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Full Length Research Paper

In vivo and *in vitro* study of the effects of entomopathogenic bacteria and their filtrates on *Meloidogyne incognita*

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Symbiotic bacteria associated with entomopathogenic nematodes produce compounds with antibacterial and antifungal activity. In the present study, *Xenorhabdus* spp. and *Photorhabdus* spp. were cultivated in culture medium (*in vitro*) and in *Galleria mellonella* (L.) infected with entomopathogenic nematodes (*in vivo*), and the effect of their filtrates on the mortality of *Meloidogyne incognita* second stage juveniles (J2s) was investigated. The bacteria filtrates obtained by media culture and hemolymph were associated with different species of entomopathogenic nematodes and placed in direct contact with J2s of *M. incognita*. The mortality of the juveniles was estimated after 24 h. After dilution of the hemolymph from infected *G. mellonella,* the filtrates obtained from the *in vivo* culture and *in vitro* culture produced an 80% and 20% mortality rate, respectively. Thus, the quality of substances with nematicidal activity was affected by the technique used to cultivate the bacteria, reflecting the difference between cultivation *in vivo* and *in vitro* and the importance of the insect host.

Keywords: *Xenorhabdus*, *Photorhabdus*, Rhabditida, root-knot nematode, control.

INTRODUCTION

Nematodes from the genera *Steinernema* (Travassos, 1927) and *Heterorhabditis* (Poinar, 1976) (Rhabditida: Steinernematidae and Heterorhabditidae) are insect parasites that depend on symbiont bacteria such as *Xenorhabdus* spp. (Poinar and Thomas, 1965) and *Photorhabdus* spp. (Boemare, Akhurst and Mourant, 1993), respectively, for the survival of the host (Fallon et al., 2002).

Xenorhabdus spp. and *Photorhabdus* spp. have a mutualistic relationship with entomopathogenic nematodes of the genera *Steinernema* and *Heterorhabditis*, respectively (Thomas and Poinar, 1979). The aforementioned bacteria thrive in the intestines of infected juveniles, and first stage juveniles (JIs) penetrate the insect and liberate bacteria in the hemocele. The bacteria quickly multiply, resulting in septicaemia and the death of the host insect in 24 to 48 h, and the nematodes feed on the bacteria. The nematode-bacteria complex has been commercially developed as an agent for the biological control of pests. Secondary metabolites produced by *Xenorhabdus* spp. and *Photorhabdus* spp. attack the immune system of the insect (Forst and Nealson, 1996) and inhibit the growth of competing fungi and bacteria (Akhurst, 1982; Chen et al., 1994; Chen et al., 1996). Therefore, symbiont bacteria prevent corpse decomposition and enable the multiplication of nematodes and symbiont bacteria (Gaugler and Kaya, 1990).

When an entomopathogenic nematode invades the circulatory system of a larva, it releases the bacteria. In this new environment that is rich in food, the bacteria

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change from a quiescent nematode resident to a highly effective pathogen of insects, while the nematode infective juveniles develop into adult and start reproducing (Bode, 2009).

Xenorhabdus species can resist the response of insect larvae to immunity and can rapidly colonise the larvae, producing compounds with antibacterial and antifungal activity (Issacson and Webster, 2002). According to Ji et al. (2004), *Xenorhabdus nematophila* shows antibacterial activity in cultures of *Agrobacterium vitis*, *Pectobacterium carotovorum* subsp. *atrosepticum*, *P. carotovorum* subsp. *carotovorum*, *Pseudomonas syringae* pv. *tabaci* and *Ralstonia solanacearum*. Hu et al. (1999) have demonstrated that bacteria use nematicides to control many species of *Meloidogyne*, including phytonematodes.

The techniques used to cultivate entomopathogenic bacteria may affect the production of substances with nematicidal activity; however, information on the effects of cultivation techniques is relatively scarce. According to Hu and Webster (2000) the crude extracts of *Xenorhabdus* and *Photorhabdus* obtained from infected insect larvae have strengthened the evidence of antibacterial, antifungal and insecticide extracted versus the levels extracted when the bacteria are grown on culture media. The host is probably one of the factors responsible for getting these activities and it is probably a feature of insect larvae to produce these molecules.

Therefore, the aim of the present investigation was to study the effect of the filtrates obtained by *Xenorhabdus* spp. and *Photorhabdus* spp. cultivated in culture medium and in *Galleria mellonella* (Linnaeus, 1758) (Lepidoptera: Pyralidae) and the effects on *Meloidogyne incognita* juveniles.

