Effects of Vesicular-Arbuscular Mycorrhizal Fungi on the Development of Verticillium and Fusarium Wilts of Alfalfa

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ABSTRACT

Hwang, S. F., Chang, K. F., and Chakravarty, P. 1992. Effects of vesicular-arbuscular mycorrhizal fungi on the development of Verticillium and Fusarium wilts of alfalfa. Plant Dis. 76:239-243.

Interactions of vesicular-arbuscular (VA) mycorrhizal fungi and two wilt pathogens of alfalfa (*Medicago sativa*), *Verticillium albo-atrum* and *Fusarium oxysporum* f. sp. *medicaginis*, were investigated under controlled conditions over a 6-mo period. The four × two factorial design used included four treatments of mycorrhizal fungi (*Glomus* spp., *G. fasciculatus*, *G. mosseae*, and nonmycorrhizal control) and two levels of pathogen inoculum (with and without) of either *V. albo-atrum* or *F. o. medicaginis*. Shoot dry weights of alfalfa plants inoculated with VA mycorrhizal fungi significantly exceeded those of nonmycorrhizal plants. Inoculation with *V. albo-atrum* or *F. o. medicaginis* significantly reduced the shoot dry weights of alfalfa. Seedlings inoculated with VA mycorrhizal fungi had a lower incidence of wilt than nonmycorrhizal ones. Propagule numbers of both pathogens were lower in the soil inoculated with VA mycorrhizal fungi than in the nonmycorrhizal soil.

Verticillium wilt of alfalfa (*Medicago sativa* L.), caused mainly by *Verticillium albo-atrum* Reinke & Berthier, has spread through the Pacific Northwest of the United States (4,25,36,37) and western Canada (28,29). This destructive disease can cause up to a 50% yield reduction by the end of the second year and shorten the productive life of an alfalfa crop to 3 yr from the customary six or more years (31). Fusarium wilt, caused by *Fusarium oxysporum* f. sp. *medicaginis* (J. L. Weimer) W. C. Snyder & H. N. Hans., is another disease of the vascular system of alfalfa. Although the disease usually progresses slowly in natural alfalfa stands, considerable losses in stand may occur over a period of several years (21).

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Accepted for publication 8 September 1991 (submitted for electronic processing).

Vesicular-arbuscular (VA) mycorrhizae are important components of intensively managed alfalfa plants because mycorrhizal infections result in increased growth, especially in soil of low fertility where the symbiont increases efficiency of nutrient absorption by roots (1,2,30). VA mycorrhizae have been reported to protect roots from certain root-infecting fungi (5,40,41); however, the effect of VA mycorrhizae on plant disease is very difficult to generalize because the interactions involving VA mycorrhizae and root-infecting fungi vary with the species of mycorrhizal fungi and plant cultivars (41). The systems most commonly studied are VA mycorrhizal fungi with Verticillium wilt and Fusarium wilt of cotton and tomato (6,7,13,16,17,29,35). Although alfalfa is the major forage crop in North America, published information on the influence of VA mycorrhizae on fungal wilts of alfalfa does not exist. Consequently, studies were conducted to investigate the interaction between the VA mycorrhizal fungi (*Glomus* spp., *G. fasciculatus* (Thaxter) Gerd. & Trappe, and *G. mosseae* (Nicol. & Gerd.) Gerd. & Trappe) and the wilt fungi (*V. albo-atrum* and *F. o. medicaginis*) of alfalfa.

MATERIALS AND METHODS

Production of wilt pathogens and VA mycorrhizal fungi. The pathogens, *V. albo-atrum* and *F. o. medicaginis*, were isolated originally from infected alfalfa plants collected in Alberta, Canada. Inoculum for each fungus was produced in 250-ml conical flasks containing 100 ml of sterile Kerr's solution (32). The Kerr's solution was inoculated with a 9-mm-diameter mycelial disk and shaken continuously at 200 rpm for 5 days at room temperature (20 ± 2 C) in natural light. The culture of each flask was filtered through two layers of cheesecloth to remove mycelium and centrifuged at 1,000 rpm for 10 min. The pellets of conidia were resuspended and diluted with sterile water to a concentration of 1×10^5 conidia per milliliter.

