

EFFECT OF CULTURE FILTRATE OF *SINORHIZOBIUM FREDII* SNEB183 ON THE ACTIVITY AND BEHAVIOR OF SOYBEAN CYST NEMATODE (*HETERODERA GLYCINES* ICHINOHE, 1952)

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Abstract. *Rhizobium* species and the soybean cyst nematode (SCN, *Heterodera glycines*) are attracted by the root exudates of soybean and colonize its roots. There is complicated relationship between the soybean, *Rhizobium* species, and SCN. This study intended to discover the effect of *Sinorhizobium fredii* Sneb183 culture filtrate on SCN second-stage larvae (J2), egg hatching and J2 chemotaxis. The mortality of J2 treated with *S. fredii* Sneb183 culture filtrate increased with the exposure duration. Additionally, *S. fredii* Sneb183 culture filtrate reduced egg hatching within cysts, neutralized the positive effect of a susceptible soybean cultivar root exudate on hatching, and postponed the peak of hatching, thereby revealing other mechanisms for reducing nematode infection and J2 numbers. *Heterodera glycines* J2 were repelled by the culture supernatant of *S. fredii* Sneb183, the movement of J2 towards the susceptible cultivar root exudate and soybean roots was disrupted by a mixture of susceptible cultivar root exudate with *S. fredii* Sneb183 culture filtrate or by dipping the root into culture filtrate. These results indicate that *S. fredii* Sneb183 may be a potential economic, eco-friendly and effective biocontrol microorganism against SCN.

Keywords: *root exudate, biocontrol, chemotaxis, mortality, susceptible*

Introduction

Plant-parasitic nematodes pose a serious threat the food production of many economically important crops. Soybean cyst nematode (SCN) (*Heterodera glycines* Ichinohe) was first reported as a pest of soybean in 1899. Currently it is one of the most damaging pathogen for soybean, causing more than 30% yield losses annually in the United States (Allen et al., 2017). The combination of the use of SCN-resistant cultivars and non-host crop rotation are important means of managing the pest population levels in the field. Nevertheless, an overdependence on resistant cultivars coupled with genetic variation in SCN field populations has led to population shifts (Mitchum et al., 2007; Niblack et al., 2008). Meanwhile, public concern over environmental hazards evoked by chemical pesticides has steadily increased (Pimentel and Burgess, 2014). Therefore, alternative control strategies are highly desirable for the control of SCN. Biological control of plant diseases is usually an eco-friendly and potential component of integrated disease management; biological control of SCN has been studied for more than 40 years (Davies and Spiegel, 2011). Zhou et al. (2017) Demonstrated that combination of multiple rhizobacterial strains applied through seed coating may be a better management method for SCN.

Rhizobia are the best known beneficial plant-associated bacteria because they can fix nitrogen, which occurs during the *Rhizobium*-legume symbiosis (Zahran, 1999; Long, 2001). Several strains of *Rhizobium* spp. inhibit the growth of several root-infecting fungi to various degrees. For example, reports determined that *Rhizobium* could inhibit spore germination, and hyphae growth of some plant pathogens, including *Phoma medicaginis*, *Macrophomina phaseolina* and *Rhizoctonia solani* (Omar and Abd-Alla, 1998; Dileep Kumar et al., 2001). Drapeau et al. (1973) isolated *Rhizobium* strain TL-3 with obviously suppressive effects on *Pyrenochaeta terrestris*, *Colletotrichum destructivum* and *Coniothyrium* sp. Antagonism toward the potato cyst nematode *Globodera pallida* and the root-knot nematode *Meloidogyne incognita* by *Rhizobium etli* G12 is associated with its ability to induce systemic resistance (Hallmann et al., 2001; Martinuz et al., 2013). Numerous observations confirm that rhizobia may function as potential inducers of plant disease resistance.

Sinorhizobium fredii Sneb183, isolated from soybean nodules, could induce systemic resistance in soybean against SCN; the number of juveniles and cysts significantly decreased as a result of Sneb183 inoculation (Tian et al., 2014). The oxygen consumption rate of J2 was decreased by 93.1% after 24 h treated with Sneb183 culture filtrate, while after 48 h treatment, J2 body fluids exuded and the conductivity was increased by 62.4% (Tian et al., 2014). The present study is conducted to evaluate the effect of *S. fredii* Sneb183 fermentation filtrate on the activity of J2, the hatching rate of eggs within cysts, and J2 chemotaxis, with the goal being to expand the potential of this strain as a new agent for biological control of soybean cyst nematode.

