Potential of *Verticillium lecanii* (*Lecanicillium* spp.) hybrid strains as biological control agents for soybean cyst nematode: Is protoplast fusion an effective tool for development of plant-parasitic nematode control agents?

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Verticillium lecanii is a potentially useful biological control agent (BCA) for soybean cyst nematode (SCN), *Heterodera glycines*. The objective of this study was to screen hybrid strains of *V. lecanii*, derived from protoplast fusion, for effective control agents of SCN, and to investigate whether the protoplast fusion technique is an effective tool for development of nematode control agents. Three parental strains (Vertalec®, Mycotal®, and B-2) and their 162 hybrid strains were screened in greenhouse pot tests. Some of these hybrid strains suppressed damage on soybean plants and reduced the density of SCN in the soil. In particular, one hybrid strain, AaF42, was observed to reduce the nematode egg density by 93.2% as compared with the control. Furthermore, this strain significantly reduced the cyst and egg density as compared with the parental strains. In conclusion, some of the hybrid strains exhibited enhanced biocontrol efficacy by protoplast fusion. Therefore, the protoplast fusion technique may be a potentially valuable tool for developing nematode-antagonistic fungi as BCA. Jpn. J. Nematol. 38 (1), 9-18 (2008).
Key words: greenhouse pot test, *Heterodera glycines*, nematode-antagonistic fungi, screen.

INTRODUCTION

The soybean cyst nematode (SCN) *Heterodera glycines* Ichinohe, is widely distributed in soybean-producing countries. The losses in total yield caused by SCN are greater than those for any other pest of soybean (Wrather et al., 2001). These nematodes have generally been controlled by rotating soybeans with nonhost crops, planting of resistant cultivars, application of an effective nematicide and organic material, and physical control techniques such as solarization. The combination of biological control with the above methods will enhance the effectiveness of the nematode control. To date, numerous studies have been conducted on the fungal antagonists of SCNs (Chen et al., 1996; Kim and Riggs, 1991, 1995; Liu and Chen, 2001, 2005; Meyer and Huettel, 1996; Meyer and Meyer, 1995, 1996; Timper et al., 1999); however, few biological control agents have been commercialized.

Verticillium lecanii (A. Zimmermann) Viégas has been studied as a potential biological control

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agent for SCN (Meyer et al., 1990, 1997; Meyer and Meyer, 1995, 1996). *Verticillium lecanii* is ubiquitously distributed in soils, although this fungus is mainly isolated from insects. In addition, it is known that *V. lecanii* has a broad host range, *e.g.*, insects, phytopathogenic fungi, and plant-parasitic nematodes (Hall, 1981; Meyer et al., 1990). Although in previous laboratory and greenhouse studies, 1 strain of *V. lecanii* was found to exhibit a high virulence to SCNs, this strain was found to be a poor colonizer of the soybean rhizosphere (Meyer et al., 1998). However, it is quite likely that other strains are more aggressive rhizosphere colonizers because *V. lecanii* possesses varied abilities among different strains (Sugimoto et al., 2003).

The basic collection method for fungal antagonists of plant-parasitic nematodes is to isolate the fungi from nematodes or other organisms living in soils. However, it is extremely difficult to collect large numbers of a single fungal species because their isolation and identification is time-consuming. Uziel and Sikora (1992) indicated that the ability of *V. lecanii* to infect the potato cyst nematode, *Globodera pallida*, could not be related to a particular trait of the host from which an isolate originated. Furthermore, they suggested that *V. lecanii* isolated from a nontarget insect could be a source of effective biological control targeted at *G. pallida*.

Protoplast fusion, which promotes the recombination of whole genomes, even between incompatible strains, is a useful tool in strain improvement and in the development of hybrid strains of entomopathogenic and phytopathogenic fungi (Couteaudier et al., 1996; Hocart and Peberdy, 1989; Lalithakumari, 2000; Pe'er and Chet, 1990; Silveira and Azevedo, 1987), and the fungal agent derived by protoplast fusion of *Trichoderma harzianum* has been commercialized as F-stop® (Eastman Kodak Co., Rochester, NY) for control of several soil-borne phytopathogenic fungi (Harman and Hayes, 1993). It is known that the hybrid strains acquire increased virulence, a wide spectrum of activity, and high rhizosphere competence as a result of such a genetic recombination (Couteaudier et al., 1996; Sivan and Harman, 1991; Viaud et al., 1998). Aiuchi et al. (2004) conducted protoplast fusion among three strains of *V. lecanii* and obtained 174 hybrid strains. Therefore, the objective of this study was to compare the relative potential of these hybrid strains of *V. lecanii* as biological control agents (BCAs) against SCNs with that of the parental strains in greenhouse pots and to determine the efficacious strains.

