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IN VITRO STUDY OF THE EFFECT OF EPIPHYTIC BACTERIA ON THE MYCELIUM GROWTH OF MOLDS ISOLATED FROM WHEAT SEEDS

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Abstract

Epiphytic microorganisms – bacteria (six strains) and molds (twenty-three strains) were isolated from wheat seeds and were co-cultured in order to estimate the bacterial effect on mold growth. The *in vitro* tests were done on malt agar media and after cultivation the size of mycelium was measured. The results revealed that the bacteria had inhibitory, stimulatory or no effect on the mold mycelium growth. The broadest inhibitory range showed the strain *Paenibacillus xylanexedens* which suppressed the growth of fourteen epiphytic mold strains isolated from wheat including the strain of *Fusarium oxisporum* and had an even more pronounced effect on the strain of *Fusarium tricinctum*. The other strain from the same genus - *Paenibacillus tundrae* exibitited activity against thirteen strains, stimulated the growth of eight strains and had no effect on the growth of two of the mold species. The other examined bacterial strains - *Micrococcus luteus*, *Brachybacterium alimentarium* and *Janibacter anophelis/ hoylei* had narrower range of inhibitory activity and suppressed the development of twelve, ten and eight mold strains respectively. The inhibitory activity of bacterial strains against some important phytopathogenic, mycotoxigenic and food born fungi makes them perspective bioactive agents with a variety of practical applications and more information for their properties warrant a further research.

Keywords: epiphytic bacteria, epiphytic molds, mycelium growth, biological activity.

INTRODUCTION

Wheat production in Bulgaria for the year 2022 is projected to reach up to 5.9 million metric tons (MMT) (Foreign Agriculture service, 2021) and with its annual yield Bulgaria ranks in 25th position in terms of production in the world (World population review - 2022). Wheat seeds transmit abundant microbial populations with diverse impact not only on the seed properties, but subsequently also on plant development and yield (Kuzniar *et al*., 2020; Johnston-Monje *et al.*, 2021). According to (Syed Ab Rahman *et al.*, 2018) phytopathogenic microorganisms and storage conditions are responsible for 25 – 50 % of the global food production loss during the post-harvest period. However, (Savary *et al.*, 2012) explained that most of these estimations inadequately reflect the true costs of crop losses because losses affect farmers, consumers and environment in much broader scale with both short and long-term consequences.

Epiphytic bacteria can synthesise enzymes that break down polymer molecule such as pectin, cellulose, hemicellulose in the seed coat and as a result they facilitate the development of other undesirable microorganisms, including molds. Development of mold fungi on seeds resulted in reduced seed germination or caused systemic or local infections (Magan *et al.*, 2004). The excessive growth of molds on the seeds is associated not only with a decrease in yield and deterioration of the technological qualities of the grain, but also with the production of mycotoxins which are harmful to human and animal health (Magan *et al.*, 2003, Gashgary *et*

al., 2019). The microflora of the wheat seed is comprised of mold species such as *Alternaria alternata*, *Drechslera sorokiniana*, *Fusarium moniliforme*, *F. avenaceum*, *F. graminearum*, *F. nivale*, *F. culmorum*, *F. equiseti*, *F. sporotirchioides*, *Cladosporium herbarum*, *Stemphylium botryosum* (Pathak & Zaidi, 2013, Senbeta & Gure, 2014). During the storage of wheat, various microorganisms can be isolated, including those which are the main reason for the deterioration of the seed quality. Some of the mold species came from the soil, some other from a plant or the field. A typical example of the field fungi are the species belonging to genus *Alternaria* and *Fusarium*. Molds from genera *Aspergillus* and *Penicillium* predominate in the group of storage fungi (Hocking, 2003, Scussel *et al*., 2016)

The pesticides are the chemicals commonly used for control of plant diseases, weeds and pests. The improper use of pesticides has a significant negative impact on environment and there is a need for development of alternative strategies. A good alternative to overuse of chemicals is the application of microorganisms as biocontrol agents (Khanzada *et al.*, 2002, Droby *et al.*, 2016, Wisnievski *et al*., 2016). The antimicrobial activity of bacteria against some phytopathogens affects the growth of spores and the development of fungal mycelium by producing organic acids, lipoproteins and enzymes (Nourozian *et al.*, 2006, Lahlati *et al.*, 2022). The antagonistic activity due to synthesis of bacteriocins of bacterial strains and their application was extendedly reviewed by Juturu & Wu (2018).

