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Original scientific paper

Application of different combinations of lactic acid, phototrophic bacteria and yeast mixtures in control of seed and seedlings pathogens of tomato and pepper

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SUMMARY

Application of three combinations of lactic acid bacteria (*Lactobacillus plantarum*, *Lactobacillus rhamnosus*), phototrophic bacteria (*Rhodopseudomonas palustris*) and yeast (*Saccharomyces cerevisiae*) with sugar cane molasses, marked as: EM1, EM5 and EM AGRO, against the phytopathogenic fungi of tomato and pepper: *Fusarium oxysporum, Alternaria alternata, Botrytis cinerea*, *Colletotrichum* sp., *Verticilium dahliae* and *Pythium aphanidermatum* was evaluated *in vitro* and *in vivo*. A combination of bacteria and yeast named EM5 showed the highest mycelium growth inhibition against *B. cinerea* (38.4%) in a double agar diffusion test. In a microdilution test, the combination EM1 showed the highest inhibitory effect on *B. cinerea* (MIC $1x10^{-3}$ µl/ml), while EM5 showed a similar inhibitory effect towards *F. oxysporum*, *A. alternata* and *Colletotrichum* sp. (MIC 10 µl/ml). The use of EM1 (in concentrations 10 and 100 µl/ml) and EM AGRO (10 µl/ml) is recommended for tomato seedling protection. EM1 (100 µl/ml), EM5 and EM AGRO (10 µl/ml) are recommended for pepper seedling protection.

Keywords: tomato, pepper, lactic acid bacteria, phototrophic bacteria, yeasts, antifungal potential

INTRODUCTION

Tomato (*Solanum lycopersicum* L.) and pepper (*Capsicum annuum* L.) are two very important vegetable crops in Serbia. In 2019, tomato production in Serbia was 111.649 tonnes on 7.880 ha (FAO, 2021), while Gvozdenović (2010) reported over 150.000 tonnes of peppers that were harvested from 21.000 ha.

Tomato and pepper crops are exposed to many phytopatogenic fungi, such as: *Alternaria alternata*, *Colletotrichum* spp., *Fusarium* spp. (Mannai et al., 2018; Rezaee et al., 2018), *Pythium* spp. (Whipps & Lumsden, 1991), *Botrytis* spp. (Williamson et al., 2007), *Rhizoctonia* spp., *Septoria lycopersici* and *Verticilium* spp., which are able to cause severe economic losses. Some of these phytopatogenic fungi can produce toxins that have harmful consequences for human health. Frequent application of synthetic pesticides, as control measures in the management of seed and seedlings diseases, is associated with resistance of these pathogens to synthetic pesticides (Rosslenbroich & Stuebler, 2000; Hahn, 2014), which increases production costs and polluting the environment.

Biological control is one of the most important alternative strategies (Karimi et al., 2012). The issues of fungal resistance, environmental pollution, and negative effects on human health can be significantly reduced by applying biological plant protection products. Several bacterial antagonists are used in plant protection, but as they live in nature close to pathogens, they need to be identified, isolated, amplified and correctly applied.

Important groups of microorganisms used in the biological control of fungal diseases are lactic acid bacteria (LAB) (Dalie et al., 2010; Laref & Guessas, 2013; Zebboudj et al. 2014). The application of plant growth promoting bacteria (PGPB), to control phytopathogens, has gained increasing attention, for example purple nonsulfur bacteria (PNSB) *Rhodopseudomonas palustris* strains have been mentioned as possible biocontrol agents (Nookongbut et al., 2019). Therefore they may be considered as commercial alternatives to chemical pesticides to manage plant diseases, provide food security and contribute to a sustainable agrosystem (Stamenković et al., 2018).

The objective of this study was to determine the antagonistic capacity of PGPB by evaluating the antifungal power of three combinations of lactic acid bacteria, a phototrophic bacterium and yeast *in vitro* and *in vivo* against the phytopathogenic fungi of tomato and pepper: *Fusarium oxysporum, Alternaria alternata, Botrytis cinerea*, *Colletotrichum* sp., *Verticilium dahlia* and *Pythium aphanidermatum*.

