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In vitro screening of antagonistic activity of microorganisms against anthracnose disease

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A B S T R A C T

Anthracnose disease can be successfully controlled by chemical pesticides in conventional fruit growing. However, in organic farming, in order to maintain environmental safety and fulfill consumer demand for pesticide-free food, the control of this disease is a major problem. The use of biocontrol agents with the antagonistic mechanism of control of many phytopathogens is an innovative alternative for cost-effective and eco-friendly production. The antagonistic activities of *Trichoderma viride* and *Bacillus subtilis* were tested *in vitro* against *Colletotrichum acutatum*, one of major plant pathogens responsible for anthracnose. The microbial antagonists inhibited mycelial growth in the dual culture. *T. viride* exhibited strong antagonism against *C. acutatum* isolates (80%). The *B. subtilis* isolate also had a strong effect on inhibiting the development of *C. acutatum* (37.5%). The results of this study identified *T. viride* and *B. subtilis* as promising biological control agents for further testing against anthracnose disease in fruits.

Keywords: Antagonistic action, *Colletotrichum acutatum*, *Trichoderma viride*, *Bacillus subtilis*.

И З В О Д

У конвенционалној воћарској производњи антракноза се може успешно контролисати коришћењем хемијских пестицида. Међутим, у циљу очувања животне средине и задовољавања растућих потреба потрошача за здравствено безбедном храном, контрола ове болести представља један од већих проблема у органској пољопривредној производњи. Употреба биолошких средстава која се одликују антагонистичким механизмом дејства против великог броја биљних патогена представља иновативну алтернативу за економичну и еколошки прихватљиву воћарску производњу. У раду су приказани резултати *in vitro* испитивања антагонистичких својстава сојева *Trichoderma viride* и *Bacillus subtilis* у борби против једног од најзначајнијих проузроковача антракнозе биљака – *Colletotrichum acutatum*. Микробни антагонисти су инхибирали раст мицелија у дуалној култури. *T. viride* је испољила снажан антагонизам у односу на изолат *C. acutatum* (80%). Изолат *B. subtilis* такође је имао снажан ефекат на инхибицију развоја *C. acutatum* (37,5%). Резултати ове студије показали су да сојеви *T. viride* и *B. subtilis* представљају обећавајуће биолошке агенсе погодне за даља испитивања у борби против антракнозе у плодовима.

Кључне речи: Антагонистичка активност, *Colletotrichum acutatum*, *Trichoderma viride*, *Bacillus subtilis*.

1. Introduction

The phytopathogenic fungi of the genus *Colletotrichum* are the most significant causative agents of fruit anthracnose disease, leading to losses of up to 80% (Grahovac et al., 2012). Common hosts include many different cultivars of small, pome, stone and citrus fruit species, as well as olives (Denoyes-Rothan et al., 2003; Talhinhas et al., 2015; Guarnaccia et al., 2017; Fu et al., 2019). The symptoms appear as water soaked lesions, which are covered with salmon-colored spore masses. There are several synthetic fungicides such as propiconazole, difenoconazole, carbendazim, benomyl, maneb, captain, etc. which are successfully used in pre-harvest anthracnose control (Ali et al., 2016). However, the most important losses occur during fruit infection after harvest, storage, transport and marketing, resulting in large economic losses. Despite the long list of fungicides available for the control of anthracnose in conventional protection, there are still problems of finding effective preparations that could inhibit high population densities in a short period of time or provide permanent resistance of host plants. In addition, the problem is further complicated by the fact that their use can have a harmful effect on the environment and human health (Saha et al., 2012). The problem is additionally complicated in organic systems since there are very few products with limited efficacy which are suitable for the control of this disease. All this together affected the business related to the export and import of food, which consequently influenced the governments of certain countries to ban their use. Therefore, finding some alternative approaches for anthracnose control that are non-toxic to the environment and without harmful implications for human health has become one of the priority research tasks (Paa et al., 2020). One such alternative is the use of microorganisms. Since species of the genera *Bacillus* and *Trichoderma* are known for their mycoparasitic and antagonistic mechanism, they could be an alternative to chemical fungicides and serve as an innovative, cost-effective and environmentally friendly approach in suppressing this disease.

Because of their resistance to most chemical pesticides, *Trichoderma* species can serve as good biocontrol agents (Harman, 2011; FRAC, 2016). They act through various complex mechanisms, relying mainly on mycoparasitism, production of antibiotics and/or hydrolytic enzymes and competition for nutrients and space (Hovel, 2003), which makes them extremely aggressive against a wide range of phytopathogenic fungi (Vinale et al., 2008).

Furthermore, some strains from *Trichoderma* genus can elicit local and systemic host immune response against pathogenic microorganisms (Shoresh et al., 2010).

