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Short communication

Effect of the entomopathogenic nematode-bacterial symbiont complex on *Meloidogyne hapla* and *Nacobbus aberrans* in short-term greenhouse trials

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ABSTRACT

Meloidogyne hapla and *Nacobbus aberrans* are plant-parasitic nematodes that form galls in the roots of infected plants and cause important economic losses. Entomopathogenic nematodes (EPNs) of the genera *Steinernema* and *Heterorhabditis* infect and kill insects via toxins produced by their symbiotic bacteria. EPNs have shown to have an antagonistic effect on different plant-parasitic nematode species in field and greenhouse trials. The aim of the present work was to evaluate, in tomato plants in greenhouse, the effect of the application of three Argentine EPN isolates, their symbiotic bacteria and cell-free supernatants, on a population of *M. hapla* and two populations of *N. aberrans*. Sixty days after inoculation, the number of galls and egg masses, the nematode reproduction factor (RF) and plant biomass were calculated. With a few exceptions, biomass was not affected by the different treatments. None of the plant-parasitic nematode populations was reduced by infective juvenile inoculation of the different EPN isolates. Bacterial action differed among populations; *M. hapla* was the most susceptible one, with a significant reduction in the number of galls, egg masses and RF caused by the application of the three bacterial strains. The most significant effect was produced by the cell-free supernatants on nematode RF, with reductions of 62–90%, caused by bacterial metabolites. The different inoculation alternatives of the EPN-bacterial symbiont complex tested in the present work (infective juveniles, bacteria and cell-free supernatant) are compared for the first time for plant-parasitic nematode species.

1. Introduction

Plant-parasitic nematodes cause great damage to crops and, therefore, are a limiting factor in agriculture (Archana and Prasad, 2014), generating worldwide economic annual losses that have been estimated at $173 billion (Elling, 2013). In regions of tropical and sub-tropical climates, crop production losses caused by nematodes were estimated in 14.6% compared with 8.8% in developed countries (Nicol et al., 2011).

The root-knot nematode (*Meloidogyne* spp.) is one of the most damaging plant-parasitic nematodes in the world. This cosmopolitan genus comprises approximately 90 valid species (Jones et al., 2013); *M. arenaria*, *M. hapla*, *M. javanica* and *M. incognita* are polyphagous species that have the most severe effects on crops (Bent et al., 2008). On the other hand, the false root-knot nematode *Nacobbus aberrans* is native to the American continent and, to date, has been found in Argentina, Bolivia, Chile, Ecuador, USA, Mexico and Peru; it has quarantine importance and is characterized by a wide host range (EPPO, 2009).

Entomopathogenic nematodes (EPNs) of the genera *Steinernema* and *Heterorhabditis* infect and kill insects with the aid of toxins produced by their symbiotic bacteria. In *Steinernema* spp., bacteria belong to the genus *Xenorhabdus* whereas in *Heterorhabditis*, bacteria are of the genus *Photorhabdus*. The infective juvenile (IJ) enters the host and releases the bacteria in the hemocoel, where bacteria reproduce and kill the insect, generally within 48 h (Dillman et al., 2012). Bacteria produce cytolysin, hemolysin and toxins, some of which induce apoptosis or necrosis in the host cells (Nielsen Le-Roux et al., 2012).

Biological control for plant-parasitic nematodes management using antagonist microorganisms is an alternative to the application of chemical pesticides (Vagelas and Gowen, 2012). More than 30 years ago, an antagonism between plant-parasitic nematodes and EPNs was observed (Bird and Bird, 1986; Ishibashi and Kondo, 1986). EPNs have shown that effect in field and greenhouse trials on different species, such as *Criconemoides* spp., *Belonolaimus longicaudatus* (Grewal et al.,

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1997), *Rotylenchulus reniformis* (Lone et al., 2014), *Globodera rostochiensis* (Perry et al., 1998) and *Meloidogyne* spp. (Khan et al., 2010, 2016; Raza et al., 2015; Kepenekci et al., 2016); the best results have been obtained with species of the latter genus (Lewis and Grewal, 2005). The application of IJs of different isolates has had a suppressive effect on *Meloidogyne* spp., both in the number of eggs (Pérez and Lewis, 2004) and egg masses (Kepenekci et al., 2016), and in the infection of second-stage juveniles (J2) in roots (Molina et al., 2007). Regarding *N. aberrans*, the only work conducted to date showed a reduction in nematode reproduction on tomato (*Solanum lycopersicum*) plants inoculated with IJs of *H. bacteriophora* and *S. rarum* (Caccia et al., 2013). Furthermore, the use of symbiotic bacteria and/or their metabolites has shown to have a nematicidal action against J2 of *Meloidogyne* spp. *in vitro* (Grewal et al., 1999; Hu et al., 1999; Aatif et al., 2012) as well as a reduction in host infection in greenhouse trials (Grewal et al., 1999; Sasnarukkit et al., 2002; Vyas et al., 2008; Kepenekci et al., 2016). Even in some treatments, the level of control was comparable to the chemical treatments (Vyas et al., 2008).

Some EPN isolates may be more effective against certain plant-parasitic nematode species, and some plant species may not be as attuned to the benefits of specific EPNs (Kenney and Eleftherianos, 2016). For this reason, it is important to consider new EPN isolates and compare the effect of different inoculation options of the nematode-bacterial symbiont complex. The aim of this work was to evaluate, under controlled conditions, the effect of the application of IJs of Argentine EPN isolates, as well as their symbiotic bacteria and metabolites, on *M. hapla* and *N. aberrans* populations and plant growth in tomato plants.

