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Trichoderma harzianum as a biocontrol agent against *Alternaria alternata* on tobacco

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Trichoderma fungi are the most popular agents used in a biological control. Therefore, our aim was to determine an impact of *Trichoderma harzianum* on the fungus *Alternaria alternata* - a causing agent of the brown spot disease on tobacco. In vitro analyses were made in several variants of double culture, in order to study the effect of diffusible and volatile metabolites. There was strong reducing effect on the development of *A.alternata* with various mechanisms of antagonistic influence. The volatile metabolites have also shown reducing effect. Some abnormalities were observed in the pathogen's morphology both in diffusible and volatile metabolites. The strong reducing effect of *T. harzianum* towards *A. alternata* can be applied in biological control of this pathogen.

Keywords: Pathogen, antagonist, inhibitory effect, diffusible/volatile metabolites, abnormalities

Introduction

The presence of fungal diseases on tobacco and its economical consequences require the use of many pesticides. Besides chemical preparations, the Integrated Pest Management involves the use of biological control of pathogens. Modern production of tobacco in Macedonia is striving to involve this kind of disease control and limited chemical usage.

The greatest data in biological control literature, refers to genus *Trichoderma*. The activity of this useful species has been recognized from 1930 and today there are modern technologies for including them in biological control of various diseases.

For a long time, *Trichoderma* species have been known as biological agents for control of plant diseases (Ranasingh, Saturabh, Nedunchezhiyan, et al., 2006). They interact with root, soil and leaf surroundings. They produce and release many components, which induce local or systemic plant resistance to abiotic stress. According to Rosado et al. (2007), the main factor for ecological success of this genus is a combination of very active mycoparasitism mechanisms and an effective defensive strategy, induced in the plants.

These fungi are used in biological control against various plant pathogens. Similar to soil pathogens, they successfully suppress leaf pathogens as well.

Biological control offers an environmentally friendly approach to the management of plant disease and can be incorporated into cultural and physical controls and limited chemical usage for an effective integrated pest management (IPM) system (Monte, 2001).

The brown spot disease plays a significant role among some economically important diseases on tobacco in Republic of Macedonia. Its causing agent is the pathogenic fungus *Alternaria alternata*. The specific symptom (brown spots) causes biochemical changes that impair the quality of raw tobacco, and result in severe economic losses on tobacco production. The occurrence of brown spot disease depends on the climate conditions, but also on other factors such as plant age,

time of leaf harvesting, susceptibility of the variety, sugar content, etc.. The use of *Trichoderma* with all mechanisms of biological control will help suppress this pathogen.

According to Mandare et al. (2008), *A. alternata* development in laboratory conditions is inhibited by *T. koningii*, *T. lingorum* and *T. virens*. Six out of 16 isolates of *Trichoderma* species suppress the development of her mycelia more than 55%. Many of them are tolerant and the third isolates are compatible with more fungicide active ingredients (Umamaheswari, Thakore, and More, 2009). This species is known due to its ability to produce many lithic enzymes and/or antibiotics (Lieckfeldt, Samuels et al., 1999). Therefore, the pathogenicity of *A. alternata* is decreased when the solution of crude chitinase is applied in tobacco seedlings. The effect is significantly bigger in crude than purified chitinase (Gang, Xing Zhong et al., 2004).

T. harzianum is a biocontrol agent in control of *A. alternata* (Roco and Perez, 2001; Izzati and Abdulah, 2008; Monte, 2001; Sempere and Santamarina, 2007). According to Roco and Perez (2001), in vitro biocontrol of *T. harzianum* towards *A. alternata* is improved in the presence of the growth regulators or foliar nutrient at concentrations similar or higher than those used at the field level. So, their presence could benefit plant from the elicitor activity of this *Trichoderma* that induces plant defense mechanisms against the pathogen.

There is a not general solution and biocontrol system must be developed for each crop. Before the application of a commercial product, it is necessary to first do various studies. The first and quickest way was to screen for antibiotic production and/or mycoparasitism in petri dishes assays (Harman, 2006). Our aim, therefore, was to determine the impact of *T. harzianum* on the pathogen *A. alternata,* in laboratory conditions.

Materials and methods

Using ordinary laboratory method, the pathogen fungus *A. alternata* was isolated from transitional part between healthy tissue and brown spots on tobacco leaves. *T. harzianum* was isolated from the root zone of rhizosphere, by a method of dilution, using Czapek agar.

In-vitro biological activity of *T. harzianum* on *A. alternata* was investigated by double cultures on the potato dextrose agar.

Trials were set up in three replications, with five Petri dishes by each variant. They were incubated at 25°C and the diameter of the colonies was measured for 10 days.

