

## Mechanisms Involved in Biological Control of Fusarium Wilt of Cucumber with Strains of Nonpathogenic *Fusarium oxysporum*

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### ABSTRACT

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Two nonpathogenic strains of *Fusarium oxysporum* (C5 and C14) introduced into soil at  $1.08 \times 10^4$  and  $5 \times 10^4$  colony-forming units (cfu) per gram of soil significantly decreased the slope values of inoculum density-disease incidence curves generated for Fusarium wilt of cucumber induced by *F. oxysporum* f. sp. *cucumerinum*. Strain C14 reduced disease significantly more than C5. A three-phase mechanism was associated with biological control of Fusarium wilt by these agents. There was a significant reduction in germination of chlamydo-spores of *F. o. cucumerinum* in the rhizospheres of cucumbers infested with C5 or C14. Competition for infection sites with *F. o. cucumerinum* was demonstrated in soil infested with C14 but not C5. Enhanced systemic resistance of the host to inoculation with *F. o. cucumerinum* or *Colletotrichum lagenarium* occurred when wounded or nonwounded roots were exposed to C14. Strain C5 significantly reduced disease induced by *F. o. cucumerinum* in similar

experiments only if roots were wounded when the agents were introduced. When roots were exposed to either C5 or C14 and microconidia of *F. o. cucumerinum* applied to transversely severed stems of cucumber, significant reduction in germination of the pathogen occurred 72-96 hr after introduction of the biocontrol agents; however, wounding of roots was necessary for C5 to induce the response. Therefore, mechanisms of suppression of Fusarium wilt of cucumber by these nonpathogenic agents involved competition in the rhizosphere and infection sites, as well as induction of enhanced resistance in the host. Apparently, the ability of C14 to compete for infection sites, penetrate through intact root tissue, colonize the host, and induce resistance reactions in the host were attributes that contributed to its superior performance in biological control in comparison with C5.

Biological control of Fusarium wilt diseases has been accomplished by introducing nonpathogenic *Fusarium* spp. in soil or infection courts (e.g., 1,7,8,12,18-21,23,26). Three hypotheses were proposed to explain mechanisms involved in the suppression of the pathogenic *Fusarium* that result in a decrease in disease incidence. Alabouvette et al (1) compared root colonization, chlamydo-spore germination, and population dynamics of *Fusarium* in conducive and suppressive soils, and concluded that suppression was due to nutrient competition between pathogenic and saprophytic *Fusarium* spp. Schneider (23) proposed that the reduced incidence of Fusarium wilt of celery induced by nonpathogenic *Fusarium* spp. was directly correlated to competition for infection sites at the root surface. Enhanced resistance induced by nonpathogenic *Fusarium* spp. (7,8,15,18,26) also was demonstrated in protection against pathogenic *Fusarium* spp. Therefore, a multi-component mode of action might be involved (6), and an examination of the possibility of diverse mechanisms in biological control induced by nonpathogenic *Fusarium* spp. is appropriate.

Our objective was to determine the nature of biological control by strains of nonpathogenic *Fusarium oxysporum* Schlecht.:Fr. by testing three hypotheses: that competition for nutrients occurs in the rhizosphere; that there is competition for infection sites on the rhizoplane; and that host resistance responses are enhanced.

### MATERIALS AND METHODS

**Soil.** Nunn sandy loam soil, used in some of these investigations, was collected near Nunn, CO. It was air-dried, passed through a 4-mm screen and stored in an outside bin. Characteristics of the soil were: pH 7.0, 0.4 mmhos/cm conductivity, low lime, 1.4% organic matter, 1  $\mu\text{g/g}$  of  $\text{NO}_3\text{-N}$ , 9  $\mu\text{g/g}$  of P, 198  $\mu\text{g/g}$  of K, 0.5  $\mu\text{g/g}$  of Mn, and 3.2  $\mu\text{g/g}$  of Fe. In other experiments, Terra-Lite potting mix (W. R. Grace & Co., Cambridge, MA) was used as a substitute for raw soil.

**Cultures and inoculum.** Nonpathogenic strains of *F. oxysporum*, C5 and C14, were originally isolated by Paulitz et al (20) from surface-disinfested, symptomless cucumber roots grown in Nunn sandy loam soil; *F. oxysporum* emend. Snyder & Hans. f. sp. *cucumerinum* J. H. Owen is pathogenic to cucumber (*Cucumis sativus* L. 'Marketer Long').

Nonpathogenic strains C5 and C14 of *F. oxysporum* and *F. o. cucumerinum* were increased from single-spores, cultured, and stored in sterile soil at room temperature. All strains were recovered from soil storage and cultured on potato-dextrose agar (PDA) (Difco Laboratories, Detroit, MI) before use.

*Colletotrichum lagenarium* (Pass.) Ell. & Halst. race 1, grown on lima-bean agar slants, was obtained from J. Kuć, University of Kentucky, Lexington (17). This also was pathogenic to the same cucumber cultivar.

Conidia of C5, C14, and *F. o. cucumerinum* were obtained by placing a small amount of soil stock cultures on petri dishes containing 2% water agar. After 1 day, germinating microconidia were transferred to PDA. Cultures were maintained at room temperature and incubated for 8 days. Dishes were flooded with sterile water, spores were gently freed from the culture surface with a brush, and the suspension was sieved through eight layers of cheesecloth to remove mycelial fragments. Spore suspensions were dispensed into 50-ml plastic tubes and centrifuged (2,500 g for 20 min). The supernatant was decanted and discarded and the pellet was resuspended in sterile water. This process was repeated at least three times. Density of the spore suspension was adjusted to the desired level as determined with the aid of a hemacytometer.

Chlamydo-spore inoculum of *F. oxysporum* was prepared as described by Paulitz et al (20). Nunn sandy loam soil (1 kg) was mixed with 20 g of rolled oats (Quaker Oats Co., Chicago, IL) and 12 g of dried bean leaves. The soil was moistened to 15% (w/w), placed in 2-L Erlenmeyer flasks, and autoclaved for 1 hr on 2 consecutive days. Mycelial disks from cultures of *F. oxysporum* were transferred into 50 ml of potato-dextrose broth in 250-ml Erlenmeyer flasks. Cultures were incubated on a rotary shaker operating at 250 rpm at 25 C. After 4 days, cultures were

sieved aseptically through eight layers of cheesecloth and washed three times in sterile distilled water. A 5-ml sample of the suspension containing a high proportion of microconidia was aseptically transferred to the soil. Erlenmeyer flasks containing infested soil, oats, and bean-leaf mixture were shaken twice weekly and incubated for 4 wk at room temperature.

