UDC 633.71
Тутун/Tobacco, Vol.62, N^{O} 1-6, 36-44, 2012
University "St. Kliment Ohridski" - Bitola
Scientific Tobacco Institute – Prilep, R. Macedonia

ISSN 0494-3244
UDK: 633.71-153.037:632.48]:632.937.1
632.937.1:[633.71-153.037:632.48
Original scientific paper

EVALUATION OF THE ANTAGONISTIC EFFECT OF *TRICHODERMA ASPERELLUM* AGAINST THE PATHOGEN *PYTHIUM DEBARYANUM*

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ABSTRACT

Investigations on *Trichoderma asperellum* species were conducted in vitro and in vivo. During the cultivation of the fungus *T. asperellum*, on nutrient medium in biculture with phytopathogenic fungus *Pythium debaryanum*, it stops the growth of the pathogenic fungus and continues to develop upon its colony. The growth of the pathogenic fungus cultivated in the presence of the antagonist is reduced for 37.27% in average, while the percentage of inhibition of fungus growth ranged between 54.55% and 72.73%. The biological control of root-rot disease on tobacco seedlings caused by this pathogen was evaluated in greenhouse conditions. Inoculation of seedlings was done with culture of the pathogen and with biculture of *P. debaryanum* and *T. asperellum*. The results have shown high percentage of infection in the control variants, while in variants treated with biculture the infection was minimum, i.e. the healthier and faster growing seedlings were obtained.

Key words: Tobacco, Pythium debaryanum, Trichoderma asperellum, antagonism.

ОЦЕНА НА АНТАГОНИСТИЧКОТО ДЕЈСТВО НА *TRICHODERMA ASPERELLUM* ПРОТИВ ПАТОГЕНОТ *PYTHIUM DEBARYANUM*

Испитувањата се вршени со видот *Trichoderma asperellum* во in vitro и in vivo услови. При одгледување на габата *T. asperellum* на хранлива подлога во двојна култура со фитопатогената габа *Pythium debaryanum*, истата го запира порастот на патогената габа и продолжува да се развива врз нејзината колонија. Порастот на патогената габа одгледувана во присуство на антагонистот е намален во просек за 37.27%, додека процентот на инхибирање на порастот на колонијата од патогената габа се движеше од 54.55% до 72.73%. Биолошката контрола на болеста полегнување на тутунскиот расад причинета од оваа фитопатогена габа, беше оценувана во заштитен простор. Извршено е инокулирање на расад со култура од патогенот и двојна култура од *P. debaryanum* и *T. asperellum*. Добиените резултати покажаа висок процент на зараза кај контролните варијанти, додека кај третираните варијанти со двојна култура, процентот на зараза беше минимален, односно расадот беше здрав и со побрз развој.

Клучни зборови: Тутун, *Pythium debaryanum, Trichoderma asperellum*, антагонизам.

INTRODUCTION

Besides the possibility of using alternative methods for plant protection from diseases, pests and weeds, so far chemicals have been mostly used in practice. Lately, however, the usage of useful microorganisms in plant protection has become more and more important, especially in greenhouse conditions or in organic production. For ecological protection of the environment and plants, increased use of biological products and reduction of chemicals is recommended.

A lot of chemical pesticides are out of use because of the harmful influence on humans health, environment pollution and low effect in pest control or occurrence of resistance in the harmful organisms to the given chemical compound (4)

Today, in the era of integral and ecological protection of plants, in order to reduce the negative consequences of chemicals, more and more attention is paid to the usage of biological protection. Biological control of plant pathogens is not a new idea. The antibiotic properties of species of the genus Trichoderma (7) have been known since the 1930's.

The term biological control is used in different fields of biology, especially in entomology and plant pathology (8). In entomology it denotes the use of living insects or pathogen microorganisms in the control of population of some harmful insects, and in phytopathology it applies to the use of microorganism antagonists to prevent the occurrence of disease. In both fields, the organisms that prevent the appearance of pests and weeds or pathogens are called biological control agents-BCA. The term biological control according to Baker and Cook, (1974), (1), means reducing of inoculum or the disease, i.e. activity of the pathogen made by one or more microorganisms with exception of the humans. In other words, biological control is control of the harmful activities of an organism by one or more other organisms (natural enemies)

