

Association of the Plant-beneficial Fungus *Verticillium lecanii* with Soybean Roots and Rhizosphere

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Abstract: Colonization of soybean roots by the biocontrol fungus *Verticillium lecanii* was studied in vitro and in situ. For in vitro experiments, *V. lecanii* was applied to soybean root tip explant cultures. Four weeks after inoculation, the fungus grew externally on at least half of the roots (all treatments combined), colonizing 31% to 71% of root length (treatment means). However, when a potato dextrose agar plug was available as a nutrient source for the fungus, root tips inoculated soon after transfer were not colonized by *V. lecanii* unless *Heterodera glycines* was present. Scanning electron microscopy of colonized roots from in vitro cultures revealed a close fungus-root association, including fungal penetration of root cells in some specimens. In the greenhouse, soybeans in sandy soil and in loamy sand soil were treated with *V. lecanii* applied in alginate prills. The fungus was detected at greater depths from the sandy soil than from the loamy sand soil treatment, but fungus population numbers were small and variable in both soils. Root box studies coupled with image processing analysis of the spatial distribution of *V. lecanii* in sandy soil supported these findings. *Verticillium lecanii* was detected randomly in the rhizosphere and soil of root boxes, and was rarely extensively distributed. These in vitro and in situ experiments indicate that *V. lecanii* can grow in association with soybean roots but is a poor colonizer of soybean rhizosphere in the soil environment.

Key words: biological control, colonization, fungus, *Glycine max*, *Heterodera glycines*, nematode, rhizosphere, soybean, *Verticillium lecanii*.

The soybean cyst nematode (SCN; *Heterodera glycines*) is the most economically important pest of soybean (Wrather et al., 1997). Various techniques, including crop rotation, planting of resistant cultivars, and nematicide application, have been used for management of this nematode, but crop yield losses persist. Nematicide use is limited for safety and economic reasons, resistance can be affected by shifts in nematode races, and crop rotation is often effective but may be restricted by the need to select certain crops, by the increase of other nematode populations, and by the ability of the eggs to remain dormant for years (Carris and Glawe, 1989; Caviness, 1992; Rodríguez-Kábana, 1992; Wrather et al., 1992; Young,

1992). New approaches for the management of this nematode would be beneficial to integrated pest management programs.

Existing management procedures could be enhanced by development of biological control strategies. One potentially useful biological control organism is the soil-inhabiting fungus *Verticillium lecanii* (A. Zimmermann) Viégas. This fungus is antagonistic to insects and other fungi (Harper and Huang, 1986; Heintz and Blaich, 1990; Hussey, 1984; Uma and Taylor, 1987) and has been sold commercially for insect control. Additionally, *V. lecanii* acts against the cyst nematodes *H. glycines*, *Heterodera schachtii*, and *Globodera pallida* (Gintis et al., 1983; Hänsler, 1990; Hänsler and Hermanns, 1981; Meyer et al., 1990; Meyer and Huettel, 1996; Meyer and Meyer, 1995, 1996; Uziel and Sikora, 1992). Research has been conducted on the use of this fungus as a biocontrol agent for *H. glycines*. In laboratory and greenhouse studies, strains of *V. lecanii* reduced SCN populations, with decreases in cyst numbers of up to 85% compared with untreated controls (Meyer et al., 1990; Meyer and Huettel, 1996; Meyer and Meyer, 1995, 1996). However, results from field studies were variable when a strain of *V. lecanii* was applied to reduce *H. glycines* population densities. Cyst numbers on soybean

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were reduced in only 1 of 2 years in micro-plot tests of *V. lecanii*, and no effect was detected after fungus application in a small-scale field test conducted in 1992 (Meyer et al., 1997). Research on the basic biology of *V. lecanii* is needed to understand the factors that contribute to this variability.

Little is known about the mechanism of suppression of *H. glycines* by *V. lecanii*, or the importance to biocontrol efficacy of such traits as rhizosphere colonization. The rhizosphere is where *H. glycines* juveniles enter soybean plants, where male vermiform stages and stationary females emerge from roots, and where nematode eggs are formed. Consequently, colonization of soybean rhizosphere may be important for suppression of *H. glycines*. Although *V. lecanii* has been isolated from soil during the course of greenhouse experiments, isolations were infrequent, and differentiation between rhizosphere and bulk soil was not attempted (Meyer and Meyer, 1995, 1996; Meyer and Huettel, 1996). Therefore, the goals of this study were to examine the association of one *V. lecanii* isolate with soybean roots, to determine whether the isolate colonized the soybean rhizosphere, and to look at dispersal of the fungus in the soil. The isolate selected for these studies has been applied in field tests and was the strain most frequently used in previous greenhouse studies.

