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EFFECT OF INFLUENCE OF BACILLUS SUBTILIS TS 01 ON THE GROWTH OF MYCOTOXIGENIC FUNGI

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Abstract: *Mycotoxin contamination is now one of the most insidious challenges to food safety. The most prevalent mycotoxigenic fungi belong to the genera Aspergillus, Fusarium, Penicillium and Alternaria. In this study the antifungal and antimycotoxigenic capability of Bacillus subtilis TS 01 in vitro was evaluated. The results of influence of the bacterium on the growth of some fungi, hyphal morphology and spore germination are demonstrated. Bacillus subtilis TS 01 strongly inhibited the growth of the fungi on potato-dextrose agar during a fourteen days after inoculation. A highest average inhibitory percentage - 85,71% was measured by Alternaria sp. The effect on growth inhibition was less pronounced by Aspergillus species. Malformation of hyphae of fungi as swellings and vacuoles were observed in the presence of Bacillus subtilis TS 01. The strain suppressed the conidial germination significantly. Altogether, these results indicate that Bacillus subtilis TS 01 could be considered as potential biocontrol agent to combat toxigenic fungal growth and subsequent mycotoxins contamination of agricultural crops in practice.*

Keywords: *Mycotoxigenic fungi, Mycotoxins, Biological control, Bacillus subtilis.*

INTRODUCTION

Mycotoxins are toxic low molecular weight natural products (secondary metabolites) produced by filamentous fungi. These toxic molecules are capable of causing disease and death in humans and animals.

The most prevalent toxigenic fungi belong to the genera *Aspergillus, Fusarium, Penicillium* and *Alternaria*. *Fusarium* and *Alternaria* usually pose a high mycotoxicological risk at pre-harvest level or in freshly harvested products that are drying, whereas toxigenic species of *Aspergillus* and *Penicillium* represent a higher risk for food and feed products in storage or other kind of processing (Tsitsigiannis, D. I., Dimakopoulou, M., Antoniou P. P., & Tjamos, E. C., 2012).

The major agriculturally important mycotoxins include aflatoxins, fumonisins, zearalenone, deoxynivalenol, ochratoxins, each of which is produced by several fungal species. *Aspergillus* species are the major producers of aflatoxins; several *Aspergillus* and *Penicillium* species produce ochratoxins; several *Fusarium* species produce fumonisins, trichothecenes; and *Alternaria* species produce alternariols (Palumbo, J. D., O'Keeffe, T. L., & Abbas, H. K., 2008).

Effective control of mycotoxigenic fungi traditionally has been through the use of chemical fungicides, modifications in cultural practices, and development of resistant cultivars. Currently the global trend is turned to safer and eco-friendly alternative approaches. Among them, biological control appears to be the most promising approach for control of mycotoxin in both pre- and post-harvested crops. Different organisms, including bacterial and fungal antagonists of mycotoxigenic fungi have been investigated, with the goal of developing other biocontrol agents for mycotoxin reduction. Several bacterial species, such as *Bacillus subtilis*, *Lactobacilli sp., Pseudomonas sp., Ralstonia sp*. and *Burkholderia sp.*, have shown the ability to inhibit fungal growth and production of aflatoxins by *Aspergillus sp*. (Haggag, W., El Habbasha, E. F., & Mekhail, M., 2014; Nesci, A. V., Bluma, R. V., & Etcheverry, M. G., 2005; Palumbo, J. D., Baker, J. L., & Mahoney, N. E., 2006; Siahmoshteh, F., Siciliano, I., Banani, H., Hamidi-Esfahani, Z., Razzaghi-Abyaneh, M., Gullino, M. L., & Spadaro D., 2017; Yin, Y., Yan, L., Jiang, J., & Ma, Z., 2008). Culture filtrates of biocontrol agents, such as *Trichoderma sp*., a strain of *B. subtilis*, and *Rhodococcus erythropolis*, different strains of *Pseudomonas fluorescens*, showed considerable

biocontrol activities against *A. flavus* and limited the production of aflatoxins (Mannaa, M., & Kim, K. D., 2016). Choudhary, D. K., Verma, S. K., Patel, A. K., & Dayaram (2014) reported that *Bacillus* bacteria inhibited *Fusarium oxysporum* and *Alternaria* species.