MATERIAL AND METHODS

Bacteria cultivated in *Galleria mellonella* hemolymphs

Infective juveniles (J3s) of *Steinernema riobrave*, *Steinernema carpocapsae*, *Heterorhabditis* sp. JPM4 and *Heterorhabditis* sp. CCA were placed separately in Petri dishes (9 cm) containing *G. mellonella* caterpillars on sterilized filter paper (porous size 25 µm) and distilled water. After 3 days, deceased caterpillars were removed, placed in a dry dish and stored at 25 ± 1ºC for 4 days.

Subsequently, the caterpillars were transferred to Petri dishes (5 cm) containing glass blades. Four *G. mellonella* caterpillars infected with *Xenorhabdus* sp. (filtered Rbv) from *S. riobrave*; *X. nematophila* (filtered Ccap) from *S. carpocapsae*; *Photorhabdus* sp. (filtered Jpm) from *Heterorhabditis* sp. JPM4 or *Photorhabdus* sp. (filtered Hsp) from *Heterorhabditis* sp. CCA were placed in each dish. After 4 days, the liquid from each dish was collected to obtain the hemolymph bacterial culture. Subsequently, the suspension was filtered twice with 5-µm and 0.22-µm Millipore syringe filters. The filtrate from each species was placed in glass tubes and was frozen at -20°C.

Bacteria cultivated in culture medium

Symbiont bacteria and associated products were isolated from *S. carpocapsae*, *S. riobrave*, *Heterorhabditis* sp. JPM4 and *Heterorhabditis* sp. CCA according to the methodology described by Akhurst (1980). Entomopathogenic nematodes were disinfected in a solution of 0.1% sodium hypochlorite. To isolate the bacteria, J3s were suspended in a nutrient broth and were steeped. Afterwards, 0.5 mL of the suspension was applied to a dish of solid culture medium containing nutrient and agar. After 2 days at 28°C, one colony from each dish was cultured in yeast and salt (YS) solid medium. The developed bacteria were transferred to YS liquid medium and were stored in a shaker at 28°C for 7 days (Kaya and Stock, 1997). Subsequently, the liquid medium was centrifuged at 8000 rpm for 10 minutes, and the supernatant was collected to obtain the filtrate used in the experiments.

Obtention of *Meloidogyne incognita* juveniles

According to the technique developed by Hussey and Barker (1973) and modified by Boneti and Ferraz (1981), tomato roots infested with *M. incognita* were collected from a greenhouse and were ground in a blender. The eggs were obtained as a suspension and were placed in a hatching chamber to obtain second stage juveniles (J2s), which were collected after four days. Using a 0.025-mm sieve and 10-cm Petri dishes, the juveniles were disinfected by immersion in 300 ppm of pentabiotic (streptomycin, dihydrostreptomycin, benzathine, procaine and potassium benzyl-penicillin) for 1 minute. Subsequently, the juveniles were washed 3 times in sterilised, distilled water.

Effect of symbiont bacteria filtrates on *Meloidogyne incognita*

In Elisa serum test dishes (96 cells) (Biosystems®), 50 µL of a suspension containing 20-30 J2s *M. incognita* per cell was added, followed by 30 µL of Hsp, Jpm, Ccap or Rbv entomobacterial filtrates, which were obtained from an *in vivo* culture. The experiment consisted of 4 filtrate treatments and 2 control treatments, which consisted of sterilised, distilled water or 50 ppm of aldicarb (Bayer S.A®). A completely randomised design was applied, and 5 repetitions were conducted.

The metabolites of *in vitro* cultures were obtained according to the aforementioned methodology; however,