MATERIALS AND METHODS

Antagonistic activity of investigated mixtures

Double agar diffusion test. To evaluate the efficiency of three combinations of lactic acid bacteria (*Lactobacillus plantarum*, *Lactobacillus rhamnosus* >10^3CFU/g), phototrophic bacteria (*Rhodopseudomonas palustris* >10^3CFU/g) and yeast sugar molasses (*Saccharomyces cerevisiae* >10^3CFU/g), marked as: EM1, EM5 and EM AGRO (property of LUMAX - doo, Belgrade, products registered commercially as soil conditioners), an *in vitro* assay was performed on potato dextrose agar (PDA) to observe mycelial development of *F. oxysporum, A. alternata, B. cinerea*, *Colletotrichum* sp. (from a collection of the Institute for Plant Protection and the Environment, Belgrade), *V. dahliae*, and *P. aphanidermatum* (from a collection of the Institute of Pesticides and Environmental Protection, Belgrade), originating from tomato and pepper seeds. Mycelial disks (5 mm diameter) from 15-day old pure cultures of the investigated fungi were placed on PDA dishes. Twenty-four hours later, different bacterial combinations were added at 3 cm distance. Petri dishes with mycelia disks alone served as the positive control (K+). The dishes were incubated at 25°C. Three replicates were used for each treatment. After 7 days, mycelia diameter was measured in two directions. The percentage of inhibition of radial growth (PIRG) was calculated following the method of Al-Al-Hetar et al. (2011):

$$PIRG\% = [(R1-R2)/R1] \times 100\%$$

where R1= radial micelial growth on the control plate, and R2 = radial micelial growth on treated plates.

The results were statistically analysed using STATISTICA v. 6 (StatSoft, Inc.).

Microdilution test in vitro. Minimum inhibitory concentrations (MIC) of three combinations of lactic acid, phototrophic bacteria and yeast, marked as: EM1, EM5 and EM AGRO against *F. oxysporum, A. alternata, B. cinerea*, *Colletotrichum* sp., *V. dahliae*,. and *P. aphanidermatum,* were determined by microdilution using 96-well microtitre plates according to Balouiri et al. (2016) in a concentration range of 10 µl/ml - 1×10^{-9} µl/ml of each mixture. Fungal spores were washed from the surface of potato dextrose (PD) plates with sterile 0.85% saline solution containing 0.1% Tween 80 (v/v). Spore suspension was adjusted to a concentration of approximately 5×10^4 in the final volume of 100 µl per well with different dilutions of bacterial suspension. Microtitar plates were incubated for 5 days at 25°C. The experiment was repeated four times. Fluconazole (0.8 mg/ml) was used as a positive control. The lowest concentrations without visible growth were defined as the minimum concentrations inhibiting fungal growth.

Effects of tested mixtures on seed and seedling infection percentage

Filter paper test. Effects of two concentrations (100 µl/ml and 10 µl/ml) on the percentage of infection of tomato and pepper seed on filter paper were examined. Sixty seeds (20 in each of three repetitions) were soaked in the two concentrations and transferred to wet filter paper for two exposure periods lasting 3 h and 4 h. The percentage of infection was assessed 7 days after treatment. Seeds soaked in sterile water were used as a negative control.

In vivo (soil test). Untreated seedlings of tomato and pepper were planted in soil substrate, watered with 3 ml of tested mixtures at concentrations of 100 µl/ml and 10 µl/ml every 4 days during three weeks. The experiment was set up in three replications with 20 plants in each variant. An untreated control was watered with the same amount of water. The presence of disease was recorded after 15 days. The results were analysed using the statistical analysis package STATISTICA c. 6 (StatSoft, Inc.).

RESULTS

Antagonistic activity of investigated mixtures

Double agar diffusion test. All tested pathogens except *P. aphanidermatum* were inhibited by the mixtures investigated (Figure 1A,B). The investigated mixtures demonstrated the highest level of inhibition against the fungus *F. oxysporum* (30.3-38.4%), followed by *A. alternata* (28.0-30.4%), while no inhibition was observed against *P. aphanidermatum*. The mixture EM 5 showed the highest degree of inhibition of micelial growth of *F. oxysporum*, *A. alternata*, *B. cinerea* and *Colletotrichum* sp., and moderate inhibition of *V. dahlia*. The degree of interactions between the tested mixtures and pathogens was high (R=0.838).

