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ANTIFUNGAL ACTIVITY OF *TRICHODERMA ASPERELLUM* AND A PROFILE OF ITS VOLATILE ORGANIC COMPOUNDS

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Abstract

Members of the genus *Trichoderma* have great biotechnological potential as the basis of biological products for crop production. These species have the ability to form conidia, which can remain viable for a long time under adverse environmental conditions; high growth rate; low inertia, which manifests itself in the rapid activation of spores and the "explosive" nature of growth on rich substrates; low demands on environmental conditions; attraction to acidic pH values and, accordingly, the ability to manifest its biological activity in acidic soils; the ability of hyperparasitism on phytopathogenic fungi, which together with the production of antibiotic substances provides effectively protection to crop plants. The present study describes the antigungal activity of *Trichoderma asperellum* and its volatile metabolites against *Fusarium oxysporum* and *Alternaria alternata.* Metabolites like 1-Octen-3-one; 1-Octen-3-o; o-Xylene; 3-heptanone; 6-methyl-; Nonanal; 1-Hexanol, 2-ethyl- were also isolated from broth cutlres of *T. asperellum.*

Key words: *Trichoderma asperellum*, Antifungal, Volatile compounds, Biotechnology, Microbiology.

Introduction

The intensification of the production of legumes and fodder crops in many industrialized countries has led to a marked increase in the prevalence and harmfulness of root rot. It has been established that root rot is caused by various phytopathogenic fungi including *Fusarium* spp. and *Alternaria* spp. (Dubey & Patel, 2000; Pereira *et al*., 2013; Pareek & Varma, 2015; Escrivá *et al.*, 2017).

The high degree of latent infection of these crops can be the reason for their low productivity, as well as pose a danger when used in the food industry and in the preparation of animal feed, sowing material and new seed reproduction (Gorobei *et al.*, 2011; Wilman *et al.*, 2014).

Chemical methods are currently the most affordable means. However, their use is limited due to the high toxicity to humans and animals. In addition, their repeated use contributes to the development of chemically resistant strains of pathogens, and the uncontrolled use of these chemicals poses a serious threat to the environment due to residual exposure (Ajith & Lakshmidevi, 2012). Therefore, it is advisable to switch to an alternative biological method of combating a number of dangerous pests that cause great damage to crops (Effmert *et al*., 2012).

The most promising microorganisms for plant protection are *Trichoderma* fungi, which have high physiological activity and inhibit the growth of a number of pathogenic fungi, thereby attracting the attention of researchers to create plant protection products and obtain valuable biologically active substances (Schuster *et al*., 2009; Mukherjee & Lal, 2013; Nosir, 2016; Kumar *et al.*, 2017; Redda *et al.*, 2018).

The improvement of the soil structure and the accumulation of humus is facilitated by the plowing of crop residues or the spreading of straw on the fields (Chitarra *et al*., 2003). At the same time, soil erosion is reduced, water and air regimes are improved, its absorbency is improved, and nitrogen fixation processes are stimulated. When decomposing crop residues , about 11-14 kg of nitrogen, up to 6,5 kg of phosphorus, 29 kg of potassium, 3,5 kg of sodium enter the soil per 1 ha. Crop residues are a source of nutrition for soil microorganisms, which largely ensure the availability of individual nutrients for agricultural plants (Ahmad *et al*., 2022).

At the same time, plant residues in the fields are a conservator and a source of phytopathogens. Without creating the necessary pool of antagonists that contribute to the formation and strengthening of soil suppressiveness, it is impossible to stabilize the phytosanitary situation in agrocenoses, preserve fertility and obtain high yields (Carballo-Mendez *et al.*, 2022).

