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IN VITRO BIOCONTROL ACTIVITY OF TRICHODERMA HARZIANUM AGAINST SOME PATHOGENIC FUNGI ON TOBACCO

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Abstract - *Biological control is an environmentally friendly approach to the plant protection from diseases. It reduces use of a chemical pesticides and it is a contemporary and reliable model which can be introduced into integrated pest management system. The biocontrol activity of Trichoderma harzianum was evaluated against some fungal pathogens on tobacco. In vitro assays confirmed the main mechanisms involved in biocontol-mycoparasitism, antibiosis and competition for food and space. T. harzianum has a biocontrol activity against all tested fungi. It reduced the pathogen growth and completely overgrowth its colony. Their relative growth in the presence of the biocontrol agent was 13,75 to 62,66%. The percentage inhibition of radial growth ranged from 37,34 to 93,83%. Inhibition of the radial growth of R. solani is 61,10%. Inhihition of the leaf pathogen A. alternata is very high -86,64%. T. harzianum showed the highest antagonistic potential (96,95%) against P. parasitica var. nicotianae, and the smallest (68,67%) – in P. debarianum. This result suggests that T. harzianum can be used in biological control of economically most important fungal diseases on tobacco.*

Keywords*: T. harzianum, pathogen, interaction, inhibition*

1. Introduction

Tobacco is a crop of a great interest and benefit for many countries. It has a big agricultural, economical and social impact. Total world tobacco production is still increasing over recent years, mostly in developing countries and toward economic objectives [29]. But tobacco yield, quality and profitability are often affected by disease.

Fungal plant pathogens are among the most important factors that cause serious losses to agricultural products every year [14]. The pathogenic fungi attack different parts of the tobacco plant and cause a various damages. The most common fungal diseases affecting the tobacco in our country, from seedling production to mature leaves are: the damping-off on seedling caused by *Rhizoctonia solani* and *Pythium debarianum*, the black shank caused by *Phytophthora parasitica var.nicotianae* and the brown spot, by *Alternaria alternata.*

The damping-off disease on tobacco seedlings is a very destructive and causesconsiderable damages in tobacco production. The pathogenic fungus attacks the plants on the ground part of the

seedling's stem. The tissue at this point necrotized, died and the following development is not enabled. Infected plants lie down on the soil as they are “cuted”,. The disease is spreading to the neighboring plants and the big infected areas appears in the seed beds. Damages and losses are obvious because of seedling's importance for quality production.

The disease is caused by pathogenic fungi *R.solani* and *P. debarianum.* These are worldwide pathogens causing the damages of many horticultural and agricultural crops. Symptomsnoticeable from the each fungi is almost the same. Only microscopic observation and then, isolation from infected material may help in the true determination of the causing agent. Although chemicals are used, control of these soil borne pathogensis almost impossible.

Black shank affects tobacco plants at all growth stages.The causing agent is a soil-bornefungus*P. parasitica var. nicotianae*.The root system and lower stalk of infected plantsare usually black, from several inches above the soil. The disease development is followedbywilting, yellowing of leaves, development of stem lesions, and plant death.

According to [29], the objective for an ideal cultural system and post-harvest management is not only for high yield, but also for leaf usability and desirable smoke quality. Therefore, the brown spot disease (caused by the pathogenic fungus *A. alternata*) plays a significant role among some economically important diseases on tobacco because of severe economic losses in tobacco production. The recognizable symptom is the presence of brown spots, mainly on tobacco leaves, but on the other parts of plant, too. Their presence causes biochemical changes that strongly reduce the quality of raw tobaccoaccompaniedby non-pleasant aroma.

The available technique of disease control is fungicidal treatment, though often are ineffective. At the same time, they cause harmful consequences to human health and environmental security [19]. Therefore, alternative control measures are focused on the use of biocontrol agents which reduce the disease and are less harmful than conventional fungicides. They have a high level of safety and minimal environmental impacts [21].

