Effects of Fumigation and Fungal Antagonists on the Relationships of Inoculum Density to Infection Incidence and Disease Severity in Fusarium Crown Rot of Tomato

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ABSTRACT

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Fusarium crown rot of tomato was used as a model to quantify the effects of fumigation and biological control agents on the relationships of inoculum density to disease incidence and subsequent disease severity. The model was chosen because the epidemic is severe when soils are treated with biocides that allow recolonization by *Fusarium oxysporum* f. sp. *radicis-lycopersici;* thus, the introduction into soils of microorganisms that are antagonistic to the pathogen should provide protection against the disease. Chlamydospores of the pathogen were formed under axenic conditions so that defined concentrations of specific inocula could be added to freshly fumigated soil. The antagonists were selected for their abilities to increase populations rapidly in freshly fumigated soil, to establish high populations in the root zone of the host, and to interact with the pathogen to reduce the incidence of infection or disease. The inoculum levels of the pathogen at which 50% of the tomato plants were infected were 300, 900, and 6,500 chlamydospores per gram of soil that had been fumigated previously, not fumigated, or fumigated and infested with antagonists, respectively. In greenhouse experiments the mean lesion length on stems increased as the inoculum density was increased in fumigated soil; lesion length, however, did not increase as the inoculum density was increased in fumigated soil with antagonists added.

Additional key words: Penicillium funiculosum, Trichoderma harzianum, Aspergillus ochraceus, Lycopersicon esculentum.

Fusarium crown rot of tomato (*Lycopersicon esculentum* Mill.), which is caused by *Fusarium oxysporum* Schlecht f. sp. *radicis-lycopersici* Jarvis and Shoemaker, is a disease which is severe when tomatoes are grown in soil treated with biocides (8,9,19). This phenomenon fits Kreutzer's (11) concept of disease trading in which dominant pathogens are controlled by soil treatments but minor pathogens are elevated to major importance because they can recolonize soil in which their competitors and antagonists have been eliminated. The traditional methods of applying fungicides directly to plants growing in previously treated soil have proved ineffective in the control of Fusarium crown rot (18). Rowe and Farley (18), however, controlled the disease with the application of captafol to freshly steamed soil before planting. The successful results were attributed to the selective action of the fungicide which inhibited reinvasion by the pathogen but did not adversely affect the recolonization of the soil by other airborne microorganisms.

Thompson (25), as early as 1929, realized that chemical agents would be useful in disease control mainly where conditions are relatively unfavorable for the pathogen and that biological agents would be more important when the environment is conducive to activity of the pathogen. In Florida, an environment conducive to the development of Fusarium crown rot is established when plastic mulch, which inhibits recolonization by airborne inocula, is applied during fumigation and maintained during the entire growing season. If antagonists of *F. oxysporum* f. sp. *radicis-lycopersici* could be introduced under the plastic before the soil is recolonized by the pathogen, it should be possible to reduce disease. Thus, the nature of the pathogen and tomato production methods provide an excellent system for a quantitative field study of biological control.

Before field studies are undertaken, however, the importance of fumigation and recolonization of soil by antagonists can be evaluated critically by quantitatively determining the relationships of the pathogen and antagonists to incidences of infection and disease in fumigated or nonfumigated soil under growth-chamber and greenhouse conditions.

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The quantification of inoculum required the development of a procedure in which defined levels of chlamydospores of the pathogen could be established in freshly fumigated soil. The present procedure used in inoculum density studies with *Fusarium* spp. involves placing plants in soil with populations of the pathogen established by assaying artificially infested, incubated soil with selective media and diluting the assayed, infested soil with noninfested soil (6). This procedure is not applicable to a system which demands freshly treated soil because populations of many microorganisms can become established during the time required for the incubation of soil infested with the pathogen. An alternative to this method is to infest soil with chlamydospores produced under axenic conditions and quantified by direct counts; a subsequent estimation of the population can be obtained by soil dilution plating.

The objectives of this study were: to determine the relationships of densities of chlamydospores of *F. oxysporum* f. sp. *radicis-lycopersici* to the incidence of infection of tomato in fumigated and nonfumigated soils, to determine the effects that selected antagonists have on the relationship of inoculum density to the incidence of infection, and to determine the effects of antagonists on disease severity. The techiques and procedures were developed so that they can be applied to any disease which is severe after fumigation due to decreased competitor populations and subsequently increased populations of the pathogen. Portions of this work have been reported previously (12,13).

