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SHORT COMMUNICATION

Effects of culture filtrates from the nematophagous fungus *Verticillium leptobactrum* on viability of the root-knot nematode *Meloidogyne incognita*

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Abstract Filtrates of three isolates of the nematophagous fungus *Verticillium leptobactrum* were evaluated for their nematicidal activity against the root-knot nematode *Meloidogyne incognita*. The filtrates inhibited egg hatching, with maximum toxicity observed for isolate HR21 at 50% (v:v) dilution, after 7 days exposure. Filtrates also inactivated second-stage juveniles (J2) at 10-50% dilutions. A scanning electron microscopy study of treated eggs showed severe alterations caused by the filtrate of isolate HR43 on *M. incognita* eggs, which appeared collapsed and not viable, suggesting the production of chitin-degrading enzymes or other active compounds.

Keywords Chitin · Egg · *Meloidogyne* · Nematode · Parasitism · *Verticillium*

Introduction

The nematodes *Meloidogyne* spp. are plant pests causing losses in a wide range of host crops, worldwide. They are managed by cultural practices, resistant cultivars or chemicals. Due to environmental and human health concerns, the use of nematicides prompts for alternative control methods, including biocontrol agents or use of natural compounds (Kerry 2000).

Among microorganisms regulating nematode densities in soil, fungi hold an important position due to their parasitic, antagonistic or predatory behaviours. Some species have potentials in biocontrol and exhibit a range of antagonistic activities, including production of nematotoxic compounds (Siddiqui and Mahmood 1996; Kerry 2000; Lopez-Llorca and Jansson 2006). Nematophagous fungi directly parasitize nematodes or secrete nematicidal metabolites affecting viability of one or more stages (Lopez-Llorca and Jansson 2006). The search for nematotoxic or antagonistic compounds in culture filtrates hasgreatly intensified in recent years, due to the number of toxins, enzymes or compounds derivable from their metabolites (Ciancio 1995; Liu et al. 2008; Lopez-Llorca et al. 2008).

Assays with culture filtrates may provide first informations about the role of a fungus in the plant rhizosphere, as in vitro studies showed toxic and inhibitory effects of several filtrates toward plant parasitic nematodes (Nitao et al. 1999). However, when tested for effects on egg viability as well as second-stage Juveniles (J2s), culture filtrates and purified substances showed different levels of efficacy (Chen and Chen 2002; Olivares-Bernabeu and López-Llorca 2002; Mukhtar and Pervaz 2003; Khan et al. 2004). Specific secondary metabolites or enzymes showing nematode-antagonistic or toxic properties are a promising research field (Shinya et al. 2008). However, due to the large number of species, climates and soil ecological types, a broad range of fungi remains thus far unexplored, in particular for soils, crops or habitats not yet fully investigated.

The objective of this study was to evaluate the activity of isolates of *Verticillium leptobactrum* Gams proceeding from the coastal, semi-arid areas of Tunisia, in which

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root-knot nematodes (RKN) are endemic. The fungus is a nematode and a fungus parasite (Siddiqui and Mahmood 1996), but few data are available on its soil biology, behavior and parasitic metabolism. The effects of filtrates from some *V. leptobactrum* isolates on *M. incognita* eggs and J2 were therefore investigated in vitro and with scanning electron microscopy (SEM), and the results are herein described.

Materials and methods

Egg preparation

Approximately 70 egg masses, hand-picked from RKN-infested tomato roots, were pooled and shaken in 4–5 ml of 1.05% NaOCl for 5 min. After the debris were allowed to settle from the suspension for 30 s, the eggs in suspension were pipetted onto an autoclaved 500-mesh sieve (pore size 25 µm) and washed four times with sterile distilled water (SDW) during 5 min. They were then sterilized with streptomycin (0.1%, w:v), ampicillin (0.1%) and chlorophenol (0.1%) solutions for 10 min each, rinsed five times with 100 ml SDW in a sterile beaker and used directly for the assay, after removal of an aliquot for counting.

Filtrate preparation

Three *V. leptobactrum* isolates (HR1, HR21 and HR 43) proceeding from RKN egg masses or soil collected in Tunisia in two localities (Bekalta and Chott Mariem) were used. The isolates were confirmed for species identification by sequencing of the 18S rDNA and the internal transcribed spacer fragments (data not shown). All isolates were cultured in Czapek-Dox broth (35 g l^{-1}), in flasks with 50 ml of liquid medium, each inoculated with a 1 cm^2 agar block chopped from a 2-week-old fungal colony growing on potato dextrose agar. All isolates were grown in stationary culture for 1 week at 25°C. For all experiments, the fungal biomass was removed by filtering through filter paper and subsequently through a 0.45 µm filter. The filtrates were used at concentrations of 1/10, 1/4 and 1/2 (v:v).

