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Evaluation of Soil Suppressiveness of Various Japanese Soils against the Soybean Cyst Nematode *Heterodera glycines* and Its Relation with the Soil Chemical and Biological Properties

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Abstract: This study aimed to evaluate the suppressive potential of different soils on soybean cyst nematodes (SCN) and to estimate the suppressive mechanism. Fifteen soils (designated as soil A to O) from different agricultural fields with varying organic inputs were added with SCN-infested soil and grown with a green soybean variety. The SCN density in the soil at 6 weeks of soybean growth was markedly different depending on the soils used, indicating a different level of disease suppressiveness. No significant correlation was observed between the SCN density and any of the soil physicochemical and biological characteristics tested. Then, to estimate a suppression mechanism, F-soil that showed the lowest density of SCN was added to the SCN-infested soil with or without streptomycin to kill bacteria and grown with soybean. SCN density was not increased by the addition of streptomycin, indicating that soil bacteria may not be involved in the suppressiveness of F-soil. In total, 128 fungal strains were isolated from the rhizosphere of F-soil and inoculated in a combination or singly in the SCN-infested soil. After repeated screenings, five strains were selected since the SCN density was consistently decreased by them. Sequence analysis showed that they were closest to *Clonostachys rosea*, *Aspergillus niger*, *Aspergillus fumigatus*, *Fusarium oxysporum*, and *Cylindrodendrum alicantinum*. All five strains significantly reduced the mobility of second-stage juveniles (J2). Further, *C. rosea* a2, *A. niger* a8, and *F. oxysporum* a25 significantly decreased hatching. Overall, the present study demonstrated that soil fungi played an important role in SCN suppression in F-soil.

Keywords: soybean cyst nematode (SCN) suppression; disease-suppressive soils; soil fungi

Citation: Yang, Y.; Wu, J.; Perry, R.N.; Toyota, K. Evaluation of Soil Suppressiveness of Various Japanese Soils against the Soybean Cyst Nematode *Heterodera glycines* and Its Relation with the Soil Chemical and Biological Properties. *Agronomy* **2023**, *13*, 2826. https://doi.org/10.3390/agronomy13112826

Academic Editor: John P. Thompson

Received: 20 October 2023
Revised: 13 November 2023
Accepted: 13 November 2023
Published: 16 November 2023

1. Introduction

The crop losses caused by soil diseases vary by region and crop. Approximately 10% of global grain production is lost annually due to soil diseases [1]. Soil diseases include those caused by fungi, bacteria, and nematodes [2,3]. Soil-dwelling plant-parasitic nematodes cause severe crop damage resulting in yield losses [4].

Soybean is a staple crop widely grown around the world and is used for a variety of products including food and animal feed [5]. However, like any crop, soybean is susceptible to various diseases and pests that reduce its yield and quality [5,6]. Among them, there are the soybean cyst nematode (SCN, *Heterodera glycines*), soybean rust, *Phytophthora* root and stem rot, bean leaf beetle, soybean aphids, cutworms, and white mold [6–9]. SCN is one of the most important diseases of soybean, and by feeding on the roots of soybean plants, it causes significant damage, resulting in yield losses of 30% or more [9].

To control SCN, a combination of cultural, biological, and chemical methods are generally used [9]. There are some soybean varieties that are resistant to this pest [10]; however, no resistant variety is known for green soybean, which is a type of early harvested vegetable soybean. Farmers can rotate with non-host crops such as corn or wheat, which can reduce the number of SCN [11,12]. The use of biological control agents, such as predatory

nematodes, fungi, and bacteria [13,14], seed treatments, and nematicides, especially soil fumigation [15], can also be used to control SCN. However, chemical methods are costly and may have negative environmental impacts [9].

Severe disease outbreaks occur during successive plantings of susceptible crop varieties, but some of the fields naturally start to suppress the disease [16]. In such disease-suppressive soils, beneficial microorganisms and organic matter effectively suppress the growth of pathogens and reduce disease occurrence [17–19]. Suppression of diseases by microorganisms and organic matter is a promising method of controlling pathogens [20]. Maintaining stable and sustainable agriculture requires controlling excessive fertilizer application and minimizing damage from diseases, so efforts are underway to clarify the mechanisms of disease-suppressive soils [21,22].

Well-structured soils, such as friable, well-aerated soils with high levels of organic matter, help to promote the activity of beneficial microbes that can compete with and suppress pathogenic microbes [23]. In some soils, populations of beneficial microbes, such as bacteria and fungi, can outcompete and suppress the growth of pathogenic microbes [24,25]. These beneficial organisms produce antimicrobial compounds or enzymes that break down the cell walls of pathogens, preventing them from causing disease [26]. Therefore, enhancing the suppressive mechanisms of soils against pathogens includes promoting the growth and activity of beneficial microbes in the soil as well as improving the ideal physical and chemical properties of the soil [27,28].

Soil-specific suppressiveness reflects previously encountered pathogens and is associated with antagonistic activity of microbial communities near roots [29]. There are two types of biological suppression: (i) general, in which soil biodiversity might be involved in suppressing pathogens through complex ecological interactions, and (ii) specific or several antagonists acting on a single pathogen [20]. These mechanisms include abiotic soil conditions in which pathogens cannot grow or survive, or ecological interactions that reduce the number or infectivity of pathogens [3,16,30].

Fungi, such as *Purpuricillium lilacinum*, *Pochonia chlamydosporia*, and *Trichoderma* spp., are most commonly used in commercial biological control products, specifically targeting harmful organisms [31,32]. For example, *P. lilacinum* colonizes SCN nematode eggs, females, and cysts, and produces various enzymes and metabolites [27,32]. These substances disrupt the life cycle of SCN and weaken the structural integrity of SCN. *Hirsutella minnesota* and *H. rhosilensis* are endoparasitic fungi that are highly effective against SCN second-stage juveniles (J2) [9,33]. The mechanism by which biological control agents control soil nematodes is largely unclear [20]. A better understanding of the natural mechanisms of regulation for nematode population density is required to facilitate the further development of biological control [26,34].

