ISSN 0494-3244
Тутун/Tobacco Vol. 67 No 7-12, 35-45, 2017 UDC:633.71-153.037:632.48]:632.937.1(497.775)
Original scientific paper

TOBACCO SEEDLINGS PROTECTION FROM DAMPING OFF DISEASE WITH *TRICHODERMA HARZIANUM* T22 (BIOPRODUCT TRIANUM P)

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ABSTRACT

Modern tobacco protection follows the principles of sustainable agricultural production and supports the use of environment-friendly methods.

Trichoderma harzianum T22 is the most powerful biocontrol agent. The aim of our research was to study its ability to control the causing agents of damping off disease in tobacco seedlings.

Trianum P is biological fungicide which contains spores of *T. harzianum* T22. It was applied in a rate of 15 g/10m^2 through three modes of application and with two additional treatments by the time of planting. Investigations were made in biolaboratory and in seedbeds, with natural infestation. The biofungicide was applied by storing the seed in suspension 48h before sowing, fungicide application over the soil and first application 15 days after sowing. All variants in seedbeds were set up with and without herbicide. In the biolab tests, treatment of infested soil was included.

Generally, the best results in the control of damping off were achieved in variants where the seed was kept in the product (with and without herbicide-in seedbeds). The poorest results were obtained with the fungicide first application 15 days after sowing. It can be concluded that *T. harzianum* T22 is biocontrol agent which can be used in protection of tobacco seedlings from the damping off disease. Biological product Trianum P has a good perspective in sustainable tobacco production.

Keywords: *Trichoderma harzianum* T22, Trianum P, damping off disease, intensity of attack

ЗАШТИТА НА ТУТУНСКИОТ РАСАД ОД БОЛЕСТА СЕЧЕЊЕ СО *TRICHODERMA HARZIANUM* T22 (БИОПРЕПАРАТ TRIANUM P)

Современото производство на тутун ги следи принципите на одржливото земјоделско производство и ја поддрржува примената на еколошко-прифатливи методи.

Trichoderma harzianum T22 е најмоќниот биоконтролен агенс. Нашата цел беше да се испита неговата способност во сузбивањето на предизвикувачите на болеста сечење на тутунскиот расад.

Trianum P е биопрепарат кој содржи живи спори на *T. harzianum* T22. Тој беше аплициран во доза од 15 g/m^2 на три начини и две дополнителни третирања до времето на расадување. Испитувањата беа извршени во биолошка лабораторија и тутунски леи –природна инфекција. Биофунгицидот беше аплициран преку: семе чувано во препаратот 48 часа пред сеидба, апликација на препаратот врз почвата и прва апликација по 15 дена по сеидба. Сите варијанти во леите беа поставени со и без хербицид. Во биолошка лабораторија беше вклучена и варијанта со инфицирана почва.

Најдобри резултати во заштитата од болеста сечење беа постигнати кај варијантите каде семето беше чувано во препаратот (во леи - со и без хербицид), а најслаби при неговата прва апликација 15 дена по сеидбата.

T. harzianum T22 е биоконтролен агенс кој може да се употребува во заштитата на тутунскиот расад од болеста сечење. Биопрепаратот Trianum P има добра перспектива во одржливото производство на тутун.

Клучни зборови: *Trichoderma harzianum* T22, Trianum P, сечење, интензитет на напад

INTRODUCTION

Production of tobacco seedlings can be seriously affected, mostly by fungal diseases such as damping off. The causing agents of the disease are the pathogenic fungi *Rhizoctonia solani* and *Pythium debarianum*. The control is difficult because of their ability to survive in the soil, the large number of hosts etc. Therefore, biological control is important segment in protection from this disease, but also the only acceptable measure in the case of dual infection and inadequate choice of fungicides.

Biological control is an environmentally acceptable measure and a real alternative to the use of pesticides.

