IDENTIFICATION OF CYCLO(L-PRO-D-TYR) FROM *BACILLUS AMYLOLIQUEFACIENS* Y1 EXHIBITING ANTIFUNGAL ACTIVITY AGAINST *FUSARIUM GRAMINEARUM* TO CONTROL CROWN ROT IN WHEAT

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Abstract. In this study, evidence for antagonism of antifungal metabolites produced by *Bacillus amyloliquefaciens* Y1 was described, as they actively inhibit growth of *Fusarium graminearum* under *in vitro* and *in vivo* condition. The culture supernatant and crude butanol extract inhibit the mycelial growth of *F. graminearum*. In addition, an antifungal compound was purified from the metabolites of Y1 and identified as cyclo(L-Pro-D-Tyr) using ^{1}H and ^{13}C NMR spectroscopic analysis. For the first time, cyclo (L-Pro-D-Tyr) displayed potent antifungal activity against *F. graminearum* under *in vitro* condition. The hyphae of this fungal pathogen became deformed with cyclo(L-Pro-D-Tyr). Exposure of *F. graminearum* to various concentrations of cyclo(L-Pro-D-Tyr) on wheat seeds significantly inhibit fungal colonization. Furthermore, during *in vivo* wheat pot experiment, Y1 results in 27% higher total yield and showed a protective effect against *F. graminearum* by reducing low discoloration symptoms on stems. The grain yield per pot was five times higher compared to control pots (water only) infected with *F. graminearum*. To our knowledge, the antifungal activity of the cyclo(L-Pro-D-Tyr) is reported for the first time against the plant pathogenic fungus *F. graminearum.* The present study demonstrated the potential of *B. amyloliquefaciens* Y1 as a biocontrol agent against the wheat crown rot fungal pathogen as well as a plant growth promoter for wheat.
Keywords: *biological control, wheat disease, chemistry, antifungal, pathogen*

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Introduction

Two major destructive fungal diseases that posing a serious threat to wheat cultivation and production are head blight and crown rot caused by the mycotoxin-producing fungal pathogen *Fusarium graminearum*. *Fusarium* crown rot (FCR) is characterized by dry rot of crown, necrosis of root tissue and basal stem, and limited grain yield. *Fusarium* crown rot has never gotten much attention as it is mainly considered to be limited in its geological distribution, but according to latest reports, it occurs in many cereal producing regions (Smiley et al., 2005 a,b). A severe intensive infection can result in more than 50% yield loss, and reduced grain quality. It is known that this fungus is harmful to animals and humans because it produces several mycotoxins in wheat such as zearalenone (ZEA) (Cowger and Arellano, 2013) and deoxynivalenol (DON), that can cause embryotoxic, neurotoxic, immunosuppressive and teratogenic effects (Cowger and Arellano, 2013; Pestka, 2007; Pestka and Smolinski, 2005).

Conventional methods such as crop rotation and inter-row sowing can be used to control diseases caused by *F. graminearum*. Crop rotation is effective, but there are also economic limitations, as *F. graminearum* can reside inside the stubble for several years (Burgess, 2005). The recent reports revealed that there is a remarkable reduction in the *Fusarium* crown rot (FCR) damage due to inter-row sowing (Simpfendorfer et al., 2012). Due to limitations, FCR has recently been reported in cereal-growing regions. Moreover, chemical management is effective but the application of chemicals such as acephate, carbendazin, chlorothalonil, chlopyriphos, Dichlorodiphenyltrichloroethane (DDT), and diazinon causes environmental pollution, affects human health, and enables pathogens to build resistance to these chemicals (Baldwin and Rathmell, 1988). Few studies have been done about the losses caused by *F. graminearum* and more information is needed to manage this pytopathogenic fungus through the application of antagonistic microorganisms (Palazzini et al., 2016).

Several bacteria are available commercially including strains of the genera *Pseudomonas* and *Bacillus*, and have been used successfully as alternative to chemical pesticides in crop production (Choudhary and Johri, 2009). *Bacillus* species are among the most important bacteria that can be most promising for plant protection (Pérez-García et al., 2011). Their most notable characteristics include their ability to survive for extended periods of time, their spore formation, the wide range of biochemically active molecules that can significantly inhibit phytopathogen growth and ease of formulating their commercial products (Broggini et al., 2005). *Bacillus subtilis, Bacillus amyloliquefaciens* and *Paenibacillus polymyxa* mostly found in soil are considered to be safe for use in environment and with mammals (Stabb et al., 1994; Zhao et al., 2015). Two new cyclic lipopeptides (3 and 4) were isolated from the culture filtrate of *Bacillus amyloliquefaciens* strain SD-32, together with two known metabolites, iso-C15 and iso-C16 bacillomycin D (Tanaka et al., 2014). They can provide higher protection to plants (Falcäo et al., 2014; Krebs et al., 1993), because of their antifungal and antibacterial activities against several phytopathogens (Ben Abdallah et al., 2015; Krebs et al., 1998). They produce various types of secondary metabolites (antibiotics) (Asaka and Shoda, 1996; Stabb et al., 1994) and most of them have been identified as low molecular weight dipeptide or cyclic peptides (Dolej and Bochow, 1996; Nakano and Zuber, 1990; Vanittanakom et al., 1986). Among cyclic peptides, diketopiperazines (DKPs) are relatively simple cyclic dipeptides consisting of two α-amino acids produced by bacteria, fungi, mammals, and plants (Ström et al., 2002).