Materials and methods

Experimental Design

Current study has been conducted to evaluate efficacy of *S. fredii* Sneb183 against *H. glycine* at Nematology Institute of Northern China, Shenyang Agricultural University, Liaoning, China during 2018.

Isolation of cysts and J2 of SCN

H. glycine was first reported as pest of soybean in 1899. Surface soil samples (2 cm depth) were removed from the rhizosphere of soybean (*Glycine max* cv Liaodou 15, a SCN-susceptible cultivar). *H. glycine* was first reported as pest of soybean in 1899. Cysts were obtained from experimental field of Nematology Institute of Northern China, and collected by following elutriation and hand-picking under a stereomicroscope. Cysts were surface-sterilized by immersion in 0.1% HgCl₂ solution for 1 minute followed by rinsing three times with sterile distilled water. The cysts were then placed in Baermann funnel at 25°C. J2 were collected from the bottom of the Baermann funnel every two or three days (Liu, 1995).

Preparation of soybean roots and root exudates

Soybean seeds ('Liaodou 15') were rinsed with distilled water, sterilized in 75% ethanol for 1 min, and rinsed several times with sterilized water. After the seeds were swollen for 4 h in Petri dish covered with sterile moist filter paper, then they were placed in sterile petri dishes filled with sand sterilized (160°C for 1 h). After one week, apical

1 cm segments of roots were rinsed with sterilized water and stored in 4°C for subsequent use.

The sterilized sand and soil (V:V=2:1) in pots 25 cm in diameter was seeded with one of the surface-sterilized soybean seeds, and plants were watered with N-free Hoagland's solution on alternate days, in which Ca(NO₃)₂ and KNO₃ were replaced with CaCl₂, K₂HPO₄ and KH₂PO₄, as recommended (Hoagland and Arnon, 1950). The soybeans were grown in a greenhouse at 28 ± 2°C with a 16/8 h light/dark photoperiod. Soybean root exudates were obtained via a slightly modified procedure of Tefft and Bone (1985) and Levene et al. (1998) such as five soybean seedlings were used for root exudate. The soybean seedling roots after grown 20 d were rinsed with tap water and distilled water, placed in sterile 50 mL centrifuge tubes containing sterile water of volume sufficient to submerge the roots, incubated in a dark room at room temperature for 24 h, one soybean seedling in each centrifuge tube. Some root exudates was stored at 4°C for subsequent egg hatch bioassays and 50 mL exudate was placed in a rotary evaporator, concentrated to 5 mL under reduced pressure at 50°C, and stored at 4°C for chemo-attraction experiments.

Preparation of Rhizobium Sneb183 fermentation filtrate

Sinorhizobium fredii Sneb183 was originally isolated from pine rhizosphere and identified by Zhao et al. (2009). Strain was stored at -80°C at Nematology Institute of Northern China, Shenyang Agricultural University, Liaoning, China. *S. fredii* Sneb183 was suspended in sterilized water and adjusted to 1.0×10⁸ cfu/ml with a hemocytometer under a microscope, then 1.0 mL of this suspension was added to 50 mL sterilized TY liquid medium (Duelli and Noel, 1997). Cultures were maintained at 28°C and 150 rpm for 168 h, and were then centrifuged at 5590 g for 20 min. The fermentation filtrate through 0.22 µm filter was used for the impact of J2 lethality and chemotaxis assays and egg hatching bioassays (Hasky-Guenther et al., 1998; Reitz et al., 2000).

J2 lethality assay

100 J2 was transferred into a 5 ml glass petri dishes containing 2.0 ml Sneb183 fermentation filtrate (Sneb183F) and sterilized TY liquid medium (TY), respectively. After that dishes were incubated at 25°C and covered by coverslip. The number of dead nematodes was determined at 12 h, 24 h, 48 h, and 72 h. Three independent experiments were done and each experiment has three replicates. J2 treated with sterilized water was used in the control treatment. The J2 were regarded dead when they were remained static once touched with hair-needle (Cayrol et al., 1989). The lethality (L) of J2 was calculated by Eq.1.

$$L = \frac{L_t}{100} \times 100 \quad (\text{Eq.1})$$

where, L_t stands for the number of dead J2 in assay.