Effect of culture filtrates on egg hatching

RKN used for this experiment proceeded from a population of *M. incognita* collected at Castellana (Italy) and mantained on tomato (Tondino liscio). RKN infested roots were dissected and maintained at 4°C for subsequent hand picking of egg masses. Egg suspensions (0.04 ml), prepared as described before, were placed into wells of a 96-well tissue culture plate (ca. 100 eggs per well) and combined with 0.196 ml of fungal culture filtrates at various dilutions. Czapek-Dox broth without fungal inoculum was used as control. Eight replicates of each dilution were prepared. The eggs were maintained for 1 week in a dark cabinet at 27 ± 1°C. During the assay, the numbers of J2 hatched on days 1, 4 and 7 were counted using an inverted light microscope and hatching percentages were evaluated with analyses of variance and Student's *t* -test.

Effect of culture filtrates on J2 motility

Hatched J2 of *M. incognita* were collected from egg masses over 2 days, and suspended in SDW (1,000 J2 ml^{-1}). J2 suspensions (0.01 ml) were placed into wells of a 96-well tissue culture plate (ca. 10 J2/well) with 0.9 ml of diluted fungal culture filtrates prepared as described, or a Czapek-Dox broth without fungal inocula as control, in eight replications. The plates were maintained at 25°C, checking the effects on J2 viability at 1, 24 and 72 h. The J2 response was observed, under an inverted microscope, adding a drop of 1 M NaOH to each well (Chen and Dickson 2000). The nematodes that responded by moving during 5 min were considered alive, whereas those not responding were considered dead. The assay was repeated twice.

SEM observations of filtrate effects

RKN eggs exposed 7 days to the 50% diluted filtrate of isolate HR43 were rinsed three times with sterile distilled water, and placed at room temperature on a sheet of freshly cleaved mica in a thin water film. The eggs were dried with a paper tip and prepared for high-vacuum SEM imaging with a physical fixation, plunging the mica sheets into liquid nitrogen cooled to its freezing point. The freeze-fixed specimens were then dried by sublimation of the frozen water in a vacuum lyophilizer (Micromodulyo freeze dryer, Thermo) to remove water without sample exposure to surface tension forces. Thereafter, the mica sheets with the eggs were glued on an aluminium stubs and examined with a Cambridge Stereoscan 360 SEM at 3 kV.

Results

Effect of culture filtrates on *M. incognita* eggs hatching

No lethal effects on *M. incognita* eggs were observed at 10 and 25% dilutions of *V. leptobactrum* filtrates. Emergence of J2 was, however, inversely related to the filtrate concentration and time intervals. A significant reduction in hatching was found for isolate HR21 at 10% dilution after a 4 days exposure. In some cases, occasional increased hatchings were also observed for the other two isolates. All

V. leptobactrum culture filtrates inhibited hatching at 50% dilutions after 7 days of exposure, as compared to the control (Table 1). No difference was observed at this time among isolates. The medium did not show ovistatic or ovicidal properties.

Effect of culture filtrates on larval mortality of *M. incognita*

J2 mortalities were proportional to the *V. leptobactrum* filtrate concentrations and the duration of exposure (Table 2). Filtrates showed a nematicidal activity toward *M. incognita* at 10% dilutions, when the J2 appeared paralysed after 24 h of exposure. Highest toxicity was observed for isolate HR43, and almost all nematodes were paralysed at 50% dilutions and 48 h exposure. Mean mortalities in the fungal filtrates differed significantly from the control, which showed no nematostatic or nematicidal effects (Table 2).

SEM observations

After 7 days exposure to *V. leptobactrum* HR43 filtrate, the *M. incognita* eggs showed extensive damages and shell degradation, whereas untreated controls displayed a smooth and undisturbed surface, with minor adhering products, probably related to some medium precipitates (Fig. 1ab). The external surface of the treated eggs was altered by a pronounced shell peeling. All treated eggs showed a shrinkage indicative of a structural collapse, together with a pronounced loss of turgor (Fig. 1c–f). In treated eggs, the integrity of the external chitinous layers appeared compromised by the filtrate activity, and in some areas their surface was interrupted or fractured, with partial release of the egg content on the mica sheet surface (Fig. 1ef).

Discussion

Fungal by-products with toxic and/or enzymatic properties are promising sources of new chemicals in plant nematode management (Nitao et al. 1999). Filtrates of *V. leptobactrum* inhibited egg hatching and were lethal to *M. incognita* J2. Egg hatching inhibition, however, required concentrations difficult to observe in natural conditions, not suitable for a direct use of filtrates. However, their activity showed the presence of one or more active compounds, worth further characterization. Results also showed an antagonistic activity on J2, with high mortality rates (>93%) at 50% dilution.