The VA mycorrhizal fungi, *G. fasciculatus* and *G. mosseae*, were obtained from B. Mosse, Rothamsted Experimental Station, England, whereas *Glomus* spp. were obtained from the rhizosphere of alfalfa collected in Alberta. All VA mycorrhizal fungi were maintained on roots of onion (*Allium cepa* L.) seedlings grown in a steam-sterilized mixture of sand and loam (1:1, v/v) in plastic pots in a growth chamber where day/night temperatures were 20/15 C and a light intensity of 300 $\mu E \cdot m^{-2} \cdot s^{-1}$ was provided by cool-white fluorescent tubes for a photoperiod of 16 hr. Two months after inoculation, the fibrous onion roots were collected, chopped (2–3 mm in length), and mixed into the steam-sterilized sand and loam soil. This mixture of soil, chlamydospores, and segmented, colonized roots was air-dried, packed in plastic bags, and stored at 4 C until used.

Inoculations and growth conditions. Seeds of alfalfa cv. Anchor were surface-disinfested in 70% ethanol for 2 min, followed by 2 min in 0.6% sodium hypochlorite, rinsed three times in sterile distilled water, and sown in flats (50 × 30 × 10 cm) containing vermiculite. One week after germination, the seedlings were transplanted to 13-cm-diameter plastic pots that contained 10 ml of mycorrhizal inoculum (500 spores per gram of soil) of either *G. fasciculatus*, *G. mosseae*, or *Glomus* spp. distributed in one layer 5 cm below the sand/loam (1:1, v/v) soil surface. The seedlings (five per pot) were placed 2.5 cm above the mycorrhizal inoculum. Nonmycorrhizal control pots did not receive any VA mycorrhizal fungal treatment except 1 g of nonmycorrhizal onion roots. One month after transplanting, inoculated treatments received a conidial suspension of *V. albo-atrum* or *F. o. medicaginis* (100 ml per pot) poured on the soil surface.

All pots were arranged randomly in growth chambers, each with a light intensity of 300 $\mu E \cdot m^{-2} \cdot s^{-1}$ (16-hr day) provided by cool-white fluorescent tubes. Temperatures (day/night) were set at 20/15 C for the Verticillium wilt study and at 22/15 C for the Fusarium wilt study because Fusarium wilt is favored by warm soil temperature (26). All seedlings were watered as needed with distilled water and fertilized with 50 ml/wk of 10% Hoagland's solution (27), minus phosphorous, beginning 1 mo after transplanting.

Evaluation of plant growth and disease severity. Seedlings were harvested at monthly intervals for 5 mo after transplanting, and shoot dry weights (dried at 70 C for 24 hr) were recorded. Disease severity, rated before harvests made at 2, 3, and 4 mo after inoculation, was determined by the wilt index described by Ebbels (20). Individual plants were rated on a scale of 0–5 where 0 = no wilt symptoms; 1 = fewer than 25% of the leaves wilted; 2 = 25–50% of the leaves wilted; 3 = 50–75% of the leaves wilted; 4 = 75–100% of the leaves wilted, including stunt (plant less than 15 cm tall); and 5 = plant dead. The individual ratings were converted to mean percent wilt using the following equation: (sum of individual plant ratings values × 100)/(5 × number of plants assessed).

At the end of the experiment, three 1-cm-diameter soil cores were removed from each of 10 pots. The three cores were combined into one sample from which 50 arbitrarily selected root segments were cleared and stained with fuchsin (38), placed on a grid of 5-mm divisions, and examined under a dissecting microscope. The percentage of root length and root segments colonized by VA mycorrhizae was determined by the gridline intersection method (23).