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More attention has paid to study about diversity and biological roles of more than 100 DKPs found in nature (McCleland et al., 2004). Four DKPs from *B. thuringiensis* strain and two from *B. endophyticus* demonstrated their antifungal activities (Sansinenea et al., 2016). Beside their antimicrobial properties, cyclic peptides are involved in root colonization and the systemic stimulation of immune system of the host plant (Ongena and Jacques, 2008). In general, the activities of cyclic peptides from *Bacillus spp*. against fungal phytopathogens have been frequently described. Synthetic DKPs has also been reported to have antibacterial and antifungal activities (Graz, 2002; Pitchen, 2002).

The present study was aimed to evaluate antifungal potential of *B. amyloliquefaciens* Y1 against *F. graminearum* under *in vitro* and *in vivo* condition. In addition, identification of an antifungal compound may have a role in its probable mechanism to control *Fusarium* crown rot in wheat.

Materials and methods

Microorganisms and culture conditions

B. amyloliquefaciens Y1 was isolated previously with accession number KP967704. *Fusarium graminearum* KACC 41040, was obtained from Korea Agriculture Culture Collection (KACC). For *in vitro* and *in vivo* study, a spore suspension was prepared by growing *F. graminearum* in carboxymethyl cellulose (CMC) broth at 25 °C in shaking incubator for 10 days.

Antifungal characterization

Antifungal activity of culture supernatant

Antifungal activities were detected by growing strain Y1 in Luria-Bertani 33 (Palazzini et al., 2016) broth at 40 °C for 7 days at 170 rpm. The culture broth was centrifuged at 7,000 × g for 20 min. The supernatant collected was filtered through Whatman filter paper No. 2 (0.45 μm) and finally with 0.20 μm syringe filter. The resulting supernatant was mixed with sterile PDA to make final concentration of 0, 10, 30, and 50%. A 5 mm diameter mycelial plug of *F. graminearum* from a 7-days-old culture was placed in the centre of the PDA plate and incubated at 28 °C in the dark. Mycelial growth was measured as colony diameter of *F. graminearum* hyphae after 6 days inoculation and growth inhibition percentage were evaluated by using the following formula:

$$\text{Growth inhibition percentage (GI \%)} = [(R - r) / R] \times 100$$

where R is the radius/ diameter of the colony on the control plates and r is the radius of the colony on the treatment plate. Experiment was carried out in triplicate and repeated three times.

Antagonistic activity of bacterial crude extract against the plant pathogens

Strain Y1 was inoculated into the LB broth at 40 °C for 10 days. The pH of Y1 culture broth was adjusted to 3 using concentrated HCl and used to obtain the crude extract by active fractionation (1:1, vol/vol) with an eluotropic series of solvents: ethyl

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acetate and n-butanol. The partially isolated butanol solvent was concentrated through a rotary evaporator to get crude extract (48 g) (Büchi, Rheinstetten, Germany). A 20% stock solution of crude extract was prepared. From the stock solution, 3, 4 and 5 mg was added on paper disc to check antifungal activity against *F. graminearum*. A 5 mm diameter mycelial plug from 7 days old culture was placed in the centre of the PDA plate. The control consists of an equal volume of methanol added to another paper disc on the same PDA plate. Mycelial growth was measured as colony diameter of *F. graminearum* and growth inhibition was evaluated by using the following formula:

$$(GI\ \%) = [(R - r)/R] \times 100$$

where R is the radius/diameter of the fungal growth towards the paper disc on the control side and r is the radius of the fungal colony growth towards the paper disc on the treatment side. Experiment was carried out in triplicate.

Extraction and purification of the antifungal compound

The butanol crude extract (48 g) was prepared from cell free culture supernatant (36 L) of strain Y1 and was dissolved in methanol and subjected to silica gel column chromatography (Kieselgel 60, 70-230 mesh, Merk, Darmstadt, Germany) with stepwise elution of $CHCl_3$: MeOH (100:0, 90:10, 70:30, 40:60, 50:50, and 0:100; v/v). Total of 3L solvent was used in each step of elution. The vacuum (EYELA rotary vacuum evaporator) was used to concentrate all fractions to a semisolid mass, and each fraction was assayed for antifungal activity. The bioactive fraction $CHCl_3$: MeOH (100:0) was further purified by HPLC (high-performance liquid chromatography) using a system with a PrepHT C18 column (7 × 300 mm, 10 µm). The SPD-10 UV-VIS detector (Shimadzu, Japan) was used to monitor elution with manual injection at 210 and 254 nm using acetonitrile and water as a mobile phase (35:65) at a flow rate of 2 mL/min. All peaks were collected and concentrated using a centrifugal evaporator (Hanil Scientific Inc, South Korea) at 40 °C. The purities of the collected fractions were further analysed using an HPLC analytical C18 column (5 µL, 4.6 × 250 mm). The pure compound with a single peak was used for antifungal activity. The active compound was used for further structural analysis.

Identification of the purified antifungal compound

The ^{1}H and ^{13}C nuclear magnetic resonance (NMR) was used to determine the structure of the purified compound. Briefly, the purified antifungal compound (approx. 12 mg) was dissolved in 0.6 mL methanol-*d*4 (CD_3OD) and used for spectral analysis. NMR spectra were recorded on a DRX 500 NMR instrument (Bruker, Rheinstetten, Germany) operating at 500 MHz for ^{1}H and 125 MHz for ^{13}C at room temperature. Chemical shifts are reported in ppm (δ) using CD_3OD as the solvent (unless otherwise indicated) and tetramethylsilane $(CH_3)_4Si$ as an internal standard.

