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Field Efficacy of *Verticillium lecanii*, Sex Pheromone, and Pheromone Analogs as Potential Management Agents for Soybean Cyst Nematode

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Abstract: A soybean cyst nematode sex pheromone (vanillic acid), chemical analogs of the pheromone, and the fungus *Verticillium lecanii* were applied in alginate prills (340 kg/ha) to microplots and small-scale field plots as potential management agents for *Heterodera glycines* on soybean. In 1991 microplot tests, treatment with *V. lecanii*, vanillic acid, syringic acid plus *V. lecanii*, or vanillic acid plus *V. lecanii* lowered midseason cyst numbers compared with the untreated susceptible cultivar control, autoclaved *V. lecanii* treatment, or aldicarb treatment. At-harvest cyst numbers were lowest with *V. lecanii* and with vanillic acid treatments. Aldicarb treatment reduced midseason cyst numbers in 1992. There were no differences among seed yields either year. In the field trials, numbers of cysts were reduced one or both years with aldicarb, ferulic acid, syringic acid, vanillic acid, or 4-hydroxy-3-methoxybenzonitrile treatments, or with a resistant cultivar, compared to an untreated susceptible cultivar. Highest yields were recorded after treatment with 4-hydroxy-3-methoxybenzonitrile (1991), methyl vanillate (1992), and aldicarb (1992). These studies indicate that some chemical analogs of vanillic acid have potential for use in soybean cyst nematode management schemes.

Key words: biological control, fungus, *Glycine max*, *Heterodera glycines*, microbial control, nematode management, sex pheromone, soybean, soybean cyst nematode, vanillic acid, *Verticillium lecanii*.

Heterodera glycines Ichinohe (soybean cyst nematode; SCN) is a major pest in soybean-growing areas worldwide. In the top soybean-producing countries, yield losses due to this nematode (a total of 3,025,400 metric tons in Brazil, Canada, China, and the United States) were greater than losses from any other single disease organism (Wrather et al., 1997). It is evident that current management techniques, such as application of nematicides, planting of resistant cultivars, crop rotation, tillage, and use of irrigation, would benefit greatly from supplemental control measures.

Application of biological control agents has been investigated for management of *H. glycines*, and much of this research has focused on fungal agents. Numerous studies have been conducted on isolation of fungi from females, cysts, and eggs of *H. glycines*, and on antagonism of fungi to SCN (e.g. Carris and Glawe, 1989; Chen and Dickson, 1996; Chen et al., 1994; Kim et al., 1992; Kim and Riggs, 1991, 1994, 1995; Liu et al., 1992, 1995; Morgan-Jones et al., 1981; Niblack and Hussey, 1986; Rodríguez-Kábana and Morgan-Jones, 1988; Stiles et al., 1993). Nevertheless, research has not yet led to the development of a successful biocontrol agent for SCN. Our study focused on *Verticillium lecanii* (A. Zimmermann) Viégas, one of the fungi known to be associated with *H. glycines* and to reduce nematode populations in laboratory and greenhouse studies (Gintis et al., 1983; Godoy et al., 1982; Meyer et al., 1990; Meyer and Huettel, 1993, 1996; Meyer and Meyer, 1995, 1996b). Mutant strains of *V. lecanii* were induced with ultraviolet light and selected for increased benomyl tolerance (Meyer, 1992), resulting in increased efficacy of certain isolates against *H. glycines* in greenhouse tests (Meyer and Meyer, 1995, 1996b). One of these mutant strains was selected for further investigation as a possible SCN management agent.