To determine the number of propagules of *V. albo-atrum* or *F. o. medicaginis* in each of 10 pots, 10 g of air-dried soil cores free of roots was placed into 100 ml of water amended with 0.2% agar in a 250-ml flask and shaken for 1 hr. One milliliter of the suspension from each flask of 100-fold dilution was spread on petri dishes containing Komada's selective medium (33). The plates were incubated under fluorescent light at room temperature. The number of propagules per gram of soil was determined by counting colonies of *V. albo-atrum* and *F. o. medicaginis* after 10 and 7 days of incubation, respectively.

Experimental design. A four × two factorial design was used with mycorrhizal inoculum consisting of *G. fasciculatus*, *G. mosseae*, unidentified *Glomus* spp., or roots of onion without mycorrhizal infection as one factor and two levels of pathogen infection (with and without *V. albo-atrum* or *F. o. medicaginis*) as the other factor. The eight treatments for the Verticillium wilt study were *G. fasciculatus*, *G. mosseae*, *Glomus* spp., *G. fasciculatus* plus *V. albo-atrum*, *G. mosseae* plus *V. albo-atrum*, *Glomus* spp. plus *V. albo-atrum*, nonmycorrhizal onion roots plus *V. albo-atrum*, or nonmycorrhizal onion roots alone. For the Fusarium wilt study, the same eight treatments were employed except that *F. o. medicaginis* was substituted for *V. albo-atrum*. Twelve replicated pots were used for each treatment. The entire experiment was repeated and homogeneity of variance was determined before pooling of data for analysis. The results of the two wilt studies were analyzed separately. Data were subjected to analysis of variance and means were compared using Duncan's new multiple range test with SAS software (39).

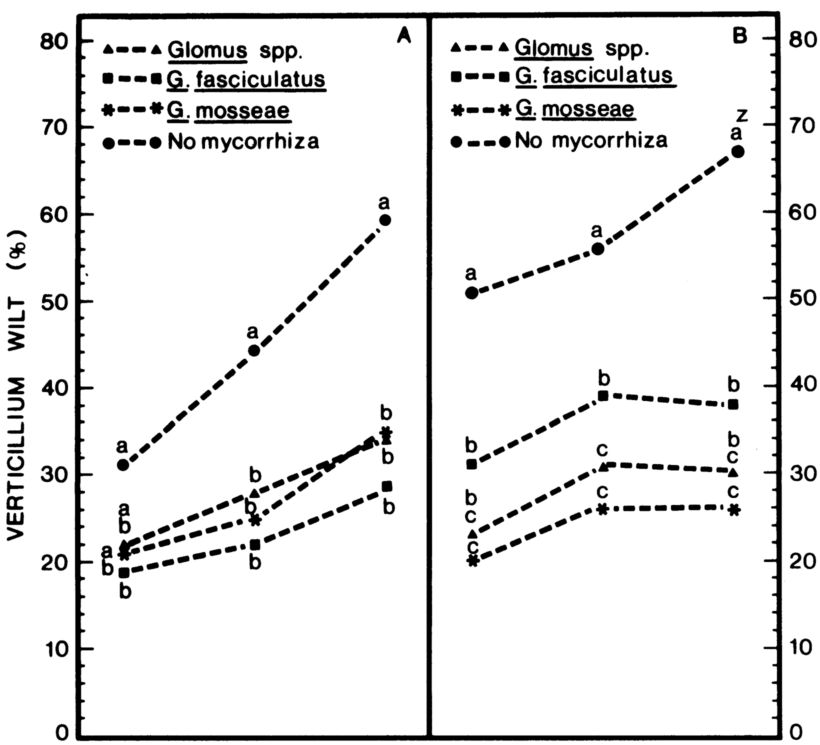

Fig. 1. Percent wilt of alfalfa plants inoculated with **(A)** *Verticillium albo-atrum* or **(B)** *Fusarium oxysporum* f. sp. *medicaginis* in the presence or absence of VA mycorrhizal fungi at 2, 3, and 4 mo after transplanting. z = Means of percent wilt within the same month after inoculation followed by the same letter do not differ significantly from each other by Duncan's new multiple range test ($P = 0.05$).

