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Growth inhibition of *Fusarium graminearum* and reduction of deoxynivalenol production in wheat grain by bacillomycin D

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ABSTRACT

Fusarium graminearum causes Fusarium head blight, which leads to the quality and yield loss of wheat. In the present work, the inhibition effects of bacillomycin D (BD) on the growth of *F. graminearum* and reduction of deoxynivalenol (DON) production in wheat grain storage were investigated. The hyphal growth and sporulation of *F. graminearum* were restrained dramatically in the presence of 75 μg/mL BD, and the inhibition rate reached to 94.6% and 97.5%, respectively. Ultrastructural observation of the hyphae showed BD caused stripping of *F. graminearum* hyphal surface and destroying of the cellular structure. Furthermore, BD could lower the free fatty acid value and total antioxidant capacity and delay the decline of wheat quality, then exhibit an effective protection for wheat infection by *F. graminearum*. BD could effectively inhibit mold growth and DON production during wheat seeds storage, thus enhancing the quality and shelf life of the kernel. The addition of BD (75 μg/g wheat) remarkably inhibited the amount of *F. graminearum* growth, and reduced the DON production to 47.5–71.5%. These results indicated that BD might be a promising natural and effective fungicide, and would have potential for reducing mycotoxins in food and feed.

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1. Introduction

Fusarium head blight (FHB), which is caused primarily by *Fusarium graminearum*, is one of the worldwide devastating fungal diseases in wheat (Jansen et al., 2005; Palazzini et al., 2016). Poor drying during the harvesting phase or storage of damp grain can facilitate rapid colonization by spoilage and mycotoxigenic mold at the post-harvest stage (Magan and Aldred, 2007). This results in a drop in the production and quality of wheat with significant economic consequences. Furthermore, grain is also prone to be contaminated by mycotoxins produced by mold. Thus, any deterioration in the quality of these commodities due to fungal spoilage or contamination with mycotoxins results in a significant impact on human and animal health.

F. graminearum can contaminate wheat with deoxynivalenol (DON), nivalenol, and zearalenone (Choi et al., 2015). DON is classified by the International Agency for Research on Cancer (IARC) as a group 3 agent (not classifiable as to carcinogenicity in humans) (IARC, 1993). However, consumption of food contaminated with DON may result in nausea, vomiting, gastrointestinal upset, dizziness, diarrhea, and headache (Pestka, 2007). In 1987, an outbreak was reported. A considerable segment of the population of the Kashmir Valley in India became sick after eating wheat bread contaminated with DON and other trichothecene mycotoxins (Bhat et al., 1989). Hope et al. (2005) reported that the growth of *F. graminearum* and DON production on wheat grain needed to achieve a_w 0.85. Thus, there is a potential risk of mycotoxins production in wheat contaminated with *F. graminearum* and stored at high temperature and humidity conditions. Mycotoxins are difficult to eliminate in foods because they are heat-resistant.

In previous years, there has been no obvious development in the use of natural and safe antimicrobial drugs for the storage of cereals. Some chemical fungicides, such as benzimidazoles, triazoles, aromatic hydrocarbons, and sterol biosynthesis inhibitors, are often used. However, their application increases the risk of toxic residues in food. Therefore, natural antifungal compounds including biological control agents (BCAs) have attracted considerable attention (Glare et al., 2012). A number of plant extracts with efficient antifungal bioactivity that can be used to control foodborne pathogens

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and inhibit biosynthesis of mycotoxins have been screened (Chitarrini et al., 2014; da Silva Bomfim et al., 2015; Hua et al., 2014). Many BCA formulations are already available for practical use (Pérez-García et al., 2011). Among them, *Bacillus* species-based BCAs are considered to have promising potential. Many strains of *Bacillus* species have been reported to secrete several antimicrobial metabolites. *Bacillus subtilis*, a Gram-positive bacterium, has been recommended as one of the safe (GRAS) organisms by FDA for the food industry (Singh et al., 2009). Bacillomycin D (BD), a lipopeptide from *B. subtilis*, was identified to have a strong in vitro antifungal activity (Moyne et al., 2001, 2004). Gong et al. (2014) studied BD for its high antifungal effect on the mycelium growth, sporulation, and spore germination of *Aspergillus flavus* and concluded that BD could be a potential antifungal agent. However, there are no reports on the effect of BD on the *F. graminearum* growth and deoxynivalenol (DON) production.

Thus, the objectives of this study were to: a) clarify the inhibition effect of BD on *F. graminearum* growth, b) investigate wheat grain changes including water content, fatty acid value, and the total antioxidant capacity during storage after BD treatment, and c) research on the number of molds and DON content after the application of BD in stored wheat grain contaminated with *F. graminearum*.