MATERIALS AND METHODS

Organisms: The fungus being investigated was *Verticillium lecanii* strain M2S1 (Nematology Lab number), deposited as Agricultural Research Culture Collection NRRL 18726. The nematode used for the study was *Heterodera glycines* race 3, which was originally isolated from Maryland soybean (*Glycine max* (L.) Merr.) fields (isolation by Beltsville Nematology Laboratory). The nematode has been maintained in gnotobiotic cultures at this location for more than 5 years. This nematode was cultured on excised soybean root tips (cv. Kent) grown on Gamborg's B-5 medium in petri dishes.

In vitro root colonization studies: Eight treat-

ments were tested: 1 and 2) Young root tips with *V. lecanii* applied as an aqueous suspension or on an agar plug, 3 and 4) Old root tips with fungus applied as an aqueous suspension or on an agar plug, and 5–8) Each of the four previous treatments with *H. glycines*. For aqueous suspension treatments, a suspension of *V. lecanii* was applied 3 mm from each root tip (1.5×10^7 colony-forming units/ml sterile distilled water). Plugs (9-mm diam.) were taken from potato dextrose agar (PDA) cultures of *V. lecanii*; each plug was placed in a hole cut in the Gamborg's B-5 medium 3 mm from a root tip. Young root tip cultures received *V. lecanii* 4 days after root tip transfer to Gamborg's B-5 medium, while old root tip cultures received the fungus 2 weeks after root tip transfer. Ten *H. glycines* females were added to each nematode-treated petri dish 2 days after excised root tips were placed onto Gamborg's B-5 medium. Six replicate petri dishes (two roots per petri dish) were used for each of the eight treatments. All cultures were maintained at 25 °C, and the extent of root colonization determined 1, 2, 3, and 4 weeks after inoculation. The lengths of the main roots and the lengths of fungus colonization observed on the root surfaces were measured with string. Because fungus colonies increased in diameter from a central propagule, root colonization was considered to have occurred only when mycelium advanced along root surfaces ahead of, or in a different direction from, an expanding colony. Number of roots per treatment equal 12, with some exceptions where roots could not be measured.

Scanning electron microscopy procedures: Roots from excised soybean root tip cultures inoculated with *V. lecanii* (as described above) were placed into 3% glutaraldehyde (in 0.05 M PO₄ buffer) for 2 hours at room temperature and transferred to 4 °C overnight. Samples were quickly rinsed in 0.05 M PO₄ buffer twice and washed three times in the same buffer for 20 min each time. Samples were subsequently dehydrated for 30-min intervals in 20%, 40%, 60%, 80%, and 100% EtOH (three times each); mounted on aluminum stubs with Avery la-

bels; coated with Au/Pd in a Hummer Sputter Coater; and viewed with a Hitachi SEM model S-570.

Soil and rhizosphere population studies: PVC rings (7.5-cm internal diameter, 2-cm height) were taped together to form cylinders 20 cm high (10 rings per cylinder), and DeWitt Pro-5 Weed Barrier was taped to the bottom of each cylinder to hold in soil and allow excess moisture to drain. Cylinders were filled with either a sandy soil (96% sand, 3% silt, 1% clay, organic matter 0.47%; pH 7.3) or a loamy sand soil (82% sand, 13% silt, 5% clay, organic matter 3.2%; pH 6.8). Soils were "untreated;" i.e., no heating or pesticide application was done to remove the microflora or microfauna. A suspension of 10,000 *H. glycines* eggs in 1 ml water was pipetted into the soil in each cylinder and up to six soybean seeds (cv. Essex) placed on top and covered with soil. *Verticillium lecanii* was incorporated into alginate prills containing wheat bran (100 g wet weight fungus and 5 g bran per liter alginate solution; Meyer and Huettel, 1996); 5 g of dry prills (each dry prill ca. 1-mm diameter and 0.001-g weight) per cylinder was placed in a ring around the soybean seeds prior to coverage with soil. Experiments were maintained in the greenhouse at 23–34 °C. Seedlings were culled to one per cylinder shortly after emergence. Control cylinders without prills were also prepared. Soil and roots from six cylinders containing prills and three cylinders without prills were harvested after 2 and 4 weeks for each soil type. The experiment was repeated once, for a total of 12 cylinders per treatment at each harvest time and six controls per soil type at each harvest time.

At harvest, rings were taken apart in sets of two starting at the top of each cylinder. From each 4-cm-deep section, 0.5 g soil and 0.1 ml of a soil dilution (made from mixing 5 g soil suspension in 100 ml water) were plated separately onto each petri dish of selected media. Additionally, root segments 2 cm long were vortexed in sterile distilled water for 30 seconds, and 1.0 ml wash water and the root segments were plated separately onto selected media. Colonies of *V.*

lecanii were identified and the locations (depth and isolation from soil, root wash, or root) recorded.