The soil inoculum was air-dried for 48 hr and colony-forming units per gram of soil from the strains were determined by plating a series of 10-fold dilutions onto acidified PDA. The soil contained approximately  $10^5$  cfu/g of soil. Examination of the inoculum by fluorescent microscopy (22) showed that the primary inoculum consisted of chlamydospores.

**Effect of strains C5 and C14 on incidence of Fusarium wilt.** To determine the effect of C5 and C14 on the inoculum density-disease incidence interactions of *F. o. cucumerinum*, the following experiments were conducted. Soil-chlamydospore inoculum of *F. o. cucumerinum* and each nonpathogenic strain were mixed together with air-dried Nunn sandy loam soil for 5 min in a twin-shell soil blender. Final levels of inoculum obtained were  $1 \times 10^2$ ,  $2.5 \times 10^2$ ,  $6.5 \times 10^2$ ,  $1.6 \times 10^3$ , and  $2 \times 10^3$  cfu/g of soil from *F. o. cucumerinum*, and  $1 \times 10^3$ ,  $2.7 \times 10^3$ ,  $7.1 \times 10^3$ ,  $1.08 \times 10^4$ , and  $5 \times 10^4$  cfu/g of soil from either C5 or C14 in all combinations. Ethazole (5-ethoxy-3-trichloromethyl-1,2,4-thiadiazole) was added to all these treatments (55  $\mu$ l/L per kilogram of soil) to inhibit pathogenic *Pythium* spp.

Water was added to the raw soil to obtain 10% moisture and the soil was mixed again by hand. A kilogram of soil of each inoculum level was placed in a 15-cm-diameter plastic pot in four replications. Cucumber seeds were sown in a 1-cm layer of potting mix over the raw soil to enhance uniform germination. Each pot was sown with 15 cucumber seeds. The pots were watered and covered with polyethylene sheets to maintain moisture during seed germination. After 5 days, seedlings were thinned to 10/pot. The pots were transferred to a greenhouse bench and arranged in a randomized complete block design. Plants were watered with tap water as needed and twice weekly with nutrient solution (Peters General Purpose 10-10-10, 1:200, Peters Fertilizer Prod., W. R. Grace & Co., Fogelsville, PA). Greenhouse temperature was maintained at 28–30 C. Disease incidence was recorded after 40–50 days.

**Germination of chlamydospores of *F. o. cucumerinum* in the rhizospheres of cucumber roots treated with C5 or C14.** Cucumber seeds were surface-disinfested by soaking for 5 min in 2% sodium hypochlorite solution and washing three times in sterile distilled water. Seeds were pregerminated by incubating at 25 C for 4 hr on autoclaved moist layers of paper towels in petri dishes. Chlamydospore inoculum of *F. o. cucumerinum* in soil was prepared as previously described to achieve an inoculum density of  $10^5$  cfu/g of soil from the pathogen in combination with a population density of  $10^4$  cfu/g of soil from C5 or C14. Samples of chlamydospore-infested soil of both the pathogen alone and with biocontrol agents (C5 or C14) were thoroughly mixed together and placed on 76- $\times$ -25-mm glass microscope slides in a 4–5 mm thick layer in four replicates. A germinated cucumber seed was placed on the upper portion of each slide, covered with a second glass slide, secured with two rubber bands, and the slides were inserted at a slight angle in moist, raw soil in plastic pots (8 cm diameter by 8 cm high). Pots were covered with plastic bags and incubated at 28 C for 36 hr to allow the roots to grow along the slides. The slides were removed from soil, the rubber bands were removed, and soil not adhering to the root was removed by gently shaking the plants. Rhizosphere soil was transferred to test tubes for processing and staining. One milliliter of 0.3% Calcofluor White M2R New (American Cyanamid Co., Bound Brook, NJ) solution was added to each tube. Tubes were covered with aluminum foil and incubated at 28 C for 5 hr. Soil was suspended by shaking, and a drop of the suspension from each treatment/replicate was placed on a microscope slide. Chlamydospore germination was observed with an Olympus BH microscope (Olympus Optical Co., Tokyo, Japan), with a blue exciter filter (BG-12) that provided 400 nm of light supplied by an epifluorescent illuminator. A barrier filter (350 nm) also was

used when viewing the slides. The numbers of germinated and ungerminated chlamydospores on each slide were recorded. At the level added to the soil, chlamydospores of C5 or C14 were not seen. From each sample, 100 chlamydospores were counted and the percentage of germination was determined. A similar technique was used previously for assays of competition in rhizospheres by fluorescent pseudomonads (10).

**Competition for infection sites.** To assay the relative number of infection sites colonized by the pathogen and the biocontrol agents either alone or in combination, colony-forming units of the organisms from roots of cucumber were assayed shortly after penetration occurred.

Cucumber seeds were treated and pregerminated. Germlings were selected for uniformity of stage of germination and placed (10 germlings per 10-cm-diameter pot) in moist Terra-Lite infested or uninfested with various population densities of *F. o. cucumerinum* and/or either strain C5 or C14 as described earlier. Plants were not watered after seeding, but plastic covers were placed over the pots for slow evaporation. Treatments were arranged in a randomized complete block design with four replications in a growth room held at  $27 \pm 2$  C with 10 white fluorescent, 40-W tubes 1–1.5 m above the pots. After seeds were germinated, roots grew at a rate of 1.5 cm/day. Seedlings were grown for 5 days. Of the 10 seedlings exposed to each combination of *F. o. cucumerinum* and C5 or C14 (four replicates), five were chosen for uniformity of length and diameter of roots. Stems were severed 3 cm above the soil surface and the attached roots were washed to remove adhering soil. Sections of stem from each treatment and replicate were bound together with masking tape, placed in a beaker covered with a nylon net, and washed in running tap water for 20 min. Of 20 roots exposed to *F. o. cucumerinum* in combination with C5 or C14, one from each of the four replications was taken for analysis, dried briefly between four layers of autoclaved paper towels, transferred to a dish containing 2% acidified water agar, and incubated for 30 hr at 25 C.

Root segments, each showing 1 day of growth, were excised 0–1.5, 1.5–3.0, and 3.0–4.5 cm from the tip of the root. The sections were dried with filtered air on autoclaved towels in a transfer hood for 3 hr and then placed on wax paper (5  $\times$  5 cm). The paper was folded and root segments were crushed into a fine powder that was distributed uniformly on acidified PDA. The number of colony-forming units of *F. o. cucumerinum*, C5, or C14 per section of root was determined. Strains were identified by colony morphology and pigmentation.

