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# ANTAGONISM OF *TRICHODERMA ASPERELLUM* TO *PHYTOPHTHORA PARASITICA VAR. NICOTIANAE*

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## ABSTRACT

Antagonistic effect of the fungus *Trichoderma asperellum* to the pathogenic fungus *Phytophthora parasitica var. nicotianae in vitro* and *in vivo* was investigated in this paper. *Trichoderma asperellum,* grown on nutrient medium in dual culture with pathogenic fungus *Phytophthora parasitica var. nicotianae* inhibits the growth of the pathogen and continues to develop on its colony. Development of the fungus grown in presence of the antagonist was reduced by 32.14%, and the percentage of inhibition was 67.86%. *T. asperellum* - biological agent for control of the soil-borne pathogen *P. parasitica var. nicotianae*, was investigated on tobacco seedlings in a protected area. Seedlings were inoculated both with pure culture of the pathogen and with dual culture grown in presence of the antagonist. In the seedlings treated with culture of the pathogen high percentage of infection was observed, while the seedlings treated with dual culture developed healthier and better.

**Keywords:** tobacco, *Trichoderma asperellum, Phytophthora parasitica var. nicotianae*, antagonism

# АНТАГОНИЗАМ НА *TRICHODERMA ASPERELLUM* ВРЗ *PHYTOPHTHORA PARASITICA VAR. NICOTIANAE*

Во овој труд е испитувано антагонистичкото дејство на габата *Trichoderma asperellum* врз патогената габа *Phytophthora parasitica var. nicotianae* во in vitro и in vivo услови. Габата *T. asperellum* одгледувана на хранлива подлога со патогенот *P. parasitica var. nicotianae* во двојна култура, го инхибира порастот на патогената габа и продолжува да се развива врз неговата колонија. Порастот на патогената габа одгледувана во присуство на антагонистот е намален во просек за 32,14%, додека процентот на инхибирање изнесува 67,86%. Биолошкиот агенс *T. asperellum* за контрола на почвениот патоген *P. parasitica var. nicotianae*, беше испитуван на тутунски расад во заштитен простор. Расадот беше инокулиран со чиста култура од патогенот и со двојна култура одгледувана во присуство на антагонистот. Кај расадот третиран со култура од патогенот имаше висок процент на зараза, додека расадот третиран со двојна култура беше здрав и со подобар развој.

**Клучни зборови:** Тутун, *Trichoderma asperellum, Phytophthora parasitica var. nicotianae*, антагонизам.

## INTRODUCTION

The soil pathogen *Phytophthora parasitica var. nicotianae* – the causing agent of black shank is a serious disease on tobacco. It attacks tobacco crop in all stages of its development. The disease appears in seedbeds and after transplanting in field, during the whole growing season. The first infections appear on root, root neck and bottom part of the stalk. The root of infested plants becomes necrotic, the stalks are dark brown and the leaves turn yellow and dry. The first occurrence of the disease was reported in Java, Indonesia in 1896 (Tashkoski, 1994), by the Dutch scientist J. van Breda de Haan. Presently it occurs in all continents in which tobacco is grown, causing severe losses to tobacco industry. In our country, losses caused by this pathogen in some plots of the regions Prilep and Strumitza approached 50-80 % (Tashkoski, 1999). According to literature data, in some tobacco producing areas the damage was 85-100 % (Fengli et al., 2011, Gallup et al., 2006), which brought into question the production of tobacco in those areas. Since the pathogen can infest tobacco in all stages of growth, protection of tobacco is of crucial importance. Application of some chemicals can reduce the percentage of infection, but they can not ensure complete and effective protection of tobacco. Chemical control of the disease can be effective only when used in combination with other measures. Therefore, tobacco protection from this pathogen requires an integral approach that includes modern agrotechniques, application of chemicals and breeding of resistant varieties. The best method for *Phytophthora* control is planting of resistant tobacco varieties (Gallup et al., 2006). Varieties with one gene of resistance as well as those with high level of partial resistance (Sullivan et al., 2005) provide a high degree of protection from the disease.

