DOI: 10.48113/496_2020_155-165

# TRICHODERMA BIOCONTROL AGENTS FOR TOBACCO SEEDLINGS PROTECTION

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**Abstract.** Tobacco production has intentions to introduce a biological control as contemporary and environmentally friendly plant protection measure.

*Rhizoctonia solani* is a very destructive pathogen, the causing agent of damping off in tobacco seedlings. Application of biological control is a good way of control, according to all moments that make difficulties in control of this pathogen. Biocontrol activity of some *Trichoderma species - T. aureoviride, T. hamatum and T. harzianum* was investigated *in vitro* - by dual culturing technique and *in vivo* – in greenhouse .

All the three species show the high antagonistic potential and percentage inhibition of radial growth of the pathogen, but the highest value is in *T. harzianum.* In conditions of artificially inoculation with the pathogen, the intensity of a disease was reduced applying the investigated biocontrol agents.

*T. harzianum* has the best reducing effect on the damping off in all application ways of this biocontrol agent. The intensity of disease was only 5.56% in the application 15 days after sowing, while in the application before sowing and mixed with seeds, there was no disease incidence.

Application of *Trichoderma* by treatment of seed has confirmed as the best application way of the biocotrol agents.

Biocontrol activity of these *Trichoderma* species enables them to be used in the biological control against R. solani in tobacco seedling protection.

**Keywords.** Biological control, *Rhizoctonia solani, Trichoderma aureoviride, Trichoderma hamatum, Trichoderma harzianum,* atagonistic potential, control.

## Introduction

Fungal diseases are among the most important factors that cause serious losses of tobacco yield each year. Damping off is a very destructive disease in production of tobacco seedlings. The most common causing agent of the disease is Rhizoctonia solani - pathogenic fungus that attacks the lower part of the stalk, after which the tissue becomes necrotic and dies, making further growth of the plant impossible. Considering the importance of good quality seedlings, the damage to the total production of tobacco is enormous.

Protection from the disease includes fungicides, but the long-term use of chemicals leads to resistance to the pathogen and very often chemical control is inefficient and uneconomic. They also cause adverse effects on human health and the environment. Therefore, scientific approach to disease control is directed toward finding alternative means.

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).

Majorities of biocontrol products are applied against seed borne and soil borne fungal pathogens, including the casual agents of seed rot, damping –off and root rot disease (Heydari and Pessarakli, 2010).

Fungi of the genus Trichoderma are one of the most popular biocontrol agents. Living in the soil and in root systems, they activate numerous biocontrol mechanisms to attack the pathogen. It is believed that antibiosis, mycoparasitism and competition for food are the main mechanisms in biological control. On the other hand, they have a stimulating influence on root and plant development.

Trichoderma spp. are broadly effective across a range of plant species. They control a wide range of plant pathogens, including fungi, oomycetes, bacteria, and one virus (Harman, 2004, 2006). They act against important soilborne plant pathogens. Trichoderma strains as an alternative to hazardous fumigants and fungicides (Monte, 2001).

Rhizoctonia root rot is difficult to control because it survives for many years as sclerotia in soil or as mycelium in an organic matter under numerous environmental conditions (Grosh et al., 2003). The fungus has a wide host range, ie, limited rotational controls, there are no resistant cultivars and the fungus can grow and survive without a live plant host – it has "saprophytic ability." It cannot be eliminated but can be suppressed to a level that doesn't cause economic loss.

Since that, the chemical control is not always effective. Therefrom, biological control is an acceptable and effective means of disease management, since microbial organisms can control resistant pests and reduce the possibility of development of further resistance (Brimmer and Boland, 2003). The use of microorganisms that can grow in the rhizosphere are ideal for use as biocontrol agents against soil-borne pathogens, since the rhizosphere is available for combined biocontrol mechanisms and the other interactions that contribute to general suppression (Heydari and Pessarakli, 2010). The efficacy of Trichoderma species against soil fungal diseases is greater than fungicides and lasts longer.

Application of biological control against R. solani is a contemporary measure of protection, against all other preventive measures which proved unsuitable and inefficient due to the large number of hosts of this pathogen, the ability to survive in soil, etc.

Many Trichoderma species have antagonistic effect against R. solani and are successful in biological control of this pathogen. Therefore, the aim of this work is to evaluate the impact of some Trichoderma species on pathogen's growth at in vitro conditions, and also their effect in reducing the severity of the damping off disease.