Determination of antifungal activity of the purified compound and its effect on hyphal deformation

The purified compound was assayed against *F. graminearum* growth. A 4% stock solution of the purified compound was prepared in methanol. A total of 1000 µL (a mixture of 300 µL conidial suspension + 700 µL CMC broth) was added to each well of

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a 24-well microplate except for the first well, which contained 600 µL of the conidial suspension of *F. graminearum* (4×10^6 spores/mL) and 1300 µL CMC. After addition of the conidial suspension, 100 µL of the compound was added to the first well. Further, serial dilution was performed to obtain the desire concentration of 2000, 1000, 500, 250, 125, 62.5, 31.25, 15.625, 7.81, 3.90, and 1.95 ppm in each well. The conidial suspension of *F. graminearum* and methanol were used as a control. The well plate was kept for one h in clean bench to evaporate methanol. The direct effect of the antifungal compound on hyphae was examined using light microscope (Olympus BX41TF, Japan).

Assessment of the purified compound to control F. graminearum on wheat seeds

Wheat seeds (n = 30) per treatment were immersed in 100 mL of DW (distilled water) for 1 h, followed by immersing 1 min in a dilute solution of NaOCl (sodium hypochlorite) for surface sterilization. They were then washed with distilled water and placed in petri dish to dry. Solutions of the purified compound at various concentration were prepared in methanol using serial dilution to obtain serial doses (2000, 1000, 500, 250, 125, 62.5, 31.25 ppm). Then, wheat seeds were soaked for 1 h in various concentration of compound and left in open petri dish to evaporate excess methanol. Seeds soaked in methanol were used as a control. A spore suspension of *F. graminearum* was prepared (4×10^6 spores/ml) and approximately 10 µl of the spore suspension was spread on each wheat seed. The inoculated wheat seeds were incubated at 28 °C on moisten cotton in petri dish, and the inhibition of fungal growth was recorded as white mycelia appeared on all seeds (30 = 100%) in the control treatment. All tests were performed in triplicate.

Glasshouse experiment

The growth promotion and biocontrol effect of strain Y1 in wheat plants were also focused. Two types of pot experiment were performed in a greenhouse at Chonnam national university, South Korea. A glasshouse potting mix was prepared by mixing soil, sand, vermiculite, and compost at a ratio of 2:1:1:0.04 (v/v), respectively. The soil was air-dried and ground to pass through a 2-mm sieve and mixed thoroughly. The potting mixture was transferred to 20 L pots containing 20-25 kg soil. A total 60 seeds per pot were sown in four rows. In one type of pot experiment, at two weeks interval, after 14,28, and 42 days of seeds sowing, each pot was amended by pouring 300 ml of water, or fertilizer media (M) (KH_2PO_4 0.08 g, KCl (0-0-60) 0.02 g, K_2SO_4 0.1 g, $CaCl_2$ 0.1 g, water soluble fertilizer 2.66 ml, blue fertilizer (20-20-20-2, 4g) per litre of water) or Y1 culture grown for 7 days at 40 °C in media known as BB media (chitin 0.5 g, gelatin 0.5 g, Yeast 0.1 g, KH_2PO_4 0.08 g, KCl (0-0-60) 0.02 g, K_2SO_4 0.1 g, $CaCl_2$ 0.1 g, water soluble fertilizer 2.66 ml, blue fertilizer (20-20-20-2, 4 g) and sugar 4 g/L of water) for a total of three times. The treatments were arranged in a randomized complete block design and replicated four times. To assess growth promotion in wheat, plant height, plant weight, root weight and grains per pot was calculated after four months.

To assess the biocontrol of crown rot in wheat by Y1, pots were amended with *F. graminearum* in the soil. One month after seed sowing, one mL of *F. graminearum* suspension containing 4×10^6 spores per mL was drench in each seedling. At 14, 28 and 42 days after seeds sowing, each pot was amended by pouring 300 mL of water (C + Fg), or fertilizer media (M + Fg), or strain Y1 culture grown in BB media

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(Y1 + Fg) or commercial fungicide (F + Fg). A total of 60 wheat tillers were randomly collected from each replicate after harvesting and assessed for the incidence of crown rot by recording the percentage of tillers with brown discoloration on the lower stems (Liu and Ogbonnaya, 2015). After harvesting the plants, reduction in yield due to crown rot was also measured by measuring plant height, plant weight/pot, root weight, and grains weight/pot.

Statistical analysis

The data were statistically analysed through SAS 9.1 software (SAS Institute, 2003). The least significant difference (LSD) was used to calculate mean values among treatments at a 5% level (p = 0.05) of significance.

Results

Antifungal characterization

The cell-free culture supernatant displayed antifungal activity at various concentrations mixed in PDA as it inhibited the growth of *F. graminearum*. The inhibitory effects increased with concentration increased (*Fig. 1a*). At 10% concentration, the cell-free culture supernatant showed more than 30% growth inhibition of *F. graminearum*. Higher concentration increased the growth inhibition, as 40 and 45% growth inhibition were recorded for the 30 and 50% concentration, respectively.

