IN VITRO INHIBITORY ACTIVITY OF CELL-FREE SUPERNATANTS OF *LACTOBACILLUS* SPP. AND *BACILLUS* SPP. AGAINST *FUSARIUM GRAMINEARUM*

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ABSTRACT

In this study, the antifungal activity of cell-free supernatant (CFS) of *Lactobacillus* spp. (*Lactobacillus plantarum* CCM 1904; L81, *Lactobacillus fermentum*; 2I3, *Lactobacillus reuteri*; 2/6, L26;) and *Bacillus* spp. (*Bacillus subtilis* CCM 2794, *Bacillus licheniformis* CCM 2206) against two strains of *Fusarium graminearum* CCM F-683 and *Fusarium graminearum* CCM 8244 were investigated *in vitro*. All tested CFS of *Lactobacillus* spp. were able to inhibit the growth of both strains of *Fusarium graminearum*. The highest inhibitory effect (IE) (56.5 %) against *F. graminearum* CCM F-683 was observed for CFS *Lactobacillus fermentum* (2I3) at the minimum inhibitory concentration (MIC) ($2.25 \pm 0.56 \text{ mg.ml}^{-1}$). CFS of *Lactobacillus reuteri* (2/6) showed the best IE (40.0 %) against *F. graminearum* CCM 8244 (2/6) at the MIC 1.25 mg.ml^{-1} . However, no inhibitory effect of *Bacillus subtilis* and *Bacillus licheniformis* CFS against both strains of *F. graminearum* were observed, even at the highest tested concentration of 5.0 mg.ml^{-1} .

Key words: *Bacillus* spp.; biological protection; inhibitory effect; *Lactobacillus* spp.; microscopic filamentous fungi

INTRODUCTION

Fusarium graminearum is one of the most important fungal pathogens of cereal grains (Fig. 1). It is the causal agent of *Fusarium* head blight (FHB) in wheat and barley [15]. FHB or scab is a plant disease of economically significant importance worldwide [9]. *F. graminearum* causes direct damage, poor quality of grains and losses of crop and contamination of the raw grains and processed wheat products with mycotoxins [25].

The most important mycotoxin produced by *Fusarium graminearum* is deoxynivalenol (DON, vomitoxin) and its acetylated derivatives. The presence of deoxynivalenol, in a host-pathogen interaction, can be a virulence factor in *Fusarium graminearum* wheat infection [21]. In addition, deoxynivalenol and other *Fusarium* secondary metabolites

Fig. 1. Sporulation of *Fusarium graminearum*, which is manifested by orange colouration (own figure)

(zearalenone, fumonisins and other) are very toxic compounds for animals and humans and can cause the so-called mycotoxicosis [17]. Mycotoxicosis are manifested as a variety of clinical signs that depend on factors such as: type and concentrations of mycotoxins; duration of exposure; species; genus; age and health status of the individual [11].

Nowadays, there are various options to eliminate the growth of *Fusarium graminearum*. In addition to chemical antifungals, adsorbents, antioxidants and biologically active substances are used [22]. According to Kokonen [12], lactic acid bacteria are involved in the biological adsorption of mycotoxins and prevent their absorption from the digestive tract into the blood of humans and animals. The ability of the genus *Lactobacillus* to suppress the growth of *Fusarium* microscopic filamentous fungi has been reported [5]. Bacterial endophytes, *Bacillus subtilis* and *Bacillus licheniformis* are biological tools to protect plants from phytopathogens. They can enter and colonize plants without causing any signs of plant disease. Endophytes penetrate plants from the soil and roots and subsequently spread into leaves, flowers and fruits [2]. *Bacillus* spp. can protect plant hosts by producing many antimicrobials molecules and induced systemic resistance [23]. The strains of the genus *Lactobacillus* and *Bacillus* can be used for the formulation of cell-free supernatants (CFS). CFS are mixtures derived from broth cultures. The separation and removal of cells can be performed using several physical and mechanical processes. CFS can be obtained through two

main operations, centrifugation and filtration, or in combination with other technologies according to the desired final product [3, 19]. Many studies of CFS deal with their utilization in medical and food sectors. The studies on the biostimulant and biocontrol properties of CFS in plants are limited to *in vitro* tests [19].

The aim of this study was to determine the inhibitory effect of CFS of various species of the genera *Lactobacillus* and *Bacillus subtilis* and *Bacillus licheniformis*.