The aim of the present study was to study the effect of bacteria on mold mycelium growth with particular interest on estimation of the perceived inhibitory effect.

MATERIALS AND METHODS

All the experimental work was done in the laboratory of the Department of microbiology and ecological biotechnologies, Agricultural University – Plovdiv. The epiphytic microflora was isolated from five wheat varieties – *Avenu*; *Apash*; *Andino*; *Enola* and *Sadovo1*. The identification of fungal (twenty-three strains) and bacterial strains (six strains) (data not presented) was based on their morphological characteristics and carried out with *Biolog Microbial Identification System* (Biolog, Inc., Hayward, Calif., USA). The strains of microorganisms used in this study are listed in Table 1.

Table 1. List of bacterial and mold strains used in the study.

Molds strains	
Fusarium sporotrichioides	*Aspergillus tereus*
Fusarium lateritium	*Aspergillus flavus*
Fusarium subglutinans	*Aspergillus carbonarius*
Fusarium oxysporum	*Aspergillus parasiticus*
Fusarium tricinctum	*Penicillium aethiopicum*
Bionectria sesquicilli	*Alternaria alternata*
Fusarium coccicicola	*Curvularia lunata*
Penicillium canescens	*Scopulariopsis candida*
Penicillium thomii	**Bacterial strains**
Penicillium brevicompactum	*Paenibacillus xylanexedens*
Penicillium griseofulvum1	*Paenibacillus tundrae*
Penicillium griseofulvum2	*Micrococcus luteus*
Colletotrichum truncatum	*Janibacter anophelis/ hoylei*
Aspergillus phoenicis	*Brachybacterium alimentarium*
Aspergillus ostianus	

The biological activity was tested *in vitro* according to the method of Mushtaq *et al.*, (2010). The experimental procedure, with slight modifications, is presented briefly. A dense bacterial suspension was obtained from nutrient broth (Biolife, Italy) culture after 24 hours cultivation at 25 ± 2 °C by centrifugation at 7000 rpm for 15 min at 4ºC. The cell pellet was diluted with physiological solution (0.9 gL^{-1} NaCl) in order to obtain the final concentration of 10^6 mL^{-1} cells according to MacFarland scale. Mold strains were cultivated on malt extract agar (Biolife, Italy) for 7 days at 25 ± 2 °C. A quantity of 100 µl of bacterial suspension was spread on the surface of the plate with malt extract agar and a disc with a diameter of 4 mm was cut from the mold mycelium and placed in the center of petri dish (d = 90 mm). Inoculated petri dishes each with one bacterial and one mold strain were incubated at 25 ± 2 °C for five days. Control dishes with mold discs were also prepared.

The diameter (cm) of the mold mycelium in the control and experimental plates was measured with electronic caliper. The inhibitory effect of the bacteria was calculated according to the following equation:

$$I = \left(1 - \left(\frac{G}{G_o}\right)\right) \times 100$$

Where: I – inhibition (%); G – mycelium size in the presence of bacteria, (cm); G_o – mycelium size in the control plates (cm).

Statistical Analysis

The experiment was carried out in triplicate and the calculation of mean values of mycelium development ± standard error and inhibition (%) was done in Excel.

RESULTS AND DISCUSSION

The broadest inhibitory effect showed the strain *Paenibacillus xylanexedens* which suppressed the growth of fourteen epiphytic mold strains isolated from the wheat and had a pronounced effect on the strain of *Fusarium tricinctum*. However, it was not active against eight of the tested mold species and among these there were some important mycotoxigenic species such as *Aspergillus flavus*. The second strain from the same genus - *Paenibacillus tundrae* exhibited activity against thirteen strains, stimulated the growth of eight strains and had no effect on two of the mold species. The other bacterial strains included in the study - *Micrococcus luteus, Brachybacterium alimentarium* and *Janibacter anophelis/ hoylei* showed narrow inhibitory activity and suppressed the development of twelve, ten and eight of the mold species respectively.

