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THE *IN VITRO* ANTAGONISTIC EFFECTS OF SOME *BACILLUS* SPP. ON THE GROWTH AND MYCOTOXIN PRODUCTION OF TOXIGENIC ASPERGILLI

Miroslava Hlebová[1], Viktória Uzsáková[1,2], Ivana Charousová[3], Lukáš Hleba[2]*

Address(es): Ing. Miroslava Hlebová, PhD.
[1]University of SS. Cyril and Methodius, Faculty of Natural Sciences, Department of Biology, Nám. J. Herdu 2, SK-91701, Trnava, Slovak Republic.
[2]Slovak University of Agriculture in Nitra, Institute of Biotechnology, Faculty of Biotechnology and Food Sciences, Tr. A. Hlinku 2, 94976, Nitra, Slovak Republic.
[3]Clinical Microbiology Laboratory, UNILABS SLOVENSKO, s.r.o., J. Bellu 66, Likavka, Slovak Republic.

*Corresponding author: miroslava.hlebova@ucm.sk

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ABSTRACT

This study aimed to investigate the antagonistic effect of three strains of the genus *Bacillus* on toxigenic fungi contaminating food commodities, with emphasis on two ochratoxigenic isolates (*A. ochraceus*, *A. westerdijkiae*) and two aflatoxigenic isolates (*A. flavus* and *A. parasiticus*). *In vitro* studies were carried out using two different methods for cultivation tested bacilli with fungal isolates – coincubation and dual culture method. The most sensitive isolate was *A. ochraceus* by both used methods. *B. mycoides* (5.72 ± 6.4 mm) and *B. subtilis* (5.08 ± 2.84 mm) were able to inhibit its growth and sporulation during ten days of cultivation (both inhibited the sporulation of *A. ochraceus* 100%) in coincubation. The most effective in the dual culture method were *B. subtilis* and *B. thuringiensis* against *A. ochraceus* (growth inhibition rate 84.40%; 90.55%) and *A. flavus* (growth inhibition rate 91.54%; 92.43%). The most effective sporulation inhibitors were *B. subtilis* and *B. thuringiensis*, which completely inhibited the sporulation of *A. ochraceus* and *A. parasiticus* after ten days of coincubation. Likewise, all tested bacterial strains showed complete inhibition of ochratoxin A synthesis in *A. ochraceus* and *A. westerdijkiae* exposure to bacterial volatiles. So, the current study illustrated that strains of the genus *Bacillus* could significantly inhibit the growth, sporulation, and mycotoxin production of toxigenic aspergilli and showing the great potential as a biocontrol agent of pre- and post-harvest food diseases caused by microscopic filamentous fungi.

Keywords: antagonistic activity, *Bacillus* spp., direct contact, mycotoxins, toxigenic aspergilli, volatiles

INTRODUCTION

Food spoilage, mainly microbial spoilage, is a major worldwide problem for the food industry, leading to the waste of edible foods and economic losses for producers and consumers (**Lorenzo *et al.*, 2018)**. Among microorganisms, filamentous fungi are the primary food contaminants, which can occur at various stages of the food chain (pre-/post- harvest, during processing, or storage) **(Sadiq *et al.*, 2019**). The development of fungi on or in food leads to changes in sensory properties of food, ranging from visual damage to noticeable changes in smell, taste, or texture, but can also have negative effects on human and animal health through the production of mycotoxins **(Badiale Furlong *et al.*, 2021)**. To prevent the deterioration of food crops and foodstuffs by fungal contaminants and their mycotoxins and thus prolong the shelf life of the products, different methods are used to eliminate them. This includes the use of fungicides, chemical preservatives, and various other chemical and physical methods. Despite their effectiveness, there are some limitations in using these methods due to their high cost, possible production of by-compounds with higher toxicity, loss of nutritional value of food **(Karlovsky *et al.*, 2016; Neme and Mohammed, 2017**). In the view of the above context, there is a growing interest in the use of biological agents as preservatives in the field, processing, and postharvest handling has achieved the attention of the scientific community because they offer the best alternative to control fungi in food. The use of antagonist microorganisms or their secondary metabolites has proven to be safe and effective in the biological control of growth inhibition of many fungal pathogens **(Ramos-Pereira *et al.*, 2021; Zhang *et al.*, 2021).**
The bacterial genus *Bacillus* is a promising candidate for a fungal antagonist in the sphere of bioprotection and bioconservation of food **(Nath *et al.*, 2015)**. The importance of this genus is associated with the biological control of numerous fungal pathogens contaminating many food commodities **(Islam *et al.*, 2019)**. There are several mechanisms for the use of *Bacillus* species to control food fungal pathogens, including competition in space or nutrients, antibiosis, and induced systemic plant resistance **(Lastochkina *et al.*, 2019).** One of these mechanisms, antibiosis, has been reported as an essential mechanism by which the genus *Bacillus* produces active metabolites that inhibit fungal contaminants. Antifungal lipopeptides, volatile organic compounds produced by species of the genus *Bacillus*, are mainly biologically active substances used to inhibit fungal growth **(Fira *et al.*, 2018; Saleh and Jaoua, 2020)**. The role of *Bacillus* strains is not limited only to inhibiting fungal growth, but some species can also interact with fungal mycotoxins, resulting in their inactivation or removal through cell wall binding. These mechanisms, which help eliminate mycotoxins, include biodegradation of mycotoxins by chemical or enzymatic cleavages and adsorption, where the prominent role is played by the cell wall components of the microorganism **(Chen *et al.*, 2018).**
This study was designed to investigate *in vitro* inhibition effects of *Bacillus* spp. by using two different methods, coincubation and dual culture method, against the growth, sporulation, and mycotoxin production of four toxigenic isolates of the genus *Aspergillus* (*A. ochraceus*, *A. westerdijkiae*, *A. flavus*, and *A. parasitcus*).