## Material and methods

**In vitro investigations.** Pathogenic fungus Rhizoctonia solani was isolated from infected plant material. Trichoderma species were obtained from the collection of Scientific Tobacco Institute - Prilep.

These investigations were conducted by the method of dual cultures. 5 mm fragments both from the 10-day culture of the pathogen and from Trichoderma species were placed in the center of each half of the Petri dish on PDA (potato dextrose agar) as nutrient medium.

Pure cultures of R. solani and each Trichoderma control agent were used as a check.

The experiment was set up in three replications, with five Petri dishes for the check and dual cultures. Incubation was performed at 25° C and the diameter of the colony was measured each day during the 7-day incubation interval.

Relative growth of the pathogen was calculated by the method of Mello and Faul (2000), based on the values of pathogen's diameter in the presence of biocontrol agent. The percentage of reduction of pathogen's growth was determined according to the formula of Mishra (2010). Estimation was made by taking the values for diameter of pathogen's colony in the presence of biocontrol agent at the time of placing the pathogen in the control Petri dishes, i.e. on the fifth day.

Atagonistic potential of the biocontrol agents was estimated according to the formula of Simoes et al. (2012).

**In vivo investigations.** These investigations were made in biological laboratory, using seedlings of tobacco variety P23. Nine pots were sown for each variant of the three biocontrol agents; 27 pots for each biocontrol agent. The trial was set up in two replications.

Each variant is the way of application of the each Trichoderma species. The ordering number of variants is:

1, 2, 3 - T. harzianum; 4,5,6 -T. hamatum and 7,8,9 – T. aureoviride.

Application of the biocontrol agent was performed in three ways:

- over the soil, before the sowing
- by seed (stored 48 hours with the biocontrol agent)
- 15 days after sowing.

The check represents inoculation with the pathogen only.

**Table 1.** Treatments and variants

| Variant | Vegetative period of tobacco seedlings | | |
|---|---|---|---|
| | Sowing | After 15 days | Inoculation |
| Before sowing | T | T | T + R |
| With seed | T | T | T + R |
| After sowing | - | T | T + R |
| Check Ø | - | - | R |

T- *Trichoderma;* R- *R. solani*

Inoculation was carried out before the rapid growth stage (along with the third i.e. second application of biocontrol agent), with suspension made of pure culture of the pathogen (one Petri dish in 100 ml distilled water /pot). Variants and the applied treatments are presented in Table 1.

The occurrence and growth of the disease was followed daily, and the percentage of infected area was evaluated 10 days after inoculation. These results are presented in Figure 8.

## Results and discussion

**In vitro investigations.** Pure cultures of pathogens and Trichoderma species are presented in Figure 1-4.

Development of the pathogen compared with the three species of Trichoderma (in controls) on the first day of incubation has an advantage. But by the second day the situation is changing towards Trichoderma. They already filll the petri box up to the third day.

There are the same situation when the pathogen develops in the presence of biocontrol agents. It lags behind in growth, while the three Trichoderma species occupy half of the petri box (Table 2). Their reducing effect is already being expressed, which is not entirely in line with any claims that inhibition of R. solani development in double cultures occurs soon after contact with the antagonist. It can be 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).

**Table 2.** Growth of colonies during incubation (mm)

| Variant | Days | | | | | | |
|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| | Diameter (mm) | | | | | | |
| *R. solani in T. harzianum* | 17.2 | 21.6 | 35.0 | 36.0 | 36.4 | 38.4 | 39.8 |
| *R. solani in T. hamatum* | 24.0 | 29.6 | 37.4 | 40.0 | 45.8 | 46.0 | 46.3 |
| *R. solani in T. aureoviride* | 1.8 | 22.6 | 32.8 | 38.6 | 40.3 | 41.7 | 42.8 |
| *Ø R. solani* | 19.8 | 46.4 | 67.6 | 88.2 | 110.0 | 110.0 | 110.0 |
| *Ø T. harzianum* | 13.0 | 53.2 | 108.0 | 110.0 | 110.0 | 110.0 | 110.0 |
| *Ø T. hamatum* | 13.8 | 58.4 | 105.4 | 110.0 | 110.0 | 110.0 | 110.0 |
| *Ø T. aureoviride* | 13.8 | 51.6 | 108.2 | 110.0 | 110.0 | 110.0 | 110.0 |

Rapid development is characteristic of the fungi of the genus Trichoderma. When the spatial and temporal advantage of the pathogen was disabled, further development has stopped after the contact with Trichoderma. Even in the completely developed culture this biocontol agent develops normally, sporulates and uses its nutrients. In this way, it reduces the pathogen's development involving known biocontrol mechanisms (Гвероска, 2009). This confirms the results of these research, i.e. this property is the basis for activation and realization of all control mechanisms that stop the development of the pathogen.