The solution of this problem is facilitated by the increasing use of the microbiological method in the protection of plants. Modern biological products have growth-stimulating activity, their producers produce a variety of biologically active substances that suppress the development of phytopathogen populations, increase disease resistance and yield of agricultural plants (Arif *et al*., 2022). The success of the application of the microbiological method depends on the effectiveness of biocontrol agents (Effmert, 2012). To create biological products, the greatest of interest are the types and strains of microorganisms with a variety of metabolic processes, unpretentiousness to cultivation conditions, high technological and ecological plasticity (Haran *et al.*, 1996; Novikova & Nazarenko, 2007; Ziganshin & Sirotkin, 2017). All these qualities are possessed by strains of the genus *Trichoderma* are natural biodestructors of plant residues and other cellulose–containing materials, occupying a special position as producers of poly functional biological products (Colombet *et al.*, 2001; Boureghda & Renane, 2011). They are able to quickly assimilate an organic substrate by actively

decomposing simple and complex compounds, accelerating tenfold the process of their mineralization and improving the physical and chemical properties of the soil. Due to the high antagonistic and hyperparasitic activity in relation to soil-dwelling pathogens, strains *Trichoderma* reduces the morbidity of plants by 2.5–3 times, increases their disease resistance, and exhibits regulatory activity by stimulating development nitrogen-fixing bacteria, contributing to the enrichment of the soil with amine nitrogen, the binding of mineral fertilizer salts, and increased mobilization of phosphorus and potassium (Sadykova *et al.*, 2009; Alamri *et al.*, 2012; Devi *et al.*, 2012; Heidi & Abo-Elnaga, 2012; Parra, Maniscalco, 2012). Of particular importance in the conditions of the northern regions is the ability of a strain of a microorganism used in agricultural technologies to maintain viability and target biological activity at low temperatures, especially when growing winter crops.

Material and Methods

Trichoderma asperellum 1M F-RKM 0786 strain isolated in 2013 from the rhizospheric soil of soybean, Almaty region, Sarkand district, and the Republic of Kazakhstan wëas used. The strains of *Fusarium oxysporum* and *Alternaria alternata* were isolated from the affected chickpea seeds of the Ikarda variety, growing in the Almaty region, Sarkand district, and the Republic of Kazakhstan in 2015. For long-term storage, the fungi were kept in test tubes with cotton-gauze plugs on potato dextrose agar (PDA) at a temperature of 4°C.

The antifungal activity of *T. asperellum* 1M F-RKM 0786 was established by the method described by Soytong and Quimio (1989). The agar disc was taken separately from the edge of the radial growth of the test cultures of *F. oxysporum* and *A. alternata* using sterile borer (d = 8 mm) and placed on one side of the plate with PDA medium. The agar disk of the *T. asperellum* 1M F-RKM 0786 antagonist strain was placed on the other side of the agar plate. In the control, an agar disk of pathogens was placed in Petri dishes containing PDA medium; on the other side, an agar disk of a clean medium without an antagonist fungus was placed (Bennett & Inamdar, 2015). Dual culture Petri dishes were incubated at 28°C for three days. The radius of the colony was measured using a ruler. The growth inhibition percentage was calculated using the following formula:

$$\% \text{ Inhibition} = A - B / A \times 100$$

where: A = Radius of the pathogen colony in the control, B = Radius of the colony in the dual culture.

The effect of volatile compouds produced by the antagonist on the growth of plant pathogens was determined by the method given by (Dennis, C., & Webster J., 1971) with a slight modification. Fungal strains were grown on PDA for 5 days. Then, a 7 mm diameter block with a grown culture of *F. oxysporum* or *A. alternata* was cut from the plates and inoculated into Petri dishes on PDA. A plate of each pathogen was placed on top of the plates containing the culture of *T. asperellum* and sealed with Parafilm to prevent the release of volatile compounds and incubated at 25°C for 5 days. In control, Petri plates containing cultures of *F. oxysporum* and *A. alternata* were placed over the Petri plates containing PDA without *T. asperellum* (Guo *et al*., 2019).

The inhibitory effect of *T. asperellum* on growth of *F. oxysporum* and *A. alternata* was calculated by the formula used for the dual culture method.

