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IN VITRO STUDY OF *TRICHODERMA* ISOLATES - POTENTIAL ANTAGONISTS OF *SCLEROTINIA SCLEROTIORUM* AND OTHER SOIL PATHOGENS CAUSING ROOT AND STEM ROTS ON PEPPER (*CAPSICUM ANNUUM* L.)

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Abstract

White mold *Sclerotinia sclerotiorum* (Lib.) de Bary is a potentially serious disease of pepper (*Capsicum annuum* L.) grown in unheated greenhouses. Control of *Sclerotinia sclerotiorum* is difficult due to lack of *Capsicum* spp. resistance towards this pathogen. Currently, among the registered fungicides there is none that can combat this pepper disease. The isolation and selection of local strains of antagonistic microbes is part of the strategy to combat pathogens by means of alternative methods. *In vitro* studies have been carried out on soil isolates of *Trichoderma* spp. regarding their antagonistic activity on the pathogen *Sclerotinia sclerotiorum*. All isolates were found to have a higher ability to compete with the phytopathogen for the food substrate. The competitive ability coefficient of *Trichoderma* spp. isolates varies widely – from 1.2 to 11. Among them, an isolate with pronounced antagonistic activity and a competitive ability coefficient of 10.8 stands out. The isolated fungus was identified as *Trichoderma viride* based on the cultural and morphological characteristics. The isolated strain was found to have high *in vitro* antagonistic activity not only against *Sclerotinia sclerotiorum* but also against various pathogens causing root and stem rot of pepper such as *Botrytis cinerea, Verticillium dahliae* and *Rhizoctonia solani*. The results of the current study showed that *Trichoderma viride* (Trv) has the potential to be used for production of biopreparation intended to control *Sclerotinia sclerotiorum* and other soil-borne pathogens in greenhouses.

Keywords: antagonistic fungi, *in vitro* screening, antifungal activity, biological control, white mold, *Capsicum annuum*

INTRODUCTION

Sclerotinia stem rot, also known as white mold, is a common and devastating disease caused by the fungus *Sclerotinia sclerotiorum* (Lib.) de Bary (1884). The disease develops in conditions of prolonged cool and wet weather and a characteristic white plaque is formed on the surface of the infected tissues. The hallmark of this pathogen is its ability to form persistent black structures known as sclerotia (Adams, 1990, Bolton, 2006, Merriman, 1976). The vegetable crops which are hosts for *Sclerotinia sclerotiorum* invasion are cabbage, common bean, celery, coriander, melon, pumpkin, soybean, tomato, pepper, lettuce and cucumber, carrot, onion, pea, squash, etc. (Kohn, 1979). The pathogen is widespread in the North and South America, Australia (Bardin & Huang, 2001), Iran (Castano et al., 2005), Europe - Bulgaria, Hungary, Poland, Romania, France, Yugoslavia (Stancheva, 2001), Moldova, Ukraine, Kazakhstan (Karadzhova, 1981), in the border areas of Belarus with Ukraine (Piven & Alifirova, 2001), in many regions of Russia - North Caucasus, Central Chernozem, Middle Volga, Urals, Altai Territory (Yakutkin, 2001). *Sclerotinia sclerotiorum* occurs throughout the growing season in different forms depending