**Bioassays for induced resistance.** Bioassays were used to test systemic resistance induced by either C5 or C14.

**Enhanced resistance induced by strain C5 or C14 to anthracnose of cucumbers.** Cucumber seeds were planted, one per pot, in Terra-Lite potting mix. Plants were grown in a growth chamber (Percival, Model E32U, Boone, IO) for 25 days at  $29 \pm 1$  C in continuous light. Plants were lifted from the growth medium and roots were washed three times in running tap water. After severing the root tips, they were immersed in a microconidium suspension ( $10^6$ /ml) of C5 or C14 for 30 min. In some treatments, roots were not trimmed, but were exposed to chlamydospore inoculum ( $10^4$  cfu/g of mix) of either C5 or C14. Appropriate controls not exposed to C5 or C14 were included.

After replanting in Terra-Lite, the second and third true leaves were inoculated with *C. lagenarium* by applying 6-mm-diameter filter disks (five per leaf) soaked in a spore suspension containing  $10^6$  conidia per milliliter as described by Biles and Martyn (8). Plants were placed in a humidity chamber in a randomized complete block design for 24 hr at 27 C. Lesions were counted 10 days later after further incubation in the growth room. There were eight plants (replicates) per treatment.

**Stem lesions induced by *F. o. cucumerinum* after exposure of roots to strain C5 or C14.** Wounded or intact cucumber roots were exposed to strain C5 or C14 or only H<sub>2</sub>O and transplanted as described above. A longitudinal wound in the stem, 2–3 cm above the soil surface, approximately 0.25 cm in length and 20–40  $\mu$ m in depth was made with a razor blade. A water agar block (2 cm diameter) containing *F. o. cucumerinum* was placed in

each wound. Two layers of paper-towel strips saturated with water were wrapped over the wound and secured with masking tape. Agar blocks without *F. o. cucumerinum* were also applied to wounds. Plants were incubated in high humidity for 10 days. The tape and paper-towel strips were removed, and plants were placed in the growth room for an additional 10 days before disease assays were obtained. At this time, girdling of the plants induced by *F. o. cucumerinum* resulted in death; whereas, if lesions were restricted, plants remained viable. Each of eight plants per treatment was considered a replicate.

**Germination of conidia of *F. o. cucumerinum* in wounded tissue after exposure to strain C5 or C14.** Cucumbers were grown in Terra-Lite for 14 days at 27 C in the greenhouse. A water suspension of chlamyospore inoculum ( $10^4$  cfu/ml) of either C5 or C14 was poured into the potting mix around the root ball. Roots of half of the seedlings were wounded with a sharp knife in the area of the root ball. Roots of the other half were not injured. At each interval of 48, 72, and 96 hr after introduction of C5 or C14, the stems of four plants in each treatment or in the control were cut transversely and a microconidium suspension of *F. o. cucumerinum* ( $10^6$  propagules per milliliter) was applied to each surface. Plant units were enclosed in plastic bags containing moist paper towels and incubated at 26 C in the dark.

After 9–10 hr, a thin disk of tissue was cut in a cross-section just below the inoculated stem and stained with 0.5% malachite green and 0.01% acridine orange, and examined microscopically for the percentage of germination of microconidia. A total of 100 microconidia were observed on each stem from each treatment in each replication, and the percentage of those with germ tubes twice their length was recorded.

**Split roots exposed to either strain C5 or C14 and *F. o. cucumerinum*.** Roots of 2-wk-old cucumber plants grown in Terra-Lite in the growth chamber were removed from the mix and washed in running tap water. Roots were trimmed and divided in two approximately equal portions. One of the portions was dipped into 20 ml of a water suspension in test tubes that contained strain C5 or C14 ( $10^6$  cfu/ml) or into sterile water only. The other half of the root system of each plant was inoculated simultaneously in a similar manner with *F. o. cucumerinum* ( $10^6$  cfu/ml of H<sub>2</sub>O) or with H<sub>2</sub>O. Ten replicate plants were included per treatment. The roots were exposed to these treatments for 20 min and then placed with roots still separated into two portions in invert V-shaped plastic plumbing devices filled with Terra-

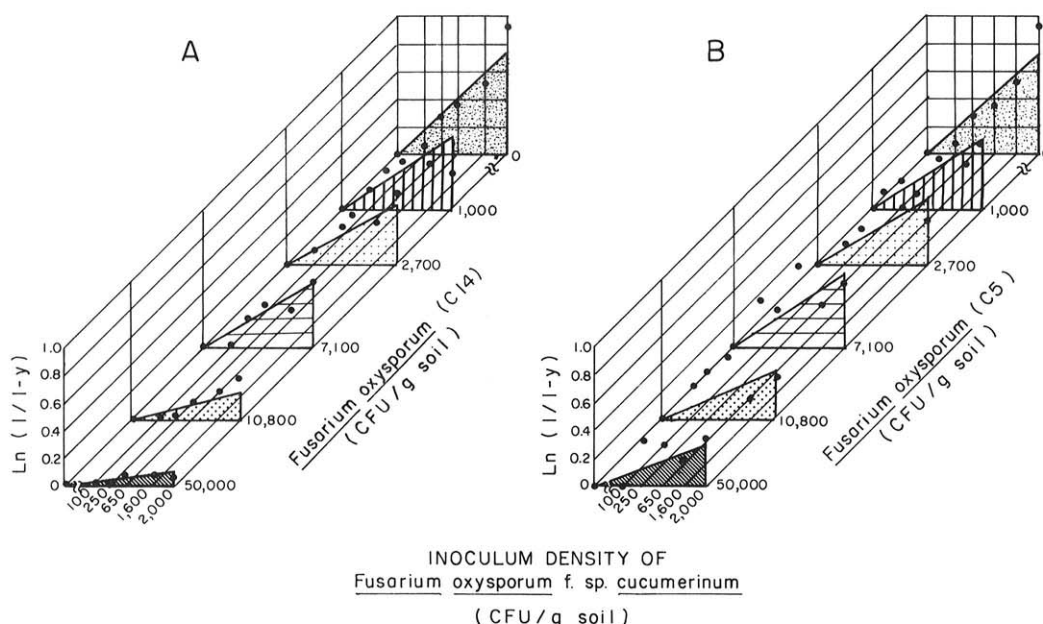
Lite. One portion was placed in one side of the inverted V and the second portion in the other. Plants were exposed to high humidity for 7 days. Incidence of Fusarium wilt was recorded over a 35-day period from the time of transplanting.

**Application of biocontrol agents in layers of raw soil.** Plastic cone-shaped containers (3.8 cm in diameter and 13.7 cm deep) were filled to a depth of 7 cm with either raw Nunn sandy loam soil or soil infested with chlamydozoospores of *F. o. cucumerinum* ( $2 \times 10^3$  cfu/g of soil). The container was filled to the 13-cm level by addition of raw soil or soil infested with chlamydozoospores of C5 or C14, so that there was a population density of  $10^4$  cfu/g of soil. One germinated cucumber seed was planted in each of 30 containers and exposed in the growth room for 35 days at 32 C. There were four replications. Plant death was recorded over time.

