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Characterization of entomopathogenic nematodes and symbiotic bacteria active against *Spodoptera frugiperda* (Lepidoptera: Noctuidae) and contribution of bacterial urease to the insecticidal effect

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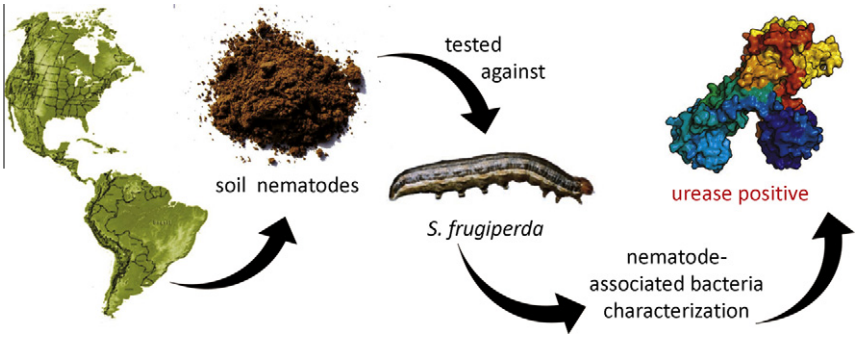
HIGHLIGHTS

- Soil nematodes from the South of Brazil were screened for pathogenicity against *S. frugiperda.*
- Symbiotic bacteria associated to these nematodes were isolated and characterized.
- Urease production by symbiotic bacteria along infection in *S. frugiperda* was evaluated.
- A positive correlation was found between bacterial urease production and entomopathogenicity.

GRAPHICAL ABSTRACT



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ABSTRACT

Entomopathogenic nematodes carrying symbiotic bacteria represent one of the best non-chemical strategies for insect control. Infective juveniles of *Heterorhabditidae* and *Steinernematidae* nematodes actively seek the host in the soil, penetrating through insect's openings to reach the hemocoel where symbiotic bacteria in the genera *Photorhabdus* or *Xenorhabdus*, respectively, are released. The bacteria replicate and produce virulence factors that rapidly kill the insect host, providing nutrients for the nematodes development and reproduction within the insect cadaver. More studies are necessary to better understand the factors implicated in the nematode-bacteria association, particularly focusing the bacterial symbionts, the final effectors of the insect death. Our group has shown that ureases are lethal to some groups of insects and may contribute to the entomopathogenic properties of the symbiotic bacteria.

The fall armyworm *Spodoptera frugiperda* (Lepidoptera: Noctuidae) is one of the major insect pests in corn (*Zea mays*) crops in Brazil, with infestations resulting in reduction up to 39% yield and losses amounting US$ 500 million annually. Native strains of entomopathogenic nematodes active against *S. frugiperda* represent a promising alternative to the intensive use of chemical insecticides to control fall armyworm population in corn plantations.

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In this study we screened soil nematodes collected in the south region of Brazil for pathogenicity against *S. frugiperda*. Symbiotic bacteria associated with these nematodes were isolated and characterized. We also evaluated urease production by the symbiotic bacteria *in vitro* and along the course of infection in *S. frugiperda* and demonstrated that urease production correlated positively to their entomopathogenicity.

1. Introduction

Entomopathogenic nematodes carrying symbiotic bacteria represent one of the best non-chemical strategies supporting insect control (Boemare et al., 1997; Forst et al., 1997; Lang et al., 2011; Zhu et al., 2011). In the soil, the infective juveniles of these nematodes (*Heterorhabditis* or *Steinernema* genera) actively seek the host in the soil, penetrate through insect's natural openings, travel to the hemocoel and release symbiotic bacterial cells (mostly species of *Photorhabdus* or *Xenorhabdus*, respectively). The bacteria multiply and release a number of virulence factors, including complexes of toxins, hydrolytic enzymes, hemolysins, and antimicrobial compounds, that kill the insect host usually within 48 h (Eleftherianos et al., 2010; ffrench-Constant and Bowen, 2000; ffrench-Constant et al., 2007), thus providing nutrients for the nematodes development and reproduction within the insect cadaver. More studies are still necessary to better understand the factors implicated in the nematode–bacteria association, particularly focusing the bacterial symbionts, which are final effectors of the insect death (Campos-Herrera et al., 2009).