Along with this mutant fungal strain, a natural product secreted by SCN, vanillic acid, also was chosen for testing as a man-

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agement agent. Vanillic acid is a sex pheromone isolated and identified from *H. glycines* that induces male coiling needed for fertilization (Huettel and Rebois, 1986; Jaffe et al., 1989). Consequently, vanillic acid acts as a bioregulator, a compound that can affect an organism's life processes. Limited in vitro studies indicated that application of at least one chemical analog of vanillic acid decreased the ability of second-stage juveniles to find or penetrate roots, while addition of vanillic acid or of various analogs to cultures appeared to inhibit mating in *H. glycines* (Stern et al., 1988). Because *H. glycines* only reproduces sexually, it was hypothesized that addition of excessive amounts of vanillic acid to soil would substantially disrupt the SCN life cycle (Huettel and Meyer, 1992; Meyer and Huettel, 1993, 1996). Under some greenhouse test conditions, application of vanillic acid in alginate prills indeed reduced nematode numbers on soybean roots (Meyer and Huettel, 1996). Certain chemical analogs of this pheromone (ferulic acid, 4-hydroxy-3-methoxybenzonitrile, isovanillic acid, and syringic acid), also were effective for reducing *H. glycines* population densities in some of those studies (Meyer and Huettel, 1996). Additional treatments applied in those greenhouse tests included a *V. lecanii*-vanillic acid combination and a *V. lecanii*-syringic acid combination. This approach tested the hypothesis that the fungus would kill eggs while the compounds decreased mating, thus resulting in a net synergistic reduction of nematode numbers (Huettel and Meyer, 1992). Both combinations did decrease populations of *H. glycines* produced on soybean roots. However, the *V. lecanii*-vanillic acid combination was not as effective as the fungus applied alone, and the *V. lecanii*-syringic acid combination did not provide enhanced control over either treatment applied individually (Meyer and Huettel, 1996).

Given the efficacy of various agents in greenhouse studies, the current research was initiated to determine effects of selected agents applied for management of *H. glycines* on soybean in microplots and in small-scale field tests. Vanillic acid, ferulic acid, 4-hydroxy-3-methoxybenzonitrile, methyl vanillate, syringic acid, one effective mutant strain of *V. lecanii,* and two *V. lecanii*-bioregulator combinations were all applied individually to test efficacy in the field.

Materials and Methods

Potential management agents: Verticillium lecanii strain M2S1 (Agricultural Research Service Culture Collection, NRRL 18726) was induced with ultraviolet light from wild type strain ATCC 58909. For the field studies, strain M2S1 was grown in potato dextrose broth (Meyer and Huettel, 1996) in flasks, carboys, or in a fermenter. Alginate prills containing fungus, test compounds, or fungus-test compound combinations were made as previously described (Meyer and Huettel, 1996; Meyer and Meyer, 1996a): 100 g wet weight fungus was added per liter of alginate slurry, and bran was supplied as a substrate for fungal growth. The inert compound pyrophyllite (hydrous aluminum silicate) was added as a carrier for bioregulator-containing prills. Fungus dry weight was ca. one-third of the total prill weight (ca. 0.3 g dry weight fungus/g dry weight prills). Autoclaved prills containing nonviable *V. lecanii* were used as "prill only" controls in microplot tests. Test compound levels were less than 2.2% (dry weight) in prills. Test compounds used were ferulic acid (4-hydroxy-3-methoxycinnamic acid), 4-hydroxy-3-methoxybenzonitrile, methyl vanillate (methyl 4-hydroxy-3-methoxybenzoate), syringic acid (4-hydroxy-3,5-dimethoxybenzoic acid), and vanillic acid (4-hydroxy-3-methoxybenzoic acid). Prills were also made with fungus-bioregulator combinations; one combination was *V. lecanii*-vanillic acid, and the other was *V. lecanii*-syringic acid. All agents were applied at planting (May or June).

Microplot tests: These studies were conducted at the Crop Genetics International farm near Ingleside, Maryland in 1991 and 1992. Microplots were 35 cm in diam., and each was lined with a cylinder of polyvinyl chloride (60 cm high) inserted 50 cm into the soil. Microplot soil was a sandy loam:

75% sand, 16% silt, 9% clay, 1.8% organic matter, pH 7.0. Mineral and nutrient levels in microplots were adjusted according to soil test recommendations. Microplots were hand-weeded.

