ISSN 0367-6315 (Print) / ISSN 2288-2162 (Online)
Korean J. Soil Sci. Fert. 49(4), 401-408 (2016)
http://dx.doi.org/10.7745/KJSSF.2016.49.4.401

Article

Identification of 2-methylbutyric Acid as a Nematicidal Metabolite, and Biocontrol and Biofertilization Potentials of *Bacillus pumilus* L1

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(Received: August 12 2016, Revised: August 26 2016, Accepted: August 29 2016)

The present study described the isolation of 2-methylbutyric acid (2-MBA) produced from *Bacillus pumilus* L1, to subsequently investigate its nematicidal activity for the control of the root-knot nematode. The results showed that 2-MBA could be purified by chromatographic techniques and was identified using nuclear magnetic resonance and liquid chromatography-mass spectrometry. Crude extract and partially purified compounds had a significant effect on the inhibition of egg hatchability and second-stage juvenile (J2) mortality. A dose-dependent effect of 2-MBA was observed for J2 mortality and egg hatchability. Egg hatchability was 69.2%, 59.9%, 32.7%, and 0.0% at 125, 250, 500, and 1000 μg mL^{-1} of 2-MBA after 4 d of incubation, respectively. Meanwhile, J2 mortality was in the range of 24.4%-100.0% after 2 d of incubation, depending on the concentrations of 2-MBA used. A pot experiment also demonstrated that treatment of *B. pumilus* L1 culture caused a significant reduction in the number of galls, egg masses, and J2 population than that of the tap water (TW) control. However, as the *B. pumilus* L1 culture concentration was decreased, the efficacy of nematode control by treatment of *B. pumilus* L1 culture was reduced compared to that of TW. *B. pumilus* L1 inoculation at different concentrations also promoted cucumber plant growth. Therefore, our study demonstrated the potential of 2-MBA from *B. pumilus* L1 as a biocontrol agent against the root-knot nematode and a plant growth promoter for cucumber plants.

Key words: *Bacillus pumilus* L1, Biocontrol, Root-knot nematode, Plant growth, 2-methylbutyric acid

Changes in plant growth parameters and degrees of nematode infection at 6 weeks after transplantation.

Treatments[b]	Fresh shoot length (cm)	Fresh shoot weight (g)	No. of gall Plant^{-1}	No. of egg mass plant^{-1}	No. of J2 in 50 g soil pot^{-1}
BC	32.7±6 b[a]	19.3±3 b	0±0 d	25.0±12 c	68.0±20 d
BC3	43.0±5 a	25.0±2 a	0±0 d	44.0±7 b	173.0±46 c
BC10	18.0±1 c	10.9±1 c	405.7±24 a	68.0±8 a	268.7±17 b
BC100	14.0±3 c	8.1±0 d	270.7±14 b	79.0±6 a	352.0±18 a
TW	14.2±0 c	6.9±0 d	131.3±19 c	67.0±4 a	356.0±28 a

[a]Each value is derived from the means of three replicates. Mean values ± SD with different letters indicate statistically significant differences at $P \leq 0.05$ when compared by LSD.

[b]BC: bacterial culture, BC3: bacterial culture of 3-fold dilutions, BC10: bacterial culture of 10-fold dilutions, BC100: bacterial culture of 100-fold dilutions, TW: tap water.

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[§]Acknowledgement: This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF), funded by the Ministry of Education (2014R1A1A2009841).

