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Biological control of the cereal cyst nematode (*Heterodera filipjevi*) by *Achromobacter xylosoxidans* isolate 09X01 and *Bacillus cereus* isolate 09B18

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HIGHLIGHTS

- *A. xylosoxidans* and *B. cereus* were first reported as agents against *H. filipjevi*.
- They caused high mortality of juveniles and reduced egg hatch.
- The two strains reduced numbers of white females in the greenhouse and field.
- The two strains increased wheat yields.

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ABSTRACT

Two bacterial strains, *Achromobacter xylosoxidans* (09X01) and *Bacillus cereus* (09B18), were isolated from cereal cyst nematodes (*Heterodera filipjevi*) in Henan, China. The two bacterial strains were subsequently evaluated for their biological control potential. The results showed that the culture filtrate of the two strains caused high mortality of the second stage juvenile (J2) nematodes and reduced *in vitro* egg hatch. Results of greenhouse and field trials demonstrated that treatments with bacterial suspensions of strains 09B18 and 09X01 significantly reduced the numbers of white females in wheat roots. The rate of reduction was 75.9% by 09B18 and 70.2% by 09X01 in greenhouse trials, and 43.5% by 09B18 and 51.1% by 09X01 in field trials. These treatments also increased wheat yields by 15.9% (09B18) and 13.2% (09X01) compared to untreated control. It is concluded that the two bacterial strains can be regarded as potential biocontrol agents.

1. Introduction

The cereal cyst nematode (CCN) is a major constraint on cereal production worldwide (Riley et al., 2010). Nematode infection interferes with water absorption and nutrient uptake by plant roots, leading to plant wilting or death (Meagher, 1977). Three species of cereal cyst nematodes, *Heterodera avenae*, *Heterodera filipjevi*, and *Heterodera latipons*, have been reported to significantly reduce wheat yields (Hajihasani et al., 2010), of which the most widely distributed and studied of these species is *H. avenae* (Gurner et al., 1980). Since *H. filipjevi* was first isolated and reported in 2010 in China (Li et al., 2010; Peng et al., 2010), it has caused serious damage and aroused concern among many researchers (Fu et al., 2011; Peng et al., 2013; Yuan et al., 2011), indicating the need for effective methods of its control.

Plant-parasitic nematodes have mainly been controlled by chemical nematicides, but in recent decades a significant number of effective nematicides and fumigants have been withdrawn from the market due to their deleterious effects on human health and the environment (Dror et al., 2003). The development of new strategies for better disease control is therefore urgently required. Biological control with bacterial strains that effectively attack the nematode is an environmental friendly method that has the potential to reduce nematode multiplication and subsequent damage to crop plants (Ashoub and Amara, 2010).

A variety of microorganisms, such as fungi, bacteria, and actinomycetes have been successfully used as biocontrol agents of nematodes on different crops (Affokpon et al., 2011; Kerry, 2000;

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Seenivasan et al., 2012; Wei et al., 2014). Bacterial species such as *Bacillus thuringiensis*, *Pasteuria penetrans* and *Pseudomonas fluorescens* have potential as biological control agents of cereal cyst nematodes (Davies et al., 1988; Saxena and Stotzky, 2001; Siddiqui et al., 2001). Most biocontrol bacteria are known to against plant parasitic nematodes by producing secondary metabolites such as enzymes and toxins, which could inhibit nematode reproduction, egg hatch or juvenile survival (Siddiqui and Mahmood, 1999). The serine protease extracted from *Bacillus* sp. culture supernatant could hydrolyze cuticle and collagen of nematode (Niu et al., 2006). Siddiqui and Shaukat (2003) reported that the secondary metabolites of *P. fluorescens* such as 2,4-diacetylphloroglucinol suppressed the damage of *Meloidogyne javanica*.

Currently, a number of commercial biological control products based on microorganisms have been developed for nematode control (Radwan et al., 2012). However, strain efficiency varies with both environmental conditions and nematode species, and bacterial strains collected from one set of environmental conditions may be less effective under other environmental conditions (Stirling, 1991). Therefore, it is important to isolate and identify local strains that are well adapted to local environmental conditions (Kiewnick and Sikora, 2006). The bacterial strains 09B18 and 09X01 examined in this study were isolated from nematode cysts collected in the study area, and were found to have high potential for providing effective control of *H. filipjevi*. The bacterial strains were deposited in the China General Microbiological Culture Collection Center (accession number CGMCC No. 8632 for strain 09B18 and CGMCC No. 8631 for strain 09X01). The main objective of this work was to evaluate the biological control potential of the two bacterial strains 09B18 and 09X01 against cereal cyst nematodes on wheat plants.

2. Materials and methods

2.1. Bacterial strain identification

Strains 09B18 and 09X01 were grown on nutrient agar (NA, Peptone: 10 g, beef extract: 3 g, NaCl: 5 g, Agar: 20 g, H_2O: 1000 ml, pH 7.0–7.2) at 30 °C for 48 h. The bacteria were then gram stained. Physiological and biochemical characteristics were determined according to Bergey's manual of determinative bacteriology (Buchanan and Gibbons, 1974).

