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INFLUENCE OF ACTINOMYCETE ISOLATES ON CEREAL CYST NEMATODE *HETERODERA FILIPJEVI* JUVENILE MOTILITY

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Summary. The effects of 126 actinomycete isolates were investigated *in vitro* on the motility of the second stage juveniles of the cereal cyst nematode, *Heterodera filipjevi* Madzhidow. Among them, isolate 3208 inhibited the motility of juveniles by 56.6% more than the negative control after one day of exposure. Motility inhibition reached 59.6% with isolate 3307 relative to the negative control after three days of exposure. All active actinomycete isolates were identified at the genus level as *Streptomyces* spp. Some of the isolates appear promising and worthy of further investigation for use as bio-control agents.

Keywords: Biological control, nematotoxic activity, *Streptomyces* spp.

Cereal cyst nematodes (CCNs) are one of the most important biotic constraints to wheat and other cereal production worldwide (Griffin, 1984; Nicol and Rivoal, 2008). One of the widespread species of CCN is *Heterodera filipjevi* Madzhidow, being reported from Russia (Balakhnina, 1989), Turkey (Rumpenhorst *et al.,* 1996), Sweden (Cook and Noel, 2002), India (Bishnoi and Bajaj, 2002), Iran (Tanha Maafi *et al*., 2003), Bulgaria, Spain (Subbotin *et al.,* 2003) Norway (Holgado *et al*., 2004), Germany (Grosse and Kohlmüller, 2004) and the USA (Smiley *et al.*, 2008). *Heterodera filipjevi* is found in 87% of the wheat growing area of the Central Anatolian Plateau (CAP) and transition zone in Turkey (Yildirim *et al.,* 2006). Recent yield loss studies showed that this nematode is of major economic importance under rain-fed wheat systems in Turkey. Yield losses of up to 50% were recorded on commonly grown cultivars in the CAP in Turkey (Nicol *et al*., 2006).

Rhizosphere bacteria that release inhibitory metabolites are important sources of biological control under natural conditions. *Bacillus megaterium* de Bary and other *Bacillus* spp., isolated from sugar beet roots, were found to be the most effective strains against the sugar beet cyst nematode (*Heterodera schachtii* Schmidt), inhibiting the hatching of eggs and infection of sugar beet roots by second stage juveniles (Neipp and Becker, 1999). Also, Nitao *et al.* (1999) reported that *Gibberella intricans* Wollenweb. (syn. *Fusarium equiseti*) inhibited egg hatching and juvenile motility of *Meloidogyne incognita* (Kofoid *et* White) Chitw. and *Heterodera glycines* Ichinohe.

Actinomycetes are soil microorganisms with high biologically active metabolite secretion activity. They are resistant to desiccation and nutrient stress; partially as a function of spore formation favoring existence in soil for a long time. Many species of *Streptomyces* inhabit the rhizosphere and effectively colonize plant roots, influence plant growth and protect plant roots from pathogens (Samac and Kinkel, 2001; Erginbaş Orakçi *et al.,* 2010). Fewer studies have been undertaken on the nematode antagonistic activity of actinomycetes. Actinomycetes have been isolated from 30% of surveyed cysts of the clover cyst nematode *Heterodera trifolii* Goffart in North Island of New Zealand (Hay and Skipp, 1993). Also, actinomycetes were reported parasitizing the vermiform stages of *Radopholus similis* Cobb and *Tylenchulus semipenetrans* Cobb on Florida citrus (Walter and Kaplan, 1990). Nematode inhibitory activity of actinomycetes was shown on adults and second stage juveniles of *Pratylenchus penetrans* (Cobb) Filipjev *et* Schuurmans Stekhoven under laboratory conditions by Walker *et al.* (1966). Chumbachi *et al.* (1999) also showed the inhibitory effect of *Streptomyces* isolates on *M. incognita* second stage juveniles under *in vitro* and green-house conditions. A *Streptomyces* sp. soil isolate from China was found highly effective against *M. arenaria* (Neal) Chitw. *in vitro* by Zeng *et al.* (2009), and highly effective strains against *Bursaphelenchus xylophilus* (Steiner *et* Buhrer) Nickle were found among marine isolates of actinomycetes (Lei *et al*., 2007).

The aim of the present study was to investigate the inhibition activity of soil isolates of actinomycetes on second stage juveniles of *H. filipjevi* under *in vitro* conditions. The identification of inhibitory activity of the actinomycete isolates could provide an important input for the use of environmentally friendly control methods against CCN under natural conditions.