RESULTS

Disease incidence. Nonmycorrhizal plants grown in soil amended with a conidial suspension of *V. albo-atrum* or *F. o. medicaginis* had higher disease severity ratings than did plants inoculated with pathogens plus *Glomus* spp., *G. mosseae*, or *G. fasciculatus* at 2, 3, and 4 mo after inoculation (Fig. 1). The severity of wilt in nonmycorrhizal seedlings increased over time, although time was not included as a factor in analysis of variance. The percentage of wilt-infected nonmycorrhizal plants increased from 32 to 59% for Verticillium wilt and from 49 to 67% for Fusarium wilt (Fig. 1).

Population of pathogens in soil and mycorrhizal colonization of roots. The numbers of propagules of *V. albo-atrum* and *F. o. medicaginis* in the soil of nonmycorrhizal alfalfa seedlings were greater than those in the soils of alfalfa plants inoculated with mycorrhizal fungi (Fig. 2). The soils of alfalfa inoculated with *Glomus* spp. had significantly fewer propagules than those of alfalfa inoculated with either *G. mosseae* for both wilt studies or *G. fasciculatus* for the Fusarium wilt study (Fig. 2).

In both studies, the number of root segments and root length colonized by *Glomus* spp., *G. fasciculatus*, and *G. mosseae* in plants infected with *V. albo-atrum* and *F. o. medicaginis* were significantly lower than those of alfalfa seedlings inoculated with either *G. mosseae*, *G. fasciculatus*, or *Glomus* spp. alone (Table 1). Similarly, the number of vesicles declined significantly when concomitantly inoculated with VA mycorrhizal fungi and *V. albo-atrum* or *F. o. medicaginis* than when inoculated with VA mycorrhizal fungi alone (Table 1). Mycorrhizal colonization and number of vesicles were highest when inoculated with *Glomus* spp. alone. Noninoculated plants were free of mycorrhizal colonization throughout the entire period of the study.

Plant growth. Shoot dry weights of alfalfa infected with *V. albo-atrum* were significantly lower than those of noninfected plants at 4 and 5 mo after transplanting (Fig. 3A). The dry weights of the mycorrhizal plants significantly exceeded those of the nonmycorrhizal plants except at 1 mo after transplanting (Fig. 3B). There were no significant differences among mycorrhizae × Verticillium interactions for shoot dry weights.

Shoot dry weights of alfalfa infected with *F. o. medicaginis* were significantly lower than those of noninfected alfalfa at 3, 4, and 5 mo after transplanting (Fig. 4A). The monthly yields of the mycorrhizal plants were significantly higher than those of the nonmycorrhizal plants at 2, 3, 4, and 5 mo after transplanting (Fig. 4B). Interaction between mycorrhizal fungi and Fusarium had no significant effect on shoot dry weights.

DISCUSSION

This study demonstrated that infection by the pathogens, *F. o. medicaginis* and *V. albo-atrum*, reduced growth of alfalfa seedlings at 2, 3, and 4 mo after inoculation. The addition of mycorrhizal fungi together with pathogens reduced the impact of the pathogens. The lower incidence of wilt and increased growth of seedlings when coinfected by mycorrhizae and pathogens indicate that alfalfa infected by mycorrhizal fungi is resistant