Fungi of the genus *Trichoderma* are broadly effective against various plant pathogens and a range of plant species. They successfully suppresssoil pathogens, as well as leaf pathogens[11,13]. There must be hundreds of separate genes and gene products involved in the biocontrol processes. The main mechanisms involved in biocontol are: antibiosis, mycoparasitism and food competition[10,12,19,14]. The use of *Trichoderma* with all mechanisms of biological control will help suppress these pathogens. There are many data aboutthe biocontrol activity of the *Trichoderma* fungi against above mentioned pathogens.

Biological control effect of *Trichoderma sp*. on *R. solani* is confirmed by Küçük and M. 16, 10,11,12,24,21]. These fungi are used in the control of many soil pathogens, including *R. solani* and *P. debarianum*[2].

[1] researched in vitro antagonism of *Trichoderma* species against six fungal pathogens, including *R. solani, P. aphanidermatum*and *P.parasitica var. nicotianae. T. viride* and *T. harzianum* have antagonistic properties against *Phytophthora nicotianae*[29]. According to [8], these two *Trichoderma* strains controlled *Phytophthora* spp. in some crops, including tobacco.

T. harzianum is a biocontrol agent used in control of *A.alternata* [22,19,23]. It is a strong BCA and the base of many commercial products [10]. It can produce lytic enzymes and antifungal antibiotic, it can be competitors of fungal pathogens and, in promoting plant growth [16].

The first and quickest screening technique for antibiotic production and/or mycoparasitism is a petri dish assays [12]. Therefore, the aim of this in vitro investigation is to take a certain knowledge about the biocontrol activity of *T. harzianum* against tobacco fungal pathogens.

2. Materials and methods

The pure cultures of pathogenic fungi on tobacco were obtained from the culture collection of Scientific Tobacco Institute-Prilep. *T. harzianum* was isolated from the root zone of tobacco rhizosphere, by a method of dilution, using Czapek agar.Then, it was transmitted and maintained inPotato-Dextrose Agar Medium (PDA), as well as pathogens.

The dual culturing technique was used for in vitro investigations of *T. harzianum* biocontrol activity against the pathogens.A 5 mm diameter mycelial disc from the margin of the Trichoderma 10 days-old culture and the pathogen were placed on the opposite sites, in the center of each half of the plate with a PDA.
Only in *P. parasitica var.nicotianae*, because of its very slow development(very fast overgrowing by the biocontrol agent), the other variant was used, also.*T. harzianum* was placed after 5 days in the plates with this pathogen.
Trials for each pathogen were set up in three replications, with five Petri dishes for the controls and dual culture plates. They were incubated at 25^{o}C and the diameter of the colonies was daily measured at 10 days, or the fulfillment of control plates for pathogens with faster development.

Interactions between pathogens and biocontrol agent were evaluated according changes in growth of mycelium in pure and mixed cultures, with a modified scale of the Index of sensitivity (0-10) [15]:

0	Good growth and stop before contact with other fungi	6	Faster growth before and stop at contact
1	Slower growth before and stop after contact	7	Faster growth before and growth over other fungi mycelium after contact
2	Slower growth before and growth over other mycelium after contact	8	Slower growth before and stop before contact
3	Good growth before and stop at contact	9	Faster growth and stop before contact
4	Good growth before and overgrowth, above other mycelium, after contact	10	Faster growth before and growth over other fungi before contact
5	Good growth before and grow beneath the colony/mycelium after contact		

Even that there are different pathogenic mechanism, spreading of the pathogen and disease, and finally, a various environmental conditions for the biological control, understanding the mechanisms which are involved and making possible to suppress them enable the use of biological control as an effective and eco-friendly measurement of tobacco disease control.