Media development: There are no highly selective media for isolating *V. lecanii* from soil. One medium, PDA ABE (PDA plus benomyl (2 g per liter), amended with the antibiotics streptomycin sulfate and tetracycline hydrochloride (0.3 g per liter) and with 6 ml 95% ethanol per liter), which was developed in our tests and selectively allows growth of *V. lecanii*, was used throughout the soil and rhizosphere population studies. Another medium found to be useful was oatmeal agar (OMA) plus dodine (1 g per liter), amended with the same antibiotics and ethanol and referred to as OMA ADE (variant of oatmeal agar plus dodine recommended by Beilharz et al. (1982) for isolation of certain fungi from soil). Variations of these media were also tested to determine whether an improved semi-selective medium could be formulated. These included adding 0.1 g cycloheximide per liter, varying the amount of benomyl, replacing benomyl with dodine, and combining benomyl and dodine. PDA ADET and OMA ADET were both found to have improved selective properties for *V. lecanii* and were used in image-processing studies (see below). For these media, PDA and OMA were amended with dodine, and with the antibiotics streptomycin sulfate and tetracycline hydrochloride, and ethanol as in PDA ABE (above). Thionin (0.1 g/liter) was added as a background stain for photography. Media used in image-processing experiments contained 3% agar. Propagules may have been detected at lower amounts than present in the soil, but numbers of colonies isolated on the media should still have been representative of population distributions in the soil.

Procedures for root box experiments analyzed with image processing: Root boxes were constructed by cutting PVC (internal diam. 7.5 cm) in half longitudinally to form half cylinders 10.5 cm long. Each root box was covered with a piece of transparent plastic (4 mm thick) and filled with the sandy soil (described under "soil and rhizosphere population studies"). Three soybean seeds (cv.

Essex) were sown in each root box and 10,000 *H. glycines* eggs added to the soil near the seeds. Prills containing *V. lecanii* were placed in a semicircle near (but not touching) the seeds, and the seeds and prills were covered with a small amount of soil. Treatments were: 1 g prills (19 root boxes), 2 g prills (19 root boxes), and controls with no prills (12 root boxes). The experiment was repeated once. Root boxes were placed tilted slightly forward (plastic sheets at ca. 50–60° angle to the soil) in tubs of steamed soil to provide moisture and to hold the soil in the root boxes. Additionally, soil was kept moist by application of water drops at the top of each root box. Drops were applied slowly so that they did not run down the inner surfaces of the plastic covers. Plants were culled to one per root box. Experiments were maintained in the greenhouse at 23–34°C. After 4 weeks, the plastic covers were removed and root images were obtained by photographing root boxes with Kodak Technical Pan Film. Three petri dishes (150-mm diam. × 15 mm high) each of OMA ADET and of PDA ADET were pressed onto the soil and roots that had been under the plastic cover in each root box. The petri dishes were placed into an incubator for 4 to 8 days and then photographed with Kodak Technical Pan Film.

Photographs were digitized with a Gateway 2000 Pentium 133 computer. Images of all six petri dishes for each root box were normalized for size to the root image and combined using an HP Apollo Series 710 workstation running Khoros Image Processing software by standard procedures (Castleman, 1979). The composite petri dish image was then combined with the corresponding root image by standard procedures (Castleman, 1979). Composite images of petri dishes from half cylinders were also combined into a single image with standard image-processing procedures (Castleman, 1979).