on the age of the plants on the root, stem, fruit, or caused seed damages (Aćimović, 1983, Chang & Kim, 2003, Grau & Hartman, 1999). The dominance of different forms of the disease is determined by environmental conditions and is not the same throughout the years and seasons (Zhatova & Trotsenko, 2018). Sclerotinia stem and fruit rot, caused by *Sclerotinia sclerotiorum* (Lib.) de Bary, is a potentially serious disease of pepper *Capsicum annuum* L., affecting both seedlings and mature plants (Pernezny and al., 2003, Jeon et al., 2006). The possibilities of controlling the disease are limited and there is no information on the resistance of *Capsicum* spp. to this pathogen (Heffer Link & Johnson, 2007, Sanogo, 2003, Tsitsigiannis et al., 2008, Winton et al., 2006). Control of *Sclerotinia sclerotiorum* is difficult under cool and moist environmental conditions. Currently, there are no fungicides registered to control this disease on pepper (Yanar et al, 1996, Yanar and Miller, 2003). The effectiveness of fungicides to control Sclerotinia stem rot (SSR) is controversial (Mueller et al., 2002), mainly due to the difficulty in achieving good fungicide coverage and the timing of application in relation to ascospore release (Kora et al., 2008). In addition, prolonged use of high-concentration chemicals can develop fungicide resistance in the pathogen (Steadman, 1979) and to impact the non-target species and environment. The use of microbial agents to control plant pathogens can be an environmentally friendly and cost-effective component of an integrated management program (Mao et al., 1997). In the search for alternatives to fungicides, several microorganisms have been reported as potentially effective biocontrol agents for managing *Sclerotinia sclerotiorum* in soybean (Murtazina et. al., 2020). A more reliable method of protection against white rot is currently the use of antagonists or hyperparasites. Such protection is believed to combine the ecology demands and the system biodiversity conservation (Katircioglu et al., 2006, Nair et al., 2005, Ordog, 1999). Several microorganisms are used as biological protective agents*:* *Pseudomonas fluorescens*, *Trichoderma harzianum*, *Talaromyces flavus*, *Coniothyrium minitans*, *Erwinia herbicola*. Myzorin (*Arthobacter mysorens*) and flavobacterin (*Flavobacterium sp.* L-30) are used as inhibitors of *Sclerotinia sclerotiorum* on sunflower (Yakutkin, 2001). The introduction of microorganisms into the environment is associated with certain difficulties, so the results of such protection are unstable over the years and across different regions (Smolińska & Kowalska, 2018). Abiotic factors affect the development of both participants – antagonist and pathogen. The effectiveness of the antagonist depends on the adaptability of the antagonist strain and the available substrate (Troian et al., 2014, Woo et al., 2014). The successful competition with the pathodgen in the soil requires at least two symbiotic microorganisms. Bin et al. (1991) were the first who used the soil bacteria together with *Trichoderma harzianum* to suppress *Sclerotinia sclerotiorum* in the soil. A mixture of *T. flavus* and *C. minitans* has been successfully used to protect sunflower from white mold (Zeng et al., 2012, Zhao et al., 2020). To date, the most promising bioagent to kill sclerotia in soil is *Coniothyrium minitans* which is available as a commercial product (Thaning et al., 2001). *Bacillus subtilis* bacteria have been recorded to control the main diseases of cherries, pumpkin, grapes, leafy greens, pepper, peanuts, potatoes, tomatoes, and walnuts (Zhang & Xue., 2010, Awais et al., 2008). *B. subtilis* has been shown to be effective against *Sclerotinia sclerotiorum* in legume trials (Tu, 1989). Wang et al. (2018) reported that the cell suspensions of *Bacillus subtilis* and its cell-free filtrates significantly reduced the mycelial growth of *Sclerotinia sclerotiorum* by 50 to 75% and suppressed sclerotia formation by up to 90% (Cook et al., 1993, Zhang & Zhuang, 2020).

The aim of the current experiment was to isolate saprophytic strains of fungi and

bacteria from the soil and to study their antagonistic activity against *Sclerotinia sclerotiorum* and other soil pathogens.

MATERIALS AND METHODS

Isolation of antagonistic soil fungi

Isolation of fungi - antagonists from soil. Soil samples from a greenhouse infected with white rot were used to isolate pure fungal cultures by the soil dilution method described by Borovikova (2003). Soil samples were inoculated onto nutrient-poor water agar. A primary screening for antagonism against the accompanying fungal microflora was performed on Petri dishes. Antagonist colonies were detected by the presence of sterile zones around them, and isolated as pure cultures on potato dextrose agar (PDA).

Test-microorganisms. Virulent fungal pathogens *Sclerotinia sclerotiorum*, *Botrytis cinerea*, *Rhizoctonia solani*, *Verticillium dahliae*, *Fusarium capsici*, *Phytophthora capsici*, *Pythium ultimum* isolated from diseased pepper plant tissues were used in the present study.

