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Ovicidal Activity of Lactic Acid Produced by *Lysobacter capsici* YS1215 on Eggs of Root-Knot Nematode, *Meloidogyne incognita*

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Lysobacter capsici YS1215 isolated from soil previously showed nematicidal potential for biological control of the root-knot nematode. In this study, lactic acid, a nematicidal compound, was isolated from culture filtrate of YS1215, and its ovicidal activity was investigated. Purification and identification of lactic acid were performed by a series of column chromatographies and identified by ^{1}H and ^{13}C NMR spectra and GC-MS analysis. Our results showed that bacterial culture filtrate containing lactic acid significantly inhibited egg hatching. The lowest egg hatch rate (5.9%) was found at a high concentration (25 µl/ml) of lactic acid at 5 days after incubation, followed by 20 (15.2%), 15 (23.7%), 10 (29.8%), and 5 (36.4%) µl/ml, while egg hatching in the control (sterile distilled water) was 44.5%. This is the first report of lactic acid as an ovicidal compound, and it may be considered as an alternative of chemical pesticide against root-knot nematodes.

Keywords: Lactic acid, *Lysobacter capsici* YS1215, bacterial culture filtrate, crude extract, egg hatch

Introduction

In agricultural crops cultivated worldwide, root-knot nematodes, *Meloidogyne* spp., are obligate plant-parasitic nematodes that infect over 2,000 plant species ranging from grasses to trees, and obtain nutrients from their host plants [5]. Because of this characteristic of the nematodes, they cause severe damage to a wide variety of crops, and lead to significant economic losses of a wide variety of crops that have been estimated to be approximately 78 billion dollar worldwide annually [24]. Chemical control of *Meloidogyne* spp. has been commonly used over the past decades. However, there are current limitations to application of soil nematicides, which are costly and harmful to the environment and human health [14]. For example, dibromochloropropane and ethylene dibromide have been withdrawn from the global market owing to their possible detrimental effects on humans and the environment [15]. Therefore, alternative control measures are necessary for the management of *Meloidogyne* spp. Biological control is a potential option against the root-knot nematodes instead of use of chemical nematicides, because it is an environmentally safe and feasible approach. Microorganisms are the main means for managing root-knot nematodes [18].

Microorganisms have been used as biocontrol agents against plant-parasitic nematodes [9]. They can directly affect the nematodes in several ways, such as in the production of nematicidal metabolites that suppress egg hatching and the survival of second-stage juveniles (J2), as well as inducing direct death of nematodes [22, 25]. For example, *Pseudomonas fluorescens* strain CHA0 suppressed *M. javanica* by producing hydrogen cyanide [21]. Similarly, avermectins produced by *Streptomyces avermitilis* showed nematicidal activity toward plant-parasitic nematodes [2]. Propionic acid, 2-methylhexanoic acid, lactic acid, maleic acid, and sulfuric acid were shown to have nematicidal

activity against plant-parasitic nematodes [1]. Amino acids produced by *Paenibacillus macerans* and from commercial sources induced J2 mortality, and reduced the formation of root galls by *Meloidogyne exigua* [16].

Lysobacter spp. are gram-negative rod bacteria naturally found in soil, and have the feature of gliding motility. They easily colonize the plant roots through artificial inoculation, which renders biocontrol more efficiently [8]. The many *Lysobacter* species have been recently recognized as biocontrol agents for different nematodes and fungal pathogens [7, 23]. For example, the egg hatching and juvenile mobility of *Heterodera schachtii* were inhibited by *L. enzymogenes* strain C3 [4]. However, there have been no reports so far on information of their nematicidal compound. *L. capsici* YS1215 was previously isolated from soil and showed antifungal activity [11]. The present study was based on the investigation of egg hatch inhibition of *Meloidogyne incognita* by bacterial culture filtrate and a purified nematicidal compound produced by *L. capsici* YS1215 under *in vitro* conditions.

