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Screening of antagonistic activity of selected microorganisms against apple rot pathogens

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MILA S. GRAHOVAC[1], JELICA S. BALAŽ[1], JOVANA A. GRAHOVAC[2], JELENA M. DODIĆ[2], BRANKICA B. TANOVIĆ[3], JOVANA G. HRUSTIĆ[3], IVANA Ž. TADIJAN[2]

[1] University of Novi Sad, Faculty of Agriculture, Trg Dositeja Obradovića 8, Novi Sad, Serbia
[2] University of Novi Sad, Faculty of Technology, Bulevar Cara Lazara 1, Novi Sad, Serbia
[3]Institute for Pesticides and Environmental Protection, Banatska 31b, Belgrade, Serbia
Corresponding author: phone: +381 21 4853723, e-mail: tadi@uns.ac.rs

Abstract

Biological control of plant pathogens is considered an attractive alternative to chemical-based treatments because it has a minimal impact on the environment. In this study, in vitro antifungal efficiency of four microorganisms (Streptomyces hygroscopicus, Saccharomyces cervisae, Bacillus cereus and Leuconostoc mesenteroides) against causal agents of apple fruit rot, was investigated. Isolates of Colletotrichum acutatum, Colletotrichum gloeosporioides and Fusarium avenaceum were obtained from apple fruit samples expressing rot symptoms. Apple samples were collected during 2012 from Ultra Low Oxygen storages in Vojvodina Province, Serbia. The test microorganisms were identified according to pathogenic, morphological and ecological characteristics, and the identification of Colletotrichum isolates was confirmed by polimerase chain reaction (PCR). Antifungal effect was tested using wells method and the obtained data were processed by factorial ANOVA. Duncan's multiple range test was used to test significance of differences ($p \leq 0.05$) between mean values of measured radius of inhibition zones. Activity of S. cervisae, B. cereus and L. mesenteroides was not satisfactory. On the other hand, S. hygroscopicus expressed the strongest antagonism against all tested fungal isolates. The studied isolate of S. hygroscopicus showed significant antagonistic properties against storage pathogens of apple fruits identifying itself as promising biocontrol agent for commercial use.

Keywords: biocontrol, *Streptomyces hygroscopicus* , apple storage pathogens, antagonists

Introduction

After harvest, losses of fruits can be very high. In developing countries these losses are over 50%, while in industrialized countries they reach over 25%. Most of these losses are caused by fungal pathogens that develop due to high amount of nutrients and water in fruits, low pH and loss of intrinsic resistance of the plant [1]. Besides *Penicillium, Botrytis* and *Monilinia*, fungi from the genera *Alternaria*, *Colletotrichum* and *Fusarium* are known as the most important storage pathogens of apple fruits [1-3]. Postharvest loss have been managed by postharvest fungicide applications, postharvest management practices and by storage at low temperature. However, the problems of pathogen resistance to many fungicides, lack of replacement fungicides and effects of fungicides on human health and the environment have promoted restricted use of fungicides and the need to find alternative methods to control postaharvest diseases. As a result, biological control has emerged as an effective tool for management of postharvest decays of fruits.

Biopesticides have several advantages over synthetic pesticides: their dagradation in the environment is much faster and they are less toxic to non-target organisms [4]. Moreover, modes of action of biological fungicides usually differ from conventional fungicides and therefore, they can reduce resistant populations of pests and pathogens [5]. Biological control of postharvest fruit pathogens is in its infancy compared to long-standing interest in biological control of soilborne pathogens [6]. Products based on microorganisms have a share of 30 % in total biopesticide market [5]. Bacteria with antifungal potential occur in many genera, such as *Bacillus, Streptomyces, Pseudomonas, Xanthomonas, Rhizobium, Enterobacter* etc [7].

Therefore, the aim of the study was to test the activity of four microorganisms (*Streptomyces hygroscopicus*, *Saccharomyces cervisae, Bacillus cereus* and *Leuconostoc mesenteroides*) as potential antagonists against isolated strains of causal agents of apple fruit rot: *Colletotrichum acutatum, Colletotrichum gloeosporioides* and *Fusarium avenaceum.*

Material and methods

Fungal pathogens

Isolates of *C. acutatum, C. gloeosporioides* and *F. avenaceum* were obtained from apple fruit samples expressing rot symptoms. Apple samples were collected during 2012 from Ultra Low Oxygen storages in Vojvodina Province, Serbia. The pathogens were isolated using standard phytopathological techniques. Infected apple fruits were surface-sterilized with 96% ethyl alcohol, cut at the turn of diseased to healthy tissue, and tissue fragments were aseptically placed on sterile potato dextrose agar medium (PDA) and incubated at 25°C for seven days. After seven days, the obtained mycelium was subcultured to sterile PDA medium to obtain a pure culture. The obtained pure cultures were incubated for three days on PDA slants at 20°C and afterwards kept in refrigerator at 4°C until use (Dhindra and Sinclair, 1995).

