Interaction of *Fusarium oxysporum* f. sp. *medicaginis* with Feeding Activity of Clover Root Curculio Larvae in Alfalfa

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ABSTRACT

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Twenty genera of fungi were isolated over a 3-yr period from feeding sites of the clover root curculio (CRC) in roots of field-grown alfalfa. *Fusarium oxysporum* f. sp. *medicaginis* was the predominant pathogenic fungus isolated, constituting 63% of all isolates at one sampling. Root diameter, root type, and depth of feeding were not related to the isolation frequencies of specific fungi. In pathogenicity tests, 89% of all *F. oxysporum* isolates tested caused wilt symptoms in alfalfa. Radial internal spread of fungi from feeding sites was usually limited to the 2 mm of tissue immediately centripetal to the feeding site. *F. o. medicaginis,* other *Fusarium* spp., and other fungi were isolated from excised head capsules of field-collected, late-instar larvae of CRC. The addition of 50 CRC eggs to a growth medium infested with *F. o. medicaginis* inoculum significantly increased the severity of Fusarium wilt in greenhouse experiments.

Additional keywords: *Sitona hispidulus*

Alfalfa (*Medicago sativa* L.) is the premier forage legume grown in the United States. It is a perennial species with disease and insect problems that are also perennial. Pathogens and insects often attack alfalfa at the same time and interact in an additive (13) or synergistic (3,8,17) manner to impair plant performance and cause economic loss (2,6,9).

Fusarium wilt of alfalfa is a systemic disease, caused by the fungus *Fusarium oxysporum* Schlechtend.:Fr. f. sp. *medicaginis* (J.L. Weimer) W.C. Snyder & H.N. Hans., that was first reported in the United States in 1927 (21). This disease occurs throughout many of the world's alfalfa-growing regions and is most severe in the warmer areas (1). Symptoms of Fusarium wilt, which include chlorosis, wilting, stunting, and death of plants, usually become evident during the second production year and increase in severity and incidence during subsequent growing seasons.

The clover root curculio (CRC), *Sitona hispidulus* (Fabricius), was first reported as a pest of alfalfa in the United States in 1876 (22). Larvae of this insect feed on alfalfa roots and predispose them to greater disease incidence and severity (4,5,7,12,15,19,20). The injury caused by the CRC on red clover (*Trifolium pratense* L.) was for many years more severe than the injury on alfalfa. However, from the late 1970s to the present, the injury on alfalfa has increased considerably in the northeastern United States (*authors' observations*). During this same period, the incidence and severity of Fusarium wilt has also increased to a level requiring host-plant resistance to grow alfalfa successfully (14).

The objective of this research was to examine the relationships between *F. o. medicaginis* and CRC in alfalfa. Field, laboratory, and greenhouse experiments were used to determine the fungal colonization of larval feeding sites, the lateral spread of fungi within the roots, the potential for CRC larvae to act as vectors of *F. o. medicaginis,* and the effect of CRC feeding on the severity of Fusarium wilt.

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MATERIALS AND METHODS

Field samples and fungal incidence. Alfalfa cultivar WL-316 was seeded in a 1-ha stand in the spring of 1984 to use in the study of various behavioral aspects of CRC. The alfalfa was harvested four times annually from 1985 through 1987. Root samples were dug each spring and fall, beginning with the fall of 1985 and ending in 1987. Samples were systematically collected along diagonal paths that traversed each quarter of the field. Twenty-five roots were obtained from each quarter of the field. Roots were washed with a forceful spray of water and kept in plastic bags for 48 hr at 8 C until assayed.

Typical feeding sites of CRC were selected on the taproot, a large lateral

root, and a fibrous root of each plant when available. Three sites per root system were assayed on each of 25 roots per quarter of the field for a total of 300 sites per sampling date. A root piece approximately 1.5 cm long was excised, surface disinfested by immersing in 70% ethanol for 10 sec and in 0.15% sodium hypochlorite for 2 min, and rinsed four times in sterile, distilled water.