MATERIAL AND METHODS

Bacterial strains, identification, growth condition and inoculum preparation

Three strains of the genus *Bacillus*, namely *B. subtilis*, *B. mycoides* and *B. thuringiensis* were used in this study. The antagonistic bacteria of the genus *Bacillus* were obtained from food samples (*B. mycoides* and *B. subtilis*) and from soil samples (*B. thuringiensis*). Before identification, the strains were cultivated on nutrient broth (Imuna, Slovakia) at 30 °C for 24 h. Identification of the bacterial strains was performed by MALDI TOF MS (Bruker Daltonics, Germany, Maldi Biotyper) using single colonies of 24 h cultures of each strain according to **Hleba *et al.* (2017).** To prepare a bacterial inoculum the bacterial strains were cultivated 24 hours at 30 °C in meat peptone broth (MPB) (HiMedia, India). Subsequently, the bacterial inoculum was prepared by diluting the bacterial suspension in sterile phosphate saline to a final concentration of 10^6 cells/ml by adjusting the density to 0.5 McFarland units.

Fungal isolates, identification, growth condition and inoculum preparation

The following toxigenic isolates of fungi were used in this study: *Aspergillus flavus*, *A. parasiticus*, *A. ochraceus* and *A. westerdijkiae*. All the fungal isolates were obtained from green coffee beans samples, marketed in Slovakia. The isolates were identified to the species level according to morphological characteristics based on microscopy used follow diagnostic literature: **Samson *et al.*, 2002**, **Chen *et al.*, 2017**, **Frisvad *et al.*, 2019** and **Yamairi *et al.*, 2019.** *Aspergillus* isolates were tested for aflatoxin B_1 and G_1, and ochratoxin A, and their production was confirmed by thin layer chromatography (TLC) **(Labuda and Tancinová, 2006)**. To prepare a fungal inoculum the isolates were cultivated on Sabouraud dextrose agar (SDA) (HiMedia, India) for 5 days at 25 °C. Then the inoculum was prepared according to **Císarová *et al.* (2020)** to a final concentration 10^6 CFU/mL by adjusting the density to 0.7-1.2 McFarland units, depending on the strain.

***In vitro* screening antagonistic activity of *Bacillus* spp. by volatiles**

The antifungal activity of volatiles potentially produced by *Bacillus* species against toxicogenic *Aspergillus* species (*Aspergillus flavus, A. parasiticus, A. ochraceus* and *A. westerdijkiae*) was tested using the coincubation method **(Ul Hassan *et al.*, 2019)**, with some modification (Fig 1). A 100 µl of the prepared bacterial inoculum suspension (10^6 cells/ml) was applied on MPA plates (Meat peptone agar; HiMedia, India) and cultivated without sealing for 24 hours at 30 °C. After 24 hours, the cover of the bacterial plate was replaced by a base SDA plate containing 5 µl of fungal inoculum (10^6 CFU/ml). Subsequently, both parts of the plates were tightly sealed with parafilm to prevent volatiles escape. The plates were cultivated for 10 days at 30 °C in the dark. The plates were cultivated in an inverted position (the plate containing the bacterial inoculum was on top). The control treatment was represented by fungus inoculated plates without bacterial inoculum. The effects of bacterial volatiles on fungal growth and sporulation were evaluated by measured diameters (Ø mm) of the growing fungal colonies at the 2^{n}d, 4th, 6th, 8th, and 10th day of cultivation with a digital caliper. All analyzes were performed in 6 replicates.
To evaluate the reversibility effects of bacteria volatiles on the fungal growth, on the 5th day of cultivation, a small piece of a fungal colony (Ø 10 mm) from the margin was removed with a sterile cork borer and transferred to fresh SDA plates. These plates were incubated for 10 days at 30 °C, and mycelial growth and sporulation were monitored (in the same way as before on 2^{n}d, 4th, 6th, 8th and 10th day) and compared with the control group (plates with fungal inoculum never exposed to bacterial volatiles).

