3-Methylthiopropionic Acid Produced by Enterobacter intermedium 60-2G Inhibits Fungal Growth and Weed Seedling Development


*Division of Applied Plant Science, Agricultural Plant Stress Research Center,
**Department of Food Technology, Institute of Biotechnology,
***Department of Agricultural Chemistry, Agricultural Plant Stress Research Center,
College of Agriculture and Life Sciences, Chonnam National University,
Gwangju 500-757, Korea

(Received for publication October 18, 2002)

A wide range of soil microorganisms produce allelopathic and antimicrobial metabolites1). We isolated from the rhizosphere of grasses a phosphate-solubilizing bacterium Enterobacter intermedium 60-2G that possesses several biological functions with potential uses in agriculture5). In this paper, we identify an antimicrobial substance from this bacterium and characterize its inhibitory effects on growth of fungi and weed seedlings.

To assess the antimicrobial activity of E. intermedium against plant pathogenic fungi, cubes of mycelium were inoculated into the center of PDA agar plates. The plates were incubated 28°C for 2-5 days until the fungal mycelium covered 30% of the plate. Ten µl of E. intermedium suspension (10⁹cfu/ml) was spotted around the fungal mycelium and incubated for 2-3 days further at 28°C. After co-incubation, the inhibition zone around the bacterial spot was measured. E. intermedium inhibited mycelial growth of phytopathogenic fungi; Didymella bryoniae, Pythium dissotacum, Pythium irregulare, Fusarium oxysporum, Rhizoctonia solani, Chaetomium globosum, Magnaporthe grisea, and Monosporascus cannonhallus (data not shown).

To isolate and identify the main compound responsible for antimicrobial activity of E. intermedium against plant pathogenic fungi, cubes of mycelium were inoculated into the center of PDA agar plates. The plates were incubated 28°C for 2~5 days until the fungal mycelium covered 30% of the plate. Ten µl of E. intermedium suspension (10⁹cfu/ml) was spotted around the fungal mycelium and incubated for 2~3 days further at 28°C. After co-incubation, the inhibition zone around the bacterial spot was measured. E. intermedium inhibited mycelial growth of phytopathogenic fungi; Didymella bryoniae, Pythium dissotacum, Pythium irregulare, Fusarium oxysporum, Rhizoctonia solani, Chaetomium globosum, Magnaporthe grisea, and Monosporascus cannonhallus (data not shown).

Isolate and identify the main compound responsible for antimicrobial activity of E. intermedium 60-2G, the supernatant of E. intermedium acidified to pH 3.0 with 1 M HCl was extracted with ethyl acetate (EtOAc-soluble acidic extract). The ethyl acetate-soluble acidic extract showed antimicrobial activity against above plant pathogenic fungi. The EtOAc-soluble acidic extract (2.045 g) was chromatographed on a column of Sephadex LH-20 with MeOH-HCl (4:1, v/v). Antifungal activity was found in fractions eluting with a Ve/Vt (elution volume/total volume) of 0.66~0.73. The active fraction (448 mg) to the silica gel adsorption column, eluted with 100% MeOH and reapplied to a silica gel column with elution by a hexane (10): ethyl acetate (4): MeOH (1) mixture. The material eluted at hexane:EtOAc:methanol (10:4:1, v/v, 164.9 mg) was further fractionated on an ODS column using stepwise elution with an increasing gradient of MeOH in H₂O. The material eluted at 40% MeOH/water eluate had antimicrobial activity and was refractionated using MeOH/water (v/v) at 36, 38, 40, 42, and 44%. Further purification by HPLC with Mundapak C₁₈ (30% MeOH) produced a colorless oil with high polarity and antibacterial activity (Fig. 1A).

The active substance in this purified fraction was identified as 3-methylthiopropionic acid (3MTPA) according to results obtained by MS, ¹H- and ¹³C-NMR, HMBC, and ¹H-¹H COSY (Fig. 2B). The EI-MS spectrum of the active substance had a molecular weight of m/z 120 (M⁺, 32.4%) and fragment ions of 105 (M⁺-CH₃, 2.7%), 74 (M⁺-SCH₃, 13.5%), 61 (M⁺-SCH₂, 100%), 47 (M⁺-SCH₃, 12.2%), 45 (M⁺-COOH, 13.5%). ¹³C-NMR spectrum (100MHz, CD₃OD, TMS) analysis showed four carbon signals; δ 176.1 (-COOH), 35.5 (C-2), 30.1 (C-3), and 15.3 (-SCH₃). ¹H-NMR (400MHz, CD₃OD, TMS) showed three proton signals of δ 2.71 (2H, t, J=7.26Hz, H-3), 2.09 (3H, s, -SCH₃) (Fig. 1B).