**Statistical analysis.** All experiments were arranged in a randomized block design and repeated at least once. When appropriate (4), disease progress or inoculum density-disease incidence data were transformed by use of  $\text{Ln } 1/(1-y)$  in which  $y$  is disease incidence. In these cases, slope values were determined by regression analysis. Data were subjected to one-way analysis of variance. When significance was obtained among treatments in the *F* test, means were separated by use of Duncan's multiple range test at  $P=0.05$ . Data from repeated experiments were pooled because variances between the trials were homogeneous. Separate analyses of each of the greenhouse experiments, however, were done because in initial trials, C5 or C14 was used in separate tests at different times. In the last greenhouse experiment, both were used simultaneously in different treatments so that statistical comparisons of the efficiencies of C5 with C14 in cross-protection could be compared directly.

## RESULTS

**Effect of strains C5 and C14 on incidence of Fusarium wilt.** Slope values of inoculum density (*F. o. cucumerinum*) infection ( $\text{Ln } 1/(1-y)$ ; in which  $y$  is disease incidence 55 days after planting and inoculation) were decreased significantly when population densities of  $1.08 \times 10^4$  and  $5 \times 10^4$  cfu/g of soil of strains C5 and C14, respectively, were added to soil in repeated experiments. However, only data from the second experiment are illustrated in Figure 1. When *F. o. cucumerinum* was applied at  $2 \times 10^3$  cfu/g of soil in combination with  $5 \times 10^4$  cfu/g of soil of C5



**Fig 1.** Effect of nonpathogenic strains of *Fusarium oxysporum* on disease incidence ( $y$ ) of Fusarium wilt of cucumber induced by *F. oxysporum* f. sp. *cucumerinum* 55 days after planting. **A**, Strain C14. **B**, Strain C5.

or C14, there was significantly less disease incidence in treatments with C14 but not with C5 in comparison with the inoculated control.

**Germination of chlamydospores of *F. o. cucumerinum* in rhizospheres of cucumber roots treated with C5 or C14.** Strain C5 or C14 added to rhizosphere soil ( $1 \times 10^4$  cfu/g) significantly reduced the germination of chlamydospores of *F. o. cucumerinum* by 23 and 13%, respectively, in comparison with the control. Differences in germination between treatments with C5 or C14 were not significant.

**Competition for infection sites.** Soil was infested with various population densities of C5, C14, or *F. o. cucumerinum* and the sections of roots of 4- to 5-day-old cucumber seedlings assayed 0–1.5, 1.5–3, and 3–4.5 cm from the root tip for the number of colony-forming units of each strain. No colony-forming units of strains of *F. oxysporum* were detected in any segment of root tissue when C5 was introduced into the soil; however, as higher levels of either *F. o. cucumerinum* or C14 were applied, an increase in colony-forming units of these strains was observed in both the 1.5- to 3- and 3- to 4.5-cm segments (Fig. 2A–C). There were significantly more colony-forming units in the 3- to 4.5-cm than in the 1.5- to 3-cm root segment. There was a significant decrease in the number of colony-forming units of *F. o. cucumerinum* recovered from the 3- to 4.5-cm segment at  $8 \times 10^4$  than at the  $1.1 \times 10^5$  cfu/g of soil treatment. In subsequent experiments, the 3- to 4.5-cm root segment was assayed for colony-forming units in root tissue.

The number of colony-forming units of *F. o. cucumerinum* or strain C14 in root segments was compared when C5 or C14 was introduced into soil at population densities ranging from  $0-1.1 \times 10^5$  cfu/g to  $0.8 \times 10^4$  cfu/g for *F. o. cucumerinum* in all combinations. In Figure 3A and C, the effects of C14 on the colony-forming units of *F. o. cucumerinum* recovered from roots in the repeated experiments are illustrated. There was a significant reduction in the slope values of inoculum density (cfu/1.5 cm of root regressions) when C14 was applied to soil at  $8 \times 10^4$ ,  $3.5 \times 10^4$ , or  $1.1 \times 10^5$  cfu/g as compared to the treatment with *F. o. cucumerinum* alone. In Figure 3B and D, the effects of *F. o. cucumerinum* on colony-forming units of C14 recovered from roots in the repeated experiments are illustrated. There was no significant reduction in slope values for any of the treatments. Similarly, there was no significant reduction in slope values (cfu/1.5 cm of root as a function of inoculum density) in treatments receiving various population densities in soil of C5 (Fig. 4A,B). Strain C5 was not recovered from the roots that received treatments with it alone or in combination with *F. o. cucumerinum*.

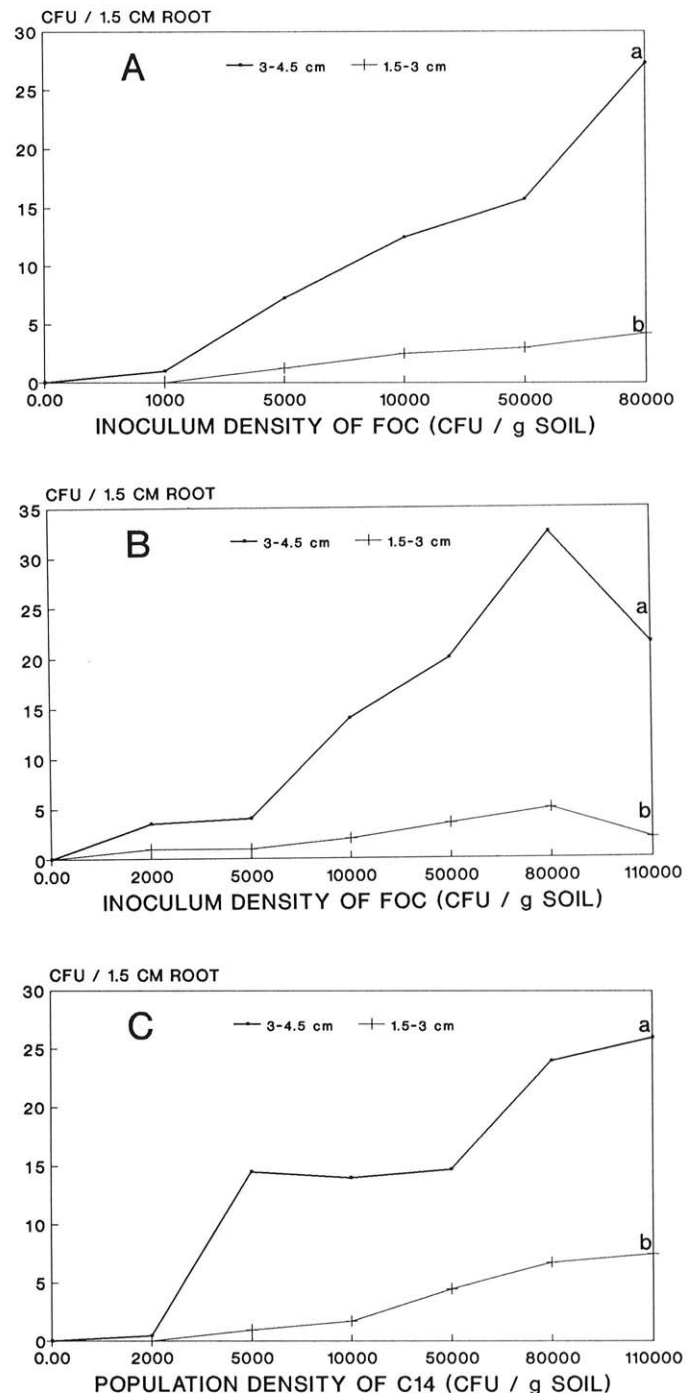
**Resistance to cucumber anthracnose by application of C5, C14, or *F. o. cucumerinum* to soil.** When wounded or intact roots were exposed to *F. o. cucumerinum*, C5 or C14, or water, the average number of lesions per leaf inoculated with *C. lagenarium* was significantly reduced by treatments of cucumber exposed to C14, but not C5, *F. o. cucumerinum*, or water (Fig. 5).

