# Isolation and Identification of Antifungal Compounds from *Bacillus subtilis* C9 Inhibiting the Growth of Plant Pathogenic Fungi

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Antagonistic microorganisms against *Rhizoctonia solani* were isolated and their antifungal activities were investigated. Two hundred sixteen bacterial isolates were isolated from various soil samples and 19 isolates were found to antagonize the selected plant pathogenic fungi with varying degrees. Among them, isolate C9 was selected as an antagonistic microorganism with potential for use in further studies. Treatment with the selected isolate C9 resulted in significantly reduced incidence of stem-segment colonization by *R. solani* AG2-2(IV) in Zoysia grass and enhanced growth of grass. Through its biochemical, physiological, and 16S rDNA characteristics, the selected bacterium was identified as *Bacillus subtilis* subsp. *subtilis*. Mannitol (1%) and soytone (1%) were found to be the best carbon and nitrogen sources, respectively, for use in antibiotic production. An antibiotic compound, designated as DG4, was separated and purified from ethyl acetate extract of the culture broth of isolate C9. On the basis of spectral data, including proton nuclear magneric resonance ($^1$H NMR), carbon nuclear magneric resonance ($^{13}$C NMR), and mass analyses, its chemical structure was established as a stereoisomer of acetylbutanediol. Application of the ethyl acetate extract of isolate C9 to several plant pathogens resulted in dose-dependent inhibition. Treatment with the purified compound (an isomer of acetylbuanediol) resulted in significantly inhibited growth of tested pathogens. The cell free culture supernatant of isolate C9 showed a chitinase effect on chitin medium. Results from the present study demonstrated the significant potential of the purified compound from isolate C9 for use as a biocontrol agent as well as a plant growth promoter with the ability to trigger induced systemic resistance of plants.

KEYWORDS : Antagonistic microorganisms, Antifungal compounds, Acetylbuanediol, *Bacillus subtilis* subsp. subtilis, Plant pathogenic fungi

## Introduction

Soil-borne bacteria that are antagonistic to plant pathogens could make a substantial contribution to prevention of plant diseases, and therefore represent an alternative to the use of chemical pesticides in agriculture [1]. Due to their role in plant health and soil fertility, soil and the rhizosphere have frequently been used as a model environment for screening of putative agents for use in biological control of soil-borne plant pathogens. Among the 20 genera of bacteria, *Bacillus* spp., *Pseudomonas* spp., and *Streptomyces* spp. are widely used as biocontrol agents and *Bacillus* spp. has been reported to produce several antibiotics [2].

Non-pathogenic plant growth promoting rhizobacteria, *Bacillus* sp., form endospores and can tolerate extreme pH, temperature, and osmotic conditions; therefore, they offer several advantages over other organisms. *Bacillus* sp. was found to colonize the root surface, increase plant growth, and cause lysis of fungal mycelia [3]. They are regarded as safe biological agents and their potential is considered high [4]. As they are bacterial antagonists, the assumption is that their antagonistic effects are due mainly to production of antifungal antibiotics [5], which appear to play a major role in biological control of plant pathogens [6].

Biosynthesis of antibiotics from microorganisms is often regulated by nutritional and environmental factors. El-Banna [7] reported that antimicrobial substances produced by bacterial species were greatly influenced by variation of carbon sources. Several abiotic factors, such as pH and temperature, have been identified as having an influence on antibiotic production from bacteria [8]. Antifungal peptides produced by *Bacillus* species include mycobacillins [9], surfactins [10], mycosubtilins [11], and fungistatins [5]. It can produce a wide range of other metabolites, including chitinases and other cell wall-degrading enzymes [12, 13], volatiles [13], and compounds that elicit plant resistance mechanisms [14]. Volatile metabolites produced from *Bacillus* sp. have been reported to inhibit mycelia growth of

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*Fusarium oxysporum*, with the highest effect on reduction of *Fusarium* wilt of onion [15]. Ryu *et al.* [16, 17] reported on promotion of growth and induction of systemic resistance (ISR) response in *Arabidopsis thaliana* against *Erwinia carotovora* subsp. *carotovora* by volatile substances (VS) (acetylbuanediol and acetoin, same as the present purified compounds) from *Bacillus amyloliquefaciens* isolate IN937a and *B. subtilis* isolate GB03. Therefore, VS-producing bacteria can be used as biocontrol agents for protection against microbial plant diseases.