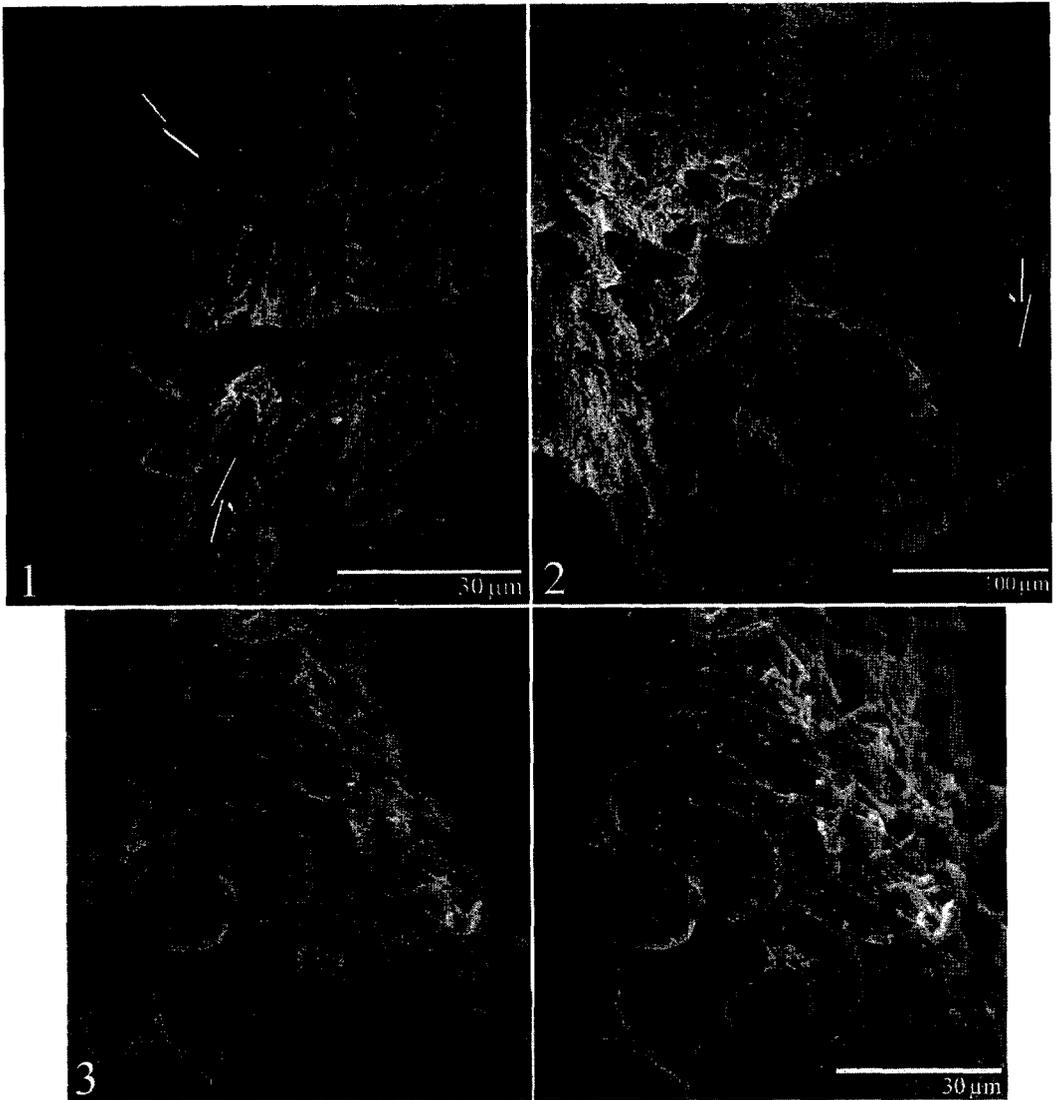
RESULTS

In vitro root colonization studies: *V. lecanii* was capable of colonizing excised soybean

root tips *in vitro* (Figs. 1–3). Scanning electron microscopy demonstrated that the fungus grew in close association with roots. Surface growth of *V. lecanii* on roots ranged from netlike (Fig. 1) to a heavy mat of mycelium (Fig. 2). *Verticillium lecanii* also colonized cells of some roots (Figs. 2,3); viability of those root cells was not known.

The length of root and number of roots colonized varied with treatment (Figs. 4A, B). When root tips were inoculated with fungus in water suspension, mean percent root lengths colonized were not significantly different from each other after 1 week (Fig. 4A). However, older roots with no SCN were the most frequently colonized. Two weeks after inoculation, percent root length colonized was still ca. 16% to 25% for three of the treatments. Young roots with SCN had a higher percentage of root length colonized (65%), but only 17% of the roots were associated with *V. lecanii*; this was a much lower proportion than found with the other treatments. At week 3, the anomalous treatment still had a low percentage of roots colonized. After 4 weeks, results from suspension-treated young roots were similar to each other. However, the number of colonized roots from the treatment with SCN had tripled, and percent root length colonized was lower on the more recently colonized roots. Young roots with SCN still had a higher mean root length colonized than did either treatment with old roots. All four treatments resulted in a minimum of half of the roots being colonized by week 4.

The results were somewhat different when the root tip cultures were treated with *V. lecanii* on a plug of PDA (Fig. 4B). One week after inoculation, younger roots were not colonized by *V. lecanii*. Old roots were colonized, but percent root length colonized on roots with SCN was lower than that recorded from all other colonized roots (Figs. 4A,B). Two and three weeks after inoculation, the two young root treatments inoculated with the fungus plugs were still not colonized by the fungus (Fig. 4B). Conversely, by week 3, percent root length colonized on older roots was similar whether a plug or suspension was used as inoculum (Figs. 4A,B).



