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Purification and antifungal characterization of cyclo(D-Pro-L-Val) from *Bacillus amyloliquefaciens* Y1 against *Fusarium graminearum* to control head blight in wheat

Qaiser Jamal[a], Jeong-Yong Cho[b], Jae-Hak Moon[c], Kil Yong Kim[a,*]

[a] *Division of Food Technology, Biotechnology and Agrochemistry, Institute of Environmentally-Friendly Agriculture, Chonnam National University, Gwangju 61186, South Korea*
[b] *Department of Food Engineering, Mokpo National University, Mokpo 530-729, South Korea*
[c] *Department of Food Science and Technology, and Functional Food Research Center, Chonnam National University, Gwangju 61186, South Korea*

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ABSTRACT

This study describes the purification, identification and antifungal characterization of cyclo(D-Pro-L-Val) isolated for the first time from *Bacillus amyloliquefaciens* Y1 (Y1) against *Fusarium graminearum*. In primary screening, Y1 inhibited more than 50% growth of *F. graminearum* in dual culture and volatile assay. Similarly, the treatment of wheat grains with crude extract delayed the growth of *F. graminearum*. Cyclo(D-Pro-L-Val) was purified from crude extract using chromatographic techniques and identified by ^{1}H and ^{13}C nuclear magnetic resonance. This compound showed potent antifungal activity against *F. graminearum*. The minimum inhibitory concentration of cyclo(D-Pro-L-Val) to inhibit any visible mycelial growth of *F. graminearum* was 250 μg/ml. The microscopic study revealed alterations of hyphae like deformation, lysis or degradation of *F. graminearum* after the addition of cyclo(D-Pro-L-Val) in fungal suspension. Moreover, growth of *F. graminearum* on wheat grains was completely inhibited by 1000 ppm concentration of cyclo(D-Pro-L-Val) after incubation for 10 days. *B. amyloliquefaciens* Y1 may be a promising alternative to chemical fungicides due to production of cyclo(D-Pro-L-Val) and other antifungal compounds as secondary metabolites to prevent growth of *F. graminearum* in wheat grains to control head blight and other post-harvest diseases.

1. Introduction

The fungal spoilage reduces 5–10% of the world's food production (Pitt and Hocking, 2009). Fungi can cause various diseases in various cereal crops like rice, wheat, maize, and soybean. Quality and yield of small grains cereals (principally wheat, barley, rye and maize) damaged by *Fusarium* spp. and other associated fungi is a well-documented problem in 19th century (Jenkins et al., 1988; McMullen et al., 1997; Snijders, 1990). Wheat is the second most important cereal crop, next to rice. It is cultivated worldwide and economically plays an important role. Wheat is known to be susceptible to a number of pathogens causing post-harvest diseases especially *fusarium* head blight (FHB) particularly *F. graminearum*, *F. culmorum*, *F. poae* and *M. nivale* (Parry et al., 1995). *F. graminearum* have been known the second most predominant after *F. culmorum* to cause diseases in wheat (Cook, 1968). It attack the spikes of small grain cereals causing FHB. Severe intensive infection can result in more than 50% yield loss and grain quality is significantly reduced. It is known that this fungus is harmful to human because of production of several toxins in wheat like deoxynivalenol (DON) and can also cause emetic symptoms disease like vomiting and nausea in animal (Vesonder et al., 1976). It also can cause black point in wheat may be responsible for post emergence "damping off" of seedlings. In harvested grains, 5% infected kernels might contain enough toxins to be harmful for humans and animals (Singh et al., 1986).

In recent years, scientific attention has been drawn alternative to chemical pesticides to control diseases in crops. More specifically, the insensible application of chemicals causes environmental pollution and affects human health and gives chances to pathogens for building-up resistance to these chemicals (Baldwin and Rathmell, 1988). In this regard, more attention was drawn towards application of microorganisms. Choudhary and Johri (2009) reported that several bacteria are now available commercially as bio control agents (BCA) including strains of the genera *Pseudomonas* and *Bacillus* which are used successfully as alternative to chemical pesticides in crop production. Antibiotic production by some bacteria plays a major role in disease

* Corresponding author.
E-mail address: kimkil@jnu.ac.kr (K.Y. Kim).