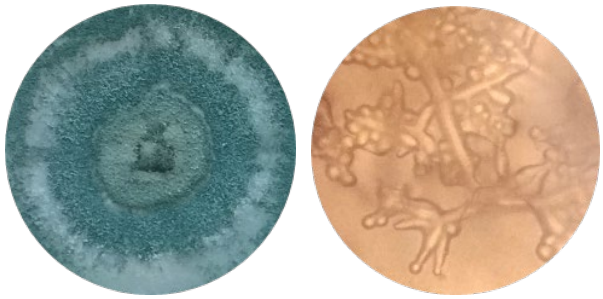
Screening for antifungal activity against phytopathogens. The antimicrobial activity of the isolated strains was determined by the Waterhouse counter-colony method (Chumakov et al., 1974) and by measuring the size of the resulting sterile zones in mm. In the center of the Petri dish, 7-day-old pure cultures of the antagonist strain and the pathogen strain were placed on the PDA (200 g potato infusion form, 200 g dextrose, 150 g agar-agar per litre) at a distance of 2 cm. The Petri dishes were kept in a thermostat at a temperature of 23-25^{o} C for 10 days. The linear growth of microorganisms (mm) and the sterile zone (mm) were measured each day until the colony reached the edge of the dish. The data from the last measurement were used to calculate the inhibition of fungal mycelium growth in the individual variants relative to the controls according to the formula of Abbott (1925): GI % = 100–(D*100/C), where: GI % - Growth inhibition of mycelium; D - Diameter of the fungal colony (mm) in the version with a separate bioagent, C - Diameter of the fungal colony (mm) in the control.

***In vitro* compatibility assay of *Trichoderma viride* and *Bacillus subtilis* isolates**. The possibility of co-cultivation of *Trichoderma viride* strain Trv and the antagonistic bacterium *Bacillus subtillis* was studied *in vitro* according to the method described by Dhingra & Sinclair (1995). A bacterial isolate from the Maritsa Vegetable Crops Research Institute (MVCRI) collection of microorganisms was used. A pure culture of bacterium was streaked on the PDA medium as a straight line along the diagonal of each Petri dish using a sterile inoculating loop. The inoculated Petri dishes were incubated for 72h in the dark at +26^{o}C. Two agar plugs (6 mm in diameter) cut from the periphery of seven-day-old pure culture of *Trichoderma viride* Trv grown in a Petri dish on PDA were placed symmetrically on either side of the streak-shaped bacterial colony at approximately 40 mm distance from it. Each variant (fungal pathogen x bacterial strain) was plated in four replicates (Petri dishes). Identically prepared PDA plates inoculated only with fungal plugs without pre-developed bacterial culture served as controls.

RESULTS AND DISCUSSION

According to the G. Samuel's interactive key, USDA-ARS (http://nt.ars-grin.gov) *Trichoderma* species can be identified by morphological and cultural characteristics. *Trichoderma viride* sensu Rifai (Rifai, 1969) refers to species with uniformly branched, not thick conidiophores and bottle-shaped phialides. To characterized and differentiate *Trichoderma* species Rifay (1969) and Bisset (2011) used the structure of conidia and macroconidia in combination with other characteristics and a description of colony growth on Czapeck-Dox medium (3 g NaNo3, 0.5 g MgSo4.7H2O, 0.5 g KCl, 0.01 g

FeSO4.7H2O, 1 g KH2PO4 per litre, supplemented with 10% glucose), as the main diagnostic feature. In the current study, from the soil samples were isolated eighteen strains of *Trichoderma* sp., which inhibited the growth of microorganisms in the Petri dishes. The identification of the isolates was performed on the several macro- and micromorphological characteristics. On the 7th day of sowing on PDA and cultivation at 23°C, the isolated fungi *Trichoderma* sp. form a dense dark-green conidial covering without coloring the substrate. The conidiophores were uniformly branched, the phialides were bottle-shaped, the conidia were spherical, with smooth walls, numerous, collected in heads (Figure 1). Macro-morphological differences of *Trichoderma* spp. isolates were manifested in the different shape of the fungal colony on the nutrient medium PDA (Figure 2).



a **b**

Figure 1. Isolate *Trichoderma* sp. on the 7th day of inoculation on PDA: **a -** dense green conidial cover; **b** - evenly branched conidiophores, bottle-shaped phialides and spherical conidia photograph (microscopic morphology of the fungus).

