IN VITRO ANTAGONISTIC ACTIVITY OF TRICHODERMA SPP. TO FUSARIUM OXYSPORUM AND FUSARIUM GRAMINEARUM

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Abstract

One of the major problems in agriculture are *Fusarium* species which cause fusariosis in wheat, corn, tomato, melon, watermelon, onion, peas, and beans. Also, *Fusarium* can synthesize thermostable mycotoxins which can lead to certain diseases if they were found in food. Currently, chemical fungicides are mostly used to prevent the occurrence of fusariosis disease, but the resistance of pathogens to such treatment is being more and more widespread. Soil microorganisms help in plant defense and growth. The rhizosphere fungi have an important role in the mutual exchange of nutrients with plants and they can establish specific interactions with plants. Such rhizosphere fungi are known as plant growth-promoting (PGP). These PGP fungi affect the better plant growth through the synthesis of certain phytohormones but they also have the function as biocontrol agents. They can inhibit the growth of phytopathogens through direct mechanisms of antibiosis, mycoparasitism, and competition.
The goal of this research was an investigation of the antagonistic effect of *Trichoderma harzianum* TR1 and *Trichoderma citrinoviride* 1V on *Fusarium oxysporum* and *Fusarium graminearum*. The antagonistic effect was examined through two tests: Dual culture test (DUAL test) and the effect of volatile organic compounds (VOCs) of *Trichoderma* strains on the growth of *Fusarium* strains. Also, the cell-wall degrading enzymatic activity of *T. citrinoviride* and *T. harzianum* was examined. The results showed that antagonistic activity of *Trichoderma* strains varies from moderate to high. Mycelial growth inhibition by *T. citrinoviride* was 44% for *F. graminearum* and 67% for *F. oxysporum*. *T. harzianum* inhibited *F. gramminearum* growth for 59% and *F. oxysporum* for 66%. Based on the results, it was concluded that *T.harzianum* and *T.citrinoviride* can be considered biocontrol agents for *F. oxysporum* and *F. graminearum*.

Keywords: *Fusarium oxysporum*, *Fusarium graminearum*, *Trichoderma harzianum*, *Trichoderma citrinoviride*, VOCs, *biocontrol agent.*

Introduction

The pesticides are very important in modern agriculture and economy and they are used to prevent spread of the plant disease. Some of the consequences of their excessive use are pollution of soil, surface water, groundwater, the emergence of resistance to pesticides, the emergence of diseases in humans and animals, etc. One of the possible solutions for solving this problem is using microorganisms as biocontrol agents. Biocontrol agents belong to the group of Plant Growth Promoting Microorganisms (PGPM) which have a number of positive effects on the plant such as the supply of nutrients to plants and phytopathogen suppression (Sousa et al., 2020). The use of biocontrol agents in agriculture are considered effective enough to compare vith chemisal fungicides (Hyder et al., 2017).

Fungi are among the most significant biocontrol agents, especially repsesentatis of the genus *Trichoderma* which have been shown as a good biofungicides. The *Trichoderma* strains have proven to be successful antagonists of *Fusarium sp*., which is one of the major pests in the field because it synthesizes persistent mycotoxins (Sharma I. P. and Sharma A. K., 2020). The genus *Trichoderma* have been known as biocontrol agents since 1920, it synthesize antibiotics and used for pest control as well as to increase yields (Chaparro et al., 2011). More than 60% of all biofungicides, are those that contain *Trichoderma* species (Abbey et al., 2019). The *Trichoderma* shows antagonistic interactions with phytopathogens based on several models like mycoparasitism, antibiosis and competition.

Volatile organic compounds (VOCs) are molecules of low molecular weight, lipophilic, easily volatile at room temperature and atmospheric pressure. Their basic role is to enable communication between fungi and other organisms. Over 300 VOCs of fungal origin have been identified so far and many of them are synthesized by different species of *Trichoderma* (Moya et al., 2018). A very important group of metabolites is peptaibol, which is synthesized in large quantities by fungi which are able to induce systemic resistance of the plant to pathogen attack (Mukherjee et al., 2012).

Trichoderma harzianum has proven to be the most effective biocontrol agent so far. It was noted that the antagonistic activity of *T. harzianum* (isolated from soil) versus *Fusarium oxysporum* is greater than 50% (Redda et al., 2018). The *T. citrinoviride* inhabits soil and it synthesizes enzymes that contribute to degradation phytopathogens cell wall.