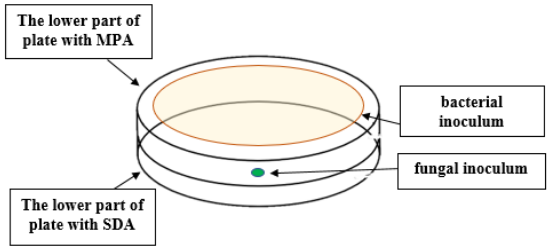

Figure 1 The scheme of coincubation method

***In vitro* screening antagonistic activity of *Bacillus* spp. by contact**

The strains of genus *Bacillus* were also evaluated *in vitro* for their antagonistic properties against toxicogenic *Aspergillus* species by using dual culture technique according to **Rajkumar *et al.* (2018)** with slight modification (Fig. 2). Firstly, the Petri dishes (Ø 60 mm) with PDA (Potato dextrose agar, HiMedia, India) were divided in half by a dividing line and then were inoculated with the bacterial inoculum (10^6 cells/ml) by spreading (comma) approximately 1 cm from the center of the plate dividing line to the left side of plates. After 24 hours of cultivation at 30 °C in the dark, the plates were inoculated with fungal inoculum (1 cm from the center of the plate dividing line). Each fungus was inoculated to the right side of plates with inoculation needle. The antagonistic activity of the *Bacillus* spp. was monitored in comparison with a control sample (plates with fungal inoculum in the absence of bacteria). The prepared plates were sealed with parafilm and cultivated at 30 °C for 8 days. The mycelial growth of fungi was recorded with a digital caliper on the 2nd, 4th, 6th, and 8th day of cultivation in mm. The Percentage of Mycelial Growth Inhibition (PGMI) of the tested species was calculated using the following equation:

$$PGMI = ((FC-FB) / FC) * 100$$

where FC is the growth of the fungal mycelium control (measured in mm from the inoculation point to the edge of the mycelial colony), FB is the growth of the fungal mycelium in the presence of bacteria (measured in mm from the inoculation point to the edge of the mycelial colony towards the antagonist). All analyzes were performed in 6 replicates.

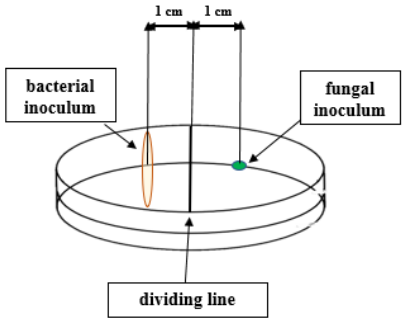

Figure 2 The scheme of dual cultivation method

Antitoxigenic effect of *Bacillus* spp.

The antitoxigenic effect of the genus *Bacillus* was evaluated only after coincubation method (including the reversibility test). For the detection of mycotoxins, the qualitative TLC (thin layer chromatography) screening method according to **Labuda and Tančinová (2006)** was used. After 10 days of cultivation (coincubation and reversibility) the ability of the genus *Bacillus* to suppress the production of aflatoxin B_1 (AFB_1) in *A. flavus*, AFB_1 and aflatoxin G_1 (AFG_1) in *A. parasiticus* and ochratoxin A (OTA) produced by *A. westerdijkiae* and *A. ochraceus* was evaluated. The used method is described in **Císarová *et al.* (2015)**. Visualization of mycotoxins AFB_1, AFG_1, ochratoxin A and their authentic standards (Sigma Aldrich, Germany) was performed directly under a UV lamp at 366 nm, where aflatoxin B_1 and ochratoxin A appeared as a blue spot and AFG_1 as a green spot.

Statistical analysis

Obtained results were evaluated by analysis of variance (one-way ANOVA) and Tukey HSD 95% multiple range test ($p<0.05$) using the program STATGRAPHICS Centurion XVI (version 16.1.11). The results of the percentage inhibition of spore germination and percentage of the mycelial growth inhibition (PMGI %) were calculated by using the MS Office Excel 2016 program.

RESULTS AND DISCUSSION

Antagonistic activity of the genus *Bacillus*

Contamination of food with various perishable microorganisms, mainly microscopic filamentous fungi, is a global problem that leads to large-scale economic losses. Nowadays, synthetic preservatives or antifungals are used to prevent their growth in food. However, these substances can affect humans and the environment, so more environmentally friendly alternatives are still being sought **(Foysal *et al.*, 2018; Morita *et al.*, 2019; Zhang *et al.*, 2020)**. Biological control of fungal growth and food deterioration by antagonist bacteria such as *Bacillus* spp. is an alternative to the chemical preservatives used. Many previous studies have reported that substances produced by some *Bacillus* species have antifungal activity against a broad range of fungi such as *Botrytis cinerea* (**Touré *et al.*, 2004**), *Fusarium graminearum* **(Wang *et al.*, 2007),** *Alternaria solani* **(Zhang *et al.*, 2020)**, including the genus *Aspergillus* **(Jiang *et al.*, 2014; Arfaoui *et al.*, 2018)**. In this study, three strains of the genus *Bacillus* (*B. subtilis, B. mycoides, B. thuringiensis*) were assayed against four isolates of ochratoxigenic (*A. westerdijkiae* and *A. ochraceus*) and aflatoxigenic (*A. flavus* and *A. parasiticus*) aspergilli using two different methods. The first coincubation method investigated the antifungal activities of potentially produced volatiles of the genus *Bacillus*. The second method was used to evaluate the antifungal activity of *Bacillus* spp. by the method of dual culture, where the tested bacilli were inoculated on one plate together with the fungi and were in contact with each other. Furthermore, there are many reports focused on the antioxygenic potential of the genus *Bacillus* **(Taniwaki *et al.*, 2018; Ren *et al.*, 2020; Higazy *et al.*, 2021)**. So, the inhibition effect of tested bacilli on the production of mycotoxins was evaluated in this study, too.

