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Investigation and application of *Bacillus pumilus* QBP344-3 in the control of *Aspergillus carbonarius* and ochratoxin A contamination

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ABSTRACT

This study was designed to investigate the antifungal activity of *Bacillus pumilus* 344-3 against toxigenic fungi. *In vitro* co-incubation assay revealed that *A. carbonarius* AC82, *A. niger* AN8 and *P. digitatum* PD43 are most sensitive fungi to bacterial antifungal compounds with zone of inhibition of 29.2 mm, 27.7 mm and 27.1 mm, respectively. The addition of *Bacillus pumilus* 344-3 culture supernatant at low concentration in the fungal growth medium stimulated *A. carbonarius* biomass, but inhibited ochratoxin A (OTA) synthesis significantly ($p \leq 0.05$). Conidial germination of *A. carbonarius* was not affected in a medium containing 10% and 20% of the bacterial culture supernatant, while it was completely inhibited in 100% bacterial extract. Storage of bacterial culture supernatant at temperature ranging from −20 °C to 100 °C for 1 h, didn't affect its antifungal potential. *In vivo* application of bacterial extract on the maize kernels, showed 95% protection against *A. carbonarius* infection. Application of *B. pumilus* 344-3 culture supernatant on the surface of maize kernels provided 99% reduction in OTA production potential of *A. carbonarius* AC82. Because of its strong activities against the growth of *A. carbonarius* AC82 and OTA-synthesis, *B. pumilus* 344-3 can be considered as a very promising biocontrol agent.

1. Introduction

Mycotoxins are secondary metabolites of toxigenic fungi which infect the food crops during pre- and post-harvest stages (Adeyeye, 2016; Balendres et al., 2019). According to the Food and Agriculture Organization (FAO) of United Nations (UN), more than 25% of the worlds crops are contaminated with mycotoxins with huge annual losses to food and feed industry (Rahmani et al., 2009). The accumulation of mycotoxins in food and feed chain is mainly due to *Aspergillus*, *Penicillium* and *Fusarium* infections (Balendres et al., 2019; Paterson et al., 2018). In a list of >400 mycotoxins, the most important due health implications are aflatoxins (AFs), ochratoxins (OT), deoxynivalenol (DON), zearalenone (ZEN), T-2 and HT-2 (Liew & Mohd-Redzwan, 2018). Ochratoxin A (OTA), synthesized by some *Aspergillus* (mainly *A. carbonarius* in some fruits and cereals) and *Penicillium* spp. is known to possess nephrotoxic, hepatotoxic, carcinogenic and teratogenic activities (Heussner & Bingle, 2015; Zahoor-ul-Hassan et al., 2012). International Agency for Research on Cancer (IARC) have classified OTA as group 2B human carcinogen (Cabañes et al., 2013; International Agency for Research on Cancer (IARC), 1993). Based on the toxicity data, European Union has set maximum limit for OTA contamination in unprocessed- and processed cereals for human consumption as 5 μg/kg and 3 μg/kg, respectively (Duarte et al., 2010; European Commission, 2010).

The common preharvest approaches to overcome contamination by mycotoxins include the application of fungicides, modification in the agricultural practices such as crop rotation, sowing and harvesting timings, development of fungal resistant cultivars and dilution of contaminated batches (Ponsone et al., 2011; Schaarschmidt & Fauhl-Hassek, 2018). Similarly, post-harvest management practices include proper storage temperature and humidity, grading and sorting of infected grains, chemicals and heat treatment, ultraviolet (UV) irradiation, etc. (Jubeen et al., 2012; Neme & Mohammed, 2017). Correct use of synthetic fungicides in agriculture warrants optimal protection against fungal infections and mycotoxins contamination. However, prolonged use of synthetic biocides results in; a) reduction in the efficacy of these agrochemicals because of emerging fungicides resistant fungal strains, and b) transfer of pesticides residues in food and feed chains, posing health risk for exposed species (Tilocca et al., 2020). These facts

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promote the need to find safer chemical-free alternatives for the control of mycotoxins in food crops and their products. In this context, the application of friendly biocontrol agents, such as yeasts, bacteria and non-toxigenic fungal strains are being explored as safer substitutes (Chulze et al., 2015).

Bacillus species are intensively being explored for their potential to inhibit fungal growth and alter their mycotoxin biosynthesis profiles (Ren et al., 2020). In this genus *B. subtilis*, *B. licheniformis*, *B. cereus*, *B. megaterium*, *B. amyloliquefaciens* and *B. mojavensis* have been studied for their antagonistic activities (Pérez-García et al., 2011; Ren et al., 2020). Unlike many other bacteria, *Bacilli* have least nutrient requirements and can readily develop resistant spores, which can survive in harsh environmental conditions (Pérez-García et al., 2011). Furthermore, the potential to synthesize a range of antifungal compounds including lipoproteins (surfactin, iturin, fengycin), isocoumarins, bacillomycin, dehydrogenase, laccase, hydrolase etc., with least-to-no toxicity and resistant to a range of pH, temperature and enzymes (Stein, 2005) rank *Bacillus* spp. among the most effective biocontrol agents.