Bacillus subtilis TS 01, isolated from soil, showed broad-spectrum antagonism against potent fungal phytopathogens some of them belonging to the genera *Fuzarium* and *Alternaria* (Todorova, S., & Kozhuharova, L., 2010). The object of this work is to determine the degree of the inhibitory effect of *B. subtilis* TS 01 on some mycotoxigenic fungi and to investigate *in vitro* its biological activity on the mycelial growth and spore germination of fungi.

EXPOSITION

Materials and methods

B. subtilis TS 01 was isolated from Bulgarian soil, identified and selected in our previous studies as antagonist against a large number of fungal and bacterial phytopathogens (Todorova, S., & Kozhuharova, L., 2010). The strain is registered in the Bulgarian National Bank for Industrial Microorganisms and Cell Cultures (No 8718/16.05.2011). *B. subtilis* TS 01 was stored on potato-dextrose agar (PDA) at 0-4°C. In experiments the culture was grown on a №3 medium containing [g l^{-1}]: glucose 5, polypepton 10, KH_2PO_4 1, $MgSO_4.7H_2O$ 0,5 (pH 7). The strain was cultivated in 500 cm^3 Erlenmeyer flasks with 50 cm^3 medium on a rotary shaker at 220 min^{-1} and 28°C for 72 h. The inoculation was performed with 2 % (v/v) 18 h old vegetative inoculum.

Fusarium graminearum, F. culmorum, *F. moniliforme, Alternaria sp., Aspergillus niger, A. fumigatus, Penicillium sp., P. scopolariopsis,* were selected to analyze inhibitory activity of the bacterial strain. The fungi were stored on PDA at 0-4°C.

Determination of the suppressive effect of *B. subtilis* TS 01 on mycelial growth

A sterile metallic ring (d=7 mm) was pressed lightly into PDA 12 mm from the edge of a petri dish (d=90 mm). 10 µl of *B. subtilis* TS 01 culture broth ($1x10^{10}$ kfu ml^{-1}) was dropped in the ring. In control plates 10 µl of sterile № 3 medium was placed in the metallic ring. A mycelial plug (d=7 mm) of an actively growing culture of corresponding fungus on PDA was placed halfway (35 mm) between the bacterium and the opposite side of the petri dish. The experiments were with 3 replicas. Both experimental and control dishes arranged in a completely random desing were incubated at 26°C while mycelial growth reached the edge of the petri dish (Ferreira, J., Matthee, F., & Thomas, A., 1990). The inhibition was calculated as a percentage by the formula:

$$\text{Inhibition ratio } (\%) = \frac{C - E}{C} x100 \qquad (1)$$

where, C is the distance between mycelial plug and metallic ring with bacterial culture, mm;
E is the distance with mycelial growth toward the bacterium, mm.

Investigation *in vitro* of the effect of *B. subtilis* TS 01 on the hyphal morphology

The fungi were previously cultivated on PDA in petri dishes at 26°C for 7 – 10 days. Plugs (d=7 mm) from actively growing cultures of fungi were transferred to the center of petri dishes, containing 15 cm^3 Czapek-Dox broth (CDB). The plates were incubated at 26°C. When mycelial growth on the surface of the liquid reached a diameter of about 10 mm, a loopful from *B. subtilis* TS 01 culture broth was transferred to a dish. Cultures in CDB without *B. subtilis* TS 01 served as controls. The experiments were with 3 replicas for each fungus and both experimental and control dishes arranged in a completely random desing were incubated again at 26°C for 3 days. Hyphal strands at the edge of the fungal colonies were examined under a light microscope “Olympus” for abnormalities.

Investigation *in vitro* of the effect of *B. subtilis TS 01* on the conidial germination

Three wells (d=7 mm) on PDA in petri dish were made at 30 mm – distance each of other. Into each well 50 µl from *B. subtilis* TS 01 culture broth was pipetted. In control plates was dropped 50 µl of sterile № 3 medium only into each well. Fungi were cultivated on PDA slants in tubes at 26°C for 7 – 10 days. Spore suspension of each fungus was prepared with 5 cm^3 sterile distilled water. The suspensions were filtered through cotton and contained about $1x10^5$ spores/cm^3). Three drops (approximately 30 µl) of each fungus suspension were pipetted around each well and spread in a radius of 15 mm with a sterile glass rod. The experiment was repeated and all dishes (experimental and control) arranged in a completely random design were incubated at 26°C for 24 h. Fungal conidia occurring within 10 mm around each well were examined under a microscope for germination in presence and in absence of *B. subtilis* TS 01. The spore was considered to be germinated when the germ tube length was a half of the length of the spore (Ferreira, J., Matthee, F., & Thomas, A., 1990).