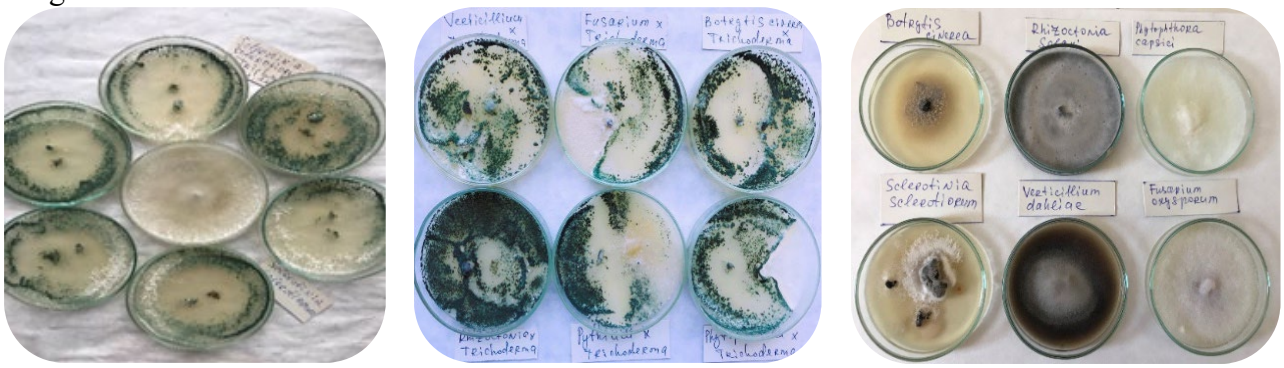
Figure 2. Macro-morphological differences of isolates *Trichoderma* spp. on the 7th day of inoculation on PDA.

Representatives of the genus *Trichoderma* were characterized by rapid growth, bright green conidia, and a branched conidiophore structure (Gams & Bissett, 1998). When a strain of *T. viride* was cultivated on a solid medium, the fungus developed a highly branched substrate mycelium, until the development of an aerial mycelium. The substrate mycelium extended radially - from the center of the colony to the periphery of the agar. Aerial mycelium was formed 30-48 hours after inoculation and it was whitish in color. From 72nd to 96th hour after inoculation, the strain occupied the entire surface of the petri dish (90 mm) with mycelium. After 72nd hour, conidia began to form over the entire surface of the aerial mycelium by forming concentric circles with a diameter of 5-7 µm.

Influence of antagonistic fungi *Trichoderma* spp. on the linear growth of *Sclerotinia sclerotiorum* (Ss) *in vitro*

In vitro evaluation of the antagonistic activity of isolates of *Trichoderma* spp. showed that most of them have a well-expressed superparasitism to *Sclerotinia sclerotiorum*. All eighteen isolates of *Trichoderma* spp. had higher possibilities to conquer the food substrate compared to the phytopathogen. The coefficient of competitive ability among the isolates varies widely – from 1.2 to 11 (Table 1, Figure 3). Twelve isolates exhibited strong antagonistic activity with a coefficient above two. Of these, an isolate with pronounced antagonistic activity and a competitive ability coefficient of 10.8 stood out. The isolate was identified as *Trichoderma viride* and selected for further studies as *Trichoderma viride* (Trv). The stain has well developed white mycelium which darkened over time. It forms hyphae with conidiophores which were 140–200 µm with elongated sterigmata that form a panicle together. Conidia were elliptical, colorless and after time became green with size of 4.5–6.5 x 2.6–3.2 µm, united in spherical or cylindrical heads. On the PDA, the fungus forms a dense

dark green conidial cover.



a **b** **c**

Figure 3. Waterhouse counter-colony method: **a** - Antagonistic activity of the isolate *Trichoderma viride* (Trv) versus *Sclerotinia sclerotiorum. Trichoderma viride* isolate occupies the surface of the culture medium, the lysis zone is clearly visible. For comparison, in the middle of the figure is a Petri dish with a pure culture of *Sclerotinia sclerotiorum*. **b** - Development of *Trichoderma viride* on colonies of the soil pathogens *Rhizoctonia solani*, *Pythium ultimum*, *Phytophthora capsici, Verticillium dahliae*, *Fusarium oxysporum*, *Botrytis cinerea*. **c** - Pure cultures of the isolated pathogens.