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MATERIAL AND METHODS

Microorganisms. The actinomycete isolates tested in this study were isolated from 43 wheat field soils representative of the Eskişehir province in the CAP in Turkey (Fig. 1). The sampled fields are not geographically close. The most commonly planted wheat cultivar in the sampled fields was Bezostaja1, which is known to be susceptible to the CCN. Wheat roots and adhering soil were used as a source of rhizosphere actinomycetes. The soil sampling procedure involved first removing litter from the surface of the soil before taking samples using an alcohol-disinfected spatula. The samples were stored in plastic bags at 4 °C until they reached the laboratory, which was no more than 16 h later. Isolation was carried out using a well known dilution plate technique (Phelan *et al*., 1979). Briefly, soil samples were suspended in sterile distilled water, diluted serially, and then pipetted on to the isolation media (Yeast extract/malt extract agar, glycerol yeast extract agar and tryptone yeast extract agar) supplemented with filter-sterilized nystatin and cyclohexamide (50 µg/ml). In total, 126 actinomycete isolates were selected from isolation plates, based on variation in colony morphology. Single colonies were sub-cultured on yeast extract/malt extract agar medium. Purified isolates were preserved on 2 fold diluted tryptone yeast extract agar at 4 °C and as spore suspensions and hyphal fragments in 15% glycerol (vol/vol) at -20 °C until used.

The isolates identified as having inhibitory activity on nematode motility were further characterized using morphological and chemotaxonomic methods. The morphological properties of the isolates were determined by light microscopy using a ×40 long working distance objective, after 21 days growth on ISP4 medium. Selected actinomycete strains were tested for spore chain morphology, colour of aerial and submerged mycelia, production of diagnostic soluble pigment and melanin, and growth on different culture media. Pigment colours of the strains were assessed in natural daylight and were compared with Inter-Society Color Council National Bureau of Standards (ISCC-NBS) Color Name Charts (United States Department of Commerce, Gaithersberg, Maryland, U.S.A.).

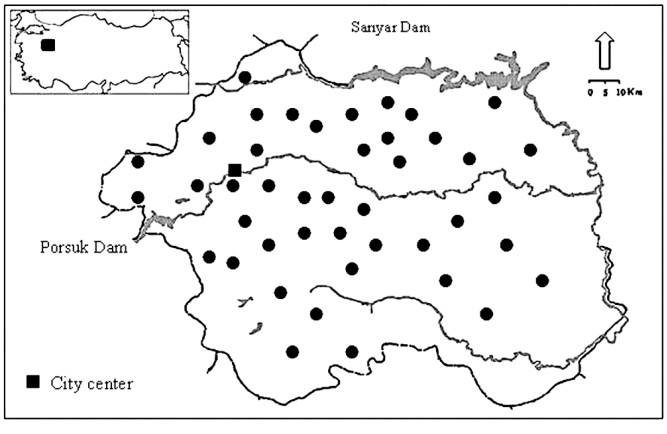

Fig. 1. Soil sampling sites in Eskişehir area, Turkey, from where actinomycetes were isolated.

For the chemotaxonomic analysis of the cell wall, the isolates were grown in triptone yeast extract broth for a week and harvested bacterial mycelia were washed twice with sterile distilled water (SDW), dried and hydrolysed. Determination of diaminopimelic acid (DAP) stereoisomers in whole cell hydrolysates as chemotaxonomic markers of actinomycetes was conducted (Hasegawa *et al*., 1983). Diaminopimelic acid isomers were analyzed by thin-layer chromatography using the solvent system methanol: water: 10 N HCl:pyridine [80:17.5:2.5:10 (v/v)] and microcrystaline cellulose TLC plates. To determine the isomeric form of DAP in the cell wall of the isolates, whole cell hydrolysates of the isolates and the commercial DAP standard (Sigma) were compared for their Rf values. The selected isolates were identified to genus level by morphological and chemotaxonomical properties according to Lechevalier (1989).

Nematodes. Second stage juveniles were used for the *in vitro* inhibitory activity test. For this purpose, newly formed cysts were extracted from infected wheat field soil samples collected in July 2002 from Konya, Karapınar (latitude 37° 42' 54" longitude 33° 32' 54"), Turkey, using the "Fenwick can" technique (Fenwick, 1940). Cysts were used straight after extraction from soil for juvenile collection. Second stage juveniles were obtained by incubating the cysts in sterile water at 18 °C.

Preparation of culture filtrates. The actinomycete isolates were grown in 20 ml GBC broth medium (10 g glucose, 7 g biosoyase, 1 g $CaCO_3$ in 1000 ml distilled water, pH 7.6) at 27 °C and under 100 rpm shaking conditions for 7 days (Walker *et al*., 1966). At the end of the incubation period, the culture broth was centrifuged at 15,000 rpm (1260 g) for 20 min (Nitao *et al.,* 1999). Four ml of supernatant was taken to be used in the nematode inhibition assay.