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suppression, including *Pseudomonas* and *Bacillus* spp. (Cook et al., 1995; Dowling and O'Gara, 1994; Fravel, 1988; Klich et al., 1994). Many strains of *Bacillus amyloliquefaciens, Bacillus cereus* and *Bacillus subtilis* have been found to interact with plants and produce beneficial effects including disease suppression (Choudhary and Johri, 2009). *Bacillus* spp. produce a variety of antibacterial and antifungal peptide antibiotics (Katz and Demain, 1977; Zuber et al., 1993).

The intensive research efforts have been initiated to identify new sources of antimicrobial (antifungal) substances due to problem of increased resistance of various pathogens towards available drugs. In this regard, natural products from microorganisms have been explored. In recent years, there has been growing attention towards the diversity and biological roles of more than one hundred diketopiperazines found in nature (McCleland et al., 2004). In 1995, small scale evaluation of cyclic dipeptides begun as they possess interesting and possibly economically beneficial biological activities (Prasad, 1995). Many derivatives have phytotoxic (e.g., cyclo(Pro-Tyr)), antibiotic (e.g., bicyclomycin) properties and antiviral (e.g., the gliotoxins and sporidesmins) properties, whereas simple members like cyclo(His-Pro), cyclo(Pro-Leu), cyclo(Asp-Pro), cyclo(Pro-Val) and cyclo(Pro-Phe) show various biological activities (Prasad, 1995). Jayatilake et al. (1996) reported antifungal activity of cyclo(L-Ile-L-Pro) isolated from *P.aeruginosa*. In 1999, cyclo(Gly-Leu) was purified from *Lactobacillus plantarum* and investigated its antifungal activity. Synthetic diketopiperazine were also reported for their antibacterial and antifungal activity by Graz (2002) and Pitchen (2002).

In the present study we have tested the effect of the *B. amyloliquefaciens* Y1 on the growth of *F. graminearum*. The aim of this study was to investigate the biocontrol potential of Y1 against *F. graminearum* and to identify the antifungal compound produced by Y1 and to determine if the compounds have the ability to prevent fungal growth in a wheat model system.

2. Material and methods

2.1. Microorganisms

B. amyloliquefaciens Y1 was isolated in our previous study from field soil of Chonnam National university (35.1764° N, 126.9081° E), South Korea and deposited with accession number KJ616752 in GenBank. It was kept in 25% glycerol solution at −70 °C for further use. The fungus; *Fusarium graminearum* KACC 41040, which was purchased from Korea Agriculture Culture Collection (KACC) and was cultured on potato dextrose agar for further study (PDA; Difco Laboratories). For minimum inhibitory concentration (MIC) assay and *in vitro* seed treatment, the spore suspension of *F. graminearum* was grown in carboxymethyl cellulose (CMC) broth at 25 °C in shaking incubator for 10 days followed by filtering through cheesecloth. Spore concentration was determined using haemacytometer.

2.2. Antifungal characterization

Strain Y1 was subjected to antifungal characterization against *F. graminearum* using dual culture and volatile assay. In dual culture assay, Y1 was inoculated on LP (Laurie Britannia1/2+potato dextrose 1/2) agar medium one day before inoculation of the fungal pathogen. The antagonist and the test pathogen were placed 4 cm apart on the same LP plate, and all cultures were incubated at 26 °C. Growth of fungal colonies was evaluated after 7 days by measuring the colony radius from the original point of inoculation in the direction of the antagonist. The antifungal volatile compounds assay was performed following the method used by Naing et al. (2014). Bacterial strain was cultured by spreading 50 µL (10^6) of bacterial culture on Laurie Britannia agar plate and incubated at 28 °C for 24 h. Mycelial plug (5 mm) taken from the actively growing culture of *F. graminearum* was placed in the center of another petri dish containing PDA. The dishes containing the mycelial plugs were inverted over the bacterial plates by replacing their covers and were sealed together with parafilm followed by incubation at 26 °C. Antifungal activity was recorded when control plate was fully covered with fungal growth.