Each root piece was cut longitudinally through the center of the feeding site. Internal tissue adjacent to the necrotic tissue was excised and transferred to 1.5% water agar at pH 2.6, with a 12-hr photoperiod at 50 $\mu E \cdot m^{-2} \cdot s^{-1}$ of cool-white fluorescent light and a temperature of 21 C. Isolation plates were checked daily for fungal growth, and fungi that could not be identified on the isolation plates were transferred to potato-dextrose agar (PDA) for identification. Selected *Fusarium* isolates from each root sampling were sent to the Fusarium Research Center at Pennsylvania State University for corroborative identification. Other parameters assessed at each sampling were root type, root diameter, and depth of feeding site. To determine the change in fungi colonizing feeding sites over time, data were subjected to analysis of variance (ANOVA) with orthogonal contrasts.

Pathogenicity tests with *F. o. medicaginis* isolates. A total of 108 randomly selected isolates from feeding sites of CRC were tested for their ability to cause wilt in alfalfa. Plants of Pioneer 526, a Fusarium wilt susceptible variety, were grown individually in Cone-Tainers filled with a commercial peat–vermiculite potting mix. Three plants were inoculated with each isolate. Plants were 6 wk old when inoculated, and disease ratings based on internal necrosis and foliar symptoms were determined after 4 mo.

Inoculation consisted of cutting across a Cone-Tainer and root mass about halfway down, which exposed several severed roots of each plant. Each cut-off Cone-Tainer, with severed roots exposed, was immersed in an inoculum suspension prepared by scraping the mycelia and spores from one culture plate into 20 ml of tap water and comminuting in a blender for 10 sec. The fungi were grown on PDA for 10 days under conditions described for field isolations. After all the suspension had been absorbed by the cut-off roots and growth medium, the two halves of the original Cone-Tainer were reassembled within another intact Cone-Tainer and maintained in the greenhouse under a natural photoperiod. Disease-severity scores on a 1–5 scale were the following: 1 = no vascular discoloration or foliar symptoms; 2 = local necrosis or vascular discoloration and no foliar symptoms; 3 = vascular discoloration, 1.0 cm or greater, above the inoculation site with no foliar symptoms; 4 = same as 3 but with leaves chlorotic and stems wilted or stunted; and 5 = dead plant. Control plants were treated the same as the inoculated plants except that tap water was used instead of a fungal suspension. Isolation of *F. oxysporum* was attempted from 10 symptomatic and 10 asymptomatic plants. A completely randomized design was maintained throughout the experiment.

Spread of fungi from feeding sites. Roots collected in the fall of 1987 were subjected to an additional assay to determine how extensively fungi spread centripetally from the feeding sites. Roots that had a distinct and isolated feeding site were selected for this assay, and only taproots and large lateral roots were used. Root segments about 1.5 cm long, with a single feeding site at about the midpoint, were surface disinfested as previously described. Segments were blotted dry with sterile paper towels. A cross section 2 mm thick was cut through the center of the feeding site of each root segment. The slice was laid flat on a sterile cutting board, and a 1-mm-wide strip through the center of the feeding site was excised aseptically. The strip was cut into 1-mm-wide subsections and serially plated onto water agar acidified to pH 2.6. All cuts were made from the back side of the root toward the feeding site to avoid contamination of root pieces with the scalpel. Root diameters ranged from 3 to 12 mm. A total of 88 feeding sites were assayed.

Feeding sites were classified as surface or deep. Surface sites were those not deep enough to bridge the cortex, and deep sites extended into the stele. After 5 days, root pieces were evaluated at 125× magnification for the presence or absence of fungi.

***Fusarium* isolation from field larvae.** One hundred late-instar larvae of CRC were manually removed from soil samples taken from the test field. The larvae were refrigerated at 8 C until killed for fungal assay. Under 7× magnification, the head capsule from each larva was excised with a sterile scalpel and placed on water agar at pH 2.6. Soil particles were visible on most head capsules. Fungi producing a red pigment in the agar were transferred to PDA for identification.