Introduction

Plant-parasitic nematodes, root-knot nematodes, are recognized as major agricultural pathogens, influencing global crop quality and quantity (Nicol et al., 2011). The typical symptoms of infected plants include galls and knots in the roots of plants, wilting, and yellowing of aboveground foliage from insufficient nutrient uptake (Adekunle and Akinlua, 2007; Siddiqui et al., 2011). These nematodes result in yield losses equating to billions of US dollars worldwide every year (Reynolds et al., 2011). For managing the presence of this pest on cultivated crops, chemical nematicides are usually more effective than other strategies. However, they have potential negative impacts on the environment and human health resulting from their toxic residues. As general awareness of the harmful effects of chemical nematicides has increased, their use has been prohibited in some areas, and farmers have reduced the use of these chemical nematicides (Reynolds et al., 2011; Elbadri et al., 2008; Pandey et al., 2000). Therefore, to overcome these problems, there is a great need for effective and environmentally friendly alternatives to control root-knot nematodes. Antagonistic microorganisms have been used previously as biocontrol agents against different plant-parasitic nematodes to develop alternatives to chemical nematicides. The main mechanism of root-knot nematode suppression by microorganisms may be the production of antibiotics, such as lipopeptides and surfactin, as well as other enzymes and toxins (Yoon et al., 2012; Tian et al., 2007; Siddiqui and Mahmood, 1999). For example, hydrogen cyanide produced by *Pseudomonas fluorescens* CHA0 suppressed the root-knot nematode, *Meloidogyne javanica*, in tomato (Siddiqui et al., 2006). Avermectins produced by *Streptomyces avermitilis* were shown to control plant-parasitic nematodes, such as *Hoplolaimus galeatus* and *Tylenchorhynchus dubius* (Blackburn et al., 1996). Several organic acids and amino acids produced by microorganisms were shown to have nematicidal activity (Oliveira et al., 2009; Abdel-Rahman et al., 2008).

Bacillus pumilus is a gram-positive, aerobic, spore-forming bacterium commonly found in soil, and recently became known as a biocontrol agent against plant pathogens. *B. pumilus* can produce a wide range of metabolites, including antibiotics, biosurfactants, and enzymes (Akbulut et al., 2013; Ahmadian et al., 2007). According to Moghaddam et al. (2014), *B. pumilus* ToIrFT- KC806241 and ToIrMA-KC806242 showed potential antagonism against *M. javanica* by reducing the number of galls and eggs in tomato plants. Similarly, *B. pumilus* reduced the gall formation and reproduction of the root-knot nematode (Akhtar and Siddiqui, 2008). Several studies have reported the nematicidal effect of *B. pumilus* on root-knot nematodes in different agricultural crops (Pinho et al., 2009; Mekete et al., 2009: Oliveira et al., 2007). However, information on active metabolites isolated form *B. pumilus* and its nematicidal activity have not yet been investigated. *B. pumilus* L1 was previously isolated from soil in which crops were grown in Gwangju Province, Republic of Korea, and was identified by 16S rRNA gene sequence analysis (Lee and Kim, 2016). The aim of the present study was to isolate and identify a metabolite produced from *B. pumilus* L1 and to evaluate its nematicidal activity against a root-knot nematode.

Materials and Methods

Bacterial culture and nematode preparation To obtain nematicidal compounds, *B. pumilus* L1 (GenBank accession number KJ206069) was cultured in 2-L Erlenmeyer flasks containing growth medium (urea 0.63 g L^{-1}; KH_2PO_4 1.2 g L^{-1}; KCl 0.3 g L^{-1}; K_2SO_4 0.05 g L^{-1}; $CaCl_2 \cdot 2H_2O$ 0.053 g L^{-1}; $MgSO_4 \cdot 7\ H_2O$ 0.015 g L^{-1}; sugar 8.0 g L^{-1}; yeast extract 1.0 g L^{-1}; crab shell powder (Purne Co, Gwangju, Korea) 1.0 g L^{-1}) at 30°C with shaking at 140 rpm for 5 d. The obtained cultural broth was used to extract nematicidal compounds.

Meloidogyne arenaria was reared routinely on tomato (*Solanum lycopersicum* L.; Cupirang; NongWooBio CO., LTD, Suwon, Gyeonggi-Do, Korea) for 2 months in a greenhouse at 25±3°C. Nematode eggs were extracted from infected tomato roots using 0.5% NaOCl solutions and then obtained by rinsing the egg suspensions with tap water using a 25-μm sieve (Hussey and Barker 1973). The eggs were incubated for 3-5 d using the modified Baermann funnel method (Southey, 1986) to obtain second-stage juveniles (J2) for 5 d at 28°C within 5 d. Eggs or J2 were surface sterilized by treatment with 0.01% streptomycin sulphate solution for 1 h before use.