2.3. Effect of crude extract on fungal hyphae

The Y1 was cultured in Luria-Bertani (LB) broth medium at 30 °C for 10 days to obtain crude extract. The culture broth was centrifuged at 7000 g for 20 min. After centrifugation, the supernatant was acidified with concentrated HCl to pH 3. The supernatant was filtered through a Whatman filter paper No. 2. The supernatant after filtration was carried out to obtain the crude extract by an active fraction (1:1, v/v) with an eluotropic series of solvents; ethyl acetate and *n*-butanol. The partially isolated *n*-butanol solvents was concentrated by a rotary evaporator (Büchi, Rheinstetten, Germany) and was found to have antifungal activity using paper disc method. To examine the effect of n-butanol crude extract on hyphae of *F. graminearum,* culture was grown at 30 °C for 7 days. The 300 µl conidial suspension of *F. graminearum* (4×10^6 spores/ml) was added to each well of a 24-well microplate and the crude extract dissolved in methanol was added with final concentration of 500, 1000 and 2000 ppm. The same volume of methanol was used as control. Finally, CMC was added to make total volume of 1 ml in each well. The mixtures of crude extract and *F. graminearum* were incubated at 30 °C for 72 h and the mycelia were observed with a light microscope (Olympus BX41TF, Japan).

2.4. Crude extract as bio-preservative

The potential of the crude extract as bio preservative was tested against *F. graminearum* on wheat grains. To avoid germination and surface contamination, seeds were soaked in 100 mL of distilled water for 5 h, and then autoclaved at 121 °C for 20 min. To test the effect, n-butanol crude extract at concentration of 500, 1000 and 2000 ppm were prepared using methanol. Total of 30 grains per treatment were used. Initially, wheat grains were soaked in crude extracts at various concentrations and then dried. Grains soaked in methanol were used as control. After drying, 10 µl conidial suspension of *F. graminearum* (4×10^6 spores/ml) was treated on each wheat grain and then transferred to petri dish for incubation at 30 °C. Growth inhibition percentage was recorded when white mycelia were found on all control grains. Growth inhibition percentage was calculated using following formula. Growth inhibition percentage=(W−w)/W×100; where W' is the number of the control grain 'w' is the number of treatment grains. All tests were done in triplicate.

2.5. Extraction and purification of an antifungal compound

The bioactive crude extract was further subjected to purify the antifungal compound. The *n*-butanol crude extract (48 g) extracted from 36 L cell free culture filtrate of Y1 was dissolved in methanol and subjected to silica gel column chromatography (Kieselgel 60, 70-230 mesh, Merk, Darmstadt, Germany) with stepwise elution of CH_3Cl: MeOH (100:0, 90:10, 70:30, 40:60, 50:50, and 0:100; v/v). All fractions of the elution were concentrated in vacuum (EYELA rotary vacuum evaporator) to a semisolid mass, and each fraction was tested for antifungal activity. The active fraction was further subjected to high performance liquid chromatography (HPLC) system with PrepHT C18 column (7×300 mm, 10 µm). The elution was monitored using a SPD-10 UV–vis detector (Shimadzu, Japan) at 210 and 254 with manual injection. Each peak was separately collected using acetonitrile and water as a mobile phase (35:65, v/v) at a flow rate of 2 ml/min. All peak fractions were collected and concentrated using a centrifugal evaporator at 40 °C. The purity of collected fraction was further analyzed using HPLC analytical C18 column (5 µl, 4.6×250 mm). The