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ANTAGONISTIC POTENTIAL OF *Lactobacillus plantarum* AGAINST SOME POSTHARVEST PATHOGENIC FUNGI

ABSTRACT: *Lactobacillus plantarum*, one of the most widespread lactic acid bacteria, exert a strong antagonistic activity against many microorganisms. The present study was conducted to determine *in vitro* and *in situ* antagonistic potential of *L. plantarum* (DSM 20174) for control postharvest decay caused by phytopathogenic fungi: *Aspergillus flavus, Colletotrichum acutatum, Colletotrichum gloeosporioides*, and *Fusarium avenaceum.* The results obtained in *in vitro* assays showed that *L. plantarum* had a stronger inhibitory effect on spore germination than on mycelia growth of all tested fungi. After 3 days of incubation, the diameter of inhibition zones ranged from 11.67 mm for *C. gloeosporioides* to 14.67 mm for *C. acutatum*. The bacterial suspension of *L. plantarum* significantly inhibited conidial germination of all postharvest pathogens (89.62–97.61%). *In situ* assays showed that treatment with *L. plantarum* efficiently inhibited necrosis ranging from 42.54% for *C. acutatum* to 54.47% for *A. flavus*. The disease incidence in *L. plantarum* treated fruits was statistically significantly lower than in the positive control for all fungi tested ($P<0.05$). The presented data demonstrate the antagonistic potential of *L. plantarum* (DSM 20174) and indicate the possibility of using this bacterial strain as a biological agent to control postharvest fungal pathogens.

KEYWORDS: antagonistic activity, biocontrol**,** *Lactobacillus plantarum*, postharvest fungal pathogens

INTRODUCTION

The postharvest losses are mainly due to pathogenic fungi which usually infect fruits through wounds made during harvest, transportation, and processing (Vero et al., 2002). Some of the postharvest fungal pathogens cause serious problems in food by producing mycotoxins and potentially allergenic spores

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(Mushtaq et al., 2010). Several methods have been used to solve postharvest losses, such as fungicide treatment and modified controlled atmosphere (Montero et al., 2010; Romanazzi et al., 2012). The development of fungicide resistance by postharvest pathogens and increasing environmental concern over fungicide residues in food have stimulated the finding of alternative means for controlling postharvest decay (Holmes and Eckert, 1999). Biological control involves the use of naturally occurring nonpathogenic microorganisms, biocontrol agents (BCAs), that are able to reduce the activity of plant pathogens and thereby suppress diseases. Several strains of *Bacillus*, *Pseudomonas* and lactic acid bacteria (LAB), as well as yeasts, have been identified and commercialized for the control of postharvest decay caused by fungi in fruits (Janisiewicz and Korsten, 2002).

LAB form an ecologically heterogeneous group of Gram-positive bacteria, nonspore-forming, immobile and catalase negative that excrete lactic acid as the major product and are generally recognized as safe organisms (GRAS) (Konings et al., 2000). The antimicrobial properties of lactobacilli are of special interest in developing strongly competitive starter cultures for food fermentation (Harris et al., 1989). Today, LAB strains play crucial roles in the manufacturing of fermented milk products, vegetables, and meat, as well as in the processing of other products such as wine (Konings et al., 2000). These bacteria produce various compounds such as organic acids, diacetyl, hydrogen peroxide, and bacteriocin or bactericidal proteins during lactic fermentations (Lindgren and Dobrogosz, 1990). Lactobacilli are able to inhibit food-borne pathogens: *Staphylococcus aureus, Salmonella typhimurium, Escherichia coli,* and *Listeria monocytogenes* (Jamuna and Jeevaratnam, 2004; Darsanaki et al., 2012). They are selected as probiotic, which are able to promote health and prevent infections against enteropathogenic bacteria (Fernandez et al, 2003). In addition, LAB strains are efficient in inhibition of mycotoxigenic fungi: *Penicillium expansum, Botrytis cinerea, Aspergillus niger, Aspergillus flavus*, and *Fusarium graminarum*, as well as phytopathogenic bacteria, such as *Xanthomonas campestris* and *Erwinia carotovora* (Lavermicocca et al., 2000; Trias et al., 2008).