In vitro *inhibition assay*. *In vitro* inhibition activity of the actinomycete isolates on motility of second stage juveniles of *H. filipjevi* was investigated using a modification of the method of Walker *et al*. (1966). Second stage juveniles were surface sterilised in 0.5% NaOCl for three minutes and washed three times with SDW (Nitao *et al*., 1999). The concentration of juvenile suspension was adjusted to approximately 25 juveniles per 0.1 ml of water. A 0.1 ml juvenile suspension was pipetted into each well of 24-well tissue plates. A 0.5 ml sample of culture filtrate of the actinomycete isolates or sterile culture medium as negative control was added to the nematode suspensions in the wells. Avermectine, which is a mixture of natural products produced by *Streptomyces avermitilis* MA8680 (NRRL-8165) (Lasota and Dybas, 1991), was used as

positive control. A 0.5 ml sample of commercial solution of Avermectin was used at 3×10^{-4} g/ml concentration, as suggested by Hague and Gowen (1987), to compare the inhibitory activity of the actinomycete isolates.

The test plates were incubated at 18 °C for 3 days and checked for motionless juveniles every day. On the third day of the experiment, juveniles in the wells showing more inactive juveniles than in the negative control treatments were stained with New Blue R stain (Shepherd, 1962) to determine the survival of the nematodes. For this purpose, culture filtrates were removed from the wells and replaced with 0.5% New Blue R stain for two hours. Then the juveniles were examined for the staining to a dark purple or black colour, which indicates nematode mortality.

Each treatment was replicated four times. The experiments were repeated three times with the isolates that gave 100% inhibition by the third day and greater than 50% inhibition by the first day of experiment against *H. filipjevi.*

Results are means of the three separate experiments. Mean juvenile motility inhibition % in the actinomycete treatments is presented as a function of the juvenile motility inhibition % in the negative control wells.

Statistical analysis. ANOVA was performed on the data of percentages of non-motile *H. filipjevi* juveniles on the first and third days of the *in vitro* experiment (SAS Institute, 1985; Cary, NC, USA).

RESULTS AND DISCUSSION

Inhibition activity in vitro. Mean non-motile juvenile scores of 15.7 and 32.8% were observed in sterile GBC broth culture medium controls at the first and third days of the experiment. Inhibition of juvenile motility by the commercial insecticide avermectine (3×10^{-4} g/ml) was 100% on both the first and third days of the experiment.