For molecular identification, genomic DNA was extracted from the tested strains in logarithm phrase using the Bacteria Genome DNA Extraction Kit (Sangon Biotech Co., Shanghai, China) as a template for polymerase chain reaction (PCR). The 16S rRNA gene was amplified, and the primers were 27F(C) AGAGTTTGATCCTGGCTCAG and 1492R(C) TACGGCTACCTTGTTACGACTT (Marchesi et al., 1998). The reaction mixture was as follows: 1 μl of template DNA (60 ng), 1 μl each of forward and reverse primers (10 μM/L), 10 μl of 2 × Taq PCR master mix (Lifefeng Company, Shanghai, China), and 7 μl of double-distilled water. The PCR program was as follows: 5 min denaturation at 94 °C, 30 cycles of 30 s at 94 °C, 1 min at 55 °C, 1 min at 72 °C, followed by chain elongation with 10 min at 72 °C, then the mixture was cooled to 10 °C. The digested PCR products were separated by electrophoresis on 2% (wt/vol) agarose gels with ethidium bromide staining. The PCR products were then cloned and sequenced (Genomics Company, Wuhan, Hubei, China), and the obtained sequences were compared to the GenBank database in the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov) using the BLAST program. Then the DNA sequences were edited with Genetyx-Win (Ver 5.0, Genetyx Co., Tokyo, Japan) and aligned with ClustalX (Ver 1.83, http://www.clustal.org). Phylogenetic trees were constructed with neighbor-joining algorithms (MEGA 4: Molecular Evolutionary Genetics Analysis, http://www.megasoftware.net).

2.2. Effect of the bacterial strains on J2 nematode larvae mortality and egg hatch

Soil samples were collected from a wheat field that was heavily infested with *H. filipjevi* in Xuchang (Henan, China). The nematode cysts were obtained using a modified Fenwick flotation can (Caswell et al., 1985). The isolated cysts were surface disinfected by immersion in 1% NaClO for 2 min, and then gently rinsed with tap water. After incubated in sterile tap water at 4 °C for 2 months, the cysts were crushed on a sterilized glass slide and eggs were suspended in sterile tap water to 2000 eggs/ml for later assessments. Other cysts were incubated at 4 °C for 2 months and 1 month at 15 °C, the second-stage juveniles (J2) were collected and were adjusted to 2000/ml in tap water for later.

The two bacterial strains were cultured on nutrient broth (NB) at 30 °C in a reciprocating shaker (180 rpm) for 48 h. The supernatants were collected from the bacterial suspensions by centrifugation at 10,000 rpm for 10 min and filtered through 0.22 μm membranes.

To assess the effects of metabolites of the antagonists on J2 mortality, 200 μl of the cell-free culture filtrates were transferred into 1.5 ml centrifuge tubes with 30 μl of suspension containing 60 juveniles. The tubes were incubated at 15 °C and the numbers of dead J2 were counted at 24-h intervals by observing movement when exposed to sodium hydroxide 4% (w/v) solution for 3 min (Chen and Dickson, 2000). The same volume of sterile NB was used as an untreated control. This experiment was replicated six times. J2 revised mortality was calculated according to the formula:

MJ (%) = $(D/T) \times 100$, where MJ represents the mortality of J2, D represents the number of dead J2, and T the total number of J2 used in the test.

RMJ (%) = $(TMJ - CMJ)/(100 - CMJ) \times 100$, where RMJ represents the relative mortality of J2, CMJ the mortality of J2 in the control group, and TMJ the mortality of J2 in each treatment.

To determine the effect of bacterial metabolites on the inhibition of egg hatch, 200 μl of the cell-free filtrate were added to centrifuge tubes with 30 μl of egg suspension containing about 60 eggs. Eggs in sterilized NB served as untreated control. Treatments and controls were both replicated six times, and the tubes were kept at 15 °C. After 1 month, the hatched J2 were counted and the inhibition rate on egg hatch (IREH) was calculated as follows:

IREH (%) = $(CEH - TEH)/CEH \times 100$, where CEH is the egg hatch rate in the control, and TEH is the egg hatch rate in the treatment groups.

2.3. Effect of the bacterial strains on nematode control in greenhouse and field tests

The highly nematode-susceptible wheat cv. Wenmai 19, provided by the Wheat Research Center from the Henan Academy of Agricultural Sciences, was used in both the greenhouse and field tests. After the two bacterial strains were cultured on NB at 30 °C in a shaker (180 rpm) for 48 h. The population density (colony forming units; CFU) of the final culture solutions were determined by dilution plating and then the solutions were diluted to about 10^8 CFU/ml with sterile water. Then, the wheat seeds were coated with 10^8 CFU/ml culture solutions for later use. Seeds dressed with equal sterile water were used as untreated control. Avermectin 60%; Syngenta Investment Co., Ltd, Beijing, China) was applied as a coat seed with 6 ml/kg as a nematicide control.

The soil for the greenhouse experiment was collected from heavily CCN-infested wheat fields in Xuchang, Henan, China (34.0447°N, 113.7415°E), which was heavily infested with