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Application methods of *Steinernema feltiae*, *Xenorhabdus bovienii* and *Purpureocillium lilacinum* to control root-knot nematodes in greenhouse tomato systems

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ABSTRACT

The efficacy of various biological control agents and their application methods was tested against the root-knot nematodes *Meloidogyne javanica* and *Meloidogyne incognita* in large, in-ground tomato production areas. Treatments included infective juveniles (IJs) of the entomopathogenic nematode *Steinernema feltiae*, nematode-infected insect cadavers and cell-free supernatant of the nematode's bacterial symbiont (*Xenorhabdus bovienii*) and the nematode-parasitic fungus *Purpureocillium lilacinum*. The treatments were tested in two 1000 m^2 greenhouses, one located in Fethiye and the other in Kepez Turkey. Galling index according to the Zeck scale, total yield and cost analyses were evaluated for each application method. The Zeck scale was the lowest (mean index = 2.37) for plants treated with a commercial product based on *P. lilacinum*, followed by *S. feltiae* (125 IJs/cm^2), *X. bovienii* (dipping + topical application) and cadaver treatments. The control group had the highest root-knot infection (mean index = 8.55). Total yield from the *P. lilacinum*-treated plants in the Kepez region was estimated at 18.800 kg/1000 m^2, which was the highest yield among all treatments in both regions. *S. feltiae* and *S. feltiae* cadaver application treatments followed the *P. lilacinum* treatment with 17.216 and 16.440 kg/1000 m^2 production, respectively. Total yield was 11.184 kg/1000 m^2 in the control. The cost analysis of each treatment was calculated based on the total harvested amount of tomatoes per plant x 4000 (the number of tomato plants in a typical 000 m^2 greenhouse). The net profit difference between *P. lilacinum* treated and non-treated (control) greenhouses in Fethiye and Kepez regions was US $ 3268 and 797 US $ for the tomato growers per 1000 m^2, respectively.

1. Introduction

Plant-parasitic nematodes cause an estimated $118 billion in annual losses to world crops (Atkinson et al., 2012). Root-knot nematodes (RKN) (*Meloidogyne* spp.) are obligate parasites of a wide range of plant species, and is the most economically important genus of plant-parasitic nematodes. RKN second stage infective juveniles penetrate into plant root tips and after moving through the root and establishing a feeding site, they cause the development of galls that drain the plant's photosynthates and nutrients. Infection of young plants may be lethal, whereas infection of mature plants decreases yield. RKN damage results in poor growth, a decline in crop quality and yield and reduced resistance to other stresses (e.g. drought, other diseases). Extensive damage can lead to total crop loss. In particular, four *Meloidogyne* species (*M. javanica, M. arenaria, M. incognita,* and *M. hapla*) are major pests of crops including field crops, pasture and grasses, ornamental and vegetable crops. They are responsible for at least 90% of all damage caused by nematodes (Castagnone-Sereno, 2002). RKNs are among the most difficult agricultural pests to control. Current management strategies include the use of chemical nematicides, organic amendments, resistant cultivars, soil solarization and biological control (Randhawa et al., 2001; Sakhuja and Jain, 2001). Limited availability and registration restrictions, the high cost of nematicide development and public demand for safer agricultural practices create the need to discover alternative methods of RKN management (Barker et al., 1994; Mnif and Ghribi, 2015).

A wide variety of soil organisms are antagonistic to plant-parasitic nematodes (Coleman and Crossley, 1996), but despite their numbers

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and commonality (Timper, 2014), relatively few have been developed commercially. *Purpureocillium lilacinum* (syn. *Paecilomyces lilacinus*) is a soil-inhabiting fungus that can parasitize nematode eggs, juveniles and females, thus reducing soil population densities of RKNs. This fungus can also become endophytic and reduces cotton aphid (*Gossypium hirsutum*) populations on cotton plants (Castillo Lopez et al., 2014). Strains of this fungus have been formulated for use as biological nematicides in several countries (EPA , 2005; Kiewnick and Sikora, 2003, 2006; Stirling, 2014). Other natural products based on metabolic by-products of fungal fermentation are used as nematicides; for example, the fermentation solids and solubles of *Myrothecium verrucaria* strain AARC-0255 (DiTera WDG, Valent Biosciences, USA).

An antagonistic interaction between entomopathogenic and plant-parasitic nematodes can also lead to reductions in RKN populations. Entomopathogenic nematodes (EPNs) in the genera *Steinernema* and *Heterorhabditis* survive in soil and are lethal insect parasites of a wide range of insect species (Kaya and Gaugler, 1993; Hazir et al., 2003), but have no direct interactions with plant-parasitic nematodes. These nematodes are mutualistically associated with bacteria in the genus *Xenorhabdus* (for *Steinernema*) or *Photorhabdus* (for *Heterorhabditis*) (Lewis and Clarke, 2012). When infective juvenile (IJ) stages of EPNs encounter a suitable host, they penetrate into the insect hemocoel via natural openings (anus, mouth or spiracles) and release their mutualistic bacteria into the nutrient-rich hemolymph. The bacteria grow rapidly and produce toxins and other metabolites that kill the host by means of inducing septicemia or toxemia within 48 h (Griffin et al., 2005). Grewal et al. (1997) reported that *X. bovienii* bacteria associated with *S. feltiae* had a key role in the suppression of RKNs and also reported that EPN-infected insects repel *M. incognita* juveniles. Plant-parasitic nematode suppression by EPNs has been documented numerous times (reviewed by Lewis and Grewal, 2005).