MATERIALS AND METHODS

Nematode inoculum:

The SCN used as the inoculum in experiment 3 was grown in the greenhouse on soybean (cv. Kurosengoku). Ten weeks after SCN inoculation, the cysts in the soil were collected using a method described by Yoshihara and Kegasawa (1989), and then crushed in order to release the eggs. The eggs were separated from the debris by passing them through a 64-μm pore sieve to remove debris. The eggs were rinsed 5 times and suspended in 50 ml sterile distilled water in an autoclaved beaker, and incubated at 28℃. After a 10-day incubation, second-stage juveniles (J2) and eggs were used as inoculum for the experiment.

Fungal culturing and inoculum:

In experiments 1 and 2, each strain of *V. lecanii* (parental and hybrid) was cultured on potato dextrose agar (PDA; Difco Laboratories) at 25℃ for 14 days before using them in the experiments. The agar disks (5-mm diameter) of each strain were added to 30 ml of wheat bran medium (1:4, wheat bran/potting soil) in 50-ml Erlenmeyer flasks, and the cultures were then incubated for 14 days in darkness at 25℃.

In experiment 3, each strain of *V. lecanii* was

cultured on PDA at 25°C for 14 days, and then the conidia were scraped from the culture with a sterile glass rod and suspended in sterilized water to obtain a conidial suspension. The suspension was filtered through the gauze to remove mycelial fragments. The conidia suspensions were pelleted by centrifugation at 3,000 rpm for 5 min and the pellets were resuspended in sterile distilled water. The concentration of each conidia suspension was adjusted to 1×10^7 conidia/ml.

Experiment 1 (First screening against soybean cyst nematode):

Three parental strains (Vertalec®, Mycotal®, and B-2) and 162 hybrid strains derived from these three parental strains (Aiuchi et al., 2004, 2008) were used in experiment 1. Two of these parental strains, Vertalec® and Mycotal® (Koppert UK Ltd., Wadhurst, East Sussex, UK), are exploited commercially in order to control aphid and whitefly, respectively. A third parental strain, B-2, isolated from green peach aphid at Obihiro was also utilized.

Soybean seeds (*Glycine max* (L.) Merr. cv. Toyohomare, susceptible to SCN) were sown in 6-cm diameter pots containing fungus-inoculated soil (95% potting soil, Takii Seed Co. Ltd., Kyoto, Japan; 5% wheat bran with fungal inoculation). The potting soil and the wheat bran with fungal inoculation were uniformly mixed. After five days, the seeds had germinated and were transplanted with soil directly into 9-cm diameter vinyl pots containing potting soil along with 45 cysts of SCN isolated from natural SCN-infested field soil using a method described by Yoshihara and Kegasawa (1989). One pot per fungal treatment was prepared.

The plants were harvested after a further eight weeks, and the cyst index of the roots and leaf symptoms were estimated visually. The control pots were inoculated with cysts only. The cyst index of the roots was scored on the basis of the number of females and cysts (hereafter referred to as “cysts”) on the roots, on a scale of 0 (no damage) to 4 (extensive damage) as follows: 0 = no cysts; 1 = several cysts can be found on the root; 2 = light attacks, no more than 20 cysts can be found on the root; 3 = moderate attacks, numerous cysts can be found on the root; 4 = severe attacks, extremely large number of cysts can be found, and the whole root is crowded (Yamada et al., 2003). The leaf symptoms were scored on the basis of the appearances of plants (5 disease classes), on a scale of 0 to 5 as follows: 0 = all leaves were healthy; 1 = less than 25% of the leaves were yellowing and/or wilting; 2 = 26-50% of the leaves were yellowing and/or wilting; 3 = 51-75% of the leaves were yellowing, wilting, and/or shedding; 4 = 76-100% of the leaves were yellowing, wilting, and/or shedding; 5 = damping-off or root rot. This means that the plants died very young and there were no leaves. The experiments were conducted in an air-conditioned greenhouse at 25 ± 1°C. Each test was performed once for a single isolate.