Compared to the other *Bacilli*, little is known about the antifungal potential of *B. pumilus* against food spoilage fungi. In the first available study, Munimbazi and Bullerman (1998) reported the antifungal spectrum of *B. pumilus* culture supernatant, its resistance of temperature and pH, and sensitivity to enzymes. Unlike the findings of Munimbazi and Bullerman (1998), the *B. pumilus* isolate of Bottone and Peluso (2003) was effective against *A. flavus* and *A. terreus,* and ineffective against *Fusarium* fungi. Also, metabolite of *B. pumilus* strain of Bottone and Peluso (2003) was inactive at pH 5.6, while those of Munimbazi and Bullerman (1998) showed activity at wider pH range of 2–10. Likewise, Agarwal et al. (2017) reported differential antifungal activity of *B. pumilus* MSUA3 against *Rizoctonia solani* and *Fusarium oxysporum*. The existing controversy in the antifungal potential of *B. pumilus* needs further exploration. In addition, none of the studies conducted so far, focused on the antifungal potential of the *B. pumilus* against *A. carbonarius* which is known as a source of ochratoxin A (OTA) on many food products including nuts, cocoa, maize, dried fruits and grapes (Taniwaki et al., 2018). Furthermore, we were also interested to investigate *B. pumilus* on agricultural commodities (through *in vivo* assay) to explore its possible application in field conditions.

This study was designed to investigate antifungal potential of *B. pumilus* 344-3 against OTA-producing strain of *A. carbonarius* AC82. *In vitro* co-incubation assays were performed to explore the effect on vegetative growth of *A. carbonarius* and its potential to inhibit OTA synthesis. Also, the effect of *B. pumilus* 344-3 culture supernatant was tested on spore germination of *A. carbonarius*. Thermostability of the bacterial compounds was tested by exposing at −20 °C to 100 °C for extended duration of 1 h. *In vivo* application of bacterial extract was tested on maize kernels to record the effect on *A. carbonarius* infection rate and OTA-synthesis.

2. Materials and methods

2.1. Microbial strains and growth media

List of microbial strains used in this study and their source have been presented in Table 1. For the identification of *B. pumilus* QBP344-3 and *B. megaterium* QBM344-1, isolated bacterial colonies were used for protein extraction using ethanol-formic acid procedure as described earlier by Ul Hassan et al. (2019a). Mass-to-charge (m/z) spectrum were obtained by Bruker Biotype (Bruker Daltonics, Bremen, Germany), which were compared with those available in the database. *Burkholderia cepacia* QBC03 (used as positive control in this study) was identified by sequencing of 16S rRNA (Zeidan et al., 2019). Filamentous fungi used in this study were identified used specie-specific PCR primers (Hassan et al., 2018; 2019b). Luria Bertani (LB) agar was prepared by adding tryptone (10 g), yeast extract (5 g), NaCl (5 g) and agar (15 g) in 1000 ml of distilled water. Nutrient broth yeast extract (NBY) was prepared by mixing mannitol (1 g), peptone (0.5 g), yeast extract (0.5 g) and meat extract (0.3 g) in 100 ml of distilled water. The mixture was added with mineral solution prepared by adding $Mo_7(NH_4)6O_{24}.4H_2O$ (123 mg) and $ZnSO_4.7H_2O$ (28 mg) in 100 ml of distilled water. Nutrient broth (NB) was prepared by adding peptic digest of animal tissue (5 g), NaCl (5 g), beef extract (1.5 g) and yeast extract (1.5 g) in 1000 ml of water. Potato dextrose agar (PDA) was obtained from Formedium (U.K.) and consisted in agar (15 g), glucose (20 g), and potato extract (4 g) in 1000 ml of water. Soft PDA was prepared by adding the half quantity of agar (7.5 g/L), while keeping all the other ingredients as PDA.

Table 1
List of microbial strains used in this study.

Name		Source
Bacterial strains	B. pumilus QBP344-3	Strawberry jam
	B. megaterium QBM344-1	Strawberry jam
	Burkholderia cepacia QBC03	Animal feed
Fungal strains	A. carbonarius AC82	Animal feed
	A. niger AN8	Animal feed
	A. westerdijkiae AW82	Animal feed
	A. parasiticus AP82,	Animal feed
	A. flavus CECT2687,	Culture Collection Center, Univ. De Valencia, Spain
	A. ochraceus CECT2948	Culture Collection Center, Univ. De Valencia, Spain
	Penicillium digitatum PD43,	Animal feed
	P. verrucosum TF11,	Animal feed
	P. camemberti PC44	Animal feed
	P. italicum PI48	Animal feed
	Fusarium verticillioides FV04,	Animal feed
	F. proliferatum FP08	Animal feed
	F. oxysporum FOx9	Animal feed
	Candida albicans	Hammad Medical Corporation, Doha-Qatar

2.2. Investigation of the antifungal spectrum of Bacillus pumilus QBP344-3

The spectrum of antifungal activities of *B. pumilus* 344-3 were tested using agar diffusion assay against 13 representative filamentous fungi and one non-filamentous ascomycete (mentioned in Table 1). With the help of sterile toothpicks, cells from *B. pumilus* 344-3, *B. megaterium* 344-1 (known for not producing diffusible antifungal compounds, and thereby used as negative control) and *Burkholderia cepacia* QBC03 (known for being a strong producer of antifungal compounds and used as positive control) colonies were transferred to the middle of LB agar Petri plates. The plates were incubated at 30 °C for 3 days to allow the production and diffusion of bacterial antifungal compounds. Six plates of uniform colony size and morphology from each bacterial species were chosen. An inoculum of each fungus was prepared by adding loopful of spores in 1 ml of saline solution having 0.005% Tween 80. After thorough mixing and counting the spores using Neubauer cell counter, the number of spores was adjusted to 1×10^6/ml. Fungal spores were suspended in molten soft PDA, and a total of 3 ml was poured around the bacterial colonies. The plates were incubated at 27 °C for 7 days. The zone of fungal growth inhibition around the bacterial colonies was measured (mm) at day 3 and day 7.