Microplot preparation: In 1991, *H. glycines* race 3 from greenhouse stock pots and from an infested field near Salisbury, Maryland was introduced into the microplots prior to planting. The soil from the top of each microplot (ca. 30 cm) was mixed in a cement mixer with infested soil from both sources, and the mixture was placed in the microplots. Each time soil was mixed, the soil in the mixer was sampled to determine at-planting cyst numbers. The experiment was conducted in a randomized complete block design with four blocks of eight treatments (five microplots in a row per treatment per block). Each of the eight treatments (Table 1) was replicated in 20 microplots; a total of 160 microplots was used. The treatments were repeated in 1992.

Microplot planting and maintenance: Twelve soybean seeds (susceptible cultivar Essex) were hand-planted in a circle in each microplot. Experimental control agents in alginate prills were applied "in furrow" (rather than mixed throughout the microplot) at 4 g per microplot, ca. 0.33 g prills near each seed (including carrier). If stand count was poor when seedlings came up, microplots were reseeded by hand for a total of at least nine plants per microplot. Microplots with larger numbers were thinned to 9 or 10 plants per microplot. Microplots were treated with fungicide (0.85 kg benomyl/ha) and insecticide (0.57 kg carbaryl/ha), as needed, and irrigated by trickle or overhead irrigation when necessary.

Microplot sampling: Cyst counts were made at planting, at midseason (ca. 6 weeks after planting), and at harvest. Soil was sampled at planting. Samples were taken at midseason and harvest by removal of two nonadjacent plants from each microplot with a trowel or spade; samples were taken to the depth of the root systems. The soil from the two samples was combined, and cyst numbers were counted from a 100-cm^3 sample of the soil and from the roots. Cysts were isolated from soil and roots with a modified centrifugal-flotation technique (Meyer and Huettel, 1996) or by collection on filter paper (Krusberg et al., 1994), and all collected cysts were counted with a stereomicroscope. Plants were harvested by hand and threshed, and seed dry weights were recorded after drying at 63 °C for at least 24 hr.

Field tests: One field, located in Laurel, Delaware, was used in 1991. In 1992, three fields were planted. Locations were at Laurel (the same field as the previous year, but in a different area); Eldorado, Maryland; and Easton, Maryland. All three fields had been planted to soybean, were naturally in-

TABLE 1. *Heterodera glycines* cyst numbers and dry weight of soybean seeds harvested from microplots.

Treatment	Preplanting cyst numbers		Midseason cyst numbers		At-harvest cyst numbers		Mean seed dry weight (g) per row of five microplots	
	1991	1992	1991	1992	1991	1992	1991	1992
Aldicarb	373	201	126 a	95 b	883 ab	395	657	660
Prill only	354	237	139 a	193 a	727 ab	339	504	557
Untreated susceptible cultivar	311	203	138 a	199 a	614 ab	740	494	862
Vanillic acid plus *Verticillium lecanii*	331	237	21 d	195 a	624 ab	382	544	596
Syringic acid plus *Verticillium lecanii*	385	126	47 bc	174 a	552 ab	494	532	721
Syringic acid	456	216	80 ab	157 a	450 bc	623	598	774
Vanillic acid	420	231	46 bc	171 a	379 c	482	555	775
Verticillium lecanii	391	253	38 cd	199 a	354 c	488	547	692

Cyst numbers per microplot were counted from 100 cm^3 soil; cysts from two root systems per microplot were added to the 100 cm^3 soil for midseason and at-harvest counts. Cyst number data are presented as least squares means per microplot sample, yield data as least squares means per row of five microplots. All numbers are back-transformed from $\log_{10}$. Means in a column with different letters are significantly different ($P \leq 0.05$) according to pair-wise contrasts of the means.

