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3-Hydroxypropionic acid as a nematicidal principle in endophytic fungi

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This paper is dedicated to Prof. Dr. H. Zähner on the occasion of his 75th birthday

Abstract

3- Hydroxypropionic acid was isolated by bioactivity-guided fractionation of extracts obtained from submerged cultures of several endophytic fungi isolated from above-ground plant organs. This compound showed selective nematicidal activity against the plant-parasitic nematode *Meloidogyne incognita* with LD_{50} values of 12.5–15 μg/ml. Activity against the saprophytic *Caenorhabditis elegans* was fivefold lower. No antimicrobial, cytotoxic or phytotoxic effects were observed. Propionic acid and D- and L-lactic acids were not active against either nematode species. Based on morphological features and ITS, 18S and 28S rDNA analyses, the producing strains were identified as *Phomopsis phaseoli* isolated from the leaf of a tropical tree, and four strains of *Melanconium betulinum* isolated from twigs of *Betula pendula* and *B. pubescens* in Germany. This is the first report of 3-hydroxypropionic acid in fungi, and of the nematicidal activity of this metabolite.

Keywords: *Diaporthe phaseolorum*; *Phomopsis phaseoli*; *Melanconis stilbostoma*; *Melanconium betulinum*; *Meloidogyne incognita*; Endophyte; Nematicidal activity; 3-Hydroxypropionic acid

1. Introduction

Based on host type, two kinds of endophytic fungi can be distinguished, viz. those infecting grasses (Graminеae) and those infecting other plants. Graminaceous endophytes are particularly well characterized for their production of toxic substances, especially alkaloids, which deter both insect and mammalian herbivores (Breen, 1994; Leuchtmann and Clay, 1997). The toxins can be isolated from the host-endophyte symbiome and in some cases also from pure cultures of the endophyte (Siegel and Bush, 1997; Bacon and White, 2000; Tkacz, 2000). The endophytes of grasses belong to a narrow taxonomic group of fungi, the Clavicipitaceae, which infect mainly above-ground organs of their host. In addition to reduced above-ground herbivory, endophyte-infected grasses can show significant protection against root damage by plant-parasitic nematodes (Kimmons et al., 1990). Further, the presence of endophytes in plant shoots can inhibit root infection by mycorrhizal fungi and fungal plant pathogens (see Latch, 1998). It is as yet unclear whether these below-ground effects are due to translocated toxins or other effects such as induced systemic resistance, or a combination

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of both (Bultman and Murphy, 2000). However, it is at least conceivable that a host plant can benefit from nematicidal compounds produced by an endophyte of aerial plant organs, and translocated into the roots.

Information in the literature about nematicidal metabolites from endophytes is scarce, especially concerning non-graminaceous endophytes. Pregaliellalactone and structurally related lactones seem to be the only compounds with nematicidal activity known from non-graminaceous endophytes and related saprophytic ascomycetes (Köpcke et al., 2002a,b). Therefore, a screening of endophytic fungi for the production of compounds active against the root-knot nematode *Meloidogyne incognita* (Kofoid and White) Chitwood was conducted. Bearing in mind a potential application of active substances as selective and ecologically safe nematicides, bioassay-guided purification of nematicidal substances was attempted preferentially from extracts containing no or only weak cytotoxic, phytotoxic or antimicrobial activities. The present paper describes the production, isolation and characterization of one such nematicidal principle, 3-hydroxypropionic acid (3-HPA), from several endophytes.

2. Results and discussion

2.1. Isolation and identification of 3-HPA

Some 500 strains of endophytic fungi were isolated from plants collected in temperate (near Buxtehude, Northern Germany), mediterranean (Dép. Var, Southern France; Tempio Pausania, Sardinia) and tropical climates (near Les Nourages, French Guyana). All isolates were screened for antimicrobial, cytotoxic, phytotoxic and nematicidal activities. Among these, 17 strains exhibited purely nematicidal activities, most of which were preferentially located in the mycelial extracts. Details of their origin and accession numbers are listed in Table 1. In order to avoid the isolation of long-chain fatty acids with nematicidal activity which are mainly located in the mycelium (Stadler et al., 1993; Anke and Sterner, 1997, 2002), those five strains with the highest activities in culture filtrate extracts were chosen for purification of the active compounds.