Experiment 2 (Second screening):

In the second experiment, 17 hybrid strains that exhibited the cyst indices of 0-2, and the leaf symptom indices of 1 or 2 in the first screening were selected; seeds of soybean (cv. Toyohomare) were sown in 6-cm diameter Jiffypots® (Sakata Seed Corporation; Yokohama, Japan) filled with 97% potting compost and 3% (w/w) wheat bran containing the fungal strains. Five days after sowing, three seedlings from separate Jiffypots® were transplanted together into one 18-cm diameter pot containing potting soil (Takii Seed Co. Ltd., Kyoto, Japan) *ca.* 266 cysts from naturally infected soil per pot.

After eight weeks, the cyst index of parasitism on the root, fresh shoot weight, fresh root weight, and leaf symptoms were evaluated. The leaf symptoms were scored on the basis of the

appearances of the individual leaves on a scale of 0 to 3 as follows: 0 = healthy, 1 = yellowing, 2 = wilting, 3 = defoliation. The leaf symptom index was calculated by using the following formula (Praveena and Naseema, 2004). Leaf symptom index = {Sum of the score for each leaf / (Number of leaves scored × Maximum disease score)} × 100. The cyst index was scored in the same manner as described in experiment 1 and was calculated by using the following formula. Cyst Index = {(Σ (Score × Number of plants of each score) / (Total number of the plants × 4)} × 100 (Yamada et al., 2003). There were three plants in each pot, and three replicate pots per fungal treatment were prepared. That is, there were a total of nine plants per fungal treatment. The experiments were of a randomized block design conducted in an air-conditioned greenhouse at 25 ± 1℃

Experiment 3 (Third screening and detailed comparison between the hybrid and their parental strains):

In the third experiment, five hybrid strains exhibiting good results (based on the overall evaluation) in the second screening and their two parental strains—Vertalec® and Mycotal®—were used in order to more vigorously investigate the potential of the strains and to compare the parental and hybrid strains in greater detail.

Soybean seeds (cv. Kitamusume) were sown in 8-cm diameter vinyl pots containing 200 g of sterilized soil (a loamy soil; 66.3% sand, 31.1% silt, 2.6% clay, pH 5.8) and were inoculated with 10 ml of a conidia suspension of each strain containing 1×10^7 conidia/ml. In controls 1 and 2, the pots were inoculated with 10 ml of sterile distilled water instead of the conidia suspension. Furthermore, one week later, the two seedlings received the same inoculum were transplanted together into one plastic pot (diameter, 16 cm; height, 20 cm) filled with 3,000 cm^3 sterilized soil, and 2,000 J2 and eggs were added into holes made in the soil around the roots of each plant; control 2 plants received no J2s or eggs. The plants were maintained in an air-conditioned greenhouse at 25 ± 1℃ for 60 days. There were two plants in each pot, and three replicate pots per fungal treatment were prepared.

Sixty days after SCN inoculation, the soybean plants were cut at the soil surface. The fresh root weights and SCN population density were then measured. In order to estimate the number of cysts and eggs, the soil and roots in each pot were mixed, and a subsample of 50 g (dry weight) of air-dried soil was used for the extraction of eggs using similar procedures described for the preparation of the nematode inoculum. Three soil subsamples per pot were collected.

Statistical analysis:

The effects of fungal treatment were examined using ANOVA and when the *F* test was found to be significant at $P < 0.05$ or 0.01, the treatment means were compared using the Tukey's HSD test. Furthermore, the Kruskal-Wallis test was used when the conditions did not permit the use of parametric statistics. All statistical analyses were performed using Statcel2 (OMS publishing Inc., Japan).