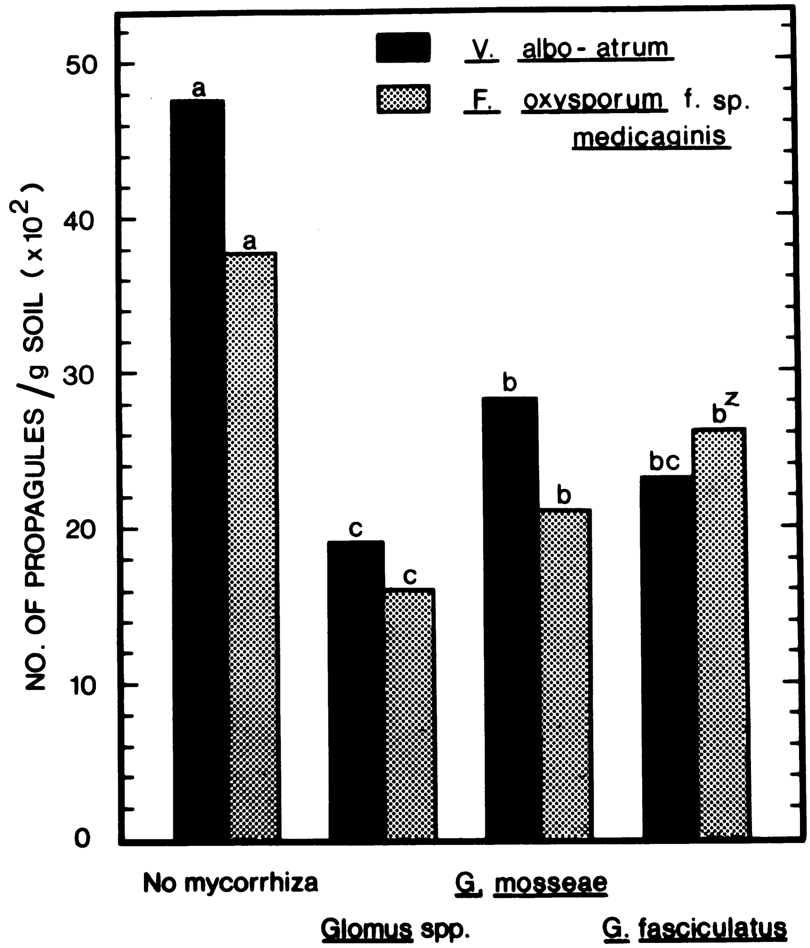

Fig. 2. Numbers of propagules of *Verticillium albo-atrum* or *Fusarium oxysporum* f. sp. *medicaginis* in the rhizosphere of alfalfa seedlings in the presence or absence of VA mycorrhizal fungi. z = Means of the number of propagules of *V. albo-atrum* or *F. o. medicaginis* followed by the same letter do not differ significantly from each other by Duncan's new multiple range test ($P = 0.05$).

Table 1. Mycorrhizal colonization of roots of alfalfa plants inoculated with *Verticillium albo-atrum* or *Fusarium oxysporum* f. sp. *medicaginis*

	Colonization (%)		
Treatment	**Root segment**	**Root length**	**No. of vesicles**
V. albo-atrum (VAA)			
Glomus spp.	91 a[z]	90 a	83 a
G. fasciculatus	81 b	53 d	30 c
G. mosseae	80 b	63 c	30 c
Glomus spp. + VAA	71 c	72 b	71 b
G. fasciculatus + VAA	58 d	27 f	16 d
G. mosseae + VAA	69 c	34 e	10 e
F. o. medicaginis (FOM)			
Glomus spp.	88 a	92 a	77 a
G. fasciculatus	78 b	55 d	31 d
G. mosseae	76 b	64 c	46 c
Glomus spp. + FOM	65 c	70 b	61 b
G. fasciculatus + FOM	51 d	25 f	15 f
G. mosseae + FOM	53 d	34 e	20 e

[z] Values represent means of 500 root segments. Means within a column for each experiment followed by the same letter do not differ significantly from each other by Duncan's new multiple range test ($P = 0.05$).

to Fusarium and Verticillium wilts. Schönbeck (41) hypothesized that in the presence of VA mycorrhizal fungi, fungal root diseases usually are reduced, although fungal shoot diseases often are enhanced. Other workers also have found increased tolerance to fungal root pathogens in VA mycorrhizal plants (3, 8–12,15,24,42–44). The severity of wilt was increased at 2, 3, and 4 mo after inoculation in our study. This indicates that seedlings are susceptible to *F. o. medicaginis* and *V. albo-atrum* even when they age.