MATERIALS AND METHODS

Tested strains

In the experiment, 9 strains of microorganisms (Table 1) were used. The strains of *F. graminearum* CCM F-683, *F. graminearum* CCM 8244, *Lactobacillus plantarum* CCM 1904, *Bacillus subtilis* CCM 2794 and *Bacillus licheniformis* CCM 2206 were obtained from the Czech Collection of Microorganisms (Masaryk University, Brno, Czech Republic). Other strains of the genus *Lactobacillus* were provided by the Department of Microbiology and Immunology of the University of Veterinary Medicine and Pharmacy in Košice.

Table 1. The tested strains

Species	Number
<i>Fusarium graminearum</i>	CCM F-683
<i>Fusarium graminearum</i>	CCM 8244
<i>Bacillus subtilis</i>	CCM 2794
<i>Bacillus licheniformis</i>	CCM 2206
<i>Lactobacillus plantarum</i>	CCM 1904
<i>Lactobacillus fermentum</i>	213
<i>Lactobacillus plantarum</i>	L81
<i>Lactobacillus reuteri</i>	2/6
<i>Lactobacillus reuteri</i>	L26

Preparation of fungal inoculum

The inoculum suspension was prepared from 10-day old culture of *F. graminearum* (CCM F-683 and CCM 8244) grown on potato dextrose agar (PDA, HiMedia Laboratories Pvt. Ltd., Mumbai, India). Twenty ml of physiological saline containing 0.1 % Tween 80 was applied on

the culture surface and a microbiological stick was used to release conidia from the mycelium. The conidia were then collected with a Pasteur pipette. The density of the fungal inoculum was adjusted to McFarland 2 (10^6 CFU.ml⁻¹) by adding saline and using a densitometer (Pliva-LaChema a. s., Brno, Czech Republic). The prepared inoculum was diluted with potato dextrose broth (PDB) to a concentration 10^6 CFU.ml⁻¹, which was used for the microdilution testing.

Preparation of cell-free supernatants (CFS) from *Lactobacillus* spp. and *Bacillus* spp.

To prepare CFS from *Bacillus subtilis* (Bs) and *Bacillus licheniformis* (B1) strains a 24-hour old culture grown on B10 agar at 35 °C was used. Using a sterile inoculation loop (mesh volume 10 µl), bacteria colonies were picked up, and 50 ml of B10 broth, in an Erlenmeyer flask was inoculated. The cell-free supernatant from individual *Lactobacillus* strains was prepared by inoculating 50 ml of MRS broth with colonies of cells grown under anaerobic conditions on MRS agar at 35 °C for 48 h, using a Gas pack system. Multiplication of *Lactobacillus* spp. and *Bacillus* spp. was performed in a thermostat under aerobic conditions, at 35 °C, by shaking the Erlenmeyer flasks on a horizontal shaker (Orbital Shaker—Biosan) at 170 rpm. To obtain CFS, the suspensions formed by *Bacillus subtilis* (Bs) and *Bacillus licheniformis* (B1) were centrifuged twice for 20 minutes at 6000 rpm, after 24 hours of incubation. Pure, filtered (Q-Max syringe filter, pore size 22 µm) CFS was used to test *F. graminearum* growth inhibition. *Lactobacilli* supernatants: *Lb. fermentum* 2I3 (L1), *Lb. reuteri* L26 (L3), *Lb. plantarum* L81 (L4), *Lb. reuteri* 2/6 (L5) and *Lb. plantarum* CCM 1904 (L6) were prepared in the same manner, after 48 hour of incubation.

Test procedure

The microdilution method was used to determine the inhibitory effect of cell-free supernatants. The experiment was performed in 96-well sterile microplates (U shaped), and PDB was used as the medium. Undiluted (100 %) sterile CFS (200 µl) was added into the well No. 1 and, subsequently, a series of two-fold dilutions was applied to wells No. 2—10. After application of the fungal inoculum (100 µl) to each dilution of CFS, the final concentrations ranged from 5 to 0.01 mg.ml⁻¹. Each *Fusarium* strain was tested in five rows (A—E). Row H, which served as a neg-

ative control, contained only individual concentrations of CFS (100 µl) diluted in PDB (100 µl). Column 11 was used as a blank containing 200 µl of PDB. Column 12, a positive control, contained 100 µl of PDB and 100 µl of inoculum. The microtitre plates were incubated for 72 h at 25 ± 2 °C, in the dark [6]. After 48-hours of incubation, 15 µl of 0.1 % resazurin solution was added into rows A—E and H [13]. The inhibitory effect was determined by measuring the absorbance of the content of wells at 635 nm using an ELISA reader (Dynex Technologies, Inc., Virginia, USA) and calculated according to the formula:

$$IE (\%) = \frac{(PC - NC) - (S - NC)}{(PC - NC)} \times 100$$

IE—the inhibitory effect,

PC—the absorbance of the positive control,

NC—the absorbance of the negative control,

S—the absorbance of the sample.