This study aimed to assess the suppressive potential of different agricultural soils with a different history of organic input in Japan in order to find a suppressive soil and evaluate its suppressive mechanism against the soybean cyst nematode *H. glycines*.

2. Materials and Methods

2.1. Soil Sample Collection

Different soils were collected in March and September 2020 from 15 farmers' fields with various pest management strategies in Tokyo, Kanagawa, Ibaraki, and Chiba prefectures. Soil was collected using a trowel from 8 to 10 different points at a depth of 0 to 15 cm and combined to make a composite sample from each field. The soil was sieved through a 5-mm sieve and stored at room temperature. Chemical analysis was performed using soils that were sieved through a 2-mm screen and air-dried.

SCN-infested soil was collected from a plastic house in Saitama, in which green soybean had been repeatedly cultivated and leaf yellowing was seen as a typical symptom of SCN infection. The soil was sieved through a 2-mm screen to get a homogenous inoculum and stored at room temperature.

2.2. Comparison of a Growth Level of Soybean Cyst Nematodes among 15 Soils

Dry soil (100 g of each soil) was added to a pot with a diameter of 9 cm and a height of 7.6 cm with or without 20% (*w*/*w*) of the SCN-infested soil (initial density of SCN measured by the real-time PCR method as described below: 1250 eggs/20 g soil), and mixed well ($n = 5$). In a preliminary experiment, each soil was mixed with the SCN-infested soil at rates of 5% and 20% and very few cysts were observed in the soils mixed at 5%. Thus, we mixed at a rate of 20% in this experiment. One seed of soybean "Yuagarimusume" was sown in a pot and grown in a Biotron LH-350s (Nippon Medical & Chemical Instruments Co., Ltd., Osaka, Japan) under controlled light conditions with a photoperiod of 12 h of light (light intensity: 1000–1700 lux) and 12 h of darkness at a constant temperature of 25 °C for 6 weeks. Soil was maintained at 60% of maximum water-holding capacity (MWHC) by watering every 1 to 3 days. In some pots, soybeans did not grow and the number of replicates was reduced in such a case. On average, 4 plants grew in each soil. After 6 weeks of cultivation, the SPAD value, an indicator of chlorophyll content in plant leaves, was measured in the upper three leaves of each pot using a SPAD meter (SPAD-502 plus, Konica-Minolta Japan, Inc., Tokyo, Japan). Additionally, the aboveground and belowground parts of the plants were dried separately at 60 °C for 24 h and weighed. After drying, 20 g of the dried soil was ground using a FastPrep-24 (Funakoshi Co., Ltd., Tokyo, Japan), and SCN density was measured with real-time PCR using the method described below. In this experiment, since it was impossible to evaluate 15 soil samples at the same time, the 15 soils were divided into 2 batches, one with 7 soils (Figure 1A) and the other with 8 (Figure 1B). In the third batch (Figure 1C), a total of eight soils that showed a higher suppressive property in the two batches were used to repeat the experiment.

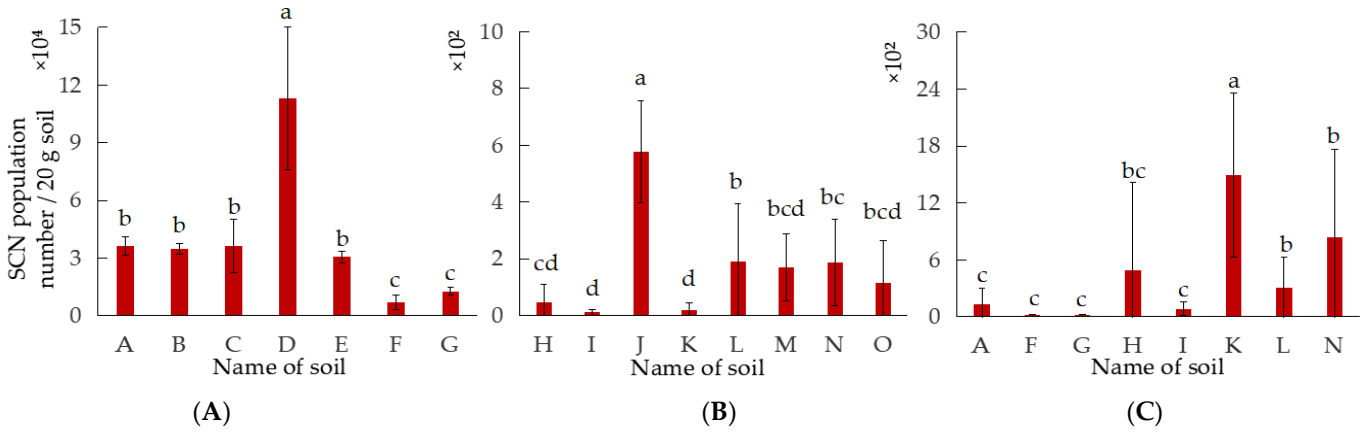

Figure 1. Density of soybean cyst nematode (SCN) in different soils after 6 weeks of soybean growth. Soybean was grown in different soils mixed with 20% SCN-infested soil ($n = 5$). (**A**): Screening of 7 different soils, planted in May 2020; (**B**): Screening of 8 different soils, planted in November 2020; (**C**): Some soils were selected from (**A**,**B**), and planted in August 2021. All treatments were evaluated using a two-way ANOVA followed by Fisher's LSD test at $p \leq 0.05$ significance, with lowercase letters denoting significant differences in each screening. Error bars represent standard deviations.