Cyst hatching inhibition assay

Plump and brown cysts were crushed and 1000 eggs were picked into glass petri dish under room temperature to test the hatching rate. The experiment included four treatments with nine replicates per treatment: 10 ml soybean root exudate (SRE), 10 ml Sneb183

fermentation filtrate (Sneb183F), a mixture of 5.0 mL soybean root exudate and 5.0 mL Sneb183 fermentation filtrate (SRE+Sneb183F), 10 mL Sterile water (Control). The treatment solution was replaced and numbers of hatched J2 were determined every three days until no additional J2s were observed in the control treatment. The hatching rate (HR) was calculated by (Eq.2).

$$HR = \frac{T.N.H.J2}{1000} \times 100 \quad (\text{Eq.2})$$

where, *T.N.H.J2* stands for the total number of hatched J2.

Chemotaxis assays

Indirect method with filter paper

The effect of *S. fredii* Sneb183 fermentation filtrate on nematode movement and chemotaxis was studied on Pluronic F-127 gel in 60 mm diameter petri dishes (Wang et al., 2009), with the bottom engraved with concentric circles of radii 1.0 and 2.0 cm (Hewlett et al., 1997; Schroeder and MacGuidwin, 2010). Filter paper ($r = 0.5$ cm) was soaked with soybean root exudate (SRE), Sneb183 fermentation filtrate (Sneb183F), an equal proportional mixture of soybean root exudate and Sneb183 fermentation filtrate (SRE+Sneb183F) was placed in the center of the dish. 50 freshly hatched J2 in 1 ml sterile water were uniformly distributed in the radius of 1-2 cm along the circumference of dish. After 24 h at 25°C, the number of J2 distributed in the different sections was counted. Sterile water was used as a control. Three independent experiments were done and each experiment has three replicates. The distribution rate, regarded as an index of chemotaxis, was calculated as follows: $DR_s = DN_s/50$. DR_s represents the distribution rate in each section and DN_s represents the distribution number in each section.

Direct method with in vitro root

A direct method for observing chemotaxis utilized 5 cm young soybean roots at the growing point instead of filter paper. Roots were soaked in Sneb183 fermentation filtrate and sterile water, respectively. The remaining operations are the same as indirect method with filter paper. After 24 h at 25°C, counted the number of J2 distribute in different sections of Petri dish and their distribution rate.

Statistical analysis

Data were analyzed using one-way analysis of variance (ANOVA). Means for each trial in each assay were separated by the Duncan multiple range test.

Results

J2 activity assay

This result strongly indicates that Sneb183 has biocontrol potential against SCN. The fermentation filtrate of *S. fredii* Sneb183 showed high nematicidal activity (*Fig. 1* and *Table 1*). When exposed to Sneb183 fermentation filtrate, J2s of *H. glycines* displayed significant time-dependent mortality. Treatment with Sneb183 fermentation filtrate for 24 h and 72 h, the lethality (L) of J2 reached to 46.66% and 86.66%, respectively. These

results indicated that *S. fredii* Sneb183 can produce some extracellular substances to kill nematodes.

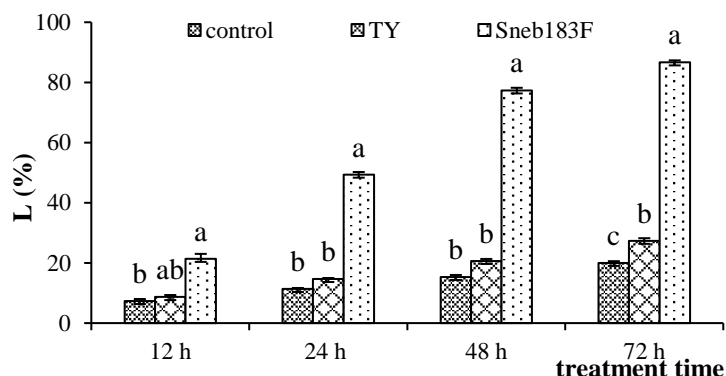


Figure 1. Lethality of *Sinorhizobium fredii* Sneb183 fermentation supernatant to J2 of *Heterodera glycines*. Different letters on bar indicates that values are significantly different according to Duncan multiple range test at $P > 0.05$. Whereas; Control (Sterile water), TY (Fermentation liquid without bacteria), Sneb183F (Sneb183 Fermentation filtrate)