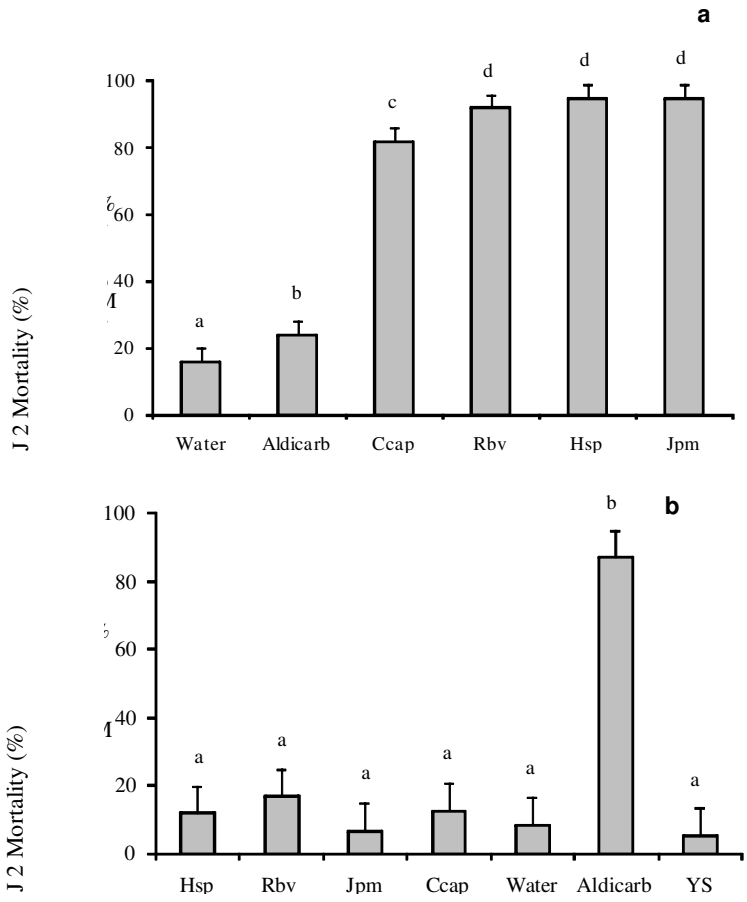

Figure 1. The activity of entomobacterial filtrates toward second stage juveniles (J2s) *Meloidogyne incognita* mortality: a) bacteria cultivated in *Galleria mellonella* hemolymph; b) bacteria cultured *in vitro*. Jpm = *Photorhabdus* sp., Hsp = *Photorhabdus* sp., Ccap = *Xenorhabdus nematophila*, Rbv = *Xenorhabdus* sp. filtrate and YS = culture medium (yeast and salt). Bars followed by letters were significantly different according to the Scott and Knott test ($p<0.05$).

50 µL of the J2s suspension per cell was added, followed by 100 µL of the bacterial suspension and 10 µL of 3000 ppm pentabiotic (Fort Dodge®). In addition, 500 ppm aldicarb, sterile distilled water and YS liquid medium were used as controls. Treatments were stored at 25 ± 1ºC and J2s mortality was evaluated 24 hours after exposure to bacterial metabolites. Specifically, J2s without movement were submitted to the Chen and Dickson test (2000) to confirm mortality. The data were submitted to an analysis of variance, and a Scott-Knott test ($p<0.05$) (Scott and Knott, 1974) was conducted to compare the averages.

RESULTS

Filtrates Ccap, Rbv, Jpm and Hsp obtained from bacteria cultured *in vivo* produced mortality rates greater than 80% in *M. incognita* J2s, which were significantly higher than that of 50 ppm of aldicarb. However, the Ccap filtrate was less efficient than Rbv, Jpm and Hsp filtrates, and the mortality rates obtained from Ccap were significantly different from those obtained from aldicarb (Figure 1a). Rocha and Campos (2003) demonstrated that the mortality rate of *M. incognita* J2s was 57% after 24 hours of exposure to 65 ppm of aldicarb. Alternatively, in the present study, 50 ppm of aldicarb produced J2 mortality rates of 24%.

When the suspension obtained from the *in vitro* culture was employed, significant differences were not observed among treatments; therefore, the metabolites did not have an effect on the survival of the juveniles. Specifically, only the aldicarb treatment produced significant nematode mortality. Similar to treatment with water or bacterial filtrates, the YS culture medium did not affect the J2s of *M. incognita* (Figure 1b). Alternatively, when Rbv, Jpm and Hsp were cultivated in *G. mellonella* (*in vivo* method), high mortality rates toward the J2s of *M. incognita* were obtained; thus, these filtrates showed potential for use in nematode suppression.

DISCUSSION

Pérez and Lewis (2004) demonstrated that *Steinernema* and *Heterorhabditis* nematodes could be used to suppress *M. incognita* and *Meloidogyne hapla*. According to Hu et al. (1999), these bacteria produce metabolites that act as nematicides toward a large number of nematodes, including some phytonematodes. Several tests conducted in the greenhouse and the field demonstrated that phytonematodes could be effectively suppressed through the application of *Xenorhabdus* spp. and *Photorhabdus* sp. (Pérez and Lewis, 2002; Pérez and Lewis, 2004).