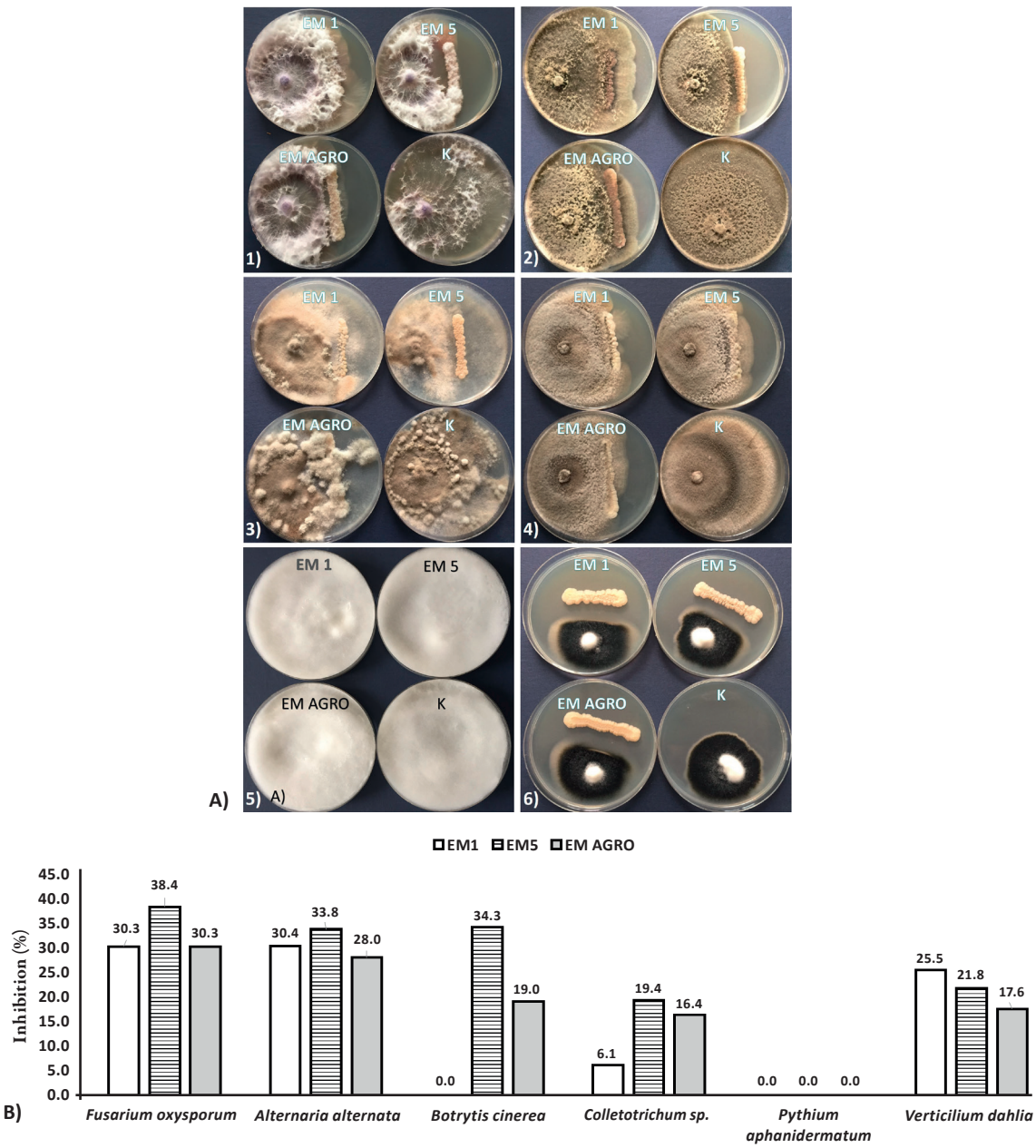


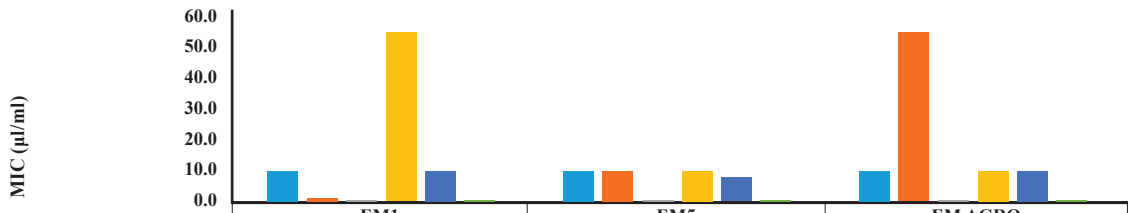
Figure 1. The effect of combinations of bacteria and yeast on mycelial growth inhibition of: 1) *Fusarium oxysporum*, 2) *Alternaria alternata*, 3) *Botrytis cinerea*, 4) *Colletotrichum* sp., 5) *Pythium aphanidermatum* and 6) *Verticilium dahlia* (A), and the percentage of micelial inhibition growth in dual cultivation test (B)

Microdilution test - minimum inhibitory concentration (MIC). The tested combination EM1 (Figures 2 and 3) showed its highest inhibitory effect on *B. cinerea* (MIC 1×10^{-3} µl/ml), *V. dahliae* (MIC 3×10^{-3} µl/ml), and *A. alternata* (MIC 1µl/ml); moderate against *F. oxysporum* and *P. aphanidermatum* (MIC 10 µl/ml), and the lowest on *Colletotrichum* sp. (MIC 55 µl/ml).

