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The activity of amino acids produced by *Paenibacillus macerans* and from commercial sources against the root-knot nematode *Meloidogyne exigua*

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Abstract The rhizobacterium *Paenibacillus macerans* was grown in tryptic soy broth and after separating the cells by centrifugation the activity of fractions of the supernatant was tested against *Meloidogyne exigua* juveniles. From HPLC analyses and spectral data, the most active fractions were found to contain alanine, glutamic acid, glycine, histidine, threonine and valine, which were probably produced by bacterial hydrolysis of proteic nutrients. Amino acids from commercial sources were then assayed to confirm these results and to evaluate their potential for the control of nematodes. LC_{50} of 26 and 283 μg ml^{-1} were shown for the nematicide aldicarb and L-cysteine respectively when tested on *M. exigua* juveniles. At a concentration 38.4 times>LC_{50}, the amino acid diminished the nematode population on coffee plants to values statistically equal to those obtained with aldicarb at a concentration 19.2 times>LC_{50}.

Keywords Rhizobacterium · Nematicide · Cysteine · Biological activity

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Introduction

The lack of efficient methods to control root-knot nematodes (*Meloidogyne* spp.) causes a loss of US$ 400 million/yr to Brazilian coffee producers (Santos 2000). A promising method of control is based on the use of rhizobacteria (Feldman et al. 1999; Ali et al. 2002), which can affect nematodes in several ways, such as the production of nematicidal metabolites. For example, *Pseudomonas fluorescens* can control *Globodera rostochiensis* in potato (*Solanum tuberosum*) by the production of 2,4-diacetylphloroglucinol (Cronin et al. 1997). Similarly, *Bacillus amyloliquefaciens* synthesises a cyclic peptide patented for nematode control (Bendzko et al. 1998). In a previous study, we investigated the effect of 69 rhizobacteria strains obtained from several plants collected in the south of Minas Gerais State, Brazil, on *Meloidogyne exigua*, a pest commonly found in Brazilian coffee plantations. The crude metabolites produced by *Paenibacillus macerans* caused a significant reduction in the number of root galls and nematode eggs per coffee plant (Oliveira et al. 2007). Thus, the present work initially aimed to purify and identify the nematicidal substances produced by such a bacterium, and the active substances and their commercial analogues were then used in tests with *M. exigua* to investigate their potential to control nematodes in coffee plants.

Materials and methods

Crude metabolite production

This work was carried out with *P. macerans* (Paenibacillaceae) isolated from lettuce roots (*Lactuca sativa*), which is on deposit at the Departament of Phytopathology-Federal University of Lavras (code D1-62-12/154). The microorganism was grown in 3.0 l of tryptic soy broth (TSB, Merck) for 10 days, at 25°C, under constant stirring (100 rpm), with no light. After bacterial cell removal by centrifugation (10,000 g, 15 min), an aliquot (2 ml) of the supernatant liquid (2.2 l) was stored at −10°C until use in the *in vitro* assay with *M. exigua*. The majority was freeze-dried and maintained at −10°C. An aliquot (70 mg) of the final residue (34.3 g) was dissolved in 2 ml of an aqueous 1% (g ml^{-1}) Tween 80 solution and tested for *in vitro* activity against *M. exigua*.