2.3. Determination of the effect of B. pumilus 344-3 culture extract on fungal mycelium mass and mycotoxins synthesis

The bacterial culture extract was prepared by adding 50 μL of BP344-3 cell suspension in a 50 ml tube containing 10 ml of LB broth. After incubation at 30 °C for 48 h, the tubes were centrifuged at 5500 rpm for

20 min at 4 °C, the supernatant was separated from the bacterial pellet and stored at 4 °C. To evaluate the effect of *B. pumilus* 344-3 culture extract on the mycelium growth of toxigenic fungi, increasing volumes of bacterial culture supernatant was added to 50 ml tubes containing PDB to achieve a final concentration of bacterial extract at 0%, 20%, 40%, 60%, 80% and 100%. The final volume of 10 ml was maintained in each tube. To inhibit the bacterial cells growth, chloramphenicol (Glentham Life Sciences, UK) at 100 μg/L was added. *A. carbonarius* (10 μL) spore suspension (10^5 spores/mL) was added in each tube. The control tubes contained 10 mL of PDB along with chloramphenicol without bacterial extract. The tubes were incubated at 26 °C with shaking at 140 rpm. After 48 h of incubation, the contents of each tubes were filtered, and mycelia were dried and weighed. The filtrate of fungal mycelia was used for the determination of OTA content using enzyme linked immunosorbent assay (ELISA) assays (RIDASCREEN Ochratoxin A Kit 30/15, R-Biopharm Darmstadt, Germany).

2.4. Investigation of the effect of B. pumilus 344-3 culture extract on fungal conidial germination

To investigate the effect of the bacterial extract on fungal conidial germination, *B. pumilus* 344-3 was cultured in LB broth as described above, and supernatant was separated from bacterial cells. The effect of bacterial extract on fungal spore germination was tested in 24-wells cell culture plates. The bacterial extract was added either at 100, 200, 300, 500 and 1000 μL, and the final volume was adjusted to 1000 μL with PDB to reach final concentration of 10%, 20%, 50% and 100% in each well of 24-wells cell culture plates. Three replicates of each bacterial extract were made. In order to compare the effect of increasing concentration of LB, appropriate controls of LB at 10%, 20%, 30% 50% and 100% with PDB were maintained. To each well, 100 μL of *A. carbonarius* spores (10^6 spores/ml) and chloramphenicol (100 μg/L) were added and plates were sealed with double layer of Parafilm®. Plates were incubated at 26 °C and checked for conidial germination after 24, 72, and 96 h using an inverted microscope (Joo et al., 2015).

2.5. Study on the thermostability of the B. pumilus 344-3 antifungal extract

To investigate the thermostability of *B. pumilus* 344-3 antifungal compound(s), *A. carbonarius* spores were allowed to germinate in the bacterial extract treated at −20 °C, −4 °C, 30 °C, 50 °C and 100 °C for 1 h. Time countdown of treatment was started when the desired temperature was attained. Increasing concentrations of each treated extracts at 10%, 20%, 30% 50% and 100% were added to triplicate wells in 24-wells plates and effect on *A. carbonarius* spore's germination was tested as described above (section 2.4).

2.6. Application of B. pumilus 344-3 extract against fungal contamination and mycotoxin synthesis on maize kernels

To investigate the possible application of *B. pumilus* 344-3 extract, cell-free bacterial culture was point applied on maize kernels (Foody's, Thailand). After disinfection by dipping into 10% NaOCl solution for 1 min, kernels were washed with sterile distilled water as described by Ul-Hassan et al., 2019. All maize kernel pieces after drying were shifted to sterile glass containers. In the control group, the maize kernels were sealed without any treatment. In a negative control group, maize was inoculated with 10 μL of *A. carbonarius* spore suspension (10^5/mL). In a positive control group, 50 μL of QBC03 extract was poured on the labelled area followed by 10 μL of *A. carbonarius* spore suspension (10^5/mL). To evaluate the effect of LB (media used for the bacterial extract), another group of maize kernels was treated with 50 μL of LB on a marked area followed by inoculation of 10 μL of fungal spores. In the experimental maize kernels, 50 μL of *B. pumilus* extract was poured on the marked area followed by 10 μL of spore suspension (10^5/mL). All containers were incubated at 26 °C for 3 weeks and replicated in thrice. Fungal growth was monitored by measuring the infection spread using polygon feature of image processing program (ImageJ, Version 1.52v). After setting the scale with the known distance in the image, the infection areas were calculated by marking the polygon. At day-21 of the experiment, 2 g of maize ears were removed at the site of fungal spore inoculation and grind to powder for OTA extraction and analysis by using RIDASCREEN® Ochratoxin A 30/15 (R-Biopharm Darmstadt, Germany) ELISA kits.

