Eur J Plant Pathol (2010) 126:417–422
DOI 10.1007/s10658-009-9550-z

# Characterisation of volatiles produced from *Bacillus megaterium* YFM3.25 and their nematicidal activity against *Meloidogyne incognita*

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Received: 7 April 2009 / Accepted: 8 October 2009 / Published online: 21 October 2009


**Abstract** A member of plant growth-promoting rhizobacteria, *Bacillus megaterium* YMF3.25, was demonstrated to be an efficient biocontrol agent (BCA) against root-knot nematode *Meloidogyne incognita*. Results from three-compartmented Petri dish tests and a pot experiment indicated that the bacterial culture could significantly inhibit the hatch of eggs and reduce infection of the nematode through production of nematicidal volatiles. After analysis by gas chromatograph/mass spectrometer and confirmation with commercial pure compounds, the nematicidal volatiles produced by the bacterium were characterised to include mainly the benzeneacetaldehyde, 2-nonanone, decanal, 2-undecanone and dimethyl disulphide, which were active against to both juveniles and eggs at the concentration of 0.5 mmol. Six compounds (phenyl ethanone, nonane, phenol, 3,5-dimethoxy-toluene, 2,3-dimethyl- butanedinitrile and 1-ethenyl-4-methoxy- benzene) with nematicidal activityies of 30%–63% also contributed to nematicidal efficacy of the bacterium.

**Keywords** *Bacillus megaterium* · Biological control · Nematicidal volatiles · *Meloidogyne incognita* · Root-knot nematodes

## Introduction

Plant-parsitic nematodes cause annual yield losses of about $ 100 billion worldwide with 70% of the damage attributed to root-knot nematodes (*Meloidogyne spp.)* (Sasser and Freckman 1987). Due to the deleterious effects of nematicides on human health and environment, development of alternative control methods is of great importance. Crop rotation with non-hosts or resistant cultivars remains the main management strategy to regulate populations of these pests, but success if often limited due to the wide host ranges of root-knot nematode species and the frequent occurrence of mixed populations (Roberts 1992). Biological control may provide an additional method for the management of these pests. Several antagonistic microorganisms are successfully used to control nematodes in agricultural fields. These microbes include *Pochonia chlamydosporia* and *Myrothecium verrucaria* (Butt et al. 2001), *Paecilomyces lilacinus* and *Hirsutella rhossiliensis* (Liu et al. 2004), and

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*Pasteuria penetrans* (Chen and Dickson 1998; Javed et al. 2008). The use of rhizobacteria that can produce nematicidal metabolites is also considered a promising tool for controlling these nematodes (Campos et al. 1998; Ali et al. 2002) and their presence in soils is abundant (Gu et al. 2007).

This study intends to evaluate the efficacy of *B. megaterium* YMF3.25 against *M. incognita* based on Petri dish and pot tests and also to characterise the nematicidal volatiles produced by the bacterium.

## Materials and methods

### Bacterial inoculum preparation

In an investigation of nematicidal bacteria, the *B. megaterium* YMF3.25 which exhibited excellent activity to nematodes through production of nematicidal volatiles (Gu et al. 2007), was isolated from the rhizosphere of tobacco in Jianshui county, Yunnan Province, China. The bacterium was stored at 4˚C in slant with BPA medium (BPA: $l^{-1}$: beef extract 3 g, peptone 10 g, NaCl 5 g, agar 20 g, pH 7.0). To produce liquid cultures for experiments, the stock culture was streaked onto BPA plates and incubated at 37˚C for 24 h. Then a loop of the fresh culture was transferred into a 500 ml-conical flask containing 200 ml of BPB medium (BPA without agar). After incubation in a rotary shaker (200 rpm) at 37˚C for 72 h, the cell concentration was determined and the resulting suspension was used in the following experiment.

### Nematode inoculum

Infested roots of tobacco were collected from a field in Songming (Yunnan Province, China) and a single egg mass was used to establish a population of *M. incognita* on the tomato variety ZZ806. Eggs for inoculum were extracted from infected tomato roots by agitating in 0.05% NaOCl for 2 to 3 min (Hussey and Barker 1973). The eggs were then collected and rinsed with tap water on nested 150- and 25-μm pore sieves. To obtain juveniles, the egg suspension was poured on gauze in a dish and incubated at room temperature. After hatching, the juveniles were collected, and a suspension of juveniles in distilled water was prepared.