The fungal behaviour appears similar to other more studied, nematode-parasitic species. Among them, *Pochonia chlamydosporia* is a common parasite of eggs, as well as an endosymbiont and soil colonizer, with potentials in biocontrol (Kerry 2000; Chen and Chen 2002;

Table 1 Effect of filtrates from isolates of *Verticillium leptobactrum* on hatching (%) of *Meloidogyne incognita* eggs

Dilution (%)	10			25			50		
Exposure (days) Isolates	1	4	7	1	4	7	1	4	7
HR43	5.8 ± 4.1	29.0 ± 13.5	80.3 ± 9.6*	6.6 ± 4.6	29.2 ± 13.2	70.0 ± 8.9	7.4 ± 3.7	30.7 ± 7.7	49.5 ± 12.4***
HR21	4.4 ± 1.7	23.6 ± 4.8**	71.2 ± 12.6	7.7 ± 2.9	33.8 ± 13.9	62.8 ± 15.2	12.0 ± 5.7	38.3 ± 8.2	50.6 ± 9.8***
HR1	6.8 ± 2.5	26.6 ± 4.6	66.5 ± 7.3	10 ± 3.6*	33.4 ± 8.2	64.5 ± 14.3	3.3 ± 3.2	33.8 ± 14.6	49.8 ± 7.4***
Control	4.2 ± 2.1	30.5 ± 4.3	67.6 ± 9.3	6.4 ± 2.4	38.1 ± 6.3	67.7 ± 9.5	7.8 ± 3.1	38.3 ± 6.5	71.1 ± 4.9

Mean and SD from eight replicates. Data significantly different from control according to Student's *t* test, at (***)$P = 0.001$, (**)$P = 0.01$ and (*)$P = 0.05$

Table 2 Effect of filtrates from isolates of *Verticillium leptobactrum* on the mortality (%) of *Meloidogyne incognita* J2

Dilution (%)	10		25		50	
Exposure (h) Isolates	24	48	24	48	24	48
HR43	61.0 ± 14.1***	86.4 ± 13.1***	70.9 ± 15.7***	90.5 ± 14.3***	89.6 ± 8.1***	98.4 ± 3.5***
HR21	59.4 ± 14.4***	70.3 ± 14.9***	74.0 ± 6.4***	88.4 ± 5.6***	89.9 ± 15.5***	93.8 ± 12.1***
HR1	33.9 ± 27.4*	59.1 ± 24.6*	60.9 ± 21.6***	73.1 ± 13.3***	93.6 ± 8.3***	97.8 ± 4.2***
Contrl	10.4 ± 9.0	32.6 ± 12.7	11.8 ± 11.2	38.4 ± 14.3	10.5 ± 9.3	24.0 ± 9.7

Mean and SD from eight replicates. Data significantly different from control according to Student's t test, at (***)$P = 0.001$ and (*)$P = 0.05$