Effect of *Bacillus* spp. on the growth and sporulation of tested fungi by bacterial volatiles

The result of the coincubation method showed that in the presence of the *Bacillus* spp. and their released volatiles, the growth of tested fungi was inhibited significantly ($p<0.05$). The strains showed different antagonistic activity on mycelia growth and sporulation of toxigenic aspergilli. *A. ochraceus* was the most sensitive isolate from the tested ochratoxigenic fungi. *B. mycoides* (5.72 ± 6.4 mm) and *B. subtilis* (5.08 ± 2.84 mm) were able to inhibit vegetative growth and sporulation during ten days of cultivation (both inhibited the sporulation of *A.*

ochraceus 100%) of this isolate significantly. After ten days of coincubation with these species, the radial growth of *A. ochrac*eus resulted in smaller colony diameters compared to the control set (13.18 ± 7.07 mm). *B. thuringiensis* showed the ability to inhibit this species only until day 6 of cultivation (Fig. 1A) with the inhibition of sporulation 33,33%. The better results for *B. thuringiensis* were noted by **Arfaoui *et al.* (2019)**. They found that *B. thuringiensis* J4F was able to inhibit 78% of *A. ochraceus* growth. Also, partial inhibition of *A. westerdijkiae* was found in treatment with *B. thuringiensis* (20.75 ± 12.44 mm) compared to a control set (23.65 ± 12.65 mm). However, *B. thuringiensis* did not show any inhibitory effect on *A. westerdijkiae* sporulation (Table 1). *B. subtilis* and *B. mycoides* were the most effective species in inhibiting the growth of this isolate. Antagonism of *B. subtilis* by volatiles was studied in work of **Petchkongkaew *et al.* (2008)**. They found that the growth of *A. westerdijkiae* tested by the coincubation method for seven days was lower, about 34%. Inhibition of growth was associated with prolongation of the lag phase of the microscopic fungus, thereby reducing its ability to compete and utilize the nutrients in the environment. Also, **Einloft *et al.* (2017)** observed the inhibitory effect of *B. safeness* RF69, *B. amyloliquefaciens* RP103, and *B. subtilis* RP242 on the growth of *A. westerdijkiae* as well as their ability to inhibit spore formation. They found that *B. subtilis* RP242 was the most effective biological control agent of fungal growth and sporulation. The results showed a 95.6% reduction in growth and spore germination after ten days of coincubation.

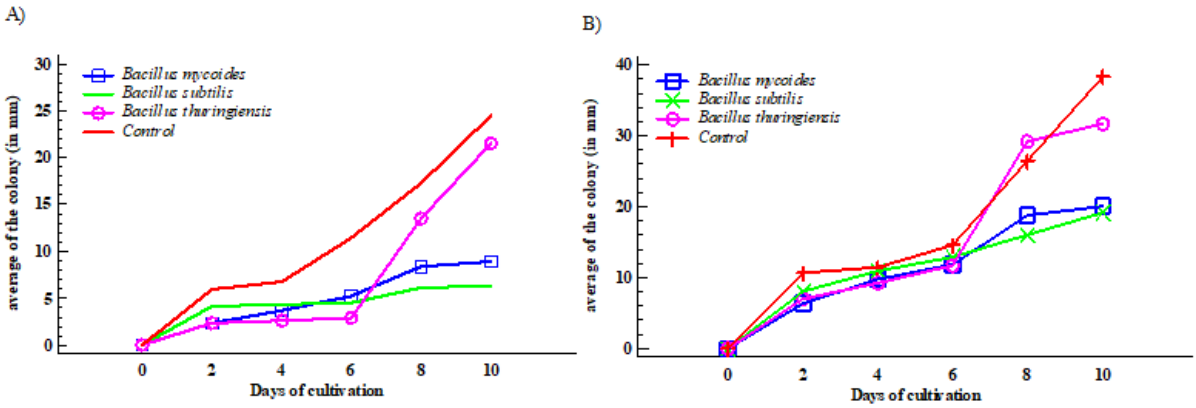

Figure 1 The antagonistic effect of the tested *Bacillus* spp. on the growth: A) *A. ochraceus* (n=6) and B) *A. westerdijkiae* (n=6) during 10 days of cultivation in the dark (in mm) at 30 °C