In addition, some species belonging to the genus *Bacillus*, besides having plant growth promotion properties (Pešaković et al., 2020), can also serve as good biocontrol agents. They are a group of aerobic or facultative anaerobic bacteria that form spores and have the ability to grow in a wide range of environmental conditions, including even adverse ones (Logan et al., 2007). Stein (2005) and Pal and McSpadden Gardene (2006) identified strains of the genus *Bacillus* as producers of antifungal and antibacterial substances such as catalytic enzymes as well as peptide antibiotics. Among the genus Bacillus, B. subtilis is the most studied species, primarily due to its biocontrol potential (Asaka and Shoda, 1996; Krebs et al., 1998; Lin et al., 2001).According to the Food and Drug Administration (1999), representatives of the species *B. subtilis* are harmless strains with "GRAS" status, making them good bio-control agents.

The objective of this study was to identify potential biocontrol agents suitable for organic management by *in vitro* evaluating the antagonistic activity of natural isolates of *Trichoderma spp.* and *Bacillus spp.* against the anthracnose disease caused by phytopathogenic fungus *Colletotrichum spp.*

2. Materials and methods

Test organisms

To determine the antimicrobial activity of native *Trichoderma* spp. and *Bacillus* spp. strains, the isolate of *Colletotrichum acutatum* (C.A.2) was used as an indicator strain. The pathogen isolate was obtained from the culture collection of the Faculty of Agriculture in Kruševac, University of Niš, Serbia. It was isolated from strawberries grown in Serbia. The isolate was identified on the basis of morphological, pathogenic and molecular characteristics.

Antagonistic organisms

The *Trichoderma viridae* and *Bacillus subtilis* isolates used in the study were isolated from soil samples. Strains were previously identified based on colony morphology and biochemical properties using standard methods. The isolates were stored on nutrient agar (NA) at 4˚C for *Trichoderma* and Luria-Bertani (LB) broth with 30% glycerol at -20 ºC for *Bacillus* until further use. The working cultures of *Trichoderma* and *Bacillus* strains were prepared by transferring stock agar plugs containing mycelium/bacteria colony onto Potato Dextrose Agar (PDA) and LB plates and incubating them for 5 days at 25˚C and at 30°C, respectively.

Antagonism assay

According to Skidmore and Dickinson (1976), dual culture testing was used for the screening of the antagonism of *T. viride* against the pathogenic isolates. For that purpose, after the incubation period of 5 days, an agar disc (6 mm) of *T. viride* was transferred to one side of PDA plates. At the same time, an agar disc (6 mm) of *C. acutatum* was transferred to the same Petri plate at a distance of 4 cm from *T. viride* and 2 cm from the periphery of the Petri plate. The effect of *T. viride* against the pathogenic fungus was tested for 10 days at 30ºC±2 until the fungus had achieved equilibrium, beyond which there was no further alteration in mycelial growth. Controls were also maintained in which the pathogenic fungus (*C. acutatum*) was grown independently on sterilized PDA medium. The radial growth of fungal colonies was measured (mm) and the percentage of growth inhibition (PGI) was calculated as follows:

$$PIG\ (\%) = (KR - R1)/KR \times 100,$$

KR - radial growth of the fungus from the center of the colony to the center of the Petri dish without antagonistic fungus; R1 - growth of the fungus from the center of the colony towards the antagonistic fungus placed in the center of the Petri dish.

The interaction between *Colletotrichum acutatum* and *Trichoderma viride* was evaluated according to the Porter (1924) and Dickinson and Broadman (1971) models. Scaling was done into five types of interaction degrees according to Skidmore and Dickinson (1976): Grade 1 - Intermingling without any macroscopic signs of interaction; Stage 2 - Growth of intermingling, where the growth of the fungi is stopped by the growth of the opposite fungi; Grade 3 – mixed growth, where the observed fungi grows on the opposite fungi above or below; Grade 4 – visual inhibition of both fungi in interaction with a narrow line of demarcation; Grade 5 – mutual inhibition of growth at a distance of >2mm).

To evaluate the antagonism between *Bacillus subtilis* and *Colletotrichum acutatum*, a double culture method was used, in three replicates according to Fokkema (1978). For this purpose, 6 mm disks of the tested pathogen (*C. acutatum*) were placed on one side of the PDA plates, while a loop of overnight bacterial culture (*B. subtilis*) was placed at a distance of 2 cm from the edge of the same Petri plate. Plates inoculated only with cultures of the pathogen *C. acutatum* served as controls. Incubation lasted 8 days at 30±2 ºC. After that, the antagonistic potential of the bacterial strain was measured as the size of the zone of growth inhibition and expressed in *mm*. Percent growth inhibition (PGI) was calculated according to Rahman et al. (2007) and Saria (1994):

PGI (%) = (R1 – R2)/R1 × 100,

R1 - radial growth of the pathogen in the control plate;
R2 - radial growth of the pathogen.