The present study was aimed at isolation of potential antagonistic soil microorganisms and purification of antifungal components. Analysis of their structure, as well as their effects on growth against tested pathogenic fungi, was conducted.

## Materials and Methods

**Source of sample and identification of antagonistic microorganisms.** Bacterial isolates were collected from agriculture crop fields and antagonistic isolates were selected according to their inhibitory efficacy against *R. solani*. Standard morphological and 16S rDNA methods were used for identification of isolates. Bacterial isolates were stored at $-70^{\circ}$C in 15% glycerol with Triptic soy broth and pathogenic fungi were maintained in potato dextrose agar (PDA) at $4^{\circ}$C for use in further studies.

**Effect on stem-segment colonization of antagonistic microorganisms.** Seedlings of Zoysia grass were inoculated with a mycelial suspension of *R. solani* AG2-2(IV) and a cell suspension ($10^{8}$ cfu/mL) of antagonistic microorganisms, and kept under greenhouse conditions at $27 \pm 2^{\circ}$C, 85% r. h., and 12 hr photoperiods. After three wk of incubation, stem-segment colonization was measured as follows:

$$\text{Stem-segment colonization (\%)} = [(0 \times A + 2 \times B + 3 \times C + 5 \times D + 8 \times E + 10 \times F)/ \{\text{Total number of stems} \times \text{highest score (10)}\}] \times 100$$

where, A (0), healthy stem; B (2), normal with little blight; C (3), blight < 10%; D (5), blight = 11~50%; E (8), blight = 51~90%; F (10), blight = 91~100%.

**Optimization of nutritional and environmental conditions for antibiotic production.** To determine the best carbon and nitrogen sources for antibiotic production, the culture was grown on medium containing different carbon and nitrogen sources. Luria broth was used as a basal medium for optimization of carbon and nitrogen sources (except peptone and in addition with 1% glucose). For determination of optimal nutritional concentrations, 0.5% to 9% of the selected carbon and nitrogen source was used. The culture temperature and initial pH for maximum production of antibiotic substances from antagonistic microorganism was determined by incubation at 20 to $40^{\circ}$C and 4.0 to 10.0, respectively.

**Extraction and purification of antibiotic substances.** The crude antibiotic substance was recovered from the culture filtrate of isolate C9 by solvent extraction with ethyl acetate. Ethyl acetate was added to the filtrate at a ratio of 3 : 1 (v/v) and shaken vigorously for 20 min. To obtain a crude extract, the organic layer was collected and evaporated to dryness in a vacuum evaporator at $40^{\circ}$C.

Different chromatographic techniques, including column chromatography, one dimensional ascending thin layer chromatography, and preparative thin layer chromatography, were used according to standard methods for further purification of the crude extract. The selected fractions were further subjected to nuclear magnetic resonance (NMR) spectroscopic analysis.

A Bruker advance 400 MHz spectrometer (Bruker Co., Billerica, MA, USA) with a 5 mm inverse probe was used to obtain NMR spectra. Proton spectra were run at a probe temperature of $25^{\circ}$C. The sample was dissolved in $CDCl_3$ at a concentration of 5 mg/mL ($^1$H NMR) and 20 mg/mL (for $^{13}$C NMR). $^1$H chemical shifts were referenced to internal $CDCl_3$ (4.7 ppm at all temperatures). Structures of the compounds were confirmed by comparison with reference data from available literature matching.

**Antifungal activity of purified compounds.**
**Agar well plate assay:** The agar well diffusion assay, as reported by Tagg and McGiven [18], was used for determination of antagonistic activity of the crude extract and purified compounds. Briefly, PDA medium (20 mL) was poured into each sterile petri dish, followed by placement of two mycelial mats (5 mm in diameter) of the tested pathogen at the same distance from the center of the plates. A well (5 mm in diameter) was made by punching the agar with a sterile steel borer on the center of the plate and crude extract from isolate C9 or purified compound (different concentration) was poured into the well. The plates were incubated for 72 hr at $27^{\circ}$C and the inhibitory activity of each concentration was expressed as the percent growth inhibition, compared to the control (solvent only used in the wells), according to the following formula:

$$\text{Growth inhibition (\%)} = (DC - DT)/DC \times 100$$

where, DC, diameter of control; and DT, diameter of fungal colony with treatment [19]: Each concentration was replicated three times and three separate tests were performed.
**Agar diffusion assay:** The agar diffusion technique described by Grover and Moore [20] was used for determination of antagonistic activity of crude antibiotics against the tested pathogen. For measurement of the amount of sterile culture supernatant, 20 mL of PDA was

poured into sterilized petri dishes; crude extracts or purified compound was added along with the molten agar in order to obtain the required concentrations. Test fungi were inoculated with (5 mm in diameter) a mycelial plug and growth was recorded after 3 to 5 days of incubation at 27°C. Percentage inhibition was computed after comparison with the control.

**Statistical data analysis.** Data were subjected to analysis of variance using SPSS software (SPSS Inc., Chicago, IL, USA). Mean values were compared using Duncan's multiple range test (DMRT) at the 5% ($p < 0.05$) level of significance.
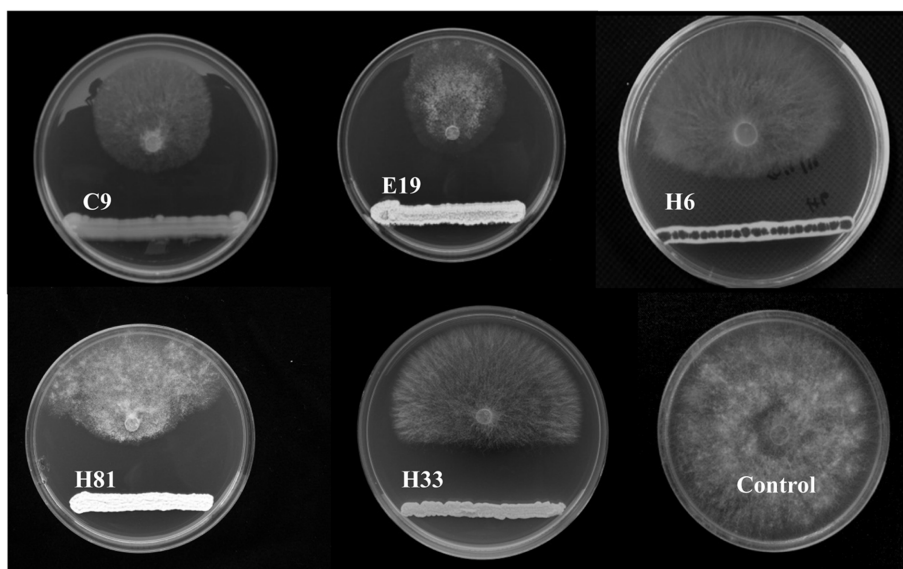


**Fig. 1.** Efficacy of *in vitro* selected antagonistic isolates against *Rhizoctonia solani* AG2-2(IV) using the dual culture assay. Each plate number represents the antagonistic isolates. Control, culture of *R. solani* AG2-2(IV) on Triptic soy agar.
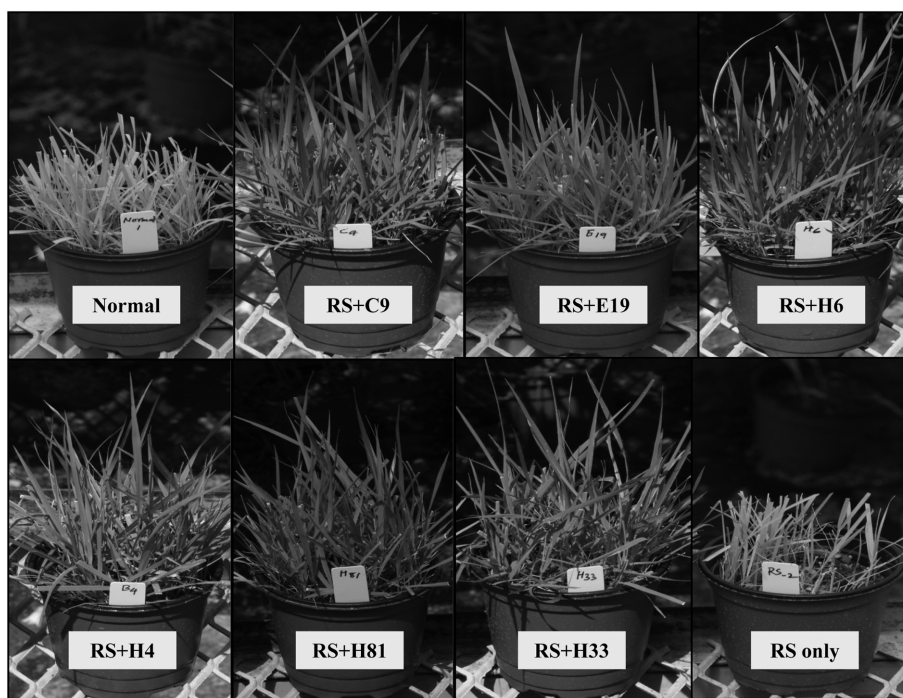