Extraction and purification of the nematicidal compound The culture broth of *B. pumilus* L1 (15 L), prepared as described above, was centrifuged at 6,000 *g* for 20 min at 4°C using a Supra 21K apparatus (Hanil Science Industrial, Korea). The supernatant was then filtered through a Whatman No. 2 and acidified with concentrated HCl to pH 3.0. The extracellular metabolite from the filtered supernatant was extracted with an equal volume of ethyl acetate. The ethyl acetate fraction was concentrated using a rotary evaporator (Büchi, Switzerland) to obtain 10.0 g of the crude extract. The crude extract dissolved in methanol was subjected to silica gel column chromatography (Kiesel gel 60, 70-230 mesh; Merck, Darmstadt, Germany), and eluted with ethyl acetate. The eluted fraction was collected, concentrated using a rotary evaporator (Büchi, Rheinstetten, Germany), and tested for nematicidal activity. The fraction (0.92 g) having the nematicidal activity was further purified by silica gel column chromatography using the same elution as stated above. The eluted fraction (0.52 g) was subsequently purified by silica gel column chromatography using *n*-hexane: ethyl acetate (100:0, 90:10, 80:20, 70:30, and 0:100; v:v). The fraction (*n*- hexane:ethyl acetate = 90:10, v/v; 0.1 g) showing

strong nematicidal activity was subsequently purified by high performance liquid chromatography (HPLC) using a C18 reversed-phase column (Symmetry Prep C18, 10 mm, 7.8×300 mm; Waters). The mobile phase of H_2O: acetonitrile (50:50; v:v) at a flow rate of 2.0 mL min^{-1} was used, and the peak was detected at 210 nm by a SPD-10 UV-VIS detector (Shimadzu, Japan).

Identification of the purified nematicidal compound The chemical structure of the isolated nematicidal compound was determined by nuclear magnetic resonance (NMR) spectroscopy and liquid chromatography-mass spectrometry (LC-MS) analyses. The compound was dissolved in methanol-d_4 (CD_3OD) and analyzed using an NMR spectrometer (VNMRS, Agilent, USA) equipped with a PFG triple-resonance cold probe at 500 MHz for the ^{1}H-NMR spectrum and 125 MHz for the ^{13}C-NMR spectrum. Chemical shifts were determined using tetramethylsilane as an internal standard. LC-MS analyses were performed using a 6410 MSD triple quadrupole mass spectrometer (Agilent Technologies, Santa Clara, CA, USA) with an electrospray ionization (ESI) source used in the negative and positive mode. Further source parameters were: 25 psi nebulizer pressure, 15 L min^{-1} drying gas flow, 4,000 V capillary voltage, and 300°C drying gas temperature.

Nematicidal activity of crude extract and purified compound Nematicidal activities of the ethyl acetate extract and purified fractions were tested at a concentration range of 125-1,000 μg mL^{-1}. To test the nematicidal activity of 2-methylbutyric acid (2-MBA), it was suspended in ethanol (0.1 g mL^{-1}). A 2-MBA solution (10, 5, 2.5, and 1.25 μL) was placed in the wells of a 24-well plate (Falcon, USA) containing 100-150 juvenile nematodes in 990, 995, 997.5, and 998.75 μL of water. Thus, the total volume of the solution in each well was 1 mL, and the concentration of 2-MBA was 1.0 mg mL^{-1}. As a control, three wells were treated with ethanol (10 μL) in the same volume. 2-MBA used for the assay of the nematicidal activity was purchased from Sigma-Aldrich Korea Cooperation, Gyunggi province, Korea. The tested plates were stored in the dark at 25±1°C. J2 mortality was calculated by counting the number of dead J2 after 2 d of incubation (Olympus SZX16, Seoul, Korea) at 50×magnification. Hatched eggs were counted after 4 d of incubation. The 2-MBA, finally identified by NMR and LC-MS spectra and used in nematicidal activity assays, was purchased from Sigma Aldrich Korea Cooperation (Gyunggi Province, Korea) owing to insufficient amounts obtained in the experiments for further assays.