Fusarium species are very important for agriculture as phytopathogens but also as mycotoxin producers and as opportunistic pathogens for humans. All *F. oxysporum* strains are saprophytes and can survive for a long time in soil with a lot of organic matter (rhizosphere). Interaction between *Trichoderma sp*. and *Fusarium sp*. is manifested through attraction, binding, twisting and lysis by hydrolytic enzymes or secondary metabolites (Mukherjee et al., 2012). During the interactions between *Trichoderma* sp. and *Fusarium* sp. it is forming apresoria which indicate mycoparasitism. The strains *T. virens*, *T. harzianum*, and *T. viride* produce about fifteen volatile compounds that have shown inhibitory effects on *F. oxysporum*.

The aim of this paper is determination the antagonistic activity of two representatives of the genus *Trichoderma*, *T. citrinoviride* and *T. harzianum* against *F. oxysporum* and *F. graminearum*. The ability to produce hydrolytic enzymes important in the manifestation of biocontrol potential was also investigated.

Materials and methods

Trichoderma citrinoviride TR1, *Trichoderma harzianum* 1V, *Fusarium oxysporum* and *Fusarium gramineraum* come from the collection of the Department for Microbial Ecology, Faculty of Agriculture, University in Belgrade, Serbia. The fungal strains were stored in Potato Dextrose Broth (PDB, Himedia, India) with 20% glycerol at -80°C. The working culture was maintained on Potato Dextrose Agar (PDA, Himedia, India), with occasional sieving on fresh medium, at a temperature of 25 °C.

1. Dual test

The nutrient media used in the dual test were: 1. Rose Bengal (RB) with the addition of streptomycin (streptomycin sulfate was added in the amount of 0.033 mg/1000 ml of media) which was prepared according to Pepper et al. (1995) and 2. PDA (Himedia, India). Fungal isolates were refreshed by sieving on PDA and RB medium and incubating at 25 °C (Binder,

Lithuania) for 3 (*Trichoderma sp*.) and 5 days (*Fusarium sp*.). Then, 0.5 x 0.5 cm mycelial discs were cut from the periphery of the colony, and placed on new PDA plates. The following treatments have been set: 1. *T. harzianum* 1V vs *F. oxysporum* 2. *T. harzianum* 1V vs *F. graminearum* 3. *T. citrinoviride* TR1 vs *F. oxysporum* 4. *T. citronoviride* TR1 vs *F. graminearum.* Each test was repeated twice. The control was represented by individual cultures of *F. graminearum, F. oxysporum, T. citronoviride* TR1, *T. harzianum* 1V on RB and PDA. The incubation lasted 5-7 days at 25 °C (Binder, Lithuania). The diameters of the colonies of *F. oxysporum* and *F. graminearum* were measured in a dual test and control Petri plates and the percentage of growth inhibition (MGI) was obtained according to the formula:

MGI (%) = ((DC − DT)/DC)) × 100, where MGI is mycelial growth inhibition, DC is the average diameter of a fungal colony of the control group, and DT is the average diameter of a fungal colony of the treatment group (Mohareb et al., 2017).

The antagonistic levels were classified as low (MGI 50%); medium (50% < MGI ≤ 60%); high (60% < MGI ≤ 75%); and very high (MGI > 75%) (De la Cruz et al., 1992).

2. Biochemical Characterization of* Trichoderma spp. *Antifungal Activity

The biochemical characterization of *Trichoderma* spp. antifungal activity included the determination of cell wall-degrading enzymes and the production of siderophores. A semiquantitative determination of cell-wall degrading enzymes (lipase, esterase-lipase, N-acetyl-β-glucosaminidase and β-glucosidase) was performed using an API ZYM kit according to the manufacturer's protocol (BioMereux, Craponne, France).

The presence of cellulase was determined using carboxymethyl cellulose (CMC) agar method in three repetitions (Romsaiyud et al., 2009). Siderophore production was detected on the Chrome azurol S (Sigma-Aldrich, St. Louis, USA) agar medium in three repetitions (Gezgin et al., 2020). The chrome azurol S (CAS) agar plates were inoculated with 5-mm-diameter mycelia discs of three *Trichoderma* isolates and incubated at 28 °C for 72 h. The appearance of yellow-orange halo zones around colonies was considered as a positive result.