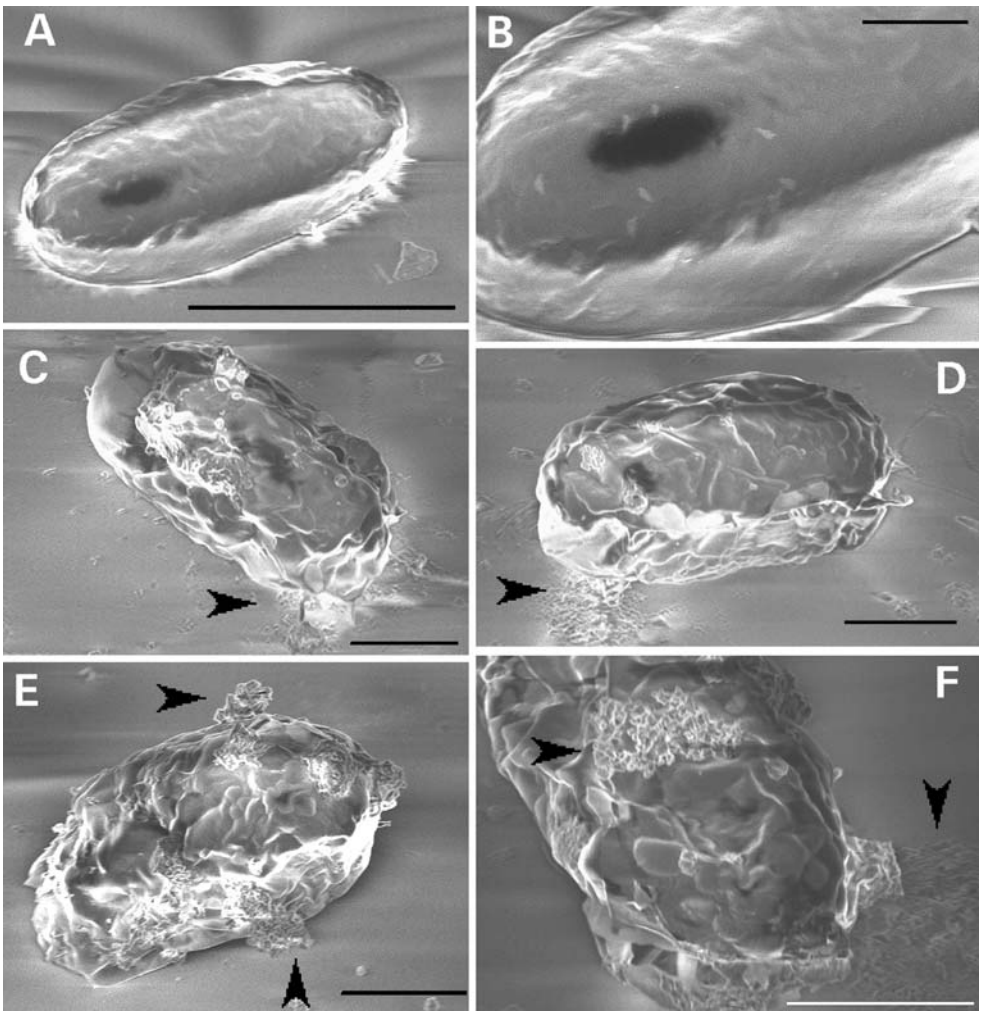

Fig. 1 Effect of *Verticillium leptobactrum* HR43 filtrate on *Meloidogyne incognita* eggs. **a** Intact egg exposed only to the control medium, showing shell integrity with minor adhering precipitates (**b**, enlarged view). Eggs exposed to *V. leptobactrum* HR43 filtrate at 50% dilution (**c–f**), show structural collapse, alterations of the external shell layers and degradation by-products (*arrow-heads*). *Scale bars*: **a** = 50 μm; **b** = 10 μm; **c–f** = 20 μm

Olivares-Bernabeu and López-Llorca 2002; Verdejo-Lucas et al. 2002; Sun et al. 2006). During its primary infection steps, this fungus produces an alkaline serine protease that specifically degrades the proteinaceous outer-vitelline membrane of the eggs (Morton et al. 2004). Fewer data are, however, available on the biochemical activities of other nematode-parasitic soil species (Lopez-Llorca et al. 2008).

Extracellular hydrolytic enzymes (proteases and chitinases) produced by biocontrol fungi are involved in complex processes leading to host cuticle penetration and cell digestion (Kerry 2000; Huang et al. 2004; Morton et al. 2004). Similarly, toxin-producing fungi affect nematodes by the production of nematicidal compounds (Dong et al. 2006).

SEM analysis confirmed the in vitro observations and revealed severe alterations in eggs exposed to the *V. leptobactrum* filtrates. Swollen, collapsed eggs showed loss of eggshell integrity due to fractures in chitin layers, and were suggestive of an enzymatic activity responsible for eggshell degradation and peeling. These effects suggest the production, by *V. leptobactrum*, of one or more chitin-degrading enzymes, through a process similar to the cuticle degradation shown by other nematode-parasites, in which several hydrolytic enzymes are involved (Lopez-Llorca et al. 2002). Chitinases and a protease purified from *P. chlamydosporia* and *V. suchlasporium*, active during egg infection, were involved in the breakdown of *Globodera pallida* eggshells (Tikhnov et al. 2002). Huang et al. (2004) also suggested a synergism of the two enzymes, and eggs of *M. javanica* treated with semi-purified proteases and chitinases from *P. lilacinus* showed significant reduction of egg development and hatching, correlated with physical and morphological changes (Khan et al. 2004). Proteases were also shown to induce a loss of the egg lipid layers and to affect the

thickness of the underlying chitin. The combined action of proteases and chitinases destroys the egg lipid layers, hydrolysing chitin and altering the vitelline layer. Consequently, the eggs lose permeability and strength, becoming deformed and swollen (Tikhonov et al. 2002).

In conclusion, experimental data suggest that direct egg penetration, as well as enzyme or toxin-based strategies, are part of the *V. leptobactrum* nematocidal behaviour, through metabolites affecting *M. incognita* eggs integrity and hatching, and active on J2 viability. Further investigations are required to identify and characterize the molecules responsible for the observed effects.

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