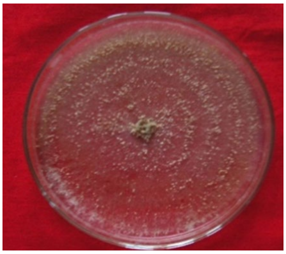

**Figure 1.** R. solani-pure culture

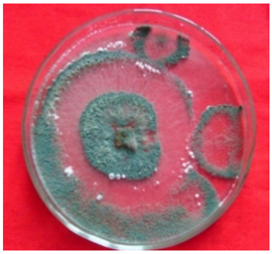

**Figure 2.** T. harzianum-pure culture

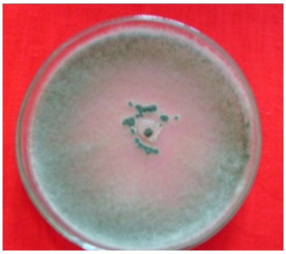

**Figure 3.** R. solani-pure culture T. hamatum-pure culture

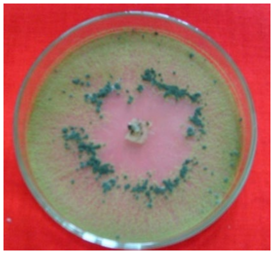

**Figure 4.** T. harzianum-pure culture T. aureoviride-pure culture

The antagonistic activity of Trichoderma sp. in culture is expressed by reducing the growth of test fungi and rapid growth of the antagonist, which multiplies on the pathogen colony (Миркова, 1982; loc cit Гвероска, 2009). Such a situation can be ascertained in these studies, too. The three biocontrol agents develop very rapidly but R. solani, slightly.

Among them, the weakest development was identified in the presence of T. harzianum and the best in T. hamatum. Their development is taking smoothly, over the pathogen culture, passing and covering it (Fig. 5-7).