Kepenekci et al. (2016) evaluated the suppressive effects of several treatments based on EPN IJs, nematode-infected insect cadaver formulations of various EPN species and the cell-free supernatants of their mutualistic bacteria grown in liquid culture in a series of experiments against the RKNs *M. incognita* and *M. arenaria* in tomatoes. Specific responses to treatments included reduced numbers of RKN egg masses, increased plant height and increased fresh and dry weights compared with the infested control plants. Of all the treatments, dipping the plant roots into *X. bovienii* bacterial supernatant just before planting to infested soil was the most promising method for *M. incognita* and *M. arenaria* control. This previous study was conducted in 40 mL plastic pots (7 cm diameter, 7 cm height) containing 320 g of sterilized loamy sand. Here, we test the efficacy of various biological control agents against RKNs in large, in-ground tomato production areas. Based on the pot experiments (Kepenekci et al., 2016), the most successfull treatments were selected for larger scale greenhouse applications. The EPN *S. feltiae*, nematode-infected insect cadavers, cell-free supernatant of the nematode's symbiont, *X. bovienii*, and nematode-parasitic fungus *P. lilacinum* were tested in two different 1000 m^2 greenhouses located in Feythiye and Kepez regions of Turkey. In addition, we conducted a cost analysis of the treatments.

2. Materials and methods

This study was conducted in two different greenhouses which had been used to produce tomatoes in natural mineral soil. The size of each greenhouse was one 1000 m^2. The first greenhouse was located in the Kepez district of Antalya and the second one was in the Fethiye region of Mugla in Turkey. These areas are the most active greenhouse production centers in Turkey. There is approximately 200 km distance between two locations.

Table 1
Primers used in molecular analyses.

Primer	Primer sequences (5′-3′)	Literature
Far	TCGGCGATAGAGGTAAATGAC	*M. arenaria*-specifik SCAR
Rar	TCGGCGATAGACACTACAAACT	Zijlstra et al. (2000)
Fjav	GGTGCGCGATTGAACTGAGC	*M. javanica*-specifik SCAR
Rjav	CAGGCCCTTCAGTGGAACTATAC	Zijlstra et al. (2000)
Finc	CTCTGCCCAATGAGCTGTCC	*M. incognita* specifik SCAR
Rinc	CTCTGCCCTCACATTAGG	Zijlstra et al. (2000)
MI-F	GTGAGGATTCAGCTCCCCAG	*M. incognita*-specifik SCAR
MI-R	ACGAGGAACATACTTCTCCGTCC	Meng et al. (2004)

2.1. Isolation and identification of native root-knot nematode or entomopathogenic nematode species in the soil of greenhouses

Two greenhouses where RKN infection was documented were selected to conduct this study. The tomato plants planted previously in the greenhouses were harvested and the Zeck scale (Zeck, 1971) of root damage was used to determine the intensity of the natural infestation. After rating, infected knots were washed carefully to obtain resident RKNs. Both morphological and molecular methods were used to identify the RKN species. For morphological identification, nematode slides were prepared according to Hooper (1986) and identified using perineal pattern morphology (Hartman and Sasser, 1985). For molecular analyses, egg masses were collected from infected tomato roots and DNA was extracted from the eggs using DNAeasy tissue and blood extraction kits (Qiagen, Hilden, Germany). The primers used in molecular analyses are provided in Table 1.

To test for the presence of native EPN populations, 20–25 soil samples of approximately 60 cc each were collected randomly from each greenhouse to a depth of approximately 20 cm. After collection, an insect baiting method (Bedding and Akhurst, 1975) using *Galleria mellonella* (Lepidoptera: Pyralidae) last instars as the host was used to sample for EPNs. Four *G. mellonella* larvae were added to each soil sample. No EPNs were found in either greenhouse.

2.2. Culture and preparation of treatments

The fungus *P. lilacinum* TR1 which was originally isolated from RKNs in tomato roots (Kepenekci et al., 2009) was obtained from the culture collection of the Ankara Plant Protection Central Research Institute. The fungus was sub-cultured on potato dextrose agar (PDA) (Difco TM, Becton Dickinson and Company, USA) and incubated at 25 ± 1 °C for 14 days. Conidia were harvested using a sterilized rubber loop attached to 1 mL borosilicate pipette at a 45° angle. The scraped material was placed into sterilized Petri plates and stored at 4 °C. The harvested fungal conidia were incorporated into sterile 0.05% Tween-80 solution and the material was stirred for complete homogeneity. The fungal dilution was prepared and the number of conidia was estimated using a hemocytometer to achieve a 1×10^8 conidia/mL concentration. *P. lilacinum* conidia were refrigerated at +4 °C and used within two weeks for the experiments. Conidia germination rates were assessed, as outlined by Ansari and Butt (2011) and always exceeded 90%.

The native isolate of *Steinernema feltiae* (isolate 09–31) was cultured in last instar *G. mellonella* at room temperature (23–24 °C) using methods described by Kaya and Stock (1997). *G. mellonella* larvae infected by the nematodes were placed on White traps (White, 1927). Collected IJs were rinsed three times in sterile distilled water and kept in 1 l tetrapak juice boxes (Gulcu and Hazir, 2012) before being stored at 10 °C. The harvested IJs were used within two weeks after emergence for the experiments.