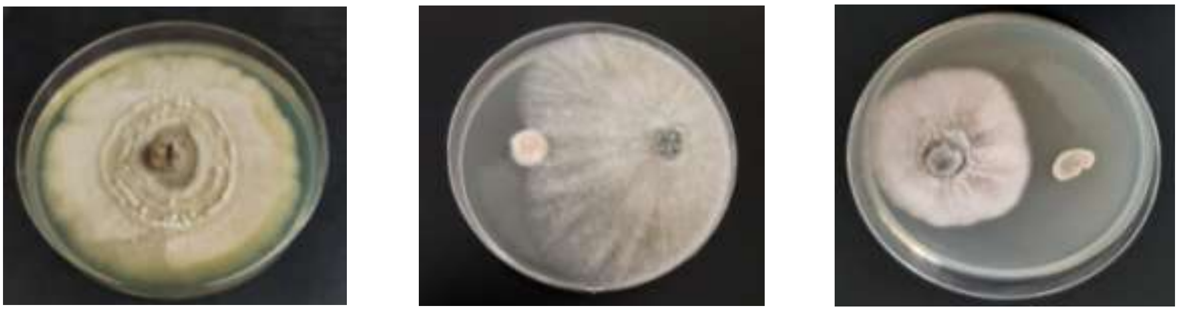
3. Results and discussions

According to the obtained data, both tested strains (*T. viride* and *B. subtilis*) inhibited the growth of *C. acutatum* mycelia (Tables 1, 3).

The present study revealed a notably antagonistic effect of *T. viride* on *C. acutatum* (80.0%) (Table 1). The radial growth of *Colletotrichum acutatum* in the control and test Petri plates is shown in Figure 1.

Table 1.
In vitro antimicrobial activity of *Trichoderma viride* strain against *Colletotrichum acutatum*

Phytopathogenic fungus	Radial growth in the test Petri plate (R1)	Radial growth in control (R)	Growth inhibition
	mm		(%)
C. acutatum	8	40	80.0



A B C

Figure 1. The effect of the antagonistic microbes *Trichoderma viride* and *Bacillus subtilis* inhibiting the mycelial growth of *Colletotrichum acutatum* isolate C.A.2: (A) control; (B) antagonistic isolate *Trichoderma viride;* (C) antagonistic isolate *Bacillus subtilis.*

When evaluating the antagonistic potential of *T. harzianum* against several soil-borne pathogenic fungi, Lone et al. (2012) determined the presence of an inhibition zone around the colonies of the examined pathogenic fungi.

Moreover, they found that the formation of inhibition zones is the result of the production of antibiotic substances by both antagonistic and phytopathogenic fungi (Lone et al., 2012).

Based on the results shown in Table 2, the interaction between *T. viride* and the phytopathogen *C. acutatum* can be graded as Grade 2: Mutual intermingling growth, where the growth of *C. acutatum* was ceased by *T. viride*.

Table 2.
Grade of interaction of *Colletotrichum acutatum* with *Trichoderma viride* strain

Phytopathogenic fungi	Grade of interaction with *T. viride*
C. acutatum	2

The antagonistic activity of native *B. subtilis* strain against *C. acutatum* is shown in Table 3. *B. subtilis* inhibited *C. acutatum* mycelium growth by 37.5%.

Table 3.
In vitro antimicrobial activity of *Bacillus subtilis* strain against *Colletotrichum acutatum*

Phytopathogenic fungi	Radial growth in the test Petri plate (R1)	Radial growth in control (R)	Growth inhibition
	mm		(%)
C. acutatum	25	40	37.5

The antagonistic activity of *B. subtilis* can be attributed to its capability to produce a wide diversity of antimicrobial metabolites.

According to Chet et al. (1990) and Than et al. (2004), a large group of bacteria from the rhizosphere is able to produce a wide range of secondary metabolites, such as siderophores, lytic enzymes, antibiotics and cyanides. The mentioned metabolites are of particular importance in their antagonistic activities against numerous plant pathogens.

In accordance with the above is the study of Milijašević-Marčić et al. (2018) who pointed out that strains of *B. subtilis* B-348 showed strong antagonistic activity against bacterial pathogens of tomato and fungal pathogen of pepper.

Han et al. (2015) reported that certain strains of *Bacillus atrophaeus*, (LB14, HM03 and HM17), produced large amounts of chitinase and protease enzymes, as well as one strain of B. *amyloliquefaciens*

(LB01) which, in addition to protease enzymes, also produced cellulase enzymes.

The study also showed that strain LB14 was the most effective in suppressing anthracnose on pepper fruits. In a similar way to our results, the authors also showed that *Bacillus* strains are capable of producing antagonistic compounds and suppressing the conidial germination of *C. acutatum* and *C. gloeosporioides*.

4. Conclusions

This study has selected natural isolates of *Trichoderma viride* and *Bacillus subtilis* as promising biocontrol agents against *Colletotrichum acutatum*. Using these strains could facilitate organic, integrated and sustainable fruit growing management. Additionally,, the results of present study represent a good basis for the development of substitute of chemical based fungicides with bio-fungicides that are composed of living microorganisms like bacteria and fungi as active ingredients and are effective against the pathogens that cause anthracnose and suitable for usage in organic agricultural production.

However, to develop a sustainable and effective pathogen control method, a number of further studies involving many biotic and abiotic factors in the ecosystems have to be carried out.Future research should, therefore, investigate the mechanisms underpinning this control aiming in the next step to undertake in vivo testing of the efficacy of the selected strains.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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