Materials and Methods

Preparation of Nematode Eggs

Eggs of *Meloidogyne incognita* were collected from the roots of infested tomato plants (*Solanum lycopersicum*) that were previously infected with the nematodes in the glasshouse (23°C, with a light period of 16 h and watered manually once a day). The plants were uprooted and roots were gently washed free of soil with tap water and cut into short lengths and placed in a bottle for collecting the eggs. After shaking root pieces with 0.5% NaOCl solution in the bottle for 3 min, the solution was poured in overlapped sieves (Chung Gye Sang Gong Sa, Korea) having 45 μm and 25 μm pores, respectively. The eggs remaining on the bottom sieve (25 μm pores) were collected along with the washed distilled water [25].

Preparation of Bacterial Culture Filtrate

Lysobacter capsici YS1215 was cultured in a liquid medium (0.02% gelatin powder (Geltec, Korea), 0.08 % crab shell powder (Purne, Korea), 0.1% L-monosodium glutamate, 0.3% sucrose (Beksul, Korea), 0.14% KH_2PO_4, 0.1% $(NH_4)_2SO_4$, 0.1% $CaCO_3$, 0.03% $MgSO_4 \cdot 7H_2O$, 0.002% $CuSO_4 \cdot 5H_2O$, 0.002% $ZnSO_4 \cdot 7H_2O$, 0.003% $FeCl_3 \cdot 6H_2O$, and 0.1% yeast extract (w/v)) with shaking (140 rpm) at 30°C for 5 days. After incubation, the culture was centrifuged at 6,000 ×*g* for 10 min, and filtered through a Whatman No. 2 filter paper to remove large particles of the culture medium before using it to purify a nematicidal compound. Moreover, the supernatant of the culture was filtered through 0.2 μm Millipore filter papers to remove bacterial cells. This resulting bacterial culture filtrate (BCF) was used to measure egg hatch inhibition of *Meloidogyne incognita* in *in vitro* experiments.

Effect of BCF on Nematode Egg Hatching

Different volumes of BCF (50, 100, 150, or 250 μl) were placed into wells of a 24-well tissue culture plate (SPL Lifesciences, Korea) by a pipette, and 30 μl of egg suspensions (approx. 300 eggs) was added into each well. The eggs were counted under a stereoscopic microscope (Olympus SZX16, Japan) at 50× magnification. A total volume of 500 μl in the well plate was obtained by adding sterile distilled water (SDW), resulting in 10%, 20%, 30%, and 50% BCFs. The non-inoculated culture medium filtrate (CMF, 50%) and SDW were used as controls. The plates were then kept in the dark at 26°C. The egg hatching, obtained as described above, was observed after 2 and 5 days of exposures under the stereoscopic microscope. All experiments were performed with three replications.

Purification and Identification of Lactic Acid

When purifying lactic acid, the activity of fractions from all columns were checked by paper disc assay on PDA plates against the fast growing fungus *Rhizoctonia solani* AG-1 (IA) (KACC 40101). The strain *L. capsici* YS1215 was grown in a medium as described above at 30°C in a rotary shaker for 5 days. The culture broth (10 L) was centrifuged at 6,000 ×*g* for 15 min. The supernatant was extracted with ethyl acetate (EtOAc, 10 L). The EtOAc soluble organic fraction was concentrated by using a rotary evaporator (Büchi Labortechnik, Flawil, Switzerland), where 1.0 g of crude extract was obtained. The crude extract was dissolved in 5 ml a methanol (MeOH), and fractionated by 60 silica gel column chromatography (Merck, Darmstadt, Germany) with stepwise elution on an increasing concentration of $CHCl_3$-MeOH (100:0, 90:10, 80:20, 70:30, 60:40, 50:50, 40:60, 0:100 (v/v)). The 70% chloroform fraction, having strong antifungal activity, was further purified by Sephadex LH-20 column chromatography (1.5 × 30 cm, 25–100 mesh; Sigma-Aldrich, Steinheim, Germany) with elution on 100% methanol as a running phase. After the LH-20 run, an antifungal activity fraction was purified using 500 mg silica columns (Strata SI-1 silica, 55 μm; Phenomenex, Torrance, CA, USA) with stepwise elution on an increasing concentration of $CHCl_3$-MeOH (100:0, 80:20, 60:40, 40:60, 0:100 (v/v)). The 40% chloroform fraction obtained by the Strata SI-1 silica was analyzed by a preparatory high-performance liquid chromatographic (HPLC) system with a C^{18} reversed-phase column (Symmetry Prep C^{18}, 3.5 μm, 7.8 × 300 mm; Waters). The mobile phase of 30% MeOH at a flow rate of 1 ml/min was used, and a single peak was detected at 210 nm by a SPD-10 UV-VIS detector (Shimadzu, Kyoto, Japan). Then 10 mg of a pure compound was obtained from 10 L of supernatant from *L. capsici* YS1215. The pure compound was further identified by ^{1}H NMR, ^{13}C NMR, and direct GC-MS.