Effect of the bacterial culture of *B. pumilus* L1 in root-knot nematode control The seeds of cucumber (*Cucumis sativus* L.) var. Eunsung- backdadaggi were obtained from Hungnong Seed Company Ltd., Jeonbuk, Korea. The cucumber seeds were sown in bed soil (Bio bed soil I, Heong Nong Seed, Pocheon-si, Gyeonggi-do, Korea) in 54×45 mm plastic cell plug trays. Four weeks after sowing, the cucumber seedlings were transplanted into pots containing 800 g of soil:sand (2:1, v:v). The cucumber plants were then grown at 24°C in an artificially illuminated room (12,000 lux) for 16 h. Each pot was drenched weekly with respective 50 mL of bacterial culture (BC), bacterial culture of 3-fold dilutions (BC3), bacterial culture of 10-fold dilutions (BC10), bacterial culture of 100-fold dilutions (BC100), and tap water (TW) for 5 weeks after transplantation. An amount of 0.5 mL of a suspension with 100 eggs and 300 J2 mixed was inoculated into four holes at 2 cm distance from the stem of each plant. Six weeks after nematode inoculation, fresh weight and length of shoots, galls, egg masses, and J2 were determined for each pot. Each treatment was arranged in a randomized block design, and replicated four times (one cucumber plant per a pot).

Results and Discussion

Extraction and purification of the nematicidal compound In the present study, the nematicidal compound was purified from the culture broth of *B. pumilus* L1 by ethyl acetate extraction and chromatographic techniques. After loading the crude compound for silica column chromatography, the fraction of ethyl acetate elution showed the nematicidal activity. After purifying it once more with silica column chromatography, the nematicidal activity was observed in the fraction of the mobile phase (*n*-hexane:ethyl acetate = 90:10, v/v). In the final purified activity fraction, it was confirmed as a clear single peak in HPLC with a C18 reversed-phase column, showing a retention time (t_R) of 13.4 min at 210 nm (Fig. 1).

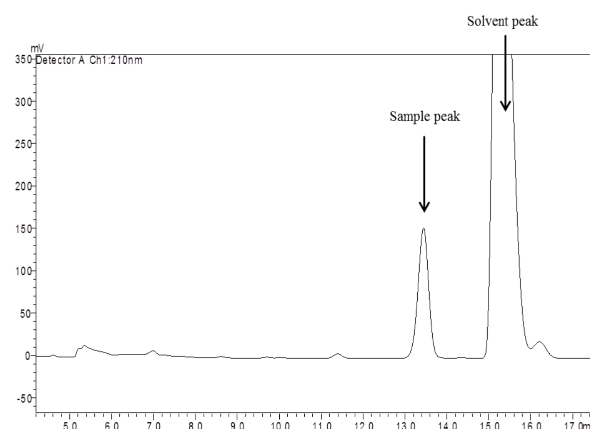

Fig. 1. High performance liquid chromatography (HPLC) chromatogram of the purified nematicidal compound from *Bacillus pumilus* L1, showing a single peak at the retention time of 13.4 min.

Structural elucidation of the purified compound The purified nematicidal compound was identified according to ^{1}H- and ^{13}C-NMR spectra (Fig. 2): ^{1}H-NMR (500 MHz, CD_3OD) δ 2.33 (1H, m, H-2), 1.70-1.61 (1H, m, H-3a), 1.52-1.43 (1H, m, H-3b), 1.13 (3H, d, J = 7.0 Hz, H-5), 0.93 (3H, t, J = 7.5 Hz, H-4); ^{13}C-NMR (125 MHz, CD_3OD) δ 179.1 (C-1), 40.6 (C-2), 26.3 (C-3), 15.6 (C-4), 10.4 (C-5). The purified compound was suggested to be methylbutyric acid. The ^{1}H- and ^{13}C-NMR spectra of the purified compound were consistent with those of (*S*)-(+)-2-methylbutyric acid, as standard compound (Sigma-Aldrich Chemical Co.). In addition, the pseudomolecular ion peaks at *m/z* 103.1 $[M-H]^-$ in the ESI (negative) spectrum (Fig. 3) were observed, indicating that the molecular weight of the purified compound was 102. The purified compound from ethyl acetate extract produced by *B. pumilus* L1 was identified as 2-methylbutyric acid (2-MBA). In previous study, 2-MBA inhibited fungal pathogen growth (Asante and Neal, 1964). To our knowledge, this is the first report of 2-MBA isolated from *B. pumilus* L1 showing nematicidal activity of *M. arenaria*. Previously, 2-MBA showing antifungal activity was isolated from *B. subtilis* HJ927 (Lee et al., 2005), and a *Pseudoalteromonas haloplanktis* INH strain (Hayashida-Soiza et al., 2008).