2.7. Statistical analysis

The data obtained from three repetition of all the experiments with 3 replicates each time (except section 2.2, where 6 replicates were maintained in each repetition), was subjected to statistical analysis by Analysis of Variance (ANOVA) test. Significant differences between groups were measured by *post hoc* multiple comparisons using least significance difference (LSD) or Duncan's multiple test at $p \leq 0.05$. In section 2.2, regarding antifungal activity of bacteria, fungal zone of inhibition (mm) was dependent variable around the bacterial colony (independent variable). Mycelium mass and OTA contents (section 2.3) were taken as dependent variables, while bacterial extract (%) was considered as independent variable. Similarly, in section 2.6, area of *A. carbonarius* spread and OTA synthesis (dependent variables) were compared with the application of bacterial culture extracts (independent variable). Statistical software IBM SPSS (IBM SPSS Version 25 for macOS; SPSS Inc., Chicago, IL, USA) was used for these analyses.

3. Results and discussion

3.1. Investigation of the antifungal spectrum of Bacillus pumilus 344-3 against mycotoxigenic fungi

In preliminary screening experiments, the antagonistic activity of *B. pumilus* 344-3 was explored against thirteen fungi from the genera *Aspergillus*, *Penicillium* and *Fusarium,* and one from *Candida*. The antifungal spectrum of *B. pumilus* showed a selective inhibitory effect among the tested fungi. Black *Aspergilli* (*A. carbonarius* and *A. niger*) and *P. digitatum* showed high sensitivity to the bacterial compounds, while others showed mild-to-no inhibition in their growth. A clear zone of fungal growth inhibition was observed around the bacterial colony (Fig. 1), as compared to the negative control (i.e., *B. megaterium*-known for not synthesizing antifungal compounds). None of the tested *Fusarium* spp. and *Candida albicans* was sensitive to bacterial compounds. *A. carbonarius*, *A. niger* and *P. digitatum* showed sensitivity to *B. pumilus* 344-3 antifungal compounds (Fig. 2). Three-weeks observations of the plates showed no change in the inhibition zones for any of the 3 species suggesting that the antifungal compounds of *B. pumilus* 344-3 are unlikely to degrade and maintain their antifungal ability over time. In line with the present study, the antifungal activities of *B. pumilus* have been reported against several *Aspergillus, Penicillium, Fusarium* and *Mucor* species (Munimbazi & Bullerman, 1998; Bottone and Peluso, 2003). *B. pumilus* studied by Munimbazi and Bullerman (1998) was ineffective against the aflatoxin producers *A. flavus* and *A. parasiticus*. In both of these studies, *A. carbonarius* was not included among the tested fungi. Jiang et al. (2014), using *B. subtilis* against ochratoxigenic strain of *A. carbonarius*, showed comparatively smaller zone of inhibition (15.4 ± 0.7 mm), as compared to our findings with *B. pumilus* 344-3 where the inhibition was 29.2 ± 0.9 mm (mean ± SD). The susceptibility of *A. carbonarius* and other fungi to *B. pumilus 344*-3 display a potential of *B. pumilus* against OTA-producing *A. carbonarius* and other food spoilage fungi. Although a precise bacterial antagonistic mechanism is not known, the possible roles include a) disruption of hyphae cell wall, b) interference of intracellular metabolic activities, or c) alteration in the expression of genes involved in growth and mycotoxins synthesis (Ren et al., 2020).

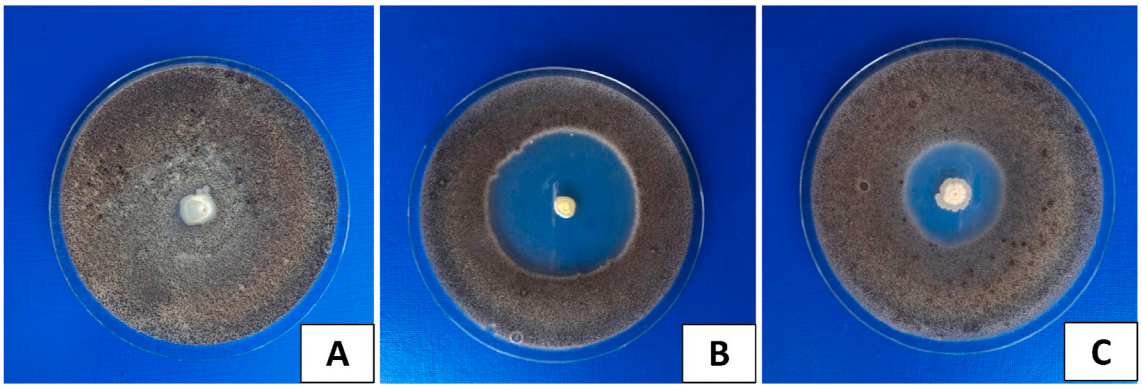

Fig. 1. Effect of the *B. pumilus* 344-3 on mycelial growth of *A. carbonarius*; **A**. Fungal growth in the presence of *B. megaterium* (bacterium not known to synthesize antifungal compounds). **B**, zone of fungal growth inhibition around *B. cepacia* (bacterium known to produce strong antifungal compounds). **C**, *A. carbonarius* growth inhibition in the presence of *B. pumilus* 344-3. This experiment was repeated 3 times with 6 replicate each time.