**Fig. 2.** Efficiency of *in vivo* selected antagonistic soil microorganisms for inhibition of stem-segment colonization by *Rhizoctonia solani* AG2-2(IV) on Zoysia grass in a greenhouse pot experiment. Normal, normal grass pot; middle pots (treated), inoculated with both (RS + C9, E19, H6, B4, H81, and H33) = *R. solani* AG2-2(IV) + selected isolate; RS only (control), inoculated only *R. solani* AG2-2(IV).

## Results

**Selection and identification of antagonistic isolate C9.**
Several soil samples were screened for isolation of antagonistic microorganisms against *R. solani*; 67 isolates were found to be antagonists against tested fungi. According to their antagonistic efficiency against *R. solani* AG2-2(IV), 19 isolates were selected for screening under greenhouse conditions. Few of these isolates were found to induce a significant reduction of the severity of disease in infected Zoysia grass. On the basis of *in vitro* performance, the top five isolates were selected for further concentration-dependent evaluation under greenhouse conditions. According to results from the dual culture assay (Fig. 1) together with *in vivo* experiments (Figs. 2 and 3), isolate C9 was finally selected as an antagonistic *Bacillus* sp. with potential for use in control of diseases caused by *R. solani* AG2-2(IV) in Zoysia grass.

According to Bergey's Manual of Systematic Bacteriology,
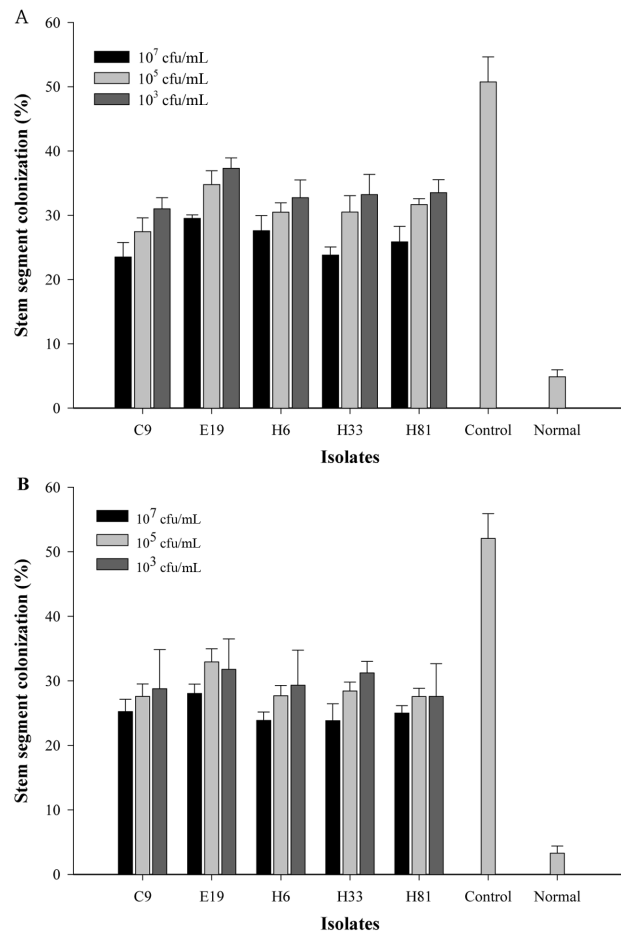
isolate C9 was classified as a bacteria belonging to the genus *Bacillus*. Further sequential analysis of the gene encoding 16S rDNA confirmed the isolate C9 as *Bacillus* sp. Comparison of the 16S rDNA gene of isolate C9 (1,200 bp) with sequences in the GenBank database revealed that the bacterium was a *Bacillus* sp. and showed 99% homology with the reference strain *Bacillus subtilis* subsp. *subtilis* (data not shown).