The results revealed that all bacterial strains suppressed the growth of *Fusarium sporotrichioides, Fusarium lateritium, Fusarium coccicicola, Penicillium griseofulvum1* and *Scopulariopsis candida* and have no effect or stimulated the growth of *Aspergillus parasiticus, Penicillium griseofulvum2, Aspergillus tereus* and *Curvularia lunata* (Table 2). In contast to the generally observed stimulating effect on *Fusarium oxysporum, Paenibacillus xylanexedens* has a significant inhibitory effect on it. The other *Paenibacillus* strain - *Paenibacillus tundrae* was also the only one bacterial strain which has a negative effect on *Penicillium aethiopicum. Paenibacillus tundrae,* similarly to the activity of *Micrococcus luteus,* restrained the growth of *Aspergillus flavus*. Each of the strains of genus *Paenibacillus* was also able to suppress the growth of *Fusarium tricinctum, Aspergillus carbonarius, Penicillium canescens, Penicillium griseofulvum1* and *Alternaria alternata*. Most of the bacterial strains restrained the growth of *Bionectria sesquicilli, Aspergillus ostianus, Penicillium thomii, Penicillium brevicompactum* and *Colletotrichum truncatum*. *Brachybacterium alimentarium* strain was the only one which suppressed the development of *Fusarium subglutinans*.

Table 2. Mycelium growth (in cm) of epiphytic molds on control malt agar plates and plates inoculated with bacterial strains (the values are represented as means ± standard error)

Molds	Mycelium growth in the control and in the presence of bacterial strains, (in cm)					
	Control	Bacterial strains				
		Paenibacillus tundrae	***Paenibacillus xylanexedens***	***Micrococcus luteus***	***Janibacter hoylei***	***Brachybacterium alimentarium***
Fusarium sporotrichioides	3,75 ± 0,02	2,82 ± 0,03	0,77 ± 0,04	3,35 ± 0,18	3,17 ± 0,12	3,12 ± 0,11
Fusarium lateritium	1,85 ± 0,02	1,57 ± 0,07	1,00 ± 0,14	1,68 ± 0,09	1,62 ± 0,08	1,38 ± 0,10
Fusarium subglutinans	4,00 ± 0,00	4,00 ± 0,06	7,03 ± 0,06	4,07 ± 0,11	4,14 ± 0,14	3,97 ± 0,12
Fusarium oxysporum	6,90 ± 0,04	7,18 ± 0,08	5,98 ± 0,13	7,13 ± 0,15	7,17 ± 0,14	7,02 ± 0,19
Fusarium tricinctum	7,85± 0,07	7,35 ± 0,20	0,68 ± 0,03	6,70 ± 0,18	7,97 ± 0,02	7,98 ± 0,02
Bionectria sesquicilli	5,60± 0,00	5,60 ± 0,08	5,00 ± 0,27	5,75 ± 0,08	5,43 ± 0,07	5,48 ± 0,06
Fusarium coccicicola	7,00± 0,04	5,37 ± 0,14	4,57 ± 0,19	5,98 ± 0,14	6,23 ± 0,11	5,52 ± 0,09
Aspergillus phoenicis	2,00 ± 0,04	2,07 ± 0,14	2,23 ± 0,07	1,97 ± 0,11	2,75 ± 0,07	3,52 ± 0,16
Aspergillus ostianus	1,90 ± 0,00	2,07 ± 0,06	0,47 ± 0,03	1,15 ± 0,08	1,95 ± 0,07	1,65 ± 0,13
Aspergillus tereus	2,15 ± 0,02	3,92 ± 0,12	6,97 ± 0,07	3,22 ± 0,09	3,50 ± 0,06	3,62 ± 0,08
Aspergillus flavus	2,15± 0,02	2,10 ± 0,15	7,02 ± 0,11	1,95 ± 0,06	2,87 ± 0,05	3,58 ± 0,14
Aspergillus carbonarius	2,50 ± 0,00	2,15 ± 0,07	2,12 ± 0,06	2,55 ± 0,02	2,85 ± 0,19	2,57 ± 0,12
Aspergillus parasiticus	1,95 ± 0,07	2,12 ± 0,05	2,75 ± 0,04	2,05 ± 0,02	2,82 ± 0,12	2,93 ± 0,07
Penicillium canescens	1,85 ± 0,02	1,37 ± 0,10	1,48 ± 0,07	2,03 ± 0,11	2,13 ± 0,07	2,27 ± 0,10
Penicillium thomii	2,35 ± 0,07	2,40 ± 0,13	2,03 ± 0,11	2,05 ± 0,06	2,22 ± 0,06	2,25 ± 0,08
Penicillium brevicompactum	4,10 ± 0,04	4,03 ± 0,07	4,97 ± 0,06	4,03 ± 0,12	3,98 ± 0,16	4,02 ± 0,14
Penicillium griseofulvum1	3,80 ± 0,00	2,93 ± 0,18	1,92 ± 0,05	3,65 ± 0,04	3,57 ± 0,04	1,57 ± 0,11
Penicillium griseofulvum2	2,05 ± 0,02	2,12 ± 0,23	6,98 ± 0,08	3,12 ± 0,07	2,57 ± 0,11	2,43 ± 0,08