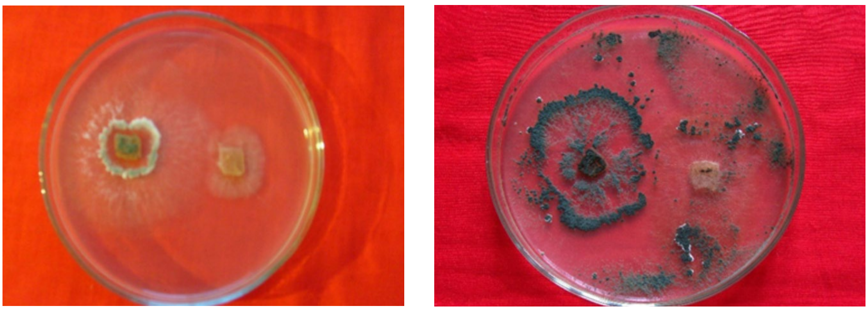

**Figure 5.** Dual culture of R. solani and T. Harzianum (on the third day and at the end of incubation)

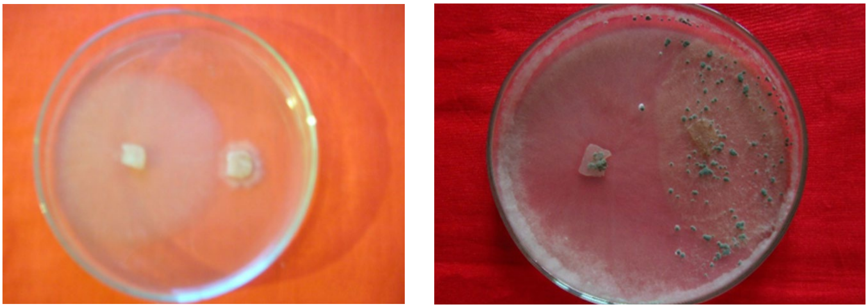

**Figure 6.** Dual culture of R. solani and T. hamatum
(on the third day and at the end of incubation)

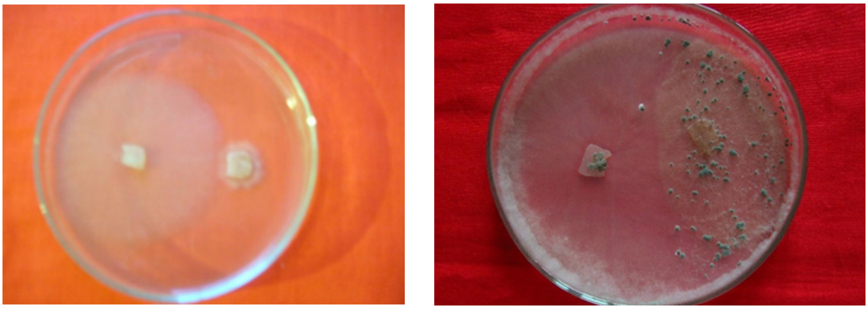

**Figure 7.** Dual culture of R. solani and T. aureoviride
(on the third day and at the end of incubation)

Trichoderma expresses a reducing effect over both volatile and diffusible. Generally, the diffusible metabolites have a bigger reducing effect than the volatile ones (Küçük and Kivanç, 2003; Gveroska and Ziberoski, 2012). According to Table 3, the pathogen has the weakest relative development in the presence of T. harzianum, and the best in T. hamatum. Consequently, the percentage of pathogen reduction is the highest in T. harzianum and the lowest in T. hamatum.

**Table 3.** Effect of the Trichoderma species on the R. solani

| Variant | Relative growth of the pathogen in the presence of Trichoderma | Percentage reduction of pathogen's growth in the presence of Trichoderma | Atagonistic potential of the biocontrol agents |
|---|---|---|---|
| *R. solani in T. harzianum* | 33.10 | 66.91 | 83.46 |
| *R. solani in T. hamatum* | 41.64 | 58.36 | 79.18 |
| *R. solani in T. aureoviride* | 36.59 | 63.40 | 81.70 |

In the in vitro conditions they are capable of activating the mechanisms of biocontrol activity. The three Trichoderma species have a strongly expressed antagonistic potential. Among them, T. harzianum is characterized by the highest value (Table 3).

In vitro results confirm that the three species of Trichoderma with their biocontrol mechanisms show antagonistic activity to the R. solani. Trichoderma sp. in vitro inhibits the 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 (Okhovvat, 1997).

***In vivo investigations.*** Trichoderma species are effective to Rhizoctonia solani. In the investigations of Singh and Chand (2006), two Trichoderma species has taken maximum reduction of a Rhizoctonia solani colony in lab conditions and maximum control of disease in bio laboratory. Biological control effect of Trichoderma sp. on R. solani is confirmed by Leach and Gaber (1970), Lewis et al. (1998), Monte (2001), Küçük and Kivanç (2003), Harman, (2000,2004,2006), Shalinni (2006). These claims are confirmed in these studies. Therefore, in vitro results were also confirmed in the in vivo conditions (Figures 8-12).

According to Fig. 8, the intensity of attack from the damping off disease in variants where Trichoderma is applied to the soil before sowing is the highest in T. hamatum, whereas in T. harzianum there is no disease occurrence.

The same situation is in Trichoderma application 15 days after sowing - the highest intensity of attack was observed in T. hamatum and the least in T. harzianum.

When the biocontrol agent is applied together with the seed, in T. harzianum has no disease at all. But in the other two species of Trichoderma there is a better situation, too. However, T. hamatum shows a smaller effect in reducing intensity compared to T. aureoviride.

