Results from the in vitro experiments indicated that *Fusarium* spp. (including *F. oxysporum* f. sp. *medicaginis*) produced a substance that inhibited development of both the Gram-positive bacterium, *C. insidiosum*, and the Gram-negative bacterium, *R. meliloti*. This substance seemed to be produced in the presence of mycelium under aerobic conditions. It was not detected when the cultures formed microconidia under anaerobic conditions. Plattner et al (7) reported that *F. oxysporum, F. solani, F. roseum,* and *F. lateritium* produced in vitro a cyclodepsipeptide, enniaten, which was a mycobactericide. However, it is not known whether the inhibitory substance produced in these experiments was the same as the one purified by Plattner et al (7).

The results of these studies indicated that a mixture of *C. insidiosum* and *F. oxysporum* should not be used while selecting for resistance in an alfalfa breeding program, since too many susceptible plants might be retained.

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