### Measurement of nematicidal activity (NA) of *B. megaterium* YMF3.25

In a three-compartment Petri dish, 3 ml of the bacterial suspension with a concentration of about $1\times10^7$ cell $ml^{-1}$ was added into one compartment and a layer of water agar (WA) was respectively poured into the other two compartments. On the surfaces of WA, about 500 juveniles and eggs of *M. incognita* were added, respectively. Plates were immediately wrapped with Parafilm to prevent the escape of the volatiles. After incubation at room temperature (20–28˚C) for 24 h, the mobile (live) and immobile juveniles were recorded by counting >200 individuals under a microscope. Those immobile juveniles were taken as dead when they could not revive within 12 h after being transferred to fresh WA and subsequently to tap water. For the egg treatment, the numbers of eggs hatched and un-hatched were determined by checking about 200 eggs under a microscope after incubation of 7 days. In control plates, the bacterial culture was replaced by an equal volume of BPB medium. Each treatment was replicated three times, and the experiment was run three times.

To confirm that the NA came from bacterial volatiles, activated charcoal, bacterial culture and nematodes (juveniles and eggs) were, respectively, added into each of the three compartments in the same Petri dish. If the NA was caused by bacterial volatiles, no effect of the culture on the viability of nematodes after the volatiles were absorbed mostly by the activated charcoal would be expected.

### Effect of *B. megaterium* YMF3.25 on *M. incognita* infection

In a greenhouse with a temperature of 20–25˚C, tomato seeds (previously sterilised with 1.5% NaOCl for 5 min) were sown in pots containing 1 kg of soil mixture (field soil: sand=1:1 v/v). Tomato seedlings with five to six true leaves were irrigated respectively with 10 ml of bacterial culture with six different concentrations ($10^4$–$10^9$ cells $ml^{-1}$). After five days, each seedling was infested with about 2,000 *M. incognita* juveniles and irrigated twice with 10 ml of bacterial suspension at days 15 and 25 of infection. After 45 days of seedling inoculation with nematodes, the number of galls and egg masses per seedling, and number of eggs per individual egg mass were

assessed according to the descriptions of Hussey and Barker (1973), Wuest and Bloom (1965). In the control treatment, an equal volume of BPB medium was used to replace the bacterial suspension for inoculation. Each treatment was replicated five times.

### Identification of nematicidal volatiles of *B. megaterium* YMF3.25

Bacterial volatiles were collected as described by Díaz et al. (2004). Briefly, 75 μm fibres (Supelco, Bellefonte, PA, USA) used for solid-phase micro-extraction (SPME) were first equilibrated with helium at 250˚C for 15 min. The extractions were then performed inside 15 ml Supelco SPME vials filled with 9 ml bacterial culture containing a stir bar and 0.1 g sodium sulphate. The vials were clamped inside a thermostatic water bath and placed on a hot stirrer. Samples were equilibrated for 1 h at 50˚C (Zeng et al. 2006) with constant magnetic stirring. The volatiles from 9 ml BPB were used as controls. After extraction, the SPME fibre was directly inserted into the front inlet of a gas chromatograph (GC, HP 6890A) connected to a mass spectrometer (MS, HP 5973, GC/MS: Agilent Technologies, USA) and desorbed at 250˚C for 2 min. GC conditions followed those described previously by Xu et al. (2004). The volatile compounds were identified, with similarity index >850, from the database search based on a comparison of the mass spectrum of the substance with GC/MS system data banks (Wiley 138 and NBS 75 k library). Each sample was tested twice.

For each detected candidate nematicidal volatile, the NA was confirmed using the same pure commercial compound (analytical reagent) instead of inoculating the bacterial culture. Based on several preliminary tests, a concentration of 0.5 mmol was used to compare the NAs among the candidates. All treatments were replicated three times.

### Data analysis and statistics

Nematicidal activity (NA) was calculated using the formula: NA=DN/SN×100%, where DN represents the number of dead nematodes (juvenile or egg) and SN represents the sum of all nematodes counted (SN >100). Data were analysed using analysis of variance (ANOVA), and the means were compared by the least significant differences (LSD) at $P = 0.05$ using SPSS 11.0 for Windows (SPSS Inc., Chicago, USA).