**Optimization of nutritional and environmental conditions for isolate C9.** Several carbon and nitrogen sources were used for optimization of nutritional conditions required for antibiotic production from isolate C9. Efficiency was measured on the basis of viable cell number. The maximum viable cell number was achieved when mannitol was used as a carbon source (Table 1); 1% mannitol was also found to be the optimum concentration (Table 2). Production of antibiotics (mycelial growth inhibition by 76.08%) reached optimum level upon supplementation of culture media with 1% mannitol (Table 1). In a similar manner, 1% soytone was selected as the optimum concentration for attainment of maximum growth and antibiotic production (mycelial growth inhibition by 94.70%) (Tables 1 and 2). For isolate C9, the viable cell number and production of antibiotic substances showed a gradual increase from $20^\circ$C until the optimum temperature reached $30^\circ$C and then decreased with increasing culture temperature (Fig. 4A and 4B). pH 7.0 was the best value for antibiotic production (Fig. 4A and 4B).



**Fig. 3.** Effect of antagonistic isolates on stem-segment colonization of Zoysia grass. A, Inoculation of *Rhizoctonia solani* AG2-2(IV) before application of isolates for 7 days; B, Inoculation of *R. solani* AG2-2(IV) after application of isolates for 7 days.

**Table 1.** Effect of different carbon and nitrogen sources on cell growth of *Bacillus subtilis* subsp. *subtilis* C9

|  |  | No. of cells ($\times 10^8$ cfu/mL) | Mycelial growth inhibition (%)[b] |
|---|---|---|---|
| Carbon sources[a] | Mannitol | $1.20 \pm 7.07$ | $76.08 \pm 2.54$ |
|  | Xylose | $0.60 \pm 6.36$ | $73.04 \pm 4.52$ |
|  | Glycerol | $0.29 \pm 2.12$ | $54.63 \pm 1.76$ |
| Nitrogen sources[a] | Soytone | $2.7 \pm 0.22$ | $94.70 \pm 1.32$ |
|  | Peptone | $2.16 \pm 0.12$ | $84.08 \pm 4.07$ |
|  | Malt extract | $2.54 \pm 0.08$ | $82.88 \pm 3.65$ |

[a]Optimum concentrations of carbon (1.0%) and nitrogen (1.0%) sources were used for culture of *B. subtilis* subsp. *subtilis* C9.
[b]The agar diffusion technique was used for inhibition of mycelial growth against *Rhizoctonia solani* AG2-2(IV).