Penicillium aethiopicum	1,85 ± 0,02	1,42 ± 0,14	2,98 ± 0,15	2,03 ± 0,06	2,15 ± 0,06	2,27 ± 0,06
Alternaria alternata	3,95 ± 0,02	2,07 ± 0,15	2,73 ± 0,09	3,97 ± 0,04	4,10 ± 0,05	4,48 ± 0,10
Curvularia lunata	4,05 ± 0,02	4,23 p ± 0,09	4,10 ± 0,07	4,18 ± 0,07	4,05 ± 0,05	4,23 ± 0,10
Scopulariopsis candida	4,00 ± 0,00	2,00 ± 0,11	1,22 ± 0,06	1,15 ± 0,04	1,12 ± 0,05	1,52 ± 0,11
Colletotrichum truncatum	7,90 ± 0,04	7,00 ± 0,23	7,77 ± 0,12	7,03 ± 0,10	7,93 ± 0,05	7,95 ± 0,03

Depending on the observed inhibition on the mycelium growth the degree of the bacterial effect was categorized in three groups as follow: 1) weak effect – when the inhibition was between 2 and 25%; 2) moderate effect - from 25 to 50%, and 3) strong effect – when inhibition on the mycelium growth was over 50%. The most varying and diverse were the estimated values of inhibition presented in the weak inhibitory effect group (Fig. 1). The lowest values of 2 – 6 % of inhibition were obtained for *Penibacillus tundrae* against *Aspergillus flavus* and *Penicillium brevicompactum*. *Micrococcus luteus* also showed only 2 % inhibition against *Aspergillus phoenicis* and *Penicillium brevicompactum* and slightly more on *Penicillium griseofulvum - 1* – 4 %. Similarly, *Janibacter hoylei* showed only 3 % activity against *Bionectria sesquicilli* and *Penicillium thomii*, but its activity reached 6% on *Penicillium griseofulvum1*. In the same group with a higher value of inhibition (23 %) it has to be mentioned *Penibacillus tundrae* which suppressed the mycelium growth of *Fusarium coccicicola, Penicillium griseofulvum1 and Penicillium aethiopicum*. The highest level of inhibition in the weak effect group was observed for *Brachibacterium alimentarium* against *Fusarium lateritium* (25 %).