One of the most widespread LAB strains used in food technology and biotechnology is *Lactobacillus plantarum*. This species synthesizes a number of substances, including benzoic acid, methylhydantoin, and mevalonolactone that have antifungal activity (Niku-Paavola et al., 1999). Lavermicocca et al. (2000) reported that the inhibitory activity of *L. plantarum* can be attributed to the organic acids phenyl-lactate and 4-hydroxy-phenyllactate.

The results of the previous investigation (Živković et al., 2014) indicated good antagonistic activity of *L. plantarum* (DSM 20174) against *P. expansum* and *Aspergillus ochraceus.* The present study was conducted to determine *in vitro* and *in situ* potential of this LAB against postharvest decay in apple fruits caused by *A. flavus, Colletotrichum acutatum, Colletotrichum gloeosporioides*, and *Fusarium avenaceum.*

MATERIAL AND METHODS

Pathogens and BCA

A. flavus, C. acutatum, C. gloeosporioides, and *F. avenaceum* were obtained from decayed apple fruits in storage and kept in the Culture Collection of Institute for Plant Protection and Environment. For conidial production, pathogens were grown on potato dextrose agar (PDA) at 25 °C. After a week, spores were harvested and suspended in 10 ml of sterile distilled water containing 0.05% (v/v) Tween 80. The concentration of spore suspension was determined with a Neubauer chamber and adjusted with sterile distilled water to 1×10^6 conidia/ml.

L. plantarum (DSM 20174) was obtained from the German Collection of Microorganisms and Cell Cultures. The bacterial strain was cultivated anaerobically in Man, Rogosa and Sharpe (MRS) broth for 72 hours at 30 °C.

In vitro assays of antagonistic activity

Antagonistic activity was determined in the dual culture overlay assays. Bacteria were inoculated in 2 cm lines on MRS agar plates and allowed to grow at 30 °C for 48 h in anaerobic jars. The plates were then overlaid with 5 ml of malt extract soft agar (2% malt extract; 0.7% agar) containing 1 x 10^6 spores of tested pathogens. After 72h of aerobic incubation at 30 °C, the zone of inhibition (ZI) was measured. The ZI was recorded as the distance between the fungal pathogen and the area of the antagonist.

For conidial germination test, 100 µl of the conidial suspension of each fungal pathogen (10^6 conidia/ml) and 100 µl of the bacterial suspension of *L. plantarum* (10^8 CFU/ml) were added into the glass tubes with 5 ml potato dextrose broth (PDB). The control consisted of suspensions of pathogens conidia in PDB. The tubes were then incubated in moist chambers for 24h at 25 °C. The percent of germination was determined by counting 100 conidia from each fungal pathogen under the microscope Olympus BX51 (Olympus Corporation Japan). Spores were considered germinated when germ tube length was equal to or greater than spore length.

In situ assays of antagonistic activity

Apple fruits (cv. Golden Delicious) were surface sterilized, wounded with a cork borer and then inoculated with 25 µl of the bacterial suspension of *L. plantarum* (10^8 CFU/ml). After 1 h, the wound was inoculated with 25 µl of the conidial suspension of *A. flavus, C. acutatum, C. gloeosporioides*, or *F. avenaceum* (1 x 10^6 conidia/ml). The positive control fruits were inoculated only with the fungal conidial suspensions, and the negative control with sterile distilled water. All apples were placed in a moist chamber and incubated at 25 °C. After 7 days the diameters of necrotic lesions were measured. The percentage

of necrosis inhibition (IN) was calculated using the formula: IN (%) = (KR-R/KR) x 100, where KR is the radius of necrosis in positive control fruit and R is the radius of necrosis in fruit treated with *L. plantarum*.

Statistical analysis

For all experiments, each treatment was done in triplicates and the entire experiment repeated twice. Data were analyzed by one-way analysis of variance (ANOVA). Mean values were compared using Tukey's multiple range test and significance was evaluated at $P<0.05$. Statistical analysis was performed using statistical software Minitab 18 (Minitab, Inc, USA).