The lowest concentration of the tested CFS that completely inhibited the growth of *Fusarium graminearum* mycelium was considered the minimum inhibitory concentration (MIC).

Statistical analysis

The statistical functions of the MS Excel software were used to evaluate the MIC results and the data obtained in this study were reported as means $\pm$ standard deviations (SD).

RESULTS

Evaluation of the inhibitory effect of cell-free supernatants

Table 2 shows the results of the evaluation of MICs and the inhibitory effect of CFS strains of *Lactobacillus* spp.: *L. fermentum* 2I3 (L1), *L. reuteri* L26 (L3), *L. plantarum* L81 (L4), *L. reuteri* 2/6 (L5), *L. plantarum* CCM 1904 (L6) and *Bacillus* spp.: *Bacillus subtilis* CCM 2794 (Bs) and *Bacillus licheniformis* CCM 2206 (B1).

The best inhibitory effect against both *Fusarium* strains was observed at a concentration of 1.25 mg.ml⁻¹, in CFS L3 (10.8 % and 7.9 %, respectively), L4 (5.4 % and 12.3 %), L5 (6.1 % and 40.0 %) and L6 (8.4 % and 12.5 %). CFS L1 was effective on *F. graminearum* CCM F-683 at MIC

Table 2. MICs (mg.ml⁻¹) and the inhibitory effect (%) of cell-free supernatants of *Lactobacillus* spp. and *Bacillus* spp. against *F. graminearum* strains

Tested strains	<i>F. graminearum</i> CCM F-683		<i>F. graminearum</i> CCM 8244	
	x ± SD [mg.ml ⁻¹]	IE	x ± SD [mg.ml ⁻¹]	IE
L1	2.25 ± 0.56	56.5	2.5 ± 0	39.1
L3	1.25 ± 0	10.8	1.25 ± 0	7.9
L4	1.25 ± 0	5.4	1.25 ± 0	12.3
L5	1.25 ± 0	6.1	1.25 ± 0	40.0
L6	1.25 ± 0	8.4	1.25 ± 0	12.5
Bs	≥ 5.0 ± 0	0	≥ 5.0 ± 0	0
Bl	≥ 5.0 ± 0	0	≥ 5.0 ± 0	0

x—mean values of MIC; SD—standard deviation; IE (%)—inhibitory effect; L1—*L. fermentum* 213; L3—*L. reuteri* L26; L4—*L. plantarum* L81; L5—*L. reuteri* 2/6; L6—*L. plantarum* CCM 1904; Bs—*Bacillus subtilis* CCM 2794; Bl—*Bacillus licheniformis* CCM 2206

2.25 ± 0.56 mg.ml⁻¹ and on *F. graminearum* CCM 8244, at MIC 2.5 mg.ml⁻¹, with inhibitory effect 56.5 % and 39.1 %, respectively. CFS from *Bacillus subtilis* and *Bacillus licheniformis* did not show a sufficient inhibitory effect even at the highest tested concentration of 5.0 mg.ml⁻¹.

DISCUSSION

The use of beneficial microorganisms in agricultural, is an increasingly and successful strategy aimed at the elimination of plant pathogens. Biological control by microorganisms is related to the restriction of the use of chemical in agriculture [1, 4]. Many species of *Lactobacillus* have antifungal activity against microscopic filamentous fungi. *Lactobacilli* species, *L. acidophilus*, *L. brevis*, *L. casei*, *L. fermentum*, *L. plantarum*, *L. reuteri*, *L. rhamnosus* and *L. sakei* are often described as producers of antifungal compounds [14]. The antifungal activity of individual strains of the genus *Lactobacillus* is associated with the synthesis of organic acids, fatty acids, esters of fatty acids, hydrogen peroxide, bacteriocins and other secondary metabolites [20]. Antifungal activity of CFS probably depends of the overproduction of substances with antifungal effects (low molecular weight and thermally stable substances, bacteriocins) [7]. However, their antifungal activity is usually poor or moderate [14]. In our experiments, CFS from *Lactobacillus fermentum* at MIC 2.25 ± 0.56 mg.ml⁻¹ was the most effective