Table 1. Effects of treatments on lethality of second stage juveniles of *H. glycines*

Treatments	Time of exposure			
	12-h	24-h	48-h	72-h
Control	7.33±2.31 ^c	11.33±1.15 ^b	15.33±2.31 ^b	20.0±2.0 ^c
TY	12.0±7.21 ^{ab}	14.67±1.15 ^b	20.67±2.31 ^b	27.33±3.06 ^b
Sneb183	21.33±6.11 ^a	49.33±3.06 ^a	77.33±3.06 ^a	86.67±2.31 ^a
ANOVA Test				
SS	304.889	2656.889	7083.556	8018.667
Df	2	2	2	2
MS	152.444	1328.444	3541.778	4009.333
F	4.831	332.111	531.267	644.357
P	0.056	0.000	0.000	0.000

Data represent the Mean±Standard deviation of lethality of J2 of *H. glycines*. Whereas; SS (Sum of square); Df (Degree of freedom); MS (Mean square); F (F-value); P (Significant value). The different letter within columns are significantly different according to Duncan's multiple range test ($P > 0.05$). Whereas; Control (Sterile water), TY (Fermentation liquid without bacteria), Sneb183F (Sneb183 Fermentation filtrate)

Egg hatching assay

The effects of the four treatments on the hatching of soybean cyst nematode eggs were determined. The results showed that SRE significantly promoted the hatching of soybean cyst nematode eggs compared with the control, the hatching rate reached to 56%. Treatment Sneb183F significantly inhibited the hatching of soybean cyst nematode eggs, the hatching rate was only 0.63%, and the inhibition rate was 98.75%. SRE+Sneb183F also showed inhibition of egg hatching, with an inhibition rate of 39.04% (Fig. 2 and Table 2), the inhibition effect is lower than that of Sneb183F. According to Fig. 3, treated with Sneb183F, J2 were not hatched out until 18d, it was almost the deadline of other treatments. It could be seen that all the treatments containing Sneb183F can significantly reduce the hatching rate of soybean cyst nematode eggs as well as delay the peak period of egg hatching (Fig. 3).

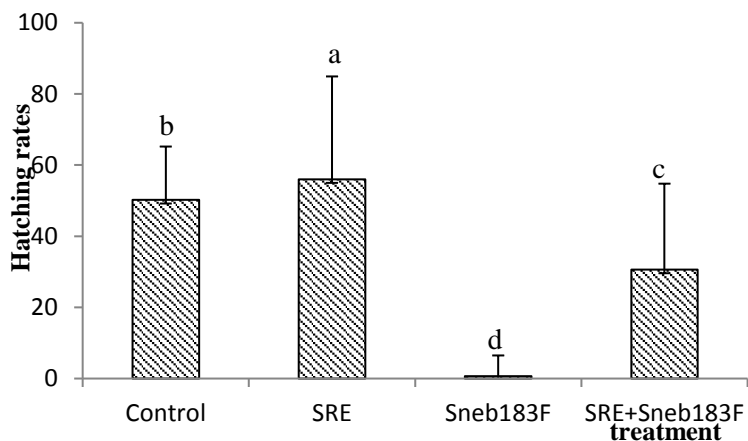


Figure 2. Effect of treatments with 4 treatments on eggs of *Heterodera glycines* hatching rate. Different letters on bar indicates that values are significantly different according to Duncan multiple range test at $P > 0.05$. Whereas; Control (Sterile water), SRE (Soybean root exudate), Sneb183F (Sneb183 Fermentation filtrate), SRE+Sneb183F (Soybean root exudate + Sneb183 fermentation filtrate)

Table 2. Effects of treatments on egg hatching of *H. glycines*

Treatments	Hatching
Control	49.67±1.50 ^b
SRE	55.27±2.89 ^a
Sneb183F	0.5±0.56 ^d
SRE+Sneb183F	30.07±2.42 ^c
ANOVA Test	
SS	5506.123
Df	3
MS	1835.374
F	437.776
P	0.000