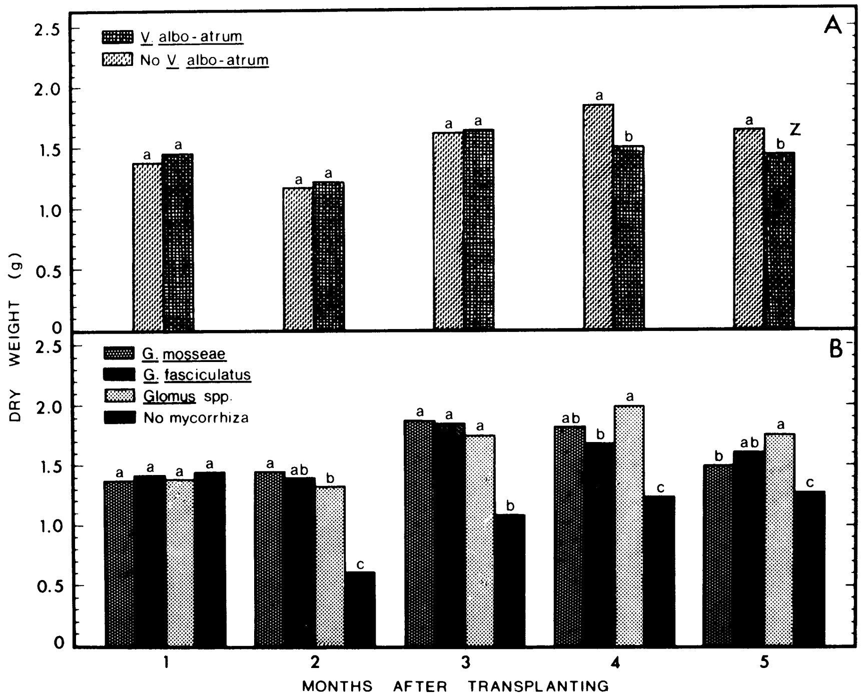

Fig. 3. Shoot dry weights of alfalfa seedlings compared at different times after transplanting after their inoculation **(A)** with or without *Verticillium albo-atrum* or **(B)** with or without mycorrhizal fungi. There were no significant mycorrhizal fungi × *Verticillium* interactions for shoot dry weight. z = Means within a month followed by the same letter do not differ significantly from each other by Duncan's new multiple range test ($P = 0.05$).