Biofungicides use beneficial microorganisms (fungi, bacteria, yeasts, viruses) (4), or products of their metabolism such as toxins, spores and antibiotics which act antagonistically on the causing agent, and plant extracts and ethereal oils in plant protection. Biopesticides have different mechanisms of functioning, and the most common are competition for food and space, antibiosis or antagonism, predatoriness or parasitism (mycoparasitism) and induced (caused) resistance of the host plant (1,4). The essentiality of the antagonistic relations is in the ability of particular microorganisms to excrete antibiotics which act inhibitory, i.e. fungistatic on the development of other microorganisms. The fungus *T. lignorum* produces the antibiotic gliotoxin which acts as a fungicide on the development of pathogenic fungi Pythium spp. and Rhizoctonia spp.; *T. viride* produces the antibiotics viridian and gliotoxin (10). The species of the genus Trichoderma produce ferments (gluconase, cellulase and chitinase) which melt the cell walls of the pathogen, they penetrate into the cell and feed on its cytoplasm (1).

Often, biopreparations are applied through the soil for preventive protection of the root, the neck of the root and the stem of the plants (7). In agricultural production for protection of plants (4), they are used for treatment of seed and tubers, for immersion of the grafts or the seedling, for watering of the seedling and foliar spraying. They are used for treatments in gardening, viticulture, fruit-growing and in other cultivated plant species.

The use of biopesticides in protection of plants is especially important in organic production and its aim is to reduce the use of chemicals without reduction of crop yield. Up to the present day, the application of microbiological products is still recommended in integral protection of plants.

Presently, according to Đorđević (2008), (4), 185 biopesticide products are registered worldwide. Most of them (72) have bacteria as an active substance, 47 are from fungi, 40 are from entomopathogenic nematodes, 24 are from viruses and 2 from protozoa. Depending on the type of organism that has been controlled, biopesticides are divided into: bioinsecticides, biofungicides, bioherbicides, etc.

The most frequently used fungi in obtaining biofunicides are the species of the genus Trichoderma. They are widespread in the nature and have the ability to parasite other

fungi (12), i.e. to colonize the plant root, which makes them excellent means for bio-control (2). Trichoderma species are saprophytic fungi (they are not pathogens) with expressed antagonism, they adjust easily and they grow very fast. They do not attack other useful microorganisms in the soil, they improve the health condition of the plant, increase its resistance and enchance plant growth.

A large number of biofungicides have been obtained from the fungi *T. harzianum, T. viride, T. polysporum* and *T. asperellum*, which are antagonists to the phytopathogenic fungi of the species Pythium, Phytophthora, Rhizoctonia, Verticillium, Sclerotinia - the causing agents of different soil diseases of plants. (1). The biofungicide Trifender WP, which was tested on tobacco seedlings, was made on the basis of *T. asperellum* (13). Using this product, a high efficiency was reached in protection of potato from the soil pathogens *P. debaryanum* and *R. solani* (16).

The aim of this investigation was to evaluate the antagonistic effect of *T. asperellum* on growth of the phytophatogenic fungus *P. debaryanum* in vitro, and to study the biological control of this fungus causing tobacco seedlings damping off in greenhouse conditions.

MATERIAL AND METHODS

The investigations were made in vitro and in vivo. The antagonistic ability of the fungus *Trichoderma asperellum* on the pathogen *Pythium debaryanum* is tested in laboratory. The pure culture of the pathogen *P. debaryanum* is derived from an infected tobacco seedling grown on a nutrient medium potato-dextrose agar, while the pure culture of the fungus *T. asperellum* on a nutrient medium potato-dextrose agar is derived from the biofungicide Trifender WP, on the basis of this fungus. For testing of the antagonistic ability of the fungus *T. asperellum* against this pathogen, the technique of biculture described by Dennis and Webster (1971), (12) is used. Fragments of 3 mm with mycelia of the pathogen fungus and the fungus *T. asperellum* were placed in Petri dishes with a diameter of 10 cm, at a distance of 3 cm. The variant with the biculture of the antagonist and pathogenic fungus was set up in three replications and 4 trials were made. Pure culture of the antagonistic and pathogenic fungus, set up in three replications, was grown separately as a check variant. The Petri dishes were incubated in thermostat at 25^{0}C, for 10 days. Readings were taken daily for 7 days, and the radial growth of mycelia colonies was regularly measured. The percentage of growth of mycelial colony of the pathogenic fungus cultivated in pure culture is estimated by the Siameto formula (11):

$$\% = \frac{\text{radius of growth in the presence of antagonist}}{\text{radius of growth in the check variant}} \times 100$$