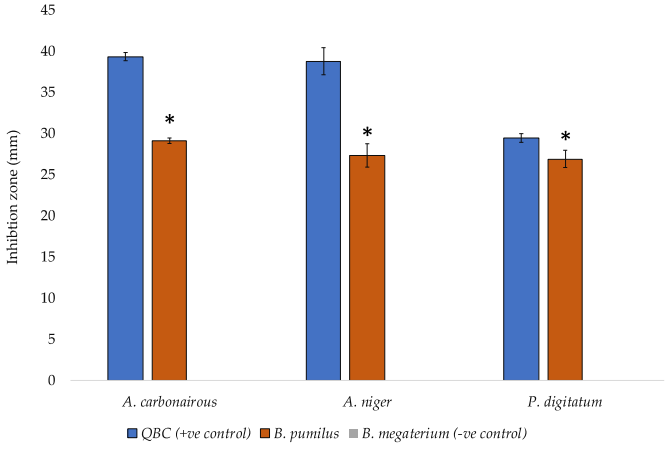

Fig. 2. Comparison of the effect *B. pumilus* 344-3 and *Burkholderia cepacia* QBC03 (positive control) on the inhibition of the mycelial growth of *A. carbonarius*, *A. niger* and *P. digitatum*. Bars shows the mean of 9 replicates obtained from three separate experiments. Error bars show the standard deviation (n = 9). Asterisks above the bars indicate significantly lower zone of inhibition as compared to their respective control.

3.2. Effect of the B. pumilus 344-3 culture extract on A. carbonarius biomass and OTA synthesis

The addition of *B. pumilus* culture supernatant at 20%, 40% and 60% in PDB inoculated with *A. carbonarius* spores, resulted in increased fungal biomass as compared to control (without bacterial extract). These findings suggest that low concentration of bacterial culture supernatant may stimulate the vegetative growth of toxigenic fungi. However, the addition of bacterial extract at a concentration of 80% showed antagonistic effect on fungal growth (Fig. 3A). Statistical analysis performed by ANOVA with *post hoc* multiple comparisons by LSD showed that at 100% concentration of the bacterial extract there was a significantly lower ($p \leq 0.05$) fungal biomass as compared to their corresponding control.

Fungal growth stimulation by bacteria is commonly observed in mycorrhizal association in the plant roots, where one bacterium selectively stimulates fungi in its environment (Schrey et al., 2012). However, this mechanism is rarely observed in case of toxigenic fungi. In a study of Nogueira et al. (2019) on the bacterial-fungi interaction, it was observed that *Klebsiella* upregulates cell wall related genes in *Aspergillus* and at the same time down-regulates filament elongation genes. A similar phenomenon was observed in our study, where biomass reduction was noted by using bacterial extract at 80% and above, suggesting a threshold level that limits *A. carbonarius* to increase its density in order to enhance its survival rate.

In the present study, the addition of bacterial extract at all concentrations resulted in significant ($p \leq 0.05$) reduction in OTA synthesis (Fig. 3B) by *A. carbonarius*, despite the fact that up to 60% concentration of bacterial extract had stimulatory effect on fungal growth. The decrease in OTA synthesis by *A. carbonarius* displayed a bacterial extract-dependent trend; with least toxins accumulation at highest concentration of extract and *vice versa*. In line with the present study, a dose-dependent reduction in OTA synthesis was observed with the addition of *Burkholderia cepacia* QBC03 culture extract to *A. carbonarius* contaminated PDB tube (Zeidan et al., 2019). A precise underlaying mechanism of OTA-synthesis inhibition needs to be explored, that might be down-regulation of key genes involved in OTA biosynthesis pathway as observed by Farbo et al. (2018). A significant reduction ($p \leq 0.05$) in the OTA synthesis potential of the *A. carbonarius* at lowest tested concentration (20%) of *B. pumilus* 344-3 extract, shows high efficacy of bacterial compounds against OTA synthesis, suggesting potential economic benefits for application at large scale. Although there was a dose dependent decrease in the OTA contents, but statistical analysis performed by ANOVA with *post hoc* multiple comparisons by LSD showed a non-significant difference among all treated concentrations. The amount of OTA synthesized (ng) per mg of the fungal biomass showed a significantly ($p \leq 0.05$) higher value of 414.81 ± 27.4 (ng/mg) in the control groups (PDB alone) as compared to all other groups. The OTA contents (ng) of tubes having 20%, 40%, 60%, 80% and 100% *B. pumilus* culture extracts in relation to fungal biomass (mg) were 33.42 ± 6.12, 14.37 ± 4.33, 10.29 ± 3.33, 3.81 ± 2.50 and 15.69 ± 5.31, which were non-significant among each other.

3.3. Effects of B. pumilus 344-3 culture extract on fungal conidial germination

Bacterial culture extract at 10% and 20% dilutions showed a non-significant effect on spore germination after 24 h of incubation. At 50% bacterial extract, 93% of the spore showed germination, while at 100% bacterial extract none of the fungal spore showed germination tube (Fig. 4). In line with the present study, Kim et al. (2019) reported up to 100% inhibition in the spore germination of phyto-pathogenic *Colletotrichum* spp. and *F. oxysporum* by the culture extract of *Streptomyces blastmyceticus*. With increasing time of incubation, there were mild-moderate morphological alternation characterized by thickened hyphae in the bacterial extract treated fungi. Apart from fungal spore germination, at 96 h of incubation, there was a significant reduction in sporulation of germinated fungi in all concentrations of bacterial culture extract as compared to their respective control. The inhibition of fungal spore germination by *Bacillus* spp. and *Burkholderia cepacia* QBC03 culture extract have also been reported by Chen, 2012 and Zeidan et al.

