Detection of *Hirsutella* spp. and *Pasteuria* sp. parasitizing second-stage juveniles of *Heterodera glycines* in soybean fields in China

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Abstract

Soybean cyst nematode, *Heterodera glycines*, is widely distributed in soybean-producing areas, and causes great yield loss in China. The second-stage juvenile (J2) is the infective stage of the nematode; therefore reducing their numbers is important in managing this nematode. In a survey of fungal and bacterial pathogens of J2, 146 rhizosphere soil samples were taken from soybean fields distributed in 11 provinces and Beijing in China during May–August 1999. J2s native in soil plus those that remained after a 2 week incubation of soil with addition of 3000 J2s were extracted and examined for fungal and bacterial infection. *Hirsutella minnesotensis* was detected in 20.5% of the soil samples, *Hirsutella rhossiliensis* in 1.4%, and *Pasteuria* sp. in 26.0%. *Hirsutella* spp. were mainly detected in the relatively low pH soils containing more organic matter from Heilongjiang province (19.2%), whereas *Pasteuria* sp. was found in high pH soils with less organic matter from Anhui (10.2%), Henan (6.8%), Jiangsu (3.4%), and Shandong (2.7%). More *Pasteuria* sp. was detected in soils after addition of baiting nematode; detection methods had no difference in *Hirsutella* spp.

Keywords: *Hirsutella* spp; Nematode pathogens; Parasitism; *Pasteuria* sp; Soybean; Soybean cyst nematode

1. Introduction

Soybean cyst nematode (SCN; *Heterodera glycines* Ichinohe) is a major yield limiting factor in soybean-producing areas in China and other parts of the world (Liu et al., 1997). The nematode directly affects yield by removing plant nutrients as it feeds and indirectly by creating wound sites for fungal invasion (Wrather et al., 1997). The second stage juvenile (J2) is the infective stage of SCN, and soil populations of this stage are directly related to yield reduction. Therefore, a biological control organism that suppresses soil populations of this nematode may improve soybean yield.

Fungal and bacterial pathogens of J2 are important factors in suppressing populations of SCN and have the potential in the biological control of the pest (Bird and Brisbane, 1988; Chen, 1997). Many fungi and bacteria capture or infect nematode juveniles, but only a few have been studied for their potential in biocontrol of nematodes. *Hirsutella rhossiliensis* Minter and Brady, *Hirsutella minnesotensis* Chen, Liu & Chen and *Pasteuria* sp. are representative nematode pathogens. *Hi. rhossiliensis* was first described as a soil saprophyte (Minter and Brady, 1980) and later as a pathogen of *Heterodera humi* Filip (Sturhan and Schneider, 1980). It has a wide host range including nematodes in the genera of *Heterodera* (Chen, 1997; Müller, 1984; Stirling and Kerry, 1983; Sturhan and Schneider, 1980), *Mesocricotopus* (Jaffe and Zehr, 1982), *Meloidogyne* (Cayrol et al., 1986), *Pratylenchus* (Stirling and Kerry, 1982), and *Xiphinema* (Birken and Donath, 1983).
lates usually have narrow host ranges (Davies et al., Sturhan, 1996). Occurrence and activity of *Hi. rhossiliensis* were also affected by soil pH (Jaffee and Zasoski, 2001; Jaffee and Zehr, 1983). However, experiments did not support the hypothesis that organic amendments enhance infection of nematodes by *Hi. rhossiliensis* (Jaffee and Zehr, 1983; Jaffee et al., 1994). Temperature affects the occurrence of *Pasteuria* spp. and their spore attachment, spore germination, sporulation, and endospore production (Ahmed, 1990; Chen and Dickson, 1997, 1998; Hatz and Dickson, 1992; Ko et al., 1995; Stirling et al., 1990). Soil water content and texture are related to spore attachment, and spore growth within the nematode (Davies et al., 1990; Mateauille et al., 1995; Oostendorp et al., 1990; Spauld, 1984; Van Gundy, 1985). Soil pH influences spore attachment (Ahmed, 1990; Davies et al., 1988; Orui, 1997).

China is the country of origin of soybeans and SCN was first reported there. Although fungi associated with cysts and females of *He. glycines* have been isolated and described (Liu et al., 1991, 1992, 1997), no survey of fungal and bacterial pathogens of SCN J2 has been conducted. To enrich nematophagous microorganism resources in China, the objective of the current study was to investigate the distribution and frequency of pathogens of SCN J2 in soybean fields.