**Table 2.** Effect of mannitol and soytone concentration on cell growth of *Bacillus subtilis* subsp. *subtilis* C9

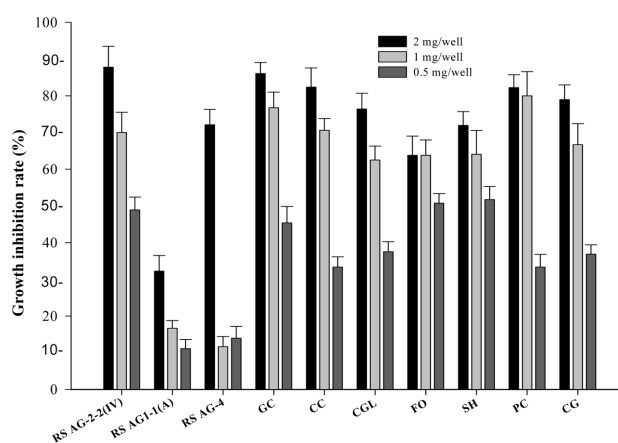| Manitol concentration (%) | No. of cells ($\times 10^8$ cfu/mL) | Soytone concentration (%) | No. of cells ($\times 10^8$ cfu/mL) |
|---|---|---|---|
| 0.5 | $0.19 \pm 4.26$ | 0.5 | $8.37 \pm 2.75$ |
| 1.0 | $0.64 \pm 5.13$ | 1.0 | $25.67 \pm 3.06$ |
| 1.5 | $0.57 \pm 2.65$ | 1.5 | $22.57 \pm 0.58$ |
| 2.0 | $0.54 \pm 1.53$ | 2.0 | $20.00 \pm 1.00$ |
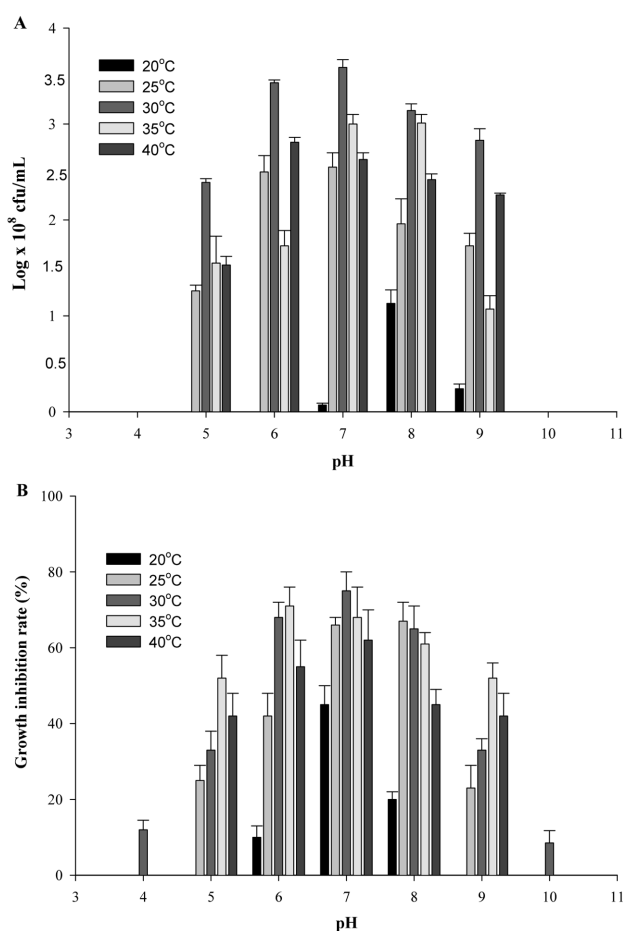
**Fig. 4.** Effect of initial pH and culture temperature on cell growth (A) and production of antibiotic substances (B) from *Bacillus subtilis* subsp. *subtilis* C9.

**Fig. 5.** Inhibition effect of antibiotic substances extracted with ethyl acetate from *Bacillus subtilis* subsp. *subtilis* C9 on mycelial growth of plant pathogenic fungi. RS AG2-2(IV), *Rhizoctonia solani* AG2-2(IV); RS AG1-1(A), *Rhizoctonia solani* AG1-1(A); RS AG-4, *Rhizoctonia solani* AG4-4; GC, *Glomerella cingulata*; CC, *Corynespora cassiicola*; CGL, *Chatomium globosum*; FO, *Fusarium oxysporum*; SH, *Sclerotinia homeocarpa*; PC, *Phytophthora cactorum*; CG, *Colletotrichum gloeosporioides*.

**Purification of antibiotic compounds from isolate C9.** Purification of antibiotic compounds from isolated C9. The ethyl acetate extract (5 g) was used for purification of antibiotic substances from isolate C9. Silica gel column (230~400 mesh ASTM; Merck, Darmstadt, Germany) and elution with concentrated dichloromethane/4% methanol gradient were performed. The 18 active fractions were collected and concentrated by evaporation. The major fractions (Fraction 6 and 7) were selected according to antibiotic activity for further purification. These fractions were again passed through a column, and the most bioactive fraction, designated as DG4, was collected.