The pathogens were identified according to pathogenic, morphological and ecological characteristics, and the identification of *Colletotrichum* isolates was confirmed by polimerase chain reaction (PCR), using species-specific primers. Species-specific primers for *C. gloeosporioides* (CgInt 5' GGCCTCCCGCCTCCGGGCGG 3') and *C. acutatum* (CaInt2 5' GGGGAAGCCTCTCGCGG 3') from the ITS1 region of the ribosomal DNA gene in combination with the conserved primer ITS4 (5' TCCTCCGCTTATTGATATGC 3') were used for the reaction, according to protocol described by Sreenivasaprasad et al. (1996) [8]. Each of 25 µl reaction mixture contained: 2.5 µl of DNA (50 ng/µl), 0.12 µl of each 10 µM primer, 0.08 µl of 10 mM dNTP, 0.5 µl of *Taq* Polymerase (5 U/µl), 1.5 µl of 25 mM MgCl, 2.5 µl of 10x polymerase buffer and 16.9 µl of sterile milliQ water. Reaction PCR mix without added DNA served as a negative control. PCR reactions were performed in Eppendorf Master Cycler and the reaction conditions were as follows: 5 min at 94°C, 30 cycles of 1.5 min at 94°C, 2 min at 55° and 3 min at 72°C, and then a 10-minute final extension at 72°C. PCR products (7 µl) were separated by horisontal gel electrophoresis in 1.5% agarose gel 0.5 × TBE buffer at 100V constant voltage for 60 minutes. Gels were stained in ethidium bromide solution (2 µg/ml) and visualised under UV light. Molecular weight of the obtained PCR product was determined according to its position in relation to 1kb DNA marker (Fermentas, Lithuania). The occurrence of amplicons about 490 bp in size was considered as positive reaction for *C. acutatum*, and of 450 bp for *C. gloeosporioides* [8]. Pathogenicity of isolates was tested on artificially inoculated injured apple fruits and pathogenicity of the reisolated isolates was confirmed the same way.

Working cultures were prepared by inoculation of 50 ml of Potato Dextrose Broth (PDB) by seven days old mycelium fragment and cultivation on horizontal shaker for 48 h at 25 °C. The data on the isolates used in the study are given in Table 1.

Table 1. Fungal isolates used in the study

Isolate code	Fungal species
KA 7	*C. acutatum*
KJ 4	*C. acutatum*
MRMCD 6	*C. gloeosporioides*
KA13	*F. avenaceum*
MRMZD 3	*F. avenaceum*

Antagonists

Four antagonistic microorganisms (*S. hygroscopicus*, *S. cervisae*, *B. cereus* and *L. mesenteroides*) were isolated from the natural environment and obtained from the Microbial Culture Collection of the Faculty of Technology in Novi Sad.

Cultivation media used to grow the strains were: Nutrient broth (Torlak) for *S. hygroscopicus* and *S. cervisae*, Muller-Hinton broth for *B. cereus* and MRS broth for *L. mesenteroides.* The pH of the media was adjusted to 7.2 ± 0.1 prior to autoclaving. The isolates were grown in a 300 ml shake flask containing 100 ml of the culture medium. The fermentation medium was inoculated with 10% (v/v) of an inoculum culture and incubated at temperature of 26±1 °C for 72 hours under standard conditions of aeration and agitation. Rotary shaker at 150 rpm was used to mix the fluids during the cultivation.

After cultivation, the sample of the cultivation medium was centrifuged at 10 000 g for 10 min and the supernatant was used for *in vitro* antagonistic activity assay.

In vitro antagonistic activity assay

Two layers of PDA medium were spread on 90 mm petri dishes. The first layer consisted of 2% PDA medium. After solidification a new layer composed of 1.2% PDA and fungal pathogen incubated in PDB for 48 h homogenized on magnetic stirrer, was added. Three wells per plate with a diameter of 10 mm were made. In each well, 100 µl of prepared antagonist was added. Activity of each antagonist was tested in four replicates for each isolate. In control plates, 100 µl of sterile, distilled water was added to wells. Experiment was repeated twice.