2.2.1. DNA Extraction from Soils

Soil DNA was extracted in duplicate with the method described by Goto et al. [35]. The soil (0.5 g) was homogenized with a ball mill (FastPrep-24: Funakoshi Co., Ltd., Tokyo, Japan) and was put into a 2 mL tube including 500 µL of 20% skim milk, 0.75 g zirconia beads (0.1 mm diameter) and 0.25 g glass beads (0.5 mm diameter), mixed thoroughly and centrifuged (12,000× *g* for 1 min, 25 °C). Then, 600 µL of lysis buffer (0.5% SDS, 100 mM Tris, 50 mM EDTA, pH 8.0) was added to the tube. Then, the tube was bead-beaten for 1 min at 5000 rpm two times. After centrifugation (12,000× *g* for 5 min, 25 °C), the supernatant (500 µL) was transferred to a new 2 mL tube, and 377 µL of 5 M NaCl and 270 µL of 10% CTAB were added to the tube. After a 10-min incubation at 60 °C with shaking at

3, 5, and 7 min, 500 µL of chloroform was added to the tube after cooling down to room temperature. Then, the tube was centrifuged at 15,000× *g* for 15 min at 25 °C and the supernatant (1.1 mL) was again transferred to a new 2 ml tube. Then, 500 µL of chloroform was added to the tube, followed by centrifugation at 15,000× *g* for 15 min at 25 °C. The supernatant was recovered to a new 2 mL tube, 600 µL of 20% PEG solution was added, mixed thoroughly, and centrifuged at 15,000× *g* for 20 min at 4 °C to precipitate DNA. Next, 500 µL of 70% ethanol was added to the tube with the precipitated DNA pellet and centrifuged at 15,000× *g* for 5 min at 4 °C. After drying the DNA pellet for 20 min using a centrifugal concentrator VC-15Sp (TAITEC Co., Ltd., Koshigaya, Japan), 100 µL of TE buffer was added to the tube to dissolve DNA.

2.2.2. Real-Time PCR

We performed quantitative PCR using a StepOne Real-Time PCR System (Applied Biosystems Japan Ltd., Tokyo, Japan) with a final volume of 10 µL containing 2.0 µL of 10 times diluted template DNA, 0.4 µL of 10 µM each primer specific to *H. glycines* [36] (SCNnew-f (5′-CTGCACATGTGAAAGGTGTA-3′) and SCNnew-r (5′-GAGCGTGCATCCATTG-3′)) and 5.0 µL of Fast SYBR® Green Master Mix (Applied Biosystems Japan Ltd., Tokyo, Japan) under the manufacturer's recommended conditions (95 °C for 10 s, then 45 cycles of 95 °C for 5 s and 60 °C for 20 s, followed by a melt curve analysis (from 60 °C to 95 °C). In this study, a calibration curve was newly prepared for the infested soil by inoculating different numbers (400, 1600, and 6400) of eggs into the 20 g of the infested soil and extracting DNA from the inoculated soils with the method described in Section 2.2. In this experiment, soil was collected from different sites (60) of the plastic house and separately measured for the density of SCN. SCN was absent in a few sites and the infested soil not including SCN was used for making a calibration curve.

2.3. Soil Physicochemical and Biological Properties

Soil analysis was conducted to examine the effect of the soil's chemical and biological characteristics on suppressiveness to SCN. Chemical properties measured in this study were exchangeable K, available P, total C, total N, and pH. Exchangeable K was extracted using a 1 mol/L ammonium acetate solution, and its concentration was measured with a flame photometer (OSK 55XC750-PLUS, BWB Technologies Ltd., Berkshire, UK) [37]. Available P was determined by the Bray II method and measured at 880 nm with a spectrophotometer (UV-1900i, SHIMADZU CORPORATION, Kyoto, Japan) [38]. Carbon and N contents were measured using a CN coder (MT-700, YANACO New Science, Tokyo, Japan) [39]. pH was determined from a 1:2.5 water-soluble extract and measured using a pH meter (744-Metrohm, Metrohm Japan Ltd., Tokyo, Japan).

For the analysis of soil respiration, 10 g of air-dried soil (passed through 5-mm sieved) was rehydrated in a 30 mL glass bottle to achieve 60% MWHC, and pre-incubated under aerobic conditions at 25 °C for one week in a Biotron LH-350s (Nippon Medical & Chemical Instruments Co., Ltd., Osaka, Japan). Constant water content was maintained throughout the incubation period. At the end of the 7-day incubation period, the rewetted soils were utilized to assess CO_2 emissions, an indicator of microbial activity [40]. The measurement of CO_2 emissions was carried out using Gas Chromatography (Shimadzu-8A, SHIMADZU CORPORATION, Kyoto, Japan) equipped with a TCD detector. Carbon dioxide emission was measured at the start and after 3 h.

2.4. Effect of the Addition of Streptomycin on the Growth of Soybean Cyst Nematode in F-Soil That Showed the Highest Suppression

In a preliminary trial, we enumerated the number of streptomycin-resistant bacteria in F-soil, in which the SCN density was the lowest in the experiments in Figure 1A,C. Serial dilutions were spread onto two types of a 1/10 dilution of nutrient agar (1/10 NA) with or without streptomycin at a final concentration of 100 mg/L. The number of bacteria was 30 ± 2 and 0 (n = 3) in 1/10 NA with and without streptomycin, respectively, indicating

that more than 99% of bacteria were streptomycin-sensitive and only less than 1% were streptomycin-resistant bacteria. This result suggests that streptomycin will kill a majority of bacteria in F-soil. Subsequently, we prepared 10 pots with a diameter of 9 cm and a height of 7.6 cm and separated into two treatments (n = 5). In treatment (i), the untreated control, 20% SCN-infested soil (the initial density of SCN: 1250 eggs/20 g soil) was added to 100 g of dry soil (F-soil), and in (ii) streptomycin treatment: a streptomycin solution (3 mg/mL) was added at a final concentration of 0.51 mg/g of soil. Then, one seed of soybean "Yuagarimusume" was sown in a pot and cultivated for 6 weeks in the Biotron LH-350s. Soil was maintained at 60% of MWHC by watering every 1 to 3 days. After 6 weeks of cultivation, SPAD values were measured in the upper three leaves of each pot. The aboveground and belowground parts of the plants were separately dried at 60 °C for 24 h and weighed. Subsequently, the SCN density was measured with the method described in Section 2.2.1. This experiment was repeated two times using the same experimental design as soybeans did not grow in some pots. The actual number of replicates in the two experiments ranged from 7 to 10.