In the recent period, efforts have been made to reduce the application of chemicals in plant protection and higher attention was paid to biological control, i.e. to the use of bioproducts. Application of bioproducts in the control of harmful pathogens in agriculture is a subject of great interest for consumers and ecologists.

Among the most popular antagonistic fungi which are used in biological control of plant pathogens are the species of the genus *Trichoderma*, the antibiotic and antifungal properties of which have been known since the 1930-ies (Mudri & Sušinjak, 2000). These species act as parasites on plant pathogens (Anonymous, 2011) and as their competitors for food. They have an antagonistic effect and cause induced resistance in the host plant (Grahovac et al. 2009).

*Trichoderma* species showed high antagonistic effect against pathogenic fungus *P. parasitica var. nicotianae.* In investigations of Chen et al., (2009), isolates of the antagonistic fungus *Trichoderma viride* grown in dual culture inhibited the growth of the pathogenic fungus for 29.12 %. The same isolates, used in the control of black shank disease, showed 56.53 % higher effectiveness compared to metalaxyl. Application of the antagonistic fungus *T. harzianum* (Fernandes et al., 2002) largely decreases the inoculi of the pathogenic fungus in the soil, thereby reducing the intensity of black shank infection. High inhibitory activity against *P. parasitica var. nicotianae* was also observed in some antagonistic bacteria isolated from tobacco rhizosphere (Fengli et al., 2011). Trichoderma species, due to their nematicidal effect (Imran et al., 2001), are used in the control of plant nematodes. This is of particular importance for the spread of black shank disease in tobacco, because the nematodes, through the injuries they made in the root system,

enable the pathogen to enter into the plant and to infect it. The nematodes control will reduce the possibilities for infection of tobacco by this pathogen. Today, a number of biofungicides are available for commercial use, obtained from *T. harzianum, T. viride, T. asperellum* and *T. polysporum* (Anonymous, 2011). In investigations of Tashkoski & Čifligaroski (2011), biofungicide Trifender WP, based on *T. asperellum,* showed high effectiveness in the control of soil-borne pathogens *Rhizoctonia solani* and *Pythium debaryanum* on tobacco seedlings grown in protected area.

The main goal of this investigation is to estimate the antagonistic effect of *T. asperellum* against the pathogenic fungus *P. parasitica var. nicotianae* in dual culture and biological control of the pathogen in tobacco seedlings.

## MATERIAL AND METHODS

Investigations were made in laboratory conditions on culture obtained from antagonistic and pathogenic fungi and on tobacco seedlings in protected area. Antagonistic effect of the fungus *Trichoderma asperellum* against pathogenic fungus *P. parasitica var. nicotianae* was estimated. Pure culture of the pathogenic fungus was obtained from infected tobacco plants and grown on potato dextrose agar, while the pure culture of the fungus *T. asperellum* on a nutritive surface potato-dextrose agar is derived from the biofungicide Trifender WP, on the basis of this fungus.

Antagonistic ability of the fungus *T. asperellum* against the pathogen *P. parasitica var. nicotianae* was tested by dual culture technique Dennis and Webster, (1971), (loc cit. Shalini and Kotasthane, 2007).