The paper disc method assessed the antifungal effect of the bacterial *n*-butanol crude extract. The Y1 crude extract at various concentrations had good inhibition effect on the pathogenic fungi (*Fig. 1b*). The crude extract (3 mg) affected the growth of the tested fungi by 13%. An increase in the inhibitory effect was found as the amount of treatment increased. The highest growth inhibition rates of 20% and 17% were obtained when 5 mg and 4 mg of crude extract were added to the paper disc respectively. The antifungal compound-enriched extract revealed interesting antifungal activities.

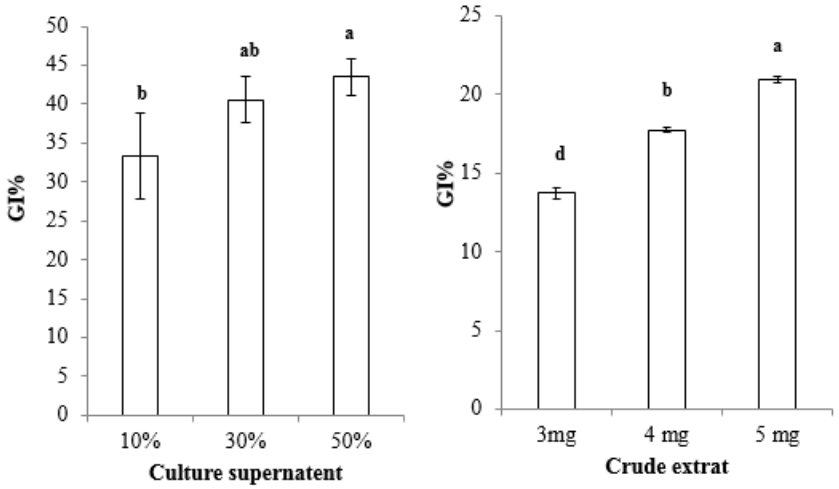

Figure 1. *Effect of Y1 culture filtrate (a) at various concentrations on growth of F. graminearum. Effect of different crude extracts (b) concentration of Y1 on growth of F. graminearum. Calculated mean in chart values are from three replicates. Error bars represent standard deviation of mean*

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Extraction and purification of antifungal compound

The Y1 crude extract displayed antifungal activity against *F. graminearum* according to paper disc assay. A bioactive fraction was found because of silica gel column chromatography. The bioactive fraction (2 g) was further subjected to prep HPLC. The purity of the compound was confirmed with a single peak on an analytical HPLC column (*Fig. 2*). The antifungal activity of pure compound was checked and confirmed by testing against *F. graminearum* using paper disc method. The 12 mg of pure active compound obtained from *n*-butanol crude extract of Y1 strain. The bioactive antifungal compound showed a single peak with a 4.93 min retention time.

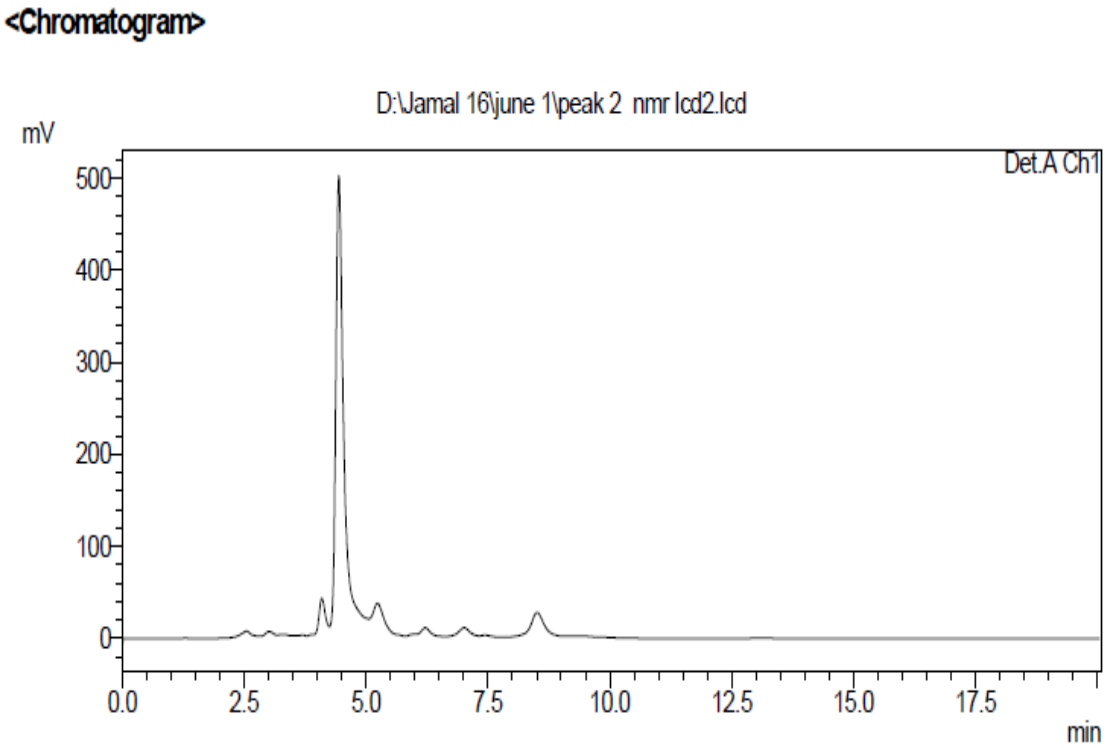

Figure 2. *The high-performance liquid chromatography (HPLC) spectrum of the purified antifungal compound from strain Y1*