$^{1}$H and $^{13}$C NMR spectral analysis was performed for identification of DG4 (compound A and B). The $^{13}$C NMR spectrum showed 12 carbon signals, including 2 carbonyl carbons, 6 methyl carbons, 2 methane carbons, and 2 quaternary carbons. Of particular interest, an unequal intensity of proton and carbon signals in a ratio (3 : 1) of compound A : compound B was observed on $^{1}$H and $^{13}$C NMR spectra, evidence of a mixture of the 2 compounds. In addition, the proton and carbon signals of compounds A and B showed similar patterns, except for a difference in chemical shifts, strongly suggesting that compounds A and B are stereoisomers of each other. Based on $^{1}$H NMR, $^{13}$C NMR, $^{1}$H-$^{1}$H COSY, TOCSY, and HMBC data, as well as mass experiments, the structure of DG4 was identified as a mixture of stereoisomers of 3,4-dihydroxy-3-methyl-2-pentanone.

**Antifungal activity of ethyl acetate extract from isolate C9.** Radial growth of tested fungi was reduced at all (0.5, 1, and 2 mg/well) concentrations of ethyl acetate extract from isolate C9 (Fig. 5). Maximum inhibition of mycelial growth by the crude extract (2 mg/well) was 87.78%, 32.22%, 72.09%, 86.05%, 82.35%, 76.39%, 63.77%, 71.91%, 82.22%, and 78.95% against *R. solani* AG2-2(IV), *R. solani* AG1-1(A), *R. solani* AG-4, *G. cingulata*, *C. cassiicola*, *C. globosum*, *F. oxysporum*, *S. homeocarpa* (Dollar spot), *P. cactorum*, and *C. gloeosporioides*, respectively.

**Antifungal activities of purified compound from isolate C9.** Using the agar well plate assay, several common plant pathogenic fungi were treated with (5 μg/well) purified compounds produced by isolate C9. Volatile organic compound (VOCs) DG4 (isomer of acetylbuanediol) exhibited strong inhibition of mycelial growth against *C. gloeosporioides* KACC 40690, *C. globosum* KACC 40308, *G. cingulata* KACC 40299, *F. oxysporum* KACC 40052, *A. niger* KACC 1700, *C. cassiicola* KACC 40964, and *R. solani* AG2-2(IV) KACC 40152 by 44.41%, 59.38%, 73.40%, 93.61%, 78.97%, 44.37%, and 79.14%, respectively

**Table 3.** Inhibition effect of DG4 (acetylbuanediol) produced by *Bacillus subtilis* subsp. *subtilis* C9 on mycelial growth of *Rhizoctonia solani* AG2-2(IV)

| Pathogenic fungi | Inhibition rate (%)[a] |
|---|---|
| *Colletotrichum gloeosporioides* KACC 40690 | 44.41 ± 1.30 |
| *Chatomium globosum* KACC 40308 | 59.38 ± 0.07 |
| *Glomerella cingulata* KACC 40299 | 73.40 ± 1.11 |
| *Fusarium oxysporum* KACC 40052 | 93.61 ± 5.90 |
| *Aspergillus niger* KACC 1700 | 78.97 ± 0.34 |
| *Corynespora cassiicola* KACC 40964 | 44.37 ± 5.82 |
| *Rhizoctonia solani* AG2-2(IV) KACC 40152 | 79.14 ± 4.09 |

[a]The agar well plate technique was used for inhibition of mycelial growth against various pathogens.
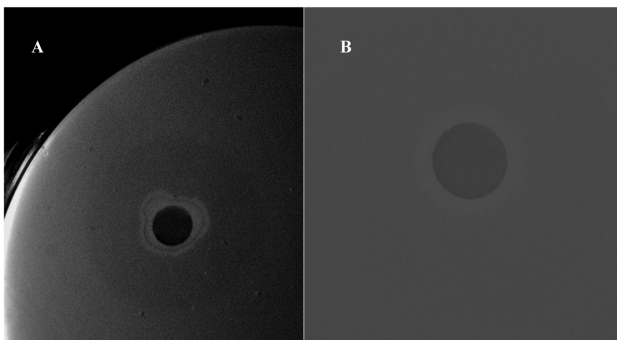


**Fig. 6.** Clear zone formation in chitin medium by cell free culture supernatant of *Bacillus subtilis* subsp. *subtilis* C9 using the paper disc diffusion method. A, Culture supernatant showed a clear zone around the paper disc; B, Control, (Triptic soy broth media) no clear zone was observed around the paper disc.

(Table 3).