Andaló et al. (2007) used filtrates to evaluate the effect of metabolites on *M. incognita* and the eggs and juveniles of *Meloidogyne javanica*, and demonstrated that high mortality rates were obtained after the nematodes were in contact with J2s for 24 h. *Galleria mellonella* hemolymphs that were not infected by nematodes were also tested, and the hemolymphs did not affect the hatching of J2 *M. incognita*. Therefore, the components of the filtrate, not the compounds present in the caterpillar's hemolymph, had a nematicidal effect. As a result, the mortality rates of *in vivo* and *in vitro* cultures were significantly different.

According to Han and Eblers (2001), phenotypic characteristics allow the distinction of two stages in the cycle of mutualistic bacteria. Stage I is characterised by the production of pigments, catalase, lecitinase, lipase, bioluminescence and antibiotic substances. In stage II, which occurs after the food reserves of the dead insect are consumed, the bacteria lose these characteristics. In the present study, the metabolites produced by bacteria cultured *in vitro* were obtained after 7 days of growth in a shaker; thus, antibiotic substances may not have been produced.

In vitro cultures have been used for the mass production of entomopathogenic nematodes and their symbiont bacteria (Ehlers, 1996); however, many factors affect the growth of bacteria and the production of antibiotics, such as the temperature, pH, nutrient concentration and the degree of agitation and aeration. However, the latter two variables are the most important parameters because they directly affect antibiotic production (Akhurst, 1982; Chen et al., 1996). Therefore, the development of bacteria and the production of secondary metabolites, which shows great commercial potential in agro-forest factories, are directly related to the method of culture (Webster et al., 1998).

Although *Xenorhabdus* spp. and *Photorhabdus* spp. are optional anaerobic bacteria, aeration is an important factor for the production of antibiotics. Namely, in a previous study, antibiotic activity was not detected in closed culture bottles that were not subjected to agitation (Akhurst, 1982; Chen et al., 1996). According to Wang (2007), the growth of the aforementioned bacteria and the production of antibiotic substances are superior when microbes are cultured in an appropriate nutritive medium in the presence of agitation, indicating that aeration is an important fermentation parameter for *Xenorhabdus* spp. and *Photorhabdus* spp.

According to Fallon et al. (2004), the *Steinernema feltiae-Xenorhabdus bovienii* complex could not suppress the development of *M. javanica* and penetration of the parasite into the roots of the host. However, Samaliev et al. (2000) demonstrated that *X. nematophilus* completely inhibited the hatching of *M. javanica* and paralysed the emergence of J2. In addition, a 74% reduction in hatching was observed when the eggs were removed from contact with the bacterial substrate, washed and stored in distilled water for 10 days.

Xenorhabdus and *Photorhabdus* produce several agents with nematicidal and antimicrobial activity, which helps maintain deceased insects for the development of entomopathogenic nematodes (Kaya and Gaugler, 1993). These antimicrobial agents include non-protein indoles, stilbene derivatives (Hu et al., 1998, 1999; Li et al., 1995), xenorhabdins, xenocoumacins (McInerney et al., 1991), proteinaceous chitinases (Chen et al., 1996) and bacteriocins (Thaler et al., 1995).

A number of substances are produced by bacteria, and many metabolites or associations among compounds may produce nematicidal effects. However, in *in vitro* cultures, interactions with the host insects are excluded, and adequate culture medium and aeration may have altered the production of nematicidal substances. For instance, Hu et al. (1999) observed that substances produced by *X. bovienii* and *Photorhabdus luminescens* bacteria were toxic to *M. incognita*.

Although filtrates obtained from *X. bovienii* cultures after 48 to 120 h caused 100% mortality rates in the J2s of *M. incognita* (Hu et al., 1999), filtrates obtained from bacteria cultured for 12 to 24 hours did not get the similar results. The development stage of bacteria is characterised by the production of various substances (Han and Eblers, 2001), and metabolites associated with adverse effects on phytonematodes may only be present in a single stage of the life cycle.

According to Crawford et al. (2010) there is a link between metabolic status and virulence in *Photorhabdus* and *Xenorhabdus* species. The hemolymph nutrient availability in an insect host can regulate the production of metabolites linked to pathogenesis through its ability to enhance the metabolic status. So, the virulence is closely related to the bacteria growing in a host and the contact with hemolymphs (Kim et al., 2005). Shrestha et al. (2010) found that derivatives of *X. nematophila* against larvae of *Plutella xylostella* (Lepidoptera: Plutellidae) cause significant immunosuppression.

Thus, in the present study, bacteria cultured *in vivo* produced filtrates that displayed nematicidal activity to the J2s of *M. incognita*. Alternatively, in the *in vitro* culture, these filtrates were not effective, resulting in a lack of J2 mortality. In this way, the form of cultivation may affect the bacterial filtrate.

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