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Article

Isolation of Antifungal Compound and Biocontrol Potential of *Lysobacter antibioticus* HS124 against Fusarium Crown Rot of Wheat

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***Fusarium graminearum* is the main cause of substantial economic loss in wheat production. The aim of this study is to investigate biocontrol potential of *Lysobacter antibioticus* HS124 against *F. graminearum* and to purify an antifungal compound. In preliminary study, n-butanol crude extract revealed destructive alterations in the hyphal morphology of *F. graminearum* and almost degraded with 1,000 μg mL^{-1} concentration. For further study, the antifungal compound extracted from the n-butanol crude extract of *L. antibioticus* HS124 was identified as *N*-Butyl-tetrahydro-5-oxofuran-2-carboxamide ($C_9H_{16}NO_3$) using NMR (^{1}H-NMR, ^{13}C-NMR, ^{1}H-^{1}H COSY, HMBC, and HMQC), and HR-ESI-MS analysis. To our knowledge, *N*-Butyl-tetrahydro-5-oxofuran-2-carboxamide may be a novel compound with molecular weight of 186.1130. The minimum inhibitory concentration value of antifungal compound was 62.5 μg mL^{-1} against *F. graminearum*. In an *in vivo* pot experiment, crown rot disease from *F. graminearum* was inhibited when wheat seeds were treated with both HS124 culture and *F. graminearum*. Growth of wheat seedling was enhanced by treatment of HS124 compared to control. Our results suggest that *L. antibioticus* HS124 characterized in this study could be successfully used to control *F. graminearum* and could be used as an alternative to chemical fungicides in modern agriculture.**

Key words: Wheat, *Fusarium graminearum*, *Lysobacter antibioticus* HS124, N-Butyl-tetrahydro-5-oxofuran-2-carboxamide, Biocontrol

Result of Minimum inhibitory concentration of *N*-Butyl-tetrahydro-5-oxofuran-2-carboxamide against *Fusarium graminearum*. A: water control; B: control treated with methanol. C: 1000 μg mL^{-1}; D: 500 μg mL^{-1}; E: 250 μg mL^{-1}; F: 125 μg mL^{-1}; G: 62.5 μg mL^{-1}; H: 31.25 μg mL^{-1}, I: 15.625 μg mL^{-1}.

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Introduction

Fusarium graminearum is considered as a serious soilborne pathogen to cause crown rot, root rot, and head blight in wheat (Scherm et al., 2013; Waalwijk et al., 2003). It has been reported that many of crown rot causal agents are several complex species of *Fusarium* (Miedaner et al., 2008; Scherm et al., 2013). This pathogen produced lesions on a basal stem showing a reddish-brown to black discoloration. Moreover, rot symptom at crown and root is recognized by whole plants or stunted individual tillers (Agrios, 2005) and also a typical pink discoloration may be observed on infected wheat plants. It is known that this fungus can produce several toxins which are harmful to human consumption and also cause emetic in animal (Prescott et al., 1986; Vesonder et al., 1976). To control the plant diseases, fungicides are extensively used in agricultural fields. They can cause various environmental pollutions and health problems. Furthermore, many important fungal pathogens become resistance to many active ingredients of chemicals (Daoubi et al., 2005; Knight et al. 1997; Russell, 1995). Therefore, biological control has been used for alternative choice to control fungal plant diseases that are non-pollution to humans and animals.