Relative development of a pathogen in the presence of biocontrol agent (% RD) was estimated by the method [17]:

$$RD = \frac{GP\ in\ in\ the\ presence\ of\ BCA}{GP\ in\ the\ control} x100 \quad (1)$$

Where GP = growth of the pathogen and BCA = biocontrol agent

Percentage inhibition of radial growth of the pathogen (% PIRG) was calculated using the following formula (2) - [18]:

$$PIRG = \frac{C - T}{C} x100 \quad (2)$$

Where C= radial growth of pathogen in the absence of biocontrol agent (control) and T= radial growth of pathogen in the presence of biocontrol agent
For *P. parasitica*, (the second case) we used the modified formula[26]:

$$PIRG = \frac{RPC - (RFP - RID)}{RPC} \text{ x100} \quad (3)$$

Where RPC= Radius pathogen colony control, RFP= radius of final pathogen colony in dual culture with Trichoderma, RIP – radius of initial pathogen colony still without the antagonist.

Degree of antagonism was evaluated according a scale [1]:
Class 1= *Trichoderma* had completely overgrown the pathogen and covered the entire medium surface,
Class 2= *Trichoderma* over grew at least two-thirds of the medium surface,
Class 3= *Trichoderma* and the pathogen each colonized approximately one-half of the medium surface (more than one-third and less than two-third) and neither organism appeared to dominate than another,
Class 4 =the pathogen colonized at least two-thirds of the medium surface and appeared to withstand encroachment by *Trichoderma,* and
Class 5 = the pathogen completely overgrew the *Trichoderma* and occupied the entire medium surface.

The antagonistic potential of the biocontrol agent (% AP) was evaluated by the formula[26]:

$$AP = \frac{\%MP + \%ATP}{2}$$

Where % MP: mycoparasitism potential of Trichoderma – determined by its growing capacity on the pathogen colony in pairs culture. This capacity is 100% against four tested pathogens. %
ATP: antibiosis potential - expressed in percentage of inhibition on the pathogen.

3.Results and disscusion

Dual culturing test

In the dual culture with biocontrol agent, *R.solani* started developmentalmost the same with a check culture, but slowly than *T.harzianum*. But, at the period of incubation, it is falling behind them (Table.1). Their contact is already realized at the second day (Fig 1). From this point, a pathogen was continuing its development (like an any attemptingtowards the opposite direction) until the fourth day and its colony take a lengthened, deformed look (Fig 2). At the contrary, the check colony fulfilght the plate on the fourth day, as well as *T. harzianum*.

Table 1.
Effect of *T. harzianum* on development of *R. solani*

Variant / day	1	2	3	4
*R. solani*Ø	18,70	52,53	82,47	110,00
R. solani	20,40	32,99	40,93	42,80
T. harzianum	30,47	68,58	105,80	110,00
*T. harzianum*Ø	29,20	82,89	94,52	110,00

T. harzianum started its development more rapidly than a pathogen. After the contact with them, it was continuing to develop without obstacles besides the presence of the pathogen (Fig. 2,3). Fulfillment of the petri dishes is achieved at control plots (Table 1). It was developed over the pathogen's colony, covering them (Fig. 4).

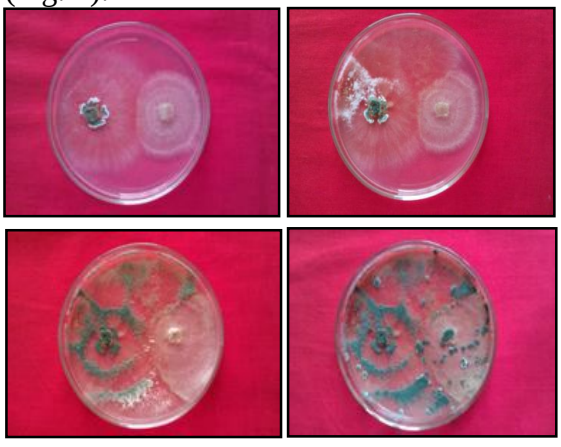

Figure 1 – 4. (upper left to down right)
Development of *R. solani* and *T. harzianum*

in dual culture

The inhibitory effect of *T. harzianum*against the growth of pathogenic fungi was through the nonvolatile and volatile metabolites and *R. solani* was one of the most sensitive fungi to all tested *T. harzianum* isolates [16]. [21] also confirmed that *T. harzianum* is the best antagonistic fungi against *R. solani.*

Isolates of *T. harzianum* can produce antifungal antibiotics and lytic enzymes with it suppreses the pathogens [10,3].[24] described phases, or more correctly, the mechanisms employed in the mycoparasitic activity.