RESULTS AND DISCUSSION

In the present study *L. plantarum* (DSM 20174) was evaluated *in vitro* and *in situ* for antagonistic activity against *A. flavus, C. acutatum, C. gloeosporioides*, and *F. avenaceum*. Results obtained in the dual culture overlay assays showed that *L. plantarum* had good antifungal activity against all tested fungi (Table 1). After 3 days of incubation, the diameter of inhibition zones ranged from 11.67 mm for *C. gloeosporioides* to 14.67 mm for *C. acutatum*.

The results of our study showed that *L. plantarum* had a stronger inhibitory effect on spore germination than *in vitro* mycelial growth of *A. flavus, C. acutatum, C. gloeosporioides*, and *F. avenaceum*. The conidia of all tested pathogens incubated in control treatment at 25 ºC were swelled and germinated, producing one germ tube. However, conidia of tested fungi were strongly limited in the co-cultivation assay with the bacterial suspension of *L. plantarum*. After 24h of co-cultivation, there was a significant inhibition of the conidial germination in all treatments with the antagonist (89.62–96.61%) (Table 1). Conidia that were ungerminated after 24h did not germinate afterward. Figure 1 (A-C) depicts the effect of *in vitro* bacterial suspension of *L. plantarum* against *A. flavus*.

Table 1. Antagonistic activity of *L. plantarum* against the postharvest fungal pathogens *in vitro.*

Pathogen	*L. plantarum*	
	Inhibition zone (mm)	Inhibition of spore germination (%)
A. flavus	12.67 ± 0.58* bc**	97.61 ± 0.60* a**
C. acutatum	14.67 ± 0.58 a	91.86 ± 0.59 b
C. gloeosporioides	11.67 ± 0.58 c	91.16 ± 1.02 bc
F. avenaceum	13.33 ± 0.58 ab	89.62 ± 1.04 c

* Data represented standard deviations of the means
** Means in columns followed by different letters are significantly different according to Tukey's multiple range test ($P<0.05$)

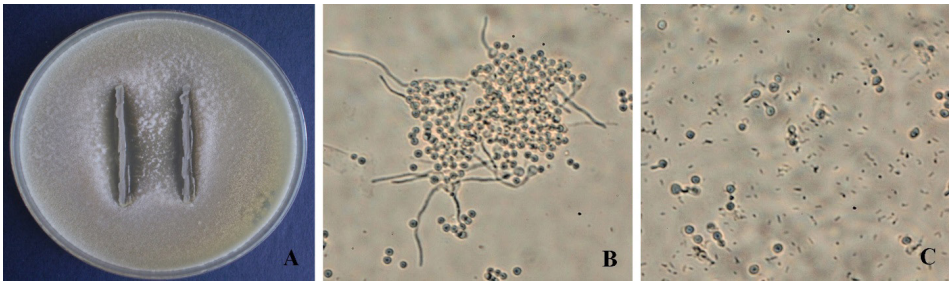

Figure 1. Effect of *L. plantarum* on *A. flavus in vitro*: A) inhibition zone; B) conidial germination of *A. flavus* in control: (magnification x400) C) inhibition of the conidial germination of *A. flavus* in treatment with *L. plantarum* (magnification x400)

The results of the previous investigation showed that *L. plantarum* (DSM 20174) had good antifungal activity against *P. expansum* (ZI =20 mm), and *A. ochraceus* (ZI =15 mm) *in vitro*. The bacterial suspension of this strain completely inhibited conidial germination of *P. expansum*, and significantly inhibited conidial germination of *A. ochraceus* (88%). In biocontrol assay, *L. plantarum* significantly reduced disease incidence caused by *P. expansum* (55%) in apple fruit. However, this LAB had moderate antifungal effect *in situ* on *A. ochraceus* (37%) (Živković et al., 2014).