3. The Effect of Trichoderma VOCs on Mycelial Growth of F. oxysporum i F. graminearum

The effect of VOCs produced by *Trichoderma* strains on the mycelial growth of *F. oxysporum* and *F. graminearum* was tested using the method of confronted cultures without contacts of the two mycelia (Dennis and Webster, 1971). The two Petri dishes containing 20 mL of PDA or RB were individually inoculated with 5-mm-diameter mycelia discs of a pathogen (*F. oxysporum* and *F. graminearum*) and an antagonist (*Trichoderma* spp.). Inoculated plates were sealed with Parafilm®, arranged to face each other and incubated at 25 °C in a microbiological incubator (Binder, Tuttlingen, Germany) in the dark, until fungi in the control plates (plates with individual fungi, negative control) reached edges of plates. The experiment was repeated three times. The effects of volatile metabolites were estimated through MGI.

4. Statistical Analyses

The data were subjected to ANOVA followed by Tukey's HSD post-hoc comparison tests to determine if there were statistically significant differences between the means ($p = 0.05$). All statistical analyses were performed using Statistica 12.0 (StatSoft, Tulsa, OK, USA).

Results and discussion

About 60% of biocontrol agents are based on different strains of the genus *Trichoderma* which was first time isolated in 1794 from soil and compost (Sood et al., 2020).

The both species of *Trichoderma* were shown the ability to inhibit mycelia growth of *F. oxysporum* and *F. graminearum* (Table 1).. The highest degree of *F. oxysporum* inhibition was recorded in the dual test with *T. citrinoviride* TR1 on RB medium (67%) while the percentage of inhibition on PDA was slightly lower. According to Sookchaoy et al., (2009) scale such activity was characterized as high in both cases. The T. *harzianum* 1V also had shown a high level of antagonistic activity. The highest degree of *F. graminearum* inhibition was observed with T. *citrinoviride* TR1 on PDA and it was characterized as a high degree. The other effects of *Trichoderma* representatives were characterized as a moderate antagonistic activity. The only exception is the response of *F. graminearum* to *T. citrinoviride* TR1 on RB and in this dual test the lowest degree of pathogen growth inhibition was recorded. This result emphasizes the importance of environmental conditions on the final result achieved by the presence of biocontrol agent.

Table 1. Growth inhibition of *F. oxysporum* and *F. gramminearum* by *T. citrinoviride* TR1 and *T. harzianum* 1V

Treatment	Percentage of inhibition RB	Percentage of inhibition PDA
T. citrinoviride TR1/*F. oxysporum*	67%	61%
T. citrinoviride TR1/*F. graminearum*	44%	63%
T. harzianum 1V/ *F. oxysporum*	66%	62%
T. harzianum 1V /*F. gramminearum*	59%	60%

In agriculture, the genus *Trichoderma* was used for control phytopathogens such as *Fusarium* (Sivan et al., 1986). Fan et al., (2020) proved that the *T. citrinoviride* strain Snef1910 shows antagonistic activity *in vitro* against pathogens that cause diseases of wheat, cotton, melon and other plants. This strain inhibits the growth of *F. graminearum* by 60.76%, *F. oxysporum* by 49.28%, *Fusarium monihforme* by 21.73%, and *Fusarium roseum* by 25.20%. Some literature data showed that antagonistic activity of *T. harzianum* versus *F. oxysporum* could be greater than 50% (Redda et al., 2018).

The zones of inhibition growth and increased colony pigmentation were observed in response of the phytopathogen to the presence of a biocontrol agent. The transparent inhibition growth zone (0.4 cm) was observed on RB medium with *T. citrinoviride* TR1 and *F. graminerarum*, which indicates antagonistic activity of diffuse non-volatile compounds and the same was also observed on PDA medium with *T. harzianum* 1V. The increased pigmentation of *F. graminearum* colonies near the zone of inhibition growth was also noticed. Both of the *Trichoderma* species have high antagonistic activity against *F. oxysporum*. The transparent zone of inhibition growth was noticed on all media in response of phytopathogens to the presence of diffuse non-volatile compounds, mostly originating from *T. citrinoviride*.