Determination of the VOC profile of *T. asperellum*: Solid-phase microextraction (SPME) was used for the determination of volatiles by gas chromatography-mass spectrometry (GC / MS). Cultures of *T. asperellum* were grown on Czapek's broth and PDA at 28°C for 5 days. A manual version of SPME was used for sampling where the cotton plugs were replaced with a cap with a hole and silicone membrane to facilitate the piercing of the septum with an SPME needle. For SPME we used CAR / DVB / PDMS (Supelco, Bellefonte, PA., USA) fiber coating, extraction time was 30 min and pre-incubation time was 15 min. All SPME sampling was performed at room temperature (25±1°C) to facilitate non-invasive sampling during fungal growth. As the SPME fiber after desorption in the GC injection port (240°C) can be considered as sterile, it was possible to sample the same vial several times during the process of monitoring volatile compound formation (Contreras-Cornejo *et al.*, 2014).

The separation was carried out using a DB-WAXetr chromatographic capillary column with a length of 30 cm, an inner diameter of 0.25 mm and a film thickness of 0.25 μm at a constant carrier gas (helium) speed of 1 ml/min. The chromatographic temperature was programmed from 40°C (5 min exposure) to 260°Cwith a heating rate of 10°C/min (10 min exposure). Detection was carried out in the SCAN m / z 10-800 mode (Hung *et al*., 2015).

Agilent MSD ChemStation software (version 1701EA) was used to control the gas chromatography system, register and process the obtained results and data. Data processing included determining retention times, peak areas, and processing of spectral information obtained using a mass spectrometric detector (Meena *et al*., 2017). For decoding the obtained mass spectra, the Wiley 7th edition and NIST'02 libraries were used where the total number of spectra in the libraries was more than 550 thousand. GC-MS analysis was performed in duplicate. The probability of identifying each compound was at least 80%.

Statistical analysis: All the experiments were performed in triplicate, and the results are presented as mean ± standard error (SE). Data were statistically analyzed using the STATISTICA 6 study guide (Khalafyan, 2007).

Results and Discussion

This study found the inhibitory effect of *T. asperellum* 1M F-RKM against the growth of phytopathogenic fungi *F. oxysporum* and *A. alternata*. The results of the antifungal activity of *T. asperellum* obtained by the dual culture method (Soytong & Quimio, 1989) are presented in Fig. 1 and Table 1.

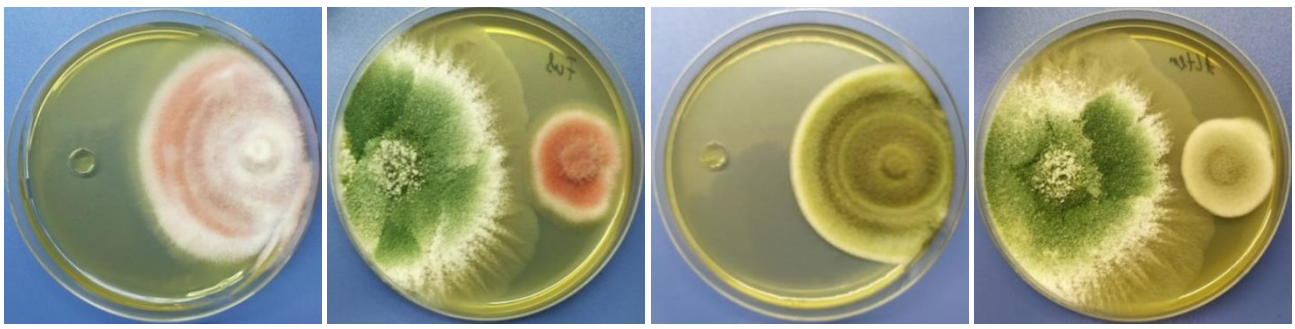

Fig. 1. Antifungal activity of *T. asperellum* 1M F-RKM.
a. Control environment (left) + *F. oxysporum*; **b.** *T. asperellum* (left)+ *F. oxysporum*; **c.** Control environment (left) + *A. alternata*; **d.** *T. asperellum* (left) + *A. alternata*