3 mm fragments with mycelia taken from the pathogenic fungus and from antagonist were placed 3 cm apart in 10-cm Petri dishes, on potato dextrose agar, in four experiments with three replications. Petri dishes were incubated in a thermostat at $25^0$C for a period of 10 days. Radial growth was measured for seven days on mycelial colony of the pathogen grown in presence of the antagonist and as a pure culture, which served as a check. The growth of mycelial colony of pathogenic fungus grown as pure culture was calculated by the equation of Siameto et al., (2010):

$$\% = \frac{\text{radial growth in the presence of the antagonist}}{\text{radial growth in the check}} \text{ x } 100$$

Percentage of inhibition of the pathogen by *T. asperellum* was calculated by the equation of Mudri & Sušinjak, (2000) and Siameto et al. (2010):

$$\text{inhibition } \% = (\text{a - b/a}) \text{ x } 100,$$

where:

a = radial growth of the pathogen in the check
b = radial growth of the pathogen in presence of the antagonist

According to Zivkovic et al., (2010), inhibition of pathogen's colony can be presented in the following scale:
0 = no inhibition, 1 = 1-25% inhibition, 2 = 26-50% inhibition, 3 = 51-75% inhibition, 4 = 76 -100 % inhibition.

Biological control of *P. parasitica var. nicotianae* was investigated on tobacco seedlings in protected area. Two experiments were conducted in three

replications. Seedlings of the variety P-66 were sown in pots in an area of 380 cm$^2$, and in later phases it was inoculated with suspensions prepared from mycelium of the fungus in the following three variants:

- Seedlings treated with pure culture of the pathogen *P. parasitica var. nicotianae*

- Seedlings treated with dual culture of the pathogen and *T. asperellum*, and

- Check – untreated seedlings

Fungal culture was grown in nutrient medium potato dextrose agar, in thermostat at 25$^0$C for a period of 10 days. A culture of the pathogenic fungus was grown separately, while in other Petri dishes, pathogenic fungus was grown in dual culture with the antagonistic fungus.

Seedlings from a 380 cm$^2$ pot were inoculated in Petri dish with inoculum prepared from the mycelia. Mycelial colony was mixed in 200 ml distilled water and the obtained suspension was used for spraying tobacco seedlings. Inoculations of tobacco seedlings were performed on 22.6.2011 in the first variant and on 25.7.2011 in the second variant.

The seedlings that were used as a check were treated with pure water. The health condition of seedlings was evaluated according to the presence of infected plants, i.e. to the percentage of infected area.

## RESULTS AND DISCUSSION

*P. parasitica var. nicotianae* is a soil-borne pathogen which can be easily isolated as a pure culture from infected plants or from the soil. When grown on potato dextrose agar it forms white substrate mycelium (Fig. 1) in which a great number of conidia and chlamydospores are made. The antagonistic fungus *T. asperellum* in the beginning is similar to the pathogenic fungus and forms white mycelia which after a few days becomes green, as a result of conidiophores and conidia formation (Fig. 2 ).