According to the growth percentage of the mycelial colony of the pathogen, the percentage of inhibition of the pathogenic fungus by *T. asperellum* was estimated, using the formula of Mudri (7) and Siameto (11):

$$\% \text{ of inhibition} = (a-b/a) \times 100$$

where:

a= radial increase of pathogen in the check variant

b= radial increase of pathogen in the presence of the antagonist

The inhibition of gowth of the pathogenic fungus colony, according to Živković (17), can be presented on a 0-4 scale, in the following categories:

0 = no inhibition,
1 = 1-25% inhibition,
2 = 26-50% inhibition,
3 = 51-75% inhibition,
4 = 76-100% inhibition

Biological control of the pathogen P. debaryanum, the causing agent of damping off

on tobacco seedlings, was checked in vivo, in protected area (biological laboratory). For this aim, two trials were set up. Tobacco seedlings of the variety P66 were grown in pots. The seedlings were inoculated at approximately 4 cm hieight (prior to rapid growth stage), by foliar spraying with suspension prepared from fungus mycelia. Three variants were tested in each trial:

- Seedlings treated with pure culture of the pathogenic fungus *P. debaryanum*
- Seedlings treated with biculture of the pathogen *P. debaryanum* and the fungus *T. asperellum*
- Check-untreated seedlings

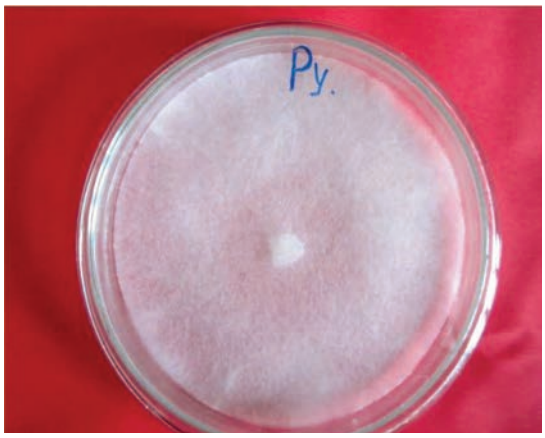
The culture of the fungi was cultivated on potato-dextrose agar, in a thermostat for 10 days. Pure culture of the pathogen was separately cultivated, and in other Petri dishes the pathogenic fungus and antagonistic fungus were cultivated together as a biculture, as was previously explained.

The inoculum was prepared by mixing the mycelia of one Petri dish in 200 ml distillated water, and the obtained suspension was used for spraying of the seedlings from a single pot with 380 cm^2 area. The seedlings were inoculated on 22.06.2011 in the first trial and on 25.07.2011 in the second trial. The control pots with seedlings were treated only with water. Each variant was set in three replications. The efficiency of protection of the seedlings was evaluated according to the occurrence of infected seedlings, i.e. according to the percentage of the infected area.

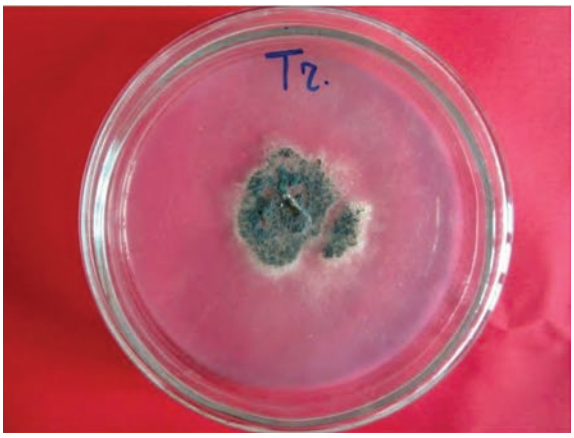
RESULTS AND DISCUSSION

Phytopathogenic fungus P. *debaryanum,* grown on potato-dextrose agar creates snow-white, web-like, airy and fast-growing mycelial colony (Picture 1). Antagonistic fungus *T. asperellum* has poorer growth and creates white-colored colony which few days later, with the formation of conidiophores with conidia, turns green (Picture 2).