2. Materials and methods

2.1. Nematodes and bacterial cultures

The origin of the nematode species and symbiotic bacteria used are indicated in Table 1. Populations of *N. aberrans* and *M. hapla* were maintained on tomato plants cultivar Platense in a greenhouse. Egg masses were extracted from infected roots and placed in Petri dishes containing distilled water; they were kept at room temperature (20 ± 2 °C) until eggs hatched, and J2 were recovered with a pipette under stereoscopic microscope for inoculation. EPN isolates were multiplied on larvae of *Galleria mellonella* (Lepidoptera: Pyralidae), following the procedure described by Koppenhöfer (2007). IJs were collected using White traps (White, 1927) and maintained in water at 25 ± 1 °C until use, for no longer than 21 days (Pérez and Lewis, 2004).

To obtain symbiotic bacteria, 100 IJs were surface sterilized in 5% NaClO for 3 min and washed with sterile water. Externally sterilized nematodes were homogenized with a stick to release the symbiotic bacteria. A drop of the homogenate was streaked on to plates with brain-heart infusion agar as growth medium. Colonies were isolated after 48 h of incubation at 28 °C. Those colonies exhibiting uniform morphology and color were subcultured by incubating them at 25 °C for 24 h. Colonies were isolated and cultured in 50 ml of brain-heart liquid medium, which was incubated for 48 h at 30 °C, with agitation at 150 rpm. Cultures were centrifuged at 20000 g for 20 min at 4 °C and the supernatant was separated. The pellet was suspended in 50 ml of sterile physiological solution (bacterial suspension). Optical density of bacterial suspension was measured, and based on calibration curves, it was diluted in physiological solution to obtain a concentration of 10^6 CFU/ml; this dose has been used in similar experiments (Samaliev et al., 2000; Vagelas et al., 2007). Each supernatant containing metabolites was diluted in an equal proportion to that of its corresponding bacterial suspension and then passed through a 0.2-µm mesh filter (Millipore) (cell-free supernatant).

Table 1
Origin of plant-parasitic nematodes, entomopathogenic nematodes and bacterial cultures involved in the present study.

Nematodes/Bacteria	Code	Locality (Department, Province)
Plant-parasitic nematode		
Meloidogyne hapla	LT	Las Tapias (San Javier, Córdoba)
Nacobbus aberrans	LUL	Lules (Lules, Tucumán)
	RC	Río Cuarto (Río Cuarto, Córdoba)
Entomopathogenic nematode/bacterial symbiont		
Heterorhabditis bacteriophora/ Photorhabdus luminescens	CBA	Córdoba (Córdoba, Córdoba)
Steinernema sp./*Xenorhabdus* sp.	LB	Villa La Bolsa (Santa María, Córdoba)
S. rarum/X. szentirmaii	RACA	Rama Caída (San Rafael, Mendoza)

2.2. Experimental design

Two experiments were conducted under controlled conditions in a greenhouse. Seeds of tomato cv Platense were germinated in plastic trays containing a mixture (3:1) of sterile soil and vermiculite (autoclaved at 1.5 atm for 30 min). Soil physicochemical properties were as follows: organic matter = 4.06%; organic carbon = 2.36%; N = 0.22%; P = 116.7 ppm; pH = 6.6. After five weeks, seedlings with four true leaves were extracted and placed in plastic pots (3.8 cm in diameter x 15 cm in height) containing 190 g sterilized soil and sand (3:1). Immediately after transplanting, for both experiments, 1.5 ml of water containing 100 J2 were inoculated on roots (Initial population = Pi); then, they were covered with the same substrate. Immediately after inoculation, depending on the experiment, IJs, bacterial suspensions, or cell-free supernatants were applied on the surface soil in each pot, as follows. In the first experiment, the effects of IJs of each EPN isolate on the different plant-parasitic nematode populations were analyzed. In each treatment (n = 7), 25 IJ/cm^2 contained in a final volume of 4 ml of water were inoculated with a pipette (Molina et al., 2007); this is the dose usually used for insect control in the field (Georgis and Hague, 1991). In the second experiment (n = 6), 4 ml of the bacterial suspension (10^6 CFU/ml) or of the cell-free supernatant, depending on the treatment, was applied. In both experiments, controls (plants inoculated only with the phytoparasitic nematode) were treated with the same amount of water or sterile culture medium (in the second experiment). The plants were grown at 25 ± 1 °C, with a 12-h photoperiod; automatic irrigation was applied daily. After 60 days, the plants were uprooted and the roots were carefully washed to remove adhered soil particles. The number of galls and egg masses was counted under stereoscopic microscope. Egg masses were removed and immersed in a 1% NaClO solution during 4 min to dissolve the gelatinous matrix (Hussey and Barker, 1973); the number of eggs was counted under light microscope. The soil of each pot was processed using the centrifugal-flotation technique (Jenkins, 1964) to obtain filiform individuals. For each replicate, the final population (Pf) of *N. aberrans* and *M. hapla* was calculated by summing the total number of eggs and the nematodes extracted from the soil; with these values, the reproduction factor was calculated (RF = Pf/Pi). After making all the observations, the roots and the aerial part of each plant were dried in a heater to estimate biomass. Both experiments had a completely randomized design and were repeated twice.

2.3. Data analysis

The variables RF and biomass of tomato plants were analyzed using Linear Mixed Models. The best model fitting heterogeneous variances was selected using the Akaike and Bayesian criteria (Zar, 1999). The number of galls and egg masses was analyzed using Generalized Linear Mixed Models, considering a Poisson distribution. In all cases, treatments and replications were defined as fixed and random effects,