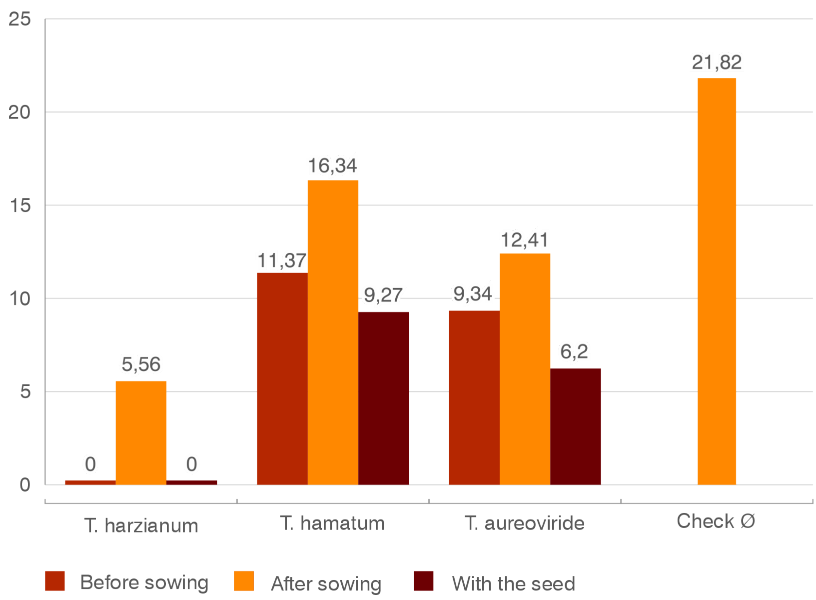


**Figure 8.** Intensity of disease attack depending on Trichoderma species and mode of application

Comparing the variants i.e. the Trichoderma application modes, the highest intensity of disease was observed 15 days after sowing. The reason for this situation is the smaller population of biocontrol agents. In this case, one less application has been performed than in the other variants. The biggest quantity of Trichoderma sp. has a good reducing effect on damping off disease in tobacco (Gveroska, 2013). Aziz et al. (1997) also found that application of Trichoderma lignorum as a seed coating or wheat bran preparation greatly reduced the number of bean seeds infested by Rhizoctonia solani, and the percentage of healthy seeds reached 92%.

Application of the biocontrol agent on the soil, before the sowing have better results than the previous one (Fig. 10, 11). These results are in accordance with findings of Heydari and Pessarakli (2010) that application of biocontrol agent to soil at the site of seed placement is one of the most- used procedures for achieve successful control.

Seed treatment has showed the best effect (Fig. 12). The lowest intensity of disease was observed in the three Trichoderma species. These application ways have the best results to reduce the damping off in seedlings. It is confirmed by Gveroska (2013). When the biocontrol agent is added as a seed treatment, the best strains colonize a root surface, even when root meter or more below the soil surface, and they can persist at useful numbers up to 18 months after application. Some strains establish robust and long-lasting colonization of root surfaces and penetrate into the epidermis and a few cells at this level (Harman, 2004).

When Trichoderma is used as seed treatment, it has been effective in protecting several major crops against fungal pathogens (Heydari and Pessarakli, 2010). The use of antagonistic microorganisms to R. solani applied as treatment of seed or soil, have been demonstrated to control a variety of cultures in the greenhouse and field studies Goes et al. (2002). These claims are confirmed in the investigations for tobacco seedlings.

**Figure 9.** Symptoms of damping off disease in tobacco seedlings


**Figure 10.** The effect of the application of T. aureoviride on attack intensity (left – check; right – before the sowing)


**Figure 11.** The effect of the application of T. harzianum on attack intensity (left–before sowing; right-after sowing)


**Figure 12.** The effect of T. harzianum on the intensity of damping off disease (left – check; right - with seed)

Biocontrol agent T. harzianum achieved the best success in control of the disease.

T. harzianum produces a high concentration of lytic enzymes - chitinases, glucanases, cellulases, which degrade cell walls, as well as survival structures such as sclerotia and chlamydospores. Therefore, it ensures its high level of mycoparasitism (Cherif et al., 1990; Elad et al., 1983; Monte, 2001). It can produce lytic enzymes and antifungal antibiotic, it can be competitors of fungal pathogens and, it promotes plant growth (Harman, 2000, 2006).

According to Küçük and Kivanç (2003), different mechanisms might be responsible for biocontrol in different plants and with different pathogens. Although there are different pathogens, obviously the most important mechanisms - competition, antibiosis and mycoparasitism and they are efficient through the mutual action. The mechanisms are integrated and what has been defined as biocontrol is the final result of different mechanisms acting synergistically to achieve disease control.

T. harzianum is a strong BCA and the base of many commercial products. It is widely used for disease control instead of chemical fungicides because it is safer to use for growers, its disease control effect last longer than those of synthetic chemical pesticides –so it is less costly than chemical fungicides-and root growth can be as good, or better, than that achieved using pesticides (Harman et al., 2004).

## Conclusions

- Relative growth of R. solani at in vitro conditions ranged from 33.10% in the presence of T. harzianum to 41.64% with T. hamatum.
- Percentage of reduction of pathogen's growth ranged 58.36% - 66.91%.
- T. harzianum showed the highest inhibition of growth of R. solani.
- In conditions of artificial inoculation with the pathogen, the intensity of disease attack was reduced by application of biocontrol agent.
- The percentage of infected area depends by the mode of application of biocontrol agent. The worst results has the application 15 days after sowing in all three Trichoderma species.

- Seed stored 48 hours in culture of the biocontrol agent is the best way of application.
- T. hamatum has the smallest effect in control of the disease. T. aureoviride has the better, but the smaller than T. harzianum.
- T. harzianum had the highest reducing effect on the occurrence of damping off disease in all modes of application of biocontrol agent.

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