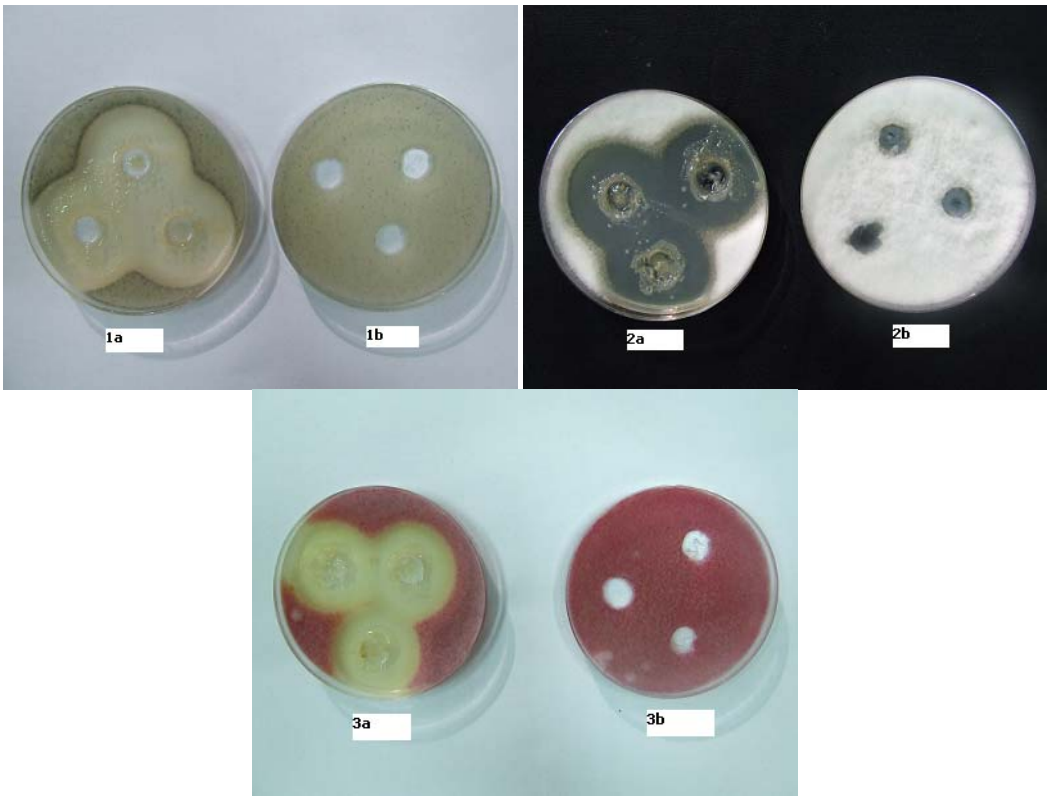

Figure 1. Inhibition zones formed around wells with 100 µl of *S. hygroscopicus* for isolates KA7 (1a), MRMCD6 (2a) and MRMZD3 (3a) and control plates for the same isolates (1b, 2b, 3b) after 48 h incubation at 25 °C.

The assesment of antagonistic activity was done after 48 h incubation at 25 °C by measuring radius of inhibition zones (mm) - zones around wells with no visible mycelial growth (Fig 1).

Data analysis

The obtained data were processed by factorial ANOVA using Software *Statistica* 10 [9]. Duncan's multiple range test was used to test significance of differences ($p \leq 0.05$) between mean values of measured radius of inhibition zones.

Results and discussion

As shown in Figure 2, significantly higher radius of inhibition zones for all tested isolates was obtained by *S. hygroscopicus* compared to all other antagonists and control, except to *S. cervisae* which expressed antagonistic activity against the isolate KA 13 on the same level of significance as *Streptomyces* spp. showed against isolates MRMCD6 and KA13. However, *S. cervisae* did not prove antagonistic activity against other tested strains. In another study with *Penicillium expansum* it has been shown that *S. cervisae* have good antagonistic activity and the strain was proposed as biocontrol agent in apple storages under commercial conditions [10]. *B. cereus* is also a commonly mentioned biocontrol agent of many phytopathogenic fungi [11-14]. In our study, *B. cereus* expressed significantly higher antagonistic activity compared to control only against *C. gloeosporioides*. *L. mesenteroides* did not show any potential as antagonist of tested fungal isolates.