MATERIALS AND METHODS

The isolate of *F. oxysporum* f. sp. *radicis-lycopersici* was obtained from a diseased tomato plant collected in a south Florida field. Cultures were stored in soil tubes according to the method of Toussoun and Nelson (26).

Pompano fine sand was treated with methyl bromide-chloropicrin (67:33%, v/v) at the rate of 1 kg of fumigant to 50 kg of soil for 2 days in a sealed container and then allowed to air in the greenhouse for 4 days.

Chlamydospores of the pathogen were used as inoculum to simulate natural conditions in which chlamydospores of *Fusarium*

spp. are the major survival structure (26). For the production of chlamydospores, macroconidia were washed from 2-wk-old cultures grown on potato-dextrose agar (PDA) (Difco, Detroit, MI 48201) at 25 C under continuous fluorescent light (3,000 lux). The macroconidia formed intercalary chlamydospores after 4 wk of incubation at 10^6 macroconidia per milliliter of water at 28 C in the dark.

The potential antagonists were isolated from recolonized soils 1 wk after fumigation. A soil dilution of 1 g of air-dried soil to 1,000 ml of water was plated on PDA which contained 1 ml of Tergitol NPX (Sigma Chemical Co., St. Louis, MO 63178) and 50 mg of chlortetracycline hydrochloride (Sigma Chemical Co., St. Louis, MO 63178) per liter of medium (PDA-TC). Conidial suspensions of each isolate were obtained by washing 2-wk-old cultures grown on PDA at 25 C under 10 hr of fluorescent light (2,000 lux) per day. Fifty milliliters of each suspension containing 2×10^4 conidia per milliliter then were added to 1 kg of freshly fumigated soil (final water concentration = 10% w/w). One-half kilogram of the infested soil was then placed in a plastic container and stored at 25 C for 1 wk, after which the population of each isolate was determined by dilution plating on PDA-TC. The remaining soil infested with an individual isolate was placed in 100-ml polypropylene beakers containing 80 g of soil per beaker. Two germinated Bonnie Best tomato seeds were placed in each beaker, and the beakers were moved to growth chambers set at 20 C and 12 hr of light (4,000 lux) per day. After 2 wk the roots were washed lightly and plated on PDA-TC. One week later the number of colonies of each potential antagonist growing from the roots was used to evaluate its ability to occupy the root environment. Those isolates which increased rapidly in the freshly fumigated soil and occupied the root environment were tested for their potential to increase the ratio of inoculum density to disease incidence in preliminary growth-chamber experiments. Of the 26 isolates which fulfilled the first two requirements, the five selected for the rest of the tests included three isolates of *Trichoderma harzianum* Rifai, one isolate of *Penicillium funiculosum* Thom, and one isolate of *Aspergillus ochraceus* Wilhelm.

Concentrations of conidia of the antagonists and chlamydospores of *F. oxysporum* f. sp. *radicis-lycopersici* were determined by counting 40 fields of a standard hemacytometer, and the desired dilutions were added to soil.

The relationships of inoculum density to infection incidence were determined by experiments done in growth chambers. Two germinated Bonnie Best tomato seeds were placed in a 100-ml polypropylene beaker which contained 60 g of thoroughly mixed infested soil layered over 50 g of autoclaved sand. A range of inoculum densities of the pathogen was used and a composite of the antagonists was added at the constant concentration of 5,000 conidia per gram of air-dried soil for each isolate. Twenty-four beakers of each inoculum combination were placed in growth chambers. After 2 wk the soil was washed from the roots; the plants were soaked in 0.6% sodium hypochlorite for 1 min and rinsed in autoclaved deionized water. The roots and lower stem were plated on Komada's (10) selective medium for *F. oxysporum* and observed after 10 days for colonies of the fungus. Populations of the pathogen in the soil from the beakers also were determined after 2 wk of incubation in the growth chamber. The populations of the pathogen were quantified by dilution plating on Komada's (10) medium. The isolates of *F. oxysporum* f. sp. *radicis-lycopersici* from soil dilutions and plant tissue were identified by the technique of Sanchez et al (20), in which the type of lesion on tomato seedlings grown in pathogen-infested water agar is used to differentiate the isolates of the pathogen from nonpathogenic and wilt-inducing *F. oxysporum* isolates.