EM5 showed a uniform inhibition capacity against *F. oxysporum*, *A. alternata* and *Colletotrichum* sp. (MIC 10 µl/ml), slightly lower against *P. aphanidermatum* (MIC 7.75 µl/ml), and the lowest against *B. cinerea* (2.5×10^{-2} µl/ml) and *V. dahliae* (MIC 2.8×10^{-1} µl/ml).

EM AGRO inhibited the mycelial growth of *B. cinerea* and *V. dahliae* with its lowest concentration (MIC 1×10^{-1} µl/ml). A slightly higher concentration was observed to inhibit *F. oxysporum, Colletotrichum* sp. and *P. aphanidermatum* (MIC 10 µl/ml), and the least effect was observed towards *A. alternata* (MIC 55 µl/ml).

This experiment demonstrated the highest susceptibility of *V. dahlia and B. cinerea* (<1 µl/ml) to all tested mixtures, while *F. oxysporum* and *P. aphanidermatum* showed



	EM1	EM5	EM AGRO
Fusarium oxysporum	10.000	10.000	10.000
Alternaria alternata	1.000	10.000	55.000
Botrytis cinerea	0.001	0.025	0.100
Colletotrichum sp.	55.000	10.000	10.000
Pythium aphanidermatum	10.000	7.750	10.000
Verticilium dahliae	0.003	0.280	0.100

Figure 2. Minimum inhibitory concentration (MIC) for three combinations of bacteria and yeast determined for phytopatogenic fungi of tomato and pepper seed and seedlings

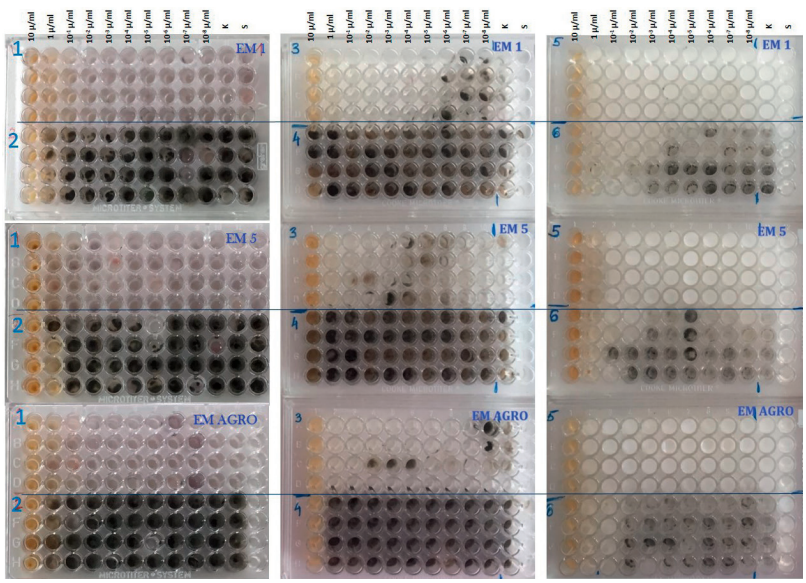


Figure 3 Minimum inhibitory concentration (MIC) of combinations of bacteria and yeast against phytopatogenic fungi: 1) 1) *Fusarium oxysporum*, 2) *Alternaria alternata*, 3) *Botrytis cinerea*, 4) *Colletotrichum* sp., 5) *Pythium aphanidermatum* and 6) *Verticilium dahlia*

satisfactory susceptibility to EM5 and EM AGRO. *A. alternata* and *Colletotrichum* sp. did not show satisfactory susceptibility to the tested combinations (EM AGRO, EM 1) (Figures 2 and 3).

Influence of tested combinations on infection percentage of seeds and seedlings of tomato and pepper

Effects of tested combinations on the percentage of infected tomato and pepper seeds (on filter paper). Experiment analysis showed that 15 of 20 tomato plants in the non-treated experiment were asymptomatic on average, while 19-20 of 20 plants (per repetition) were asymptomatic in the treated plates (Figure 4).

An analysis based on concentration and exposure time of seedlings to combinations revealed that an average of 15 pepper seedlings were asymptomatic in the non-treated control, while the number ranged from 15-20 (20 seedlings per repetition) in treatments. Only seedlings treated with EM AGRO at 100µl/ml concentration were infected as high as control seedlings, while all other treatments showed a significant decrease in infection (Figure 5).