fested with *H. glycines* race 3, and were exhibiting problems caused by this nematode. Soil types were as follows: Eldorado-loamy sand, 84% sand, 10% silt, 6% clay, pH 5.4, organic matter 1.0%; Laurel-sand, 92% sand, 4% silt, 4% clay, pH 5.9, organic matter 1.2%; Easton-silt loam, 26% sand, 58% silt, 16% clay, pH 7.0, organic matter 2.6%. In 1991, the experiment was conducted in a randomized complete block design with five blocks of eight treatments (Table 2) per block. *Verticillium lecanii* treatments were not tested because the means to produce sufficient quantities of fungus were not available at that time. Two 6.1-meter rows were replicated five times for each treatment, with a buffer row on the outside of each pair of treated rows. Rows were 76 cm wide and 76 cm apart. A 1.5-m-wide alley of untreated plants ran between blocks. In 1992, the tests were conducted in a randomized complete block design with five blocks of 11 treatments (Table 2) per block.

Field planting: Cultivars Duke (resistant to race 3) and Essex were used both years. Soybean seeds were planted with either a hand-push or a tractor-mounted planter, except for seeds in aldicarb-treated plots, which were planted by hand so that aldicarb could be applied in-furrow beneath the seeds. Seeds were planted at 33/m. Experimental control agents were applied by hand in-furrow or with a hand-push applicator at 340 kg/ha (300 lb/a). Preemergence herbicides (0.57 kg/ha linuron, 1.42 kg/ha metolachlor) were applied. Plots were hand-thinned to ca. 20 plants/m of row.

Field sampling: Cysts were counted at planting, midseason, and harvest, as in the microplot tests. For at-plant cyst counts, 20 soil cores were collected to a depth of 30 cm from the two rows, and the 20 cores from each plot were mixed before extraction and enumeration. In 1991, field plots were sampled at midseason and harvest by collecting 20 cores and four soybean plants per treatment plot. In 1992, 20 cores and eight plants were sampled from each plot. To count cysts, 100 cm^3 of the mixed soil core sample from each plot was washed along with the collected root systems from that plot. Only the two Maryland fields were available for at-harvest cyst numbers and soybean yields. Soybeans were harvested and seed dry weights were measured.

Verticillium sampling: To isolate *Verticillium* from soil, 5 g was taken from each of the samples used for cyst counts, and mixed in 500 ml water. Each sample (all microplots and field plots) was plated onto two plates of potato dextrose agar with antibiotics and benomyl (PDA ABE 100; Meyer and Meyer,

TABLE 2. *Heterodera glycines* cyst numbers and dry weight of soybean seeds harvested from field plots.

Treatment	At-planting cyst numbers		Midseason cyst numbers		At-harvest cyst numbers		Mean seed dry weight (g) per plot	
	1991	1992	1991	1992	1991	1992	1991	1992
Aldicarb	61	64	24 c	85 b	420 ab	190 bcd	109 b	2236 a
Methyl vanillate	127	77	39 abc	153 a	371 abc	261 abc	153 ab	2089 ab
Vanillic acid plus *Verticillium lecanii*	NT	74	NT	163 a	NT	239 abcd	NT	1938 abc
Verticillium lecanii	NT	85	NT	138 a	NT	189 cd	NT	1922 abc
4-hydroxy-3-methoxy-benzonitrile	82	85	24 c	177 a	194 d	301 ab	183 a	1911 abc
Ferulic acid	80	63	52 ab	150 a	200 cd	235 abcd	134 ab	1903 abc
Syringic acid plus *Verticillium lecanii*	NT	79	NT	151 a	NT	203 abcd	NT	1858 abc
Resistant cultivar	86	66	22 c	88 b	63 e	156 d	149 ab	1793 abc
Syringic acid	70	69	31 bc	145 a	356 abcd	254 abc	138 ab	1753 abc
Vanillic acid	81	78	33 abc	164 a	269 bcd	310 a	128 ab	1680 bc
Untreated susceptible cultivar	111	74	64 a	148 a	532 a	230 abcd	111 b	1545 c

Cyst numbers in each plot were counted from 100 cm^3 soil; in 1991, cysts from four root systems per plot were added to the 100 cm^3 soil for midseason and at-harvest counts. In 1992, cysts from eight root systems per plot were added. Data are presented as least squares means per plot, back-transformed from $\log_{10}$. Means in a column with different letters are significantly different ($P \leq 0.05$) according to pair-wise contrasts of the means. NT = not tested.