Pathogenicity tests were conducted with the two isolates of *F. oxysporum* obtained from the head capsules. The fungi were cultured for 10 days on V8 agar (16) at 21 C, at 30 $\mu E \cdot m^{-2} \cdot s^{-1}$ with a 12-hr photoperiod. Conidia were collected by flooding the plates with sterile distilled water and agitating gently with a glass rod. Conidial concentration was adjusted to 2×10^6 per milliliter, determined with the aid of a hemacytometer. Roots of 1-mo-old plants of Pioneer 526 were cut and immersed in the inoculum suspension for 30 min, and the plants were then repotted and kept in the greenhouse until wilt symptoms developed. Control plants were treated similarly with water. Twenty plants were inoculated per isolate. The presence or absence of chlorosis, wilting, stunting, and vascular necrosis were determined 30 days later.

Interaction of *F. o. medicaginis* and CRC. Pioneer 526 alfalfa was grown in the greenhouse in 700-ml clay pots containing the commercial peat–vermiculite potting mix. Three plants were grown per pot. Plants were 62 days old when eggs of CRC were added to the growth medium and 66 days old when *F. o. medicaginis* (from our collection and known to be pathogenic) was added. At this time, plants ranged from 22 to 30 cm in height and were well nodulated.

Eggs of the CRC were collected by the technique of Newton (18). Prior to egg collection, gravid females were maintained at 8 C in the dark for 90 days. Eggs were transferred singly with a brush to a filter paper and then rinsed with water onto the surface of the growth medium in the center of the pot. Inoculum of *F. o. medicaginis* was produced by growing fungus cultures on V8 agar for 10 days at 21 C under continuous cool-white fluorescent light at 30 $\mu E \cdot m^{-2} \cdot s^{-1}$. Plates were flooded with distilled water and agitated gently to dislodge conidia and some hyphae. Collections from one plate of each of seven isolates of *F. o. medicaginis* were combined and diluted to a concentration of 3×10^4 conidia per milliliter. Each pot received 25 ml of suspension, which was poured onto the surface of the growth medium in the center of the pot. Treatments were the following: control (water), *F. o. medicaginis,* 50 CRC eggs, *F. o. medicaginis* plus 25 CRC eggs, and *F. o. medicaginis* plus 50 CRC eggs. Each treatment was replicated eight times, with each replicate consisting of one pot with three plants, and the experimental design was completely randomized.

Plants were evaluated for wilt symptoms and vascular necrosis 77 days after the addition of the fungus. Evaluation of wilt symptoms consisted of a visual rating of individual plants, where 1 = no symptoms, 2 = wilted leaves, 3 = wilted stems, 4 = one or more dead stems, and 5 = dead plant. The presence or absence of *F. oxysporum* was determined by isolation attempts from six randomly selected symptomatic plants and from six asymptomatic control plants.

The experiment was repeated; however, the treatment of *F. o. medicaginis* plus 25 eggs was omitted from the second experiment because it was ineffective in the first. Data were subjected to ANOVA, and treatment means were compared by the Bonferroni multiple comparison technique (10).

RESULTS

Field samples and fungal incidence. Twenty genera of fungi were identified

as colonizers of CRC feeding sites in roots of field-grown alfalfa. Several other fungi were isolated but not identified. Of the genera with species known to be pathogenic to alfalfa, *Fusarium* predominated. *F. oxysporum* was the species most commonly isolated from the feeding sites. *F. solani* (Mart.) Sacc., *F. avenaceum* (Fr.:Fr.) Sacc., and *F. acuminatum* Ellis & Everh. occurred less frequently. The incidence of *Fusarium* spp. versus other fungi associated with feeding sites is given in Table 1. Genera of other fungi isolated from feeding sites were *Alternaria, Aspergillus, Cephalosporium, Chaetomium, Cladosporium, Colletotrichum, Cylindrocladium, Epicoccum, Mucor, Myrothecium, Paecilomyces, Penicillium, Phoma, Pythium, Rhizoctonia, Rhizopus, Thielaviopsis, Trichoderma,* and *Verticillium.*

The frequency of fungi associated with CRC feeding sites did not change from spring to fall sampling; therefore only fall sampling data are presented in Table 1. The only significant changes in fungal incidence over time were the decrease in *F. oxysporum* and the increase in fungi other than *Fusarium* spp. The occurrence of *F. solani* did not change over time. No relationships were found among root types, root diameters, depth of feeding injury and the species of fungi isolated.