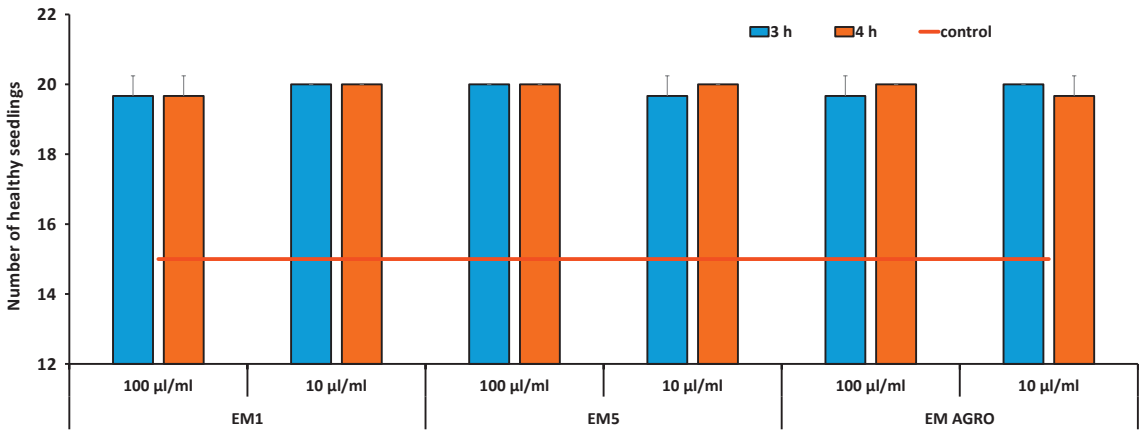


Figure 4. Effects of bacteria and yeast combination, treatment concentration, and exposure time on the number of asymptomatic tomato seedlings

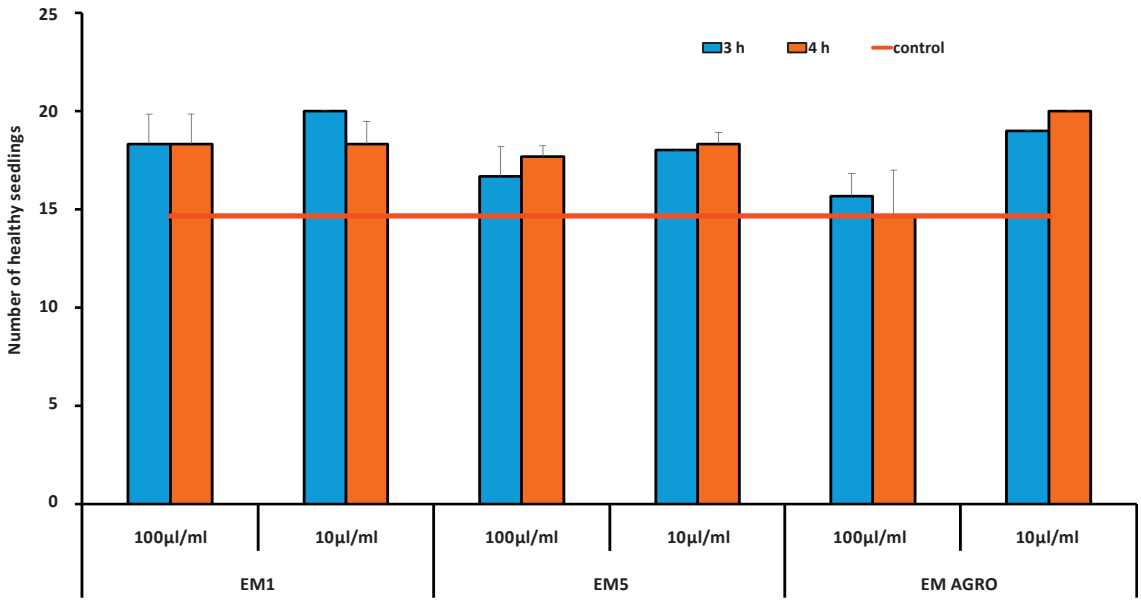


Figure 5. Effects of treatment, concentration, and time of exposure on frequency of asymptomatic pepper seedlings

Effects of tested combinations on the percentage of infection of tomato and pepper seedlings (soil test). The EM1 and EM5 treatments applied at both concentrations completely suppressed the occurrence of *Fusarium* sp. The treatment with EM AGRO completely suppressed the occurrence of *Pythium* sp. in tomato seedlings (Figure 6A). The most effective treatment was EM1 at both concentrations as it managed to suppress the occurrence of fungi of the genus *Fusarium*, as well as fungi of the genus *Pythium,* which appeared in 5% of the samples, while 20% appeared in control samples). Data analysis (Figure 6B) showed a statistically significant increase in the number of asymptomatic plants (14-18) treated with any of the three combinations, while an average of 9 asymptomatic plants were observed in the non-treated control.

The infection rate of *Fusarium* in non-treated control was 85%, while treatments with EM1 and EM5 at 100 µl/ml concentration completely suppressed these phytopatogenic fungi in pepper seedlings (Figure 7A). The highest efficacy in suppressing fungi of the genus *Pythium* was observed in the treatments EM1 and EM AGRO at 10 µl/ml concentration. Data analysis showed that pepper seedlings treated with any of the three combinations showed statistically significant 17-18 asymptomatic plants of the 20 tested, while an average of 4 asymptomatic plants were observed in the control treatment (Figure 7B).