Structure Identification of the Purified Compound Produced by Strain YS1215

The structure of the purified compound was mainly determined by the nuclear magnetic resonance analysis. The compound (approximately 3 mg) was dissolved in 0.5 ml of methanol-d_4 (CD_3OD) and then subjected to spectral analysis. The spectra of ^{1}H

and ^{13}C NMR were measured on a 500 MHz NMR spectrometer (VNMRS, Agilent, USA) equipped with a PFG triple-resonance cold probe at 24°C at the Korea Basic Science Institute (Gwangju, Korea). Chemical shifts were reported in ppm (δ) using CD_3OD as a solvent and tetramethylsilane as an internal standard. The molecular weight of the compound was identified by gas chromatography mass spectrometry (GCMS-QP2010, Shimadzu, Japan). The GC-MS equipped with HP-5 MS column (30 m × 0.25 mm id, film thickness 0.25 μm) was used with helium as the carrier gas, a flow rate of 1 ml/min, and an injector temperature of 220°C.

Effects of Lactic Acid on Egg Hatching

Commercial lactic acid for this experiment was purchased from (Sigma-Aldrich Co., Korea). Different concentrations of lactic acid (5, 10, 15, 20, or 25 μl/ml) were applied to approximately 300 eggs in the plates. SDW was used as a control. The plates were kept in a dark incubator at 26°C. Hatched larvae at different concentrations were then counted at 2 and 5 days of exposure, under the stereoscopic microscope. All experiments were performed with three replications. The data were subjected to analysis of variance using SAS 9.1 software (SAS Institute, 2003). The mean values were compared by the least significant difference test at $p \leq 0.05$.

Results

Effect of BCF on Nematode Egg Hatching

As shown in Fig. 1, inhibition of egg hatching responded to the increasing concentrations of BCF and the time of incubation. The BCF significantly induced inhibition of the egg hatching at 5 days after incubation. As the concentration of BCF increased, the egg hatching decreased significantly as compared with hatching in SDW and CMF. At 5 days, inhibition of the hatching was 10.5%, 32.8%, 58.7%, and 64.0% in 50%, 30%, 20%, and 10% of BCF, respectively. The egg hatching in BCF at 5 days increased in comparison with 2 days of incubation in all treatments. The egg hatching in 50% BCF decreased 9.3% and 21.3% at 2 days, and 59.5% and 75.2% at 5 days compared with the CMF and SDW, respectively.

Structure Identification of the Purified Compound Produced by Strain YS1215

For identification of chemical structure, the purified compound was subjected to ^{1}H and ^{13}C NMR, and GC-MS analyses. The data were as follows: ^{1}H NMR (500 MHz, CD_3OD): δ 1.40 (3H, d, J = 7.0 Hz, H-3), 4.18 (1H, q, J = 7.0, 13.5 Hz, H-2), as shown in Fig. 2; ^{13}C NMR (500 MHz, CD_3OD): δ 21.5 (C-3), 70.1 (C-2), 182.2 (C-1), as shown in Fig. 3. Therefore, the formula was suggested to be $C_3H_6O_3$. When the purified compound was analyzed by GC-MS, it