Our group described for the first time the insecticidal properties of plant ureases and urease-derived peptides (Carlini et al., 1997; Follmer et al., 2004; Mulinari et al., 2007). The mode of entomotoxic action of these polypeptides is not yet completely elucidated (Carlini and Polacco, 2008), with distinct mechanisms and signaling pathways being triggered by the whole molecule or its derived peptide(s) (Staniscuaski et al., 2009, 2010). In the case of intact urease, several ureolysis-independent toxic effects are known and these can be cumulative or synergistic to the enzymatic release of highly toxic ammonia by the enzyme. Our initial studies have suggested that lepidopterans apparently are not susceptible to ureases (Carlini et al., 1997) and that bacterial ureases are devoid of insecticidal activity (Follmer et al., 2004). Contrasting with our observations, Martin et al. (2009) reported that urease-positive strains of *Bacillus thuringiensis* Berliner were better-fitted as biological control agents against the gypsy moth *Lymantria dispar* Linnaeus than urease-negative isolates. Thus a re-evaluation of the entomotoxicity of bacterial ureases and the susceptibility of lepidopterans to ureases is needed.

The fall armyworm *Spodoptera frugiperda* (J.E. Smith) (Lepidoptera: Noctuidae) is one of the major insect pests in corn (*Zea mays* Linnaeus) crops in Brazil, with infestations resulting in reduction up to 39% yield and losses amounting US$ 500 millions annually depending on climate conditions and stage of development of the attacked plants, among other factors (Cruz et al., 1999; Cruz and Turpin, 1983; Mendes et al., 2011). Native strains of entomopathogenic nematodes active against *S. frugiperda* represent a promising alternative to the intensive use of chemical insecticides to control fall armyworm populations in corn plantations.

In this study we screened soil nematodes collected in the south region of Brazil for pathogenicity against *S. frugiperda*. Symbiotic bacteria associated with these nematodes were isolated and characterized. We also evaluated urease production by the symbiotic bacteria and its potential contribution to entomopathogenicity.

2. Materials and methods

2.1. Insects and nematodes

S. frugiperda larvae were provided by the Entomology Laboratory, Embrapa Trigo, Passo Fundo, RS, Brazil. The insects were reared on artificial diets (Burton and Perkins, 1972) at 25 ± 2 °C, a relative humidity of 60 ± 10% and a 14/10 h light/dark cycle. The colony was managed as described by (Parra, 1998). Newly hatched larvae were transferred to glass vials containing the solidified diet and closed with cotton balls, and allowed to develop until pupation. The pupae were put in plates with vermiculite inside cages for the emergence of adults. One male and one female were then transferred to individual cages with the internal walls covered with filter paper (Whatmann n°1) and with free access to a 10% v/v honey solution.

Entomopathogenic nematodes (EPN) were from a collection kept by the Insect Pathology Laboratory from Embrapa Trigo, Passo Fundo, RS, Brazil. The EPNs were cultured in *Tenebrio molitor* Linnaeus (Coleoptera: Tenebrionidae). For that a suspension of 20 infective juvenile nematodes in 500 μL of distilled water was applied to *T. molitor* larvae, kept in 90 mm Petri dishes with filter paper-covered bottoms. The plates were maintained at 23 ± 2 °C, 60 ± 10% relative humidity during 4 days until death of the larvae. Dead insects were transferred to White traps in incubators at 23 ± 2 °C for emergence and capture of EPNs (White, 1927). Recovered infective juveniles were counted under a stereoscopic microscope and used for bioassays within 7 days.

2.2. Screening of nematode entomotoxicity

Eight EPN isolates (NEPETs 05, 06, 07, 12, 15, 30, 32 and 33) were evaluated for their pathogenicity toward *S. frugiperda* larvae. The bioassays were run in 12-well plates experimental units at 28 ± 2 °C, 60 ± 10% relative humidity, 14/10 h light/dark cycle, with the bottom of cells covered with filter paper (Whatmann n°1). *S. frugiperda* 5th instars starved for 24 h were placed inside the cells with small pieces of maize leaves and 100 infective juveniles (in 300 μL of distilled water) per insect larva were added to the center of the cells. Control groups received 300 μL distilled water instead. Mortality of insects and typical signs of EPN infection were registered daily during 5 days. Experiments in quadruplicates were totally randomized with 48 insects for each EPN isolate.

EPNs were identified taxonomically according to Adams and Nguyen (2002).

2.3. Bacteria isolation

Entomopathogenic bacteria (EB) were isolated from the insect's hemolymph according to the method of Poinar and Thomas, 1966. Each of the eight EPN isolates was incubated with *S. frugiperda* hemolymph in a BOD chamber at 23 ± 2 °C for 20 h and then the suspensions were plated in nutrient agar. After 24 h, bacterial colonies were plated in NBTA agar and the process was repeated every 24 h until isolated colonies were obtained. For the bioassays, the isolated bacterial colonies were inoculated in liquid LB medium and let multiply for 16 h at 28 ± 2 °C and then the cell concentra-