P. debarianum started to grow as well as*T.harzianum*but it has grown very quickly and passed its half of the plate. (Table 2, Fig. 5). But, after the contact with a biocontrol agent, its development became very poor. It can be seen a change of the pathogen's colony color but in a dense, too. It's become yellow to green and look like "melted" (Fig 6,7).[5] founded that culture filtrates of *T.harzianum*cause changes of the mycelial growth andinhibition of zoospore germination.The mycoparasitism was evident in this case. It was concluded there was the lytic activity fromβ-(l, 3) -glucanase against cell walls of *Pythium* spp.[4].

Table 2.
Effect of *T. harzianum* on development of *P. debarianum*

Variant / day	1	2	3	4	5	6
P. debarianumØ	24,87	78,20	110,00			
P. debarianum	29,73	65,73	68,93			
T. harzianum	25,60	55,47	74,47	81,00	90,13	110,00
T. harzianumØ	25,31	65,67	94,76	110,00		

Besides a slower growth, *T. harzianum* grow well.It continued to grow after the contact, over the pathogen's colony and very soon fully covered them (Table 2, Fig 8).

P. parasitica var. nicotianae has a very slow development in the control plates. The mycelial growth rate is very small and it doesn't fill the plates in the period of incubation.

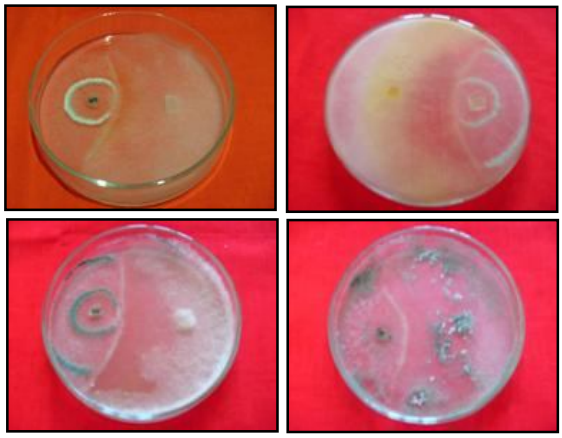

Figure 5 – 8. (upper left to down right) Development of *P. debarianum*and *T. harzianum* in dual culture

Therefore, it was "captured" and overgrown by a biocontrol agent before it can really develop. Even it was allowed to grow before the biocontrol agent was put onto plates with them, it stopped the development very soon, i.e. after the contact with them (Table 3). It is realised at the second day, as well as in the other pathogens. *T. harzianum* developed as well as in the check and filled the plates, besides the pathogen presence (Fig 9-12). *Phytophthora* changes its colony color before it was covered by the biocontrol agent (Fig 10,11). At the end of incubation it is very hard to recognize them (Fig 12).

These results are in contrary to [10] who stated that *T.harzianum* isolate T 22 has not ability to control *Phytophthora* spp.because it has no mechanism to intercept or attack zoospores.But, the presented biocontrol activity of *T. harzianum* against *P. parasitica var. nicotianae* is in agreement with [20] who

founded that competition, mycoparasitism and antagonism are mechanisms of *Trichoderma* spp. against *P.nicotianae.*

Table 3.
Effect of *T. harzianum* ondevelopment of *P. parasitica var. nicotianae*

Variant / day	1	2	3	4	5	6	7	8	9	10
P. parasitica var. nicotianaeØ	+	13,02	22,24	28,04	36,69	43,42	56,67	65,04	73,55	77,09
P. parasitica (at the same time with *T.harzianum*)	+	10,60								
P. parasitica (with *T.harzianum* after 5 days)	39,86	41,43	43,57	44,57						
T.harzianum	19,29	55,71	83,14	110,00						
T.harzianumØ	21,21	67,04	94,36	110,00						