FIGS. 1-3. Scanning electron micrographs of soybean roots from root tip explant cultures. Fig. 1. Hyphae of *Verticillium lecanii* (arrows) growing in close association with root surfaces. Fig. 2. Soybean root covered with a thick mat of *V. lecanii* mycelium and with hyphae inside root cells (one example indicated by arrow). Fig. 3. Stereo pair of the soybean root from Fig. 2, demonstrating growth of *V. lecanii* hyphae in root cells. To attain the three-dimensional view, a stereo viewer can be placed on the complementary stereo images so that the left and right lenses are aligned above the left and right images, respectively. The same effect can also be achieved by entraining the left and right eyes on the left and right figures, respectively.

Young roots inoculated with plugs were not colonized at all until week 4, and then only if SCN was present on the roots (Fig. 4B). At that time, results from plug-treated young roots with SCN were not significantly different from any other treatment with colonized roots (Figs. 4A,B). No colonization was observed on young roots treated with an agar plug but without SCN. By week 4, fungus

inoculation method and presence or absence of *H. glycines* appeared to be of little importance in determining colonization of older roots.

Colonization studies in soil: When plants were grown in soil-containing cylinders in the greenhouse, isolation of *V. lecanii* varied with the soil type. Numbers of colonies isolated on OMA ADE are presented as an ex-