In **diffusible metabolites,** investigations were maintained with the variants:

- Placing the fragments of test fungus and pathogen on the separated half of a media
- Placing the fragments at the same time, with near-contact
- Temporal and spatial advantage of pathogen growth (up to filing the half of a medium) and then place the *T. harzianum* fragment.

In **volatile metabolites** investigations, fragments of test fungus and pathogen were placed in the center of two separate PDA plates sealed with parafilm, so, the test fungus was in the under plate and, the pathogen was in upper plate. There were two controls (*T. harzianum* and *A. alternata*) in an inverted position.

After the period of incubation, micro observations were made of the pathogen in the two kinds of double cultures.

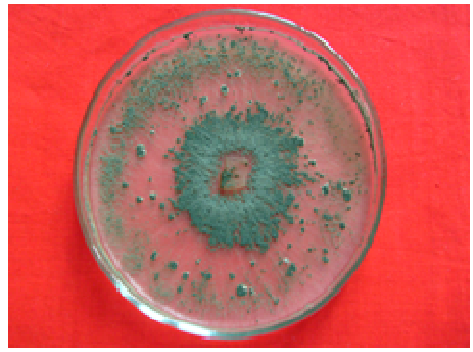
Results

The colony of pathogen fungus *A. alternata* has mostly a grey or greenish color, with aerial mycelia (Figure 1). There are also differences among the isolates about color, and sporulation and conidia size. The investigated isolate has middle intensity of sporulation.

The first, *T. harzianum* forms a colony with white mycelia, but it became green when forming conidia and conidiophores. There are conidia formed densely over the center and undulating concentric rings toward the edge (Figure 2).

FIGURE 1. *ALTERNARIA ALTERNATA*, PURE CULTURE

FIGURE 2. *TRICHODERMA HARZIANUM*, PURE CULTURE



In double cultures with fragments on separated half of a medium, *A. alternata* developed more slowly than *T. harzianum*, from first to the last day of incubation (Table.1). On the second day diameter of the colony is 22,60 mm, but that of *T. harzianum* is 58,60 mm, so, it is approaching to the pathogen (Figure 3).

A. alternata develops with a minimal amount (only 2-3mm) until the third or fourth day. The growth is seen only in some cases when it develops a lengthened deformed look as it makes an effort to develop towards the opposite site), until it is surrounded by *T. harzianum*. Also, it has very light color (Figures 3 - 6).

TABLE 1. EFFECT OF *T. HARZIANUM* ON DEVELOPMENT OF *A. ALTERNATA* (DIFFUSIBLE METABOLITES)

Diameter (mm)		Days									
		1	2	3	4	5	6	7	8	9	10
Fragments on half-medium	*A. alternata*	12.40	22.60	27.80	**29.20**						
	T. harzianum	18.60	58.60	103.40	**110.00**						
Near-contact fragments	*A. alternata*	11.60	18.60	**22.80**							
	T. harzianum	15.20	54.80	104.60	**110.00**						
Check Ø	*A. alternata*	12.40	26.40	39.60	49.20	59.60	70.40	83.00	100.20	104.00	107.40
	T. harzianum	17.60	59.40	106.20	**110.00**						

After the contact with a pathogen, *T. harzianum* continues to develop without obstacles. There are few differences between double cultures and check, the opposite than a pathogen (Table 1). *A. alternata* become captured by the colony of *T. harzianum* (Figures 4 - 6).

At the end of the investigation, it is noted that the whole plate is filled by *Trichoderma*, as it develops over the pathogen's colony, covering it (Figure 7).

In the case of near-contact pathogen and antagonist fragments, *A. alternata* has a very short developing time, but *T. harzianum* develops more rapidly and surrounds the pathogen on the second day (Table 1; Figures 8, 9). The third day it is fully surrounded by the antagonist, when it stops developing. It has smaller dimensions, and like in previous case, it has irregular colony form. *T. harzianum* develops without obstacles, suppresses the *A. alternata* from developing. It is developing over the pathogen's colony at the same time (Figures 10, 11).