The inhibition activity of *Bacillus* spp. on the growth and sporulation of aflatoxigenic aspergilli was much more potent, especially for *A. flavus* isolate. Results showed that among all tested toxinogenic fungi of the genus *Aspergillus, A. flavus* was the most sensitive to the action of bacterial antagonists. The mycelial growth of *A. flavus* reached the smallest averages throughout all periods of cultivation (10 days), mainly in the presence of *B. subtilis* (1.60 ± 1.87 mm) and *B. thuringiensis* (0.85 ± 1.15 mm) (Fig. 2A). **Nayak *et al.* (2020)** tested the antifungal activity of 4 isolates of *Bacillus* spp. against the growth and sporulation of two strains of *A. flavus* (A2, A28). Their results showed that *Bacillus vallismortis* BC5 was the most potent in mycelial growth inhibition of *A. flavus* (92.3%), followed by *B. subtilis* BC6 (90%) and *B. amyloliquefaciens* BC1 and BC2 (88.6% and 89.3%). However, in our study, their efficacy did not correlate with inhibition of sporulation. *B.* subtilis showed no inhibitory effect on sporulation, and the effect of *B. thuringiensis* on *A. flavus* sporulation was minimal (16.67%) (Table 1). On the contrary, in their study, **Gong *et al.* (2014)** found the high inhibitory activity of *B. subtilis* on the sporulation of *A. flavus*. They found that bacillomycin D produced by *B. subtilis* fmbJ strain (CGMCCN 0943) inhibited sporulation of *A. flavus* from 85.72% to 98.10%.

In this study, 100% inhibition of sporulation was observed in treatment with *B. mycoides*, but this species had only a mediatory inhibition effect on *A. flavus* growth after ten days of cultivation (18.72 ± 13.98 mm) in comparison with a control set (29.55 ± 10.20 mm). On the other hand, *B. subtilis* and *B. thuringiensis* in comparison to the control and *A. flavus* species had better inhibitory effects on the sporulation of *A. parasiticus* (100%), but these species inhibited its growth only moderately (Fig. 2B). The best inhibitory effect for *A. parasiticus* was found in treatment with *B. mycoides* (6.11 ± 3.78 mm) during the whole cultivation period (10 days). However, *B. mycoides* was not able to inhibit *A. parastiticus* sporulation (Table 1). **Also, Chaves-Lopez *et al.* (2015)** evaluated the antifungal activity of the volatile organic compounds (VOCs) produced by 75 different food-borne *Bacillus* species against *Aspergillus niger Aspergillus flavus, Aspergillus parasiticus, Aspergillus clavatus, Fusarium oxysporum* f. sp. lactucae and *Moniliophthora perniciosa*. In their study, the species *A. clavatus* MG103 and *A. parasiticus* MG51 showed the least susceptibility. Similar to our study, the growth of these species was not affected by *Bacillus* spp. volatiles production.

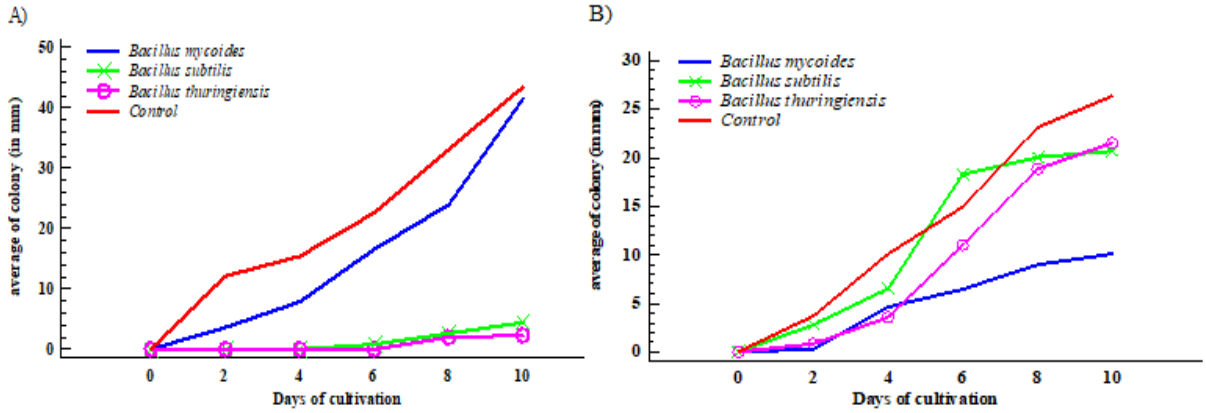

Figure 2 The antagonistic effect of the tested *Bacillus* spp. on the growth: A) *A. flavus* (n=6) and B) *A. parasiticus* (n=6) during 10 days of cultivation in the dark (in mm) at 30 °C

Table 1 Inhibition of sporulation of tested aspergilli by *Bacillus* spp. after 10 days at 30 °C of coincubation method

Tested bacilli	Tested aspergilli			
	A. ochraceus	*A. westerdijkiae*	*A. flavus*	*A. parasiticus*
	Inhibition of sporulation (%)			
Bacillus subtilis	100.00	50.00	0.00	100.00
Bacillus mycoides	100.00	66.67	100.00	0.00
Bacillus thuringiensis	33.33	0.00	16.67	100.00
Control of tested fungi	–	–	–	–

Legend: –: no effect (without presence of *Bacillus* strains)

The cell wall of fungi is necessary for their homeostasis, and various environmental or antifungal stresses can cause modifications such as suppression of sporulation, insufficient development of hyphae, or pigmentation losses **(Garcia-Rubio *et al*., 2020).** In this study, similar changes were observed. Compared to the control, some fungal isolates whose sporulation was partially or entirely suppressed showed an observed change or loss of mycelial pigmentation (Fig. 3).