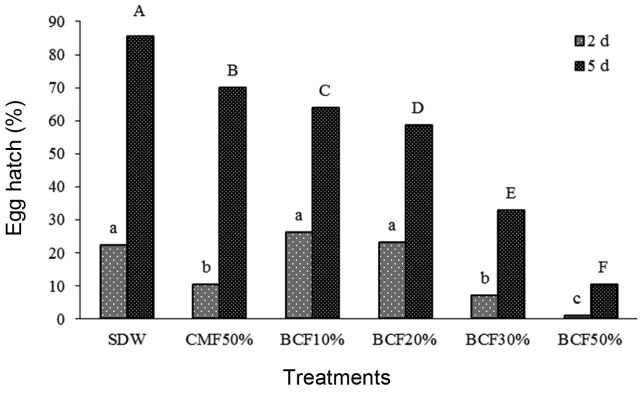

Fig. 1. Effects of various concentrations of bacterial culture filtrate (BCF) on the egg hatching of *Meloidogyne incognita* after 2 and 5 days of exposures at 26°C.

SDW = sterile distilled water; CMF = non-inoculated culture medium filtrate; different concentrations (10%, 20%, 30%, and 50%) of BCF were used. Bars labeled with different letters indicate significant difference at $p \leq 0.05$ (n = 3) of least significant difference tests.

was confirmed as lactic acid *via* a library search (NIST08s) using a match factor (Figs. 4A and 4B). The molecular ion at m/z 73 was a base peak. The observed fragments were *m/z* 66 (12.5%), 88 (5.5%), 101 (2.5%), 117 (83.5%), 133 (10.0%), 147 (90.0%), 191 (25.0%), and 219 (10.0%).

Effect of Lactic Acid on Egg Hatching

With increasing concentration of lactic acid, the egg hatching decreased proportionally (Fig. 5). The egg hatches of 16.2%, 11.8%, 9.0%, 5.7%, and 1.5% were found in 5, 10, 15, 20, and 25 μl/ml after 2 days of incubation while egg hatching was 18.0% in sterile distilled water. Meanwhile, at 5 days after incubation, the egg hatching was 36.4% at 5 μl/ml, 29.8% at 10 μl/ml, 23.7% at 15 μl/ml, 15.2% at 20 μl/ml, and 5.9% at 25 μl/ml, resulting in 8.1%, 14.7%, 20.8%, 29.3%, and 38.6% decreases of egg hatching compared with SDW control (44.5%), respectively.

Discussion

Lysobacter spp. are known to produce a wide variety of antimicrobial compounds, and have potential as biological control agents of various plant pathogens such as bacteria, fungi, and nematodes [12]. In this present study, we investigated the ability of *Lysobacter capsici* YS1215 and the purified compound to inhibit *Meloidogyne incognita* egg hatching. When bacterial culture filtrate was applied to the eggs of *M. incognita*, ovicidal action increased with increasing the concentration of BCF, whereas when BCF was diluted,