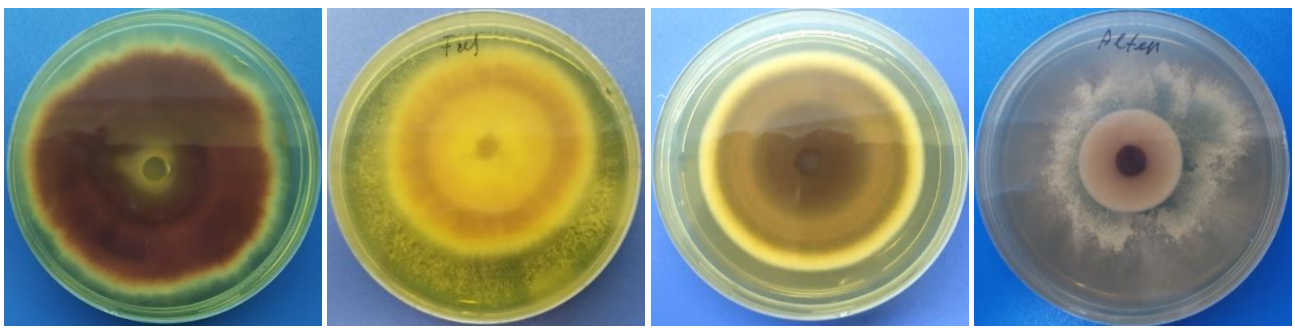

Fig. 2. Inhibitory effect of volatile compounds produced by *T. asperellum* against phytopathogenic fungi
a. Growth of *F. oxysporum* in control environment; **b.** Growth of *F. oxysporum* exposed to volatile compound produced by *T. asperellum*; **c.** Growth of *A. alternata* in control environment; **d.** Growth of *A. alternata* exposed to volatile coumpounds produced by T. *asperellum.*

Table 1. Inhibitory effect of the fungus *T. asperellum* 1M F-RKM against phytopathogens *F. oxysporum* and *A. alternata*.

Test pathogen	Radial pathogen growth, cm		Inhibitory effect, %
	With an antagonist	Without antogonist	
	Method 1		
F. oxysporum	0,75 +-0,15	3,2+-0,5	76,6
A. alternata	1,0+-0,1	2,8+-0,1	64,2
	Method 2		
F. oxysporum	2,5+-1,0	3,5+-1,0	28,6
A. alternata	1,0+-0,5	3,0+-0,5	66,7

Table 2. Volatile organic components formed by the fungus *T. asperellum* 1M F-RKM 0786 when grown on a liquid nutrient medium.

Peak	Red time	Area %	Compound
1	8,4	14,5	o-Xylene
2	9,9	12,1	3- Heptanone, 6 methyl
3	10,8	26,1	1-Octen- 3-ol
4	12,3	7,5	nonanal
5	13,0	19,9	1-Octen- 3-ol
6	13,6	12,2	1-Hexanol, 2-ethyl-
7	15,1	7,7	1 Nonanol

As can be seen in Fig. 1, pathogenic fungi grew faster in the Petri dish and the colony radii averaged 3.2 cm (*F. oxysporum*) and 2.8 cm (*A. alternata*), while in dual cultures, the radius of the pathogen colonies was significantly smaller i.e. 0.75 cm for *F. oxysporum* and 1.0 cm for *A. alternata*. Inhibition of growth of phytopathogenic fungi *F. oxysporum* and *A. alternata* were 76.6% and 64.2%, respectively (Table 1). In a similar study, a decrease in the radial growth of *F. solani* by *T. viride* was 46.6% - 73.3%, and a decrease in the growth of *A. alternata* by 69% have been reported (Naglot *et al.*, 2015). Awad *et al*., (2018) also observed similar antifungal activity of *T. viride* against *F. solani*where reduction in growth of *F. solani* was 29.76%.

