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Antagonistic effect of chitinolytic *Pseudomonas* and *Bacillus* on growth of fungal hyphae and spores of aflatoxigenic *Aspergillus flavus*

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ABSTRACT

Use of biological control methods has become a promising strategy to counteract deleterious fungal contaminations and substantial post harvest losses. One of the major biological control methods is found in the use of fungal cell wall-degrading enzymes such as chitinases, which hydrolyze the homopolymer of β-1,4-1inked N-acetylglucosamine residues exposed on the fungal cell surface. In this study, antagonistic effects of the chitinolytic enzyme producing isolates of *Pseudomonas fluorescens* PB27, *Bacillus cereus* B1 and *Bacillus thuringiensis* K1 against the aflatoxigenic fungus *Aspergillus flavus* were investigated. Germination of the spores of *A. flavus* was inhibited by up to nearly 20% after co-incubation with *P. fluorescens* PB27 for 72 h, whereas the fungal spores were less affected when incubated with either *B. cereus* B1 or *B. thuringiensis* K1. When *P. fluorescens* or its extracted extracellular enzyme preparation was sprayed on the surface of hazelnuts, the growth of *A. flavus* was reduced nearly 25%. Scanning electron microscopy observations of hyphae and spores revealed that these enzymes induce numerous ultrastructural morphological changes during spore germination and mycelium growth. Since chitinolytic enzymes have not been shown to be toxic to humans or the environment, based on the observations in this study, an understanding of the functional properties and antagonistic effect of this natural antifungal agent can be practical in the management of post-harvest losses caused by food pathogenic and aflatoxin producing fungi.

1. Introduction

Contamination due to mycotoxin-producing fungi and product rot constitute two primary sources of post-harvest losses in fruit and vegetable crops (Castoria, Wright, & Droby, 2008). The post-harvest losses due to fungal growth and subsequent spoilage is estimated to be in excess of 10% or higher in developing countries (Magan & Aldred, 2007). Applying synthetic fungicides during storage typically results in a reduction or elimination of fungal contamination, thereby reducing spoilage and increasing the storage duration (Eckert & Ogawa, 1988). However, in recent years, the application and general safety of synthetic antimicrobial agents used in crop protection has been reassessed due to their effect on human health and the environment, the difficulty in the effective removal of residues from food and feed products, and fear

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from their overuse leading to increased pathogen resistance to fungicides (McGrath, 2009). These concerns have stimulated interest to find safer and more environmentally sustainable antifungal bio-alternatives for the control post-harvest contamination. The use of antagonistic microorganisms (bacteria, yeast and various molds) and their metabolic products has the potential to become prime biological control strategy available to postharvest preservation against a wide range of fungi (Anand, Chandrasekaran, Kuttalam, Senthilraja, & Samiyappan, 2010; Castario et al., 2008).

Filamentous fungi are well adapted for growth on and through solid or semi-solid substrates in the ecosystem. They can tolerate harsh environmental conditions by having strong, insoluable homopolymer of *N*-acetyl-D-glucosamine residues linked by β-1-4 bonds as the main structural component of the cell walls of hyphae and spores in flamentous fungi (Adams, 2004; Shahidi & Abusaytoun, 2005). In nature, degradation of chitinous materials is achieved by the coordinated action of hydrolytic exo- and endo-acting chitinases (EC 3.2.1.14) (Eijsink, Vaaje-Kolstad, Varum, & Horn, 2008). Chitinases cleave the β-1,4-glycosidic bonds between the C1 and C4 of two consecutive *N*-acetylglucosamines and are capable of hydrolyzing chitin down to its *N*-acetyl-β-d-glucosamine oligomers and monomers (Anil, Seshagirirao, & Podile, 2007). High concentrations of chitinolytic enzymes can degrade the cell wall of pathogenic fungi and inhibit conidial germination by interfering with the synthesis of the cell wall polysaccharides of chitin and β-1,3-glucans (Liu, Huang, Buchenauer, & Kang, 2010; Liu, Cai, Xie, Liu, & Chen, 2010). It has been suggested that chitinolytic microorganisms and chitinolytic enzymes have the potential applications in the bio-control of plant pathogenic fungi.