Nematicidal activity of crude extract and 2-MBA The ethyl acetate extract and purified fractions had a significant effect on egg hatchability and J2 mortality, showing dose-dependent results (Fig. 4). As the purity of crude extracts increased, hatchability was reduced, whereas J2 mortality increased as compared with that of the control. Therefore, nematicidal activity was dependent on both treatment concentration and purity. A dose-dependent effect was observed for J2 mortality and egg hatchability treated with the isolated 2-MBA (Fig. 5). At 4 d after incubation with 2-MBA, egg hatchability was 69.2%, 59.9%, 32.7%, and 0.0% at 125, 250, 500, and 1,000 μg mL^{-1}, respectively, compared to that of the control (84.2%). Egg hatchability decreased with increasing 2-MBA concentrations, indicating that hatch inhibition of

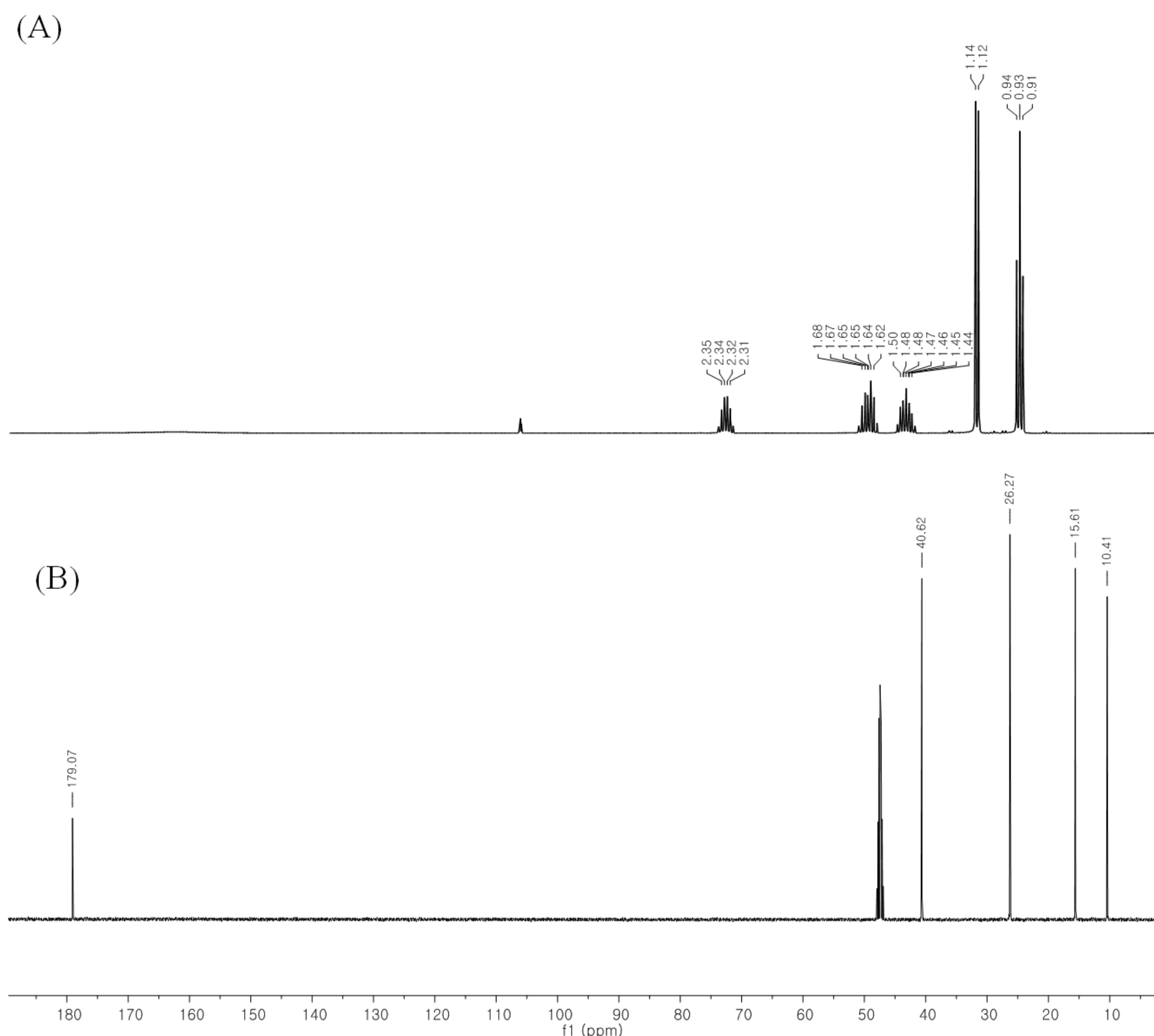

Fig. 2. ^{1}H- (A) and ^{13}C- (B) nuclear magnetic resonance (NMR) spectra of the purified compound in methanol-d_4.