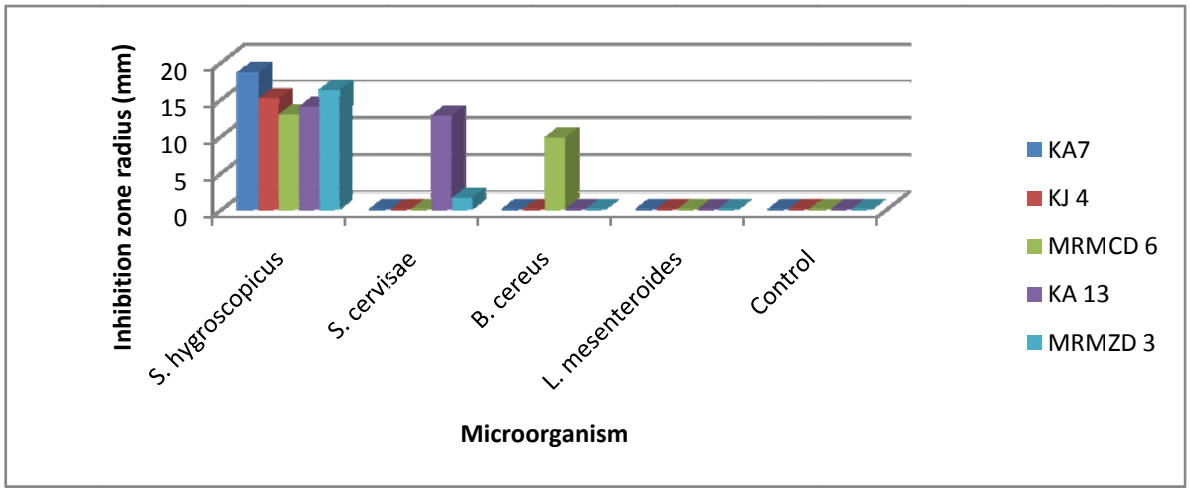

Figure 2. Mean values of inibition zone radius (mm) after 48 h incubation at 25 °C

The results presented in Table 2. indicate that differences observed between radius of inhibition zones as the consequence of activity of different antagonists, different susceptibility of investigated fungal isolates as well as interaction between these two factors, were significant. The biggest source of variation of radius of inhibition zones was the activity of different antagonists.

Table 2. Results of factorial analysis of variance: sources of variation of radius of inhibition zones after 48 h incubation at 25 °C.

Source of variation	SS	Degr. of - Freedom	MS	F-value	p-value
Intercept	2416.19	1	2416.19	1143.31	0.00
Antagonist	4930.87	4	1232.72	583.31	0.00
Isolate	97.27	4	24.32	11.51	0.00
Antagonist*Isolate	1200.52	16	75.03	35.50	0.00
Error	264.17	125	2.11		

SS – sum of squares; MS – mean square

In *in vitro* assays with antagonistic microorganisms, inhibition zones over 11 mm indicate that the applied antifungal agent is potentially highly efficient [15]. In our study, inhibition zones caused by *S. hygroscopicus* were over 11 mm for all fungal isolates, which indicates that this microorganism is potentially highly efficient antifungal agent.

Nowdays many researchers use *Streptomyces* species to obtain different high value products used as biocontrol agents. Marten et al. (2001) reported that *Rhizovit* R containing a strain of *Streptomyces rimosus* is used in the control of a wide range of fungi, such as *Pythium* spp., *Phytophthora* spp., *Rhizoctonia solani*, *Alternaria brassicola*, and *Botrytis* sp. Liu et al. (2004) also reported that *S. rimosus* showed a high antagonism activity against *Fusarium solani*, *F. oxysporium* f sp. *cucumarinum*, *Verticillium dahliae*, *R. solani*, *Fulvia fulva*, *Botrytis cinerea*, *A. alternata*, *Sclerotinia sclerotiorum* and *Bipolaris maydis* [16].

Streptomyces is the largest antibiotic producing genus and the number of antimicrobal compounds reported to be produced by this microorganism is constantly growing [17]. Many authors reported antagonistic activity of actinomycetes against plant pathogenic fungi [15, 18-21]. In some studies, hundreds of strains of actinomycetes were tested for antifungal activity,

and only a share of about 10% exhibited antifungal activity against important phytopathogens among which *C. gloeosporioides* was investigated [19,22]. Therefore, the interest in finding more efficient strains regarding biocontrol of fungal plant pathogens is increasing. In our study, only one isolate of *S. hygroscopicus* was tested and showed the greatest potential as antagonist of investigated apple pathogens among four different microorganisms included in the assay.

Conclusion

The results of the study indicate that isolate *S. hygroscopicus* has the greatest potential as antagonist of storage pathogens of apple *C. acutatum*, *C. gloeosporioides* and *F. avenaceum*, and should be included in further investigations as potential microorganism for production of a biofungicide intended to be used for protection of apple fruits from storage pathogens.

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