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Improvement in biocontrol activity of *Bacillus subtilis* UTB1 against *Aspergillus flavus* using gamma-irradiation

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ABSTRACT

Bacillus subtilis UTB1, a biocontrol bacterium isolated from Iranian pistachio nuts, has revealed to have antagonistic activity against aflatoxin-producing *Aspergillus flavus* R5. The strain UTB1 produces lipopeptide compounds and is able to degrade aflatoxin B1. In this study, a random mutagenesis generated using different doses of gamma irradiation (0.1–3 KGy) was applied on *B. subtilis* UTB1 to improve its antagonistic activity against *A. flavus* R5. Five hundred bacterial colonies were selected randomly after irradiation, and their effects against *A. flavus* R5 were assessed in a plate assay. Forty-five colonies (9%) exhibited higher inhibition activity as compared to the non-irradiated wild type. Eight colonies out of the 45 were selected based on different polymorphism patterns obtained by repetitive element sequence polymorphism-PCR (ERIC and BOX) analyses; six of which could significantly inhibit the fungal growth utilizing washed cells and cell-free supernatants as compared to the parental strain. According to thin-layer chromatograms, the production of lipopeptides including surfactin, fengycin and iturin families increased in these six mutants. A considerable inhibition of the fungal growth was observed using bioautography analysis, which associated with iturins production. *A. flavus* sporulation and aflatoxin content decreased significantly in pistachio nuts treated with mutants M419 and M464 as compared to the strain UTB1. These results suggest that both mutants M419 and M464 could be promising biocontrol candidates against *A. flavus* in pistachio nuts.

1. Introduction

Aspergillus flavus is one of the major food spoilage fungi due to the production of secondary metabolites, aflatoxins that are extremely toxic chemicals with carcinogenic, mutagenic and teratogenic effects (Zhang et al., 2008). Aflatoxin production occurs when *A. flavus* or *Aspergillus parasiticus* invades nuts, cottonseed and corn under favorable conditions of temperature and relative humidity (Diener and Davis, 1967; Koehler et al., 1985; Laciakova et al., 2008). Pistachio nuts are among the commodities with the highest risk of aflatoxin contamination (Pittet, 1998). Iran is the largest pistachio nut producer (FAO, 2001; Cheraghali and Yazdanpanah, 2010) and aflatoxin contamination has been reported in Iranian pistachio nuts (Mojtahedi et al., 1979) especially during the harvest/postharvest period and storage stage. The traditional chemical control methods are neither economical nor effective at all times. Moreover, fumigation as well as other chemical control methods may have risks to human health and the environment (Droby, 2006). These disadvantages encouraged emphasis on biocontrol methods in recent years (Droby, 2006). In nature, aflatoxin-producing molds share the same habitat with other microorganisms such as lactic acid bacteria, *Bacillus subtilis*, *Bacillus pumilus*, *Burkholderia cepacia* and many molds, which can inhibit aflatoxin production. This inhibition may result from many factors including competition for space and nutrients in general, competition for nutrients required for aflatoxin production, and production of antiaflatoxigenic metabolites by co-existing microorganisms (Munimbazi and Bullerman, 1998).

The biocontrol potential of *B. subtilis* strains is based on their ability to produce antibiotic compounds such as lipopeptides including the fengycin, iturin and surfactin families (Stein, 2005;

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Ongena et al., 2009). Lipopeptides of the iturin (iturin A, mycosubtilin and bacillomycin) and fengycin families have displayed strong antifungal activity against a wide range of plant pathogens (Moyne et al., 2004; Romero et al., 2007a,b; Arrebola et al., 2010a; Yánez-Mendizábal et al., 2011). The involvement of the iturins family of polypeptides produced by *Bacillus* spp. in inhibition of growth of aflatoxin producing fungi has been reported in previous studies (Klich et al., 1993; Moyne et al., 2001; Zhang et al., 2008; Cho et al., 2009).

B. subtilis UTB1, isolated from Iranian pistachio fruits, showed antagonistic activity against *A. flavus* in a previous study (Farzaneh et al., 2012). It was found that this strain could considerably reduce aflatoxin B1 from nutrient broth culture and pistachio nuts by 86% and 95%, respectively (Farzaneh et al., 2012). Although strain UTB1 is able to degrade aflatoxin, its ability to inhibit fungal growth is not considerable. In the present study, we were interested in improving the antagonistic activity of strain UTB1 against *A. flavus* R5. Mutagenesis by either physical mutagens (gamma or UV irradiation) or chemical mutagens is used as a tool for improvement in potentiality of biocontrol agents and/or antifungal metabolites producers (Haggag and Mohamed, 2002). Random mutagenesis has been reported to improve production of antifungal metabolites and antagonistic potential of biocontrol agents (i.e. *Trichoderma* spp. and *Gliocladium* spp.) to control a broad spectrum of plant pathogens (Haggag and Mohamed, 2007). Liu et al. (2005) and Li et al. (2007) created mutants of *B. subtilis* strains using a random mutagenesis (low-energy ion beam implantation) with higher antifungal lipopeptides production over the parental strain against *Gibberella zeae* and *Rhizoctonia solani*, respectively.