Table 1. Antagonistic activity of *Trichoderma* sp. isolates against *Sclerotinia sclerotiorum* in *in vitro* conditions.

No	Colony diameter on the 7th day, mm		Coefficient of competitive-ability	Type of antagonistic activity
	Sclerotinia sclerotiorum	Antagonist, isolate		
1	8.33	90.00	10.8	hyperparasitism
2	41.86	52.33	1.25	antibiotic action
3	33.67	62.59	1.85	antibiotic action
4	30.37	80.67	2.66	hyperparasitism
5	27.33	71.66	2.62	hyperparasitism
6	29.25	68.00	2.32	hyperparasitism
7	35.00	70.00	2.00	hyperparasitism
8	41.56	49.00	1.19	antibiotic action
9	23.67	55.19	2.33	hyperparasitism
10	33.33	65.67	1.97	antibiotic action
11	44.37	85.67	1.93	antibiotic action
12	34.33	83.57	2.43	hyperparasitism
13	20.67	79.33	3.34	hyperparasitism
14	20.00	79.00	3.95	hyperparasitism
15	16.67	74.33	4.46	hyperparasitism
16	14.33	80.33	5.61	hyperparasitism
17	23.33	76.67	3.29	hyperparasitism
18	32.27	87.00	2.70	hyperparasitism

Symbols: Superparasitism – development of the antagonist on the colony of the pathogen; antibiotic action – a well-defined zone of lysis between the colonies of the pathogen and the antagonist.

The competitive activity coefficient of the isolate against *Sclerotinia sclerotiorum* was 10.8. An antagonistic activity of an isolate above three is considered as a good technological indicator characterizing the producer strain in its use for production of a biological preparation to combat phytopathogens. The fast growth is one of the most important competitive advantages that antagonistic fungi have over the phytopathogenic fungi. The colonies of *T. harzianum*, *T. viride* and *T. longibrachiatum* always grew faster than *S. sclerotiorum* in a single or mixed culture (Matroudi et al., 2009).

The control placement of sclerotium of *Sclerotinia sclerotiorum* on nutrient medium (PDA) with a culture of *Trichoderma* spp. confirmed the activity of the antagonist strain against the pathogen. One week after planting the entire surface of sclerotia was parasitized by the antagonist fungus (Figure 4).

Figure 4. Test for the antagonistic activity of the strain *Trichoderma viride* (Trv) against *Sclerotinia sclerotiorum.* Colonization of sclerotia.

To produce a biopreparation, it is necessary to select a strain that has antagonistic activity against several pathogens belonging to one ecological group by the nature of their distribution in the soil and the substrate, which is a source of nutritional elements. In this sense, a *Trichoderma viride* strain selected to produce a biopreparation to combat white rot on pepper should have high antagonistic activity also against development of other soil pathogens causing root and stem rot on pepper (Shternshis et al., 2022, Borisov, 2004, El-Katatny, 2006). This increases the possibilities for biological control of pepper diseases in greenhouse conditions with only one biological preparation.

The results from the current study showed that in laboratory conditions the antagonistic activity of the strain (Trv) was high in relation to the soil pathogens that form sclerotia - *Sclerotinia sclerotiorum*, *Rhizoctonia solani*, *Verticillium dahliae* and *Botrytis cinerea* with an estimated coefficient of antagonistic activity above 7.4 (Table 2). *Trichoderma viride* (Trv) can be used as producer strain in a biopreparation against soil-borne pathogens in greenhouses. The results of introducing antagonists into the soil and substrate depend on many factors. In addition to abiotic factors, the effect of the application of the antagonist is also influenced by the structural composition of the microorganisms in the rhizosphere (Zhatova, 2018, Santhanam et al., 2019). In order to suppress the source of infectious in the soil, sometimes not one, but two microorganisms have been used. According to some authors, induction of bacteria into the soil stimulated the development of *Trichoderma* fungi, which actively colonize the sclerotia of the pathogen *Sclerotinia sclerotiorum* (Lugtenberg & Kamilova 2009, Mukherjee et al., 2014, Soylu et al., 2005, Vargas et al., 2014, Whipps, 2001, Zhang, 2020). In this work, the experiments aimed to study the possibilities of co-cultivation of *Bacillus subtilis* bacterium with the isolate (Trv), which showed an extremely high antagonistic activity against sclerotia-forming fungi. It was found that when the strains *Trichoderma viride* and *Bacillus subtilis* were grown together on culture medium (PDA), they grew without mutual inhibition of growth (Table 3, Figure 5).