Antagonistic bacteria are considered as biological control agents due to their rapid growth and long term survival in the rhizosphere producing plant growth promoting compounds and antibiotics (Strange, 2003). The previous studies reported that antagonistic bacteria which are strongly active against phytopathogenic fungi are the members of *Bacillus*, *Lysobacter*, *Pseudomonas* (Abdulkadir and Waliyu, 2012; Beneduzi et al., 2012; Gómez et al., 2015; Jamal et al., 2015). *Lysobacter* is known to produce metabolites or other substances that have been shown to be effective in the growth suppression of plant pathogenic fungi. The genus *Lysobacter* is gram-negative bacteria and characterized by gliding motility (Christensen and Cook, 1978; Hans, 2006). Several species of *Lysobacter* have been reported as biocontrol agents against phytopathogens; *L. enzymogenes*, *L. capsici*, *L. gummosus*, *L. daejeonensis*, *L. yangpyeongensis*, *L. defluvii*, *L. niabensis*, *L. niastensis* and *L. antibioticus* (Christensen and Cook, 1978; Weon et al., 2006; Weon et al., 2007; Yassin et al., 2007). One of *Lysobacter* species, *L. antibioticus* is a species having a potential to produce antibiotics for biocontrol ability. Ji et al. (2008) demonstrated that *Lysobacter antibioticus* strain 13-1 inhibited the growth of a bacterial leaf blight pathogen caused by *Xanthomonas oryzae* pv. *oryzae*. In previous study, *Lysobacter antibioticus* HS124 was isolated from rhizosphere soil in Korea (Ko et al., 2009). This bacterium showed antifungal activities against several phytopathogenic fungi where 4-hydroxyphenylacetic acid was purified as an antibiotic compound. Furthermore, several lytic enzymes such as chitinase and β-1,3-glucanase were produced by the HS124 (Ko et al., 2009). In disease management, the HS124 may be used as a biological control agent, providing an alternative to the use of chemical synthetic pesticides. The objectives of the current study were to isolate an antifungal compound from *L. antibioticus* HS124, to characterize its antagonistic activity against *F. graminearum*, and to investigate the potential of *L. antibioticus* HS124 to control crown rot of wheat plants in *in vivo* pot experiment.

Materials and Methods

Microbial species and culture conditions *Lysobacter antibioticus* HS124 used in this study was isolated from rhizosphere soil in Korea (Ko et al. 2009). The fungus, *Fusarium graminearum* KACC 41040, obtained from Korea Agriculture Culture Collection (KACC) was cultured on potato dextrose agar media (PDA; Difco Laboratories) for further study. For the further studies such as minimum inhibitory concentration (MIC) assay and seed treatment in *in vivo* pot experiments, the spore suspension of *F. graminearum* was prepared with culturing it with carboxymethyl cellulose broth at 25ºC in shaking incubator for 10 days followed by filtering through cheesecloth. Spore concentration was determined using haemacytometer.

Production of antifungal compound HS124 was cultured in Luria-Bertani (LB) broth medium at 30ºC for 10 days. The culture broth was centrifuged at 7,000 *g* for 20 min. After centrifugation, the supernatant was acidified with concentrated HCl to pH 2 and then filtered through a Whatman filter paper No. 2. The filtrate was extracted with same volume of n-butanol. The n-butanol soluble organic fraction was concentrated by using a rotary evaporator (Büchi Labortechnik, Flawil, Switzerland). The n-butanol crude extract was obtained, tested for antifungal activity, and purified by column chromatography to isolated antifungal compounds.

Effect of the crude extract on *F. graminearum* hyphae To examine the effect of the crude extract against *F. graminearum* hyphae, fungal pathogen was cultured on potato dextrose broth media at 25ºC for 7 days. The crude extract was dissolved in methanol to make the stock solution. The different crude extract concentrations were prepared and added to the tissue culture microplates and then mixed with the *F. graminearum* culture to make a final concentration of 250, 500 and 1,000 μg mL^{-1}. The same volume of methanol was used as control. The mixture of *F. graminearum* and the crude extract was incubated at 25ºC for 72 h and mycelia were observed under the light microscope (Olympus BX41TF, Japan). All tests were done in triplicate for morphological observation of mycelia.

Purification and identification of antifungal compound The crude extract was purified by silica gel column

chromatography. A stepwise elution was carried out using a mixture of chloroform-methanol (from 10:0 to 8:2, v/v). Purified compounds obtained after fractional collection were then concentrated and tested for antifungal activity. The active fraction was further purified by high performance liquid chromatography (HPLC) system with Prep C18 column (7 × 300 mm, 10 μm). The elution was monitored using a SPD-10 UV-VIS detector (Shimadzu, Japan) at 210 nm with manual injection. Each peak was separately collected using acetonitrile and water as mobile phase (40:60) at a flow rate of 3 mL min^{-1}. All peak fractions were collected and concentrated using the evaporator at 40ºC. The purity of collected fractions was analyzed using analytical C18 column (4.6 × 250 mm, 5 μL). The fraction showing a single peak was analyzed for determining chemical structures.