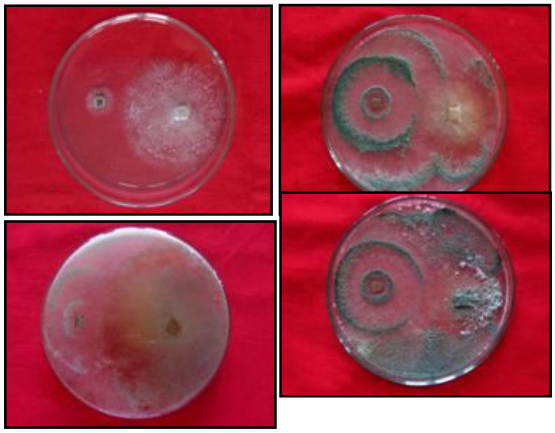

Figure 9–12. (upper left to down right) Development of *P.p. var. nicotianae* and *T. harzianum* in dual culture

It was previously stated that *Trichoderma* spp. are effective against leaf pathogens, too. Accordingly, *T. harzianum*developed more vigorously than *A.alternata*in the control plates, as well as in the dual culture(Table 4).Because of that, *Trichoderma* passed the own half of medium and approached to the pathogen's colony as early as in the second day. (Fig 14). Futher growth of *A.alternata* is seen only in some cases when it makes an effort to develop towards the opposite site (until the third or fourth day).The pathogen's colony had got a white zone and a very light colorwith reduced number of conidia.This is in accordance with the statement of [22] that diffusible as well as volatile substances induced morphological abnormalities in fungal structure.*A. alternata* was "captured", surrounded and covered by *T. harzianum* (Fig. 15, 16).

Table 4.
Effect of *T. harzianum* on development of *A. alternata*

Variant / day	1	2	3	4	5	6	7	8	9	10
A. alternata Ø	12,53	26,93	39,67	49,47	59,53	71,47	84,11	99,60	105,13	107,73
A. alternata	12,13	21,60	27,73	28,80						
T. harzianum	19,13	59,53	101,40	110,00						
T. harzianum Ø	18,27	59,20	105,60	110,00						

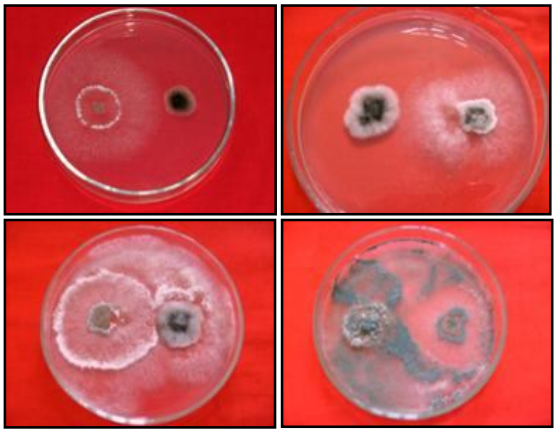

Figure 13–16. (upper left to down right) Development of *A. alternata* and *T. harzianum* in dual culture

The incidence of mycoparasitism against *A. alternata* is of a big importance in biological control. The pathogenicity of *A. alternata* is decreased when the solution of chitinaseis applied in tobacco seedlingsand there is a significantly better effect of crude than purified chitinase [6].

Interactions between pathogens and biocontrol agent

According to the presented datain Table 1-4, and a scale of the Index of sensitivity (0-10), *T. harzianum* showed index 7 in the cases of three pathogens- *R. solani, P. parasitica var. nicotianae* and *A. alternata*, whose *Is* was 1 (Table 5).After plating *T. harzianum* started to grow faster than these pathogens (especially in the case of *Phytophthora*).After contact (which is being very soon-at second day) *Trichoderma* continued to grow over other fungi mycelium. Mycoparasitism was evidenced in the all investigated pathogens.