against *F. graminearum* CCM F-683 (56.5 %) and CFS from *Lactobacillus reuteri* at MIC 1.25 mg.ml⁻¹ against *F. graminearum* CCM 8244 (40.0 %). Several literature sources point to antifungal efficacy of *Lactobacillus* and *Bacillus* bacteria, which produce substances capable of inhibiting the growth of microscopic filamentous fungi *Fusarium* [8, 10]. *Bacillus subtilis* and *Bacillus licheniformis* can enhance growth of the plant and biomass production through synthesis of various phytohormones, fixation of nitrogen, solubilisation of phosphate and production of ammonium ion. Large number of metabolites of these endophytes activate plant and protect them against harmful bacteria and fungi [24]. The antifungal properties of the *Bacillus subtilis* are mainly attributed to the lipopeptides (fengycin, surfactin and mycosubtilin), which are produced by various strains of this species [16]. In our study, we did not observe an inhibitory effect of CFS from *Bacillus subtilis* even at the highest tested concentration of 5 mg.ml⁻¹ against *F. graminearum*. According to Mihalache et al., [16] the combination of lipopeptides from *B. subtilis* showed potent inhibitory activity at concentrations as low as 5 µg.ml⁻¹ against the phytopathogenic fungi *F. oxysporum*. This antifungal activity is related to the inhibition of spore germination and the irreversible damage of the hyphae cell wall. However, the results of antagonism test showed that the strain *Bacillus licheniformis* could reduce and inhibit the mycelium growth of various plant pathogens *in vitro* and *in vivo* [18].

CONCLUSIONS

Cell-free supernatants of non-pathogenic microorganisms, such as strains of *Lactobacillus* spp. and soil microorganisms *Bacillus subtilis* and *Bacillus licheniformis* deserve a special attention in the future as fungicides. The obtained partial results point to the possibility of their practical application in agricultural practice, as well as to the further direction of scientific research. The use of these bacterial strains creates a space for the study of the synergistic effect of their mutual combinations, possible with other products of natural origin.

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REFERENCES

1. Ciancio, A., Pieterse, C. M., Mercado-Blanco, J., 2016: Editorial: Harnessing useful rhizosphere microorganisms for pathogen and pest biocontrol. *Front. Microbiol.*, 7, 1620. DOI: 10.3389/fmicb.2016.01620.
2. Compant, S., Mitter, B., Colli-Mull, J. G., Gangl, H., Sessitsch, A., 2011: Endophytes of grapevine flowers, berries, and seeds: identification of cultivable bacteria, comparison with other plant parts, and visualization of niches of colonization. *Microb. Ecol.*, 62, 188—197. DOI: 10.1007/s00248-011-9883-y.
3. Doran, P. M., 2013: *Unit operations. Bioprocess Engineering Principles*. Elsevier: Amsterdam, The Netherlands, 445—595.
4. Gaiero, J. R., McCall, C., Thompson, K., Day, N. J., Best, A. S., Dunfield, K. E., 2013: Inside the root microbiome: bacterial root endophytes and plant growth promotion. *Am. J. Bot.*, 100, 1738—1750. DOI: 10.3732/ajb.1200572.
5. Gomah, N. H., Zohri, A. N. A., 2014: Inhibition of fungal growth and Fusarium toxins by selected cultures of lactic acid bacteria. *J. Microbial. Biochem. Technol. S.*, 7, 1948—5948. DOI: 10.4172/1948-5948.S7-001.
6. Hadacek, F., Greger, H., 2000: Testing of antifungal natural products: methodologies, comparability of results and assay choice. *Phytochem. Anal.*, 11, 137—147. DOI: 10.1002/(SICI)1099-1565(200005/06)11:3<137::AID-PCA514>3.0.CO;2-I.
7. Hudáček, J., Zálán, Z., Chumchalová, J., Halász, A., 2007: Antifungal effect of lactobacilli on *Fusarium* and *Aspergillus* moulds. *Chem. Listy (Chemical Letters)*, 101, 730—737.
8. Husain, A., et al., 2017: Antifungal activity of lactic acid bacteria isolated from soil rhizosphere on *Fusarium* species infected chilli seeds. *Am. Sci. Res. J. Eng. Technol. Sci.*, 29, 182—202.
9. Chen, Y., Corby Kistler, H., Zhonghua, M., 2019: *Fusarium graminearum* trichothecene mycotoxins: Biosynthesis, regulation, and management. *Annu. Rev. Phytopathol.*, 57, 15—39. DOI: 10.1146/annurev-phyto-082718-100318.
10. Khan, N., et al., 2018: Antifungal activity of *Bacillus* species against *Fusarium* and analysis of the potential mechanisms used in biocontrol. *Front. Microbiol.*, 9, 2363. DOI: 10.3389/fmicb.2018.02363.
11. Khatoon, A., ul Abidin, Z., 2018: Mycotoxicosis—diagnosis, prevention and control: past practices and future perspectives. *Toxin Rev.*, 39, 99—114.
12. Kokkonen, M., 2010: Biodegradation of mycotoxins. *Food Microbiol.*, 2, 17.
13. Kumar, P., Mishra, S., Kumar, A., 2016: Antifungal efficacy of plant essential oils against stored grain fungi of *Fusarium* spp. *J. Food Sci. Tech.*, 53, 3725—3734. DOI: 10.1007/s13197-016-2347-0.
14. Lipińska, L., Klewicki, R., Klewicka, E., Kołodziejczyk, K., Sójka, M., Nowak, A., 2016: Antifungal activity of *Lactobacillus* spp. bacteria in the presence of xylitol and galactosyl-xylitol. *BioMed Res. Int.*, 1—8. DOI: 10.1155/2016/5897486.
15. Miedaner, T., Bolduan, C., Melchinger, A. E., 2010: Aggressiveness and mycotoxin production of eight isolates each of *Fusarium graminearum* and *Fusarium verticillioides* for ear rot on susceptible and resistant early maize inbred lines. *Eur. J. Plant. Pathol.*, 127, 113—123. DOI: 10.1007/s10658-009-9576-2.
16. Mihalache, G., Balaes, T., Gostin, I., et al., 2018: Lipopeptides produced by *Bacillus subtilis* as new biocontrol products against fusariosis in ornamental plants. *Environ. Sci. Pollut. Res.*, 25, 29784—29793. DOI: 10.1007/s11356-017-9162-7.
17. Moretti, A., Panzarini, G., Somma, S., Campagna, C., Ravaglia, S., Logrieco, A. F., Solfrizzo, M., 2014: Systemic growth of *F. graminearum* in wheat plants and related accumulation of deoxynivalenol. *Toxins*, 6, 1308—1324. DOI: 10.3390/toxins6041308.
18. Nigris, S., Baldan, E., Tondello, A. et al., 2018: Biocontrol traits of *Bacillus licheniformis* GL174, a culturable endophyte