The structure of the purified compound was confirmed using nuclear magnetic resonance (NMR). ^{1}H and ^{13}C NMR spectra were measured in CD_3OD with a Bruker AMX-500 spectrometer (VNMRS, Agilent, USA) at 500 MHz for ^{1}H NMR spectra and 125 MHz for ^{13}C NMR spectra. Chemical shifts were calculated using tetramethylsilane (TMS) as the internal standard. ^{1}H and ^{13}C NMR assignments were supported by $^{1}H-^{1}H$ correlation spectroscopy (COSY), heteronuclear multiple-quantum coherence (HMQC), and heteronuclear multiple-bond correlation (HMBC) experiments. The high resolution mass spectrometry (HRMS) with SYNAPT G2 HDMS quadrupole time-of-flight (QTOF) mass spectrometer equipped with an electrospray ion source (Waters, U.K.) by the Ochang branch of the Korean Basic Science Institute was elucidated to confirm their structure of the compound with NMR analysis.

Determination of MIC The purified compound was tested for MIC values against *F. graminearum* using broth microdilution technique. A stock solution of 2000 μg mL^{-1} of the purified compound was prepared in methanol. The conidial suspension of *F. graminearum* (10^6 spores mL^{-1}) was added to each well of a 24-well the microplate. After addition of suspension, the purified compound was added to each well containing conidial suspension to obtain desired concentration of 1000 μg mL^{-1}, 500 μg mL^{-1}, 250 μg mL^{-1}, 125 μg mL^{-1}, 62.5 μg mL^{-1}, 31.25 μg mL^{-1}, and 15.625 μg mL^{-1}. The methanol was used as a control. The MIC values were taken as the lowest concentration of the purified compound, resulting to pigment reduction and low turbidity in the well after incubated at 25 ± 2°C for 5 days. The effect of the purified compound on inhibiting the growth of *F. graminearum* was also observed under light microscope.

Evaluation of seeds treated with HS124 for control of Fusarium crown rot of wheat To investigate the ability of HS124 to control Fusarium crown rot (FCR) of wheat *in vivo*, a pot experiment was conducted. Wheat seeds were disinfected with 1% Sodium hypochlorite (NaOCl) for 1 min and then washed in sterilized distilled water for 3 times. The pots were watered for 7 days before sowing. Before sowing, wheat seeds were drenched with 10 days grown HS124 grown in GCM medium for 1 h in orbital shaker at 100 rpm. After drenching, one ml of *F. graminearum* conidia suspension (10^5 spores mL^{-1}) was sprayed on seeds. Ten seeds were sown per a pot filled with commercial grade bedding soil (12-cm in diameter). The seeds treated with sterile distilled water were served as control. The pots were kept in an artificially illuminated room for 16 h photoperiod. All the assays were performed in triplicate. After 6 weeks of sowing, assessment of FCR disease and growth parameter of plants was observed.

Results and Discussion

Effect of the crude extract on *F. graminearum* hyphae The growth of *F. graminearum* was affected by the butanol crude extract from HS124. The antifugal activity of the crude extract against *F. graminearum* increased with the increase of its concentration. The microscopic study revealed alterations in the hyphal morphology of *F. graminearum*, including loss of apical growth, deformation, and lysis. Furthermore, 500 and 1,000 μg mL^{-1} of the crude extract showed more damage of *F. graminearum* mycelia compared to 250 μg mL^{-1}, while control treatment showed normal morphology under the light microscope (Fig. 1). This present study demonstrated that the morphology of *F. graminearum* hyphae may be affected by the antibiotics contained in the crude extract from HS124. The antagonistic bacteria may mediate biocontrol activities by one or more types of mechanisms of diseases suppression (Weller, 1988). Many studies reported that primary mechanism of pathogen inhibition is by producing secondary metabolites e.g., antifungal metabolites and antibiotics, Fe^{+} chelating siderophores, ammonia and hydrogen cyanide (Lovic et al., 1993; Weller, 2007). Heat-stable antifungal metabolites isolated by thin-layer chromatography from *L. enzymogenes* Strain C3 inhibited the growth of *F. graminearum* (Li et al., 2008). Liu et al. (2004) reported that the crude extract from *Bacillus substilis* JA showed strong inhibitory activity against *Rhizoctonia solani* and *F. graminearum*. A similar result was reported that inhibition of spore germination and hyphal growth of *Colletotrichum gloeosporioides* was inhibited by treatment of crude extract from *Streptomyces cavourensis* SY224 (Lee et al., 2012). Furthermore, the previous study has been reported the effect of *Paenibacillus ehimensis* KWN38 against soilborne phytopathogenic fungi which had strong fungal growth inhibition (Naing et al., 2014).