Purification and identification of the nematicidal substances

As described above, 16.5 g of the freeze-dried metabolites obtained were successively washed with hexane (6×230 ml), ethyl acetate (6×230 ml) and methanol (6×230 ml) to give four fractions that were concentrated in a rotary evaporator and finally dried in a freeze-dryer. An aliquot (0.5%) of each fraction was dissolved in 2 ml of an aqueous 1% (g ml^{-1}) Tween 80 solution and tested for *in vitro* activity against *M. exigua*. Half of the fraction soluble in methanol (9.77 g) was eluted through a silica gel 60 (0.040–0.063 mm, Merck) column (3×10 cm) with ethyl acetate, methanol, water and 0.1 M HCl (300 ml each), to give four new fractions, which were concentrated as described above; 3.6% of each one was also dissolved in 1% Tween 80 (2 ml) to be used in the *in vitro* assay with *M. exigua*. The fraction eluted with methanol (1.03 g) was passed through an Amberlite XAD-16 (Sigma-Aldrich) column (2×20 cm) with deionised water and methanol (300 ml each). After concentration as described above, 1.5% of each fraction was dissolved in distilled water (1 ml) and used in the *in vitro* assay with *M. exigua*. Substances present in the fraction eluted with water (614 mg) through Amberlite XAD-16 were separated by several elutions of 20 μl of an aqueous 3.0 g ml^{-1} solution of such fraction through a Gemini C18 HPLC column (5 μm, 110 Å, 4.6×250 mm, Phenomenex, USA). A UV detector was set at 215 nm and water at a flow rate of 1.0 ml min^{-1} were used for the mobile phase. After concentration to dryness, aliquots (10%) of the 12 resulting fractions were dissolved in 600 μl water and used in the *in vitro* assay. Aliquots (nearly 1 mg) of fractions eluted between 2.35–3.50 min (fraction A, 26.9 mg) and 3.50–4.00 min (fraction B, 5.4 mg) were mixed with KBr to obtain infrared (IR) spectra (4,000–400 cm^{-1}) on a Scalibur Digital Series spectrometer. Hydrogen nuclear magnetic resonance (^{1}H NMR) spectra were recorded on a Varian Inova 500 MHz NMR spectrometer. Fractions (nearly 5.0 mg) were dissolved in D_2O and chemical shifts were calculated relative to the solvent peak. About 1.0 mg of A and B were dissolved in 1.0 ml of water to obtain mass spectra on an Agilent 1100 LC/

MS Trap ESI-MS. The same amount of each sample was also dissolved in 1.0 ml of a sodium citrate buffer to be analysed on a Shimadzu LC 10A/C-47A high performance chromatograph equipped with a Shim-pack column (100×60 mm, 5 μm, Shimadzu, Japan), using previously described methodology for the analysis of amino acids (Regina et al. 1998).

Amino acids from commercial sources

DL-alanine, L-Phenylalanine, L-threonine and DL-valine (>99.0%, Merck, Germany); L-aspartic acid, L-glutamic acid, Glycine, L-glutamine, L-hystidine, L-isoleucine, L-lysine and DL-tryptophan (>98.5%, Vetec, Brazil); L-arginine, L-ornitine and L-serine (>98.5%, Inlab, Japan); L-asparagine (>98.5%, Reagen, Brazil); L-cysteine (>97.0%, Sigma, Germany); L-cystine (>97%, Carlo Erba, Italy); L-leucine (>97.0%, Pfanstiehl, Germany); DL-methionine (>99.0%, Proquímicos, Brazil); L-proline (>98.5%, US Bioch. Co., USA); and L-tyrosine (>98.5%, Synth, Brazil) were purchased from commercial sources and used as received.

In vitro assay

Assays were performed in 96-well polypropylene plates as described by Amaral et al. (2003). Briefly, *M. exigua* (Heteroderidae) eggs were extracted from coffee (*Coffea arabica*) roots infected with the nematode in accordance with the Hussey and Baker (1973) technique, modified by Boneti and Ferraz (1981). Second-stage juveniles (J2) of the nematode were hatched from the eggs, collected and used; <2 day-old J2 were employed in the *in vitro* assays and 20 μl of an aqueous suspension containing approximately 25 J2 and 100 μl of the samples dissolved in aqueous Tween 80 or water were poured into each 300 μl well. For the assays with crude metabolites or fractions obtained before the fractionation on Amberlite XAD-16, 30 μl of an aqueous 3.0 mg ml^{-1} Pentabiótico solution, a mixture of antibacterials produced by Fort Dodge, Brazil, were also poured into the wells to prevent bacterial growth. After 48 h at room temperature, one drop of an aqueous 1 M NaOH solution was added, and J2 that changed their body shape from straight to curled or hook-shaped within 3 min were considered to be alive, whereas the nematodes not responding to the addition of NaOH were considered dead. This experiment was performed with six replicates per treatment, employing aqueous 1% (g ml^{-1}) Tween 80 solution (or water) and aldicarb (50 μg ml^{-1}) as negative and positive controls, respectively. For the aldicarb solution preparation, 8.6 g of Temik 150 (150 g of aldicarb kg^{-1}), from Rhône-Poulenc Agro-Brasil Ltda, was suspended in water and filtered through a filter paper. The resulting solution was diluted with water to the desired concentration. All values of dead J2 were converted to percentage prior to analysis of variance (ANOVA), followed by separation of treatment means in accordance with the Scott and Knott (1974) test ($P \leq 0.05$). Statistical analyses were done using SISVAR software (Ferreira 2000).