Pathogenicity test with *F. o. medicaginis* isolates. Of the 108 isolates tested, 89 caused root vascular discoloration or wilt symptoms in inoculated alfalfa plants. The total number of plants killed was 47 (15%), and 30 isolates each killed at least one plant. Nineteen isolates caused either no symptoms or a root discoloration limited to the inoculation site. *F. oxysporum* was recovered from 10 randomly selected symptomatic inoculated plants. No control plants were symptomatic, nor was *F. oxysporum* recovered from any of 10 randomly selected control plants.

Spread of fungi from CRC feeding sites. Radial proliferation from feeding sites into root tissues by either *Fusarium* spp. or other fungi was limited. Of the 88 feeding sites assayed, 21 had fungi other than *Fusarium* spp., 62 had a *Fusarium* sp., and five had both. The 1-mm-wide piece of root tissue immediately internal to the feeding site yielded a *Fusarium* sp. in 75% of the assays and yielded another fungus in 30% of the assays. Fungal colonization was much lower in the next innermost segment, with a *Fusarium* sp. present in 30% and other fungi in 5% of the assays. The presence of any fungus in any other root segment was less than 5%.

***Fusarium* isolation from field CRC larvae.** Fungi grew from each of the 100 excised head capsules. *Fusarium* grew from 21 capsules, with six yielding more than one species. *F. o. medicaginis* was isolated from two capsules. Both isolates caused vascular necrosis and wilting in inoculated alfalfa plants. *F. o. medicaginis* was recovered from all symptomatic plants. *F. solani* and *F. avenaceum* were also isolated from head capsules.

Interaction of *F. o. medicaginis* and CRC. The roots of two plants that were not part of the experimental design were examined 19 days after CRC eggs were deposited on the soil surface, to determine if insect survival was satisfactory. There was extensive feeding injury on nodules, and minor gouging was apparent on the taproot. This preliminary examination was made to ensure that CRC eggs had hatched and that larvae were feeding.

Results from the two experiments were similar, and data from the first experiment are presented in Table 2. No wilt symptoms occurred in control plants or in plants receiving only insects, nor could *F. oxysporum* be isolated. Feeding injury by CRC larvae was moderate to severe on fibrous, lateral, and taproots; but in the absence of *F. o. medicaginis* this did not cause foliar symptoms. The addition of 25 CRC eggs to *F. o. medicaginis*-infested soil did not increase the wilt severity over that caused by the fungus alone. The combination of 50 eggs plus *F. o. medicaginis* significantly increased the wilt-severity score. Feeding by CRC larvae breached the root periderm, and internal root necrosis was severe in some plants (Fig. 1). *F. oxysporum* was reisolated from all symptomatic plants checked.

DISCUSSION

Data from this survey of CRC feeding sites and pathogenicity tests clearly established that *F. o. medicaginis* was a common colonizer of these sites and the predominant fungal pathogen present. The decline in frequency of *F. oxysporum* and the increase of fungi other than *Fusarium* spp. over time could have resulted from a greater frequency of older sites in each succeeding sample. It is likely that more saprophytic fungi would become established in older rather than in younger sites. Some sites callus over, whereas others do not and therefore provide colonization sites for secondary fungi. *F. o. medicaginis* may not be a strong competitor and may not be able to prevent colonization by other fungi over time. *F. solani* may be more competitive than *F. o. medicaginis,* which could explain why it was isolated with relatively equal frequency throughout the sampling period. It is also possible

Table 1. Frequency of fungi isolated from feeding sites of clover root curculio larvae in alfalfa roots[y]

Fungi	Fall 1985	Fall 1986	Fall 1987	Significance of frequency change[z]
Fusarium oxysporum	63	56	36	**
Fusarium solani	17	13	12	NS
Fusarium avenaceum	8	7	2	IO
All other	16	23	48	*

[y] Data are percentages of fungi isolated from 300 feeding sites on randomly selected plants at each sampling period.
[z] Significant at $P = 0.05$, ** = significant at $P = 0.01$, NS = not significant, and IO = insufficient no. of observations.