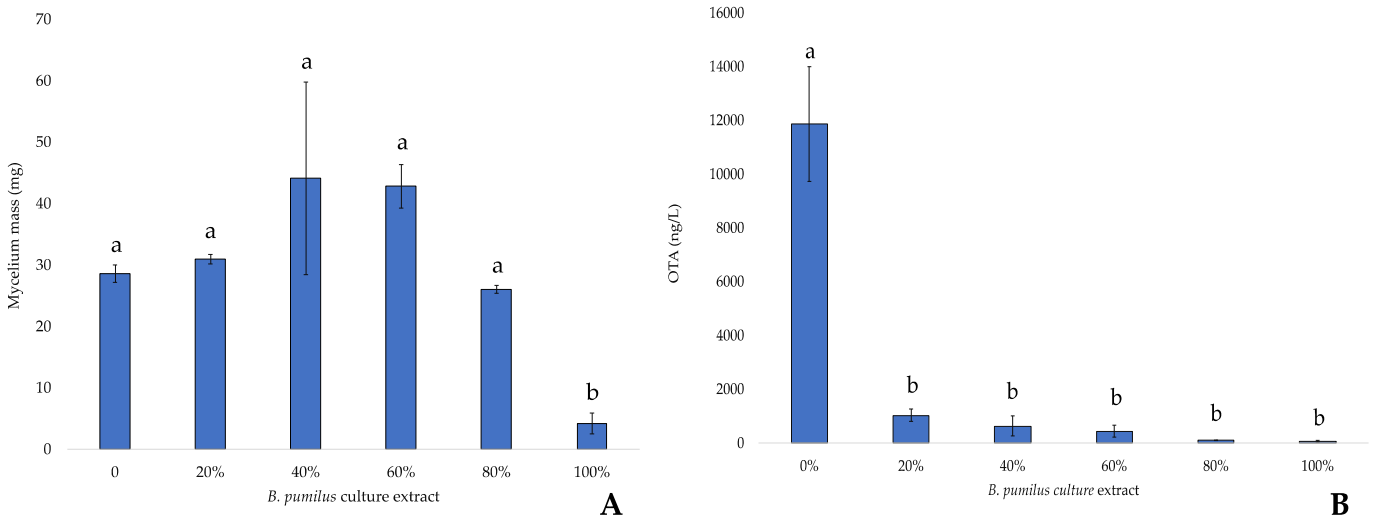

Fig. 3. Effect of *B. pumilus* 344-3 culture extract on *A. carbonarius* biomass and OTA synthesis. **A**; fungal biomass was increased with addition of bacterial extract at 20%, 40% and 60%, while significant reduction was seen at 100% bacterial extract. **B**; effect of bacterial culture extract on OTA synthesis by *A. carbonarius*. OTA synthesis was significantly inhibited by the addition of all levels of bacterial culture extract, in spite of growth stimulatory effect of low levels of bacterial extract (A). Each bar represents mean value of 9 observation (from three independent experiments), obtained by subtracting biomass from their corresponding control having similar concentration of LB media without bacterial extract. ANOVA with *post-hoc* multiple comparison by LSD test was performed. Error bars show the standard deviation (n = 9).

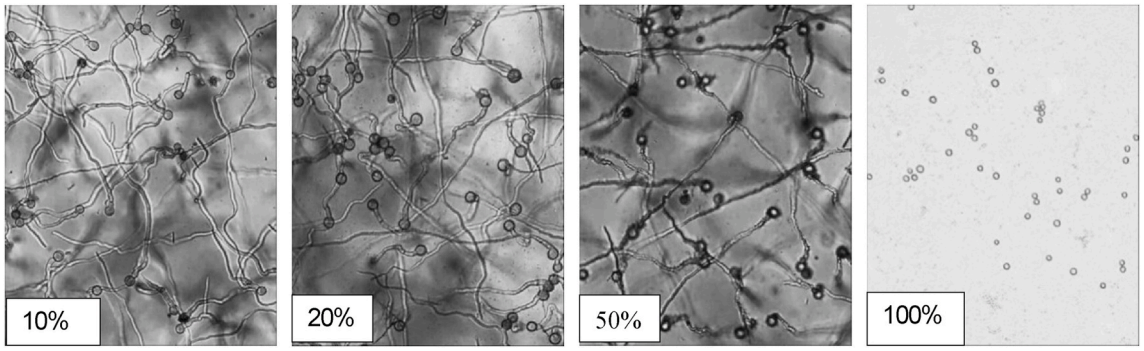

Fig. 4. Effect of different dilutions of *B. pumilus 344-3* extract on conidial germination after 24 h of incubation. *A. carbonarius* spores were suspended in 24-wells plates having bacterial extract at different dilution in PDB. Respective control wells were maintained by adding LB media in PDB in place of bacterial extract. Bacterial extract at 50% resulted in 7% reduction of fungal spore germination, while there was complete inhibition in spore germination at 100% bacterial extract. Images were taken using inverted microscope at 400X.

(2019), respectively.