Biological control offers an environment-friendly approach to the management of plant disease and can be incorporated into cultural and physical measures with limited usage of chemicals for effective integrated managament (IPM) system (Monte, 2001).

Biological control is a potential alternative to the use of chemicals which are harmful to the environment, humans and other living organisms including beneficial natural enemies (Chet and Inbar, 1994). The most detailed description of the use of biological control is the supression of pathogens by a single antagonist in a single crop system. Biological control means application of biological processes to reduce the level of inoculum in order to reduce the risk of disease intensity.

There are numerous literature data and reliable, accepted solutions for biological control of diseases caused by phytopathogenic fungi by application of the fungi of the genus *Trichoderma*, which is considered a reliable alternative that can replace the chemical control (Cuervo-Parra et al., 2015).

Trichoderma spp. are found in soil and root system and are beneficial to plants (Goes, 2002; Contreras-Cornejo et al., 2009). The most important benefit of *Trichoderma* is that they directly attack and control the causing agents of the diseases (Chet et al., 2006).

They are opportunistic, avirulent plant symbionts as well as parasites of other fungi. Colonizing the root, *Trichoderma spp*. activates numerous mechanisms that attack pathogens and stimulate the growth of plants and roots. On the other hand, those agents have a stimulating effect on the development of root and plant. In the complex of actions, antibiosis, mycoparasitism and food competence are considered the main mechanisms for biological control (Harman, 2004, 2006; Benitez et al., 2004; Howell, 2003).

Trichoderma is effective in the control of numerous plant pathogens (Harman, 2004, 2006).

It acts against seed borne and soilborne fungal pathogens, including the causing agents of seed rot, damping off and root rot disease (Heydari and Pessarakli, 2010).

Nowadays there are advanced techniques for application of *Trichoderma* in agriculture. There are numerous preparations, but the best known among those that use antagonistic microorganisms are the preparations based on various strains of *Trichoderma*. *T.harzianum* T22. is widely accepted because of its characteristics: efficient control of a large number of soil diseases, improved growth and development of plant root and increased absorption of water and nutrients from the soil, rapid growth on roots of all cultivated plants, good development in various soil types, tolerance to wide temperature and pH range and compatibility with a large number of active ingredients. According to Harman (2004), it is more secure for the farmers and provides longer and cheaper protection compared to pesticides application.

Biological product Trianum P is based on spores of *Trichoderma harzianum* T22, in form of water soluble powder or granules for direct use. It is registered as a biostimulator and plant protection agent in many countries, including the Republic of Macedonia.

The appropriate time and model of application of the biocontrol agent is very important for successful biocontrol. Development of feasible and efficient delivery system for application of appropriate microorganisms in the ecosystem is an important component of biocontrol technology (Lewis et al., 1998).

Handelsman and Stabb (1996) reported that colonization is essential in biocontrol and they found a relationship between the population size of the biocontrol agent and the degree of disease suppression. According to Heydari and Pessarakli (2010), one of the criteria for successful biocontrol is to determine the factors of successful colonization and expression of biocontrol traits. Delivery system must ensure that biocontrol agent will grow well and achieve its purpose. So, delivery and application processes must be developed for each crop (Harman, 1996). Therefore, our aim was to determine how long the biocontrol agent retains in the soil, to evaluate the colonisation by various modes of application and how its quantity affects the damping off disease in seedlings.

The purpose of this study is to determine the effect of *T. harzianum* T22 on tobacco, i.e. the effect of the biofungicide Trianum P in the control of damping off disease. The research should also identify the most efficient model of application in the standard way of sowing.

The results of these investigations will contribute to commercial use of this biocontrol agent in protection of tobacco seedlings, IPM of tobacco and sustainable tobacco production.

MATERIAL AND METHODS

Investigations of the influence of Trianum P on the intensity of damping off disease in tobacco seedlings were carried out in Scientific Tobacco Institute-Prilep, in biolaboratory and field conditions.