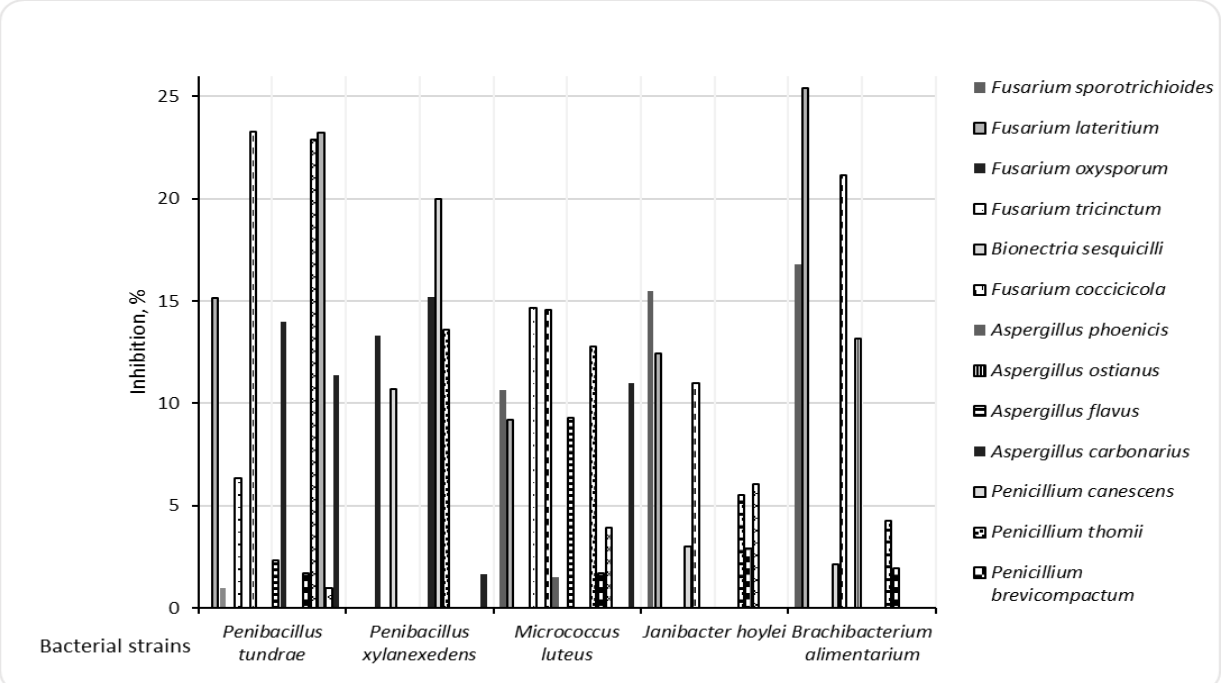

Figure 1. Bacterial strains which exhibited a weak (2 – 25 %) inhibitory effect on the mycelium growth of molds strains

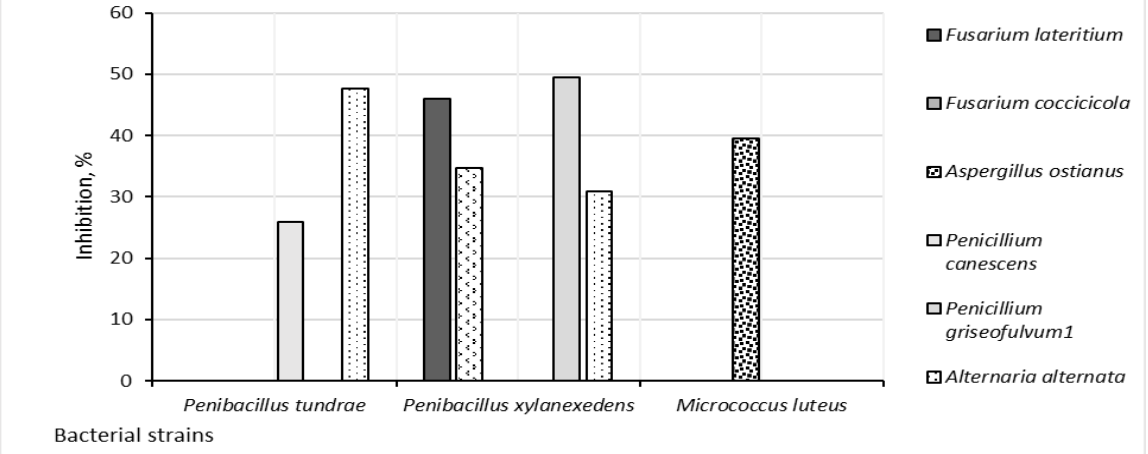

Figure 2. Bacterial strains which exhibited a moderate (26 – 50 %) inhibitory effect on the mycelium growth of molds strains

The most compact and unvarying group (Fig. 2) was the moderate inhibition effect group (from 25 to 50%). In this group with the highest values of inhibition (49 %) on the mycelium growth and also against more numbers of mold species (four species) it has to be mentioned *Penibacillus xylanexedens.* However, the inhibitory effect of *Penibacillus tundrae* on the mycelium growth of *Alternaria alternata* has reached 48 % and *Micrococcus luteus* suppressed *Aspergillus ostanius* with 39 %. It seemed that none of the estimated values for inhibitory activity of *Janibacter anophelis/ hoylei* and *Brachybacterium alimentarium* towards mold growth complied with the specified value range for the moderate inhibitory group and they had to be omitted from the graph.