The growth of mycelia from the fungi cultivated on a nutrient medium was monitored for a period of 7 days. From the results of the laboratory analysis on the growth of mycelial colony of phytopathogenic fungus *P. debaryanum* and antagonistic fungus *T. asperellum*, cultivated as a pure culture and together in biculture, the percentage of growth of the colony and the percentage of inhibition of the pathogen by the antagonist were estimated.



Pc. 1. *P. debaryanum* – pure culture



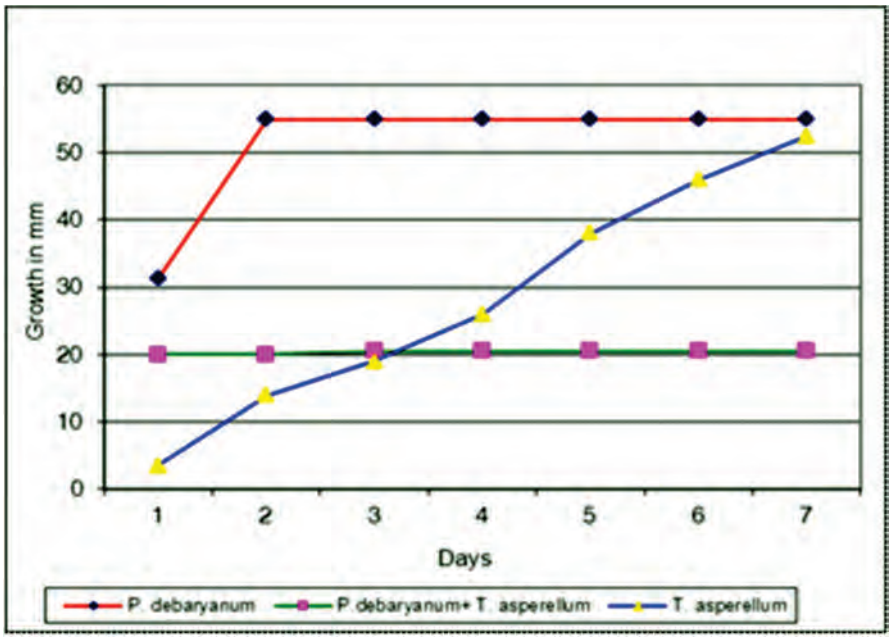
Pc. 2. *T. asperellum* - pure culture

In Table, a scheme is presented of the daily growth of fungi cultivated as a pure culture and as a biculture of the pathogen and the antagonist. The mean values of the replications from four trials are presented.

Table1. Development of the colony during incubation in mm

Variant	Days of incubation						
	1	2	3	4	5	6	7
Pythium debaryanum	31.30	55.00	55.00	55.00	55.00	55.00	55.00
P.debaryanum+ T. asperellum	20.00	20.00	20.50	20.50	20.50	20.50	20.50
Trichoderma asperellum	3.50	14.00	19.00	26.00	38.00	46.00	52.50

Phytopahogenic fungus *P. debaryanum* cultivated as a pure culture showed very fast development. 24 hours after the sowing it reached a growth of 31.30 mm, and on the second day it could be seen that the whole Petri dish was filled, i.e. the radius of the mycelia colony was 55.00 mm. The antagonistic fungus *T. asperellum* cultivated as a pure culture showed poorer growtht in comparison to the phytopahogenic fungus. The radius of its colony was 3.50 mm on the first day, and on the seventh day it reached 52.50 mm. The pathogenic fungus cultivated in the presence of the antagonist, the first day had a good growth which reached 20.00 mm. On the succeeding days, the fungus had a very poor growth. On the third observation day a minimum growth of mycelial colony was measured - only 20.50 mm, which remained unchanged until the end of observation on the 7th day. Such a poor growth of the pathogen fungus was due to the antagonistic activity of T. asperellum which inhibited its growth. The growth of fungal colony in the period of seven days after incubation is presented on Graph. 1.



Graph 1. Daily growth of the mycelial colony

On the seventh day of observation, there were no considerable differences in the growth of mycelial colony of the fungi grown in pure culture. In all four trials almost the same results were obtained (Table 2). Phytopathogenic fungus *P. debaryanum* reached the highest growth with a radius of 55.00 mm. Similar was the development of the antagonist fungus, with 50.00 mm to 55.00 mm radius. The smallest growth was observed in the pathogenic fungus grown in the presence of the antagonist, where the radius of the colony ranged from 15.00 mm in the fourth trial to 25.00 mm in the third trial. In the first and the second trial, the radius of the colony was 22.00 and 20.00 mm, respectively.