3.4. Investigation on thermostability of B. pumilus 344-3 antifungal extract

After 24 h of incubation of fungal spores in thermal-treated *B. pumilus* extract, there was 100% inhibition in spore germination. These observations suggested high stability of bacterial compounds even at 100 °C (Fig. 5). On the other hand, in the control wells (thermal treated LB broth), spore germination was 55–65%. Thermostability of bacterial culture extract against fungal spore germination have been reported in the several studies (Munimbazi & Bullerman, 1998; Chen, 2012). In our previous study (Zeidan et al., 2019), culture extract of *Burkholderia cepacia* showed reduced activity against *A. carbonarius* on treatment at 40 °C and above. Similarly, the only study with *B. pumilus* reported no alteration in the antifungal potential of culture extract even treated at autoclave temperature (121 °C) for 15 min (Munimbazi and Bullerman (1998). However, in the study of Chen, 2012, *Bacillus* culture extract showed stability at temperature 100 °C and was completely unstable at 121 °C. In general, antifungal molecules of *Bacilli* are quite stable at high temperature even at long storage of up to 30 day (Petatán-Sagahón et al., 2011; Toral et al., 2018).

3.5. Application of B. pumilus 344-3 extract in the protection of maize kernels

After 7 days of infection (Fig. 6), a significantly ($p \leq 0.05$) higher area (9.21 ± 0.83 cm^2) of maize kernels was contaminated by the fungi in the experimental groups without bacterial extract (LB alone) as compared to treated kernels. In the treated group, *B. pumilus* 344-3 extract showed a significant ($p \leq 0.05$) reduction in fungal growth and spread by 0.923 ± 0.091 cm^2, while least spread (0.47 ± 0.07 cm^2) was noted on the kernels inoculated with QBC extract. The findings of this experiment showed high potential of *B. pumilus* extract to effectively control fungal growth on the surface of maize kernels, accounting 95% reduction in the fungal spread as compared to the control kernels. Jiang et al. (2014) reported 80% reduction in the *A. carbonarius* growth on grapes berries inoculated with *B. subtilis* culture supernatant giving protection until 80 days of the experiment. A similar high protection (88.7%) of grapes berries against *A. carbonarius* infection was observed by Senthil et al. (2011) by *B. subtilis* culture supernatant. In field trials, application of *Bacillus amyloliquefaciens* cell suspension on

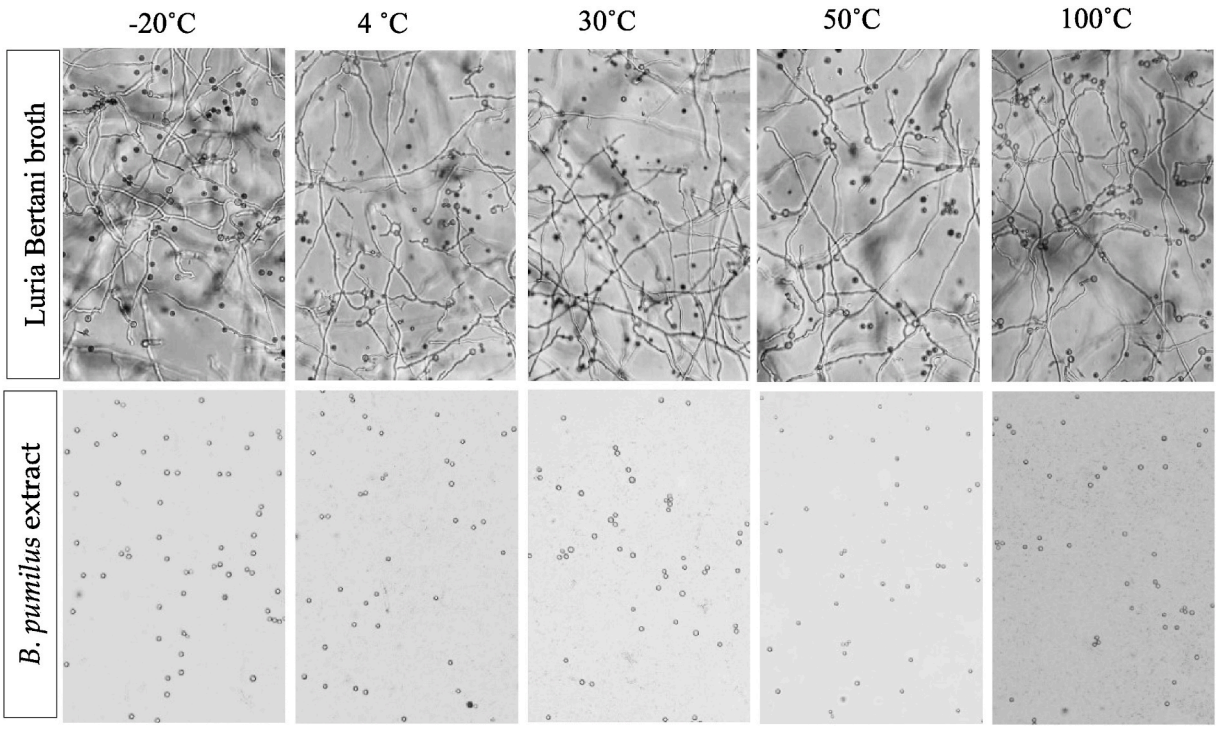

Fig. 5. Comparison between *A. carbonarius* conidial germination in LB media (control) and *B. pumilus 344-3* extract treated at different temperatures. Fungal conidia were suspended in bacterial extracted treated at different temperature. After 24 h of incubation, conidial germination was observed under inverted microscope at 400X. Temperature treatment for 1 h, showed no effect on *B. pumilus* antifungal compounds.