RESULTS AND DISCUSSION

B. subtilis TS 01 significantly inhibited mycelial growth of fungi on PDA. The inhibitory effect was observed during a month at various intervals of time. The inhibitory activities were measured after 3 and 14 days and the results were shown in Fig. 1.

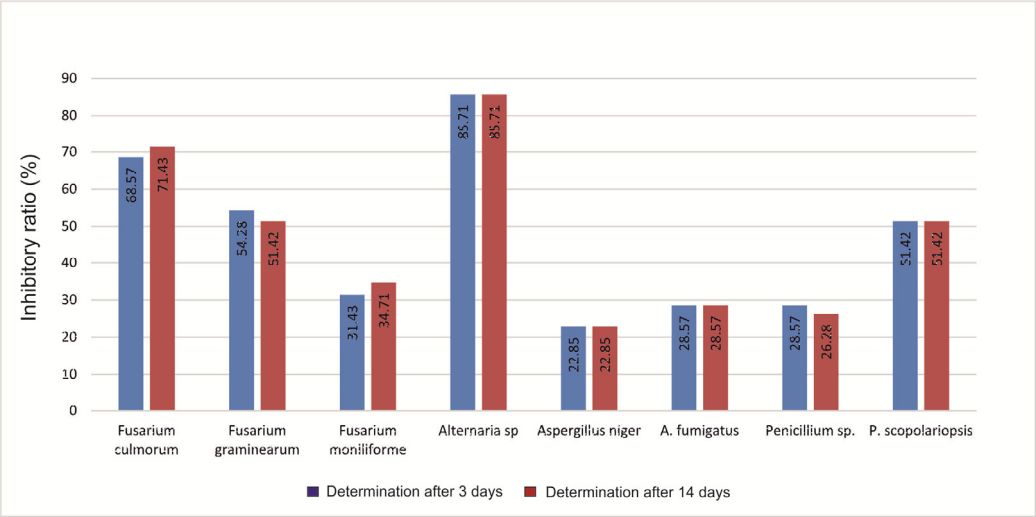


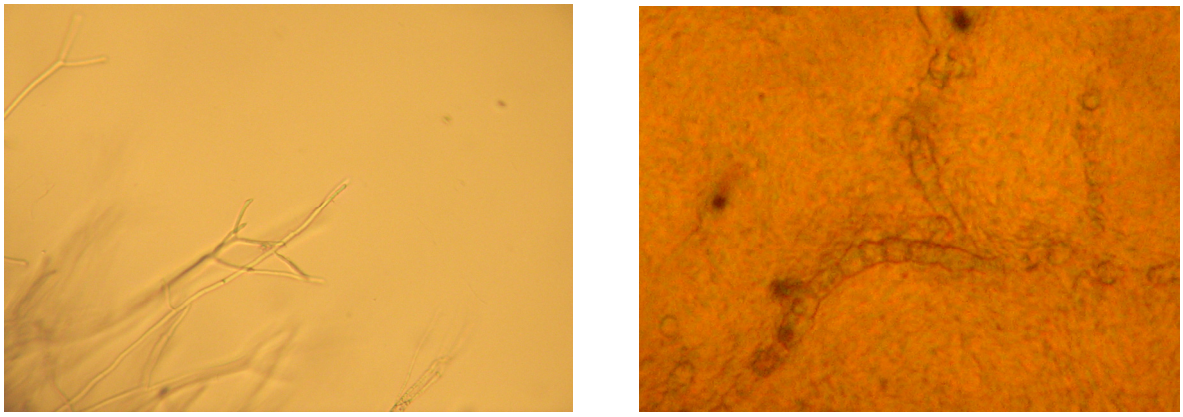
Fig. 1. Inhibition of mycelial growth of some mycotoxigenic fungi by culture broth of *B. subtilis* TS 01

The inhibitory ratio of *B. subtilis* TS 01 culture medium was significantly over 50 % at half of tested fungi. The inhibitory percentage after 3 days was highest by *Alternaria sp.* – 85,71 % and *F. culmorum* - 68,57 %. By *F. graminearum* it was 54,28 % and by *P. scopolariopsis* - 51,42 %.