LC_{50} determination

Aqueous L-cysteine solutions at 833.3, 416.6, 208.3 and 104.2 μg ml^{-1}, and aqueous aldicarb solutions at 41.7, 33.3, 25.0, 16.7 and 8.3 μg ml^{-1}, were submitted to the *in vitro* assay as described above. Water was used as a control and no Pentabiótico solution was added to the wells. Mean values of dead J2 were converted to percentage, corrected $\{\text{correctvalue} = 100[(\text{value} - \text{controlvalue})/\text{controlvalue}]\}$ and submitted to a probit analysis on the POLO-PC programme (LeOra Software 1987).

Test with coffee plants

A mixture of soil and sand at a ratio of 1:1 (v/v) was disinfested with methyl bromide (150 ml m^{-3}) and poured into 3 l pots. One 6 month-old coffee plant

Table 1 Effect of *Paenibacillus macerans* crude metabolites on *Meloidogyne exigua* second-stage juveniles (J2)

Treatments	Dead J2 (%) [a]
1% Tween 80 (control)	15.2a
Tryptic soy broth (control)[b]	18.3a
Metabolites[c]	98.4b
Freeze-dried metabolites[d]	97.2b

[a] Means of six replicates with the same letter do not differ significantly ($P \leq 0.05$). [b] Sterile culture medium freeze-dried and redissolved in water. [c] Supernatant liquid obtained after bacterial cells removal from the culture medium. [d] Supernatant liquid freeze-dried and redissoved in water.

Table 2 Effect on *Meloidogyne exigua* second-stage juveniles (J2) of fractions obtained by solvent washings of *Paenibacillus macerans* crude metabolites

Treatments	Dead J2 (%)[a]
1% Tween 80 (control)	14.2a
Hexane fraction	24.0b
Ethyl acetate fraction	30.5b
Methanol fraction	53.0c
Insolube fraction	61.3d

[a] Means of six replicates with the same letter do not differ significantly ($P \leq 0.05$).

(*Coffea arabica* cv. Catucaí Amarelo) (Rubiaceae) was planted per pot. After 30 days, each plant was inoculated with 2,000 *M. exigua* eggs (20 ml of an aqueous suspension) through four equidistant cylindrical holes (0.5 cm×4.0 cm deep) in the substrate, around the plant. Just after inoculation, aqueous L-cysteine solutions (100 ml) at 2,830, 5,442 and 8,490 μg ml^{-1} were also added through the same holes. After 90 days in a greenhouse, above ground parts of the plants were removed and roots were gently washed with water to count galls. Eggs were then extracted from roots as described by Boneti and Ferraz (1981) and counted by the use of an inverted microscope. This experiment was carried out in a randomised block design (six blocks) employing the following controls: (1) plants with no inoculation or treatment; (2) plants inoculated with *M. exigua* and treated with 100 ml of 500 μg ml^{-1} aldicarb aqueous solution; (3) plants inoculated with *M. exigua*. Except for the conversion of values to percentage, statistical analyses were performed as described for the *in vitro* assay.

Results and discussion

When used in the test with *M. exigua* second-stage juveniles (J2), *P. macerans* crude metabolites maintained the activity previously observed (Oliveira et al. 2007) regardless of whether they were freeze-dried (Table 1).

Although all fractions obtained by solvent washings of the freeze-dried crude metabolites increased *M. exigua* mortality, values of dead J2 observed for the hexane and ethyl acetate soluble metabolites were