We applied a random mutagenesis using gamma irradiation on *B. subtilis* UTB1 in order to make it a good candidate for controlling *A. flavus*. The aim of this study was to obtain an effective *B. subtilis* strain with improved biocontrol characteristics, high antifungal activity against *A. flavus* under *in vivo* and *in vitro* conditions and high aflatoxin degradation in pistachio nuts.

2. Materials and methods

2.1. Bacterial and fungal isolates

B. subtilis UTB1 (UTBSP1), has been known as a biocontrol strain against *A. flavus* R5 through antibiosis and degradation of aflatoxin B1 (Farzaneh et al., 2012), isolated from mature pistachio fruits of Rafsanjan orchards, the main pistachio producing region in Iran. *A. flavus* R5 isolated from peanut is able to produce aflatoxins B1 and B2. The fungal isolate was obtained from Department of Plant Protection, University of Tehran, with proven pathogenicity on pistachio nuts (Alibakhshi et al., 2011).

2.2. Screening of bacterial mutants

2.2.1. Gamma ray mutagenesis

For random mutagenesis of *B. subtilis* UTB1, ten different doses of gamma irradiation (0.1, 0.3, 0.5, 0.7, 1, 1.2, 1.5, 2, 2.5 and 3 KGy) were applied as described previously (Afsharmanesh et al., 2013). Briefly, the glass vials containing the frozen stock of *B. subtilis* UTB1 were directly exposed to a gamma irradiator equipped with a ^{60}Co source (Gamma cell instrument Issledovatel, PX-30 model, Russia). A plate count method was used to estimate colony forming units (CFU) on nutrient agar (NA) medium after 24 h of incubation for each irradiated sample. Three replications were applied for each dose.

Following the application of ten different doses of gamma irradiation, 500 colonies were selected randomly and then grown overnight in a 9-mL tube containing tryptic soy broth (TSB; Merck, Germany) at 30 °C and 150 rpm. Afterward, their ability to inhibit fungal growth was evaluated.

2.2.2. Inhibition of fungal growth

Inhibition of fungal growth by 500 selected colonies was evaluated against *A. flavus* R5 in a dual culture assay as described previously (Palumbo et al., 2006; Afsharmanesh et al., 2013). After incubation of 10-cm-dual culture plates (50% PDA, 50% NA) at 30 °C, radial growth of mycelium from the edge of each plug was measured and recorded after 7 d. The experiment was conducted two times in a completely randomized design with three replicates for each colony. Data analysis was performed by ANOVA using the SAS (V9.1) software (SAS Institute Inc., Cary, NC), and the means for each treatment were separated by Fisher's least significant difference test at $P = 0.01$.

2.2.3. Bacterial motility assays

Swimming and swarming motilities were analyzed according to the method described by Connelly et al. (2004). Luria–Bertani (LB) plates containing 0.3% agar (swimming) and 0.7% agar (swarming) were incubated at 37 °C for 18 h and finally the diameters of the bacterial colonies (UTB1 and 45-screened mutants) were measured. The experiment was conducted twice in a completely randomized design with three replicates for each colony. Data analysis was performed by ANOVA by means of the SAS (V9.1) software and average values were compared by Fisher's least significant difference test at $P = 0.01$.

2.2.4. Rep-PCR fingerprinting analysis of bacterial mutants

A repetitive element sequence polymorphism-PCR (rep-PCR fingerprinting) assay was designed to determine genetic diversity among the wild strain UTB1 and 45 colonies showing more inhibition activity against *A. flavus* R5. The PCR analysis was performed using ERIC and BOX primers (Versalovic et al., 1994) as described earlier (Reyes-Ramirez and Ibarra, 2005; Afsharmanesh et al., 2013). Genomic DNA of the bacterial isolates was extracted by using Biospin Bacteria Genomic DNA Extraction Kit (China). Amplifications were performed in a PCR reaction containing: 2x PCR master kit (CinnaGen, Iran), 10 pmol each of ERIC1R and ERIC2 primer or BOX A1R primer (Bioneer, South Korea) and 10 ng of genomic DNA. The thermal cycling conditions were 35 cycles of 94 °C for 30 s, 45 °C (ERIC) or 50 °C (BOX) for 1 min, 72 °C for 1 min. Amplicons were electrophoresed in 1% agarose gels at 90 V. Amplification was carried out twice for each of 45 selected mutants using ERIC and BOX primers.

The 16S rRNA gene of strain UTB1 and eight selected mutants displaying genetic diversity with strain UTB1 (M419, M425, M455, M458, M464, M523, M562 and M600) was amplified utilizing primers 1486R-P and 41-F (Stackebrandt and Goodfellow, 1991) to confirm that the mutants were obtained by irradiation of the wild type. The PCR products were purified by means of illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare UK Limited) which was used directly for sequencing (Macrogen Co., Netherlands). Homology studies were carried out using the NCBI program BLAST. Subsequently, the multiple sequence alignment was performed using AlignX of Vector NTI Advance10 software (Invitrogen Co, USA, 2005) based on ClustalW algorithm to compare the 16S rRNA sequence of *B. subtilis* UTB1 with the eight selected mutants.

2.2.5. Inhibition of A. flavus growth by washed cells and cell-free supernatants

Antifungal activities of eight selected mutants showing genetic diversity with strain UTB1 (M419, M425, M455, M458, M464, M523, M562 and M600) were tested by both washed cells and