## Results

### Nematicidal activity of *B. megaterium* YMF3.25

In the sealed three-compartmented Petri dish with the bacterial culture, the juveniles became less active over 1–12 h and activity completely ceased at 24 h. The nematicidal nature of volatiles produced by this bacterium was lethal for these immobile nematodes as they could not be revived when transferred to the fresh WA and tap water. The NA to juvenile was 100% after 24 h incubation while the control with BPB showed the NA at 1.35% of juveniles (Table 1). The bacterial volatiles also showed strong inhibition to egg hatching. After incubation at six days, the egg hatch was inhibited completely (NA=0), while the control had a hatch rate of 83% (NA=17%) (Table 1). In the treatments with activated charcoal, the NAs were 7.3% for juveniles and 19.4% for eggs, due to the absorption of the nematicidal compounds (Table 1). These results indicated that the *B. megaterium* YMF3.25 exhibited its strong nematicidal activity by production of toxic volatiles.

### Effect of *B. megaterium* YMF3.25 on *M. incognita* infection

Results from the greenhouse experiment indicated that the *B. megaterium* YMF3.25 exhibited a significant reduction in numbers of galls, egg masses and eggs per individual egg mass compared to the control in all tested concentrations (Table 2) ($P \leq 0.05$). Concentrations of $10^{7–9}$ cells $ml^{-1}$ were more effective compared to the lower concentrations ($P \leq 0.05$).

**Table 1** Nematicidal activity of *B. megaterium* YMF3.25 to *M. incognita* tested in Petri dishes

| *M. incognita* | Mean NA%±SD | | |
|---|---|---|---|
| | BS | BS+AC | BPB |
| Juvenile | 100±0 | 7.3±0.23 | 1.35±0.12 |
| Egg | 100±0 | 19.4±0.41 | 17±0.31 |

BS: Bacterial suspension, AC: Activated charcoal

**Table 2** Effect of *B. megaterium* YMF3.25 on *M. incognita* in pots

| Concentration of bacterium | Evaluation items No. gall/plant | No. egg mass /plant | No. egg/ egg mass |
|---|---|---|---|
| Control | 83.2±1.35 a | 272.2±2.31 a | 374.1±2.59 a |
| $1\times10^4$ | 71.7±2.31 b | 265.7±2.12 b | 354.3±1.87 b |
| $1\times10^5$ | 51.3±1.78 c | 259.3±2.38 b | 324.5±1.57 b |
| $1\times10^6$ | 27.2±1.21 d | 231.5±1.87 c | 227.8±1.34 c |
| $1\times10^7$ | 19.5±0.87 e | 164.5±1.28 d | 157.2±1.17 d |
| $1\times10^8$ | 16.3±1.09 e | 134.1±1.24 e | 143.5±1.25 d |
| $1\times10^9$ | 15.4±1.98 e | 128.5±1.17 e | 138.8±1.41 d |

Means in the column followed by the same letter do not differ significantly at $P \leq 0.05$, according to the method of multiple comparisons.

Number is means of replicates±SD

### Identification and confirmation of nematicidal volatiles of *B. megaterium* YMF3.25

By GC/MS analysis, 32 peaks from the bacterial culture were observed in total ion current chromatograms, while 15 peaks were detected in the BPB medium. After a comparison with the mass spectrum of the substance from the GC/MS system data bank (Wiley 138 and NBS 75 k library), 17 kinds of volatiles produced by the bacterium were identified (similarity quality >85%). These included benzeneethanol, benzeneacetaldehyde, decanal, propanone, 2-nonanone, phenyl ethanone, 2-undecanone, nonane, hexadecane, dimethyl disulphide, phenol, 2-pentylfuran, 2,3-dimethyl-butanedinitrile, propyl-benzene, 3,5-dimethoxy-toluene, 2,6,10-trimethyl-dodecane and 1-ethenyl-4-methoxy-benzene.

These 17 organic compounds were taken as the nematicidal candidates and their nematicidal efficacy was measured *in vitro*, using commercial compounds as described before (Table 3). Five from the 17 tested candidates (benzeneacetaldehyde, 2-nonanone, decanal, 2-undecanone and dimethyl disulphide) exhibited strong nematicidal activities (NAs>80%) to both juveniles and eggs at the concentration of 0.5 mmol. The activity of 2-nonanone and 2-undecanone was even higher (NAs=100%). Therefore, these five volatiles possibly had an essential role in nematode control. Furthermore the nematicidal effect of six other compounds (phenyl ethanone, nonane, phenol, 3,5-dimethoxy-toluene, 2,3-dimethyl-butanedinitrile and 1-ethenyl-4-methoxy- benzene) was 30%–63%. The remaining six volatiles (benzeneethanol, propanone, hexadecane, 2-pentylfuran, propyl-benzene and 2,6,10-trimethyl-dodecane) were not considered as potential nematicides due to low NA values (NAs<10%).