1995). Most samples were also plated onto this medium containing 0.5% Rose Bengal (PDA ABE 100 Rose Bengal). *Verticillium* isolates were then transferred to fresh PDA ABE 100 or PDA ABE 100 Rose Bengal media for growth-rate comparisons with the applied mutant strain.

Statistical analysis: The number of cysts and the dry weight of seeds were analyzed for each time period as a one-factor mixed design using PROC MIXED (SAS Institute, Cary, NC). For the microplot data, cyst numbers were analyzed per microplot, and yields were analyzed per row of five microplots. In all analyses, treatment was the fixed factor. For the microplot data and the 1991 field data, block was the random factor. For the 1992 field data, site and site × block were the random factors. Using the treatments in common for 1991 and 1992 field yields, both years were also analyzed together with year, year × site, and year × site × block as the random factors. To correct for variance heterogeneity, $\log_{10}$ transformations were made. The back-transformed least-squares means (in number of cysts or seed weights) and pair-wise contrasts of the means were determined. In the 1992 field tests, cyst counts from all three field sites were included at-plant and midseason, but at-harvest results were from Maryland fields only.

Results

Microplot tests: Several differences in cyst numbers occurred among the treatments in 1991 (Table 1). Treatment with viable *V. lecanii,* vanillic acid, syringic acid plus *V. lecanii,* or vanillic acid plus *V. lecanii* resulted in lower midseason cyst numbers than those counted from untreated susceptible cultivar controls, prill-only treatment, or aldicarb. At-harvest numbers were lower with *V. lecanii* and vanillic acid treatments. In 1992, a significant reduction in cyst numbers was recorded only in the aldicarb treatment at midseason. Seed yields did not reflect differences in nematode numbers in 1991 (Table 1), nor were there significant yield differences among treatments in 1992.

Field tests: There were no differences among cyst numbers at planting (Table 2). In 1991, midseason cyst numbers were lowest in plots treated with aldicarb, syringic acid, 4-hydroxy-3-methoxybenzonitrile, and the resistant cultivar. By the end of the season, plots treated with vanillic acid, ferulic acid, and 4-hydroxy-3-methoxybenzonitrile, and the plots planted with the resistant cultivar, had lower cyst numbers than the untreated susceptible control. Aldicarb- and syringic acid-treated plots no longer had reduced cyst numbers. In 1992, only aldicarb-treated plots and plots with the resistant cultivar had lower cyst numbers midseason. At-harvest cyst numbers did not differ significantly from the untreated susceptible control, but vanillic-acid treated plots had higher cyst numbers than aldicarb, *V. lecanii,* or resistant cultivar treatments. Plots treated with 4-hydroxy-3-methoxybenzonitrile also had higher cyst numbers than the latter two treatments.

In the 1991 field test, seed yields (Table 2) were lowest with the untreated susceptible control and with aldicarb treatment, and highest with 4-hydroxy-3-methoxybenzonitrile treatment (ca. 65% higher than untreated susceptible control; yield increases calculated from back-transformed least squares means in Table 2). In 1992, yields were lowest with the untreated susceptible cultivar control and with vanillic acid treatment. Aldicarb treatment (45% greater yield than untreated susceptible cultivar control when calculated from back-transformed least squares means) was ranked the highest, and methyl vanillate treatment ranked second highest in yield (35% greater yield than control). Fungus-bioregulator combination treatments did not differ significantly from yields with individually applied agents. When the bioregulator treatments for the two years were combined for analysis, the treatments that resulted in significantly higher yields than those obtained from the untreated susceptible cultivar were 4-hydroxy-3-methoxybenzonitrile and methyl vanillate (both 36% higher yield than susceptible cultivar controls) and aldicarb (27% yield increase).