Table 5.
Index of sensitivity of some fungal pathogens on tobacco and *T. harzianum*

Pathogen / *T. harzianum*	Index of sensitivity (*Is*)	
R. solani	1	7
P.debarianum	6	4
P. parasitica var. nicotianae	1	7
A. alternata	1	7

In the case of *P. debarianum, T.harzianum* showed *Is* 4 because of a very fast growing of the pathogen, who has an index 6.At the beginning, it developed faster than a biocontrol agent, but stop at contact. Besides the presence and very quick growth of the pathogen, *T. harzianum* has good growth and overgrowth the pathogen. *Trichoderma* sp.in vitro inhibits growth and sporulation of several soil-borne plant pathogenic fungi.Trichodermal antagonism may involve mycoparasitism, antibiosis - antimicrobial metabolites and volatile compounds and competition for food and space[20].

Because of competition, *Trichoderma* overgrowth the parasite [24].But, *Trichoderma* can sense the presence of target fungi and appeared to grow tropically towards them. When paired with a target fungus, the endochitinase and exochitinase is activated. The diffusion of this enzyme catalyzes the release of cell wall fragments of target fungi. These fragments are highly potent inducers of enzymes and induce a cascade of physiological changes within the fungus, including an enhancement in *Trichoderma* growth [12].

Relative development of a pathogen in the presence of biocontrol agent

Each pathogen in double culture was developed weaker than in check (Table 1-4).But, the relative growth in the presence of biocontrol agent differs between them. It ranges from 13,75 to 62,66%. The growth of *R. solani* in the presence of antagonist is ranged from 37,6 to 51,1% [17]. In this assay, Rhizoctonia's growth is 38,91%, which is in accordance with this.The weakest growth was estimated in *P. parasitica var.nicotianae* and, the highest, in *P. debarianum* (Figure 1).

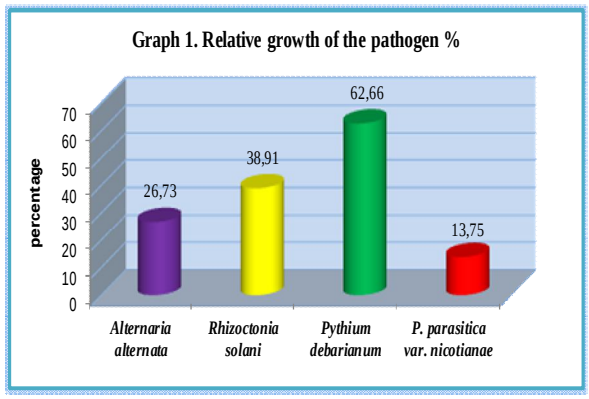

Figure 1. Relative growth of the pathogen (%)

Inhibition of radial growth of the pathogen

Accordingly to the last values of pathogen's colony diameter in double culture, radial growth of pathogens are inhibited in the presence of *T. harzianum*.The percentage of inhibition varies between pathogens (Figure 2).

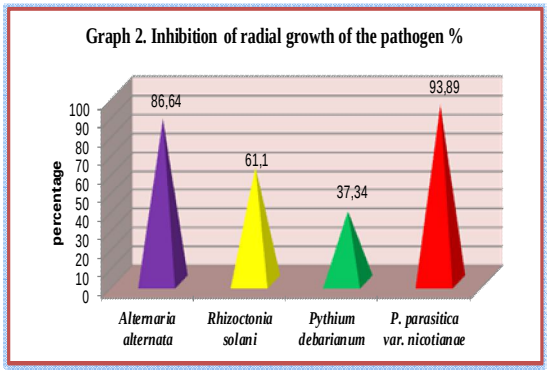

Figure 2. Inhibition of radial growth of the pathogen (%)

The inhibition of radial growth of *R. solani* is 61,10%.This result is in agreement with results of [20] who estimated that *T. harzianum* cause 61-63% reduction of mycelial growth of *R. solani*.Simillar of this, Siameto et al [25] estimated that different isolates ofT.harzianum have different radial growth inhibition of *R.solani*.It ranged from 4,31 to 71,37%.

The control plates were filled with *R. solani* in 4 days and the pathogen was overgrown by antagonist in this period.Therefore, these results are in accordance with the data obtained by [21] who found that *T. harzianum* after 4 days showed antifungal activity of 63% against *R. solani*.