Table 2. Growth of the colony of fungi on the 7th day of incubation

Variant	Radial growth of the colony in the trials				Average in mm
	1	2	3	4	
Pythium debaryanum	55.00	55.00	55.00	55.00	55.00
P.debaryanum+ T. asperellum	22.00	20.00	25.00	15.00	20.50
Trichoderma asperellum	55.00	55.00	50.00	50.00	52.50

The pathogen fungus grown as pure culture has a higher growth of mycelial colony (37.27% in average) than in the presence of the antagonist fungus (Table 3). In all four trials a higher growth of the colony was measured. In the fourth trial, the growth of the colony was 27.27% higher, which is the smallest growth, while the highest growth of the pathogen (45.45%) was recorded in the third trial.

Table 3.Percentage of growth of the colony of *P. debaryanum*

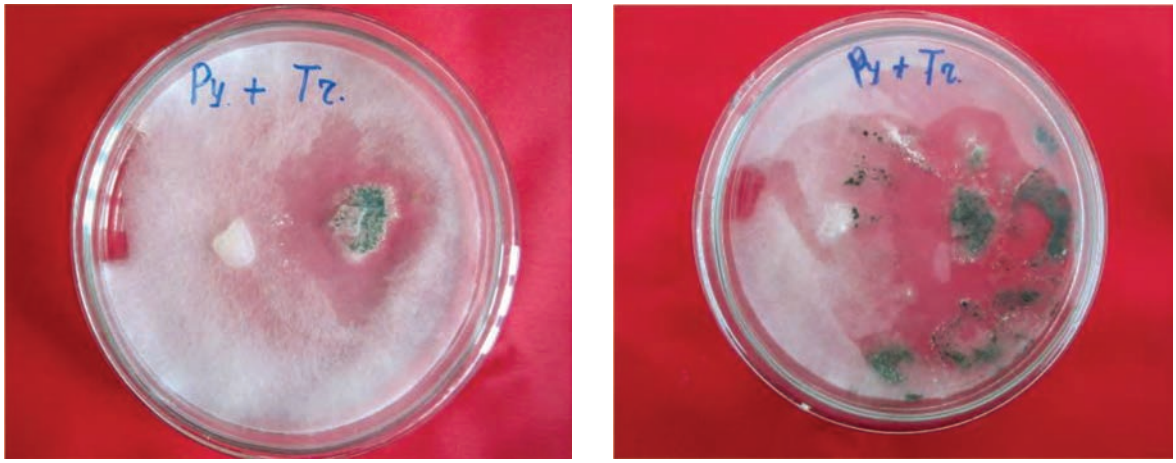
Variant	Radial growth of the colony in the check, mm	Radial growth of the colony in the presence of the antagonist, mm	% of growth of the colony
I Trial	55.00	22.00	40.00
II Trial	55.00	20.00	36.36
III Trial	55.00	25.00	45.45
IV Trial	55.00	15.00	27.27
	Average		**37.27**

From the data obtained by measuring the radial growth of the mycelial colony of the pathogen in the check variant and in the one grown in biculture, the percentage of inhibition of growth of the colony of pathogenic fungus by the antagonist was estimated. The percentage of inhibition averaged 62.73% (Table 4).

Table 4. Inhibitory effect of *T. asperellum* on *P. debaryanum*

Variant	Radial growth of the colony in mm in the check variant	Radial growth of the colony in mm in the presence of the antagonist	% of inhibition	Index
I Trial	55.00	22.00	60.00	3
II Trial	55.00	20.00	63.63	3
III Trial	55.00	25.00	54.55	3
IV Trial	55.00	15.00	72.73	3
	Average		**62.73**	3

The highest percentage of inhibition of growth of the mycelial colony in the phytopathogenic fungus *P. debaryanum* (72.73%) was obtained in the fourth trial, and the lowest percentage of inhibition (54.55%) was recorded in the third trial. In the other two trials, the inhibition of growth of the colony was 60.00% and 63.63%. From the data presented in the table, it can be seen that the antagonist fungus *T. asperellum* showed high percentage of inhibition of growth of the colony from the pathogenic fungus. During the cultivation of the antagonistic fungus on nutrient medium in biculture with the pathogenic fungus, the antagonist not only inhibited the growth of the pathogen but it also continued to develop on its colony (Picture 3).