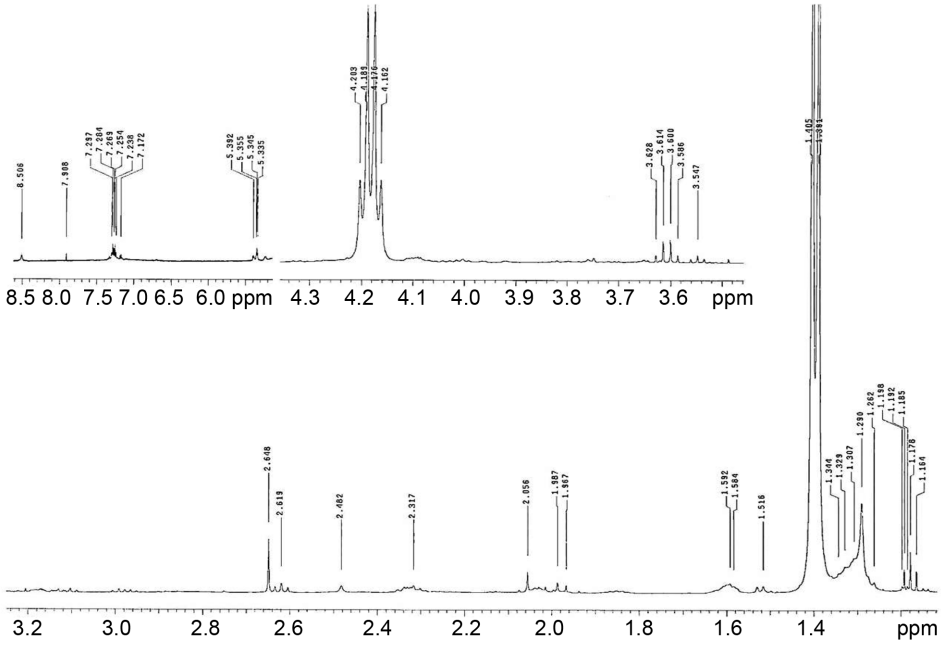

Fig. 2. ^{1}H nuclear magnetic resonance spectrum of the purified compound produced by *Lysobacter capsici* YS1215 in methanol-d_4 (CD_3OD) at 500 MHz.

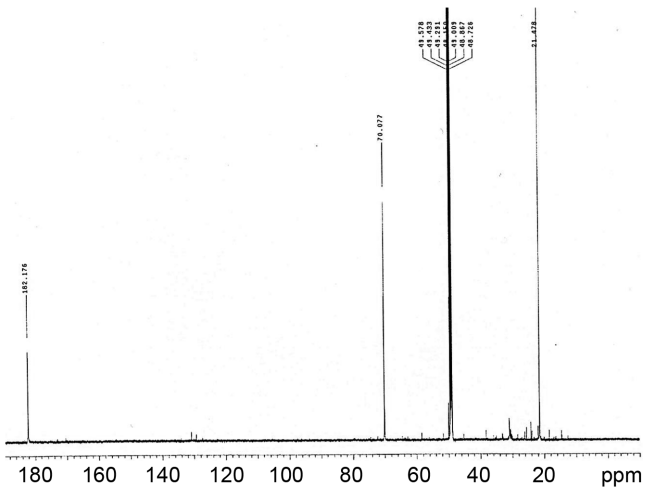

Fig. 3. ^{13}C nuclear magnetic resonance spectrum of the purified compound produced by *Lysobacter capsici* YS1215 in methanol-d_4 (CD_3OD) at 500 MHz.

the rate of egg hatch inhibition decreased compared with the high concentration (Fig. 1). Similarly, the use of culture filtrates of variable bacterial strains has frequently been reported on egg hatch inhibition of pathogenic nematodes. For example, the filtrates of *Verticillium leptobactrum* inhibited egg hatching of *M. incognita* [17]. The culture filtrate of *Bacillus firmus* reduced the egg hatch rate of *M. incognita* [13]. Fungal culture filtrates of *Verticillium lecanii* (*Lecanicillium* spp.) significantly inhibited the hatching of mature eggs, but when the culture filtrates were diluted, the inhibitory effect was low [19]. The result suggests that high concentrations of the culture filtrates are required for feasible inhibition of the egg hatching although the culture filtrates affected the hatching. It is somewhat difficult to apply in field conditions; however, their activity showed the presence of one or more active compounds that are worth further characterization.

Culture filtrates contain many metabolites produced by microorganisms during incubation to influence egg hatch inhibition. The crude antibiotic extracted from *Bacillus subtilis* significantly exerted an inhibitory effect of 88% on the hatching of *Meloidogyne incognita* eggs at 50% concentration after 72 h of exposure when compared with control [10]. Other similar results on the mortality of *M. javanica* using ethyl acetate and hexane fractions at different concentrations were reported [20]. These research could easily describe the role of microorganisms producing metabolites in the expression of the nematode. However, it is not known whether the ovicidal or nematicidal activity was due to a single compound or to more than one active compound in the crude extracts. These findings would help to some extent in future isolation and characterization of active compounds produced by *L. capsici* YS1215.