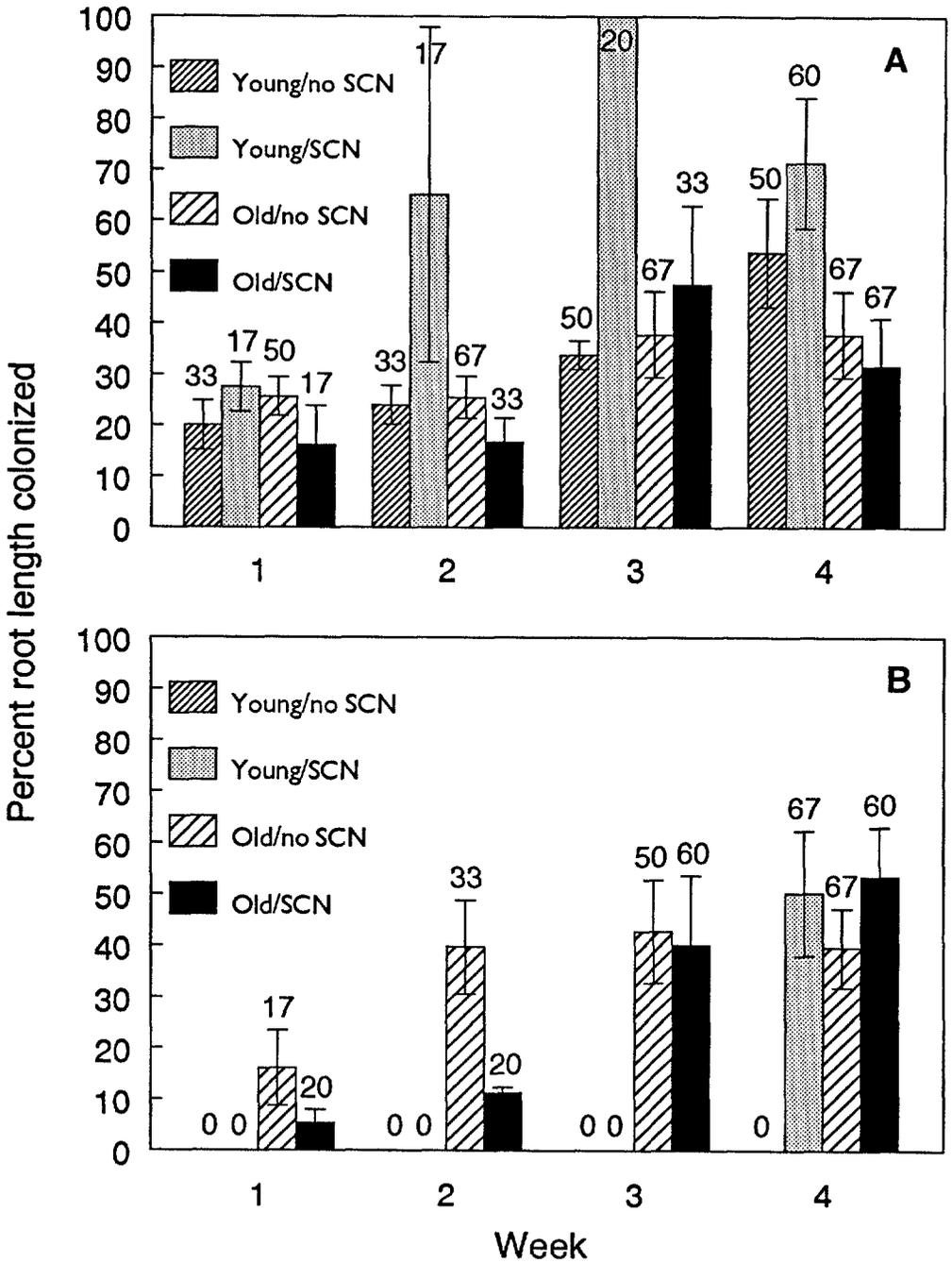


FIG. 4. Mean percentage of soybean root length externally colonized by the fungus *Verticillium lecanii*, and percentage of total roots colonized, in root tip explant cultures treated with *V. lecanii* in aqueous suspension (A) or with *V. lecanii* on potato dextrose agar plugs (B). For treatments in which roots were colonized, only the colonized roots were included when calculating the means for the y-axis. Lines indicate standard error; numbers above (or in) bars indicate percentage of roots colonized. Treatments were young roots (cultures treated with fungus 4 days after root tip transfer) and old roots (cultures treated with fungus 2 weeks after root tip transfer), each with and without soybean cyst nematode (SCN).

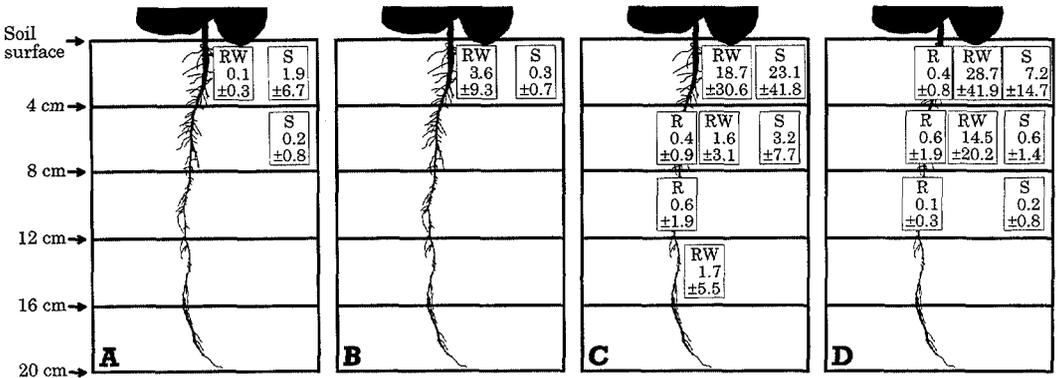


FIG. 5. Diagrams of soybean roots demonstrating locations and numbers of *Verticillium lecanii* isolations (means \pm standard deviations). Results shown are isolations on the medium OMA ADE. Samples at each time period were taken from 12 20-cm-high cylinders that were each subdivided into 4-cm-high sections. Horizontal bars indicate tops and bottoms of sections. R = isolations from washed roots (number of colonies per root segment), RW = isolations from root washes (number of colonies per ml root wash), and S = isolations from soil (number of colonies per gram of nonrhizosphere soil). When no number is given for R, RW, or S, no *V. lecanii* was detected with this medium. A) Loamy sand soil, 2-week harvest. B) Loamy sand soil, 4-week harvest. C) Sandy soil, 2-week harvest. D) Sandy soil, 4-week harvest.

ample (Figs. 5A–D) because this medium was used for all treatments, and because tests with various media demonstrated that detection was generally more successful on media with dodine than on media containing benomyl. The results obtained from OMA ADE represent the general trends observed when all media were combined. Data are reported as number of colonies per root segment on plated roots, number of colonies per ml for root washes, and number of colonies per g of soil for nonrhizosphere soil.

In greenhouse experiments with cylinders containing loamy sand, most main roots had grown to 20 cm or more by 2 weeks. When the 12 cylinders treated with prills were combined for each time period, *V. lecanii* was primarily isolated from the 0- to 4-cm section (Figs. 5A,B). Prills were present in the 0- to 4-cm section and may have been a primary source of fungus isolations in that area. Even in that section, mean numbers of colonies isolated were very low (Figs. 5A,B). When results from all media were combined, the fungus was isolated from the 0- to 4-cm section from all 12 cylinders after 2 weeks, and from 11 cylinders after 4 weeks. Below 4 cm, *V. lecanii* was detected from roots or root washes of only three plants at each time period. Soil isolations below 4 cm

were made from four cylinders (2 weeks) and two cylinders (4 weeks). *Verticillium lecanii* was isolated from the 12- to 16-cm section from only one root system (including roots and root washes), and from soil of only two cylinders at that depth. The colonies were not isolated in numbers that would influence the overall results. No isolations were made in loamy sand from the 16- to 20-cm section.