FIGURES 3-6. DEVELOPMENT OF THE COLONIES OF *A.ALTERNATA* AND *T.HARZIANUM* IN DOUBLE CULTURE

FIGURE 3

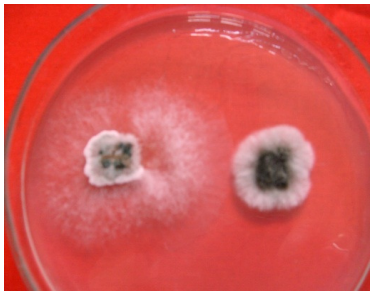


FIGURE 4

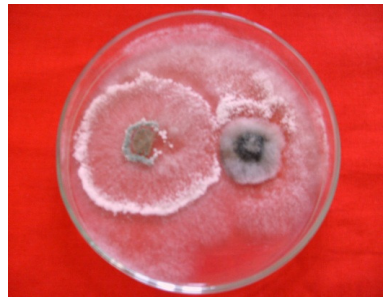


FIGURE 5

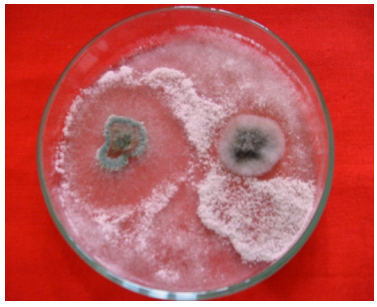


FIGURE 6

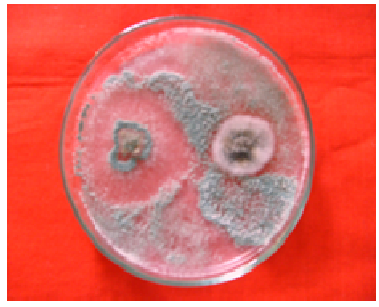
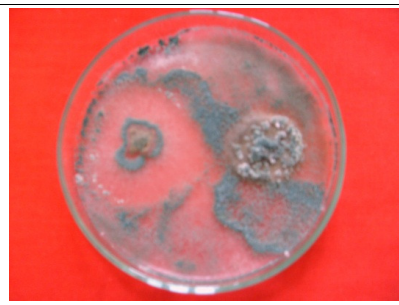


FIGURE 7. THE DOUBLE CULTURE AT THE END OF INCUBATION



FIGURES 8-11. DEVELOPMENT OF THE COLONIES *OF A. ALTERNATA* AND *T. HARZIANUM* IN DOUBLE CULTURE WITH NEAR-CONTACT FRAGMENT

FIGURE 8

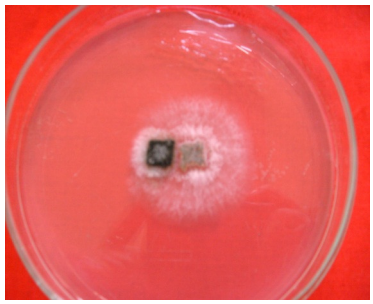


FIGURE 9

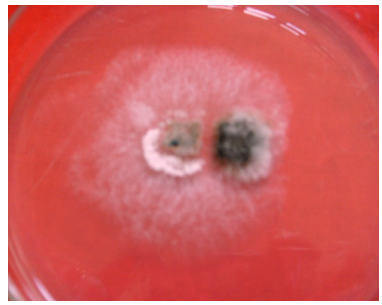
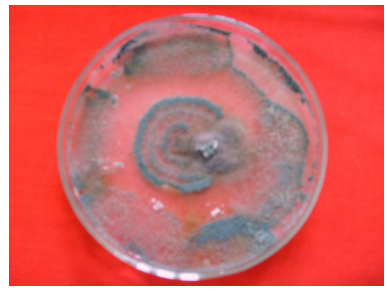


FIGURE 10

FIGURE 11



FIGURES 12-14. DEVELOPMENT OF THE COLONIES OF *A. ALTERNATA* AND *T. HARZIANUM* IN TEMPORAL AND SPATIAL ADVANTAGE OF PATHOGEN GROWTH

FIGURE 12

FIGURE 13

FIGURE 14

In temporal and spatial advantage of *A.alternata* growth, it continues to grow until it is in contact with the antagonist. From this moment, there is an attempt to grow towards the opposite side, which is visible in its deformed characteristic (Figure 12). At the same time, *T. harzianum* develops without obstacles (Figures 13, 14).

In investigations about Trichoderma's influence with volatile metabolites, it is noticeable that the pathogen grows more slowly than the antagonist does, as in the previous case. However, the growth slows and becomes stagnant about the fifth day, compared to check, which continues to grow and get up bigger diameter (Table 2). *T. harzianum* doesn't show a difference in dual cultures and check - normal and inverted position, too (Table 2).