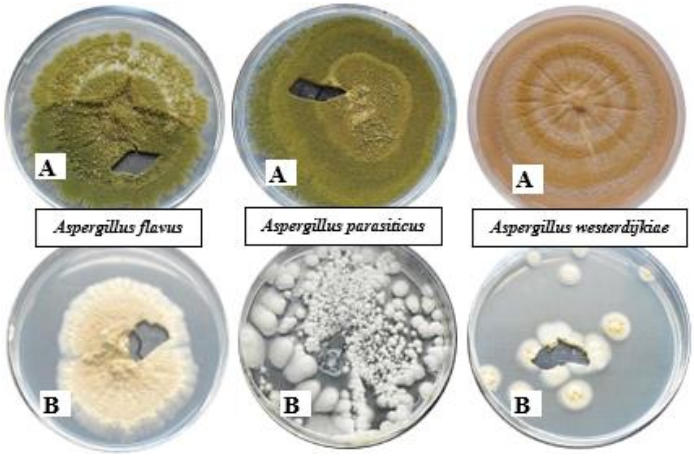

Figure 3 Changes observed in mycelial pigmentation: A) normal fungal mycelium (never exposed to bacterial volatiles) (control); B) change of mycelial pigmentation after treatment with volatiles of tested bacilli

After five days of coincubation with individual species of bacilli, the cultured plates were opened, and one small pieces of fungal mycelia were removed and subsequently cultivated on fresh SDA plates for another ten days without the presence of tested bacteria. However, upon transfer to the fresh SDA media, in the absence of bacterial volatiles, all the fungal isolates showed normal growth and sporulation, comparable with control sets. Our results showed that the inhibition was poor and that the coincubation of fungi with the volatiles of tested bacilli during the 5-days was insufficient for more significant inhibition of their growth and sporulation. For this reason, the obtained results are not presented in this work. Similar results were obtained by **Ul Hassan *et al*. (2019)** with *Bacillus* spp. against tested fungi and **Fiori *et al.* (2014)** who treated toxigenic *A. carbonarius* MPVA566 and *A. carbonarius* AN6 with antagonistic yeast *Candida friedrichii* 778. Also, in the study of **Saleh *et al*. (2021**), similar reversibility in mycelial growth and sporulation was observed in toxigenic fungi included *A. flavus* CECT, 2687after removal from volatiles of *B. megaterium* BM344-1. So, these results suggested that the microbial volatiles effect was insufficient and that bacterial volatiles were continuously required to allow effective biocontrol of spoilage fungi.

Effect of *Bacillus* spp. on the growth of tested fungi by contact

In the dual culture method, only the effect of *Bacillus* species on the growth of toxigenic fungi was observed. The results of dual culture method showed that antifungal ability of bacilli had a certain inhibitory potential on the growth of all tested fungal isolates. Significant differences ($p<0.05$) between the antagonistic effect of tested bacilli were found (Table 2). *B. mycoides* showed the best inhibitory ability against toxigenic aspergilli.

Table 2 Antifungal activity (colony diameter in mm ± SD) of *Bacillus* spp. on the growth of tested aspergilli (n=6) by dual culture method, after 8 days of incubation in the dark at 30 °C

Tested fungi	Tested bacilli			Control of tested fungi
	Bacillus subtilis	*Bacillus thuringiensis*	*Bacillus mycoides*	
Aspergillus ochraceus	3.59 ± 2.24^{a}	2.17 ± 2.25^{a}	3.92 ± 0.55^{a}	23.00 ± 0.00^{b}
Aspergillus westerdijkiae	$13.30 \pm 1.59^{b*}$	18.52 ± 0.97^{c}	5.73 ± 5.34^{a}	17.28 ± 0.28^{b}
Aspergillus flavus	3.05 ± 3.07^{a}	2.73 ± 4.04^{a}	34.58 ± 1.34^{b}	36.00 ± 0.00^{b}
Aspergillus parasiticus	27.83 ± 11.70^{ab}	36.00 ± 0.00^{b}	21.34 ± 7.38^{a}	36.00 ± 0.00^{ab}

Legend: * - data in the columns followed by the same letter are not significantly different in 95.0% Tukey HSD test, SD – standard deviation