Identification of the purified antifungal compound

The structure of the purified antifungal compound was identified according to its ^{1}H- and^{13}C-NMR spectra (*Fig. 3a* and *b*). Peak data were as follows. ^{1}H NMR (500MHz, CD_3OD, δ, *J*/Hz): 4.37 (1H, dt, 5.0, 2.0, H-2), 2.11 (1H, m, H-4a), 1.80 (1H, m, H-4b), 2.09 (1H, m, H-5a), 1.82 (1H, m, H-5b), 3.49-3.61(1H, m, H-6), 7.04 (2H, d, 8.5, H-2',6'), 6.71 (2H, d, 8.5, H-3',5'), 3.06 (1H, ddd, 11.0, 6.5, 1.0, H-7'a), 4.05 (1H, ddd, 11.0, 6.5, 2.0, H-8'), ^{13}C-NMR (125 MHz, CD_3OD): δC (ppm) 170.8 (C-1), 60 (C-2), 46.062 (C-3), 29.4 (C-4), 37.831 (C-5), 45.9 (C-6), 127.6 (C-1'), 132.1 (C-2',6'), 116.2 (C-3',5'), 157.7 (C-4'), 37.7 (C-7), 57.9 (C-8') and 167 (C-9'). From the NMR spectra, the compound was identified as cyclo(L-Pro-D-Tyr) (*Fig. 4*). The NMR spectrum was found to be similar to that reported by Kumar et al. (2013a).

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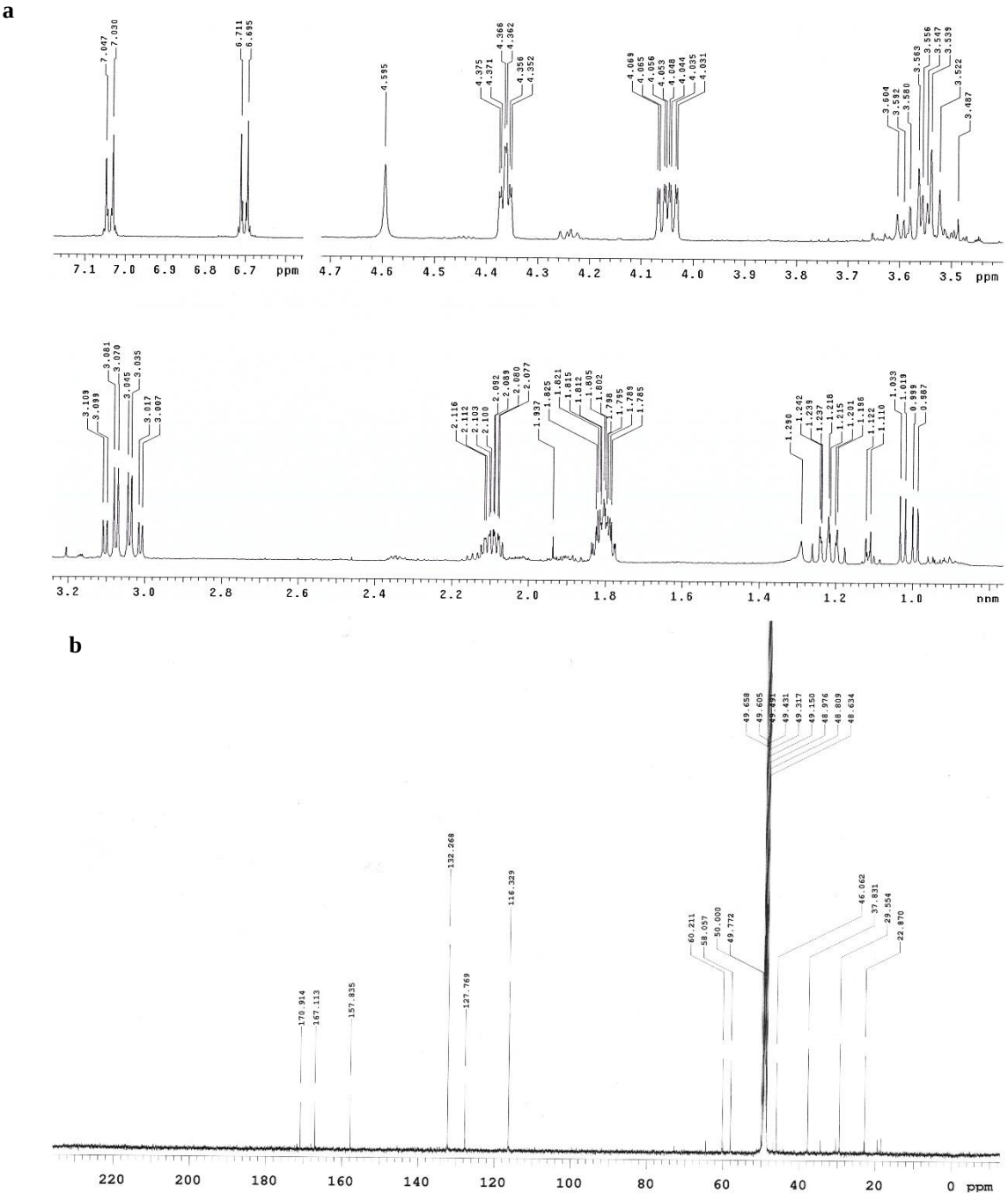