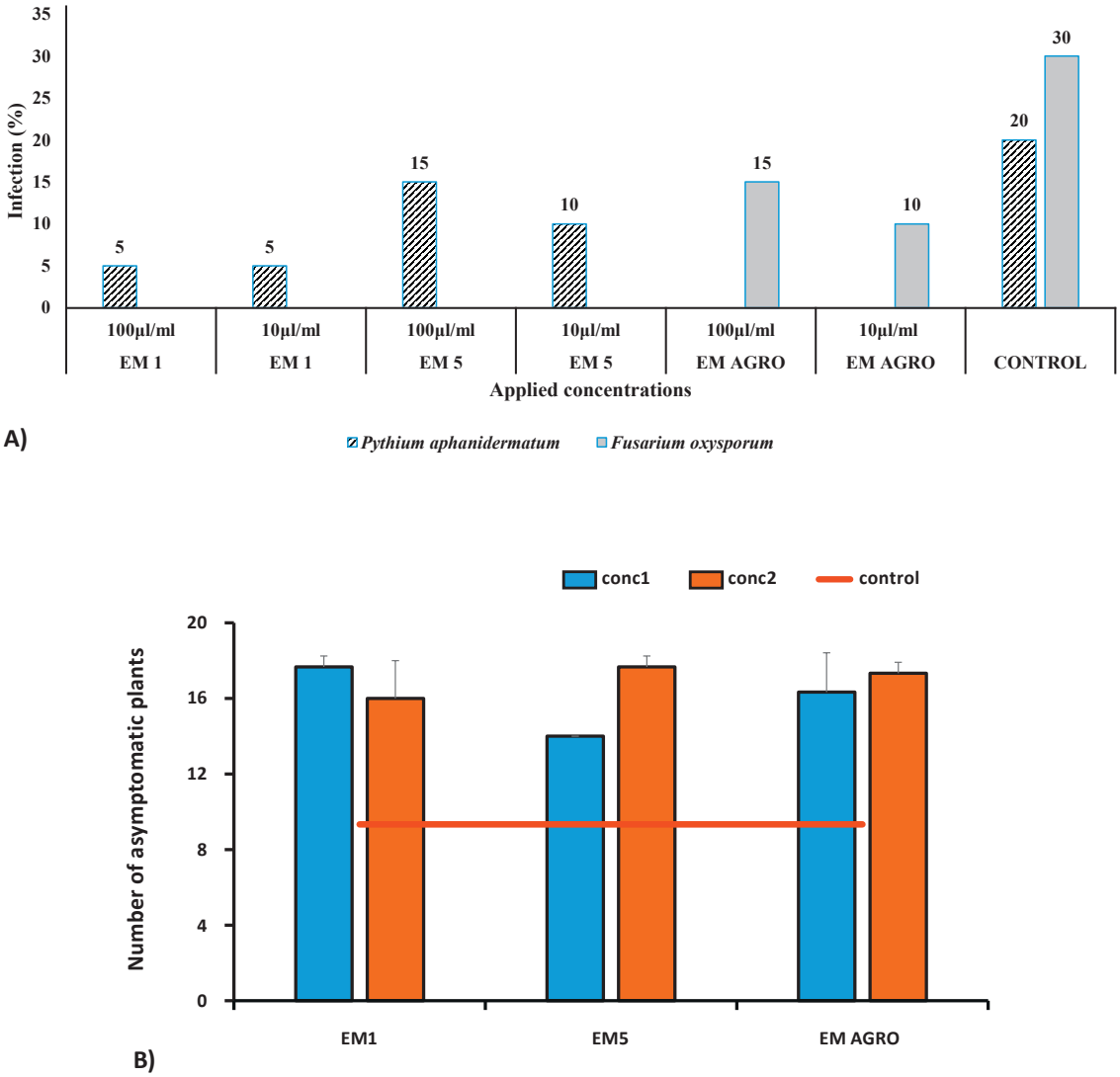


Figure 6. Effects of combinations on infection rate of tomato plants (A) and the number of asymptomatic plants (B)