In greenhouse experiments, 5-wk-old tomato (cultivar Walter) transplants were placed individually in 15-cm-diameter plastic pots containing fumigated soil. A 0.102-mm (4-mil)-thick plastic film was placed over the soil to simulate the plastic mulch used in production fields. The transplant was planted through a 3-cm-diameter hole in the center of the plastic film. Fifty milliliters of a suspension containing 5×10^5 conidia of each antagonist were poured into the planting hole and over the transplant's crown and roots. Ten milliliters of chlamydospore suspensions of the pathogen were injected into the soil at each of two points approximately 7 cm from the plant. The plants were fertilized with half-strength Hoagland's (7) solution every 2 wk and watered when necessary. After 12 wk the root weight, infection incidence (as determined by plating the roots and crown on Komada's [10] medium), disease incidence (as determined by the presence of lesions), and lesion length were recorded.

The data presented in this paper are means of experiments repeated two times. Each replicate consisted of 48 plants per treatment in the growth-chamber studies and 15 plants per treatment in the greenhouse studies.

RESULTS

Percentages of infection, percentages of diseased tomato plants, and mean lesion lengths increased with increasing inoculum levels of the pathogen (Fig. 1, Table 1). In the growth-chamber experiments, the ratio of inoculum density to infection was lowest in the fumigated soil and highest in the fumigated soil with antagonists added (Fig. 1A).

Slopes of the data from the growth-chamber experiments determined by linear regression analyses of $\log_{10}$ ($\log_e 1/[1-X]$), in which X is the proportion of infected plants, on $\log_{10}$ inoculum density were 0.82 ($r = 0.98$), 0.98 ($r = 0.97$), and 0.99 ($r = 0.99$) with fumigated soil, nonfumigated soil, and fumigated soil plus antagonists, respectively (Fig. 1B). The inoculum densities required for 50% infection of plants (ID_{50}) in each soil in the growth-chamber experiments were interpolated to be approximately 300, 900, and 6,500 chlamydospores per gram of air-dried soil in fumigated soil, nonfumigated soil, and fumigated soil plus antagonists, respectively.

When the initial inoculum density of the pathogen was 500 chlamydospores per gram of air-dried soil, populations increased after 2 wk to 4,000 propagules per gram of fumigated soil, remained constant in nonfumigated soil, and decreased to 50 propagules per gram of soil that had been fumigated and amended with fungal antagonists.

In the greenhouse experiments, the fungal antagonist amendment reduced significantly the mean lesion length and the incidence of disease (Table 1). The analysis of variance also showed that the inoculum density of the pathogen and the interaction of the pathogen inoculum density with the antagonists significantly affected disease incidence and mean lesion length ($P = 0.01$). There were no significant correlations between treatments and root weight or percent infection. In all of the treatments, including the controls, the incidence of infection was over 90%.

DISCUSSION

The relationships of inoculum density to disease incidence have been applied in the quantification of several soilborne diseases caused by *Fusarium* spp. (1,4,6,22). Baker (2) proposed that biological effects of antagonists on disease could be quantified by the analyses of curves derived by plotting the disease severity to the inoculum density of the pathogen. In this study the ID_{50} in nonfumigated soil was 900 chlamydospores per gram of soil, which is similar to the ID_{50}s for other diseases caused by *Fusarium* spp. in nontreated soil (4,6), even though different experimental designs were used. The ID_{50} in the fumigated soil was 300 chlamydospores per gram of soil; however, the pathogen inoculum density increased from 500 chlamydospores to 4,000 propagules per gram of soil during the experiment. Guy and Baker (6) reported a similar ID_{50} when the pathogen population increased due to a chitin soil amendment. High ID_{50}s (2,000 or more conidia per gram of soil) were reported by Abawi and Lorbeer (1) in steam-treated and nontreated soils. However, the method for determining disease severity was based on percent emergence rather than on the percentage of infected or disease tissue criterion used in other investigations. Also, conidia were used as the inoculum source rather than chlamydospores, as in other studies. Their data, however, still indicate that the lower ID_{50} occurred in treated soil.

The position and slope of the curve derived by the log-log transformation of inoculum density to disease severity affect the ID_{50} of a particular disease system. Guy and Baker (6) found that adding organic materials to the soil altered the ID_{50} by shifting the position of the curve rather than changing its slope, which they attributed to the relative changes in infection rates being directly correlated with inoculum density. A similar shift was noted in this study in the soils that were fumigated, nonfumigated, or fumigated and amended with antagonists. In all soils the slope was approximately 1.0.