The antifungal activity of *L. planatarum* has also been reported by other investigators. Trias et al. (2008) isolated *L. plantarum* from fresh fruits and vegetables and tested *in vitro* their potential as BCA against phytopathogenic fungi, *P. expansum*, *B. cinerea*, and *M. laxa*. All tested microorganisms except *P. expansum* were inhibited by one isolate of *L. plantarum*. Prema et al. (2010) investigated the antifungal activity of *L. plantarum* strain from grass silage. Agar plate assay showed that *Aspergillus fumigatus* and *Rhizopus stolonifer* were the most sensitive among molds. No inhibitory activity could be detected against *Penicillium roqueforti*. Sathe et al. (2007) tested the antifungal spectrum of LAB strains against *F. graminearum, R. stolonifer, S. oryzae, R. solani, B. cinerea*, and *S. minor* in the overlay method. The isolate identified as *L. plantarum* had a strong activity against all six spoilage fungi. Our results are in agreement with the results of Gerez et al. (2009) who reported that *L. plantarum* and other strains of lactobacilli were able to inhibit the conidial germination and mycelial growth of fungi from the genera *Aspergillus*, *Fusarium*, and *Penicillium*, the main contaminants in bread.

The antifungal activity of LAB strains are certainly a complex phenomenon and still partially unknown. There are few reports of low molecular weight of antifungal peptides synthesized by LAB, which inhibit spoilage and pathogenic fungi with insufficient information on their precise mechanism of action (Schnurer and Magnussion, 2005). Several studies have reported that the antifungal activity of LAB is not only related to the production of organic acids and hydrogen peroxide. Rather, it is a combined effect of several interrelated factors (Laitila et al., 2002). Cabo et al. (2002) have suggested that *in vitro* antifungal activity of LAB is due to a synergistic effect of lactic acid produced

by the bacteria and acetic acid from the MRS growth medium. This dual culture system is based on diffusion of the inhibitory substances into the agar, and consequently lactic acid will also contribute to the inhibition (Strom et al., 2002). The antimicrobial effects of different lactobacilli including *L. plantarum* against plant pathogenic fungi were greatly influenced by the substrate and pH of cultivation (Karunaratne et al. 1990; Gourama and Bullerman, 1995; Stiles and Holzapfel, 1997).

The results obtained in *in situ* assays showed that treatment with *L. plantarum* efficiently protected apple fruits from decay and inhibited necrosis ranging from 42.54% for *C. acutatum* to 54.47% for *A. flavus* (Figure 2). No lesion developed in negative control fruits inoculated with sterile distilled water. The disease incidence in *L. plantarum* treated fruits was statistically significantly lower than those in the positive control for all fungi tested ($P<0.05$). Figure 3 (A–C) presents the effect of the bacterial suspension of *L. plantarum* on apple fruits affected by *A. flavus* infection *in situ*.

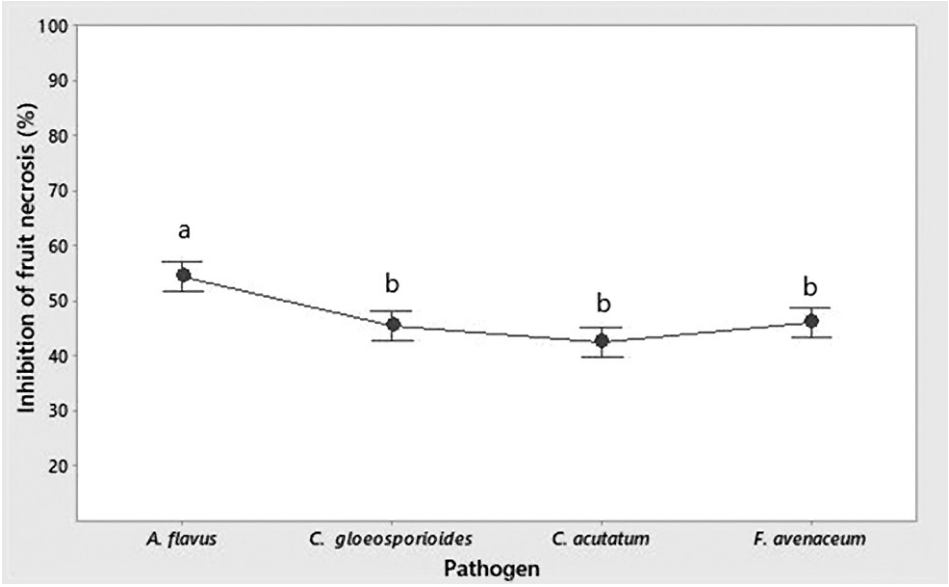

Figure 2. Inhibition of apple fruit necrosis induced by postharvest fungal pathogens using *L. plantarum.*