Table 2. The development of Fusarium wilt in alfalfa following individual and combined applications of *Fusarium oxysporum* f. sp. *medicaginis* and clover root curculio eggs

Treatment	Mean wilt severity score[z]
Untreated control	1.0 a
Curculio eggs (50/pot)	1.0 a
F. o. medicaginis	1.8 b
F. o. medicaginis + eggs (25/pot)	1.8 b
F. o. medicaginis + eggs (50/pot)	2.7 c

[z] Rating system: 1–5, where 1 = healthy plant and 5 = dead plant. Each value is the mean score of 24 plants. Numbers not followed by the same letter are significantly different at $P = 0.05$ according to the Bonferroni multiple comparison technique.

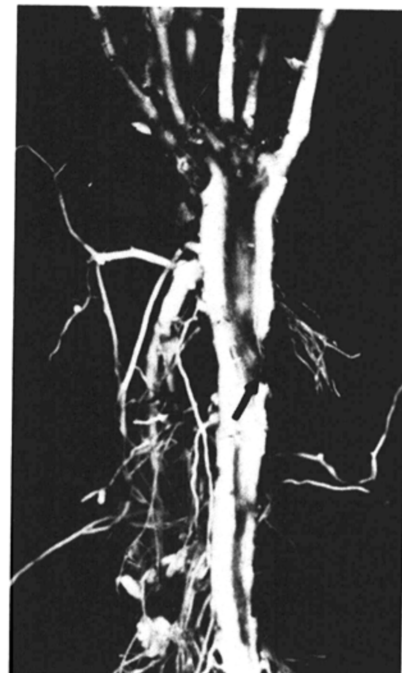

Fig. 1. Root of an alfalfa plant from a pot to which eggs of the clover root curculio and inoculum of *Fusarium oxysporum* f. sp. *medicaginis* were added. Note central root necrosis and feeding wound caused by curculio larval feeding (arrow).

that the colonization of feeding sites by saprophytic fungi could provide protection against pathogenic types.

It has been recognized for some time that feeding wounds caused by CRC larvae in the roots of alfalfa (3,6,8,9) and other legumes (4,5,11,12,15,19,21) serve as infection courts for soilborne pathogens. The presence of infested soil particles on the head capsules of CRC larvae suggests that the larvae may function as vectors of pathogens that infect alfalfa roots. The high frequency of *F. o. medicaginis* in CRC feeding sites suggests a close relationship between the two in the field studied.

The radial proliferation of fungi from the feeding wound into more internal root tissue was quite limited. However, this assay was done in 1987, when most of the sites were relatively recent, which may explain why greater colonization of the root tissue did not occur. *F. o. medicaginis* does not generally cause cortical rot but preferentially colonizes the vascular system of roots. The opportunity for vertical spread of the fungus within the plant is of greater importance than cortical invasion for a wilt pathogen such as *F. o. medicaginis*. CRC larvae carry *F. o. medicaginis* on their head capsules, and their feeding often penetrates into the xylem. Both factors likely contributed to the increased disease severity detected. *F. o. medicaginis* persists in the soil mainly as chlamydospores (1), and the *F. o. medicaginis* isolated from CRC head capsules could have originated from chlamydospores in the soil particles on the larval head capsules.

Pathogenicity tests showed that 89% of the 108 *F. oxysporum* isolates caused wilt symptoms in alfalfa, which verifies their identity as *F. o. medicaginis*. It should be noted that these isolations were made from alfalfa roots and may not reflect the actual soil population ratios.

The significant increase in Fusarium wilt that occurred with CRC feeding on plants inoculated with *F. o. medicaginis* in the greenhouse test indicates that this insect could increase Fusarium wilt in the field. The difference in efficacy of 25 versus 50 eggs per pot suggests that a critical insect population threshold may be needed before significant interaction with the fungus takes place. Our data did not allow us to determine a threshold, because we did not know the hatching or survival rate of CRC in our experiments.

In our survey we found a close association between *F. o. medicaginis* and CRC feeding. Based on this relationship, we expect that increased feeding by CRC larvae in the field would increase Fusarium wilt. The increase in CRC activity in alfalfa since the early 1970s probably resulted from changes in the quantity and type of insecticides used to control the alfalfa weevil and the potato leafhopper. CRC was probably being controlled in alfalfa by insecticides targeted at these other pests, but in red clover insecticides were not in general use, and CRC activity was always high. Fusarium wilt probably would not have increased in severity to present levels if the level of CRC activity had not also increased.

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