- of *Vitis vinifera* cv. Glera. *BMC Microbiol.*, 18, 133. DOI: 10.1186/s12866-018-1306-5.
19. Pellegrini, M., Pagnani, G., Bernardi, M., Mattedi, A., Spera, D. M., Gallo, M. D., 2020: Cell-free supernatants of plant growth-promoting bacteria: A review of their use as biostimulant and microbial biocontrol agents in sustainable agriculture. *Sustainability*, 12, 9917. DOI: 10.3390/su12239917.
 20. Perczak, A., et al., 2018: The efficiency of lactic acid bacteria against pathogenic fungi and mycotoxins. *Arch. Ind. Hyg. Toxicol.*, 69, 32—45. DOI: 10.2478/aiht-2018-69-3051.
 21. Ponts, N., 2015: Mycotoxins are a component of *Fusarium graminearum* stress-response system. *Front. Microbiol.*, 6, 1—5. DOI: 10.3389/fmicb.2015.01234.
 22. Rashidi, N., Khatibjoo, A., Taherpour, K., Akbari-Gharaei, M., Shirzadi, H., 2020: Effects of licorice extract, probiotic, toxin binder and poultry litter biochar on performance, immune function, blood indices and liver histopathology of broilers exposed to aflatoxin-B1. *Poult. Sci.*, 99, 5896—5906. DOI: 10.1016/j.psj.2020.08.034.
 23. Ryan, R. P., Germaine, K., Franks, A., Ryan, D. J., Dowling, D. N., 2008: Bacterial endophytes: recent developments and applications. *FEMS Microbiol. Lett.*, 278, 1—9. DOI: 10.1111/j.1574-6968.2007.00918.x.
 24. Verhagen, B. W. M., Trotel-Aziz, P., Jeandet, P., Baillieul, F., Aziz, A., 2011: Improved resistance against *Botrytis cinerea* by grapevine-associated bacteria that induce a prime oxidative burst and phytoalexin production. *Phytopathol.*, 101, 768—777. DOI: 10.1094/PHYTO-09-10-0242.
 25. Xu, X. M., Nicholson, P., 2009: Community ecology of fungal pathogens causing wheat head blight. *Annu. Rev. Phytopathol.*, 47, 83–103. DOI: 10.1146/annurev-phyto-080508-081737.

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