B. mycoides showed the best inhibitory ability against ochratoxigenic aspergilli. The percentage of mycelial growth inhibition (%) calculated after all days of cultivation (8 days) with comparison to control sets was 67.36 % for *A. westerdijkiae* and 82.96 % for *A. ochraceus* (Fig. 4). From the tested fungi, *A. ochraceus* was the most sensitive to the action of all *Bacillus* species (Fig. 5B). The most potent inhibition against this isolate exhibited *B. subtillis* (growth inhibition rate 91.54%) followed by *B. thuringiensis* (growth inhibition rate 90.55 %) and *B. mycoides* (growth inhibition rate 82.96%). The sensitivity of *A. flavus* isolate was lower compared to *A. ochraceus*, but its growth was significantly inhibited by *B. thuringiensis* (growth inhibition rate 92.43 %) and *B. subtilis* (growth inhibition rate 91.54%) compared to the control sets (Fig. 5A). Similar results with *Bacillus subtillis* against *S. rolfsii* obtained by authors **Rajkumar *et al*. (2018)** and **Nayak *et al*. (2020)** in their study found that *Bacillus* spp. were able to inhibit the growth of *A. flavus* up to more than 90%. A very poor inhibition effect was observed using the dual culture method with *B. thuringiensis* and *A. westerdijkiae*. *B. thuringiensis* inhibited the growth of this isolate only up to the sixth day of cultivation (Ø 8.97 mm) compared to control (Ø 13.71 mm). After the cultivation period (8 days), the diameters of *A. westerdijkiae* colonies were larger (18.52 ± 0.97 mm) than the colony diameters of control sets (17.28 ± 0.28 mm). So, its growth was moderately stimulated by *B. thuringiensis.*

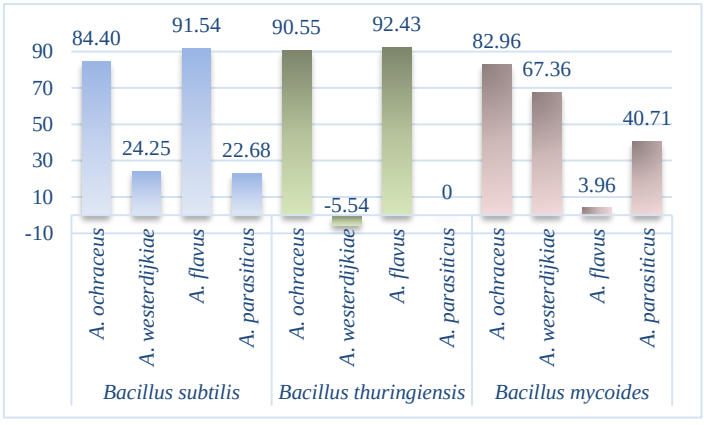

Figure 4 The percentage of mycelial growth inhibition PMGI (%) of the tested fungi by *Bacillus* species after 8 days of dual culture in the dark at 30 °C)

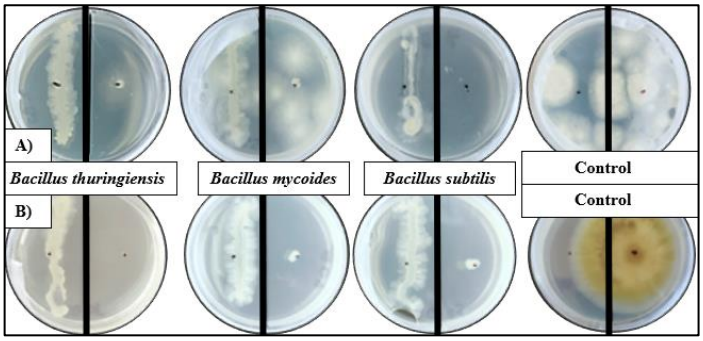

Figure 5 Effect of tested species of the genus *Bacillus* on the growth of A) *A. flavus* and B) *A. ochraceus*, by the dual culture method, after 8 days of cultivation in the dark at 30 °C

Based on the results, can be concluded that the coincubation method was better than dual culture method used. In the case of aflatoxigenic aspergilli, the difference between two used methods in the action of *Bacillus* spp. were observed. Significant and stronger antagonistic effects of selected species of the genus *Bacillus* could be seen mainly in the case of *A. flavus* and *A. parasiticus* used coincubation method in comparison with the dual culture method. The different effects of bacteria in the case of coincubation method, which were resulted in a higher inhibition of fungal growth, may be due to the use of a more suitable culture medium for bacteria, and fungi, respectively. The more appropriate used medium in coincubation method may have affected the potential production of *Bacillus* spp. volatile compounds, and since the bacteria did not come into direct contact with fungi, can be assume that the highest growth inhibition was due to their production. Also, **Morita *et al.* (2019)** studied the antifungal effect of *Bacillus pumillus* volatiles by cultivating fungi and bacteria on three different media: Trypt-Soya agar (TSA), Lauria-Betani agar (LBA) and TM Enterprise agar (TMES). They found that the bacterium grown showed the strongest inhibition effect against *Penicillium italicum* only on TMEA medium. **Tyc *et al.* (2017)** report, that the volatile organic compounds produced by *Bacillus* spp. can easily evaporate and diffuse through pores filled with air and water. Due to these physicochemical properties, volatile substances are ideal candidates for cooperation or antagonism between soil microorganisms that do not live directly next to each other.