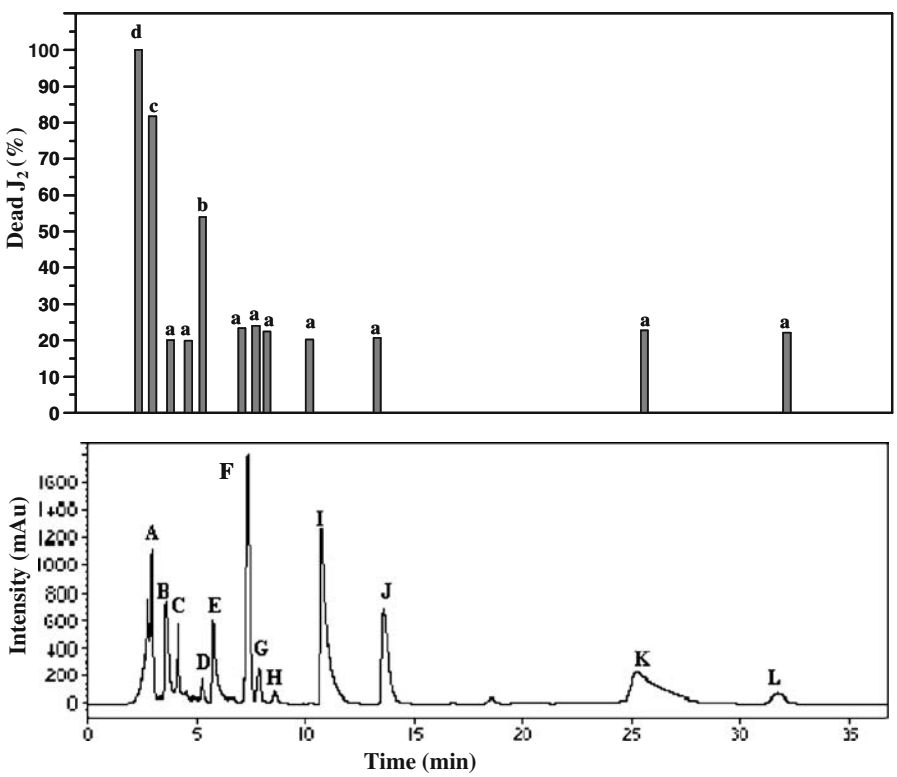

Fig. 1 Chromatogram obtained by HPLC-UV analysis of the methanol fraction resulting from elution through Amberlite XAD-16 of some *Paenibacillus macerans* metabolites (lower panel) and *in vitro* assay with *Meloidogyne exigua* second-stage juveniles (J2) (upper panel). Columns in the upper panel with the same letter do not differ significantly ($P \leq 0.05$)

Table 3 Effects of amino acids from commercial sources at two concentrations on *Meloidogyne exigua* second-stage juveniles (J2)

Treatment[a]	Dead J2 (%)[b]	
	1,000 μg ml^{-1}	2,000 μg ml^{-1}
Water (control)	13.7a	13.7a
DL-alanine	21.7b	25.8b
L-aspartic acid	25.7b	26.3b
L-glutamic acid	25.0b	21.2b
L-arginine	24.7b	27.0b
L-asparagine	23.7b	25.2b
L-cysteine	100.0c	100.0f
L-cystine	23.5b	25.0b
L-phenylalanine	22.3b	25.0b
Glycine	24.0b	24.5b
L-glutamine	24.8b	74.3d
L-hystidine	24.0b	24.7b
L-isoleucine	25.0b	48.0d
L-leucine	24.2b	27.8b
L-lysine	23.5b	25.3b
DL-methionine	25.7b	28.5b
L-ornitine	23.7b	24.7b
L-proline	25.0b	25.8b
L-serine	24.3b	27.8b
L-tyrosine	26.0b	26.5b
L-threonine	25.0b	36.3c
DL-tryptophan	23.5b	25.5b
DL-valine	25.3b	28.0b

[a] All substances were dissolved in water. [b] Means of six replicates with the same letter in a column do not differ significantly ($P \leq 0.05$).

very close to the one obtained for 1% Tween 80. The most promising fractions were those soluble in methanol and insoluble in all solvents used (Table 2).

The hexane, ethyl acetate and insoluble fractions (Table 2) were stored for future work, whereas the methanol fraction underwent silica gel column chromatography to give four new active fractions. Since the methanol-eluted fraction caused the highest percentage of dead J2, it was used in the following step. After its elution through Amberlite XAD-16, only the water fraction could affect *M. exigua* J2. Consequently, it underwent fractionation on an HPLC system. Among the twelve new fractions, A and B were the most active (Fig. 1).