**Stem inoculation with *F. o. cucumerinum* after exposure of roots to C5 or C14.** Wounded or intact roots were exposed to C5, C14, or water and were stem-inoculated with *F. o. cucumerinum*. When roots were not wounded, the percentage of death (Fig. 6) induced by girdling of stems by the pathogen was significantly reduced only by treatment with C14 in comparison with inoculated controls. When roots were wounded, a significant reduction in proportions of dead plants was observed by exposure to either C5 or C14.

**Germination of microconidia of *F. o. cucumerinum* in wounded stem tissue after exposure to strain C5 or C14.** When wounded or intact roots were exposed to C5, C14, or water, there was a significant reduction in the percentage of microconidium germination of *F. o. cucumerinum* on cut surfaces of plant stems when roots were exposed to either C5 or C14, 72 and 96 hr after wounding of the roots (Fig. 7). Only strain C14, 72 and 96 hr after inoculation, induced significant reduction in the percentage of microconidium germination of *F. o. cucumerinum* on cut cross-sections of stems when roots were not wounded.

**Split roots exposed to either strain C5 or C14 with *F. o. cucumerinum*.** Disease incidence curves resulting from exposure of the split root system to C5 or C14, plus *F. o. cucumerinum*, or the pathogen alone are plotted over time as a function of  $\text{Ln}(1/1 - y)$  in Figure 8. Rate of disease progress was reduced significantly only when divided root systems were exposed to the pathogen and C14.

**Application of biocontrol agents in layers of raw soil.** Disease progress curves resulting from exposure of cucumber roots to C5 or C14 in an upper layer of soil and to *F. o. cucumerinum* in the lower level are plotted over time as a function of  $\text{Ln}(1/1 - y)$  in Figure 9. Disease progress was significantly reduced



**Fig 2.** Effect of different population densities of *Fusarium oxysporum* f. sp. *cucumerinum* (FOC A and B) and a nonpathogenic strain of *F. oxysporum* (C14) (C) on the number of colony-forming units in cucumber roots 1.5–3 and 3–4.5 cm above the root tips. Slope values with the same letters are not significantly different ( $P = 0.05$ ) from each other.

only in the treatment in which strain C14 was present in the layer above the pathogen.

### DISCUSSION

Three hypotheses (competition for nutrients, competition for infection sites, and induced or enhanced resistance) were tested to explore mechanisms involved in the suppression of *Fusarium* wilt of cucumber by nonpathogenic *Fusarium* spp.

French (2) and Italian (9) workers presented evidence that suppression of *Fusarium* wilt diseases was attributable to nutrient competition between pathogenic and antagonistic *Fusarium* spp. Many of these studies were done in steamed soils. When raw soil was used, suppressiveness was explained by elevation of soil fungistasis in suppressive as compared with conducive soil (3), i.e., a larger biomass competing for nutrients could inhibit germination of chlamydospores of the pathogen. The bioassay used in the present study was designed specifically to measure germination of chlamydospores in the presence or absence of biocontrol agents in rhizospheres in raw soil (10). When C5 or C14 was added to raw soil, germination of chlamydospores of *F. o. cucumerinum* in rhizospheres of cucumber was reduced in comparison with controls. Antagonism, as observed in this experiment, is

conventionally attributed to antibiosis or competition (5,6). Because antibiosis was not demonstrated to be a factor in this biocontrol system (20) and nitrogen and/or available iron (22) was not limiting, reduction in germination could be attributed to carbon competition (1-3,10).

Schneider (23) ascribed suppression of *Fusarium* wilt of celery induced by nonpathogenic *Fusarium* spp. to competition for infection sites. To test this hypothesis, a technique was developed whereby the relative number of infections in roots was measured shortly after penetration by the pathogen (*F. o. cucumerinum*) or biocontrol agents (C5 or C14). From previous observations (25), chlamydospores germinated and penetrated approximately 40 hr after the first contact with a root tip. In this study, cucumber roots grew about 1.5 cm/day at 25 C. Predictably, no infections by *F. o. cucumerinum* or C14 even at higher inoculum densities were detected on the first day of root growth (0-1.5 cm above the root tip) and few in the 1.5- to 3-cm section; but 25-30 cfu/1.5 cm developed on the third day (Fig. 2A-C). Therefore, the 3- to 4.5-cm section was chosen for the development of assays to quantify competition for infection sites. The asymptotic portion of the curve reached a value of approximately 25-30 cfu/1.5 cm of root for both *F. o. cucumerinum* and C14, which could be a reflection of the relative maximum number of penetration sites.