The intensity of the attack was monitored in conditions of natural infestation.

Assessment of disease severity was made by measuring the infested area in each replication of the variants. The intensity of disease is expressed in percentage of infested area- the mean value of the three replications.

Biolaboratory tests

The experiment in the biological laboratory was set up in 3 replications for each variant including the check. The area of one replication was $0.3m^2$. Seed was sown in the norm of 0,6 g/m^2. The experiment in the biological laboratory was performed twice.

Trianum P biological fungicide was applied at a recommended rate of 15 g/10 m^2 (0,5 g for $0,3m^2$). According to the instructions, the preparation was previously dissolved in 1:5 ratio and added to the water by mixing. 800 ml water was used per replication.

The soil in Variant 4 was previously infested with a pure culture of the causing agents of disease - *Rhizoctonia solani and Pythium debarianum (*one Petri dish per pathogen for $0,3m^2$).

Seed for Variant 3 was prepared for sowing the previous day. 5 g of the product was dissolved in 25 ml water. The seed was stored in the suspension for 24 hours.

The occurrence of damping off was observed after the second treatment, but according to the applied method of work, the third treatment was done prior to evaluation.

Intensity of the damping off disease i.e. the percentage of infested area was estimated after the third treatment.

Seedbeds tests

Investigations in seedbed conditions were performed with the following variants:

- Ø Check - standard treatment with herbicide and nutrition; no fungicide application
1. Treatment after 15 days
2. Treatment after 15 days (post-sowing - herbicide)
3. Pre-sowing
4. Pre-sowing (post-sowing - herbicide)
5. Seed treated with Trianum P
6. Seed treated with Trianum P (post-sowing - herbicide)

Seed amount of 6.75 g/10 m^2 i.e. 0.67 g/m^2 was used for planting. All variants were set up in 3 replications and each replication was 3.33 m^2.

Since the purpose of this research was to determine not only the effect of the preparation, but also the possible application in the standard way of sowing (with herbicide), all the variants were replicated, i.e. set up with the use of herbicide.

Herbicide Gamit (a.i. clomazone) in a rate 0.07 ml/m^2 was used after sowing.

The application of Trianum P biopreparation

Trianum P biological fungicide was applied at a recommended rate of 15 g/10 m^2 (1.5 g/m^2) using 2.5-5l water/m^2.

- Soil in variants 3 and 4 was treated prior to sowing with Trianum P at the same rate, i.e. 5 g/3.33m^2 using 10 l of water.

- Seed in variants 5 and 6 was prepared for sowing the previous day as well as in the biolaboratory tests.

Variants 1 and 2 were treated 15 days after sowing, at the same rate as other variants.

During the growing season, two more treatments were carried out on the seedling at 15-20 day intervals, by which all stages of seedling growth were covered. Thus, seedling of the variants 1 and 2 received 2 treatments and that of other variants 3 treatments.

Health condition of tobacco seedling was monitored throughout the growing season.

The intensity of damping off disease

The first assessment on the intensity of disease was made 10 days after the third treatment and the second assessment a few days before planting.

The effectiveness of Trianum P in the control of damping off disease was calculated by Abbott's formula (from the values for the intensity of disease attack in variants and check in the second estimation).

RESULTS AND DISSCUSION

Biolaboratory tests

The highest intensity of attack in the first experiment was recorded in the check. Among the variants with application of Trianum P, the lowest value was found in the variant where the seed was stored in the preparation (Table 1). The highest value was found in the variant where the first treatment was done after 15 days of sowing.