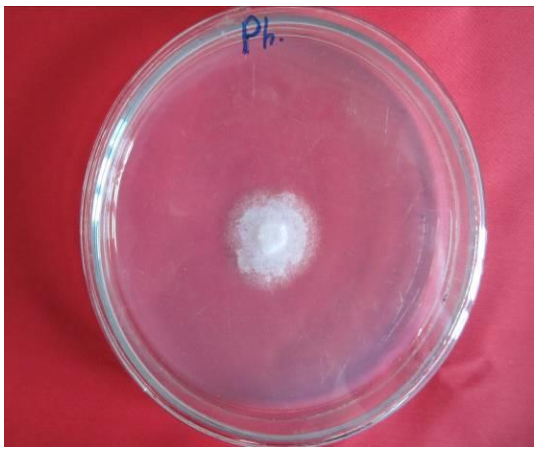

Fig. 1. Pure culture of *P. parasitica var. nicotianae*

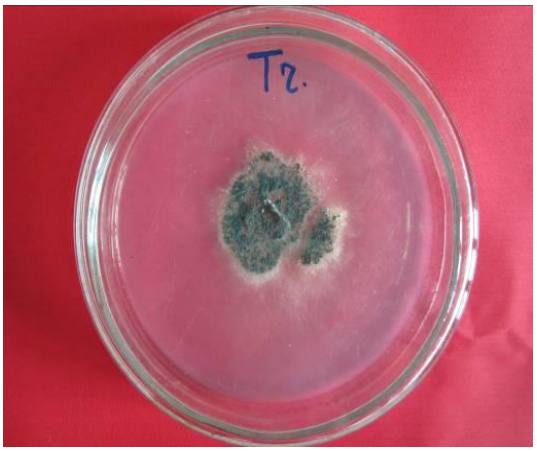

Fig. 2. Pure culture of *T. asperellum*

Percentage growth of pathogen's colony and percentage of its inhibition by the antagonist were calculated according to the results of measurements of colony's growth. Daily growth of fungi both in pure culture and in dual culture is presented in Table 1 and Fig. 3, in which mean values from the three replications in four trials are given.

**Table 1. Fungal growth (mm) during the incubation period by days**

| Variants | Days of incubation | | | | | | |
|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| ***P. parasitica var. nicotianae*** | 2,50 | 7,50 | 10,70 | 15,00 | 18,70 | 25,00 | **31,20** |
| ***P. parasitica var. nicotianae +T. asperellum*** | 2,50 | 6,00 | 9,00 | 10,00 | 10,00 | 10,00 | **10,00** |
| ***Trichoderma asperellum*** | **3,50** | **14,00** | **19,00** | **26,00** | **38,00** | **46,00** | **52,50** |

24 hours after sowing, radial growth of the pathogenic fungus *P. parasitica var. nicotianae* grown in pure culture was 2.50 mm, while seven days after, at the end of observations, it increased to 31.20 mm. Growth of the antagonistic fungus *T. asperellum* in pure culture was similar to that of the pathogenic fungus. Colony radius on the first day of observation was 3.50 mm and on the seventh day it increased to 52.50 mm. When pathogen was grown in dual culture with the antagonist, radial growth of the colony was 2.50 mm on the first day, increasing gradually up to the fourth day, when it amounted to 10 mm and stayed unchanged to the end of observation. Poor growth of mycelial colony is due to the presence of *T. asperellum* and its antagonistic effect on pathogenic fungus.