Sathe et al. (2007) reported that the suspension of *L. plantarum* delayed the growth of *A. flavus, F. graminearum, R. stolonifer*, and *B. cinerea* in cucumber. LAB isolated from yogurt and milk showed inhibitory activity against *F. oxysporum* and provided a protective effect to tomato plants (Hamed et al., 2011). Prusky et al. (2006) suggested that acidification of fruit tissue can reduce the postharvest decay caused by pathogens, such as *P. expansum* and *A. alternata*. The combination of different organic acids, such as lactic and propionic, has been reported to have a synergistic fungistatic effect (Adams and Hall, 1988).

Figure 3. Effect of *L. plantarum* on *A. flavus* decay on apple fruits *in situ*: A) positive control; B) treatment with *L. plantarum*; C) negative control.

In situ, the antimicrobial action is often the sum of many factors. In many cases, not only extracellularly produced compounds but also viable cells are needed for the maximum action.

CONCLUSION

Postharvest fungal pathogens are the main cause of substantial economic losses in stored fruits and might also be regarded as sources of mycotoxins, involving serious health problems. LAB strains are important organisms recognized for their fermentative ability as well as their health and nutritional benefits. One of the most widespread LAB, *L. plantarum,* produces several antimicrobial agents and exerts strong antagonistic activity against many microorganisms, including food spoilage organisms and pathogens. In this context, *L. plantarum* may be considered as an alternative for synthetic fungicides. The presented data exhibit *in vitro* and *in situ* antimicrobial activity of *L. plantarum* (DSM 20174) against *A. flavus, C. acutatum, C. gloeosporioides,* and *F. avenaceum*, and indicate the possibility of using this bacterial strain as a BCA to control these postharvest fungal pathogens in apple fruits.

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АНТАГОНИСТИЧКИ ПОТЕНЦИЈАЛ *Lactobacillus plantarum* ПРЕМА НЕКИМ СКЛАДИШНИМ ФИТОПАТОГЕНИМ ГЉИВАМА

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РЕЗИМЕ: *Lactobacillus plantarum* једна је од најраспрострањенијих млечнокиселинских бактерија која испољава антагонистичку активност према великом броју микроорганизама. Циљ студије био је да се у *in vitro* и *in situ* огледима утврди антагонистички потенцијал *L. plantarum* (DSM 20174) према складишним фитопатогеним гљивама: *Aspergillus flavus*, *Colletotrichum acutatum*, *Colletotrichum gloeosporioides* и *Fusarium avenaceum*. Резултати *in vitro* огледа показују да је

L. plantarum испољио јачи инхибиторни ефекат на клијање спора него на пораст мицелије тестираних гљива. Зоне инхибиције су варирале у распону од 11,67 mm за *C. gloeosporioides* до 14,67 mm за *C. acutatum*. Бактеријска суспензија *L. plantarum* је значајно инхибирала клијање конидија свих тестираних складишних патогена (89,62–97,61%). У *in situ* огледима *L. plantarum* је ефикасно инхибирао појаву некрозе у опсегу од 42,54% за врсту *C. acutatum* до 54,47% за врсту *A. flavus*. Инциденца појаве болести код плодова третираних овим биоконтролним агенсом била је статистички значајно нижа у односу на позитивне контроле свих испитаних патогена ($P<0,05$). Добијени резултати указују да *L. plantarum* (DSM 20174) има антагонистички потенцијал и да се може користити као биоконтролни агенс против складишних фитопатогених гљива.

КЉУЧНЕ РЕЧИ: антагонистичка активност, биоконтрола, *Lactobacillus plantarum*, складишне фитопатогене гљиве