TABLE 2. EFFECT OF *T. HARZIANUM* ON DEVELOPMENT OF *A. ALTERNATA* (VOLATILE METABOLITES)

Diameter (mm)		Days									
		1	2	3	4	5	6	7	8	9	10
A. alternata		9.62	22.22	30.84	38.89	45.02	50.02	54.02	56.49	57.47	58.38
T. harzianum		29.96	67.11	106.67	110.00						
Check Ø (inverted position)	*A. alternata*	9.53	16.67	24.00	32.47	40.87	49.33	59.47	65.40	72.73	79.87
	T. harzianum	19.67	61.53	104.73	110.00						

In the colony of *A. alternata* we can see wet zones of degradation. Also, it is a noticeably lighter circle in the later stages of pathogen incubation (Figures 15, 16).

FIGURES 15, 16. EFFECT OF *T. HARZIANUM* ON *A. ALTERNATA* (VOLATILE METABOLITES)

FIGURE 15

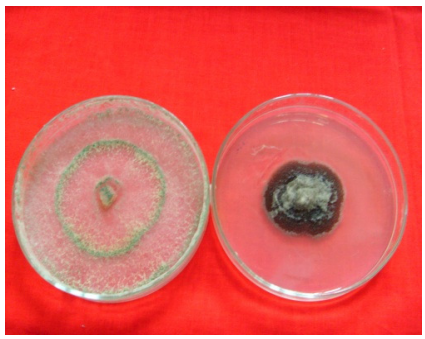
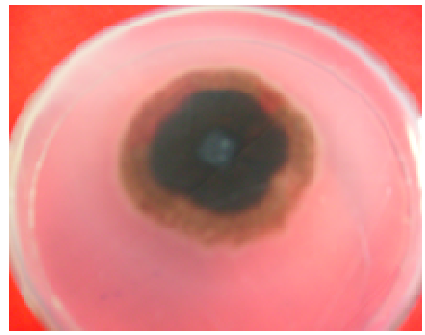


FIGURE 16



Through the incubation period of dual cultures we see a change of the *A. alternata* colony look**.** From the beginning of incubation, there is obvious discoloring in diffusible metabolites (in volatile this is later noticeable), and a loss of sporulation.

Microscopic observations of the pathogen after the interval of incubation, from both of dual colonies cases showed deformations of hyphae, which exhibit a longer distance between septa and have empty ends. There is a small number or absence of conidia than in the check (Figures 17-19).

FIGURE 17. *A. ALTERNATA*, CHECK

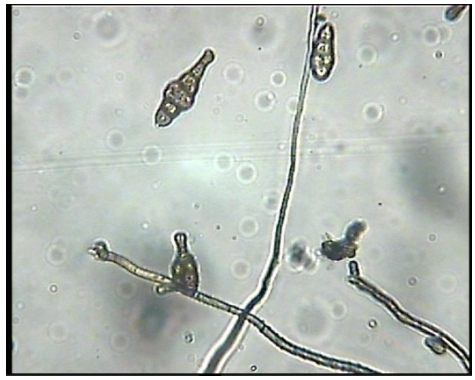
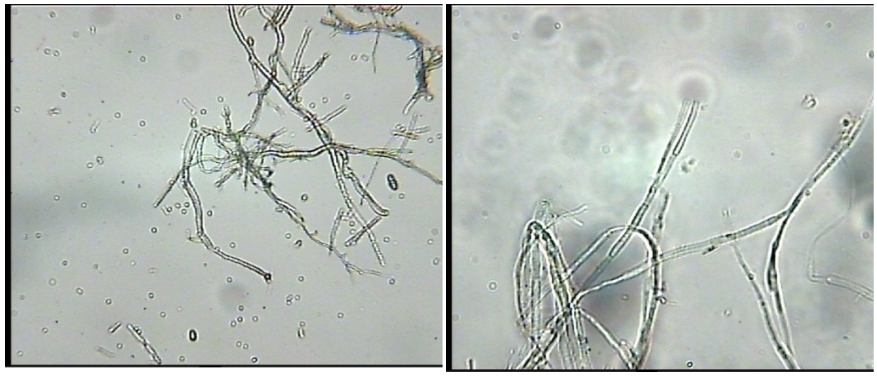


FIGURE 18. *A. ALTERNATA*, DIFFUSIBLE METABOLITES

FIGURE 19. *A. ALTERNATA*, VOLATILE METABOLITES



Discussion

One of the key elements of sustainable agriculture is the ecological approach to solving the problems with plant pathogens, by the application of biocontrol agents. The genus *Trichoderma* is most important in achieving that and, at the same time, sustaining a favourable environment, instead of using chemicals.

Fungi of the genus *Trichoderma* have long been recognized for their ability to act as biocontrol agents against plant pathogens. During this time, research has described their mechanisms of action and how they might be used for various purposes (Harman, 2006).