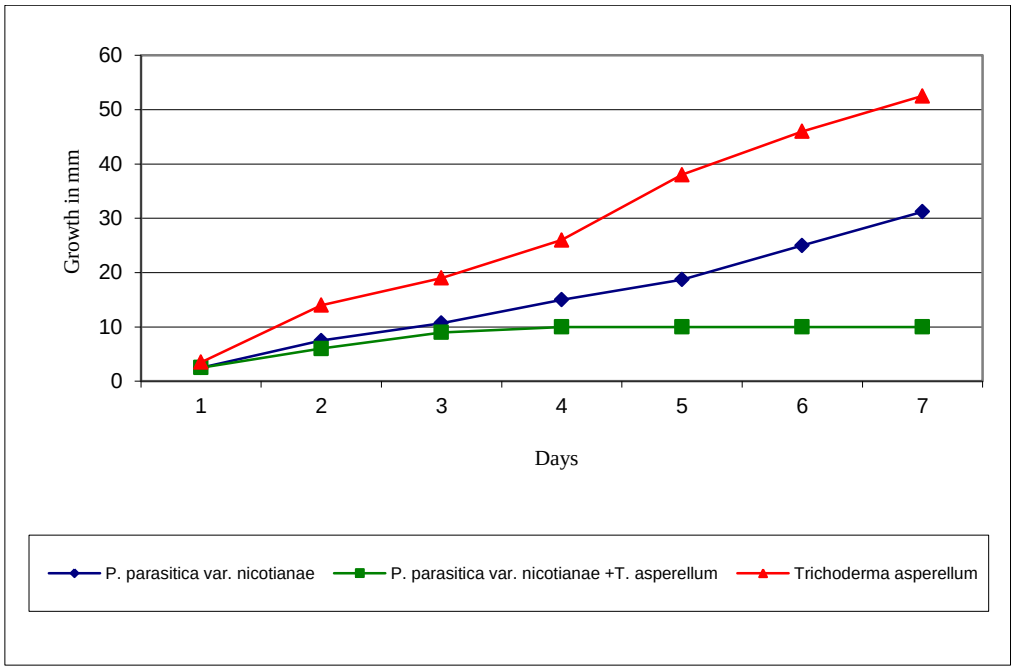


Fig.3 Daily growth of the colony

Similar results were obtained in all four trials on the seventh day of observation. No major differences were observed in growth of the fungal colony (Table 2). In the pathogen *P. parasitica var. nicotianae*, radial colony growth ranged 30.00 mm - 35.00 mm in the third trial, and in the fungus *T. asperellum* from 50.00 mm in the first and fourth trial to 55.00 mm in the second and third trial.

**Table 2. Colony growth of the fungus on the 7th day of incubation**

| Variants | Radial growth of the colony (mm) by trials | | | | Average, in mm |
|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | |
| ***P. parasitica var. nicotianae*** | 30,00 | 30,00 | 35,00 | 30,00 | **31,25** |
| ***P. parasitica var. nicotianae +T. asperellum*** | 10,00 | 10,00 | 10,00 | 10,00 | **10,00** |
| ***Trichoderma asperellum*** | **50,00** | **55,00** | **55,00** | **50,00** | **52,50** |

Unlike this, on the seventh observation day, pathogenic fungus grown in dual culture with the antagonistic fungus had an increase of only 10 mm in all trials.

According to the results, pathogenic fungus *P. parasitica var. nicotianae* grown in pure culture had 32.14 % higher mycelial growth than when it was grown in presence of the antagonist (Table 3). Inhibition of its growth by the antagonist reached 67.86 % (Table 4). Chen et al., (2009) also reported that the isolate TG050609 of *T. viride* showed 29.12% inhibitory effect on the colony of *P. parasitica var. nicotianae* grown in dual culture.

**Table 3. Percentage growth of the colony of pathogenic fungus *P. parasitica var. nicotianae***

| Variants | Radial growth of colony in the check, mm | Radial growth of colony in presence of the antagonist, mm | Colony growth % |
|---|---|---|---|
| **Trial I** | 30,00 | 10,00 | **33,33** |
| **Trial II** | 30,00 | 10,00 | **33,33** |
| **Trial III** | 35,00 | 10,00 | **28,57** |
| **Trial IV** | 30,00 | 10,00 | **33,33** |
| **Average** | | | **32,14** |

**Table 4. Inhibitory effect of *T. asperellum* upon *P. parasitica var. nicotianae***

| Variant | Radial growth of colony in the check, mm | Radial growth of colony in presence of the antagonist, mm | Inhibition % | Index |
|---|---|---|---|---|
| **Trial I** | 30,00 | 10,00 | 66,67 | **3** |
| **Trial II** | 30,00 | 10,00 | 66,67 | **3** |
| **Trial III** | 35,00 | 10,00 | 71,43 | **3** |
| **Trial IV** | 30,00 | 10,00 | 66,67 | **3** |
| **Average** | | | **67,86** | |

The results obtained during investigation confirmed the high antagonistic effect of the fungus *T. asperellum* to *P. parasitica var. nicotianae*. The antagonist not only inhibits the growth of this pathogenic fungus, but successfully develops on its colony, suppressing its further growth (Fig. 4 and Fig. 5).