2.5. Isolation and Screening of Fungi from F-Soil

2.5.1. Isolation

In order to search for antagonistic fungi, a growth experiment was conducted using F-soil, in which the SCN density was the lowest in the experiments in Figure 1A,C. After allowing green soybeans to grow for 6 weeks in 5 pots as previously mentioned, each plant was gently removed from a pot, and the rhizosphere soil was collected by shaking, thoroughly mixed, and used for the dilution plate method. From each pot, two soil samples (1 g each) were collected and suspended in 9 mL of sterile saline solution (0.85% NaCl). After shaking for 30 s on a vortex machine, 1 mL was taken and serially diluted up to 10^{-5} dilution. Aliquots (0.1 mL) were spread onto three Petri dishes including Rose Bengal agar medium [41]. A total of 30 Petri dishes were used for each dilution. After 3 days of incubation, fungal colonies with different types of morphology were selected, purified, and stored on potato dextrose agar medium (PDA, Eiken Chemical Co., Ltd., Tokyo, Japan). A total of 128 fungal strains were obtained from the rhizosphere of F-soil.

2.5.2. Screening

Wheat bran (5 g) was added with 4 mL of tap water, mixed, and then autoclaved at 121 °C for 15 min. A small piece of fungal colony formed on PDA was inoculated in the wheat bran and incubated at 25 °C. After 1 week, 0.2 g of wheat bran inoculated with each fungal strain was thoroughly mixed with 100 g (dry basis) of the SCN-infested soil. Green soybeans were grown in the Biotron with one seed per pot (n = 5). Soil was maintained at 60% of MWHC by watering every 1 to 3 days. After 6 weeks of growth, the SCN density was measured with the method described in Section 2.2.1. A total of 128 fungal strains isolated from F-soil were screened in a total of 11 experiments. In the first 5 experiments, 3 to 5 fungal strains were combined and mixed with 100 g of dry soil infected with SCN. In the next six experiments, fungal strains were individually inoculated with the SCN-infested soil.

2.5.3. Phylogeny

DNA Extraction from Fungi

Each fungal strain was cultured for 3–4 days in 10 mL of potato dextrose broth medium (PDB, Becton Dickinson, and Company, Claix, France) put in a 50 mL Erlenmeyer flask, and the culture solution was transferred in a 1.5 mL microtube. The microtubes were centrifuged at 14,000× g for 3 min, and the supernatant was discarded. After the above steps were repeated 2–3 times to collect enough fungal biomass, 1 mL of sterilized distilled water was added. The mixture was then centrifuged at 12,000× g for 3 min to remove the culture components.

The mycelium mat at the bottom of the tube was transferred to a 2.0-mL tube containing 0.2 g zirconia beads (diameter 0.1 mm, autoclaved at 121 °C, 15 min), 200 µL of sterile distilled water and 20 µL of 10 × TE buffer were added and bead beaten at 5500 rpm for 1.5 min 2 times. Then, 50 µL of 20% skim milk solution, 200 µL of extraction buffer (250 mM NaCl, 0.5% SDS, 0.2 M Tris, 25 mM EDTA, pH 8.0), and 30 µL of 3 M sodium acetate (pH 5.2) were added and vortexed. Then, 500 µL of chloroform was added and centrifuged at 15,000× *g* at 4 °C for 15 min. The supernatant (400 µL) was transferred to a new 1.5 mL tube, and 40 µL of 5 M ammonium acetate and 300 µL of isopropanol were added. The mixture was vortexed, incubated for 5 min at room temperature, and then centrifuged at 15,000× *g* at 4 °C for 15 min. After the supernatant was discarded and 500 µL of sterile distilled water and 300 µL of 20% PEG were added and vortexed again, the mixture was incubated on ice for 15 min, and centrifuged at 15,000× *g* at 4 °C for 15 min to collect DNA particles. The DNA pellet was washed with 70% ethanol and centrifuged at 15,000× *g* at 4 °C for 2 min. It was then dried with VC-15Sp (TAITEC Co., Ltd., Koshigaya, Japan) for 20 min. The DNA was suspended in 100 µL of TE buffer. After 10 times dilution, the resulting DNA was used as a template for PCR.

Sequencing

PCR targeting the 18S rDNA was performed in a TaKaRa PCR Thermal Cycler Dice® Touch (Applied Takara Bio Inc., Shiga city, Japan) with a final volume of 20 µL containing 1 µL of 10 times diluted template DNA, 4 µL of the fungal primer combination (NS3/NS4), 10 µL of 2 × GoTaq® PCR Master Mix (Promega Japan, Tokyo, Japan), and 5 µL sterile deionized water under the manufacturer's recommended conditions (94 °C for 2 min, followed by 30 cycles of 94 °C for 30 s, 50 °C for 45 s, and 72 °C for 3 min). The primer set used was NS3 (5′-GCAAGTCTGGTGCCAGCAGC-3′) and NS4 (5′-CTTCCGTCAATTCCTTTAAG-3′) [42].

PCR products were sent to Fasmac Co., Ltd. (Atsugi, Japan) for sequencing. Closely related species were searched by NCBI-Blast.

2.6. Effect of Fungal Strains on Soybean Cyst Nematode J2 and Hatching

2.6.1. Effects of Fungal Strains on J2

A piece of fungal colony (5–10 mm^2) from PDA was inoculated into 10 mL of a 10 times-diluted PDB (1/10 PDB) medium in a 50 mL Erlenmeyer flask and cultured for 1 week. After centrifuging 2 mL of the culture medium at 15,000× *g* for 2 min, 100 µL of the supernatant was taken and placed in each well of a 96-well plate (n = 3), together with a suspension of 50 J2, prepared as described below (n = 3). After 1, 2, and 3 days, the number of J2 that were immobile was recorded. If there was no movement for 10 s, the J2 were considered immobile.