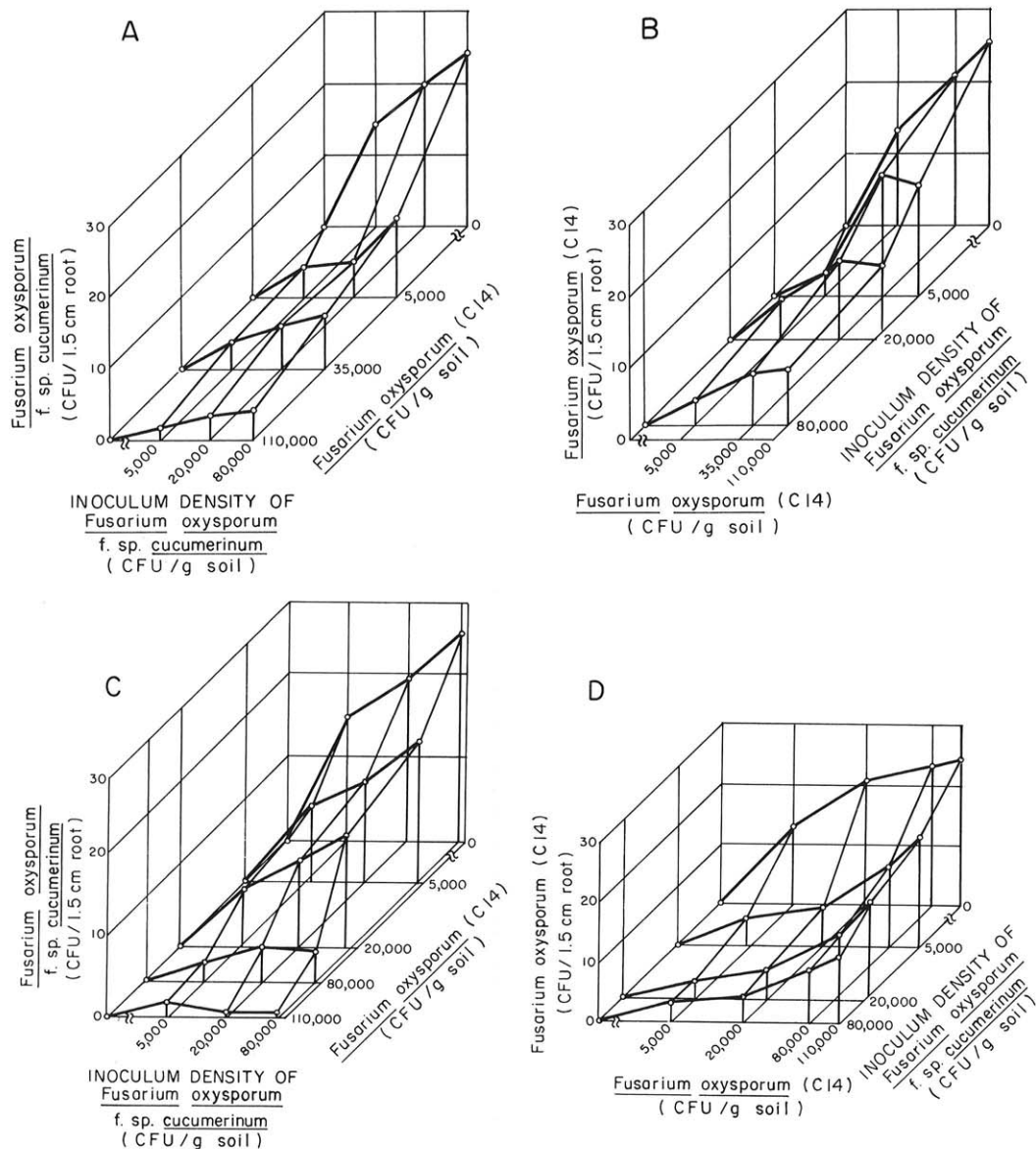
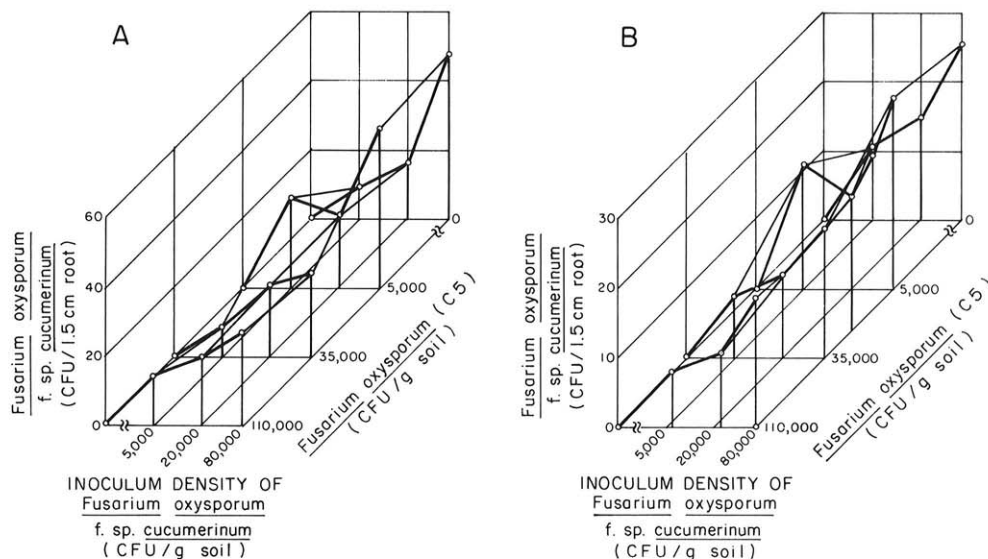
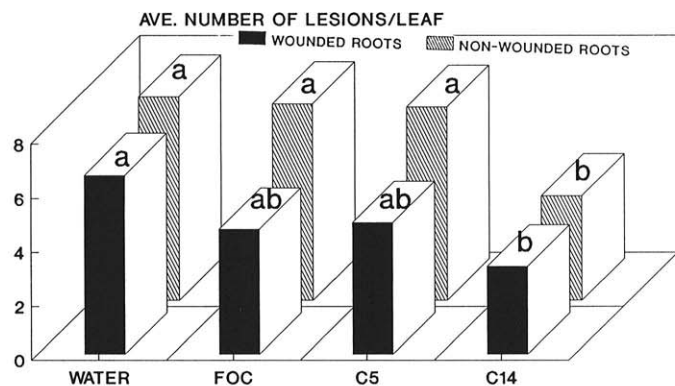


Fig. 3. Effect of different population densities of *Fusarium oxysporum* f. sp. *cucumerinum* and a nonpathogenic strain of *F. oxysporum* (C14) on the number of colony-forming units in cucumber roots 3-4.5 cm above the root tips. A,B, Experiment 1. C,D, Experiment 2.