Pseudomonas and *Bacillus* strains have been intensely investigated as a biological control method against fungal species often found in postharvest goods (Anand et al., 2010; Bagnasco, De La Fuente, Gualtieri, Noya, & Arias, 1998; Liu, Huang, Buchenauer, & Kang, 2010). The major objectives of this study were to further determine the effects of chitinolytic enzyme treatment on spore germination, mycelium growth, and hyphal morphology of *Aspergillus flavus in vitro*, and then evaluation of an application of the observed chitinolytic treatment on the surfaces of hazelnuts and pistachio nuts. Ultrastructural changes in chitin, spores, and hyphae were investigated to determine the mode of antagonistic action of the chitinase-producing cultures *Pseudomonas fluorescens* PB27, *Bacillus cereus* B1, and *Bacillus thuringiensis* K1 in controlling aflatoxin-producing fungal contamination.

2. Materials and methods

2.1. Microorganisms, culture conditions and medium used

P. fluorescens PB27 was isolated from soil and *B. cereus* B1 and *B. thuringiensis* K1 were isolated from degrading shrimp shells as described earlier (Bogo Jensen, Baloda, Boye, & Aarestrup, 2001; Ferreira da Silva, Cabral, & Gomes, 2002). The chitinolytic activity of each species was first tested on agar plates containing artificially colored (Eosin Y, Crocein scarlet and Remazol Brilliant Blue) chitin as the sole carbon source (chitin 0.5% and 1.5% agar). The activity was observed as clearing zones around the chitinase-producing colonies. The microorganisms were kept frozen in 1% glycerol (cryoprotective agent) at −80 °C. Before use, the organisms were resuscitated by incubation in tryptic soy agar (TSA, 3% tryptic soy broth, 1.5% agar) at 30 °C for 24–48 h. The indicator mold strains (*A. flavus* and *Penicillium* spp., previously isolated from contaminated foods) were obtained from the culture collection of the Worobo lab at Cornell University, USA. The microbial cell concentration of the cultures was determined by serial dilution in peptone water (0.1%) followed by 30 °C incubation and enumeration on TSA for *P. fluorescens* and *Bacillus* species, and potato dextrose agar (PDA, 3.9%) for fungi.

2.2. Molecular identification of microbial isolates

P. fluorescens PB27 had been previously identified based on the morphological, cultural, biochemical, and physiological characteristics of the organism in accordance with earlier studies (Basaran, 2010). For confirmation of the strain PB27, *B. cereus* B1, and *B. thuringiensis* K1, PCR amplification of the 16S rDNA was performed using two standard primers: 16 rRNAFor (5′-AGAGTTTGATCCTGGCTCAG-3′) and 16 rRNARev (5′-GGTTACCTTGTTACGACTT-3′) (Durak, Churey, Danyluk, & Worobo, 2010). Using the basic alignment tool (BLAST) program, the genus and species were confirmed *via* homology analysis of the 16S rRNA gene sequence of similar nucleotide sequences. 16S rDNA sequence analysis of the selected bacilli K1 and B1 strains exhibited 99.99% and 98% DNA sequence identity to database entries associated with known *Bacillus* species, respectively.

2.3. Evaluation of the effect of co-cultivation of bacterial species with Aspergillus spp.

Following a 3-day incubation at 30 °C on PDA, the *Aspergillus* spp. spores were removed from the plate surface and serially diluted to approximately $1–5 \times 10^5$ spores/ml. One-half milliliter each of *A. flavus* fungal spores, potato dextrose broth (PDB, 2.4%), and 1×10^3 bacterial cells (*P. fluorencens* PB27, *B. cereus* B1 or *B. thuringiensis* K1) were combined in sterile 2 ml Eppendorf tubes and the suspensions were divided into duplicate tubes containing 1:2 PDB/peptone water. The mixtures were incubated at 25 °C for 48 h. To determine total bacterial and fungal counts, the samples were serially diluted with peptone water (0.01% w/v), and plated and enumerated on either TCA (2.3%) or PDA following a 24–48 h incubation at 30 °C. For cell and spore counts, at least three replicate plates were averaged for each tested dilution. The results were expressed as colony forming units per ml (CFU/ml). Scanning electron microscopy (SEM) was used to examine *Aspergillus* mycelia after co-cultivation and compared to mycelia grown without co-cultivation.

2.4. Optimization of enzyme production and preparation of crude extracellular enzyme

The nutritional medium requirement for chitinolytic enzyme production by selected bacterial species was first optimized. The cultures were grown in varying media formulations