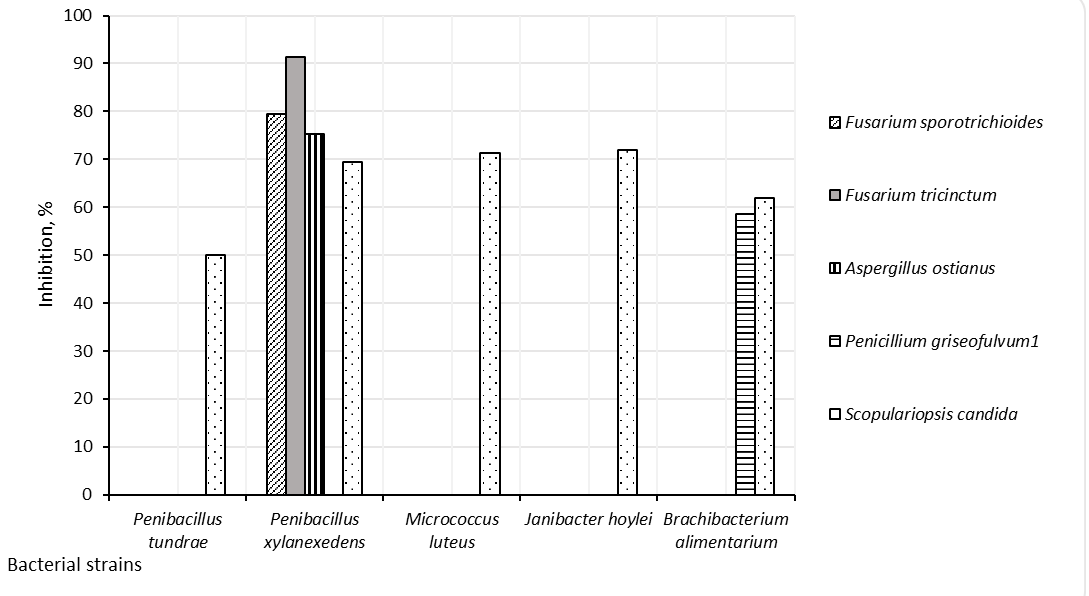

Figure 3. Bacterial strains which exhibited a strong (more than 50 %) inhibitory effect on the mycelium growth of molds strains

In the strong inhibitory effect group (Fig. 3) *Penibacillus xylanexedens* remained the bacterial strain which showed the highest values of inhibition (70 – 91 %) against numbers of molds such as *Fusarium sporotrichioides*, *Fusarium tricinctum*, *Aspergillus ostianus*, and *Scopulariopsis candida* and was also active against *Penicillium griseofulvum*, but the value

of 49 % had placed the effect in the moderate group. The inhibitory activity of other bacterial strains did not exceed 72 %. All of bacterial strains were invariably active only against *Scopulariopsis candida* and only *Brachibacterium alimentarium* showed also inhibitory activity of 59% against *Penicillium griseofulvum1*.

According to He et al. (2008) and Naing *et al.* (2014) bacterial stain from genus *Paenibacillus* showed good biological activity against fungal phytopathogens. The bacteria could cause structural deformations and lysis of hyphae, could reduce the number of sporangia and prolong the period of mold spore formation and mycotoxin production. A number of authors suggested that the inhibitory effect of bacteria of the genus *Paenibacillus* is due to the synthesis of antifungal metabolites, antibiotics and enzymes that degrade chitin in fungal cells (Raaijmakers *et al.*, 2002, Grady *et al.*, 2016, Ren *et al.*, 2020). *Micrococcus luteus* also has a pronounced inhibitory effect on the level of development of some economically important species of molds. The data from the current study are in agreement with those of Aruwa *et al.*, (2016), who found that the strain of *Micrococcus luteus* was active against several species belonging to the genus *Colletotrichum*, but also against *Fusarium oxysporum*. In this study, *Micrococcus luteus* strain showed an inhibitory effect against *Colletotrichum truncatum*.

CONCLUSION

The effective use of bacteria as biocontrol agents requires their preliminary isolation, identification and suitable characterization of biological properties (Solanki *et al*., 2021). The most suitable for application as bioagents are considered microorganisms which are isolated from local crops and their intended use follows the regional principle. The argumentation for such approach is based on the observations that such strains are well adapted to the environment, they can easily survive and as a result they possess and retain better antimicrobial activity (Campbell, 1994). In general, the antimicrobial activity of bacteria is associated with the synthesis of secondary metabolites such as organic and fatty acids, bacteriocins (Lahlati *et al*., 2022).

The strains of *Paenibacillus tundrae*, *Paenibacillus xylanexedens* and *Micrococcus luteus* from the current study showed inhibitory effect on the development of fungal mycelium of molds of the genus *Aspergillus* sp., *Alternaria* sp., *Colletotrichum* sp., *Penicillium* sp., *Fusarium* sp., and *Scopulariopsis* sp. One of the most important features of the studied strains was their broad inhibitory activity against the isolated epiphytic molds. However, their effects on molds were relatively diverse and could be strain-to-strain dependent and relatively species limited. Further work with bacterial strains could reveal the mechanisms of their antifungal activity and the possible practical application in their use as biocontrol agents.

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