Antibiosis, mycoparasitism and food competition are the main mechanisms in biological control (Ghildyal and Pandey, 2008; Umamaheswari et al., 2009; Ranasingh et al., 2006).

Ghildyal and Pandey (2008) estimated that *Trichoderma sp.* is produced diffusible and volatile metabolites. The production of volatile and non-volatile antibiotics by the species of *Trichoderma* also was reported by Dennis and Webster (1971, loc cit. Ubalua and Oti, 2007).

Investigations into the biological activity of *Trichoderma* have shown it has a strong reducing effect towards *A. alternata* development, since it develops without obstacles. *T. harzianum* develops more rapidly than *A. alternata* in single as well as in double cultures. The intensive development of *Trichoderma* gives it a significant advantage in competition with pathogens for nutrient elements and space, even as it develops the system of mycotoxins (Barbosa, Rehm et al., 2001).

The contact between two fungi begins as early as at second day, with pathogen stagnation coming after, when *Trichoderma* is developing and spreading. According to Almeida et al. (2007), inhibition of pathogen in double cultures begins soon after contact with the antagonist.

Trichoderma sp. develops exactly on other fungi's hyphae, coils around them and degrades the cell's walls. This action of parasitism restricts the development and activity of pathogenic fungi. Additionally, or together with mycoparasitism, some *Trichoderma* species release antibiotics (Harman, 1996).

It was concluded that *Trichoderma* sensed the presence of target fungi and appeared to grow tropically towards them. However, it was noticed that when they are together, endochitinase gene is activated before they come into contact, while activation of exochitinase occurs only after contact. Also, the degraded cell wall fragments of target fungi are highly potent inducers of enzymes, induction, an enhancement in *Trichoderma* growth (Harman, 2006).

According to Figure 7, the whole petri plate is filled with *T. harzianum*, thus, it develops even over the culture of *A. alternata*. In-vitro antagonistic activity of *Trichoderma sp.* is expressed by suppressing test-fungi growth and its rapid growth, which is multiplying over the pathogen's colony (Mirkova, 1982).

When the fragments are placed in near-contact, *Trichoderma* has a space advantage and bigger opportunities to stop pathogen development, and to develop its mechanisms of antagonistic action. In a short time, it significantly reduces *A. alternata*. The pathogen develops until the moment of contact with the fungus-antagonist, simultaneously it sporulates and changes the colony's color, acting as a super–parasite (Mirkova, 1983).

In the third case of double cultures, even when *A.alternata* has a significant space and time advantage, *T. harzianum* has shown to have an antagonistic influence. This effect is produced because of competition for food and space, mycoparasitism and possible antibiosis (Sempere and Santamarina, 2007).

Avoiding direct contact with an antagonist has given the pathogen an opportunity for greater development. However, it has also shown that *T. harzianum* expresses a reducing effect over both volatile and diffusible metabolites as in the previous cases. Generally, the diffusible metabolites have a bigger reducing effect than the volatile ones (Küçük and Kivanç, 2003). Ghildyal and Pandey (2008), came to the same conclusion. According to them, three species of *Trichoderma* with their own diffusible and volatile metabolites have shown the greatest rate of inhibition on *A. alternata* among the investigated pathogens.

At the time of incubation of double cultures (in diffusible and volatile metabolites), discoloring of the colony and the loss of a sporulation both occured. This was confirmed by microscopic examination. In addition, hyphae deformations were noticeable, including greater distances between septa, and empty ends. The *A. alternata* conidia germination was strongly suppressed by chitinase derived from *Trichoderma spp.* for eight hours, resulting in the complete

inhibition of germination, abnormalities, or breaking the germination tubes (Gang et al., 2008).

Hyphal shrinking, cell shortening and septa thickening were found by Barbosa et al. (2001) in Cladosporium herbarum, in the presence of *Trichoderma.* Exactly as we observed, spores of *Trichoderma* were visible in the microscopic preparations.

Morphological abnormalities in the pathogens's structure are confirmed by Ghildyal and Pandey (2008). Induction of deformities is causing by ability of *Trichoderma* to develop the direct interaction with a pathogen and produce antimicrobial substances as well as mycoparasitism involving a physical contact and production of hydrolytic enzymes, toxic components and antibiotics.

Conclusion

This study confirms in-vitro biological activity of *T. harzianum* towards *A. alternata*. It has shown strong reducing effect on the development of *A.alternata* with various mechanisms of antagonistic influence.
The diffusible metabolites have shown bigger reducing effect than volatile. This biocontrol agent caused abnormalities in the pathogens's morphology.
The strong reducing effect of *T. harzianum* towards *A. alternata* can be applied in biological control of this pathogen.

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