The results of the antifungal activity of volatile compounds produced by*T. asperellum* are presented in Fig. 2 and Table 1. The radii of the colonies of pathogenic fungi were smaller and averaged 2.5 cm for *F. oxysporum* and 1.0 cm for *A. alternata* (Fig. 2). The inhibition in growth of phytopathogenic fungi *F. oxysporum* and *A. alternata* by volatile compunds produced by *T. asperellum* was 28.6% and 66.7%, respectively (Table 1).

Comparing the results of a study of the inhibitory effect of the fungus *T. asperellum* 1M F-RKM, carried out by two different methods, we can conclude that the percentage of growth inhibition of *F. oxysporum* obtained by the first method is 2.7 times higher than that of the second method. Regarding the inhibitory effect of *T. asperellum* against *A. alternata*, in this case, the percentage of pathogen inhibition was 2.5% higher in the second method (Morath *et al*., 2012).

Thus, according to the results obtained by two different research methods, the average inhibitory effect of the fungus *T. asperellum* 1M F-RKM 0786 against phytopathogenic fungi *F. oxysporum* and *A. alternata* is 52.6% and 65.45%, respectively.

In his work, Harman, 2006 noted that volatile components could affect the physiological processes of fungi, which act as a defense system and play an important role in the interaction of *Trichoderma* fungus with other microorganisms (Rahnama *et al*., 2016)

Our analysis for the presence of volatile and semi-volatile compounds formed by the fungus *T. asperellum* 1M F-RKM 0786, when cultivated on a liquid nutrient medium revealed such compounds as: 1-Octen-3-one; 1-Octen-3-o; o-Xylene; 3-heptanone; 6-methyl-; Nonanal; 1-Hexanol, 2-ethyl- (Table 2).

It should be noted that compounds such as 1-octene-3-ol and 3-Heptanone; 6-methyl-, whose content is respectively 13.8 and 12.1% of the total volatile compounds of the strain *T. asperellum* 1M F-RKM 0786, have antifungal activity (Jeleń *et al*., 2014; Inayati *et al*., 2019). Such volatile compounds of the strain *T. asperellum* 1M F-RKM 0786 such as o-Xylene, 1-Octen-3-ol, and 1-Nonanol are confirmed by the results of several researchers, as metabolites formed by fungi of the genus *Trichoderma* (Lee *et al*., 2016; Li *et al*., 2018; Inayati *et al*., 2019).

Conclusion

According to the indicators of linear growth rate, antagonistic and hyperparasitic activity at 4-8°C, high colonization rate of wheat and corn crop residues, a promising psychrophilic strain of *T. asperellum* G-034 was selected for the development of laboratory samples of biological products based on it and conducting field experiments (Wen *et al*., 2019). Small-scale field tests have shown the high efficiency of the *T. asperellum* G-034 strain for accelerated decomposition of maize residues and soil improvement (Amin *et al.*, 2010). When conducting laboratory control studies field experience revealed the active decomposition of corn crop residues under the influence of soil micro-organisms antagonists, and most importantly, under the influence of highly active psychrophilic strain-producer of *T. asperellum* G-034, leading to a complete loss in 12 months. Intact state of plant residues due to biodegradation of more than 80% of cellulose contained in them and more than 20% of lignin providing mechanical strength. The maximum loss of biomass by cropresidues of corn for 12 months amounted to more than 70% (Weikl *et al*., 2016)

The producer strain of *T. asperellum* G-034 after overwintering in the field, it was in an active state in the amount of 104 CFU / g, leading to an increase in titer with a seasonal increase in temperature, an increase and expansion of the bioavailability of the trophic base. Micromycetes of the genus *Trichoderma*, possessing high hyperparasitic and antagonistic activity against soil-dwelling pathogens, synthesize a wide range of biologically active substances, increase the disease resistance and productivity of plants (Kolombet *et al.*, 2001). The results obtained by us are consistent with the data of other authors, and also allow us to significantly expand the possibilities of using *Trichoderma* rods for the decomposition of plant residues and biocontrol of soil-dwelling phytopathogenic species at low temperatures typical for the northern regions.

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