Data represent the Mean±Standard deviation egg hatching of *H. glycines*. Whereas; SS (Sum of square); MS (Mean square); Df (Degree of freedom); F (F-value); P (Significant value). The different letter within columns are significantly different according to Duncan's multiple range test ($P > 0.05$). Whereas; Control (Sterile water), SRE (Soybean root exudate), Sneb183F (Sneb183 Fermentation filtrate), SRE+Sneb183F (Soybean root exudate + Sneb183 fermentation filtrate)

Chemotaxis assays

The influence of fermentation filtrate of *S. fredii* Sneb183 on the chemotaxis of J2 was determined by an indirect method utilizing filter paper. The distribution rate of J2 in different region of dish varied in different treatment (Fig. 4 and Table 3). In the control treatment, the nematodes placed within 1-2 cm area originally were evenly distributed in three sections after 24 h. At 0-1 cm, the distribution rates of J2 in the root exudate and in the *S. fredii* fermentation filtrate treatments were the highest and the lowest, respectively. The majority of J2 were attracted to the central area where soybean root exudate-impregnated filter paper was placed. The opposite happened at 2-3 cm, treatment with fermentation filtrate of *S. fredii* Sneb183 or in the equal mixture of fermentation filtrate and root exudate, most J2 were repelled to the region of 2-3 cm. Overall, the effect of J2

attracted by root exudate was remarkably reduced with the addition of fermentation filtrate. Therefore, *S. fredii* Sneb183 fermentation filtrate is repellent to *H. glycines* J2.

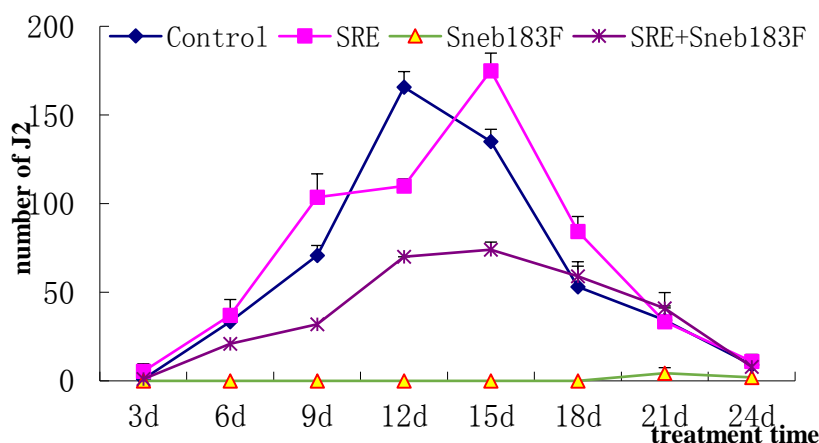


Figure 3. Effects of 4 treatments on the number of *Heterodera glycines* eggs hatching at different treatment times. Whereas; Control (Sterile water), SRE (Soybean root exudate), Sneb183F (Sneb183 Fermentation filtrate), SRE+Sneb183F (Soybean root exudate + Sneb183 fermentation filtrate)

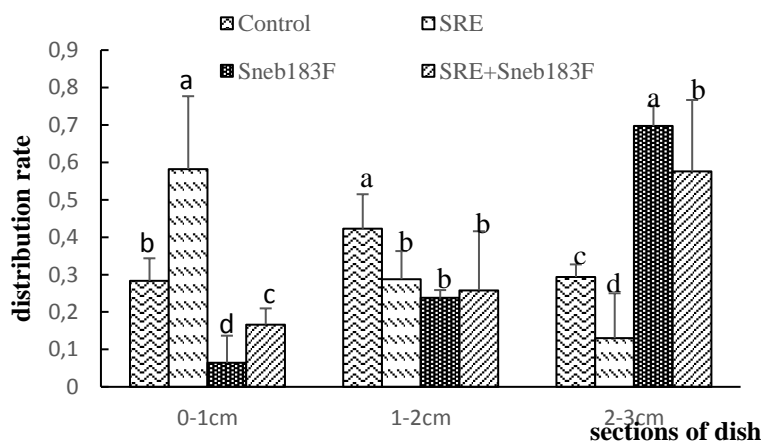


Figure 4. Effect on chemotaxis of *Heterodera glycines* J2 to filter paper treated with four treatments. Different letters on bar indicates that values are significantly different according to Duncan multiple test at $P > 0.05$. Whereas; Control (Sterile water), SRE (Soybean root exudate), Sneb183F (Sneb183 Fermentation filtrate), SRE+Sneb183F (Soybean root exudate + Sneb183 fermentation filtrate)

In the direct method of chemotaxis with roots in vivo, the distribution rates of J2 significantly differed among treatments with Sneb183F and sterile water. At original region (1-2 cm), the distribution rate of J2 in the two treatments is not much different. The young root treated with sterile water strongly attracted J2, the distribution rate of J2 in the sterile water reached 0.6 in the region of 0-1 cm, whereas the treatments with fermentation filtrate was only 0.15, which was significantly different from that in the control treatments (Fig. 5 and Table 4).