Cysts of SCN were obtained with the sieving method from the infested soil and then pre-incubated in tap water for 1 week at 25 °C. Then, 100 µL of tap water and 100 µL mung bean leachate, as described below, were added to each well of a 96-well plate, and 1–2 pre-incubated cysts were placed in each well. The plate was incubated at 25 °C for 3 days, and hatched J2 were recovered using a pipette. The mung bean leachate was prepared by growing mung bean (*Vigna radiata* 'Green mappe'; Nakahara Seed Co., Fukuoka, Japan) in the infested soil for 2 weeks, adding enough distilled water to make the soil saturated, collecting the drainage water after 24 h and filtering through a 0.2 µm membrane filter.

2.6.2. Effects on Soybean Cyst Nematode Hatching

Cysts were immersed in a 0.5% sodium hypochlorite solution for 3 min, rinsed with sterile deionized water to obtain surface-sterilized cysts, and broken open with tweezers to release eggs. Fungal strains were inoculated into 1/10 PDB medium, cultured for a week, and then the tip of the growing colony (5–10 mm^2) was scraped off with a sterile tip. A 500 µL mixture of the collected eggs and each strain was placed in a 24-well plate (n = 3) and 500 µL of 8 mM $ZnCl_2$ solution, as a hatching stimulus [43], was added. The

number of J2 was counted after 2, 4, 7, and 14 days of incubation. At each counting time, the solution in a well was carefully removed with hatched J2, and 500 µL of fresh 4 mM $ZnCl_2$ solution was added to each well. Based on the cumulative number of hatched J2 and the number of eggs that were initially put in a well, the hatching percentage was calculated. This experiment was repeated in time and combined data were statistically analyzed.

2.7. Statistical Analysis

The effects of all treatments and nematode numbers were analyzed using either a one-way or a two-way ANOVA, followed by Tukey's HSD and Fisher's LSD tests for mean comparison ($p \leq 0.05$) to identify significant differences among mean values using the Excel Statistics 2002 software (Social Survey Research Information, Tokyo, Japan).

3. Results

3.1. Evaluate the Suppression Potential of Different Soils on Soybean Cyst Nematodes

Some chemical and biological properties of the initial soils are shown in Table 1. The growth status of soybean after 6 weeks are also shown in Table 1. Then, the density of SCN in the soils after 6 weeks of soybean growth was evaluated.

Table 1. Comparison of initial soil properties and soybean growth status among different soils used in Figure 1. Soil properties were analyzed using soils before soybean cultivation. Soybean was grown in two independent experiments. After 6 weeks, the growth status of soybeans was observed two times (one with 7 different soils (Figure 1A) and the other with 8 different soils (Figure 1B)).

Soil Samples	Exchangeable K (g/kg)	Available PO_4-P (mg/kg)	Total C (g/kg)	Total N (g/kg)	C/N	pH (H_2O)	Basal Soil Respiration (mg/kg/h)	Aboveground Dry Weight (g)	Belowground Dry Weight (g)	SPAD Value
Figure 1A										
A	0.25	18.0	104	14.3	7.30	5.3	2.60	1.45	0.46	51.4
B	0.26	93.0	95.0	6.40	14.8	5.2	1.50	1.03	0.57	47.8
C	0.52	121	105	7.96	13.2	6.3	4.30	0.85	0.41	46.9
D	0.35	47.2	104	7.53	13.8	5.5	1.90	1.16	0.72	47.1
E	0.27	224	85.3	7.69	11.1	4.9	3.00	1.17	0.43	44.1
F	2.09	233	38.0	4.46	8.50	7.4	1.90	0.48	0.17	47.8
G	1.21	236	63.6	6.04	10.5	7.4	2.70	1.00	0.38	47.3
Figure 1B										
H	0.27	70.8	57.1	4.95	11.5	7.1	1.70	1.00	0.16	45.0
I	0.20	52.8	34.6	3.74	9.30	7.3	1.21	0.84	0.18	41.6
J	0.09	90.2	33.7	2.92	11.5	6.4	1.60	1.13	0.15	45.9
K	0.24	17.8	55.5	5.02	11.1	6.4	0.98	0.87	0.09	43.1
L	0.11	182	41.5	3.56	11.7	6.1	1.18	1.00	0.15	40.8
M	0.21	48.6	38.8	4.63	8.40	5.8	1.79	1.25	0.24	44.4
N	0.09	55.8	30.9	3.62	8.60	6.1	0.86	0.86	0.13	41.2
O	0.02	15.5	69.4	6.66	10.4	4.7	0.55	1.52	0.31	45.7

There was a highly significant correlation between the Ct values and the number of eggs inoculated into the soil: Ct value = $-3.241 \times \log_{10}$(No. of eggs/20 g soil) + 35.395. Using this equation, we estimated SCN density in different soils, although DNA extraction efficiency is more or less different depending on the kind of soil [44]. Among seven soils, the density of SCN was significantly ($p \leq 0.05$) lower in F and G soils than in the others, indicating their ability to suppress SCN (Figure 1A). There existed a significant positive correlation between aboveground weight, belowground weight, and SCN density ($p \leq 0.01$) (Table 2), indicating that greater aboveground weight and belowground weight were associated with higher SCN density. However, no significant correlation was found between SPAD values and SCN density (Table 2). In the next screening using the other eight soils, the density of SCN was lower in soils H, I, and K (Figure 1B). There was no significant correlation between SPAD values, aboveground weight, belowground weight, and SCN density (Table 2). In the third experiment using some soils from two previous screening experiments, suppressiveness was again observed in A, F, G, and I soils, as

the SCN densities in the soils were significantly lower than those in the others ($p \leq 0.05$) (Figure 1C).