RESULTS

Experiment 1:

A total of 165 strains—three parental and 162 hybrid—of *V. lecanii* were used in the first experiment. The cyst index was 0 for 14 strains, 1 for 44 strains, 2 for 38 strains, 3 for 10 strains, 4 for 56 strains. Of these strains, 15 (14 hybrid strains and one parental strain) caused a notable reduction of cysts on soybean roots. The cyst index of both parental strains, Vertalec® and Mycotal®, was 4, and that for B-2 was 0. In addition, the leaf symptom index was 1 for 14 strains, 2 for 32 strains, 3 for 75 strains, 4 for 41 strains. The 3 parental strains, namely,

Vertalec®, Mycotal®, and B-2, weakly suppressed the leaf symptoms (the leaf symptom indexes were 4, 3, and 3 respectively).

Experiment 2:

One of the 17 strains selected for this experiment, only one hybrid strain of Vertalec® and Mycotal®, AaF42, significantly ($P < 0.05$) reduced the number of cysts on the root as compared with the control (Table 1). The hybrid strain AaF42 also exhibited the lowest leaf symptom index and tended to increase the fresh root weights and shoot weights, although no significant differences were observed among all the treatment.

Experiment 3:

All strains of *V. lecanii* significantly ($P < 0.01$) reduced the number of cysts and eggs as compared to control 1, which was not treated with the fungus (Table 2). Furthermore, four hybrid strains (AaF17, AaF23, AaF42, and AaF103) significantly ($P < 0.01$) reduced the number of cysts and eggs as compared with their parental strains Vertalec® and Mycotal®. The cyst and egg densities were the lowest in pots inoculated with the hybrid strain AaF42, and the reductions in the number of cysts and eggs were 82.8% and 93.2%, respectively as compared to control 1. AaF42 also reduced the number of cysts and eggs 75.2% and 89.3%, respectively, as compared to those caused by the parental strain Vertalec®, and then 69.8% and 84.2%, respectively, as compared to those

Table 1. The effects of various strains of *Verticillium lecanii* on the cyst index, growth, and leaf symptom index of soybean plants in pots infested with *Heterodera glycines*.

Strains[1]	Cyst index[2]	Fresh weight (g/plant)		Leaf symptom index
		Root	Shoot	
AaF11	44.4 ± 48.1 [ab]	0.7 ± 0.5	4.7 ± 3.9	44.8 ± 48.7
AaF17	19.4 ± 12.7 [ab]	1.7 ± 1.6	7.9 ± 1.2	22.2 ± 2.2
AaF23	36.1 ± 17.3 [ab]	1.7 ± 0.5	8.2 ± 1.2	21.5 ± 5.1
AaF26	58.3 ± 14.4 [ab]	0.5 ± 0.6	3.2 ± 2.6	44.1 ± 25.6
AaF28	5.6 ± 4.8 [ab]	1.3 ± 0.3	7.3 ± 0.4	19.3 ± 10.5
AaF30	47.2 ± 37.6 [ab]	1.1 ± 0.8	5.8 ± 4.2	35.6 ± 28.4
AaF39	58.3 ± 28.9 [ab]	0.5 ± 0.2	4.0 ± 1.9	42.2 ± 23.4
AaF42	2.8 ± 4.8 [b]	1.9 ± 1.0	9.8 ± 2.1	7.4 ± 3.4
AaF46	30.6 ± 31.6 [ab]	0.9 ± 0.2	5.3 ± 1.9	26.7 ± 20.3
AaF48	41.7 ± 30.0 [ab]	0.8 ± 0.5	4.5 ± 2.5	36.3 ± 28.1
AaF49	63.9 ± 42.8 [ab]	0.5 ± 0.6	4.0 ± 4.1	51.4 ± 42.2
AaF63	44.4 ± 25.5 [ab]	0.9 ± 0.5	5.9 ± 2.5	23.0 ± 16.7
AaF64	55.6 ± 19.2 [ab]	0.9 ± 0.4	5.0 ± 2.0	29.9 ± 9.1
AaF80	30.5 ± 31.6 [ab]	1.2 ± 0.5	7.1 ± 2.7	36.3 ± 18.0
AaF88	22.2 ± 25.5 [ab]	1.4 ± 0.4	8.0 ± 2.8	20.0 ± 19.7
AaF103	25.0 ± 36.3 [ab]	1.0 ± 0.6	5.9 ± 1.2	10.4 ± 8.4
BbF17	61.1 ± 25.5 [ab]	0.9 ± 0.4	5.2 ± 2.2	23.0 ± 14.3
Control	88.9 ± 12.7 [a]	0.4 ± 0.4	3.1 ± 2.4	47.4 ± 47.2
	$P < 0.05$	NS[3]	NS	NS

The values are the means ± standard deviation of three replicates.