Figure 3. *^{1}H (a) and ^{13}C (b) nuclear magnetic resonance (NMR) spectra of the purified antifungal compound cyclo(L-Pro-D-Tyr) from strain Y1*

Determination of antifungal activity of the purified compound and its effect on fungal hyphae

Antifungal activity assay showed that various concentration of cyclo(L-Pro-D-Tyr) inhibited growth of *F. graminearum*, as evidenced by decreasing turbidity in wells with increasing treatments concentration. Microscopic examination revealed that the hyphae of *F. graminearum* were altered because of the cyclo(L-Pro-D-Tyr) treatment. The results clearly highlight that 125 ppm affected the mycelia growth of *F. graminearum*,

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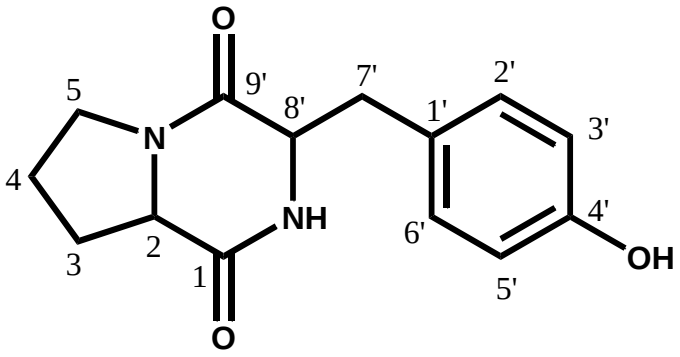
and the effects increased with increasing concentration of compound: at 250 ppm and 500 ppm, there was more visible hyphae deformation. Alterations in the shape, including deformation and degradation of hyphae, were observed under light microscopy (*Fig. 5*).



$C_{14}H_{16}N_2O_3$
Exact Mass: 260.1161

cyclo(L-Pro-D-Tyr)

Figure 4. *The structure of purified compound cyclo(L-Pro-D-Tyr) from strain Y1*

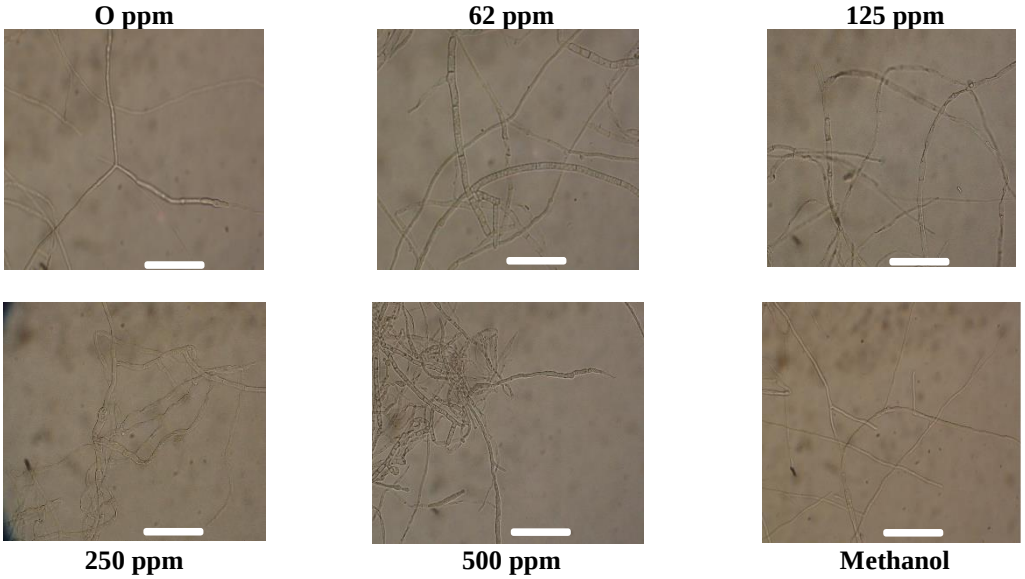


Figure 5. *Microscopically evident of changes in hyphae morphology of F. graminearum when treated with cyclo(L-Pro-D-Tyr). The carboxy methyl cellulase (CMC) containing F. graminearum conidial suspension only as control 0, 62, 125, 250, 500 ppm of Cyclo (L-Pro-D-Tyr) and methanol incubated for 5 days at 30 °C. All bars = 100 µm*

Assessment of the purified compound to control F. graminearum on wheat seeds

The ability of secondary metabolites of strain Y1 to inhibit the growth of *F. graminearum* on wheat seeds would be of major concern to the agricultural economy. Thus, the ability of cyclo(L-Pro-D-Tyr) produced by strain Y1 to protect wheat seeds

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from diseases caused by *F. graminearum* was investigated. Wheat seeds soaked in various concentration of cyclo(L-Pro-D-Tyr) demonstrated marked delay and inhibited seeds colonization compared to the control (*Fig. 6*). White mycelia were observed in the control wheat seeds 2 days after fungal inoculation, whereas seeds treated with 125 ppm or more of cyclo(L-Pro-D-Tyr) were found with low or no fungal growth.