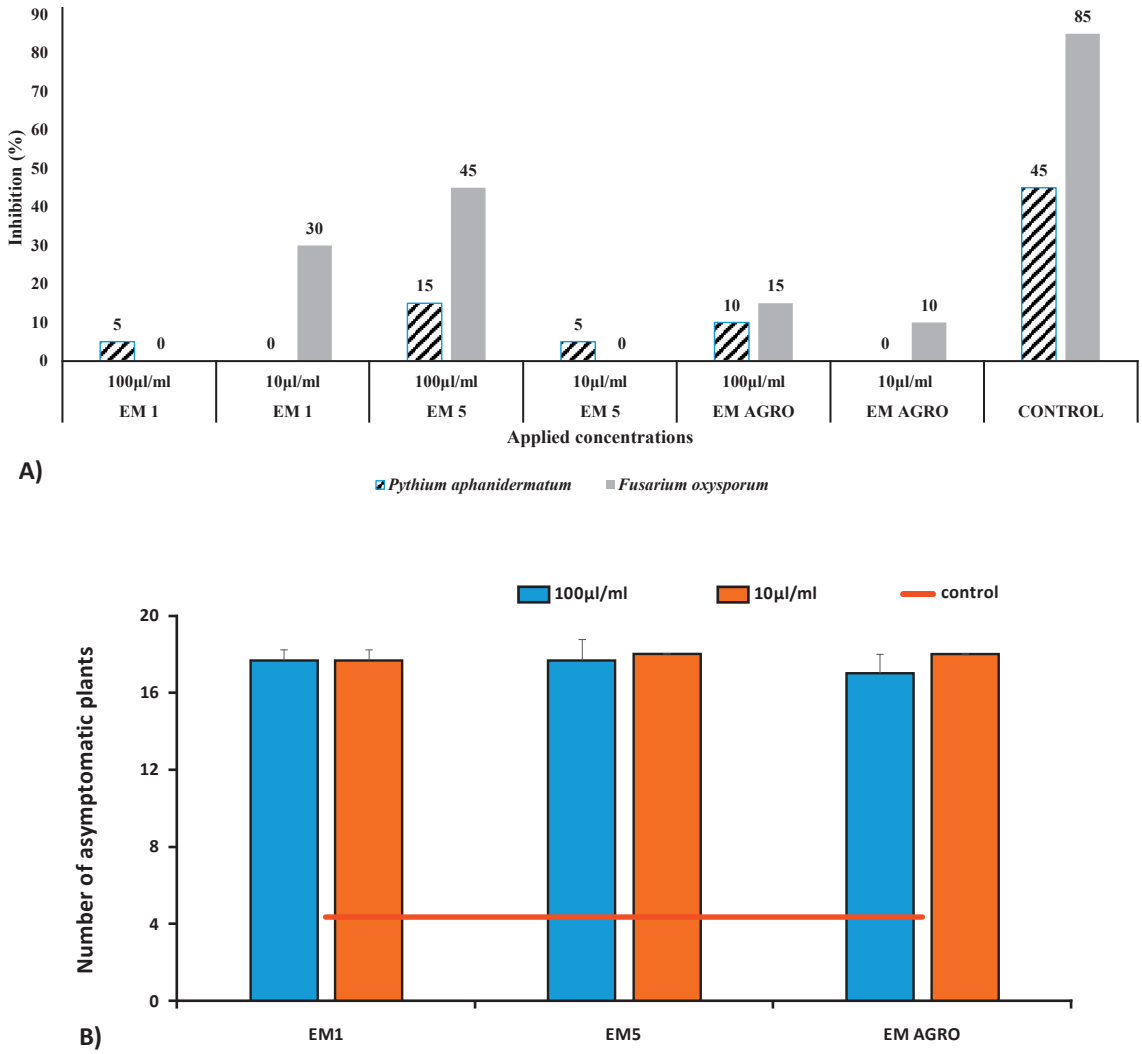


Figure 7. Effects of tested concentrations on the infection rate of pepper plants (A) and number of asymptomatic plants (B)

DISCUSSION

New, alternative strategies for biological control using lactic acid bacteria have been explored to understand the relation between pathogens and antagonistic bacteria in order to control many phytopatogenic casual agents, for example: *Fusarium* spp. (Lavermicocca et al., 2000), *A. alternata* (Zabouri et al., 2021), *B. cinerea*, *Monilinia laxa,* and *Penicillium expansum* (Trias et al., 2008).

Elsewere, the application of *R. palustris* as a plant growth promoting bacteria (PGPB), was shown to influence plant growth and combat plant patogens, such as *Magnaporthe oryzae* (Nookongbut et al., 2020).

Nally et al. (2012) published important data about the antifungal activity of yeast, *S. cerevisiae,* against *B. cinerea* on grapes, while Chand-Goyal and Spotts (1997) and Spadaro et al. (2004) examined it on apples not only at room temperature, but also in a refrigerated chamber. Several reports have mentioned the potential use and applications of different genera and species of antagonist yeasts to control *B. cinerea* on grape tissues (Lima et al., 1999; Castoria et al., 2001; Zahavi et al., 2000; Schena et al., 2000; Masih et al., 2001; Sesan et al., 1999). Other researchers have also reported biocontrol potentials of *S. cerevisiae* against *Penicillium roqueforti* in stored wheat (Petersson & Schnurer, 1995), *Macrophomina phaseolina* and *Fusarium solani* in tomato (Attyia & Youssry, 2001),

Monilia fructicola in apples (Zhou et al., 2008) and *A. alternata* in *Pinus silvestris* (Payne et al., 2000).