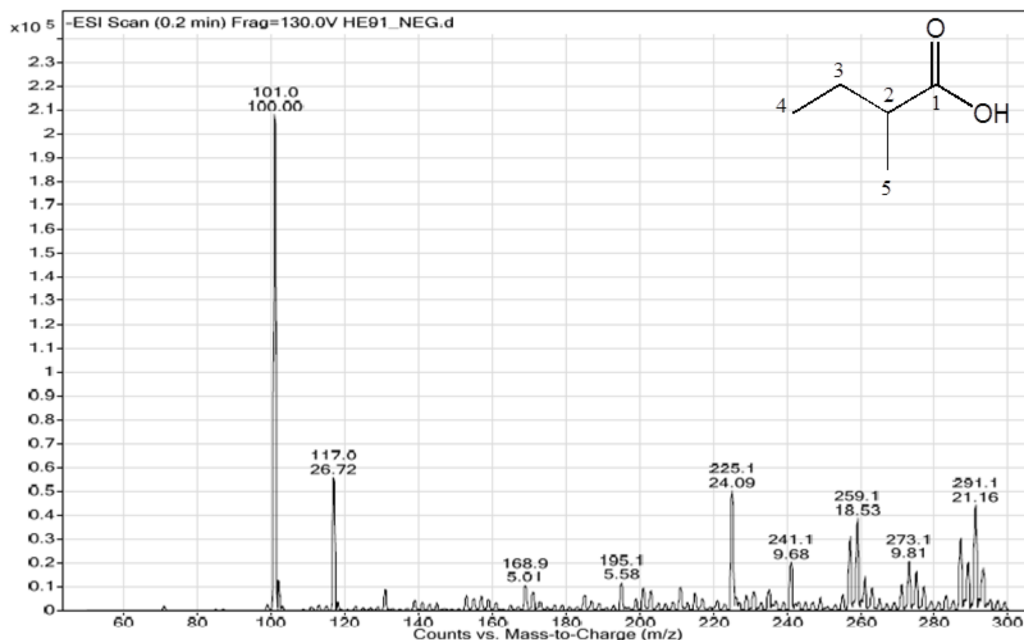

Fig. 3. Liquid chromatography-mass spectrometry spectra and chemical structure of 2-methylbutyric acid, showing pseudomolecular ion peaks at *m/z* 101.0 $[M-H]^-$ in the ESI (negative) spectrum.

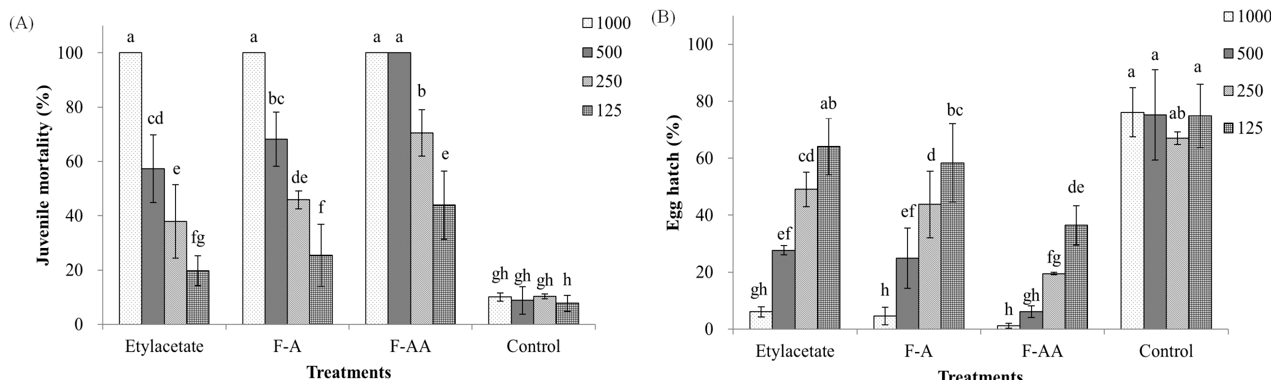

Fig. 4. Nematicidal activities of ethylacetate extract and fractions obtained from ethylacetate extract against second-stage juvenile (J2) mortality (A) and hatch (B) of *Meloidogyne arenaria* for 2 d (J2) or 4 d (eggs) after treatment. Mean values ± SD with different letters indicate statistically significant differences at $P \leq 0.05$ when compared by LSD.