Following optimization of culture conditions, the highest nematicidal activity was obtained in YMG medium in a stirred aerated 20-l fermentor with strains E00145, E00153 and E01036, and in shaken 5-l Erlenmeyer flasks with 2 l medium for E99382 and E01051. Bioassay-guided purification as described in Section 3.2 revealed the same active substance in all five strains, at yields of 4.6 (E99382), 21 (E00145), 60 (E00153), 500 (E01036) and 100 (E01051) mg/l liquid culture.

The structure of the nematicidal principle was determined as 3-hydroxypropionic acid (3-HPA) by NMR spectroscopy (500 MHz for ^{1}H and 125 MHz for ^{13}C NMR) in $CDCl_3$ containing 5% CD_3OD (with the $CHCl_3/CDCl_3$ signals at 7.26 and 77.0 ppm, respectively, as reference). Only two signals appearing as triplets (both with the coupling constant $J = 6.2$) and with the same intensity could be observed at 4.61 and 2.95 ppm in the ^{1}H NMR spectrum, while the ^{13}C NMR spectrum contained three signals at 30.8, 69.8 and 171.7 ppm. 2D experiments showed that the two triplets coupled with each other (COSY), that the protons giving rise to the signals at 4.61 and 2.95 ppm were attached to the carbons which gave the signal at 69.8 and 30.8 ppm, respec-

Table 1
Nematicidal activity towards *M. incognita* of culture filtrate and mycelial extracts from 17 endophytic fungi

Strain and host origin	Activity LD_{90} (μl/ml)	
	Culture filtrate extract	Mycelial extract
E99048 from needle of *Abies procera* (N. Germany)	250	50
E99052 from two-year-old bark of *A. procera* (N. Germany)	>250	125
E99172 from shoot, *Asparagus officinalis* (S. France)	250	250
E99191 from inflorescence of *Pistacia terebinthus* (S. France)	>250	125
E99192 from leaf of *P. terebinthus* (S. France)	250	50
E99196 from leaf of *Prunus spinosa* (S. France)	>250	250
E99218 from leaf of *Acer* sp. (S. France)	250	50
E99233 from one-year-old twig, *Abies nordmanniana* (N. Germany)	>250	125
E99270 from twig of *Quercus suber* (Sardinia)	>250	250
E99382 from leaf of rainforest tree (French Guyana)	25	50
E99385 from leaf of rainforest tree (French Guyana)	>250	50
E99389 from leaf of rainforest tree (French Guyana)	>250	125
E99390 from leaf of rainforest tree (French Guyana)	250	50
E00145 from one-year-old twig of *Betula pendula* (N. Germany)	2.5	25
E00153 from three-year-old twig of *B. pendula* (N. Germany)	25	>250
E01036 from one-year-old twig of *B. pubescens* (N. Germany)	5	20
E01051 from one-year-old twig of *B. pubescens* (N. Germany)	10	20

The amount (μl) needed to kill 90% (LD_{90}) of the nematodes in 48 h is given.

tively (HMQC), and that the carbon at 171.7 ppm was not protonated. In a HMBC experiment, both proton signals gave long-range correlations to each other's carbons as well as to the carbon at 171.7 ppm. In the IR spectrum, signals for OH (3425 cm^{-1}) and CO (1705 cm^{-1}) were seen. These data permit the unequivocal identification of 3-HPA. The purity of 3-HPA was confirmed by HPLC chromatography showing only one peak.

This is the first report on the occurrence of 3-HPA as a secondary metabolite in fungi, and on its nematicidal activity. 3-HPA is known from some bacteria capable of CO_2 fixation, e.g. the phototrophic green non-sulphur *Chloroflexus aurantiacus* or the autotrophic *Metallosphaera sedula* and other Crenarchaeota, in which the 3-hydroxypropionic acid cycle replaces the Calvin cycle responsible for CO_2 fixation in cyanobacteria and plants (Menendez et al., 1999; Herter et al., 2001).