There are no reports in literature about combined antagonistic effects of lactic acid, phototrophic bacteria and yeast. The results of this study showed that a combination of different lactic acid bacteria (*L. plantarum*, *L. rhamnosus*), phototrophic bacteria (*R. palustris*) and yeast (*S. cerevisiae),* marked as EM5, demonstrated a strong antifungal effect against *F. oxysporum, A. alternata* and *B. cinerea.* The combination EM5 showed the highest rate of spore inhibition towards *F. oxysporum, A. alternata, B. cinerea*, *Colletotrichum* sp. and *P. aphanidermatum*. All three combinations, at both concentrations and exposure times, showed significant decrease in infection of tomato seeds on filter paper. The treatment EM1 applied at 10 µl/ml concentration over 3 h exposure time, and EM AGRO concentration of 10 µl/ml and 4 h exposure time achieved complete symptom suppression on pepper seeds on filter paper. Both concentrations of all three tested combinations reduced the percentage of tomato and pepper infection with the phytopatogenic fungi *Fusarium* sp. and *Pythium* sp.

Both concentrations of EM1 treatment showed significant efficacy on tomato, and 100 µl/ml concentration on pepper, as well as the lower concentration (10 µl/ml) of EM AGRO on tomato, and the lower concentration (10 µl/ml) of EM5 and EM AGRO on pepper.

Determination of efficacy of biological agents is of paramount importance for preserving ecosystem and human health, and represents the first step towards implementation of alternative, non-pesticide methods in plant protection.

A combination of bacteria and yeast named EM5 stood out in our current *in vitro* experiments as the combination with the highest antifungal potential.

In situ experiments on tomato and pepper seedlings showed a high potential of all combinations used, especially the lower concetrations (10 µl/ml), while the lowest rate of seedlings infection was achieved by applying the combination of EM1 (10 µl/ml-3 h) and EM AGRO (10 µl/ml-4 h).

The use of EM1 (at both concentrations) and EM AGRO (10 µl/ml) is recommended for tomato seedling protection. EM1 (100 µl/ml), EM5 and EM AGRO are recommended to be used at lower concentration (10 µl/ml) for pepper seedling protection.

The results obtained from *in situ* and *in vitro* experiments represent the basic principles for synthesizing biological plant protection products based on the tested combinations of bacteria and yeast, which could safely reduce the infection potential of important phytopathogenic fungi: *F. oxysporum*, *A. alternata*, *B. cinerea*, *Colletotrichum* sp. and *P. aphanidermatum*.

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Primena različitih kombinacija smeša mlečno kiselinskih, fototrofnih bakterija i kvasaca u suzbijanju patogena semena i klijanaca paradajza i paprike

REZIME

U radu je ispitivan antifungalni uticaj tri kombinacije smeša mlečno kiselinskih bakterija (*Lactobacillus plantarum*, *Lactobacillus rhamnosus*), fototrofnih bakterija (*Rhodopseudomonas palustris*) i kvasaca (*Saccharomices cerevisiae*) sa melasom šećerne trske označenih kao: EM1, EM5 i EM AGRO, *in vitro* i *in vivo* na fitopatogene gljive paradajza i paprike: *Fusarium oxysporum*, *Alternaria alternata*, *Botrytis cinerea*, *Colletotrichum* sp., *Verticilium dahliae* i *Pythium aphanidermatum*. Kombinacija bakterija i kvasca EM5, je u eksperimentima dvojne kultivacije ispoljila najviši stepen inhibicije porasta micelije *B. cinerea* (38.4%). U mikrodilucionom testu, kombinacija EM1 ispoljila je najveći inhibicioni efekat na *B. cinerea* (MIC 1×10^{-3} µl/ml), dok je EM5 pokazala ujednačen efekat inhibicije prema *F. oxysporum*, *A. alternata* i *Colletotrichum* sp. (MIC 10 µl/ml). Za zaštitu rasada paradajza preporučuje se upotreba EM1 (u koncentracijama 10 i 100 µl/ml) i EM AGRO (10 µl/ml). Za zaštitu rasada paprike preporučuje se upotreba EM1 (100 µl/ml), EM5 i EM AGRO u nižoj koncentraciji (10 µl/ml).

Ključne reči: paradajz, paprika, mlečno kiselinske bakterije, fototrofne bakterije, kvasci, antifungalni potencijal