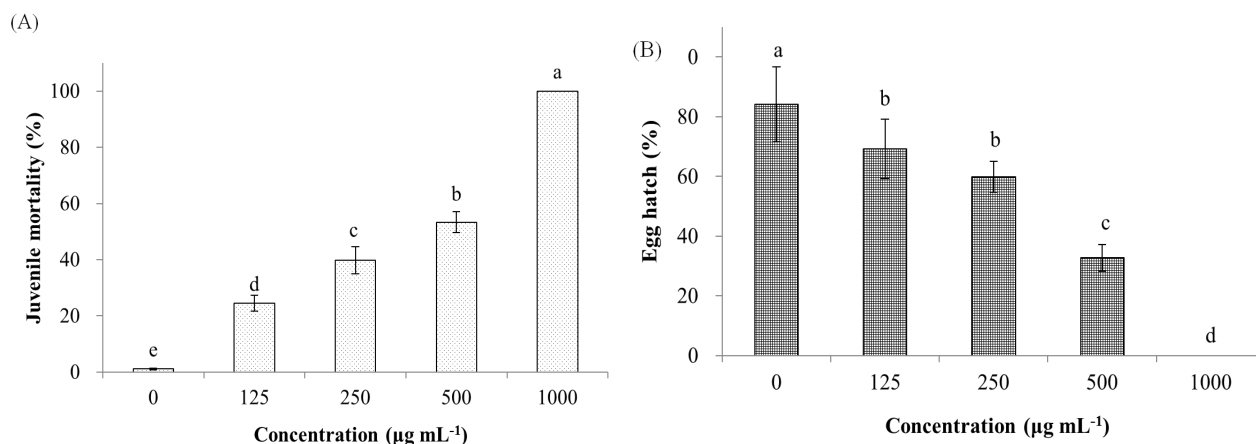

Fig. 5. Effect of 2-methylbutyric acid from *Bacillus pumilus* L1 culture on second-stage juvenile (J2) mortality (A) and hatch (B) of *Meloidogyne arenaria* at 26°C for 2 d (J2) and 4 d (eggs) incubation. Mean values ± SD with different letters indicate statistically significant differences at $P \leq 0.05$ when compared by LSD.

2-MBA was dose dependent. Meanwhile, evaluation of 2-MBA exposed differences in toxicity against *M. arenaria* J2. Briefly, J2 mortality was 24.4%-100.0% after 2 d of incubation, depending on the concentration of 2-MBA used. Overall, J2 mortality was significantly ($P < 0.05$) associated with increasing 2-MBA concentrations.

Various metabolites with nematicidal activity produced by microorganisms have been reported (Meyer et al., 2009; Mayer et al., 1997). Bacterial crude ethyl acetate extract had a significant effect on hatchability and J2 mortality of *M. incognita* (Lee et al., 2013). Fervenulin and isocoumarin produced by *Streptomyces* sp. CMUMH021 significantly increased J2 mortality of *M. incognita* (Ruanpanun et al., 2011). Organic acid induced reduction in plant-parasitic nematode survival by 94%-100% when a rate of 0.88 mg butyric acid g^{-1} sand was treated (Browning et al., 2004). A metabolite of *Streptomyces cacaoi* GY525 showed nematicidal activity against *M. incognita* (Yoon et al., 2012).