Purification and identification of antifungal compound The butanol crude extract of cell free filtrate of HS 124 showed significant antifungal activity towards *F. graminearum*.

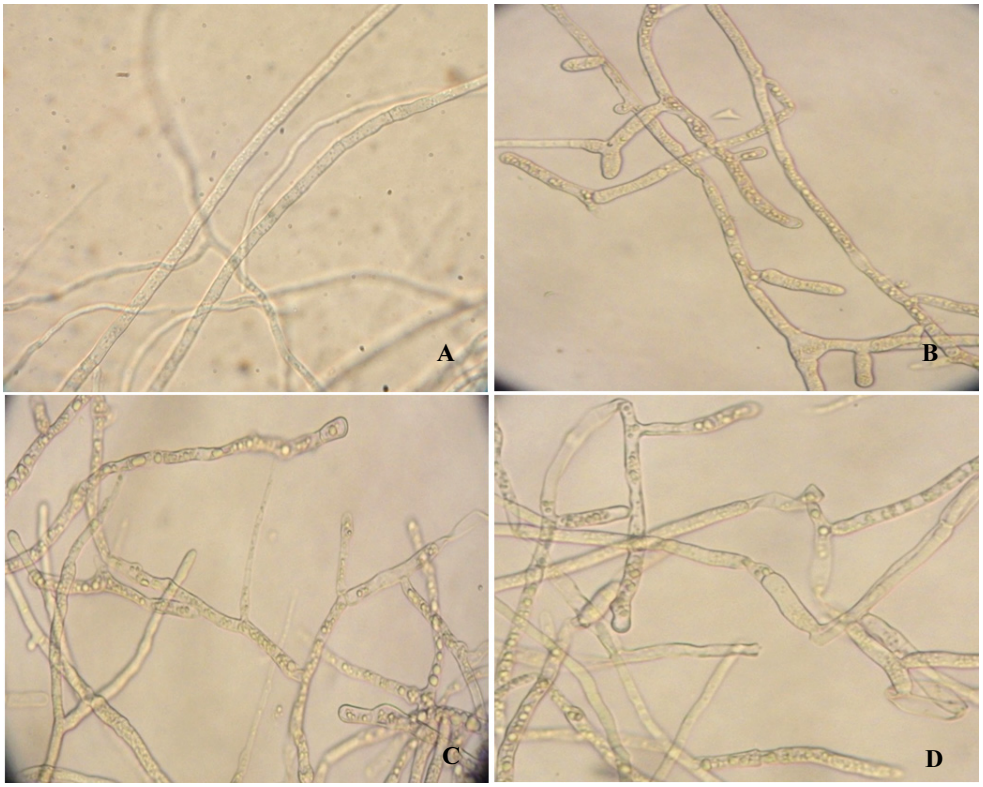

Fig. 1. Light microscopy examination on effect of crude extract from *Lysobacter antibioticus* HS124 on hyphal morphology of *Fusarium graminearum*. A : treated with methanol as a control, B : treated with 250 μg mL^{-1} of the crude extract, C : treated with 500 μg mL^{-1} of the crude extract and D : treated with 1,000 μg mL^{-1} of the crude extract.

By silica gel column chromatography, the fraction having antifungal activity was obtained and then further purifiedusing C18 prep HPLC column. The purified compound having a single peak was presented as retention time (t_R) of 4.672 min at 210 nm (Fig. 2). The purified compound was further subject to spectroscopic analysis for identification i.e NMR and HRMS. The ^{1}H and ^{13}C NMR recorded signals at (500MHz, CD_3OD): δ0.97 (H-3), 1.42 (H-2), 4.18 (H-2), 4.29 (H-1);

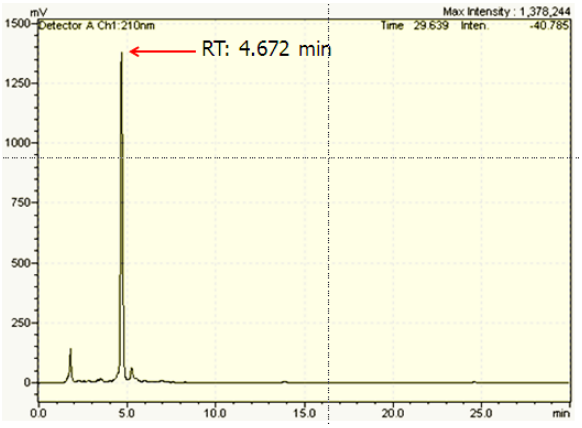

Fig. 2. High performance liquid chromatography (HPLC) spectrum of the purified antifungal compound from *Lysobacter antibioticus* HS124, showing a single peak at retention time of 4.672 min.