Table 3. Effect filter paper chemotaxis assay of second stage juveniles of *H. glycines*

Treatments	Filter paper Chemotaxis assay		
	0-1cm	1-2cm	2-3cm
Control	0.29±0.05 ^b	0.42±0.03 ^b	0.29±0.03 ^c
SRE	0.58±0.05 ^a	0.29±0.01 ^a	0.13±0.05 ^d
Sneb183F	0.06±0.00 ^d	0.24±0.03 ^b	0.70±0.03 ^a
SRE+Sneb183F	0.17±0.03 ^c	0.26±0.07 ^b	0.57±0.10 ^b
ANOVA Test			
SS	0.453	0.059	0.600
Df	3	3	3
MS	0.151	0.020	0.200
F	96.454	10.224	57.152
P	0.000	0.004	0.000

Data represent the Mean±Standard deviation of filter paper chemotaxis assay of *Heterodera glycines* J2. Whereas; SS (Sum of square); Df (Degree of freedom); MS (Mean square); F (F-value); P (Significant value). The different letter within columns are significantly different according to Duncan's multiple range test (P>0.05). Whereas; Control (Sterile water), SRE (Soybean root exudate), Sneb183F (Sneb183 Fermentation filtrate), SRE+Sneb183F (Soybean root exudate + Sneb183 fermentation filtrate)

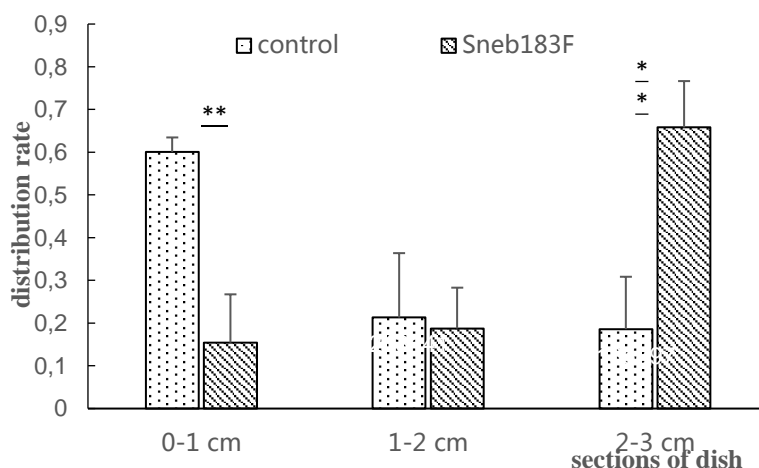


Figure 5. Effect on chemotaxis of *Heterodera glycines* J2 to *Glycine max* root treated with sterile water or *S. fredii* Sneb183 fermentation filtrate. Different letters on bar indicates that values are significantly different according to Waller-Duncan test at P>0.05. Whereas; Control (Sterile water), Sneb183F (Sneb183 Fermentation filtrate)

Table 4. Effect of In-vivo Chemotaxis assay of second stage juveniles of *H. glycines*

Treatments	In-vivo Chemotaxis assay		
	0-1cm	1-2cm	2-3cm
Control	0.60±0.33	0.21±0.15	0.19±0.12
Sneb183F	0.15±0.11	0.19±0.10	0.66±0.10
ANOVA Test			
SS	0.299	0.01	0.334
Df	1	1	1
MS	0.299	0.01	0.334
F	43.037	0.062	24.950
P	0.03	0.816	0.008

Data represent the Mean±Standard deviation of in-vivo chemotaxis assay of second stage juveniles of *H. glycines*. Whereas; SS (Sum of square); Df (Degree of freedom); MS (Mean square); F (F-value); P (Significant value). Whereas; Control (Sterile water), Sneb183F (Sneb183 Fermentation filtrate)