The attenuation of isolates of *Fusarium* spp. grown on artificial media must be considered in quantifying the relationship of inoculum density to disease incidence and severity. As an isolate becomes less virulent, more inoculum will be required to cause disease regardless of the particular treatment. The axenic production of chlamydospores of a pathogen reduces the chances of attenuation because the isolate does not reproduce vegetatively between experiments; thus, the possibility of genetic variation is minimized. Changes in virulence of the fungus were not observed in any of the experiments. The germination rate of chlamyspores of *F. oxysporum* f. sp. *radicis-lycopersici* on PDA was not significantly different from 100% ($P = 0.05$) for up to 1 yr after their formation. French (5) reported that chlamydospores formed from macroconidia of *F. oxysporum* f. sp. *batatas* remained virulent for 7 yr when stored in water.

The application of a broad spectrum biocide to a soil creates a biological vacuum which disrupts the stability of a soil community. The early recolonization pattern of treated soil involves a shift to an early successional pioneer stage consisting of a few species which occur in large numbers (28). Thus, the community is of low diversity and readily may be invaded by new species if the environmental conditions are not severe. In this study the pathogen population remained stable, as determined by dilution plating, in the more advanced seral stages of the nonfumigated soil. In the pioneer successional stage of the fumigated soil, the pathogen was able to compete and increase inoculum density. The limited ability to compete as a saprophyte is characteristic of other pathogenic *Fusarium* spp. (15,17,24).

The ability of other plant pathogens to compete as saprophytes in treated soil also may be reduced with the reestablishment of the microbial community (3,16,21,27). A decrease with time of the percentage of isolations of *Verticillium albo-atrum* from seedlings of *Senecio vulgaris* was attributed to the recolonization of autoclaved soil by airborne propagules of other microorganisms (21). This decrease was similar to that observed by Rowe and Farley (18) with Fusarium crown rot of tomato. They found that when saprophytic airborne propagules colonized steamed soils disease severity was reduced.

The observed decrease in the population of the pathogen from an initial inoculum density of 500 chlamydospores per gram of fumigated soil amended with antagonists to 50 propagules per gram of soil may have been caused either by the production of toxins or by parasitism of the pathogen by the antagonists. The

TABLE 1. Effect of initial inoculum density of *Fusarium oxysporum* f. sp. *radicis-lycopersici* and a composite of five antagonists on mean lesion length and percentage of plants with lesions under greenhouse conditions

Inoculum density (chlamydospores per pot)[x]	Antagonists	Mean lesion length (cm)	Plants with lesions (%)
0	−	0.00[y]	0[y]
500	−	0.98	50
5,000	−	1.73	69
50,000	−	2.22	70
mean		1.23a	47a
0	+[z]	0.14	4
500	+	0.73	33
5,000	+	0.68	33
50,000	+	0.96	44
mean		0.63b	28b

[x] Twenty milliliters of a chlamydospore suspension was injected into the soil 7 cm from the plant.

[y] Means in same column with different letters differ significantly ($P = 0.05$) as determined by *t*-test; percentage data analyzed after transformation to arcsine $\sqrt{x}$.

[z] + = conidia of each of five isolates (three isolates of *Trichoderma harzianum*, one isolate of *Pennicillium funiculosum*, and one isolate of *Aspergillus ochraceus*) were added to the crown area of the transplant at 5 × 10^5 conidia of each isolate per pot.