13C-NMR (125MHz, CD_3OD): δ 64.9 (C-1), 55.7 (C-2), 30.3 (C-2'), 24.5 (C-3), 18.7 (C-3'), 28.9 (C-4), 12.3 (C-4'), 179.7 (C-5), 172.7 (C-6). The structure of the purified compound corresponded to *N*-Butyl-tetrahydro-5-oxofuran-2-carboxamide as analyzed by HR-ESI-MS analysis based on the $[M+H]^+$ ion. HR-ESI-MS analysis demonstrated that this compound had molecular weights of 186.1130 Da with the formula $C_9H_{16}NO_3$ (Fig. 3 and 4). To our knowledge, this compound is first report as an antifungal compound purified from *L. antibioticus* and showed antifungal activity against *F. graminearum*. In previous study, Ko et al. (2009) explained that the 4-hydroxyphenylacetic acid isolated from *Lysobacter antibioticus* HS124 strongly inhibited growth of *Phytophthora capsici*. Moreover, genus *Lysobacter* has been widely studied to synthesize many natural products, including lysobactin, tripopeptin, xanthobaccin, maltophilin, dihydromaltophilin, phenazine, lactivicin (Xie et al., 2012), HSAF (Li et al., 2008), and WAP-8294A2 (Zhang et al., 2011).

Determination of MIC MIC value of the purified compound against *F. graminearum* was determined using a serial dilution method. As shown in Fig. 5, N-Butyl-tetrahydro-5-oxofuran-2-carboxamide inhibited the conidial germination (Fig. 5C, D, E and F). The result of MIC evaluation demonstrated that the *N*-Butyl-tetrahydro-5-oxofuran-2-carboxamide had the MIC value of 62.5 μg mL^{-1} against *F. graminearum*