Table 2. Antagonistic activity of *Trichoderma viride* strain (Trv) against pathogens isolated from diseased pepper plant tissues.

No	Colony diameter on the 7th day, mm			Coefficient of competitive-ability	Type of antagonistic activity
	Pathogen		Antagonist (Trv)		
1	*Sclerotinia sclerotiorum*	8.33	90.00	10.8	Well defined zone of lysis. Rapid growth, Superparasitism (development on the colony of the pathogen).
2	*Botrytis cinerea*	10.83	80.00	7.40	
3	*Rhizoctonia solani*	10.83	80.00	7.40	
4	*Verticillium dahliae*	5.00	80.00	16.00	
5	*Phythophthora capsici*	23.33	71.66	3.07	
6	*Pythium ultimum*	60.00	68.00	1.13	
7	*Fusarium oxysporum*	35.00	70.00	2.00	

Table 3. The influence of *Bacillus subtillis* bacterium on the linear growth of *Trichoderma viride* Trv and *Sclerotinia sclerotiorum* (in Petri dishes on PDA).

Colony diameter, mm	Lysis zone, mm	Degree of impact
Trichoderma viride		
50,83	2,50	-
Sclerotinia sclerotiorum	Lysis zone, mm	Degree of impact
26,25	18,75	+++

Legend: (-) – absence of lysis zone; (+)- lysis zone is 5-10 mm; (++)- 11-15 mm; (+++) - over 16 mm. The bacterium occupies the entire surface of the petri dish (90 mm). Strong predominance of the bacterial colony on the surface of the dish.

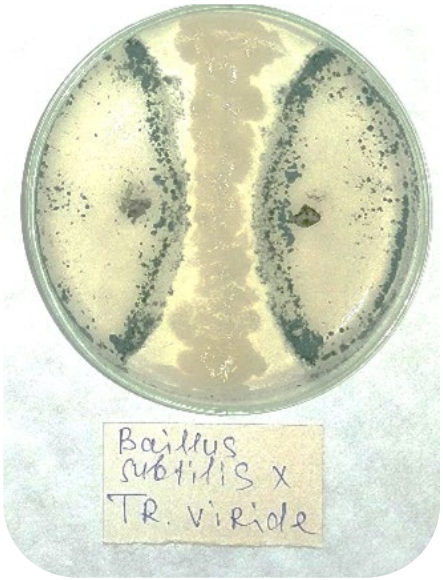


Figure 5. Colony growth of strain Trv and *B. subtilis* on PDA medium without mutual suppression.

This can serve as a basis for their investigation as producer strain or in combination for preparations intended to combat soil-borne pathogens. The aim of this research was to address the challenge of finding the suitable candidates among the potential biological control agents (BCA) from native regions with white rot-infested soils. This issue is becoming increasingly important as each soil type and region has potential antagonistic microorganisms that can be exploited in future work. White mold of pepper caused by *S. sclerotiorum* has recently become a problem that could potentially be controlled using antagonistic microorganisms isolated from native soil types. Therefore, the study of indigenous BCAs strains is of paramount importance in developing effective means to combat this dangerous disease. Local BCAs may perform better under the local conditions from which they were isolated due to their adaptation to those local environmental conditions.

CONCLUSION

The current study, identified by cultural and morphological characteristics a strain *Trichoderma viride* isolated from the soil which has a high *in vitro* antagonistic activity against pathogens causing root and stem rot on pepper - *Sclerotinia sclerotiorum*, *Botrytis cinerea*, *Verticillium dahliae* and *Rhizoctonia solani*. The coefficient of antagonistic activity of the isolate was above 7.4. This suggests that the fungus can be used as a producer strain in the production of a biopreparation to combat sclerotia-forming pathogens. When co-cultivated on the nutrient medium with bacterium *Bacillus subtilis* both participants can grow without mutual inhibition. This can serve as a basis for their study as producer strains that can be used as a combination in the creation of preparations against soil pathogens.

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