Picture 3. Colony of P. debaryanum in the presence of the antagonist (biculture)

The results obtained in this investigation confirm the possibility for use of the genus Trichoderma species in biological control of plant diseases. The antagonistic effect of 15 isolates of *Trichoderma harzianum* on five soil phytopathogenic fungi by using biculture was studied by Siameto (11). All the isolates showed a serious antagonistic effect on the growth of mycelia of the pathogenic fungi in comparison with the check. The maximum inhibition of growth (73.33%) was recorded in Pythium spp.

Biological control of the pathogenic fungus *P. debaryanum* was investigated in biological laboratory, on tobacco seedlings cultivated in pots. A pure culture of the pathogenic fungus and culture where the pathogen was cultivated in biculture, together with the antagonist, were used as an inoculum. In both trials two days after the inoculation the first symptoms of infection on the seedlings inoculated with pure culture of the pathogen appeared. In the next days, the infection spread very fast in this variant, reaching up to 50%. Contrary to this, in the check variant there was a poor percentage of naturally induced infection, while in the seedlings treated with biculture of the pathogen and the antagonist, very small percentage of infected plants could be seen. 10-15 days later, the seedlings treated with pure culture of the pathogenic fungus were totally destroyed. The seedlings cultivated in the presence of the antagonistic fungus were not infected and they had better growth compared to the control (Picture 4 and Picture 5).

Pc. 4. Inoculated seedlings (Left- Pythium, right- Pythium+Trichoderma)-I trial

Pc. 5. Inoculated seedlings (Left- Pythium, right- Pythium+Trichoderma)-II trial

Two biological control agents, *Bacillus subtilis* and *Trichoderma harzianum*, were tested by Maketon et al. (5), alone or in combination, for control of three tobacco diseases *(Ralstonia solanacearum, Pythium aphanidermatum* and *Cercospora nicotiana*). The results showed that the two biological agents applied together give higher efficiency, which is equal to chemical treatments in the control of these diseases, than being applied separately. The high efficiency of biofungicides in control of Pythium species has been confirmed in several investigations (6,15). By application of biofungicides on the basis of *T. harzianum*, the yield of the treated crops was increased for 13% and according to Tran (14), the cultures treated with Trichoderma grow better and give higher yields than the untreated.

The fungus *T. harzianum* through the product TRI 003, was used in the investigations of Parađiković and co. (9), for control of the pathogens *P. debaryanum* and *R. solani*, causing agents of damping off on tomato seedlings, where better results were achieved than by the use of the standard fungicides Previcur and Dithane. The same product gave higher efficiency in protection of salad seedlings, compared to the standard products (2,3).

In investigations made by Tashkoski (13), Trifender WP based on the fungus *T. asperellum* achieved 80% efficiency in the protection of tobacco seedlings from the pathogen *P. debaryanum* grown in protected area.

Analyzing the results obtained by the investigations of tobacco seedlings made in vitro in laboratory conditions and in vivo in proteced area, the fungus *T. asperellum* was not only a good antagonist inhibiting the growth of the pathogenic fungus colony, but it also appeared to be a real mycoparasite. The antagonistic fungus through its mechanisms of acting - antagonism and predatoriness (mycooparasitism), inhibited the infestation of tobacco seedlings by the pathogenic fungus *P. debaryanum*.

CONCLUSION

Phytopathogenic fungus *P. debaryanum* cultivated as pure culture on potato-dextrose agar as a nutrient medium has 37,27% faster growth than when cultivated in the presence of the antagonist.

The fungus *T. asperellum* showed high antagonistic activity upon the growth of mycelial colony of the pathogenic fungus. The percentage of inhibition of growth of the pathogenic colony was between 54.55% and 72.73%, or 62.73% in average.

The tobacco seedling inoculated with pure culture of the pathogenic fungus was completely destroyed, while the seedlings inoculated with culture of the pathogen cultivated in the presence of antagonist the percentage of infected plants was very low. Seedlings cultivated in the presence of the antagonistic fungus were not infected by the pathogen and they had a better growth and development in comparison to the control.

The fungus *T. asperellum* appeared to be a real antagonist and mycoparasite of the phytopathogenic fungus *P. debaryanum*, which can be used in the future, through biological products, for protection of tobacco seedlings in commercial production.

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