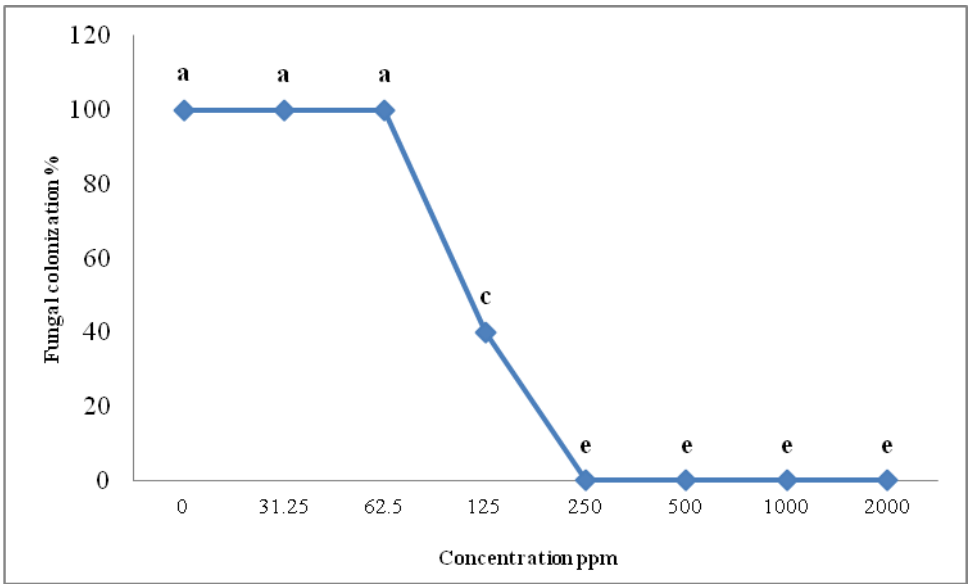

Figure 6. *F. graminearum colonization on wheat seeds with various concentration of cyclo(L-Pro-D-Tyr). Means with the same letter are not significantly different at P = 0.05 when compared by LSD*

Glasshouse experiment

The plant growth-promoting ability of strain Y1 was assessed *in vivo* using pot experiment with fertilizer media and bacterial inoculum. The results obtained from the pot experiment are shown in *Table 1*. The results indicated that at four months after sowing seeds, there were significant differences between the control and strain Y1 treatments. For vegetative growth, total yield significantly improved with the application of strain Y1, as the plant height, weight and root weight were higher with the Y1 treatment. Strain Y1 also caused an increase in the reproductive growth of wheat. The numbers of grains per spike were significantly higher in strain Y1-treated pots compared to all other treatments.

In the biocontrol of FCR, strain Y1 was found to reduce the percentage of disease in plant. The results showed that harvesting plants after four months, the strain Y1 group had the higher plant stand average against crown rot (*Table 1*). Pots treated with strain Y1 displayed high values for plant height, plant weight, root weight, and total grains per pot due to the better response of the plants to the effects of *F. graminearum* in the soil. *F. graminearum* exhibited crown rot symptoms in the control and reduced wheat yield, as the C (without *F. graminearum*) had higher values for plant height, plant weight, root weight, and total grains per pot compared to C + Fg (with *F. graminearum* spores) (*Table 1*). The level of control of crown rot in the pots treated with fertilizer medium was significantly lower (55%) compared to the pots treated with strain Y1 (28%). Total

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grain weight per pot increased with the application of medium (40 g) compared to the control only C + Fg (9 g) but was lower compared to the application of strain Y1 (48 g).

Table 1. *Effect of Y1 culture on crown rot severity of wheat in glass house experiment*

Treatment	Disease incidence %	Plant height (cm)	Plant weight/pot (g)	Root weight (g)	Grains weight/pot (g)
C	NR	61.6 ab	71 bc	1.13 b	21 c
Y1	NR	67.4a	174 a	1.64 a	68 a
M	NR	61.5 ab	148 ab	1.21 b	42 b
C + Fg	65 a	49.1 b	37d	0.98 bc	9 d
Y1 + Fg	28 c	64.24 a	148 ab	1.52 a	48 ab
M + Fg	55 b	56.1 a	126 b	0.93 c	40 b
F + Fg	8 d	66.6 a	137 ab	1.03 bc	42 ab

Means with the same letter are not significantly different at P≤ 0.05 when compared by LSD
NR = Not Recorded, C = Water control, Y1 = *Bacillus amyloliquefaciens* Y1 culture, M = Fertilizer medium, C + Fg = Water + *F. graminearum*, Y1 + Fg = *B. amyloliquefaciens* Y1 + *F. graminearum*, M + Fg = Fertilizer medium + *F. graminearum*, F + Fg = Fungicide + *F. graminearum*

Discussion

In *in vitro* study, the culture supernatant and crude extract of *B. amyloliquefaciens* Y1 indicated fungicidal activity against *F. graminearum*. The increase in concentration of culture supernatant and crude extract displayed more growth inhibition. Several studies have reported that antagonistic bacteria showed antifungal properties by producing secondary metabolites in their culture broth against pathogens (Blin et al., 2013; Ehteshamul-Haque and Ghaffar, 1993; Liu et al., 2017). Increase in concentration of secondary metabolites produced by *B. amyloliquefaciens* Y1 were likely involve in increases activity (Jamal et al., 2015).