In published literature, other strains of *B. subtilis* have been reported to show the ability to inhibit fungal growth and production of aflatoxins by *Aspergillus sp.* (Haggag, W., El Habbasha, E. F., & Mekhail, M., 2014; Nesci, A. V., Bluma, R. V., & Etcheverry, M. G., 2005; Palumbo, J. D., Baker, J. L., & Mahoney, N. E., 2006; Siahmoshteh, F., Siciliano, I., Banani, H., Hamidi-Esfahani, Z., Razzaghi-Abyaneh, M., Gullino, M. L., & Spadaro D., 2017; Yin, Y., Yan, L., Jiang, J., & Ma, Z., 2008). In our study *B. subtilis* TS 01 manifests less pronounced inhibitory action against molds *A. niger* - 22,85 %, *A. fumigatus* and *Penicillium sp.* - 28,57 %. There is little inhibition of mycelial growth of molds *A. niger* and *Penicillium sp.* but spore formation is significaltly suppressed.

The inhibitory effect of *B. subtilis* TS 01 was stable and kept in the same values by all fungi. The percentage of inhibition on *F. culmorum* and *F. moniliforme* after 14 days was even increased to 71,43 % and 34,71 % respectively. There was a faintly resumption in fungal growth only by *F. graminearum* and *Penicillium sp.* and the percentage was decreased to 51,42 % and 26,28 % respectively.

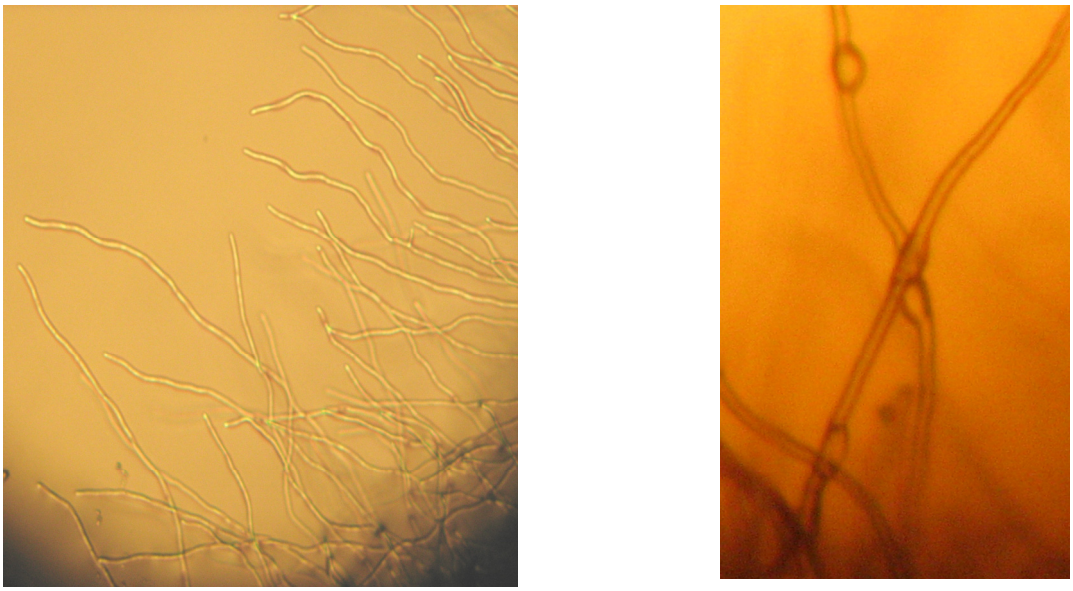
The effect of *B. subtilis* TS 01 on the hyphal morphology is shown in Fig. 2.