The biocontrol agent achieved the smallest percentage inhibition of radial growth in *P. debarianum*.[18] reported that inhibition of radial growth of *P. aphanidermatum* by two isolates of *T.harzianum*was 60.3%to 69.8%, but in the other (isolate *T.harzianum*-4)was 38.5%, which is similar to the shown inhibition of *P. debarianum* growth -37,34%. *P. aphanidermatum*is one the causing agent of damping-off in tobacco seed beds [27].

Percentage of reduction in the leaf pathogen *A. alternata* was very high-86,64%. Similar of this, [7]founded that *T. harzianum* showed 65,88 % inhibition of *A.alternata* due the production of diffusible metabolites.

Degree of antagonism

T. harzianum has shown an antagonistic abilities against each pathogen.It has completely overgrown the pathogen and covered the entire medium surface. This antagonism of *T. harzianum* against the pathogens is characterized by class 1.It is the highest degree of in vitro antagonism by the used scale.Belonging to class 1 of our isolate of *T. harzianum* is of a big importance for successful biocontrol of pathogens, especially in a case of *Phytophthora* as a destructive parasite.Inoculation with the two pure cultures of *P. parasitica* from anatgonism class 1did not induce disease during the 12 days [1].

Antagonistic potential

Antagonistic potential (resulted from mycoparasitism and antibiosis potential) has a very high value (above 80%) in the three investigated pathogens (Figure 3).

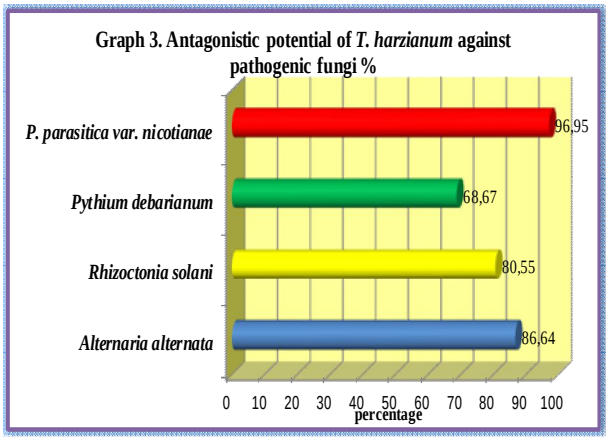

Figure 3. Antagonistic potential

T. harzianum has the highest potential against *P. parasitica var. nicotianae, but* the smallest antagonistic potential was against *P. debarianum*.[18] stated that metabolites released in culture filtrates of *T. harzianum* strongly inhibited pathogen growth, which suggest that antibiotic compounds play an important role in biocontrol activity.But, in some cases purified antibiotics mimic the effect of the

whole agent [3]. The combination of hydrolytic enzymes and antibiotics results in a higher level of antagonism that obtained by other mechanisms alone. It is also noted that direct interaction between the pathogen and BCA must be established before the synthesis of hydrolytic enzymes, toxic compounds and/or antibiotics.Establishment the interaction and the cell-wall degradation is needful because of lower synergizam when the enzymes were added after the antibiotics.Individual strains may produce antibiotics in addition or in conjunction with mycoparasitism[9]. Therefore, the conclusion that all pathogens developed until the contact with biocontrol agent, are in accordance with these findings.

According to [11], different mechanisms might be responsible for biocontrol in different plants, and with different pathogens. Although there are different pathogens, obviously the most important mechanisms are involved in the shown biocontrol activity - competition, antibiosis and mycoparasitism and they are efficient through the mutual action.Themechanisms are integrated and what has been defined as biocontrol is the final result of different mechanisms acting synergistically to achieve disease control [14].

4. Conclusions

In conclusion, this study confirmed that *T. harzianum* has a biocontrol effect against the four investigated fungal pathogens. It reduced development of the certain pathogens through the main mechanisms - mycoparasitism, antibiosis and competition for food and space. The shown biocontrol activity of *T. harzianum* ensures a possibility to use it as a biological control agent against the most important fungal diseases in tobacco. This offers incorporation into cultural practice and environmentally friendly approach to the control management of tobaccofungal diseases.

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