2.2. Biological activity of 3-HPA

When living nematodes were incubated directly with purified 3-HPA, this substance showed at least fivefold higher activity against L2 larvae of the plant-parasitic species *M. incognita* than against adult saprophytic *Caenorhabditis elegans* (Maupas) Dougherty (Table 2). Dead *M. incognita* larvae were stiff, turgid and straight, but showed no obvious fine structural abnormalities as compared to living control larvae (Fig. 1). The structurally related D- and L-lactic acids (LA) and propionic acid (PA) were also tested but showed no activity against *M. incognita*, although both LA stereoisomers were weakly active against *C. elegans* with LD_{50} values of 125 μg/ml. In the migration test, in which L2 larvae of *M. incognita* had to move across a Petri dish to reach the roots of lettuce seedlings, at 50 μg 3-HPA/ml no larvae had reached the roots after 48 h, whereas in the control experiment without 3-HPA 32% of the larvae were found at the plant roots.

Neither 3-HPA nor the other acids showed any antibacterial or antifungal activity in the agar diffusion assay at 100 μg/disk. No cytotoxic activity at concentrations up to 100 μg/ml was observed with any of the cell lines tested. Phytotoxic activity towards

Fig. 1. Living L2 larva of *M. incognita* incubated in water without 3-HPA (left) and larva killed after 12 h incubation in 50 μg 3-HPA/ml water (right). Bar = 50 μm.

Setaria italica (L.) P. Beauv. or *Lepidium sativum* L. was not observed for 3-HPA and D- or L-LA at concentrations up to 300 μg/ml. At higher concentrations (600 μg 3-HPA/ml), the length of the roots of *S. italica* was reduced by 80% while the shoots were not affected. PA (same concentration) reduced the root length by 80% and the shoot length by 50%.

2.3. Identification of the producers

Strain E99382 (Fig. 2) was isolated from the leaf tip of a broad-leaved tree near Les Nourages (French Guyana). On YMG agar it developed black stromata bearing pycnidia and one to several perithecial necks. Within the pycnidia, filamentous β-conidia (23.0–28.0×1.0–1.2 μm) were produced, identifying this isolate

Table 2
Nematicidal activity of 3-HPA, L- and D-lactic acids (L-LA and D-LA), and PA in comparison with ivermectin against *M. incognita* and *C. elegans* after 24 h

Compound	*M. incognita*		*C. elegans*	
	LD_{90} (24 h) (μg/ml)	LD_{50} (24 h) (μg/ml)	LD_{90} (24 h) (μg/ml)	LD_{50} (24 h) (μg/ml)
3-HPA	35–50	12.5–15	>150	75
L-LA	>150	>150	>150	125
D-LA	>150	>150	>150	125
PA	>150	>150	>150	>150
Ivermectin	1.25–2.5	Not tested	0.25–0.5	Not tested

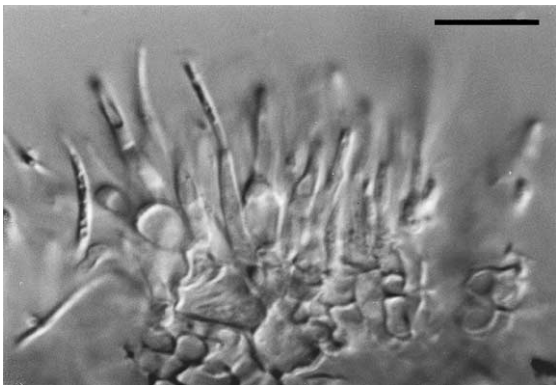

Fig. 2. Microscopic features of E99382 (*P. phaseoli*). Fertile layer dissected from a pycnidial stroma (four week on YMG) with phialides producing elongated β-conidia. Bar = 10 μm.