2. Materials and methods

2.1. Microorganism cultures

The *F. graminearum* strain was obtained from the China Committee for Culture Collection of Microorganisms (CCCCM). *B. subtilis* fmbJ strain (CGMCCN 0943) is a wild-type strain isolated and characterized in our laboratory. Culture media used in this study were prepared as follows: PDA (200 g of potatoes were cut into small size and boiled for half an hour into 1.5 L distilled water and liquid potato extract was collected through a cheesecloth. Then, 20 g of glucose and 20 g of agar were added to 1.0 L liquid potato extract), PDB (prepared with the same component as PDA but without agar). The strain *B. subtilis* fmbJ was cultured in beef extract medium (BEM; 3 g/L beef extract, 10 g/L peptone and 5 g/L NaCl, pH 7.2) for seed culture at 37 °C. Components of the modified Landy medium (Landy et al., 1948) were used as fermentation medium (20 g/L glucose; 1 g/L yeast extract; 5 g/L L-glutamic acid; 1.0 g/L KH_2PO_4; 0.16 mg/L $CuSO_4 \bullet 5H_2O$, 0.5 g/L $MgSO_4 \bullet 7H_2O$; 0.15 mg/L $FeSO_4 \bullet 7H_2O$, 0.5 g/L KCl, 5.0 mg/L $MnSO_4$, pH 7.0). The fermentation conditions of BD were 33 °C, 180 rpm, and cultured 72 h. All components of culture media and chemicals in this study were purchased from Sinopharm Chemical Reagent Co., Ltd, Nanjing, China.

2.2. Preparation of spray drying products containing bacillomycin D

Maltodextrin and talcum powders were added into fermented supernatant as spray-drying excipients in the preparation of BD powder. The prepared feedstocks were subsequently spray-dried using a spray-dryer (LPG-10 L, Changzhou Yongchang Granulating Drying Equipment Co., Ltd, China), with the following operating conditions: aspirator setting 50 Hz; inlet temperature 130 °C; outlet temperature 70 °C; atomization frequency 500 Hz; feed rate 50 mL/min. After spray-drying, the powder was stored in tightly sealed glass vials, then the spray-drying yield was noted. The concentration of BD in powder was analyzed by high performance liquid chromatography-mass spectrometry/mass spectrometry (HPLC-MS/MS) as described in previous work (Qian et al., 2015).

2.3. Effects of bacillomycin D on hyphal growth and sporulation of F. graminearum

The hyphal growth of *F. graminearum* in the presence of BD was determined by growth rate assay according to Gong et al. (2014) with minor modifications. A series of concentrations of BD (225, 112.5, 75, 37.5, and 18.75 μg/mL) were added in PDA plates. The plate without BD was regarded as the control. Then, 5 mm hyphae discs of *F. graminearum* were placed in the center of each PDA plate. After incubation at 28 °C, the hyphae diameter of each plate was measured by the decussating method (Zhao et al., 2014) when the hyphae in the control plate reached the edges of the plate and the percentage of hyphae growth inhibition was determined. Meanwhile, the spores were observed by a haemocytometer after washing with 10 mL sterile saline solution (0.85% NaCl) containing Tween 80 (0.1% v/v). All treatments were replicated three times and the test was conducted two times. The relative inhibition percentage of hyphae growth and sporulation compared to the control was calculated using the following formula:

$$Inhibition(\%) = \frac{d_0 - d}{d_0} \times 100\%$$

where d_0 is the colony diameter and sporulation of treated without BD, and d is the colony diameter and sporulation of treated with BD.

2.4. Observation of cellular ultrastructure of F. graminearum

100 μL of 10^6/mL spore suspensions of *F. graminearum* was added in a fresh PDB culture treated with 0 μg/mL and 18.75 μg/mL of BD. After incubation at 28 °C for 3 days, *F. graminearum* hyphae were harvested for observations of scanning electron microscopy (SEM, S-3000N, Hitachi, Japan) and transmission electron microscopy (TEM, H-7650, Hitachi, Japan).

The hyphae of *F. graminearum* were fixed in 2.5% glutaraldehyde at 4 °C for 24 h, rinsed 4 times with phosphate buffer (0.1 M, pH 7.2) and subsequently fixed with 2% osmium tetraoxide for 2 h at room temperature. Then, the hyphae were dehydrated in a graded series of ethanol concentrations (30%–100%) for 15 min each, CO_2 dried and sputter coated with gold (ES-2030 Hitachi, Japan). Samples were carried out using a SEM operating at 30.0 kV (Qian et al., 2016).

TEM analysis was performed according to a method from Sun et al. (2015). Briefly, after treating with 2.5% glutaraldehyde, 2.0% osmium tetraoxide, ethanol, acetone, and epoxy, specimens of 100 nm thickness were cut using a microtome (HM505E, Carl Zeiss International, Germany). Then, samples were kept in a desiccator until observed with a TEM operating at 80.0 kV.

2.5. Sterilization treatment and water content adjustment of wheat

Wheat grain used in this study was obtained from Huai'an, Jiangsu Province, China. The wheat (2015 harvest year) was treated with 12 kGy gamma irradiation (Nanjing Xiyue Irradiation Technology Co., Ltd, China) and stored at 4 °C. At this irradiation dose, the wheat was free of fungal contamination (Hope et al., 2005). Irradiated wheat was respectively adjusted to the treatment water content levels, 12%, 14% and 16% by the addition of sterile distilled water (Rani et al., 2013).

2.6. Spore inoculation and bacillomycin D treatment on wheat

Fifty grams of wheat were inoculated with 1 mL of 10^6/mL spore suspension and thoroughly mixed. Appropriate amounts of BD powders and inoculated wheat grain (50 g) were mixed and shaken

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