The molecular formula of the active substance was deduced from the LC-EI-MS and ¹³C-NMR data to be C₄H₈O₃S. The ¹H-NMR spectrum showed signals corresponding to thiomethyl proton at δ 2.1 (H, s, -SCH₃), and two methylene protons at δ 2.71 (3H, t, J=7.26 Hz, H-3) and 2.09 (3H, t, J=7.26 Hz, H-3). The presence of cross peaks between H-3 and -SCH₃ was observed in a HMBC spectrum and the connection of C₂ to C₃ was indicated in ¹H-¹H COSY spectrum. To our knowledge this is the first account of 3MTPA production from E. intermedium.

3-MPTA is a known metabolite of many pathovars of Xanthomonas campestris being produced from methionine4,6,7). Even though 3-MPTA from X. campestris pathovars was toxic to plants, this compound was not considered as having a key role in pathogenesis because...
Fig. 1. HPLC chromatography on ODS column of the active fraction (A) and structure of 3-methylthiopropanoic acid (B) from *E. intermedium*.

(A) Column: μ Bondapak C18, Detection : 254 nm, Mobile phase L: 30% MeOH, and Flow rate: 1 ml/minute. (B) Direct-EI-MS spectrum of the active substances isolated from *E. intermedium* and structure of 3-methylthiopropanoic acid (C₄H₈O₂S) as deduced from analysis with MS, ¹H- and ¹³C-NMR, HMBC, and ¹H-¹H COSY techniques.

Low levels of methionine were detected in the host tissue⁹,¹⁰). In our studies, we examined whether 3-MTPA would effect seedling development. Weed seeds were surface sterilized by soaking in 3% NaOCl solution for 50 minutes followed by rinsing with running water. Aliquots of EtOAc-soluble acidic extract (0, 3, 12, 18, 24, and 30 mg) were applied to discs (diameter 2.5 mm) and the solvent
was removed by volatilization under laminar flow air stream. The discs were placed on the water-1% agar plates and the seeds were placed on the disc. The petri dishes were placed in a growth chamber at 30°C for seven days. Daily measurements of shoot and root length showed severe inhibition of seed germination and root and shoot development at concentrations above 12mg for partially purified extracts.

Inhibition of root development was observed even at concentrations lower than 12mg/disc (Fig. 2). When purified 3-MTPA was tested with several weed species, the inhibitory effect was less than with the crude extract, suggesting that more active allelochemicals(s) were produced by E. intermedium. Generally allelopathy is caused by the movement of allelochemicals through the soil from the host to the root systems of the target plants2,8). Allelochemicals active against higher plants suppress seed germination, causing injury to root growth and other meristems to inhibit seedling growth3). Our observed inhibition of root elongation by crude extracts from E. intermedium and purified 3MTPA is consistent with the effect of an allelochemical as discussed by CHENG2).

We confirmed that the purified 3-MTPA had antifungal activity in assays using F. oxysporum as a model fungus. A concentration series of the purified 3-MTPA in 200μl of sterile potato dextrose broth (Difco Laboratories, Detroit, MI. USA) in 96 well plates was inoculated with 10μl of Fusarium oxysporum (1×10⁶ conidia/ml). At defined times, growth of F. oxysporum was determined by measuring optical densities of 600nm in an ELISA reader.
optical densities at 600 nm in an ELISA reader apparatus. The 3MTPA strongly inhibited the growth of *F. oxysporum* with an LD<sub>50</sub> of about 50 μg (Fig. 3).

The results of our studies suggest that *E. intermedium* 60-2G is a promising candidate for control of both fungal diseases and growth of weedy seedlings. Such use of the beneficial bacterium would reduce the applications of chemical fungicides and herbicides that may be hazardous to the environment and which with time may become ineffective because of the targets developing resistance. Microorganisms such as *E. intermedium* 60-2G that colonize the rhizosphere and excrete beneficial metabolites are ideal for use as biocontrol agents. Their presence in the rhizosphere provides front-line defense for roots against attack by pathogens and delivery of allelochemicals into the weed root system.

Acknowledgement

This paper is dedicated to Dr. KI-YOUNG SEONG who passed away on January 27, 2002. This work was supported by grant R11-2001-092-01005-0 from the Korea Science and Engineering Foundation through the Agricultural Plant Stress Research Center (APSRC) at Chonnam National University.

References