In the sandy soil, root lengths were a minimum of 20 cm in all cylinders at 2 weeks and at 4 weeks. More colonies were detected from the sandy soil than from the loamy sand soil, but the pattern between the soil types corresponded in that most isolations were from the 0- to 4-cm section (Figs. 5C,D). However, some isolations were made on OMA ADE from roots, root washes, and soil at 4–8 cm, and from roots, soil, or root washes to a depth of 16 cm (2 weeks) or 12 cm (4 weeks).

When results from all media were combined, the fungus was isolated from the 0- to 4-cm section from all 12 cylinders after 2 weeks, and from 11 cylinders after 4 weeks. Below 4 cm, *V. lecanii* was detected from roots or root washes of 11 plants (2 weeks) and 12 plants (4 weeks) in the sandy soil. Soil isolations below 4 cm were made from 8 cylinders (2 weeks) and 10 cylinders (4

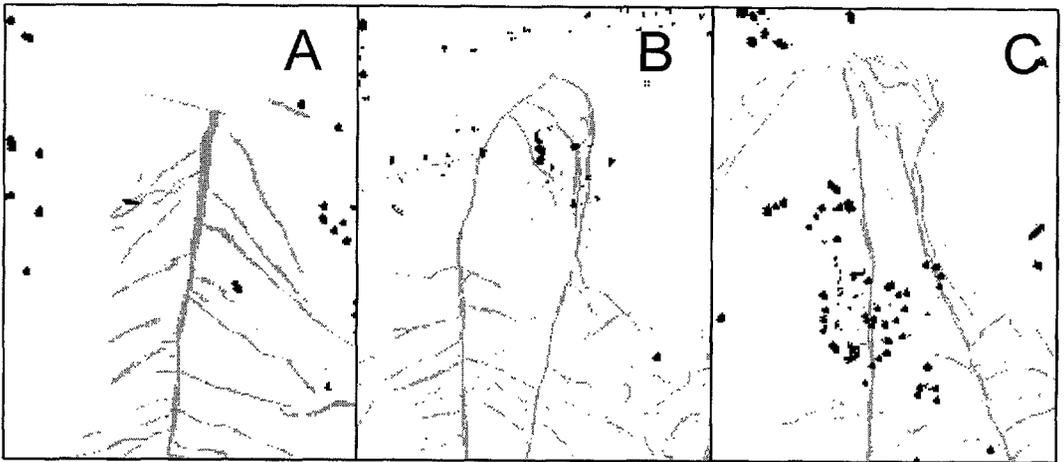


FIG. 6. Examples from root boxes showing location of *Verticillium lecanii* relative to soybean roots. Images are from areas 7.5 cm wide \times 10.5 cm high. Light gray = soybean roots, black = *V. lecanii*. Results varied from few colonies on roots (A) to some root colonization close to the top of the root box (B) to colonization farther down the roots (C). In no case was colonization of roots extensive.

weeks). Despite some isolations throughout the entire length of the root system, the apparent rhizosphere colonization was not detected from many plants; the fungus was isolated from only three root systems at the 16- to 20-cm depth and was not isolated from soil in that area.

In the experiments analyzed with image processing with root boxes 10.5 cm in height, treatments with 1 g prills and 2 g prills gave the same results. *Verticillium lecanii* was found in association with major soybean roots, but in no cases did *V. lecanii* colonize the soybean rhizosphere extensively (Figs. 6A-C). The distribution of *V. lecanii* in the root boxes was not limited to regions containing the main roots; however, there was only extensive spatial distribution in one root box. When data from 18 root boxes were combined into one image, colonies were not heavily clustered in any areas but, rather, were distributed randomly throughout the entire root box (Fig. 7).

Verticillium lecanii was not isolated from controls of any soil experiments.