Effect of the bacterial culture of *B. pumilus* L1 on root-knot nematode control In the pot experiment, the number of galls and egg masses in plants, and J2 in soil were observed 6 weeks after *M. arenaria* inoculation (Table 1). Bacterial culture (BC) treatment proved the most effective at controlling the nematode infestation, followed by BC3 treatment (bacterial culture of 3-fold dilutions). With the increase in BC dilution, nematode control efficacy decreased compared to that of TW. Following inoculation with *B. pumilus* L1 culture, the number of galls and egg masses per plant displayed a reduction of 100.0% and 100.0% (BC and BC3), or 62.7% and 34.3% (BC and BC3) compared to that of TW, respectively, whereas the final J2 populations were 80.9% and 51.4% lower. However, BC10 and BC100 treatment showed respective increases of 50% and 106% in the galls, and 18% increase in egg masses under BC100 treatment. These results indicate that for controlling nematodes with bacterial culture, appropriate amounts of it are necessary for effective treatment. The highest shoot weight of cumber plants were obtained for the BC3 treatment (25.0 g), which was significantly higher ($P \leq 0.05$) than those obtained by the BC (19.3 g), BC10 (10.9 g), and BC100 (8.1 g), and TW (6.9 g) treatments. The fresh shoot length increased significantly in the BC and BC3 treatments than that of TW, whereas those of BC10 and BC100 did not significantly increase.

The present study showed the effectiveness of *B. pumilus* L1 using the pot experiment when *B. pumilus* L1 culture was added into potted soil containing cucumber plants inoculated with *M. arenaria*; the disease was significantly reduced and plant growth was promoted. Insunza et al. (2000) also reported that *B. cereus* suppressed nematode infection and promoted plant growth in glasshouse trials. In addition, when a *B. subtilis* culture was applied to tomato plants, the formation of egg masses and galls by *M. incognita* was significantly reduced (Gautam, 1995). Treatment of *B. pumilus* ToIr-MA into soil reduced the number of galls and eggs of *M. javanica* in tomato plants under pot conditions, and enhanced plant growth (Moghaddam et al., 2014). Studies on the reduction of nematode infection by rhizobacteria reported that the mode of reducing action could include parasitism, production of toxins, antibiotics, or enzymes, competition, and the induction of systemic resistance with independent and combined performances (Nguyen et al., 2013; Khan et al., 2008; Tian et al., 2007; Siddiqui and Mahmood, 1999). Bacterial culture of *B. megaterium* could significantly reduce infection of the nematode through the production of nematicidal volatiles (Huang et al., 2010). Similar effects have been shown for secondary metabolites of this bacterium that reduced hatching of *M. graminicola* in rice plants (Padgham and Sikora, 2006). These findings are in agreement with our results.

Promotion of plant growth may also be related to the suppression of nematode infection by *B. pumilus* L1 treatment in the soil. Most studies on plant growth enhancement by microorganisms suggest that the primary mechanism may be attributed to the nematicidal effect of the inoculated bacterial strain and bacterial metabolites that antagonize pathogens, resulting in healthier and larger root systems (Kokalis-Burelle

Table 1. Changes in plant growth parameters and degrees of nematode infection 6 weeks after transplantation.

Treatments[b]	Fresh shoot length (cm)	Fresh shoot weight (g)	No. of gall Plant^{-1}	No. of egg mass plant^{-1}	No. of J2 in 50 g soil pot^{-1}
BC	32.7±6 b[a]	19.3±3 b	0±0 d	25.0±12 c	68.0±20 d
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TW	14.2±0 c	6.9±0 d	131.3±19 c	67.0±4 a	356.0±28 a

[a]Each value is derived from the means of three replicates. Mean values ± SD with different letters indicate statistically significant differences at $P \leq 0.05$ when compared by LSD.

[b]BC: bacterial culture, BC3: bacterial culture of 3-fold dilutions, BC10: bacterial culture of 10-fold dilutions, BC100: bacterial culture of 100-fold dilutions, TW: tap water.

et al., 2006; Gierth et al., 2004). Moghaddam et al. (2014) reported that significant enhancement in root and shoot length and dry root and shoot weight was recorded with *B. pumilus* ToIr-MA use than that of the control.

Conclusions

Purification, identification, and the nematicidal activity of 2-MBA, isolated for the first time from *B. pumilus* L1, were determined in this study. 2-MBA exhibited strong nematicidal activity against *M. arenaria*. Moreover, through pot experiments, it was revealed that the effect of *B. pumilus* L1 culture on nematode control in cucumber plants was associated with the amount of treatment. Therefore, this study suggests that 2-MBA may be an alternative to chemical nematicides as a natural product for the biocontrol of root-knot nematodes, and *B. pumilus* L1 may be one of potential agents.

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