2. Materials and methods

2.1. Nematode preparation

Cysts of SCN race 4 cultured in the greenhouse were collected and crushed in a 40-ml tissue homogenizer. Eggs were separated from debris by centrifugation in 35%(w/v) sucrose solution at 2500g for 3 min and then washed with water, sterilized with 0.1% NaOCl for 3 min and washed again with sterile water at least twice. Following the method described by Liu and Chen (2001b), eggs were incubated in the hatching solution (0.4M ZnCl$_2$) at 25°C; the J2s that hatched within 2 days were collected by sieving, rinsed with sterile water, and adjusted to 1000 J2 per ml sterile water.

2.2. Collection of soil samples

Soil samples were collected from fields in the main soybean-producing regions across Northeast and Huanghuai River Valley in China, including Heilongjiang, Jilin, Liaoning, Beijing, Hebei, Henan, Shandong, Jiangsu, Shanxi, Anhui, Gusu, and Xinjiang. At least 10 soil cores were taken from the soybean rhizosphere at

Xiphinema (Ciancio et al., 1986), and Rotylenchus (Tedford and Jaffee, 1995) as well as bacteria-feeding nematodes and soil mites (Tedford et al., 1994). Surveys for *Hi. rhossiliensis* indicated that it was present in 25% of the sugarbeet fields in Germany (Müller, 1982), 48% of sugarbeet fields in California (Jaffee et al., 1991), 85% of potato fields in northeastern Netherlands (Velvis and Kamp, 1995), and 43% of soybean fields in Minnesota (Liu and Chen, 2000). Up to 80% of *Mesocricton xenoplax* Raski was infected by the fungus in California peach orchard soils (Jaffee et al., 1988) and 90% of J2s of *Heterodera schachtii* Schmidt in oil-radish fields in Germany (Müller, 1982). *Hi. rhossiliensis* was first observed to infect SCN J2s in a soybean field in Minnesota in 1995 and was considered to be responsible for suppression of the nematode in the site (Chen, 1997). *Hi. minnesotaensis* was initially isolated from SCN J2 in 1996 in a soybean field in Minnesota (Chen et al., 2000). In laboratory and greenhouse tests, the fungus also infected nematodes in the genera *Aphelechoides*, *Mesocrictonema*, *Belonolaimus*, *Hoplolaimus*, *Steinernema*, and *Heterorhabditis* (Chen et al., 2000; Liu and Chen, 2001a; Ma and Liu, 2000). In a recent study, Liu and Chen (2000) detected this fungus in 14% of soybean fields in Minnesota.

Endospore-forming *Pasteuria* spp. have been investigated worldwide and demonstrated to play important roles in suppressive soils, especially of root-knot nematode species (Bird and Brisbane, 1988). Four *Pasteuria* species have been described with the type species, *Pasteuria ramose* Metch. infecting cladoceran water fleas (Metchnikoff, 1888). The other three species, *Pasteuria penetrans* (Thorne) Sayre & Starr, *Pasteuria thornei* Sher & Allen, and *Pasteuria nishizawai* Sayre et al., are pathogens of root-knot nematodes (*Meloidogyne* spp.), root-lesion nematodes (*Pratylenchus* spp.) and cyst nematodes (*Heterodera* spp. and *Globodera* spp.) (Altibalenja et al., 2000; Chen and Dickson, 1998). An isolate of *Pasteuria* from *Belonolaimus longicaudatus* Rau in Florida, USA has been proposed as a new species, *Pasteuria usgae* (Bekal et al., 2001; Giblin-Davis et al., 2003). In addition, another one from *Heterodera goettingiana* Liebscher in Germany (Sturhan et al., 1994) is probably a new species. *Pasteuria* sp. attacking *He. glycines* has been detected in some sites in Japan (Nishizawa, 1987), Illinois USA (Noel and Stanger, 1994), Korea (Lee et al., 1998), and China (Peng and Zhang, 1999). Although spores of some isolates of *Pasteuria* sp. can attach to different genera of nematodes, most isolates usually have narrow host ranges (Davies et al., 1990; Hewlett and Dickson, 1994; Weikelheide and Sturhan, 1996).

Environmental factors have important effects on multiplication, sporulation, activity, and nematode infection of *Hirsutella* spp. and *Pasteuria* sp. The sporulation of *Hi. rhossiliensis* is influenced by temperature, soil pH, water content, and organic matter (Jaffee and Zehr, 1983; Velvis and Kamp, 1995); the infection rate of *He. schachtii* by the fungus was higher in loamy soils at low water potentials than in coarse sand at high water potentials (Jaffee et al., 1990; Tedford et al., 1992).
depth of 5–20 cm across each field in a zigzag pattern and pooled to form one 500 cm³ composite sample.