Inhibitory effect of *Bacillus* spp. on mycotoxin production

Bacillus spp. volatiles not only inhibited the vegetative growth but also affected the mycotoxin production of toxigenic fungi. Although some species of the genus *Bacillus* did not completely inhibit the growth of the tested fungi, but they were able to suppress the production of their mycotoxins, especially in the case of inhibition of ochratoxin a (OTA) production. All three bacterial isolates inhibited OTA production by *A. westerdijkiaae* and *A. ochraceus* totally (100%) in comparison with control sets. Successful inhibition of mycotoxin biosynthesis was also observed in the case of reversible action of *Bacillus* spp., when 5 days exposure of microscopic fungi to bacterial volatiles was sufficient to completely inhibit their toxigenic potential (Table 3). The effect of bacterial VOCs, specifically of *B. licheniformis* 350–2 on OTA synthesis was tested after the 10th day of coincubation in study of **Ul Hassan *et al.* (2019)**. Their results showed complete inhibition of OTA synthesis by toxigenic fungi *A. westerdijkiae* BA1, *A. carbonarius* MG7 and *P verrucosum* MC12. Nevertheless, *A. ochraceus* MD1 (21.84 µg / kg) and *A. niger* MC5 (29.32 µg / kg) were still able to produce OTA in the presence of bacterial volatiles, although the levels of OTA produced were significantly lower than the levels of OTA production of the unexposed control (*A. ochraceus* MD1 - 87.21 ± 6.32 µg / kg, *A. niger* MC5– 75.44 ± 7.90 µg / kg). **Einloft *et al.* (2017)** also showed a reduction in the production of *A. westerdijkiae* OTA production by bacterial antagonists of *B. safensis* RF69, *B. amyloliquefaciens* RP103 and *B. subtilis* RP242 ranging from 62 to 96%. Some other authors have confirmed the inhibition of ochratoxin A by volatiles of *Bacillus* spp. **(Jiang *et al.*, 2017; Shukla *et al.*, 2020; Higazy *et al.*, 2021).**

Table 3 Effect of bacterial volatiles produced by *Bacillus* spp. on the mycotoxin biosynthesis of tested toxigenic aspergilli (n = 6) after coincubation and reversibility method

Coincubation					
Tested aspergilli	mycotoxin	*Bacillus subtilis*	*Bacillus mycoides*	*Bacillus thuringiensis*	Control
A. ochraceus	OA	nd	nd	nd	6/6
A. westerdijkiae	OA	nd	nd	nd	6/6
A. flavus	AFB_1	1**/6*	1/6	0/6	6/6
A. parasiticus	AFB_1 AFG_1	3/6 3/6	0/6 0/6	2/6 1/6	6/6
Reversibility					
A. ochraceus	OA	nd	nd	nd	6/6
A. westerdijkiae	OA	nd	nd	nd	6/6
A. flavus	AFB_1	2/6	1/6	0/6	6/6
A. parasiticus	AFB_1 AFG_1	4/6 4/6	0/6 0/6	2/6 2/6	6/6

Legend: nd – mycotoxin not detected (below the limit of detection of the TLC method), OA – ochratoxin A, AFB_1 – aflatoxin B_1, AFG_1 – aflatoxin G_1, ** - number of mycotoxins produced isolates, * - number of tested isolates

B. thuringiensis demonstrated the best antitoxigenic potential against AFB_1 production by *A. flavus*. Its production was completely inhibited even in the case of tested reversibility. **Kong *et al.* (2014)** found that aflatoxin accumulation was totally (more than 98 %) inhibited and cause the down-regulation of the aflatoxins genes by co-cultivation with *B. megaterium*. The biosynthesis of AFB_1 and AFG_1 produced by *A. parasiticus* totally inhibited only *B. mycoides* in all repetitions. *B. subtilis* inhibited the production of AFB_1 and AFG_1 produced by *A. parasiticus* at least. In the case of coincubation was able to suppress the production these aflatoxins only in half replicates, and in the case of a reversible effect the production of these mycotoxins was observed in up to 4 replicates. Better results with *B. subtilis* obtained **Siahmoshteh *et al.* (2018).** In their study *B. subtilis* was able to suppress *A. parasiticus* aflatoxins production up to 100 % and the degradation of aflatoxins has been proved after two days of co-cultivation the fungal strain with bacteria.

CONCLUSION

In summary, the results obtained in this study represent the promising characteristics of *Bacillus* spp. to be used as biocontrol agents of toxigenic fungi of genus *Aspergillus*. The antagonistic activity of *Bacillus* spp. was evaluated using two different methods (coincubation and dual culture technique), where some differences were observed, especially in the case of growth inhibition of aflatoxigenic isolates. Our findings demonstrated that the coincubation method showed increased inhibitory efficiency. Among all fungal isolates, we observed the strongest inhibitory effect in the case of *Bacillus* spp. on the growth of *A. ochraceus* in both coincubation and dual culture methods, respectively. This fungal isolate was indicated as the most sensitive compared to the related species (*A. westerdijkiae*) and the aflatoxigenic species. Antitoxinogenic properties of *Bacillus* spp. by using coincubation method were very effective, mainly in inhibition of ochratoxin production, but also had the potential to inhibit aflatoxins synthesis. Our research confirmed the antagonistic abilities of *Bacillus* spp. against microscopic fungi, contaminating many crops and food. Their bioprotective character and their volatile compounds have also been confirmed in many in vivo and in situ assays. Therefore, these results suggest the potential application of *Bacillus* spp. for the preservation of food commodities, and further studies should be carried out to develop the commercial usage of the selected biocontrol agents of the genus *Bacillus*.

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