[1] The hybrid strains, AaF and BbF, were derived from protoplast fusion of Vertalec × Mycotal.

[2] The different letters in the column indicate significant difference ($P < 0.05$, Kruskal-Wallis test on ranks followed by Tukey's HSD test).

[3] NS: not significant at $P = 0.05$ (Tukey's HSD test).

Table 2. The effects of various strains of *Verticillium lecanii* on the density of *Heterodera glycines* cysts and eggs, and the growth of soybean roots in pots.

Strains [1]	Cysts/50 g soil	Eggs/g soil	Eggs/cyst	Fresh root weights (g)
AaF17	12.3 ± 4.4 ^{de}	18.7 ± 7.5 ^{d}	73.3 ± 17.0 ^{e}	1.10 ± 0.09 ^{bc}
AaF23	26.7 ± 9.2 ^{cde}	39.3 ± 15.6 ^{d}	78.2 ± 16.6 ^{e}	0.93 ± 0.08 ^{ab}
AaF42	11.9 ± 3.6 ^{e}	16.8 ± 8.3 ^{d}	70.9 ± 29.4 ^{e}	1.52 ± 0.21 ^{c}
AaF80	27.8 ± 7.3 ^{cd}	65.0 ± 22.3 ^{cd}	114.9 ± 13.5 ^{cd}	0.85 ± 0.22 ^{ab}
AaF103	13.0 ± 4.6 ^{de}	27.0 ± 11.6 ^{d}	100.9 ± 19.0 ^{de}	0.83 ± 0.14 ^{ab}
Mycotal	39.4 ± 9.4 ^{bc}	106.0 ± 29.3 ^{bc}	133.3 ± 12.3 ^{bc}	0.62 ± 0.08 ^{ab}
Vertalec	47.9 ± 11.6 ^{b}	157.4 ± 54.5 ^{b}	161.1 ± 20.6 ^{ab}	0.72 ± 0.03 ^{ab}
Control 1 (without fungus) [2]	69.1 ± 17.2 ^{a}	248.6 ± 75.7 ^{a}	179.2 ± 25.5 ^{a}	0.60 ± 0.09 ^{ab}
Control 2 (untreated) [3]	ND [4]	ND	ND	1.00 ± 0.10 ^{ab}

The values are the means ± standard deviation of three replicates. The different letters in the columns indicate significant differences ($P < 0.01$, Tukey's HSD test).
[1] The hybrid strains, AaF were derived from protoplast fusion of Vertalec × Mycotal.
[2] Control 1: SCN was inoculated but fungus was not.
[3] Control 2: Neither SCN nor fungus was inoculated.
[4] ND: not detected.

caused by the parental strain Mycotal®. Out of seven strains, six strains significantly ($P < 0.01$) reduced the number of eggs/cysts as compared with control 1. Moreover, only the hybrid strain AaF42 significantly ($P < 0.01$) increased the fresh root weight as compared with both parental strains and even control 2, which was not inoculated with SCN.

DISCUSSION

Some of the hybrid strains developed by protoplast fusion exhibited higher level of nematode control efficacy against SCNs than the parental strains. In particular, the SCN density in the pot treated with the hybrid strain AaF42 was significantly reduced as compared with control 1 in experiment 3. Furthermore, the hybrid strain AaF42 significantly reduced the SCN density in the pot and increased plant growth as compared with the parental strains (Table 2). Re-isolate the hybrid strains of V. lecanii from the cysts, females, or plants were not conducted at the end of this experiment. However, AaF42 was detected from eggs in cyst and the roots of soybean in another pot test (Shinya et al., unpublished results). In addition, it was observed that some hybrid strains (including AaF42) infected the eggs, females, cysts in another in vitro test and SEM observation. Hocart and Peberdy (1989) describe how the interaction between a fungal BCA and its target organism is complex and is likely to be under the control of a large number of genes. This characteristic will apply to the biological control of plant-parasitic nematodes. Therefore, as a result of the recombination of whole genomes, some of the hybrid strains may have gained various advantages as BCAs. Such whole genome recombination via protoplast fusion of the nontarget entomopathogenic strains, Vertalec® and Mycotal®, may have increased the biocontrol efficacy against SCNs in these effective hybrid strains.