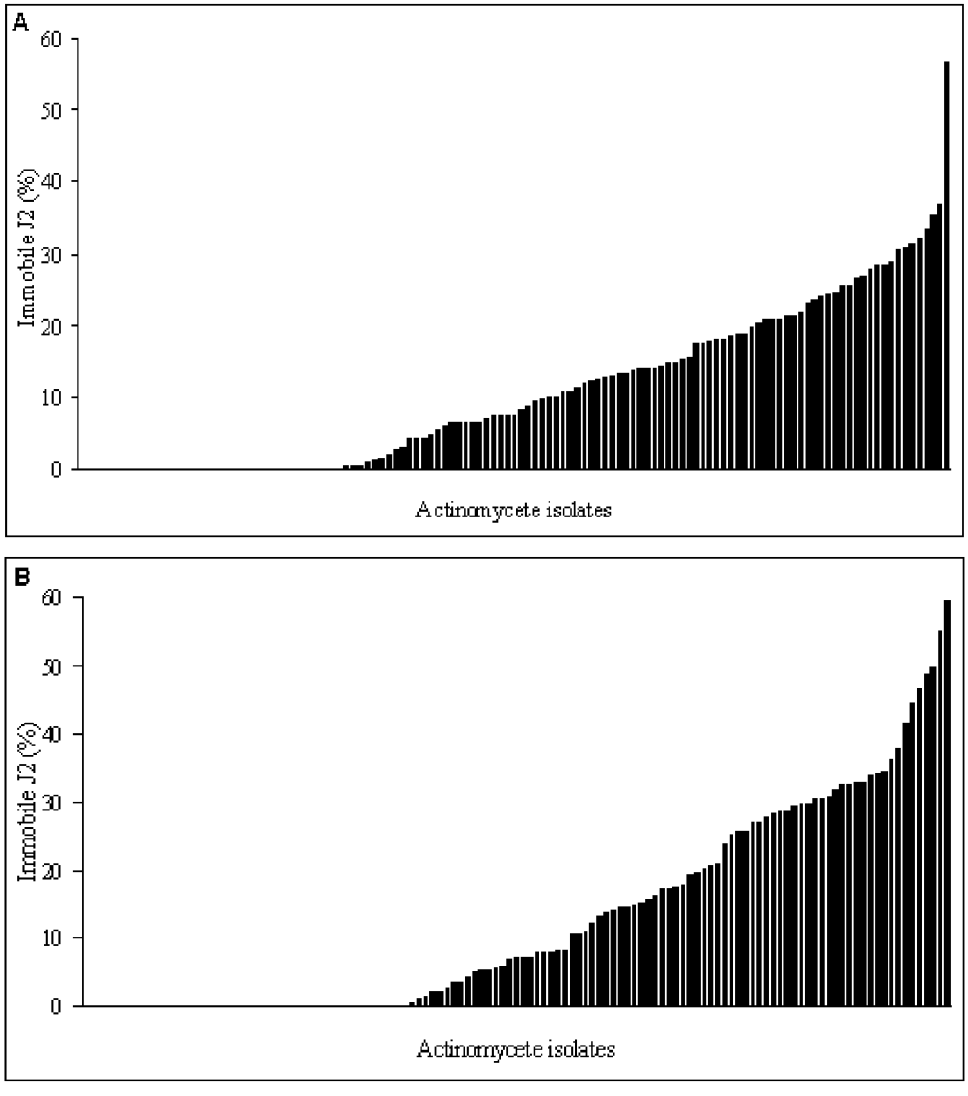

Fig. 2. *In vitro* inhibition rates of motility by actinomycete culture filtrates relative to the negative control against second stage juveniles of *Heterodera filipjevi* on the first (A) and third (B) days of exposure.

The rate of inhibition of juvenile motility by the actinomycetes increased with increase of exposure time to the culture filtrates. The greatest *in vitro* inhibition of motility was 56.6% (isolate 3208) on the first day of the experiment and 59.6% (isolate 3307) on the third day of the experiment (Figs 2A and B). The motility inhibition of *H. filipjevi* juveniles by isolate 3208 was greater on the first day than the third day (inhibition rate of 10.6% according to negative control), suggesting there was an initial effect from which the nematodes recovered.

Greater motility inhibition activity on the first day against *H. filipjevi* was found with isolates 3102, 3105, 3208, 3312 and 3702. Mean inhibition rates were 35.3, 28.4, 56.6, 33.3 and 36.8% higher than of the negative control, respectively. Isolates 3209, 3307, 3310 and 3702 had greater inhibition rates on the third day of the experiment, when motility inhibitions were 48.6, 59.6, 55.1 and 49.6% greater than the negative control. There were significant differences in activity between isolates and from positive and negative control treatments on both the first and third days ($P< 0.01$). The most active isolates are from different areas.

Chen *et al*. (2000) reported 100% paralysis of second stage juveniles of *H. glycines* after 24 hours incubation in *Paecilomyces lilacinus* culture filtrates. The inhibition activity of isolates 3307 and 3310 on the third day and 3208 on the first day of exposure to culture filtrates was not 100% but was substantial. Although many more studies have been undertaken on nematode antagonistic activity of fungi, our results with actinomycetes appear promising. Walker *et al*. (1966) found 12 ± 7 and 10 ± 5% inhibition of motility rates of *Pratylenchus penetrans* after one day of exposure to actinomycete isolates. A marine isolate of *Streptomyces* sp. caused 78.9% inhibition of motility of *Bursaphelenchus xylophilus* at 10-fold diluted fermentation filtrates (Lei *et al*., 2007). Another *Streptomyces* sp. isolated from soil, at a 10-fold dilution of the fermentation broth, was highly effective against second stage juveniles of *Meloidogyne incognita* as it inhibited motility by 75-93.7% after one day of exposure (Zeng *et al*., 2009). These results are comparable with the inhibition rates obtained with actinomycetes in the present study (44.1-72.3% after one day). However, non-motile juveniles did not appear dead when observed after staining with New Blue R after 3 days of the experiment, either in actinomycete treatments or in positive control wells. So we can argue that the isolates tested and 3×10^{-4} g/ml Avermectine solution do not have a lethal effect on *H. filipjevi* juveniles.

Characterization of Actnomycetes. *Streptomyces* species were differentiated from non-streptomycete genera by analysis of the cell wall isomeric form of diaminopimelic acid. According to chemotaxonomical analysis of the whole cell hydrolysates, all of the studied isolates showed L-diaminopimelic acid in their peptidoglycan. The cell wall type of the isolates was confirmed as belonging to type I. The spore chain types of the active isolates were also observed. Based on the type of cell wall components and morphological characteristics examined, all actinomycete isolates used in the experiments were confirmed as belonging to the genus *Streptomyces*.

Because of the distance between sampled fields, the selection of colonies with different morphology and the observed different spore chain morphology in the morphological investigation, we can argue that the screened isolates are not close. Future studies will focus on molecular characterization of the selected isolates.

Physico-chemical properties and pH values of the culture extracts of the isolates that have higher inhibition rates of *H. filipjevi in vitro* will be further investigated. It would also be informative to test them against different plant pathogenic nematode species. Those isolates with greater *in vitro* inhibition activity on the juvenile motility of *H. filipjevi* are potential agents for the biological control of CCN under field conditions.

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