2.3. Examination of soil samples

Two methods were used to detect pathogens of SCN juveniles, respectively. In the direct method, J2 naturally present in soil were extracted and examined. In the baiting method, additional J2 were added to increase the chance of detecting pathogenic fungi or bacteria. The additional SCN J2s (3000) were added to the surface of 50 cm³ soil in a 250 ml beaker. The beakers were covered with aluminum foil and maintained at room temperature for 2 weeks before extraction and examination (Liu and Chen, 2000).

SCN J2s were extracted by sucrose flotation and centrifugation (Jenkins, 1964). Nematodes extracted from each soil sample were transferred to one well of a 6-well tissue culture plate and examined using an inverted microscope (100–400×) for infection. The first 100 J2s encountered were examined for attached spores and infection. Hi. rhossiliensis, Hi. minnesotensis, and Pasteuria sp. were distinguished by morphology of the conidia attached to nematodes. Conidia of Hi. rhossiliensis are oval or ellipsoid, 6–11 μm long and 4–5 μm thick (Minter and Brady, 1980) (Fig. 1A); those of Hi. minnesotensis are glubose or subglubose, 4–6 μm in diameter (Chen et al., 2000) (Fig. 1B); Pasteuria sp. spores are cup-shaped, 5–6 μm long and 4–5 μm thick. J2 colonized by fungal mycelia or encumbered with Hirsutella conidia or Pasteuria spores was considered to be pathogenic. To confirm the identity of fungi, 5–15 J2s with attached fungal conidium (a) or filled with mycelia were transferred with a bamboo needle onto potato dextrose agar plates (PDA; Oxoid, Basingstoke, Hampshire, England) containing 100 mg streptomycin and 50 mg clortetracycline per liter of medium. After mycelia grew out from the juveniles, a piece of 2–3 mm of the culture was transferred to corn meal agar (CMA; Difco, Detroit, MI) for purification. Mycelia and conidia were observed using a microscope (100–400×). An infected SCN J2 with conidia or mycelia was further observed using scanning electron microscopy (SEM) following Eisenback and Hirschmann’s method (Eisenback and Hirschmann, 1979).

2.4. Soil factor analysis

Soil pH and organic matter (%) were determined using protocols of Institute of Soil, Chinese Academy of Sciences (Anonym, 1978). The density of SCN eggs and J2s were measured using the same method as nematode preparation.

2.5. Statistical analysis

Data were analyzed using SPSS 12.0 for Windows. Relationships between occurrence of Hirsutella spp. and Pasteuria sp. in SCN infested soil and soil factors were tested using nonparametric correlation analysis.

3. Results

A total of 146 soil samples were collected from 11 provinces and Beijing suburbs. More than one third of soil samples were collected from Heilongjiang Province (Table 1). Hi. minnesotensis and Pasteuria sp. were the predominant pathogens of He. glycines J2 in China, whereas Hi. rhossiliensis was detected in only two out of 146 soil samples. Other fungi infecting or colonizing small numbers of J2 included Aureobasidium sp. in seven samples, Aremonium sp. in four samples, Phoma sp. in three samples, Paecilomyces sp. in three samples, Arthrobotrys sp. in two samples, and Trichoderma sp., Chaetomium sp., and Monilia sp. in one sample each.

By combination of two detection methods, infection of J2s by Hirsutella spp. and Pasteuria sp. was observed in 21.9 and 26% of total samples, respectively (Table 1). Occurrence of Hirsutella spp. was mainly in Heilongjiang (52.8%), whereas Pasteuria sp. was frequently detected in Henan (76.9%), Anhui (57.7%), Jiangsu (55.6%), Shandong (23.5%), and sparsely detected in Heilongjiang, Beijing, Jilin, and Liaoning. No samples contained a combination of either Hi. rhossiliensis, Hi. minnesotensis, or Pasteuria sp.

Hirsutella spp. and Pasteuria sp. may have different requirements for soil environment. Hirsutella spp. were frequently encountered in the North China and Pasteuria sp. in the South. The occurrence of Hirsutella spp. and Pasteuria sp. was related with soil pH and organic matter. Hirsutella spp. occurred in soils with relatively low pH and high organic matter; whereas Pasteuria sp. was found in ones with relatively high pH and low organic matter.