Moreover, the growth of plants inoculated with the hybrid strain AaF42 was higher than that in plants inoculated with any other strain (Table 1), and the hybrid strain AaF42 significantly increased root weight as compared with control 2, which was not inoculated with SCN (Table 2). One possibility is that the hybrid strain AaF42 might lead to an enhancement in

plant growth by producing substances such as nitrogen, phytohormones, compounds promoting the mineralization, etc. The plant growth-promoting effect might confer an additional advantage on the fungus as a BCA. There is another possibility that AaF42 might trigger the defense response of soybean to pathogens. However, more studies without the presence of nematodes are required in order to decide whether this strain possesses these effects.

In experiment 3, it was observed that several strains of *V. lecanii* reduced the number of eggs/cysts (Table 2). There are two interpretations for this result. One hypothesis is that *V. lecanii* infected the females of SCN and reduced their fecundity. The other is that *V. lecanii* infected J2, and this result in females were less mature and had fewer eggs. However, *V. lecanii* was weak parasite of J2 of SCN in another *in vitro* test (data not shown), although *V. lecanii* colonizes the eggs, cysts, and the females of SCNs (Meyer and Wergin, 1998). Therefore, the latter hypothesis would be untrue from these points of view. On the other hand, the former would be supported by the SEM observation (Meyer and Wergin, 1998). They hypothesized that *V. lecanii* produces a natural substance that kills the eggs of SCN and causes infection and death of the females before the full complement of eggs is produced. Furthermore, Kerry (1990) demonstrated that *Verticillium chlamydosporium* reduces the fecundity of *Heterodera schachtii*. Thus, it is suggested that *V. lecanii* can influence the SCNs in several ways. It will be necessary to conduct detailed *in vitro* tests in order to reveal the fungi's various modes of action.

There was a relationship between the nematode control efficacy of the hybrid strains and the combinations of the parental strains in this study. All of the effective hybrid strains were hybrids of Vertalec® and Mycotal®. No hybrid of B-2 was effective in SCN control. B-2 exhibited the lowest effect on SCN in another *in vitro* test (Shinya et al., unpublished results). On the other hand, Mycotal had the highest ability to infect eggs of SCN in these three parental strains in this *in vitro* test. Furthermore, Aiuchi et al. (2008) demonstrated that mtDNA and genomic DNA types of Vertalec × Mycotal were of the Mycotal type, while those of Mycotal × B-2/Vertalec × B-2 were of the B-2 type. There is a possibility that these genetic biases were reflected in the characteristics of the hybrid strains. Therefore, hybrid strains of Mycotal × B-2/Vertalec × B-2 may have the characteristics like B-2. This would be the reason why no hybrid of B-2 was effective in control of SCN. From now on, a more detailed study will be needed to solve this issue.

The steps used in the selection of strains to determine their potential as BCAs generally begin with simple *in vitro* tests in the laboratory as it is difficult to screen large numbers of strains in pot tests in the greenhouse (Kerry, 2001). However, in the present study, all the steps used in the screening of the strains were conducted in soil in the greenhouse. In experiment 1, a large number of strains were screened by pot tests in order to exclude strains that are poor competitors in soil. As a result, several potentially useful strains were selected, and then more rigid experiments (experiments 2 and 3) were conducted. These greenhouse trials enabled us to detect the highly pathogenic strains to the nematode in the soil. Further studies using unsterilized field soil in the greenhouse and field are required in order to determine the potential of the strains as BCAs.

In conclusion, our results indicate that protoplast fusion in *V. lecanii* gives rise to a wide range of hybrid strain characteristics. As a result, some of the hybrid strains possessed enhanced appropriate characteristics (such as the ability to attack SCN and high rhizosphere

competence) as BCAs and enhanced biocontrol efficacy, although further investigations to assess their impact on nontarget organisms are needed. Therefore, the protoplast fusion technique may be a potentially valuable tool for the development of nematode-antagonistic fungi.

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Received September 27, 2007
Accepted December 20, 2007