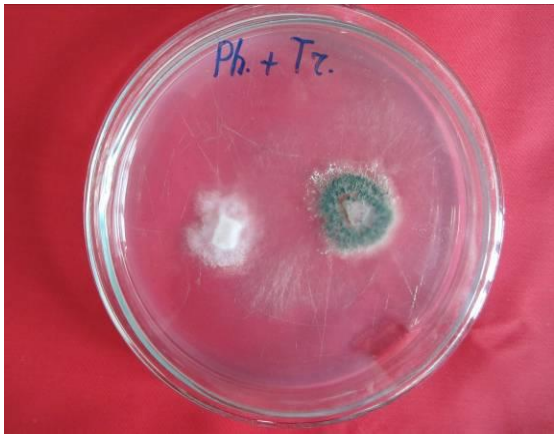


Fig. 4. Dual culture of *P. parasitica var. nicotianae* and *T. asperellum*

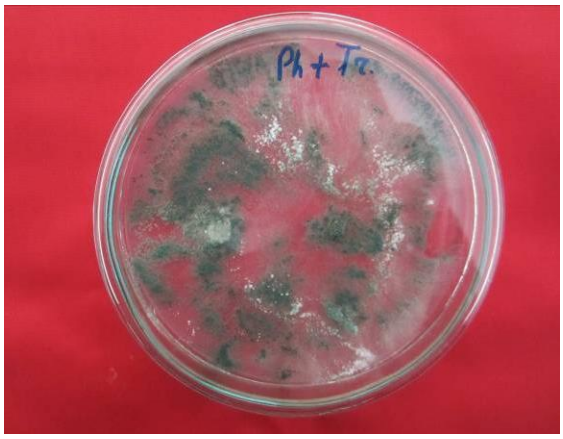


Fig. 5. Growth of *T. asperellum* on the colony of *P. parasitica var. nicotianae*

Good results were obtained with investigations of *T. asperellum* on tobacco seedlings in protected area for biological control of the phytopathogenic fungus *P. parasitica var. nicotianae*. Seedlings were inoculated with inoculi made from pure culture of the pathogen and from dual culture of the pathogen and the antagonistic fungus.

The first symptoms of infection in seedlings treated with pure culture of the pathogen in both trials occurred 2-3 days after inoculation. The infection spread very rapidly and in only a few days more than half of the seedlings were destroyed. In seedlings treated with inoculum obtained from the dual culture very small percentage of infected plants could be observed. Unlike this, low ocurrence of disease from natural infection was observed in the untreated check. 10-15 days after inoculation, seedlings treated with inoculum from pure culture were completely destroyed, while in seedlings treated with dual culture not only infection did not spread but they had better growth and development compared to the check ( Fig. 6 and Fig. 7).


Fig. 6. Inoculated seedlings (left - in pure culture , right – in dual culture ), I trial

Fig. 7. Inoculated seedlings (left - in pure culture , right – in dual culture ), II trial

High effectiveness of *Trichoderma* species in the control of *P. parasitica var. nicotianae* on tobacco was confirmed in investigations of Chen et al., (2009). *T. viride* isolate TG050609 showed 56.53 % higher effectiveness than metalaxyl.

In this investigation, made in laboratory conditions on pathogen culture

and tobacco seedlings in protected area, *T. asperellum* proved to be good antagonist and true mycoparasite which inhibits the growth of pathogenic fungus *P. parasitica var. nicotianae*. Through its mechanisms – mycoparasitism and antagonism, it protects tobacco seedlings from infection by this soil-borne pathogen.

## CONCLUSIONS

The soil-borne pathogen *P. parasitica var. nicotianae* - the causing agent of black shank disease on tobacco, grown in pure culture on nutrient medium potato-dextrose agar has 32.14 % higher growth compared to that grown in dual culture with antagonistic fungus.

Inhibitory effect of the fungus *T. asperellum* on the growth of mycelial colony of the pathogenic fungus was 67.86 %.

Tobacco seedlings inoculated with pure culture of the pathogenic fungus were infected and completely destroyed, while the seedlings inoculated with dual culture of the pathogen and the antagonist showed a very low percentage of infected plants. Seedlings grown in presence of the antagonistic fungus had faster growth and better development compared to the check variant.

*T. asperellum* proved to be a true antagonistic fungus and mycoparasite to the fungus *P. parasitica var. nicotianae* and it can be used for biological control of this phytopathogen in tobacco production.

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