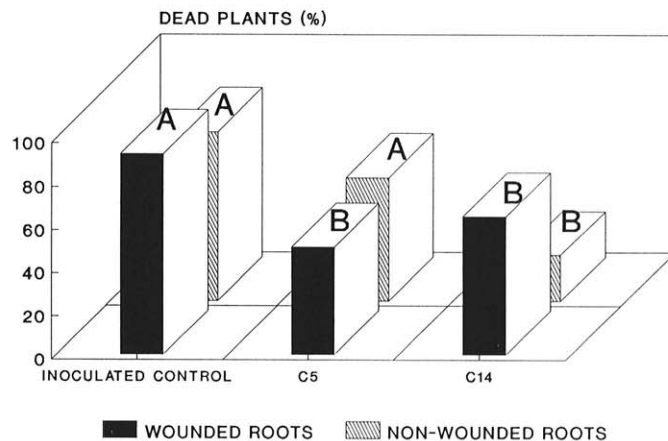
**Fig 4.** Effect of different population densities of *Fusarium oxysporum* f. sp. *cucumerinum* and a nonpathogenic strain of *F. oxysporum* (C5) on the number of colony-forming units in cucumber roots 3–4.5 cm above the root tips. **A**, Experiment 1. **B**, Experiment 2. There were no significant differences ( $P = 0.05$ ) among the slope values of the various treatments.



**Fig 5.** Average number of anthracnose lesions 10 days after leaves were inoculated with *Colletotrichum lagenarium* and either wounded or nonwounded roots exposed to *Fusarium oxysporum* f. sp. *cucumerinum* (FOC) or C5, C14, or H<sub>2</sub>O. Means with the same letters (a or b) are not significantly different ( $P = 0.05$ ) from each other.

As increasing population densities of strain C14 were added to soil, the number of colony-forming units per 1.5 cm of root of *F. o. cucumerinum* decreased (Fig. 3A,C). As colony-forming units of the pathogen decreased, increased numbers of colony-forming units per 1.5 cm of C14 were recovered from roots (Fig. 3B,D). Therefore, a decrease in infections induced by C14 was influenced by the inoculum density of the pathogen, but a significant reduction in infection occurred at population densities of C14 above  $8 \times 10^4$  cfu/g of soil when compared with controls not infested with the agent. No significant decrease in infections by *F. o. cucumerinum* was observed when C5 was added to soil at any level (Fig. 4) and this organism was not detected in 1- to 3-day-old root sections. Therefore, competition for infection sites occurred with strain C14, but no evidence for the operation of this mechanism was obtained for strain C5. Thus, competition for infection sites may involve occupation and penetration of these sites on the rhizoplane.

Most of the evidence for enhanced resistance induced by non-pathogenic *Fusarium* spp. was obtained by separation of a biocontrol agent from an infection court and a subsequent decrease of disease with challenge inoculation by the pathogen (8,15,18). The basic mechanisms involved in this hypothesis are recognition by the host upon ingress of the biocontrol agent, initiation of



**Fig 6.** Percentage of dead plants 20 days after stems were inoculated with *Fusarium oxysporum* f. sp. *cucumerinum* (FOC) and either wounded or nonwounded roots exposed to C5, C14, or H<sub>2</sub>O. No symptoms were observed in uninoculated controls. Means with the same letters (A or B) are not significantly different ( $P = 0.05$ ) from each other.

host resistance reactions, and subsequent suppression of infection by the pathogen (11,13,16,24). In this study, five methods were used to test for the presence of enhanced resistance in the host induced by the biocontrol agents.

A method (8,15,17) was used to assay the numbers of cucumber anthracnose lesions on leaves that resulted after introduction of a biocontrol agent to either wounded or nonwounded roots. A significant reduction in the number of lesions in comparison with water treatment of roots occurred only by exposure of wounded or nonwounded roots to C14 (Fig. 5).

The method for detection of enhanced resistance by the application of a biocontrol agent to roots with subsequent challenge inoculation of stems with *F. o. cucumerinum* was used (18). Strain C14 applied to roots significantly reduced death of stem-inoculated cucumbers infected with *F. o. cucumerinum*, whether roots were wounded or not; whereas, C5 was only effective in reducing disease incidence in comparison with uninoculated stems when introduced through wounded roots (Fig. 6).

In another experiment (Fig. 7), both C5 and C14 applied to wounded roots significantly reduced microconidium germination of *F. o. cucumerinum* placed on cross-sectional areas of severed

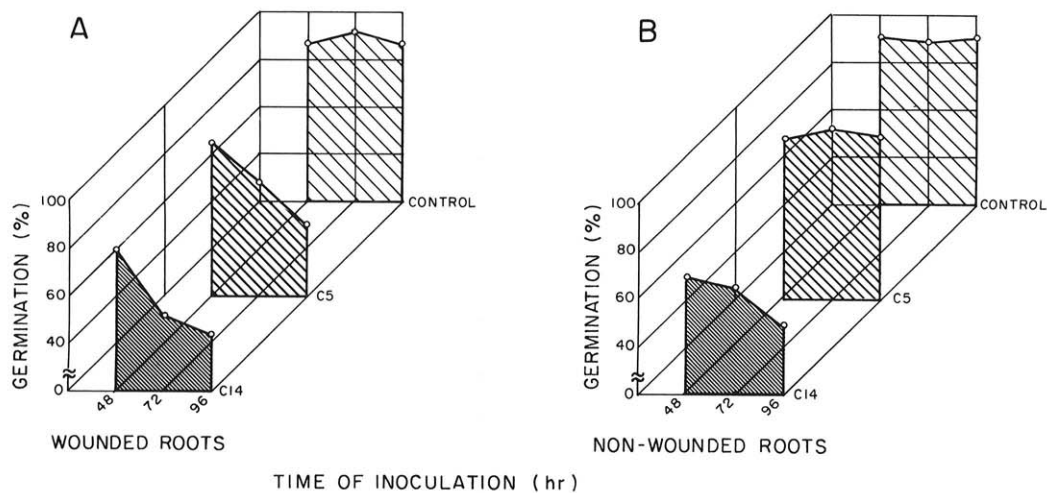


Fig 7. Effect on microconidium germination of *Fusarium oxysporum* f. sp. *cucumerinum* when placed on severed stems of cucumbers 48–96 hr after wounded (A) and nonwounded (B) roots were exposed to either strain C5 or C14. Means with the same letter are not significantly different ( $P = 0.05$ ) from each other.