Discussion

The *Rhizobium*-legume symbiosis is a special mutualistic relationship (Hirsch et al., 2001). In which beneficial bacterial symbionts provide the nitrogen source for soybean as a result of rhizobia-soybean coevolution (Coba de la Peña et al., 2018). Using *Rhizobium* as a biocontrol agent against *H. glycines* should not only provide nitrogen for soybean plants but also ease to colonize the rhizosphere of soybean. Some studies on the use of *Rhizobium* strains to control nematodes and other pathogens indicate that rhizobia may become excellent biological control agents for disease control. We obtained *Sinorhizobium fredii* Sneb183 out of more than 300 rhizobial strains isolated from the roots of different plants and rhizosphere soils to study their potential against *H. glycines*. A mixture of *Sinorhizobium fredii* Sneb183 and other strains greatly reduced SCN reproduction and significantly promoted plant growth (Zhou et al., 2017).

In this study, we evaluated *S. fredii* Sneb183 action against *H. glycines* by investigating its suppression of juvenile survival and egg hatching and its disruption of host-finding behavior. The fermentation filtrate from *S. fredii* Sneb183 had obvious nematicidal activity against J2, with lethality directly correlated with the duration of exposure, reaching a maximum of 84% at 72 h. Although numerous in vitro studies have discovered the antibiotic or nematicidal activity of bacterial culture filtrates (Sikora, 1992; Li et al., 2005), reported the production of rhizobial compounds that inhibit the growth of other organisms are few. Chakraborty and Purkayastha (1984) discovered toxic substances in the fermentation liquid of *R. japonicum*, and identified one as rhizobitoxine; fermentation liquid of *R. japonicum* inhibited *Macrophomina phaseolina* at 48 h.

Nematode egg hatching in soil is known to be affected by host root exudate, as well as by some chemicals, enzymes, toxins and metabolic by-products of microorganisms (Schmitt and Riggs, 1991; Hu et al., 2017). Previous studies have shown that egg hatching may be stimulated by exudates from roots of susceptible cultivars (Yang et al., 2016). In our current study, the root exudate of soybean 'Liaodou 15' stimulated egg hatching, but the supernatant from *S. fredii* Sneb183 culture inhibited egg hatching strongly and reduced the stimulatory effect of Liaodou 15 root exudate on egg hatchability, thereby possibly reducing the speed at which nematode populations can establish within a root. Using microorganisms to inhibit egg hatching may be an effective strategy resulting in reduced nematode populations in soils and lowered disease severity in infected plants.

Plant-parasitic nematodes were attracted by root exudates to search, locate, penetrate and establish a feeding site in host roots (Yang et al., 2016). Their movement toward host roots may be disturbed by regulating their behaviour. We found that the attraction of J2 to host roots significantly declined during treatment with supernatant from *S. fredii* Sneb183 culture. Whether an indirect or direct assay was employed, the fermentation filtrate not only strongly repelled J2 but also effectively weakened J2 chemotaxis to root exudate and soybean roots.

Nematodes dwelled in soils containing millions of soil microbes. Inevitably, nematodes exposed to fungi and bacteria interacted with them (Tian et al., 2007), including rhizobia. In general, plant-parasitic nematodes inhibit nodulation and nitrogen fixation of roots of leguminous plants (Hussey and Barker, 1976). Horiuchi et al. (2005) reported that the microbivorous nematode *Caenorhabditis elegans* mediates the interaction between roots and rhizobia positively, leading to nodulation through transfer of the rhizobial species *Sinorhizobium meliloti* to the roots of the legume *Medicago truncatula*. Some plant-pathogenic nematodes like *Meloidogyne* spp. have positive

effects on the symbiosis between rhizobia-legume interactions by enhancing the number of nodules and the amount of nitrogen fixed (Baldwin et al., 1979).

Conclusion

It is concluded that *Sinorhizobium fredii* Sneb183 culture filtrate showed nematicidal potential toward soybean cyst nematode (*Heterodera glycines*). The results of current research reveal a unique interaction between *S. fredii* Sneb183 and *H. glycines*, possibly providing a foundation as a biocontrol agent for new prospects of future utilization of rhizobia. However, further study is needed to determinants of *S. fredii* Sneb183 responsible for the activity of nematicide, mechanism of action and screening of active components.

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Conflict of interests. The authors have no conflict of interests.

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