**Enzymatic action of isolate C9.** The chitin containing agar assay described by Patil *et al.* [21] was performed for confirmation of chitinase production from the selected isolate. A significant clear zone (35 mm in diameter) was observed after placement of cell free culture supernatant of isolate C9 by a paper disc (Fig. 6).

## Discussion

An efficient antagonistic soil microorganism, C9, isolated from agricultural crop fields and identified as *Bacillus subtilis* subsp. *subtilis* C9, exhibited remarkable antagonistic activity against various plant pathogenic fungi. Most *Bacillus* spp. produce many kinds of antibiotics, including bacillomycin, fengycin, mycosubtilin, and zwittermicin, which are effective for suppression of the growth of target pathogens [22]. The antagonistic activity of the selected isolate C9 against several pathogenic fungi may be the antibiotic effect.

Many factors play important roles in the process of antibiotic production and consequently affect the antagonistic activity of the bacterial species. In this work, nutritional (carbon and nitrogen) composition and environmental conditions (pH and temperature) influenced consideration of isolate C9 for use in production of antibiotic substances with antifungal actions. Results of this study are consistent with those of previous studies, where the culture media had a significant influence on production of biosurfactant from *B. subtilis* and the highest levels of growth inhibition occurred in the presence of (2%) glucose [23]. In the present study, mannitol (1%) and soytone (1%) were selected as the optimum sources of carbon and nitrogen, respectively, for use in production of antibiotic substances. A previous study found that mannitol increased inhibition of the pathogen and was the sole carbon source for antibiotic production [24]. This finding is in agreement with that of Besson *et al.* [25], who reported that mannitol works as a suitable source of carbon for use in synthesis of the antibiotic, iturin A, from *Bacillus subtilis*. Yu *et al.* [26] reported that, compared with inorganic nitrogen sources, organic nitrogen sources were associated with relatively higher antifungal antibiotic production from microorganisms. The initial pH and culture temperature are critical factors for microbial growth and metabolic biosynthesis, which affect antibiotic production [27]. Findings from the present study demonstrated that the optimum pH and temperature for production of antibiotic substances from isolate C9 was 7.0 and 30°C, respectively, which was similar to the findings of EL-Abyad *et al.* [28], and Gong *et al.* [29].

Ethyl acetate crude extract and fraction DG4 (isomer of acetylbuanediol), which was selected from isolate C9, were found to induce significant inhibition of mycelial growth of various pathogenic fungi (Table 3, Fig. 5). These volatile compounds (acetylbuanediol) were previously isolated from *B. subtilis* GB03 and *B. subtilis* IN937a, and were found to induce significant reductions in disease severity of *Arabidopsis* caused by *Erwinia carotovora* subsp. *carotovora* [17]. In particular, this VOC, acetylbuanediol, was released exclusively from *Bacillus* spp. [30] and initiated the highest level of growth promotion of plants [16]. Application of (RR) and (SS) isomers of acetylbuanediol, similar to our purified compound DG4 (acetylbuanediol) from isolate C9, was found to trigger ISR and protect the *Arabidopsis* seedling from pathogen infection [17]. A similar effect was observed when Zoysia grass inoculated with pathogen was drenched with the selected isolate C9. It was found to induce significant suppression of *Rhizoctonia* stem-segment colonization and to promote grass health under greenhouse conditions. To the best of our knowledge, few reports have been published with regard to stimulation of ISR by VOCs from microorganisms. Findings from the present study demonstrated elicitation of ISR by volatile chemicals released from isolate C9 (plant growth promoting

rhizobacteria), as well as a new role for bacterial VOCs in initiation of plant defense responses and suppression of disease severity.

Microbial VOCs may play a role in initiation of biochemical changes of either primary or secondary plant metabolism and can also inhibit protein synthesis (bind with DNA and inhibit transcription processes) of pathogens. The chitinolytic natures of some selected microorganisms are useful for control of microbial pathogens. The inhibitory effect of the present purified compound may be due to its ability to inhibit spore germination, or synthesis of β-(1, 3)-D-glucan, or inhibition of an integral component of fungal cell walls, leading to alteration of the permeability of fungal cell membranes. The chitinolytic enzyme produced by isolate C9 may be involved in biological control of plant pathogens, particularly *R. solani* AG2-2(IV).

## Acknowledgements

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