Table 2. Correlation (p-value) of soil chemical and biological properties and soybean growth status with the density of soybean cyst nematodes (SCN) in Figure 1. A single regression analysis was conducted separately to investigate the relationship between soil properties and SCN density for 7 different soils (Figure 1A) and 8 different soils (Figure 1B). The symbol "**" indicated a statistically significant suppressive effect at $p \leq 0.01$.

Soil Samples	Exchangeable K (g/kg)	Available PO_4-P (mg/kg)	Total C (g/kg)	Total N (g/kg)	C/N	pH (H_2O)	Basal Soil Respiration (mg/kg/h)	Aboveground Dry Weight (g)	Belowground Dry Weight (g)	SPAD Value
Figure 1A	0.30	0.17	0.19	0.83	0.24	0.35	0.66	**	**	0.86
Figure 1B	0.23	0.41	0.29	0.17	0.58	0.75	0.48	0.59	0.88	0.37

3.2. Effect of Soil Physicochemical and Biological Properties on the Growth of Soybean Cyst Nematodes

Based on the chemical and biological properties, regression analysis was performed to reveal the relationship with the suppressive capacity. There were no correlations in the parameters (Table 2).

3.3. Effect of the Addition of Streptomycin on the Growth of Soybean Cyst Nematodes

Streptomycin treatment showed a significantly lower density of SCN (0.39×10^4 eggs/20 g soil) compared to the untreated control (1.18×10^4 eggs/20 g soil) ($p \leq 0.05$).

3.4. Evaluate the Suppression Potential of Different Fungal Strains on Soybean Cyst Nematodes

A total of 128 fungal strains were obtained from F soil, in which the SCN density was the lowest in the experiments in Figure 1A,C. The results of various combinations using fungal strains in five screening experiments indicated that in Trial 5, the SCN density was significantly suppressed by each fungal combination ($p \leq 0.05$) (Figure 2). In Trial 1 and Trial 3, some fungal combinations showed lower densities of SCN compared to those in the control, although there were no significant differences.

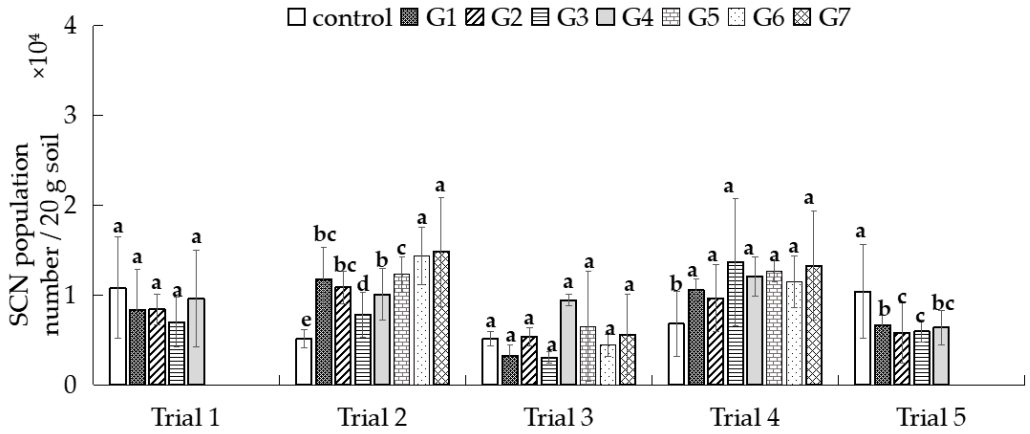

Figure 2. Effect of combinations of fungal strains on soybean cyst nematode (SCN) density. A total of 128 fungal strains from F-soil, in which the SCN density was the lowest in the experiments in Figure 1A,C, were individually cultured in autoclaved wheat bran for 1 week. In each trial, a combination of 3–5 fungal strains (group 1 (G1) to group 7 (G7)) was mixed with 100 g of dry soil added with SCN-infested soil, and in the control, only autoclaved wheat bran was added ($n = 5$). Soybeans were then grown for 6 weeks. The experiment was conducted in five trials, testing different

fungal combinations: Trial 1 (August 2021)—13 strains in 4 combinations (G1 to G4), Trial 2 (September 2021)—32 isolated in 7 combinations (G1 to G7), Trial 3 (December 2021)—28 strains in 7 combinations (G1 to G7), Trial 4 (January 2022)—35 strains in 7 combinations (G1 to G7), Trial 5 (March 2022)—20 strains in 4 combinations (G1 to G4). All treatments were evaluated using a two-way ANOVA followed by Fisher's LSD test at $p \leq 0.05$ significance, with lowercase letters denoting significant differences. Error bars represent standard deviations.

Subsequently, we tested the efficacy of individual fungal strains consisting of combinations used in Trials 1, 3, and 5, in which SCN density was lower. Among 35 fungal strains tested in Trials 6 to 10, 14 strains exhibited suppressive effects on the population increase of SCN ($p \leq 0.05$) (Table 3). Sequence analysis showed that the 14 fungal strains were categorized into 5 species: a2 was closest to *Clonostachys rosea* (similarity = 100%), a8 was *Aspergillus niger* (100%), a18 was *Aspergillus fumigatus* (100%), a25 was *Fusarium oxysporum* (100%) and f0 was *Cylindrodendrum alicantinum* (100%). In the repeat experiment (Trial 11 in Table 3), we once again confirmed the suppressive capacity of the five strains against SCN, as their densities were significantly lower compared to those in the control ($p \leq 0.05$).

Table 3. Suppression percentage (%) of soybean cyst nematode (SCN) density by individual fungal strains and their phylogeny. The SCN density at 6 weeks of soybean growth was expressed by percentage; 0% was the density in the control (without fungal inoculation) and the percentage values indicate the decrease in SCN density by fungal strains. Vertical lines " | " separate different fungal species. The symbol "-" indicates that the strains were not used in the experiment. The symbol "*" indicates a statistically significant suppressive effect at $p \leq 0.05$.

