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

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A wide range of soil microorganisms produce allelopathic and antimicrobial metabolites[1]. We isolated from the rhizosphere of grasses a phosphate-solubilizing bacterium *Enterobacter intermedium* 60-2G that possesses several biological functions with potential uses in agriculture[5]. 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 $\mu$l of *E. intermedium* suspension ($10^9$ cfu/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 irregulrare*, *Fusarium oxysporum*, *Rhizoctonia solani*, *Chaetomium globosum*, *Magnaporthe grisea*, and *Monosporascus canonballus* (data not shown).

To 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-$CHCl_3$ (4:1, v/v). Antifungal activity was found in fractions eluting with a $V_e/V_t$ (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 $H_2O$. 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 $\mu$Bondapak $C_{18}$ (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, $^1$H- and $^{13}$C-NMR, HMBC, and $^1$H-$^1$H 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 ($M^+-CH_3$, 2.7%), 74 ($M^+-SCH_3$, 13.5%), 61 ($M^+-SCH_2$, 100%), 47 ($M^+-SCH_3$, 12.2%), 45 ($M^+-COOH$, 13.5%). $^{13}$C-NMR spectrum (100 MHz, $CD_3OD$, TMS) analysis showed four carbon signals ; $\delta$ 176.1 (–COOH), 35.5 (C-2), 30.1 (C-3), and 15.3 (–SCH3). $^1$H-NMR (400 MHz, $CD_3OD$, TMS) showed three proton signals of $\delta$ 2.71 (2H, t, $J$=7.26 Hz, H-3), 2.57 (2H, t, $J$=7.26 Hz, H-2), and 2.09 (3H, s, $-SCH_3$) (Fig. 1B).

The molecular formula of the active substance was deduced from the LC-EI-MS and $^{13}$C-NMR data to be $C_4H_8OS$. The $^1$H-NMR spectrum showed signals corresponding to thiomethyl proton at $\delta$ 2.1 (H, s, $-SCH_3$), and two methylene protons at $\delta$ 2.71 (3H, t, O=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 $-SCH_3$ was observed in a HMBC spectrum and the connection of $C_2$ to $C_3$ was indicated in $^1$H-$^1$H 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 methionine[4,6,7]. 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

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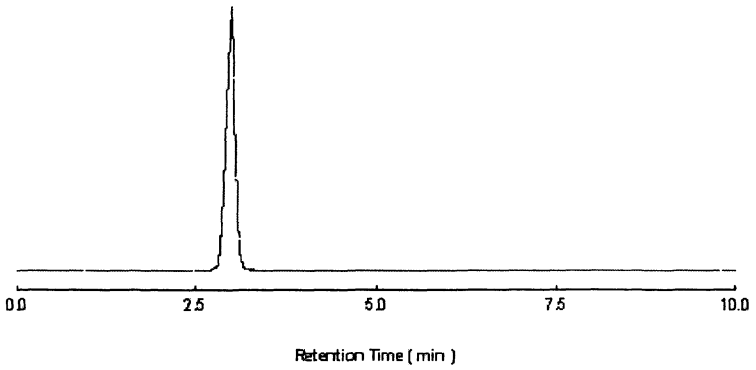
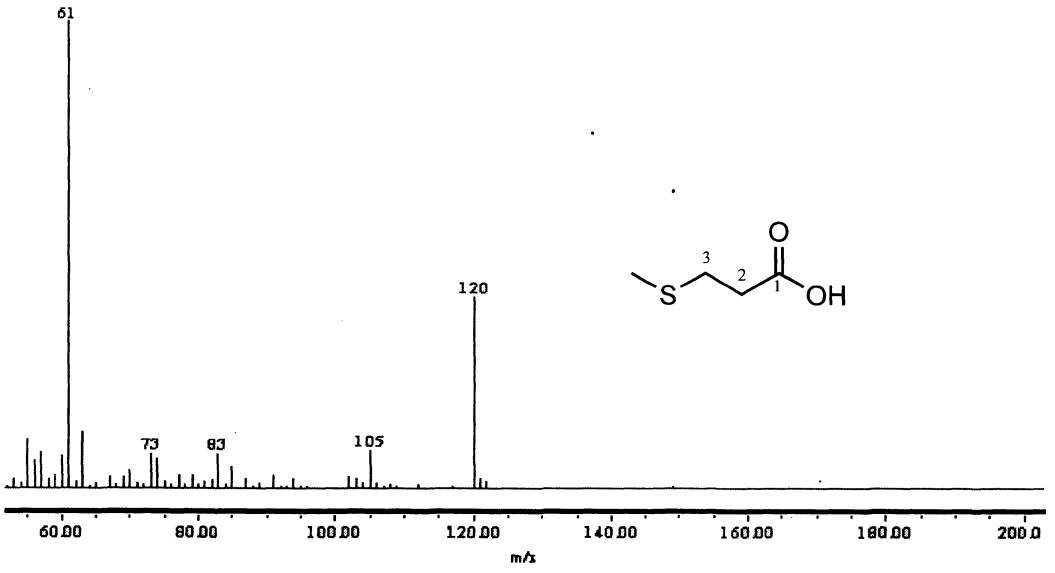
Fig. 1. HPLC chromatography on ODS column of the active fraction (A) and structure of 3-methylthiopropanoic acid (B) from *E. intermedium*.