DISCUSSION

Plant roots produce large amounts of organic material, stimulating growth of many microorganisms; the rhizosphere may have

50 to 100 times more microorganisms than soil outside the rhizosphere (Foster et al., 1983). Many nematode-antagonistic fungi are associated with the rhizosphere of crop

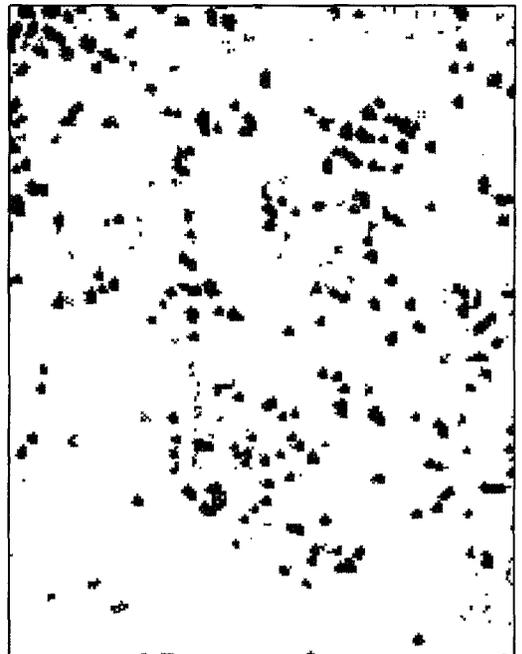


FIG. 7. Combined spatial distribution of *Verticillium lecanii* in 10.5-cm-high root boxes. Figure is a composite of 18 replicates. White = no *V. lecanii* detected, gray = one colony detected, black = two colonies detected at a single location.

plants. For example, nematophagous fungi have been isolated from the rhizosphere of pea, white mustard, barley, and citrus (Gaspard and Mankau, 1986; Persmark, 1997; Persmark and Jansson, 1997); the number of propagules of nematode-trapping fungi in the pea rhizosphere was 19 times greater than in root-free soil. This increase corresponded to the increased number of nematodes in the rhizosphere (6 to 290 times more nematodes than in the rest of the soil). It is likely that nutrients from plant roots, and presence of nematodes in the rhizosphere, are factors contributing to the fungus-rhizosphere associations. Studies with soybean demonstrated that selected fungi isolated from SCN cysts were able to colonize soybean roots (Stiles and Glawe, 1989), which suggests that the roots might be used for nutrition. Because *V. lecanii* has been isolated from soil (Domsch et al., 1980) and is antagonistic to SCN, we hypothesized that it might also grow in association with soybean roots. The *in vitro* studies reported here demonstrated that *V. lecanii* growth can be stimulated by the presence of soybean roots. However, the fungus did not aggressively colonize all of the available roots, even in the tissue cultures. Roots from one treatment were not colonized at all, suggesting that the enhanced growth on some root surfaces in tissue culture treatments was due to nutritional factors rather than to a physical attraction to the agar-root interface.

In vitro, soybean root colonization by *V. lecanii* was generally not dependent on the presence of SCN, with one exception: *H. glycines* appeared to enhance fungus growth on young roots when the PDA plug was present as a nutrient source, indicating that the nematode may have provided additional nutrients for the fungus.

The tissue culture studies indicated that the fungus would grow in association with about half to two-thirds of the available roots (with the exception of one treatment) and that the presence of SCN might enhance the root association. In the soil, fungus isolations from roots and root washes varied with soil type; colonies were detected below 4 cm

from few specimens in loamy sand, but from nearly all of the specimens in sandy soil. Below 8 cm in depth, *V. lecanii* in the sandy soil treatments tended to be isolated from root systems rather than soil, suggesting that the fungus may have been colonizing the roots to some extent. However, the detected populations were low. While the low number of colonies may have been partly caused by lack of a semi-selective medium, the trend was clear; the fungus was not found in high numbers or from many plants at lower depths. More accurate pinpointing of colonies with the image-processing techniques corroborated that the fungus was not extensively colonizing root systems.

In earlier greenhouse tests on soybean, *V. lecanii* was isolated from a few greenhouse pots (Meyer and Meyer, 1995, 1996; Meyer and Huettel, 1996) but was not isolated from microplot or field plot soil (Meyer et al., 1997). The results from the tissue culture and greenhouse studies combined indicate that *V. lecanii*, while capable of colonizing soybean roots in tissue culture, was not an aggressive rhizosphere colonizer in soil. There are several possible reasons for this result. Abiotic factors, such as temperature, moisture, and soil composition, could affect growth of the fungus; formulation and delivery might alter ability to colonize the rhizosphere; or soil microflora and microfauna could render *V. lecanii* noncompetitive in soybean rhizosphere in the soil. Similar results have been found with strains of the biocontrol fungus *Verticillium chlamydosporium*. While some strains of this fungus were able to colonize rhizosphere under sterile conditions, rhizosphere colonization in nonsterilized soil varied with fungus strain and with crop plant (Bourne et al., 1994; Kerry, 1995).

Lack of aggressive rhizosphere colonization may be one reason for the variable results found in microplot studies when *V. lecanii* was applied in alginate prills (Meyer et al., 1997). Environmental conditions and the populations of the soil microfauna and microflora may have to be within narrow parameters for the fungus to become estab-

lished in the rhizosphere and decrease nematode populations.

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