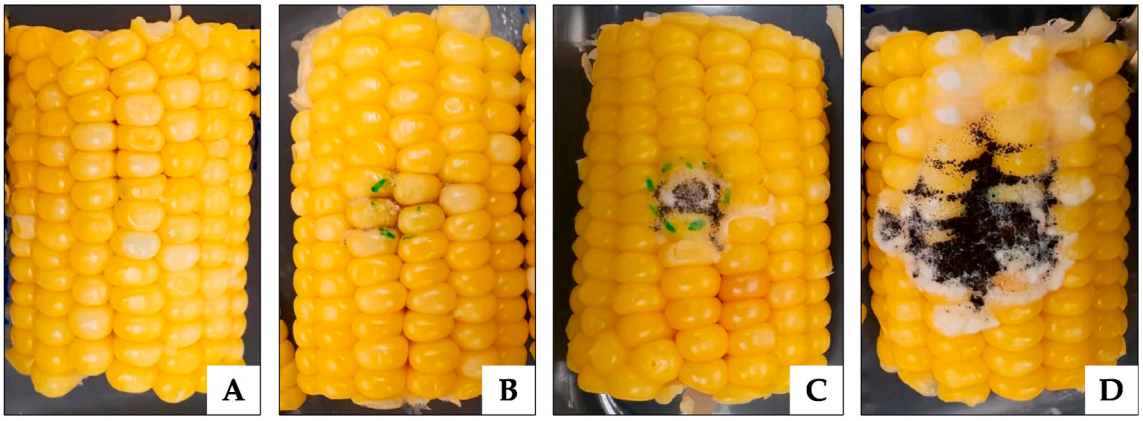

Fig. 6. Fungal growth inhibition by *B. pumilus* 344-3 extract on the surface of maize kernels. Bacterial extract was applied on the surface of maize kernels at marked area, followed by inoculation of *A. carbonarius* spores. **A**, disinfected maize kernel without bacterial extract and fungal spore. **B**, maize kernel applied with QBC culture extract and *A. carbonarius* spores. **C**, maize kernel applied with *B. pumilus* 344-3 culture extract and *A. carbonarius* spores. **D,** surface disinfected maize kernel applied with LB media and *A. carbonarius* spores. After 7-day of incubation, *B. pumilus* inoculated kernels showed least fungal growth (C) as compared to the kernels without any bacterial extract (D). Experiment was repeated three times with 3 replicate of each treatment each time.

Botryosphaeria dothidea infected peach trees resulted in 57.5% decrease in disease severity index (Li et al., 2016). These findings highlight the biocontrol potential of *Bacillus* compounds against toxigenic and phytopathogenic fungi.

The effect of bacterial extract on the mycotoxin synthesis potential of *A. carbonarius* on maize kernels was quantified by analyzing the OTA contents of kernels. A significantly higher ($p \leq 0.05$) OTA concentration (2.27 ± 0.03 mg/g) was found in the kernels inoculated with *A. carbonarius* without bacterial extract as compared to those infected with fungi in the presence of *B. pumilus* culture supernatant (0.03 ± 0.01 mg/g). The results depicted 99% inhibition in OTA synthesis potential of the fungi by *B. pumilus* culture supernatant. This reduction in OTA contents of *A. carbonarius* infected kernels in the presence of *B. pumilus* culture extract is mainly due to the inhibition in the fungal growth (95%). However, there are other mechanisms (less likely applicable in the present situation) such as down regulation of mycotoxins biosynthesis genes by microbial molecules (Farbo et al., 2018). In line with the present study, application of two yeast starins of *Kluyveromyces thermotolerans* resulted in up to 100% reduction in OTA synthesis by balck *Aspergilli* on grape berries (Ponsone et al., 2011). Likewise, El-Shanshoury et al. (2018) also reported 100% inhibition of OTA and AFB1 accumulation on grape berries treated with living cells or culture supernant of *Pseudomonas aeruginosa, B. vallismortis* and *B. amyloliquefaciens*.

3.6. Conclusion

In this study, we have investigated a novel strain of *B. pumilus* 344-3 effective against growth and OTA-synthesis by *A. carbonarius*. *In vivo* antagonistic efficacy based on protection of maize kernels highlights the field application of bacterial molecule against OTA synthesis in key food commodities (fruits, nuts, vegetable and other cereals). The investigation on precise nature of *B. pumilus* 344-3 compound (s) and their characterization will allow to develop artificial mixture for their field application at low production cost. Finally, the biocontrol agents particularly those isolated from the foods could be more appropriate for environmental sustainability, safety and possess the potential to replace the traditional fungal control strategies of pesticides and chemicals application. Moreover, the current issues of pesticides residues in food chain from agricultural application and emergence of pesticides resistant fungal strains would be widely addressed by the application of novel and safe microbial compounds and their mixtures.

CRediT authorship contribution statement

Nayla Salah Higazy: Methodology, Validation, Formal analysis. **Aya Ehab Saleh:** Methodology, Validation. **Zahoor Ul Hassan:** Conceptualization, Methodology, Validation, Formal analysis, Writing - review & editing. **Roda Al Thani:** Writing - review & editing, Conceptualization, Methodology, Validation. **Quirico Migheli:** Conceptualization, Writing - review & editing. **Samir Jaoua:** Formal analysis, Conceptualization, Methodology, Validation, Writing - review & editing, Resources, Supervision.

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