Closest Species	***Cylindrodendrum alicantinum***		***Clonostachys rosea***		***A spergillus fumigatus***		***F usarium oxysporum***			***A spergillus niger***				
	f0	**h**	**a2**	**a27**	**a17**	**a18**	**a20**	**a23**	**a25**	**a8**	**a10**	**a21**	**a22**	**a26**
Trial 6	30 *	34 *	-	-	-	-	-	-	-	-	-	-	-	-
Trial 7	71 *	52 *	-	-	-	-	-	-	-	-	-	-	-	-
Trial 8	-	-	31 *	-	-	-	-	-	-	50 *	-	-	-	-
Trial 9	-	-	-	24 *	25 *	49 *	39 *	49 *	31 *	-	21 *	34 *	16 *	31 *
Trial 10	-	-	65 *	64 *	8	36 *	15 *	34 *	79 *	60 *	7	22 *	58 *	72 *
Trial 11	16 *	-	36 *	-	74 *	56 *	-	-	18 *	-	-	-	-	-

3.5. The Effect of Suppressive Fungi on Second-Stage Juveniles (J2)

Compared with the control, the percentage immobility of J2 was significantly higher for each fungal strain ($p \leq 0.05$), with a nearly 40% increase for a8, a25, and f0 strains, and an approximately 15% increase for a2 and a18 strains (Figure 3).

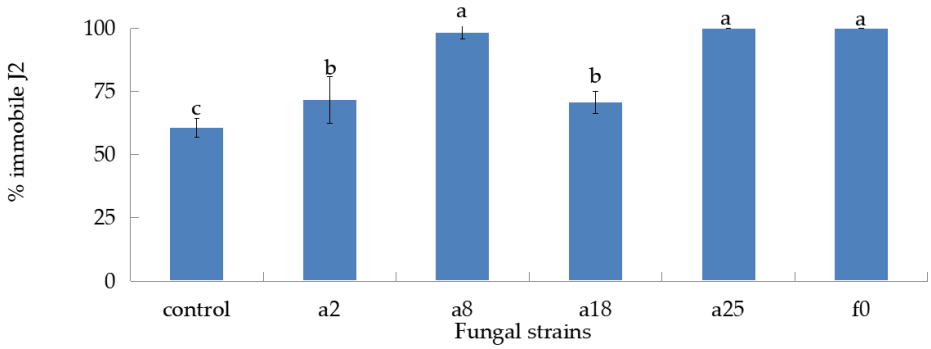

Figure 3. Effect of individual fungal strains on the immobility of second-stage juveniles (J2) at 3 days of incubation. Approximately 50 hatched J2 were placed in each well containing fungal culture filtrate ($n = 3$). All treatments were evaluated using a one-way ANOVA followed by Fisher's LSD test at $p \leq 0.05$ significance, with lowercase letters denoting significant differences. Error bars represent standard deviations.

3.6. The Effect of Suppressive Fungi on Hatching of Soybean Cyst Nematode

Compared with the control, hatching of J2 from eggs of SCN was significantly lower in fungal strains a2, a8, and a25 than in the control ($p \leq 0.05$), representing a reduction of 50%, 57%, and 32%, respectively, while the fungal strains a18 and f0 did not affect hatching (Figure 4).

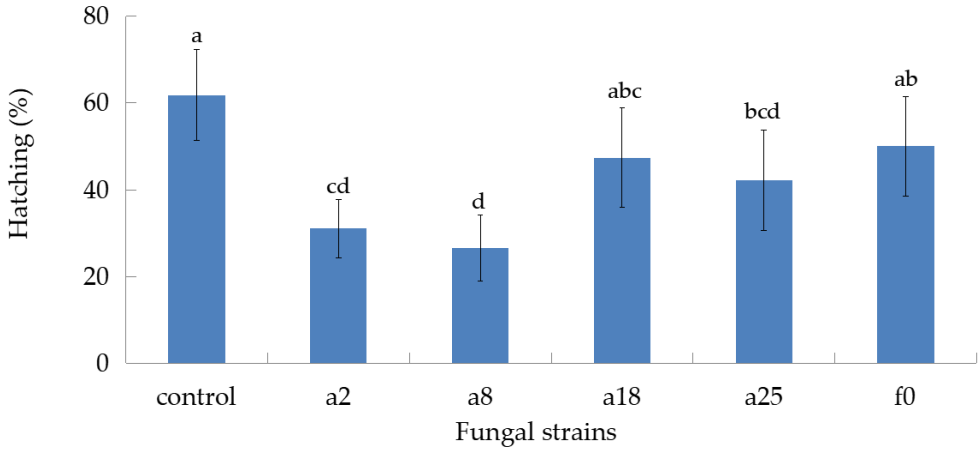

Figure 4. Effect of individual fungal strains on soybean cyst nematode hatching. Approximately 50 surface-sterilized eggs were placed in each well containing fungal culture (n = 3). The hatched J2 was recorded at 2, 4, 7, and 14 days. Every time, the number of hatched J2 was counted and removed from the well. Based on the cumulative number of hatched J2 and the number of eggs that were initially put in a well, the hatching percentage was calculated. All treatments were evaluated using a two-way ANOVA followed by Fisher's LSD test at $p \leq 0.05$ significance, with lowercase letters denoting significant differences. This figure represents the average values of two independent experiments ($n = 3 \times 2$). Error bars represent standard errors.

4. Discussion

This study aimed to assess the suppressive effects of different soils on SCN and uncover potential suppression mechanisms. Among the 15 soils evaluated, 4 soils exhibited potential for disease suppression, shedding light on the soil's ability to suppress the population increase of SCN. To estimate suppression mechanisms, we measured some chemical and biological properties in the soils. There was no consistent correlation in the two trials between the soil characteristics and SCN density (Table 2). Consequently, we considered additional factors contributing to the suppression of SCN density.