I II

Fig. 2. Hyphae of *F. moniliforme*: I – control – in absence of *B. subtilis* TS 01; II – in presence of *B. subtilis* TS 01

It was observed that the hyphae of fungi growing in the absence of the bacterial strain did not show any growth aberrations. The normal hyphae (Fig. 2-I) were thin with smooth hyphal walls and no swelling and vacuolation in the protoplasm were observed in the control dishes. After introduction of *B. subtilis* TS 01 to the culture broth of fungi however, abnormal hyphae obviously distinguishing themselvs from ones of the controls were observed (Fig. 2-II). Hyphal tips of the fungi become malformed. The hyphae were thickened. Many swellings and vacuoles occurred in the hyphae by the presence of *B. subtilis* TS 01 (Fig. 3). In some instances, extensive degradation of huphae were also observed. Shorter and more nodes in fungal mycelium were formed when the mycotoxigenic fungi and bacterium were incubated simultaneously.



I II

Fig. 3. Hyphae of *A. niger*: I – control – in absence of *B. subtilis* TS 01; II – in presence of *B. subtilis* TS 01

It was observed that germination of fungal spores was hindered profoundly by the *B. subtilis* TS 01. In the presence of the bacterium for 24 h conidia were swollen but did not germinate (Fig. 4-II). In most of them vacuoles were visible. In untreated control spores a germination of 80 – 100 % was observed (Fig. 4-I).

The strain strongly inhibited the germination of conidia of *Alternaria sp*. - germination of 0 % (Table 1). Germination of conidia under 50 % was observed by *F. graminearum* – 21,6 %, *A. niger* – 22 %, *F. culmorum* – 33,36 %.

B. subtilis TS 01 synthesizes a complex of antibiotic substances with antifungal and antibacterial activity. Mycelial malformatios and the vacuolar appearance of the mycelium and spores that occurred in this study probably was due to the antibiotic substances produced by the bacterium. The failure of conidia of fungi to germinate after 24-h exposure to *B. subtilis* TS 01 indicated that the antifungal substances produced by this bacterial strain is not only fungistatic but also fungicidal to spores of the fungi.

Table 1. Effect of *B. subtilis* TS 01 on germination of conidia of some mycotoxigenic fungi

№	Fungi	Germinated conidia (%)	
		Controls	**In the presence of *B. subtilis* TS 01**
1	*Fusarium culmorum*	100	33,36
2	*F. graminearum*	99,4	21,6
3	*F. moniliforme*	82	70
4	*Alternaria sp.*	82	0
5	*Aspergillus niger*	100	22
6	*A. fumigatus*	94	78
7	*Penicillium sp.*	92	50
8	*P. scopolariopsis*	100	90

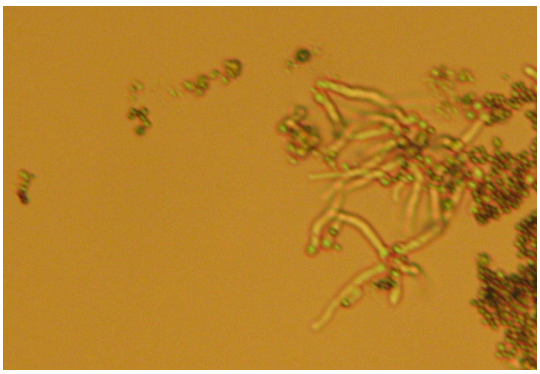
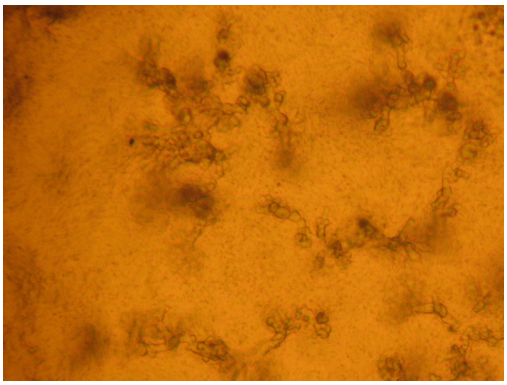
I  II 

Fig. 4. Conidia of *A. fumigatus*: I – control – in absence of *B. subtilis* TS 01; II – in presence of *B. subtilis* TS 01

CONCLUSION

B. subtilis TS 01 is strongly suppressing *in vitro* the development of some mycotoxigenic fungi. The strain inhibits germination of the spores and growth of hyphae and provokes degenerative variations in the structure.

B. subtilis TS 01 is appropriate as a biocontrol agent agains mycotoxigenic fungi which pose serious phytopathological and mycotoxicological risks at preharvest and postharvest stages, as well as in processed food products.

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