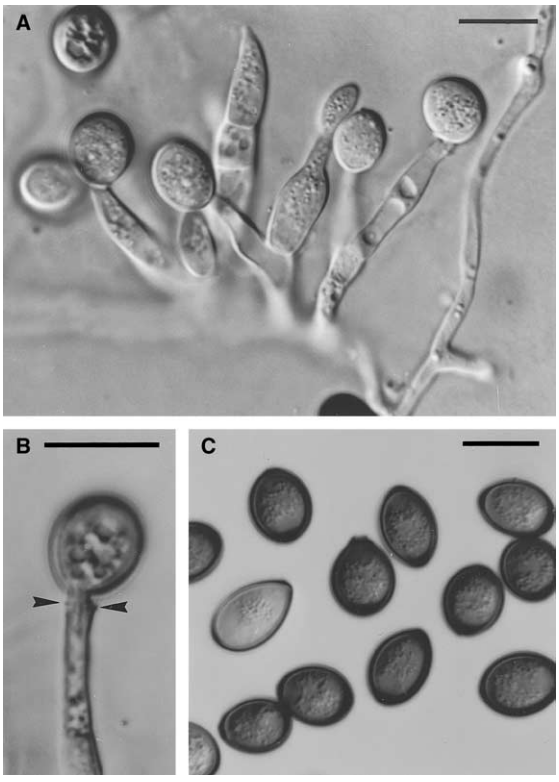

Fig. 3. Microscopic features of E01051 (*M. betulinum*). (A) Conidiophores dissected from a black acervulus (CMA, 2 wk). (B) Detail of conidiophore apex. The arrowheads indicate annellations. (C) Mature conidia with melanized walls and slightly truncated ends marking the former point of attachment to the annellide. Bars = 10 μm.

as a member of the genus *Phomopsis*. Given the notoriously variable appearance of *Phomopsis* spp. in culture (Kulik, 1984), a GenBank search with the nuclear ribosomal ITS sequence of E99382 (602 nucleotides; GenBank accession number AY577815) was carried out. The results revealed several species of *Phomopsis* or their teleomorphs (*Diaporthe*) as the closest matches, with sequence identities of the 10 closest hits ranging from 99.2% to 92.1%. The most similar sequence was that of *D. phaseolorum* var. *sojae* (S.G. Lehman) Wehmeyer obtained by Zhang et al. (1998) from Brazil (AF001020), followed by several other sequences of *D. phaseolorum* (Cooke and Ellis) Sacc. Considerable ITS sequence variations exist within this taxon which may, in fact, be a species complex (Zhang et al., 1998). On the basis of its ITS sequence, E99382 clearly belongs to the *D. phaseolorum* (anamorph: *Phomopsis phaseoli* (Desmaz.) Sacc.) complex.

Strains E00145 and E00153 were isolated from twigs of *Betula pendula* Roth, and E01036 and E01051 from *B. pubescens* Ehrh., growing near Buxtehude, Northern Germany. These four strains were indistinguishable in their colony characteristics on YMG agar, producing black acervuli containing one-celled ellipsoidal or irregularly elongated chlamydospore-like conidia (9.5–14.0×6.0–8.5 μm) which had a thick smooth melanized wall with a basal scar and were produced from annellidic structures (Fig. 3). These features correlated well with the description of *Melanconium* spp. by Grove (1937) and Sutton (1964), especially the *M. betulinum* Schumach. and Kunze/*M. bicolor* Nees species complex (Grove, 1937). Confirmation of species identification was sought by means of their ITS and partial 18S and 28S rDNA sequences which differed between E01051 and the other three strains as shown in Table 3. A meaningful GenBank search was possible only with the partial 28S rDNA sequences for which *Melanconis stilbostoma* (Fr.) Tul. and C. Tul. (AF408374) was the closest match (99.7% identity for E01051, 100% for the other three strains), followed by other, related *Melanconis* spp. (see Castlebury et al., 2002). These search results therefore confirmed the microscopic species identification because *M. stilbostoma* is the teleomorphic state of *Melanconium betulinum* and *M. bicolor* (Grove, 1937).

The five endophytes producing 3-HPA thus belonged to two different families (Diaporthaceae and Melanconidaceae), both of which are firmly placed within the Diaporthales (Ascomycota) according to Castlebury et al. (2002).