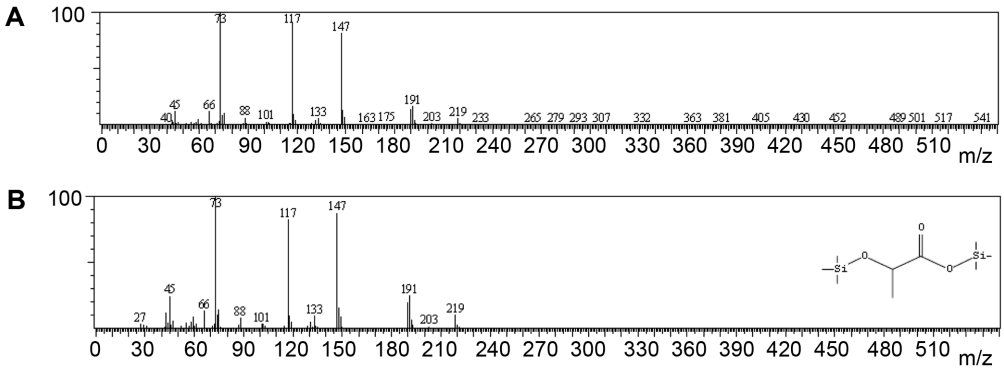

Fig. 4. Direct gas chromatography-mass spectrometry-electrospray ionization spectrum of the purified compound produced by *Lysobacter capsici* YS1215.

The purified sample was derivatized with trimethylsilyl for analysis by GC-MS. (**A**) The purified compound; (**B**) lactic acid.

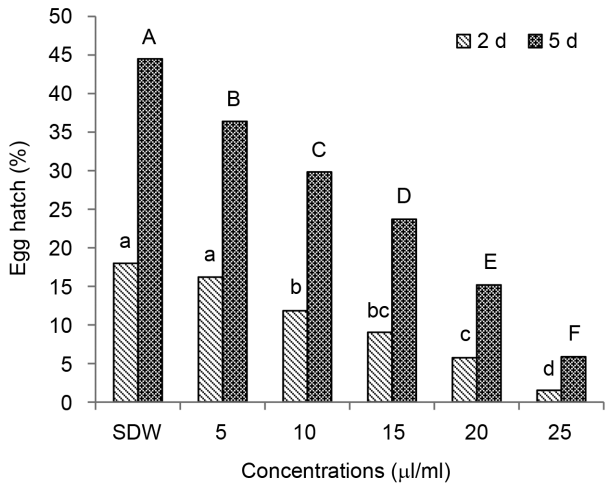

Fig. 5. Effects of various concentrations of lactic acid on egg hatching of *Meloidogyne incognita* after 2 and 5 days of exposures at 26°C.

SDW = sterile distilled water. Bars labeled with different letters indicate significant difference at $p \leq 0.05$ ($n = 3$) of least significant difference tests.

In this study, lactic acid (2-hydroxypropanoic acid) having antifungal activity on *Rhizoctonia solani* AG-1 (IA) was isolated by column chromatography and identified by NMR spectra and GC-ESI-MS analysis. Lactic acid also showed various inhibitory effects on the egg hatching of *Meloidogyne incognita* by different concentrations. The rate of egg hatch inhibition was 38.6% at 25 μl/ml compared with the SDW control. Coherently, lactic acid showed nematicidal effect against plant-parasitic, free-living and predacious nematodes [1]. Lactic acid was inhibitory to the survival or reproduction of *Aphelenchus avenae*, *Aphelenchoides goodeyi*, *Helicotylenchus pseudorobustus*, and *Xiphinema americanum* [6]. Similarly, organic acid is reported to have nematicidal activity, which resulted in reduction of plant-parasitic nematodes by 94–100% following 2 day exposure when a rate of 0.88 mg butyric acid/g sand was treated [3]. In our investigation, the results can be used as a basis for explaining the ovicidal effects of metabolites produced by *L. capsici* YS1215 *in vitro*. Therefore, pot and field experiments with lactic acid are necessary to validate these results and obtain more information about the nematode suppression mechanisms of *Lysobacter capsici* YS1215, singly or as beneficial interaction with host plants.

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