Recently, natural products have been investigated to report biocontrol mechanism of antagonistic microorganisms. In this study, we purified and identified cyclo(L-Pro-D-Tyr) as natural antifungal product from the culture supernatant of Y1 based on ^{1}H and^{13}C-NMR spectra. To our knowledge, this is the first report of cyclo(L-Pro-D-Tyr) from *B. amyloliquefaciens* strain as an antifungal compound. Cyclo(L-Pro-D-Tyr) is a diketopiperazine (DKP) compound (De Rosa et al., 2003) previously purified from body fluids, sponges, microorganisms, and a variety of tissues (Rudi et al., 1994; Ström et al., 2002). Cyclo(L-Pro-D-Tyr) has been isolated from *Callyspongia* sp., a specie of marine sponge in the South China Sea (Huang et al., 2010). Kumar et al. identified cyclo(L-Pro-D-Tyr) in the secondary metabolites of *Bacillus* spp. and reported its antifungal and antibacterial properties (Kumar et al., 2013a).

Both synthetic and natural DKPs displayed biological activities, including antiviral (Sinha et al., 2004), antifungal (Houston et al., 2004), antitumor (Nicholson et al., 2006) and antibacterial (Kwon et al., 2000). The antimicrobial activities of cyclo(L-Pro-D-Tyr) has been less explored; however, here we reported its antifungal activity against *F. graminearum*. The bio-preservative properties of the cyclo(L-Pro-D-Tyr) was also evaluated on growth suppression of *F. graminearum* on wheat seeds. To the best of our knowledge, there is no previous report of DKP cyclo(L-Pro-D-Tyr) to be used as bio-preservative to report antagonistic mechanism to control *F. graminearum* causing foliar disease on wheat seeds. The bio-preservative property of DKP, cyclo(L-Pro-D-Leu) to

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inhibit growth of *A. flavus* and *A. niger* on soybeans was also reported (Kumar et al., 2013b). Previously, we identified cyclo(D-Pro-L-Val) in Y1 as antifungal metabolites against *F. graminearum* (Jamal et al., 2017). *Bacillus subtilis* L1-21 was also reported to control this pathogen *in vitro* (Munir et al., 2018). These findings showed that Y1 could control wheat foliar disease by producing DKPs and other antifungal metabolites.

The effectiveness of strain Y1 under *in vivo* condition was also evaluated for growth promotion of wheat plants and reduction in wheat crown rot. It has also been reported that many strains of *B. subtilis*, *B. amyloliquefaciens*, and *B. cereus* interact with plants and suppress plant pathogens (Choudhary and Johri, 2009; Jamal et al., 2017). *Pseudomonas fluorescens* LY1–8 was also reported to control the FHB and FCR in greenhouse and field (Wang et al., 2015). Application of *Chlonostachys rosea* ACM941 (10^8 cfu/ml) reduced the disease index in wheat by 30–46% (Xue et al., 2014b). The wheat stalks with several *Fusarium* species was successfully control with *Clonostachys rosea* (Palazzini et al., 2013). Moreover, biocontrol species of *Chlonostachys* suppress the disease on maize debris (Luongo et al., 2005), soybean debris and wheat, (Xue et al., 2014a) and floral crops (Morandi et al., 2008).

Bacteria suppress disease by producing a number of antibiotics (Nelson et al., 2014; Roitman et al., 1990; Xu et al., 2018). Cyclic dipeptides were reported to influence the quorum sensing signalling and has strong inhibitory effect against *Vibrio anguillarum* (Campbell et al., 2009; Fdhila et al., 2003; Yan et al., 2004). Similarly, our *in vitro* study demonstrated that cyclo(L-Pro-D-Tyr) act as an antibiotic to suppress growth of *F. graminearum*. The production of cyclo(L-Pro-D-Tyr) as an antifungal compound by Y1 in its culture broth may partially explain its biocontrol mechanism in wheat pot experiment. The production of cyclic peptides responsible for antimicrobial activity of the *B. licheniformis* N1 strain in the control of tomato gray mold, tomato late blight, and pepper anthracnose were also investigated (Kong et al., 2010). The biocontrol of *Fusarium* dry rot in wounded potato tubers is due to an antibiotic, pyrrolnitrin, produced by *B. cepacia* in its culture broth (Burkhead et al., 1994).

The strain Y1 not only control wheat crown rot but also improve plant growth, as evidenced by increase in plant weight, plant height, and grain yield per pot. The present study agree with the findings of Huang and Wong that *Burkholderia cepacia* (A3R) reduced wheat crown rot significantly in under glasshouse and field condition and increase grain yield (Huang and Wong, 1998). Many plant-growth promoting bacteria improve plant growth by producing antimicrobial compounds (Sophereth et al., 2013; Xu et al., 2018). Briefly, the current and our previous study (Jamal et al., 2017) provides enough evidence to consider Y1 strain as a promising biocontrol agent to control FHB and FCR in wheat by producing antifungal DKPs cyclo(L-Pro-D-Tyr) and cyclo(D-Pro-L-Val) in its culture broth.

Conclusions

In conclusion, isolation of DKP cyclo(L-Pro-D-Tyr) from *B. amyloliquefaciens* Y1, is a new finding in biocontrol literature with its antifungal characterization against *F. graminearum*. Both *in vitro* and *in vivo* study resulted that application of Y1 suppressed growth of *F. graminearum*. Cyclo(L-Pro-D-Tyr) isolated from Y1 can serve as a role model to uncoil the antagonistic mechanism of biocontrol agent. In pot experiment, addition of Y1 significantly inhibited pathogen growth and enhanced wheat plant growth. Thus, our result indicated that Y1 can serve as successful substitute over

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synthetic chemicals which are detrimental to control pathogenic fungi causing serious health and environmental concerns. In addition, these biocontrol agents may serve as a potential antagonist to manage other important wheat diseases in the greenhouse and fields.

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