2.4. Ecological considerations

P. phaseoli is known as the causal agent of pod and stem blight in legumes, but this fungus is also capable of living as a symptomless endophyte for prolonged periods, sporulating only on dying or dead plant material (Kulik, 1984). Similarly, *M. betulinum* and other *Melanconium* spp. are known as endophytes and as causal agents of diebacks and cankers of various broad-leaved trees, including *Betula* spp. (Sieber et al., 1991; Belisario, 1999; Elamo et al., 1999). All five 3-HPA producers thus have to be placed within the diffuse boundary zone between the ecological concepts of endophytism and phyto-

Table 3
GenBank accession numbers and details of the ITS, 18S and 28S rDNA sequences of the four *Melanconium* isolates obtained in the present study

Type of sequence	GenBank accession numbers		Differences
	E00145, E00153, E01036 [a]	E01051	
ITS (including primers ITS5 and ITS4)	AY577811 (624 nt [b])	AY577814 (623 nt)	13 nt
18S (including primers NS1 and SR4)	AY577809 (819 nt)	AY577812 (819 nt)	9 nt
28S (including primers SR0R and SR3)	AY577810 (619 nt)	AY577813 (619 nt)	2 nt

[a] The sequences of E00145, E00153 and E01036 were identical to each other.
[b] nt = nucleotides.

pathogenicity. Several species complexes of fungi, including *Phomopsis*, are known to contain both virulent and endophytic (avirulent) strains (e.g. Mostert et al., 2000), supporting the notion that the latter may arise from the former by deletion of pathogenicity determinants (Freeman and Rodriguez, 1993; Saikkonen et al., 1998).

Since 3-HPA has no antimicrobial, cytotoxic and only weak phytotoxic activities, it may well have passed unnoticed during previous screenings of endophytic fungi. Endophytes producing this substance may be rather common, and both *P. phaseoli* and *M. betulinum* are cosmopolitan. Further studies should examine the presence, concentration and possible translocation of this metabolite into roots of host plants artificially infected with 3-HPA-producing endophyte strains. To our knowledge, no thorough investigations have been performed as yet on the translocation of nematicidal or insecticidal substances of fungal origin from infected shoots into roots and their anti-feeding effects on root-attacking pests.

3. Experimental

3.1. Organisms and culture conditions

Endophytes were isolated from surface-sterilized living plant material as described by Köpcke et al. (2002a), cultivated on YMG, and incorporated into the Culture Collection (Department of Biotechnology, University of Kaiserslautern). For initial screening, fungi were inoculated into 1000 ml flasks with four indentations, containing 500 ml YMG medium (4 g yeast, 10 g glucose, 10 g malt extract/l; pH 5.5 before autoclaving) and incubated at 22 °C with shaking (120 rpm). The culture fluid was separated from the mycelium by filtration through filter paper (No. 595; Schleicher and Schuell, Dassel, Germany) using a Büchner funnel. Culture fluid and mycelium were extracted separately with ethyl acetate and methanol, respectively. After evaporation of these solvents, the samples were dissolved in methanol to give a 100-fold volumetric concentration relative to the original culture.

Fermentations were carried out in a 20 l fermentor (Biolafitte C6) containing YMG medium with aeration (3.3 l air/min) and stirring (120 rpm) at 22 °C. A well-grown culture (200 ml in a 500 ml Erlenmeyer flask incubated on a rotary shaker at 22 °C) in the same medium was used as inoculum. Depending on the duration of the fermentation, 50 ml samples were taken daily or at longer intervals. The presence of glucose was assessed with Diabur-Test 5000 test strips (Roche Diagnostics, Mannheim, Germany). The production of nematicidal compounds was followed using the microtitre plate assay as described below. Ethyl acetate extracts of 50 ml aliquots of the culture filtrate were dried over Na_2SO_4, followed by evaporation of the solvent and dissolution of the residue in 0.5 ml methanol. For each assay, a 30 μl aliquot of extract was used.

3.2. Isolation of the active compounds from cultures in shaken flasks and fermentors

After the complete consumption of the carbon source, the fermentation was stopped, and culture filtrate and mycelium were separated by filtration. The culture filtrate of strain E00145 (15 l) was extracted with 10 l ethyl acetate followed by evaporation of the organic phase until an oily extract was obtained. This crude extract (8.5 g) was subjected to chromatography on the size-exclusion resin Sephadex LH20 (Pharmacia, Uppsala, Sweden) in MeOH. Final separation was achieved by HPLC on Nucleogel GPC 50-10 in 2-propanol yielding 420 mg active compound. Strain E99382 showed the best production in shaken flask culture. Culture filtrate (9 l) was obtained from five 5-l Erlenmeyer flasks each containing 2 l medium, and was extracted with an equal volume of ethyl acetate. Upon concentration, the organic phase yielded 700 mg crude product which was further processed as above. The yield of 3-HPA was 25 mg. The isolation procedures for strains E00153, E01036 (20-l fermentation) and E01051 (5-l Erlenmeyer flask) were the same as above.