Previous studies emphasized the significance of soil microorganisms, specifically bacteria and fungi, in regulating SCN density [25]. To evaluate the possible contribution of bacteria and fungi, F-soil was chosen due to its consistent suppressive effects and added with streptomycin. Streptomycin, an antibacterial agent, can suppress bacterial activity in the soil, but fungi may not be affected [45]. The results revealed that the streptomycin treatment showed a significantly lower density of SCN, implying no involvement of bacteria and a potential involvement of fungi in SCN regulation. This finding is supported by previous studies, which suggested a limited role of bacterial activity in suppressing SCN growth [6,19]. Next, we investigated the potential contribution of fungi to the SCN suppression mechanism since earlier studies have implicated fungi in suppressing SCN growth through various mechanisms, such as the production of suppressive metabolites and resource competition [41,46].

We isolated fungal strains obtained from F soil and inoculated them into infested soil to evaluate their suppressive potential to SCN. The results demonstrated a significant reduction in SCN density by some strains (Table 3). Previous studies have confirmed the suppressive abilities of certain fungi, such as *Purpureocillium lilacinum*, *Pochonia chlamydosporia*, and *Trichoderma* spp. against SCN [31,32]. However, these fungi were not isolated in this study and instead, fungal strains belonging to *Clonostachys rosea*, *Aspergillus*

niger, Aspergillus fumigatus, and *Cylindrodendrum alicantum* were isolated as agents with a disease-suppressive property. Although not reported as biological control agents with direct evidence for SCN suppression, *C. alicantum* is an endophytic fungus found in pine trees [47], and these species including *C. rosea* [48], *A. niger* [49], and *A. fumigatus* [50] were reported to suppress root-knot nematodes (*Meloidogyne* spp.).

Among the fungal strains isolated, some studies exhibited intriguing attributes and mechanisms that could be involved in their disease-suppressive effects. For instance, *C. rosea* antagonizes *Rhizoctonia solani* [51], *Verticillium* wilt [52], and *Meloidogyne* spp. [53]. It intervenes directly in the life cycle of root-knot nematodes through penetration, parasitism, and nutrient uptake, resulting in reduced proliferation of *M. incognita* eggs and J2 [48,53]. In this study, *C. rosea* a2 significantly reduced the number of mobile SCN J2 (Figure 3) and significantly decreased hatching (Figure 4), suggesting that similar suppressive mechanisms might be observed in SCN. Similarly, *A. niger* demonstrates suppressive effects against *Fusarium* spp. [54], *Botrytis cinerea* [55], and plant-parasitic nematodes like *Meloidogyne* spp. [49,56]. Metabolites like oxalic acid and citric acid produced by the fungus contributed to restricting the production of *M. incognita* eggs and J2 [49,57]. In this study, *A. niger* a8 significantly reduced the mobility of J2 and decreased hatching, indicating that the same metabolites could affect SCN. On the other hand, *A. fumigatus*, which is widely distributed in soil, effectively suppresses species of *Alternaria* spp. [58], *Colletotrichum* spp. [59], and plant-parasitic nematodes including *Meloidogyne* spp. [50]. The release of toxic metabolites like gliotoxin and penicillic acid by *A. fumigatus* plays a crucial role in suppressive root-knot nematodes [50,58]. In this study, *A. fumigatus* a8 reduced the mobility of J2, indicating a potential role of the toxic metabolites in the suppressive effect on J2 mobility. *Cylindrodendrum alicantum* was reported as an endophytic fungus in pine trees and to exhibit suppressive effects against various plant pathogenic fungi and nematodes, including *Meloidogyne* spp. [47]. While its exact mechanisms remain unclear, they likely involve antibiotic production, competitive resource utilization, and modulation of plant immune responses [48]. In this study, treatment of SCN with *C. alicantum* f0 resulted in 100% immobility of J2. These findings emphasized the role of fungi in suppressing SCN and provided important clues for investigating their potential in controlling SCN. This also implies that these fungi may exert their suppressive effects by suppressing key life cycle stages, including hatch and activity of J2 [48,53].

The isolated *Fusarium oxysporum* a25 exhibited a suppressive effect on SCN population by 46% (Table 3), a high immobility rate of J2 (100%) (Figure 3), and a high hatch suppression rate (58%) (Figure 4), agreeing with previous studies indicating its suppressive effects on SCN [60–62]. *Fusarium oxysporum* itself could produce bioactive substances like enniatins [63], beauvericins [64], and fumonisins [65], which might have suppressed the molting process of SCN, thereby affecting its normal growth and development. Furthermore, *F. oxysporum* could potentially have generated signaling molecules such as plant hormones [61], jasmonic acid [66], salicylic acid [67], and others that would activate plant immune responses [67]. These compounds could have played a role in the plant-nematode interaction, affecting juvenile-to-adult transition and the developmental stages [68,69] of male and female nematodes, and could have weakened the reproductive and survival capabilities of SCN [60–62].

Considering that the suppressive efficacy and specificity might have varied depending on specific fungal strains, host plants, and environmental conditions, further research is necessary to elucidate the molecular-level details of nematode development and interactions with antagonistic microbes. This would provide insights into the mechanisms underlying the specific suppressive roles of these fungal strains against SCN.

5. Conclusions

This study highlights the significant role of soil fungi in suppressing soybean cyst nematode (SCN). The findings suggest that soil fungi play a crucial role in SCN suppression. These findings provide valuable insights into the functional significance and potential

applications of soil fungi in promoting sustainable agriculture. *Clonostachys rosea*, *Aspergillus niger*, *Aspergillus fumigatus*, and *Cylindrodendrum alicantinum* were found to suppress SCN first, although the exact suppressive mechanisms are not yet fully determined. Future research should focus on unraveling the mechanisms of action of these fungal strains and exploring their practical effectiveness in field conditions.

Author Contributions: Conceptualization, K.T. and Y.Y.; investigation, Y.Y. and J.W.; writing-original draft preparation, Y.Y.; writing-review and editing, K.T. and R.N.P. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Data Availability Statement: All available data is presented in the publication.

Acknowledgments: We would like to express sincere gratitude to the farmers who generously allowed the collection of samples for the research.

Conflicts of Interest: The authors declare no conflict of interest.

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