The infra-red spectra of fractions A and B resembled the spectra of amino acids, with bands at 3,500–2,600 and 1,587 cm^{-1} (Silvesrtein et al. 1991). Consequently, it was analysed on an HPLC system specially designed for the identification of amino acids. It then became clear that in fraction A alanine, glutamic acid, glycine and threonine were present, while fraction B was composed of valine and histidine. This result was in accordance with the ^{1}H NMR analyses, since spectra obtained were clearly the sum of the NMR data for such substances. Furthermore, peaks at m/z 112 $[M+Na]^+$ and 128 $[M+K]^+$, 120 $[M+Na]^+$ and 142 $[M+K]^+$, 76 $[M+Na]^+$ and 114 $[M+K]^+$, corresponding to alanine, threonine and glycine, respectively, were observed during the ESI-MS analysis of fraction A. However, glutamic acid could not be detected in the positive ionisation mode. For fraction B, peaks at m/z 118 $[M+H]^+$ and 140 $[M+Na]^+$, 156 $[M+H]^+$ and 178 $[M+Na]^+$, were detected for valine and histidine, respectively.

Since the sterile culture medium (tryptic soy broth) could not increase *M. exigua* mortality (Table 1), *P. macerans* probably produced enzymes that hydrolysed peptides and proteins present in the medium to amino acids, which were not totally consumed by the microorganism. It may seem unusual that amino acids universally employed as nutrients can affect *M. exigua*, but Barbosa et al. (1999) have already observed that L-3,4-dihydroxyphenylalanine is active against *Meloidogyne incognita* and *Heterodera glycines*. Similarly, Osman (1993) reported the activity of L-arginine and L-glutamic acid against *Meloidogyne javanica*, while Talavera and Mizukubo (2005) discovered the action of DL-methionine against *M. incognita*. It is also important to mention that in cotton plants (*Gossypum hirsutum*) the amount of amino acids produced in the roots after inoculation with *M. incognita* J2 was 50% superior for the nematode-resistant cultivars (Lewis and McClure 1975).

Table 4 Effect of L-cysteine and aldicarb on nematode eggs and root galls of *Meloidogyne exigua*-inoculated coffee plants

Treatment	Galls/root [a]	Eggs/root [a]
Water (control)	41.3a	986.3a
Aldicarb (500 μg ml^{-1})	9.5c	34.6c
L-cysteine (8,490 μg ml^{-1})	5.1c	24.6c
L-cysteine (5,442 μg ml^{-1})	24.1b	224.6b
L-cysteine (2,830 μg ml^{-1})	27.5b	384.0b

[a] Means with the same letter in a column do not differ significantly ($P \leq 0.05$).

The results mentioned above suggest that amino acids could be related to the mode of action in plants and microorganisms against plant parasitic nematodes. Thus, some of these substances, obtained from commercial sources, were submitted to the *in vitro* assay with *M. exigua* J2 (Table 3). Although all of them statistically increased J2 deaths in comparison with the control (water), only L-cysteine seemed promising for further studies to evaluate the *in vivo* action against *M. exigua*.

A more detailed study on the nematicidal activity of L-cysteine revealed an LC_{50} of 283 μg ml^{-1} against *M. exigua*, while under the same conditions an LC_{50} of 26 μg ml^{-1} was observed for the commercial nematicide aldicarb. These results appeared to be very interesting, especially because the ability of L-cysteine to reduce the population of *M. incognita* in sunflower (*Helianthus annuus*)(Osman and Viglierchio 1981) and tomato (*Lycopersicon esculentum*) had already been reported (Setty et al. 1977; Reddy et al. 1975). Therefore, L-cysteine was used in an experiment with *M. exigua*-inoculated coffee plants. At concentrations 19.2 times greater than the LC_{50}, aldicarb (500 μg ml^{-1}) reduced galls and eggs per root of coffee plants more efficiently than the amino acid (5,442 μg ml^{-1}). L-cysteine only gave values statistically equal to those obtained with aldicarb when its concentration was increased to 8,490 μg ml^{-1}. Nevertheless, no statistical difference was observed between the two lowest amino acid concentrations (Table 4).

In conclusion, universally distributed amino acids are at least partially responsible for the nematicidal activity observed for the crude metabolites produced by *P. macerans* in tryptic soy broth. Thus, the use of protein or peptide in culture media for screening protocols to select nematicide-producing bacteria must be carefully planed to avoid amino acids interference. Although most of these substances show only very weak activity against *M. exigua*, an involvement of amino acids in modes of action in plants and microorganisms against nematodes cannot be rejected, especially for L-cysteine that shows a LC_{50} value against *M. exigua* approximately 10 times higher than that for the commercial nematicide aldicarb, and can significantly reduce the population of this nematode in coffee plants. Consequently, further studies should be carried out to understand the role of amino acids in plant protection against nematodes.

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