3.3. Chemical characterization of 3-hydroxypropionic acid

FT-IR spectra were recorded in KBr using a Perkin–Elmer Spectrum One spectrometer. ^{1}H and ^{13}C NMR data were obtained with a Bruker DRX500 spectrometer. HPLC analyses were performed using a HP

Series 1100 LCD-MSD (Hewlett-Packard) fitted with a LiChroCART Superspher 100 RP-18 column (125×2 mm; 4 μm particle size), applying a gradient from water to acetonitrile in 20 min at a flow rate of 0.8 ml/min.

3.4. Biological assays

Antimicrobial activity was determined by the agar diffusion assay as described previously (Anke et al., 1996). Test organisms were *Bacillus brevis* (ATCC 9999), *Micrococcus luteus* (ATCC 381), *Mucor miehei* (Tü 284), *Paecilomyces variotii* (ETH 114646) and *Nematospora coryli* (ATCC 10647). Spores or cells of these organisms were suspended in cooling agar, and filter paper disks (6 mm diameter) containing the test substances were added to the surface of the test plates, followed by incubation at 27 or 37 °C. Cytotoxic activities against HL 60 cells (ATCC CCL 240), HeLa S3 cells (ATCC CCL 2.2) and Colo-320 cells (DSMZ ACC 144) were determined according to Zapf et al. (1995). Inhibition of growth of germinating seeds of *Setaria italica*, *Lepidium sativum* and *Lactuca sativa* was tested as described by Anke et al. (1989). Tests for nematicidal activity were carried out as described by Stadler et al. (1993) for *C. elegans* (1000 nematodes/ml) and Mayer et al. (1996) for *M. incognita* (250 L2 larvae/ml). The test substances were placed as methanolic solutions in 24-well plates, and nematode suspensions were added following solvent evaporation. The widely used antihelminthic drug ivermectin (Sigma) was employed as a positive control. The migration assay with *M. incognita* (350 L2 larvae/test) and *Lactuca sativa* seedlings was carried out according to Lung (1989). Larvae were placed on one side of a 5.5 cm Petri dish containing an aqueous sludge of Sephadex G-150 with an appropriate concentration of 3-HPA, and were allowed to migrate to the opposite side of the dish seeded with three 48 h-old lettuce seedlings. The number of larvae adhering to the lettuce roots was counted after a further 48 h incubation at room temperature.

3.5. Identification of fungi

For microscopic examination, the endophyte strains were grown at 22 °C on YMG agar and cornmeal agar (Difco, Detroit, USA). The methods and reagents for DNA extraction and amplification were essentially as described by Köpcke et al. (2002a). The primers used for ITS amplification were ITS5 (5′-GGAAGTAAAAGTCGTAACAAGG) and ITS4 (5′-TCCTCCGCTTATTGATATGC) according to White et al. (1990). For the amplification of a 5′ segment of the 18S rDNA gene, primers NS1 (5′-GTAGTCATATGCTTGTCTC) and SR4 (5′-AAACCAACAAAATAGAA) were used (White et al., 1990; http://www.biology.duke.edu/fungi/mycolab). A 5′ segment of the 28S rDNA gene was amplified with the primers LR0R (5′-GTACCCGCTGAACTTAAGC) and LR3 (5′-CCGTGTTTCAAGACGGG) described by Vilgalys and Hester (1990). Deviations from the protocol of Köpcke et al. (2002a) were that a GeneAmp® PCR System 9700 was employed (Applied Biosystems, Foster City, CA, USA) and that the PCR amplification cycle consisted of 30 s at 94 °C, 1 min at 50 °C (ITS and 28 S) or 38 °C (18S), and 1 min at 72 °C. PCR products were sequenced externally by MWG Biotech (Ebersberg, Germany) using the same primers as for amplification. Each sequence was obtained in duplicate from each of two separate PCR amplifications. Database searches were performed with the FASTA function of the GCG® Wisconsin® Package.

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