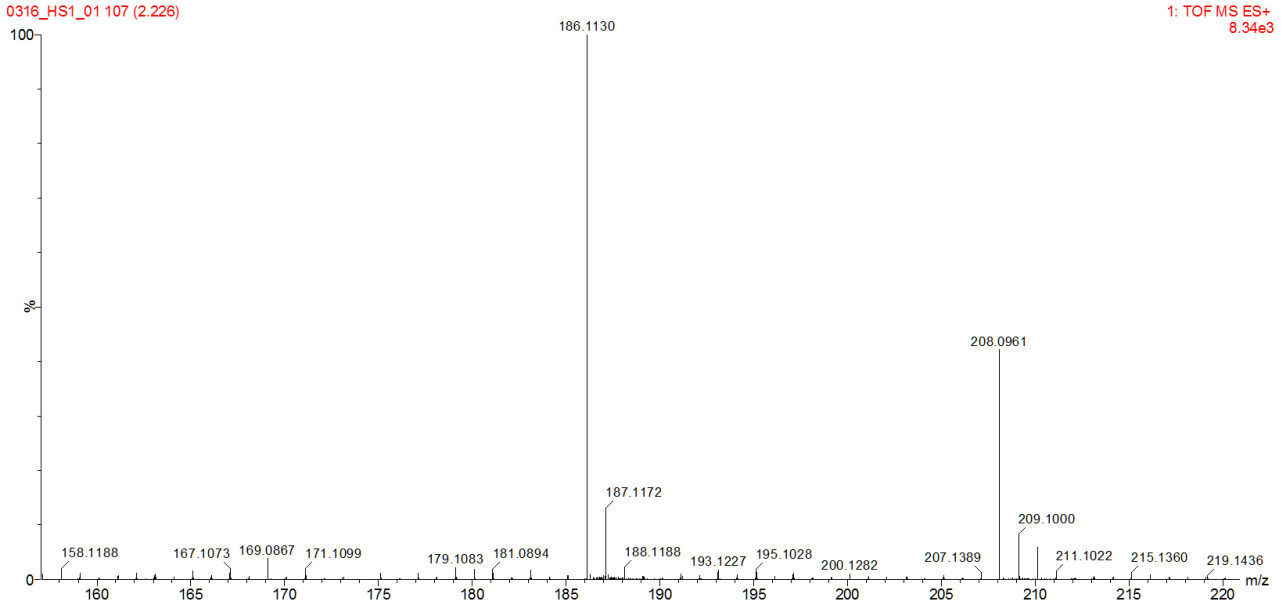

Fig. 3. The high resolution mass spectrometry (HRMS) spectrum of *N*-Butyl-tetrahydro-5-oxofuran-2-carboxamide produced by *Lysobacter antibioticus* HS124, showing main ion peaks at 186.1130.

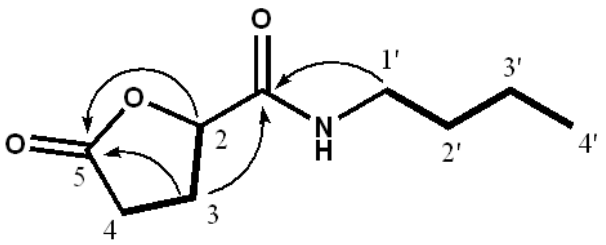

Fig. 4. Chemical structure of *N*-Butyl-tetrahydro-5-oxofuran-2-carboxamide ($C_9H_{16}NO_3$).

(Fig. 5G). The effect was reduced with decreasing concentration of the compound. Mycelial growth and conidial germination were not affected at 31.25 μg mL^{-1} and 15.625 μg mL^{-1} (Fig. 5H and I). In previous reports, benzoic acid isolated from *Bacillus subtilis* GDYA-1 showed the MIC value of 12 μg mL^{-1} (Yoon et al., 2012). In addition, *Paenibacillus elgii* HOA73 produced 3,4-dihydroxybenzoic acid which inhibited any visible mycelial growth of *R. solani*, showing the MIC of 64 mg mL^{-1} (Nguyen et al., 2015). Furthermore, the pigment produced by *F. graminearum* in broth culture was clearly reduced with increasing compound concentration (data not shown). The pigments are produced by various fungal pathogens such as melanin, which represents virulence factor to promote fungal pathogenicity of several pathogenic fungi (Cao and Yang, 2006; Jacobson, 2000). Accordingly, the previous study reported that *Bacillus subtilis* G8 produced antifungal compounds that inhibit the mycelial growth and pigment production of various pathogenic fungi (Liu et al., 2008). Thus, this result could be possible to support biocontrol potential of HS124 against *F. graminearum* for reducing the disease level.

Evaluation of seeds treated with HS124 for control of Fusarium crown rot of wheat In *in vivo* pot experiment, wheat seeds sprayed with *F. graminearum* spore suspension had a similar germination rate when compared to seeds that were not treated with *F. graminearum* (Fig. 6). This result revealed that *F. graminearum* inoculated by spraying method had no effect on seed germination rate which is according to study reported by Jung et al. (2013). Wheat seeds treated with HS124 culture broth have positive effects on the seedling growth compared to non-treatment as a control (Fig. 6A). Although the seeds without the treatment of HS124 culture broth showed symptom of discoloration from brown to black on basal stem as symptoms of Fusarium crown rot disease (Fig. 6B), the healthy seedling was observed in the treatment of HS124 culture broth (Fig. 6C). Previous studies have shown that application of *Lysobacter* spp. reduced diseases caused by different plant pathogens in several crops (Gómez Expósito et al., 2015). Furthermore, many biocontrol agents not only suppress plant pathogenic diseases but also induce the promotion of plant growth (Johansson et al., 2003). Kloepper and Schroth (1981) reported that the emergence of canola seeds coated with PGPR before planting was resulted in increase of 10-40% compared with untreated. Further research revealed that *Bacillus subtilis* increased leaf length of rice (Jia et al., 2014). Former reports demonstrated that many genera of bacteria were known to be plant growth promoting rhizobacteria (PGPR) like *Bacillus* (Castro et al., 2008; Sivasakthi et al., 2014), *Paenibacillus* (Kim et al., 2016) and *Lysobacter* (Ekici and Yuen, 2004), which can promote plant growth and control plant diseases.