## Discussion

Plant growth-promoting rhizobacteria (PGPR) associated with the roots can stimulate plant growth. *Bacillus megaterium* could promote plant growth by increasing the availability of phosphorus in soil. The P-solubilising *B. megaterium* RC07 when used as a biofertiliser could enchance the growth and quality of

**Table 3** Nematicidal activity of bacterial volatiles against *M. incognita* in Petri dishes

| Candidate of volatiles | NAs (%) | |
|---|---|---|
| | Juvenile | Egg |
| 2-nonanone | 100.00±0.00 a | 100.00±0.00 a |
| 2-undecanone | 100.00±0.00 a | 100.00±0.00 a |
| decanal | 96.33±2.14 ab | 84.36±1.78 b |
| dimethyl disulfide | 91.35±2.37 b | 84.51±1.57 b |
| benzeneacetaldehyde | 87.24±2.31 b | 80.54±1.64 b |
| phenyl ethanone | 63±0.71 c | 61.78±1.82 c |
| phenol | 54.23±2.11 c | 57.81±1.86 c |
| 3,5-dimethoxy-toluene | 45.73±2.21 d | 34.15±1.47 d |
| nonane | 43.17±0.43 d | 39.57±1.47 d |
| 2,3-dimethyl-butanedinitrile | 34.56±1.17 e | 24.84±0.83 e |
| 1-ethenyl-4-methoxy-benzene | 31.25±1.73 e | 19.36±1.79 e |
| hexadecane | 9.25±0.24 f | 7.12±0.42 f |
| propyl-benzene | 8.63±1.31 f | 5.34±1.12 f |
| propanone | 7.35±1.02 f | 6.34±0.63 f |
| 2-pentylfuran | 7.12±0.21 f | 4.23±0.32 f |
| benzeneethanol | 7.02±0.24 f | 4.15±0.21 f |
| 2,6,10-trimethyl-dodecane | 5±0.25 f | 3.12±0.63 f |

Number is means of replicates±SD

Means in the column followed by the same letter do not differ significantly at $P\leq0.05$, according to the method of multiple comparisons.

sugar beet in the greenhouse (Ramazan et al., 2006). De Freitas et al. (1997) reported that the isolate of *B. megaterium* was effective by phosphate-solubilising. A recent study indicated that *B. megaterium* promoted growth and altered root-system architecture through an auxin- and ethylene-independent signalling mechanism in *Arabidopsis thaliana* (López-Bucio et al. 2007).

The potential of *B. megaterium* as a biological control agent (BCA) against plant parasitic nematodes has been reported previously on *M. chitwoodi* (Al-Rehiayani et al. 1999) and *Heterodera schachtii* (Neipp and Becker 1999). Crude metabolites produced by *B. megaterium* caused a significant reduction in the number of root galls and/or nematode eggs of *M. exigua* (Oliveira et al. 2007). However, until now, there was no report characterising the nematicidal volatiles produced by *B. megaterium*.

Results of this study indicated that *B. megaterium* YMF3.25 could be a potential BCA against *M. incognita*. The NAs of its liquid culture to juveniles and eggs was 100% after an incubation period of 24 h and six days respectively (Table 1). Its nematicidal effect was also demonstrated in the pot test. Unlike other antagonists which parasitised nematode eggs (e.g. *Paecilomyces lilacinus* and *Pochonia chlamydosporia*) (Butt et al. 2001) or juveniles (e.g. *Pasteuria penetrans*) (Davies et al. 1988), this species attacked nematodes by producing several nematicidal volatiles which were identified. Their nematicidal potential was demonstrated by testing the respective commercial products. When methyl-bromide, an effective soil fumigant for parasitic nematodes and other pathogens, is no longer used due to its destructive potential to stratospheric ozone (Ristaino and Thomas 1998), the products of this bacterium may provide an alternative use. In the pot test despite the significant statistical difference from the control, the treatment plants still maintained considerable nematode inocula, which would damage the existing crop and reduce the yield. Therefore, further studies are needed to reduce nematode inocula and increase the crop yield in the field by optimising the conditions for bacterial fermentation and formulation development, to develop an integrated management system for root-knot nematodes.

**Acknowledgements** This work was supported jointly by the National Key Sciences and Technology Programme for Water Solutions (2008ZX07102-004), NSFC (30970100, 30760002) and projects from the Department of Science and Technology of Yunnan Province (2008GA012, 2006C0005M).

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