Similar with our result, some authors showed that certain strains like *T. harzianum* Q710613 can inhibit *F. graminearum* by 73-75% so it possesses high antagonistic activity on PDA medium (Tian et al., 2016), *T. harzianum* can inhibit the growth of *F. oxysporum* by 53% (moderate antagonistic activity) (Sundaramoorthy and Balabaskar, 2013) whereas *T. citrinoviride* inhibits *F. oxysporum* growth by 50% and 56%, with mild to moderate antagonistic activity (Redda et al., 2018).

The effect of VOCs on the growth of *F. graminearum* and *F. oxysporum*

The VOCs emitted by *Trichoderma* in the presence of *F. oxyposrum* and *F. graminearum* resulted in inhibition of phytopathogen mycelia growth in the range of 13–36% for *F. oxysporum* and 26–41% for *F. graminearum* (Table 2). The antagonistic activity between *T. harzianum* 1V and *T. citrinovirie* TR1 differs by two phytopathogens. Thus, *T. harzianum* 1V showed a better percentage of mycelial growth inhibition for *F. oxysporum* and *F. graminearum* compared to *T. citrinoviride* TR1. Macroscopically observed, increased pigmentation was noticed on the RB medium for phytopathogens in the central part of the colony while it was lost at the edges of colony.

Based on the results, it is concluded that the degree of inhibition caused by VOCs varies and that all obtained values could be classified into low antagonistic activity. However, it is undeniable that inhibition is present.

Table 2. Growth inhibition of *F. oxysporum* and *F. gramminearum* by *T. citrinoviride* TR1 and *T. harzianum* 1V volatile organic compounds

Treatment	Percentage of inhibition PDA	Percentage of inhibition RB
T.citrinoviride /F.oxysporum	13%	23%
T. citrinoviride /F. graminearum	39%	32%
T.harzianum / F.oxysporum	36%	31%
T. harzianum /F.gramminearum	26%	41%

Semiquantitative analyses of *Trichoderma* spp. enzymatic profiles showed the ability of these fungi to produce lipase and esterase-lipase and β-glucosidase at a moderate level as well as high amounts of N-acetyl-β-glucosaminidase (Table 3). The results showed appearance of bright area around the colonies that confirmed the cellulolytic activity of *T. harzianum* 1V and *T. citrinoviride* TR1.

Table 3. Biochemical characteristics of Trichoderma spp.

Enzymes	*Trichoderma citrinoviride* tr1	*Trichoderma harzianum* 1v
Lipase	2	2
esterase-lipase	2	2
N-acetyl-β-glucosaminidase	3	3
β-glucosidase	2	3
cellulase	+	+
siderophores	+	+

1—low production, 2—moderate production, and 3—high production according to the API ZYM reading color scale; + positive react

The results showed appearance of bright area around the colonies that confirmed the cellulolytic activity of *T. harzianum* 1V and *T. citrinoviride* TR1. The production of those enzymes is especially important since chitin and glucan, common constituents of fungal cell-walls, are susceptible to chitinase, N-acetyl-β-glucosaminidase, and β-glucosidase (Karličić et al., 2021). Also, N-acetyl-β-glucosaminidase is already well known as inhibitor of *Botrytis cinerea* spore

germination (Lorito et al., 1994), Literature data confirm *T. citrinoviride* as producer of strong cellulases (Park et al., 2019). The CAS assay confirmed the ability of *Trichoderma* spp. to produce siderophores which are an important mechanism of biocontrol (Chen et al., 2021).

Conclusion

Both tested biocontrol agents (*T. citrinoviride* TR 1 and *T. harzianum* 1V) showed a high percentage of antagonistic activity against phytopathogens (*Fusarium oxysporum* and *Fusarium graminearum*). The results of cellulolytic activity showed that *T. citrinoviride TR 1* and *T. harzianum 1V* produce cellulase. In the DUAL test, different results were obtained depending on the medium on which they were grown. As a response of the phytopathogen to the presence of the biocontrol agent, transparent zones of inhibition and increased pigmentation of the phytopathogen colony were observed. The test of inhibition by VOCs originating from *T. citrinoviride TR1* and *T. harzianum 1V*, showed positive results and their percentage of antagonistic activity was low. Mycelial growth inhibition values vary from 25.89% to 40.77% for *F. graminearum* and from 12.77% to 36.36% for *F. oxysporum*. The *T. citrinoviride TR1* and *T. harzianum 1V* have been shown to be very potent biocontrol agents with high antagonistic activity when it comes to non-volatile compounds and as bicontrol agents with low antagonistic activity when it comes to volatile organic compounds.

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