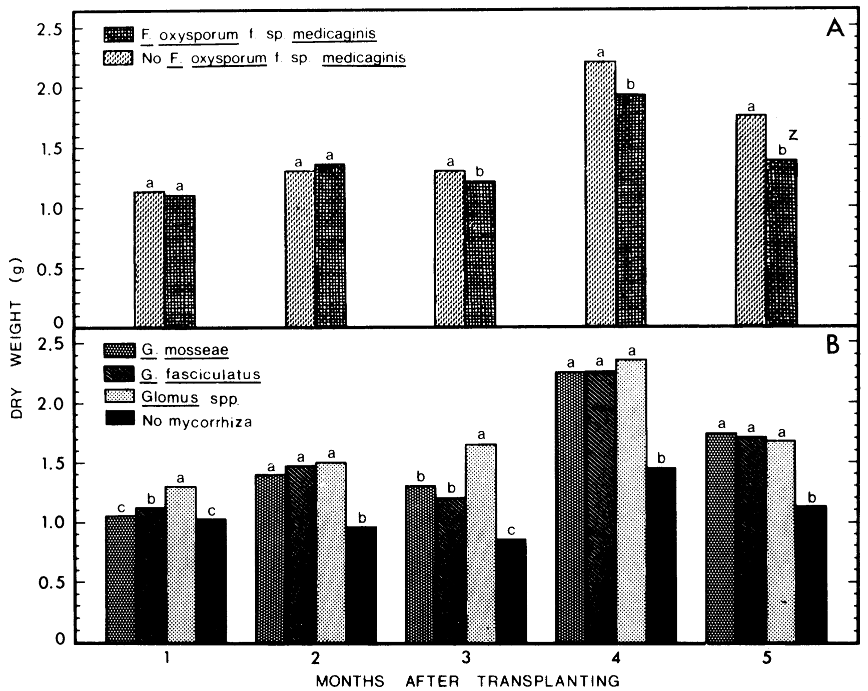

Fig. 4. Shoot dry weights of alfalfa seedlings compared at different times after transplanting after their inoculation **(A)** with or without *Fusarium oxysporum* f. sp. *medicaginis* or **(B)** with or without mycorrhizal fungi. There was no significant mycorrhizal fungi × *Fusarium* interactions for shoot dry weight. z = Means within a month followed by the same letter do not differ significantly from each other by Duncan's multiple new range test ($P = 0.05$).

Gerdemann (22) questioned whether altered disease resistance by VA mycorrhizal plants was attributable to improved plant nutrition or to other, more direct, mechanisms. In this study, lower disease severity, increased plant growth, and reduction in density of *F. o. medicaginis* and *V. albo-atrum* in the soil were associated with the presence of VA mycorrhizal fungi. It appears that the increase of nutrient absorption by mycorrhizal roots cannot alone account for increased tolerance to Fusarium and Verticillium wilts. Dehne (14) and Dehne and Schönbeck (18) observed that mycorrhizal roots were more lignified and contained more polysaccharides than nonmycorrhizal ones, especially in the stellar tissue. This may be responsible for the restriction of the mycorrhizal fungi to the root cortex. The same mechanisms of resistance may be effective against parasitic soilborne pathogens invading the host root. Roots colonized by mycorrhizal fungi also exhibit greater chitinolytic activities. These enzymes can be inhibitory against certain fungal pathogens (19).

Gerdemann (22) suggested possible mechanisms of disease tolerance induced by VA mycorrhizal fungi on host plants, such as production of antibiotics or other inhibitory compounds, altered root exudates, or changes in the microbial rhizosphere population. In this study, the population density of *F. o. medicaginis* and *V. albo-atrum* was reduced significantly in the presence of *Glomus* spp., *G. mosseae*, and *G. fasciculatus*. This suggests that the decreased pathogen inoculum in pots of VA mycorrhizal treatments could be attributable to lower disease severity that resulted in less production of secondary inoculum. Although it is now known that ectomycorrhizal fungi produce antibiotics or other inhibitory compounds in vitro (34), no information is available for VA mycorrhizal fungi. The effect of VA mycorrhizae on *F. o. medicaginis* and *V. albo-atrum* and on the development of disease should be investigated further to determine whether this effect occurs on the root or in the substrate. Because *F. o. medicaginis* and *V. albo-atrum* populations might be disseminated throughout the substrate, interaction between VA mycorrhizal fungi and the pathogens might occur far from the root between extra matrical hyphae and *F. o. medicaginis* and *V. albo-atrum* propagules.

This study suggests that VA mycorrhizae act to some degree as a biological control agent against *F. o. medicaginis* and *V. albo-atrum* wilts of alfalfa. The mechanism of tolerance appears attributable not only to improved plant nutrition by the mycorrhizal fungi but also to other factors associated with VA mycorrhizal fungi. This work emphasizes the necessity of further investigations on the possible production of antimicrobial compounds produced by VA mycorrhizal fungi or their hosts and on the environmental conditions that could enhance disease resistance.

ACKNOWLEDGMENTS

We thank L. J. Piening, D. Gaudet, L. M. Dosdall, and H. Philip for their valuable suggestions on the manuscript, and D. Aiello, N. T. Cowle, and M. Herbut for their technical assistance.

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