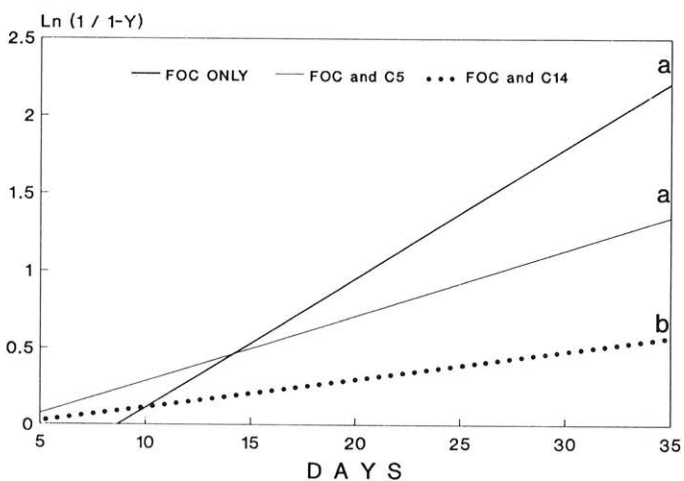


Fig 8. Disease incidence progress ( $y$ ) of Fusarium wilt of cucumber when one-half of the roots were exposed to *Fusarium oxysporum* f. sp. *cucumerinum* (FOC) and the other half to strain C5 or C14. No disease developed in treatments without *F. o. cucumerinum* or when split roots were only inoculated with C5 or C14. Slope values with the same letter are not significantly different ( $P = 0.05$ ) from each other.

stems, 72 and 96 hr later. However, when roots were not wounded, only C14 significantly inhibited conidium germination at 72 and 96 hr in comparison with the inoculated control.

Further evidence for enhanced resistance induced by C14 was obtained by exposure of one-half of the wounded root system to the agent with simultaneous inoculation of the other half with *F. o. cucumerinum* (Fig. 8). Strain C14 significantly enhanced resistance. Although disease progress over time was slower when roots were exposed to C5 than in controls, these differences were not significant.

The split-root technique complements other methods for testing the development of enhanced resistance, because roots are infection courts for the pathogen as well as the initial site of biocontrol activity of the agent (potential inducer). It was possible to use another technique that exposed intact roots alternately to the potential inducer and then to the pathogen by horizontal layering of the soil (Fig. 9). Hypothetically, either pathogen or the potential inducer would not be disseminated by mycelial growth because raw soil is fungistatic, and separation of pathogen and the agent is likely. In this case, when an upper layer of soil was infested with strain C14, significantly fewer plants wilted when *F. o. cucumerinum* was in the deeper layer than in soil without the added biocontrol agent. Again, disease incidence in

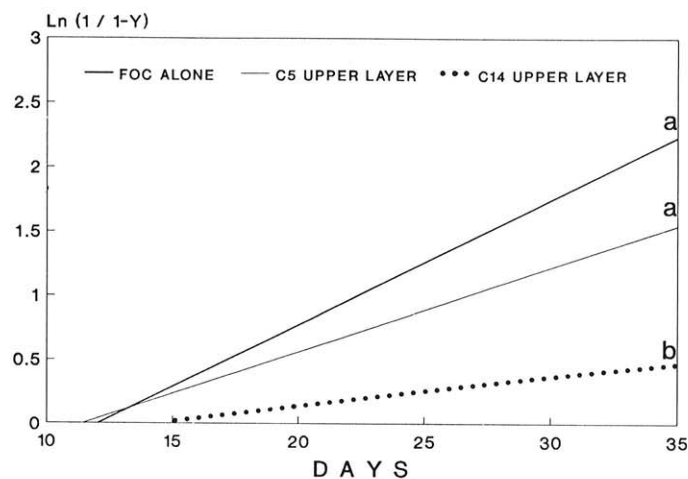


Fig 9. Disease incidence progress ( $y$ ) of Fusarium wilt of cucumber when a layer of soil was infested with *Fusarium oxysporum* f. sp. *cucumerinum* (FOC); and a layer above this inoculation was infested with strain C5 or C14. No disease developed in treatments without FOC or when layers were only infested with C5 or C14. Slope values with the same letter are not significantly different ( $P = 0.05$ ) from each other.

plants similarly exposed to C5 was not significantly reduced compared to inoculated controls.

In all experimental tests of the hypothesis for enhanced resistance, however, C14 was effective whether roots were wounded or not. Therefore, this strain has genetic potential for penetration of intact root tissue (Fig. 2C), and can subsequently induce resistance reactions in the host. In contrast, in such experiments, as well as in those measuring colonization of living root tissue, C5 evidently could not penetrate intact roots, compete for infection sites (Fig. 4A,B), and significantly enhance resistance (Figs. 5, 8, and 9), unless roots were wounded (Figs. 6 and 7A).

The three mechanisms examined in this study could be factors to consider in assays for comparative efficiencies of nonpathogenic fusaria as biocontrol agents. When either strain C5 or C14 was present, chlamydospore germination of *F. o. cucumerinum* was inhibited in the rhizosphere. A substantial increment of control resulting from this mechanism, however, is not likely because penetration by the pathogen occurs in motile infection courts. Relatively large decreases in germination and successful infection originating from chlamydospores would be necessary to reduce disease because roots constantly encounter inoculum as they move through soil (4).

Evidently, sites susceptible to infection by the pathogen on

the rhizoplane can be protected by an agent actively competing for these sites. This mechanism has more potential impact on the efficiency of biocontrol than competition for nutrients in the rhizosphere. Efficiency values probably are influenced by spatial relationships, i.e., relative proximities of the pathogen to penetration sites vs. proximity of the biocontrol agent and a so far ill-defined attribute of the agent to penetrate such sites. Apparently, there are a definitive number of infection sites to be protected (Fig. 2A,B), but relatively larger population densities of strain C14 were required to protect roots when high inoculum densities of *F. o. cucumerinum* were applied. Obviously, efficiency of control in the short-term analog system by competition for infection sites is dependent on the ratio of inoculum density of the pathogen to population density of the biocontrol agent (Fig. 3A-D).

After penetration by a nonpathogen such as strain C14, enhancement of host resistance may have a substantial influence on disease development. The hypothetical attributes associated with an efficient agent that induces biocontrol with this mechanism are: ability to penetrate and colonize host tissue without pathogenicity; induction of resistance reactions in living cells capable of producing such reactions; capacity for repeated penetration of moving infection courts (4) to provide continuous induction of resistance reactions produced by the host (26); and efficient suppressive activity in environments that are conducive for high inoculum potentials of the pathogen.

In the greenhouse tests (Fig. 1A,B), C14 was a more efficient biocontrol agent than C5. The basic attributes of these strains, reviewed above, may explain their relative efficiencies in inducing biological control.

Such attributes of nonpathogenic fusaria could be useful for screening of superior biocontrol agents. The agents should compete efficiently for infection courts; penetrate and colonize intact root tissue at high frequencies; and induce substantial resistance reactions in the host without pathogenicity. Strain C14 has these attributes.

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