A.




B.

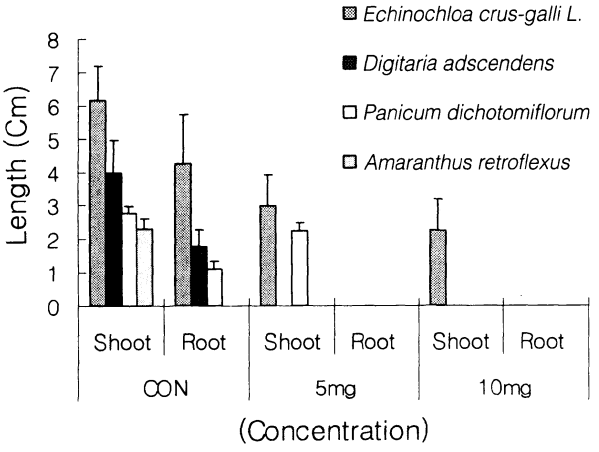



(A) Column: $\mu$ Bondapak C18, Detection : 254 nm, Mobile phase L: 30% MeOH, and Flow rate: 1 ml/minute. (B) Direct-EI-MS spectrum of the active substances isolated from *E. intermedium* and structure of 3-methylthiopropanoic acid ($C_4H_8OS$) as deduced from analysis with MS, $^1$H- and $^{13}$C-NMR, HMBC, and $^1$H-$^1$H COSY techniques.

low levels of methionine were detected in the host tissue[9,10].

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

Fig. 2. Effect of EtOAC-soluble acidic extract from the strain 60-2G on growth of common weeds.



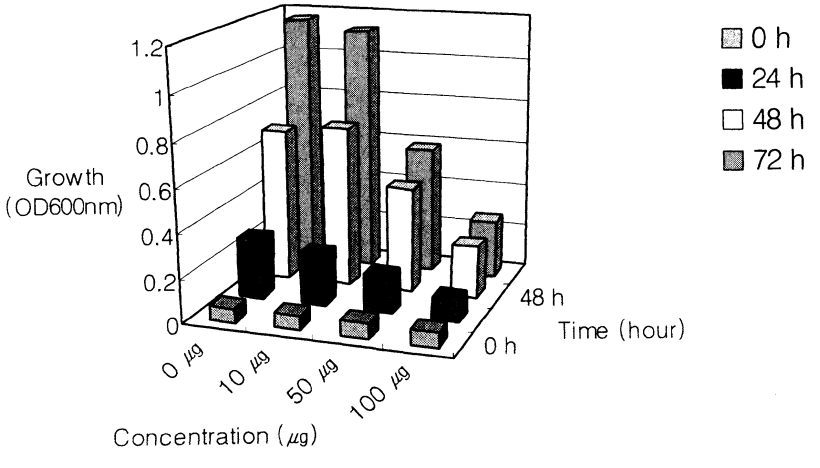

From 5-liter of cell cultures grown in LB, 2.599 g of soluble acidic fraction of EtOAc was obtained. The crude soluble acidic fraction was assayed on water agar plate with common weeds. Aliquots of EtOAc - soluble acidic extract (0, 5, and 10 mg/disc) were applied to discs (diameter 2.5 mm) and the solvent was removed by volatilization under laminar flow air stream. The discs were placed on the water 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.

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 12 mg for partially purified extracts.

Inhibition of root development was observed even at concentrations lower than 12 mg/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 plants[2,8]. Allelochemicals active against higher plants suppress seed germination, causing injury to root growth and other meristems to inhibit seedling growth[3]. 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 CHENG[2].

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 200 $\mu$l of sterile potato dextrose broth (Difco Laboratories, Detroit, MI. USA) in 96 well plates was inoculated with 10 $\mu$l of *Fusarium oxysporum* ($1\times10^6$ conidia/ml). At defined times, growth of *F. oxysporum* was determined by measuring

Fig. 3. Effect of 3-MTPA on growth of *Fusarium oxysporum*.




Different concentrations of the purified 3-MTPA were added in 200 $\mu$l of sterile potato dextrose broth in 96 well plates and 10 $\mu$l of *F. oxysporium* ($1\times10^6$ conidia/ml) was added in each well. At defined times, the growth of *F. oxysporium* 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 $LD_{50}$ of about 50 $\mu$g (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.

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