In the 1991 microplot study, *Verticillium* was isolated at planting from two petri dishes containing soil samples taken from the cement mixer. *Verticillium* was not isolated at midseason or harvest in 1991, but was isolated at midseason 1992 from one microplot treated with *V. lecanii*. *Verticillium* isolates were obtained at planting (1992) from seven plots in the Eldorado field: two plots treated with vanillic acid, two with syringic acid, one plot with 4-hydroxy-3-methoxybenzonitrile, one with methyl vanillate, and one with *V. lecanii*. In midseason 1992, *Verticillium* was isolated from one *V. lecanii*-treated Eldorado plot and one syringic acid-treated Laurel plot. None of the soil isolates grew as rapidly on the test media as the highly benomyl-resistant strain of *V. lecanii* and, therefore, were likely not the test strain.

Discussion

The small-scale field studies indicated that at least two of the tested bioregulators, 4-hydroxy-3-methoxybenzonitrile and methyl vanillate, have potential to increase soybean yields when applied as management agents for *H. glycines*. Syringic acid and 4-hydroxy-3-methoxybenzonitrile reduced *H. glycines* populations in greenhouse studies (Meyer and Huettel, 1996) and substantially decreased cyst numbers in the field during 1991, although syringic acid did not reduce cyst numbers in the microplot tests. Ferulic acid and vanillic acid, both of which reduced nematode numbers in some greenhouse trials (Meyer and Huettel, 1996), also decreased cyst numbers 1 year in the microplot or field tests. Methyl vanillate, which had no effect on cyst numbers in greenhouse studies (Meyer and Huettel, 1996) or in the field, was one of the most effective agents for increasing yields. Reasons for the increased yields without concomitant population decreases are unclear. It is possible that the agents affect the nematodes very early in the season and then leach from the prills and soil, allowing the nematode populations to subsequently increase. Studies on volatility of the compounds and diffusion in soil would aid in determining how long they are effective. Number of eggs per cyst might also be affected, although this would not be expected with compounds that affect mating and not egg viability. Research on direct effects of the compounds on plant growth would also determine whether application of these substances in alginate prills could increase seed yields in the absence of SCN.

Treatments with syringic acid, vanillic acid, *V. lecanii*, or *V. lecanii*-bioregulator combinations were efficacious for reducing SCN numbers in the greenhouse studies but did not increase yields in the field tests, even though treatments including *V. lecanii* resulted in decreased cyst numbers in the 1991 microplot tests. This strain of *V. lecanii* has been difficult to isolate from soil, even in greenhouse pots, so inability to obtain the strain from the field may or may not reflect persistence in the soil. More information about the fungus is required to determine why it was ineffective in this study. No synergistic interactions of fungus-bioregulator combinations were measured, corroborating greenhouse test results (Meyer and Huettel, 1996).

Although field tests demonstrated some yield differences among treatments, no significant differences in yields were observed among microplot treatments. This may be at least partly due to the fact that the most effective analog treatments in the small-scale field tests were not applied for 2-year microplot tests; however, aldicarb did not substantially increase microplot yields, either. It is possible that, with sufficient irrigation, fertilization, and pest control, *H. glycines* did not severely affect the soybean plants.

The results with certain bioregulators are promising; however, many variables remain to be studied. Large amounts of alginate prills were applied; lower application levels and less expensive formulations need to be tested. The agents do not necessarily decrease nematode populations, so they would undoubtedly be most useful when combined with other management techniques (resistance, etc.), which do have this effect. These studies do suggest that some chemical analogs of a *H. glycines* sex pheromone have

potential for use in SCN management schemes.

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