The baiting method greatly improved the detection of Pasteuria sp. but not Hirsutella spp. A Pasteuria sp. was observed in 17 of 146 samples (11.6%) by the direct detection and 32 samples (21.9%) by the baiting method.
Hirsutella spp. were observed in 24 of 146 samples (16.4%) by both methods, although they were not detected in the same soil samples (Table 2). The average percentage of SCN J2s infected by Hirsutella spp. was higher by the direct method (29.7 and 28.3%) than by the baiting method (21.0 and 15.2%), respectively. A high percentage of infection (>60%) by Hirsutella spp. and Pasteuria sp. was observed in about 2.7% of the total soil samples. In a few samples, all J2s were infected by Hirsutella spp. and Pasteuria sp.

4. Discussion

This study used two methods to detect occurrence of Hirsutella spp. and Pasteuria sp. in soybean fields in China. The baiting method was almost twice as effective as direct observation in detecting Pasteuria sp., whereas there was no significant difference in detecting Hirsutella spp. by the two methods. This finding differs from a previous report comparing methods for detecting Hirsutella spp. in soybean fields. The predominance of Hi. minnesotensis in our survey and the predominance of Hi. rhossiliensis in Liu and Chen’s (2000) survey may explain differences in detection efficiency with the baiting method. Perhaps Hi. minnesotensis does not recover from soil disturbance as well as Hi. rhossiliensis. As a consequence, baiting soil after disturbance may not detect additional conidia.

Although the latitude and climate of Heilongjiang are similar with those of Minnesota, Hi. minnesotensis was the prominent pathogen of SCN J2 in Heilongjiang, but Hi. rhossiliensis was the predominant fungus in Minnesota. Reasons for this difference remain unknown.
A Pasteuria sp. attacking He. glycines was only detected in a few sites in Japan, Illinois, Korea, and China (Lee et al., 1998; Nishizawa, 1987; Noel and Stanger, 1994; Peng and Zhang, 1999), but the bacterium was commonly found in a few sites in Japan, Illinois, Korea, and China (Lee et al., 1998; Nishizawa, 1987; Noel and Stanger, 1994; Peng and Zhang, 1999), but the bacterium was commonly detected in the soybean fields in the Huanghuaihai River Valley. Further studies are needed to determine the role of the bacterium in regulating the SCN populations in this region.

The occurrence of Hirsutella spp. was mainly in the cooler Northeast regions and Pasteuria sp. in the warmer Huanghuaihai River Valley, indicating that environmental factors may be relevant to the occurrence of the two pathogens. Hi. rhossiliensis optimal sporulation and infection temperature range is 20–25 °C (Jaffee and Zehr, 1983; Tedford et al., 1995). Pasteuria species have different optimum temperatures for growth ranging from 20 to 30 °C and do not develop at temperatures below 10 °C (Hatz and Dickson, 1992; Ko et al., 1995). As temperatures increased, the attachment ratio of Pasteuria spores increased and reached the highest at 30 °C (Ahmed, 1990; Hatz and Dickson, 1992; Orui, 1997). The relatively high temperature favored germination of Pasteuria spores, and increased their infectivity and amount of endospore production (Hatz and Dickson, 1992; Stirling, 1981).

Furthermore, the occurrence of nematophagous species of Hirsutella and Pasteuria was related with soil factors. Soil pH for Hi. rhossiliensis was 4.0–7.0, and optimum was 5.5–7.0 (Jaffee and Zehr, 1983); the activity of alginate-pelletized Hi. rhossiliensis from pellets at high pH was negatively correlated with soil pH, which may be due to a reduction in growth of Hi. rhossiliensis from pellets at high pH (Jaffee and Zasoski, 2001). Spore attachment of the Pasteuria sp. was greater at pH 9 and decreased as pH decreased (Ahmed, 1990), which may be related to interaction with the spores which are negatively charged (Afolabi et al., 1995). In general, organic amendment increase the quantity of nematophagous microbes directly or indirectly (Van Den Boogert et al., 1994), but infection by Hi. rhossiliensis was inhibited (Jaffee et al., 1994). Our study suggests that Hirsutella spp. are apt to occur in soils with more organic matter than does Pasteuria sp. As Pasteuria is an obligate pathogen and survives in soil as a highly resistant resting spore (Bird et al., 1990; Williams et al., 1989), it may explain why Pasteuria has no requirement for soil nutrient.

We tried several times to culture the Pasteuria sp. by adding the SCN J2s with attached Pasteuria spores around the roots of soybean; however, no cysts with Pasteuria spores were found after 2 months incubation in the greenhouse. It is possible that juveniles with attached spores could not infect the roots, or failed to complete their life cycle. Establishment of an axenic culture of Pasteuria is needed for species identification. Although P. nishizawai was has been described from He. glycines, we cannot confirm the identity of the Pasteuria sp. isolated in the present study because all stages in life cycle were not observed.

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