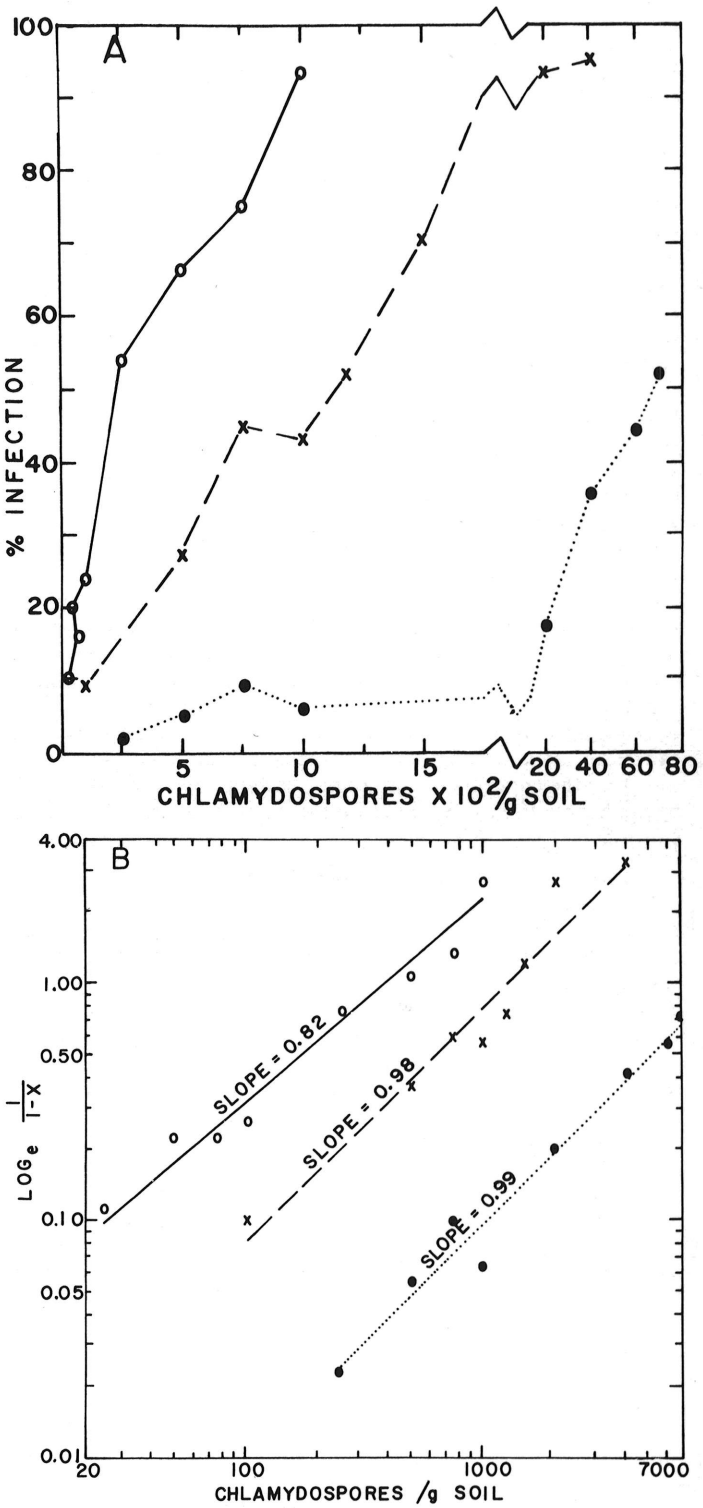

Fig. 1. The relationship of percentages of infection of tomato (cultivar Bonnie Best) under growth-chamber conditions to densities of chlamydospores of *Fusarium oxysporum* f. sp. *radicis-lycopersici* in fumigated soil (O——O), nonfumigated soil (×——×), and fumigated soil amended with *Trichoderma harzianum, Aspergillus ochraceus,* and *Penicillium funiculosum* (●·····●): **A,** proportion of infection (arithmetic) and inoculum density (arithmetic), and **B,** proportion of infection adjusted for multiple infections (logarithmic) and inoculum density (logarithmic).

increase in the ratio of inoculum density to infection in fumigated soils with antagonists added, as compared to fumigated only or nonfumigated soils, may have been due to the adverse effect of the antagonists on the growth of the pathogen or the successful competition of the antagonists at potential infection sites. The reduction in the ability of the pathogen to cause infection is evident by the increase in the ID_{50} in the growth-chamber experiments and the decrease in the disease incidence and lesion length in the fumigated soils amended with the antagonists. The correlation of increased numbers of infections and the increase in disease severity has been reported in other diseases caused by *Fusarium* spp. (14,22,23).

The reduced infection in the growth-chamber experiments and the reduced lesion length in the greenhouse experiments were due to the partial restabilization of the treated soil and root environment by addition of the fungal antagonists. The observation that nearly all of the plants including the uninoculated controls were infected in the greenhouse studies was attributed to the long duration (12 wk) of the experiment and the ability of the pathogen to spread from airborne inoculum. Rowe et al (19) found that under greenhouse conditions treated soil was rapidly reinfested by the pathogen via airborne microconidia.

Reduction of the severity of Fusarium crown rot of tomato with biological control is dependent upon the reestablishment in freshly treated soil of a microbial community that impairs the reinvasion by *F. oxysporum* f. sp. *radicis-lycopersici*. The host-pathogen model employed in this study allowed the development of a system for the selection of antagonists, and the quantification of a biological control procedure under growth-chamber and greenhouse conditions. This information provides a qualitative and quantitative basis for the application of antagonists in the field in south Florida. In addition to the field experiments, information is needed on the effects of the addition of antagonists on recolonization by other species of microorganisms, and on rates of recolonization by artificially and naturally introduced microorganisms. The interactions of several microbial populations can best be understood at the community level of the ecosystem and therefore the concepts of community stability and succession will be applicable to future studies on the quantification of biological control.

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