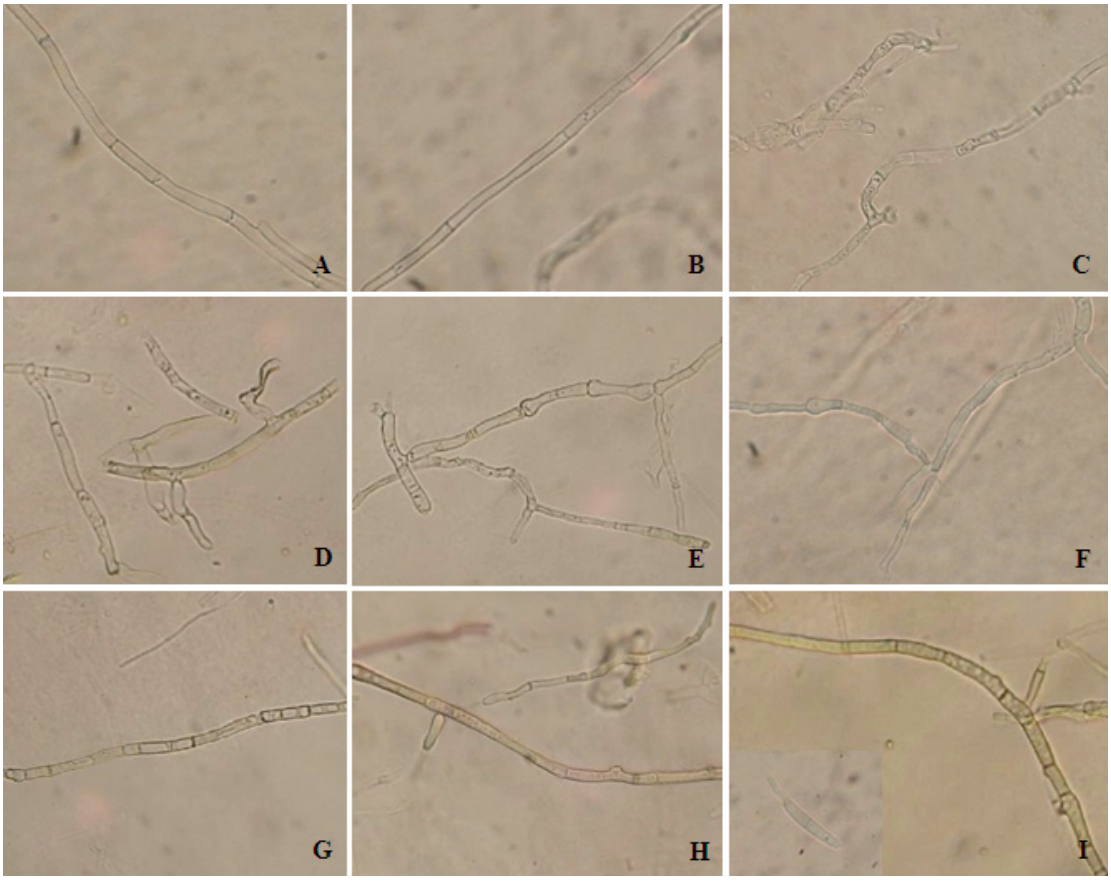

Fig. 5. Minimum inhibitory concentration of *N*-Butyl-tetrahydro-5-oxofuran-2-carboxamide against the hyphal growth of *Fusarium graminearum* with different concentrations at 15.625-1000 μg mL^{-1}. A: treated with water as a control, B: treated with methanol as a control, C: 1000 μg mL^{-1}, D: 500 μg mL^{-1}, E: 250 μg mL^{-1}, F: 125 μg mL^{-1}, G: 62.5 μg mL^{-1}, H: 31.25 μg mL^{-1}, I: 15.625 μg mL^{-1}.

Fig. 6. Effect of *Lysobacter antibioticus* HS124 on fresh length and disease occurrence in wheat seedlings. A : A six-week-old plant treated with the HS124 (left) and that treated with sterilized distilled water as a control (right), B : A six-week-old plant having crown rot symptom after the treatment of *Fusarium graminearum* and C : A six-week-old plant having no crown rot symptom after the treatment of both the HS124 and *F. graminearum*.

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