Table 1. The influence of Trianum P on the intensity of damping off disease (Ist experiment)

	Variant	Percentage of infested area			Mean value
		Replication			
		I	II	III	
Ø	Check	1,20	8,23	6,7	5,38
1	First treatment after 15 days	2,56	1,53	6,00	3,36
2	Treatment of soil before sowing	2,23	3,53	3,90	3,22
3	Seed treated with the biopreparation	0,3	1,43	5,4	2,37
4	Treatment of infested soil	8,06	0,4	0	2,82

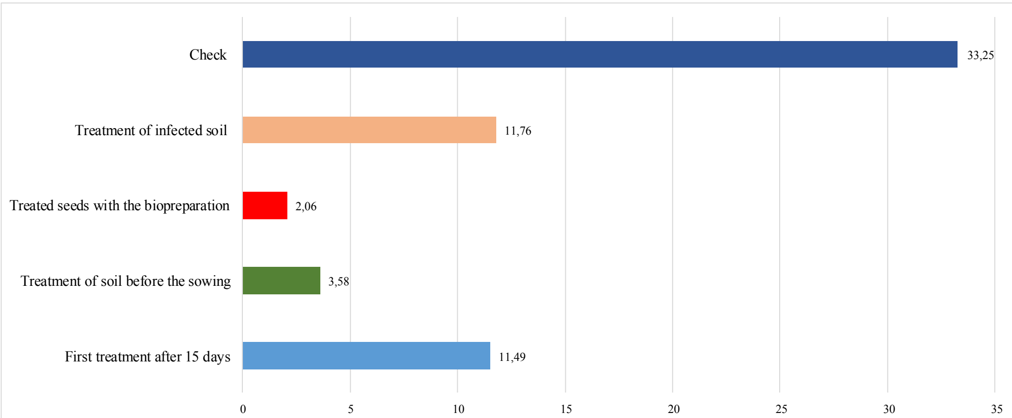
In the second experiment, the intensity of attack was much higher than in the first experiment (Table 2). However, the lowest percentage of infested area was detected in variant 3, when the seed was stored in the preparation before sowing. Also, when the soil was treated before sowing, the intensity of disease was low (3.93%). Delayed treatment of seedlings did not produce good results (20.15%). The same situation was observed in Variant 4, when the preparation was applied in infested soil. Results obtained for the variants in biolaboratory test are presented in Figures 1-4.

Figures 1-4. Variants in biolaboratory test

Table 2. Influence of Trianum P on the intensity of damping off disease (IInd experiment)

	Variant	Percentage of infested area			
		Replication			Mean value
		I	II	III	
Ø	Check	13,33	80,00	90,00	61,11
1	First treatment after 15 days	7,66	2,80	50,00	20,15
2	Treatment of soil before the sowing	7,46	4,33	0	3,93
3	Treated seeds with the biopreparation	4,80	0,30	0,13	1,74
4	Treatment of infested soil	7,66	2,80	50,00	20,15

The effectiveness of the biopreparation Trianum P applied by various application models in the biolaboratory test (mean value of the two trials) is presented in Graph 1.



Graph. 1 The effectiveness of variants in biolaboratory test

Treatment of infested soil didn't give satisfactory results. This is understandable bearing in mind the principle of action of this preparation. Actually, its activity is particularly pronounced in the root zone and is closely related to the development of the root system. For effective control, antagonist must be previously established in plant ecosystems and remain active in target pathogens during periods favourable for plant infection. (Heydari and Peskarli, 2010).

If species of the genus *Trichoderma* are applied directly in the soil or by seed, they grow up simultaneously by the root system of treated plants (Harman, 2000, Howell et al., 2000). There is abundant and constant quantity of root exudations from the root tips during root development but there was no increase after shoots emerged and initiated the photosynthesis .(Handelsman and Stabb, 1996). Constant maintenance of the population of the biocontrol agent is necessary for full expression of the biocontrol mechanisms and realization of biofungicidal activity. Hence, the greater quantity of the biocontrol agent *Trichoderma* has greater efficiency in reducing the damping off disease in tobacco seedlings (Gveroska, 2013 a).

Seedbeds tests

In seedbed conditions, the disease is manifested with very low intensity in variants where the biocontrol agent, i.e. biological preparation was applied (Table 3). The disease does not occur when biocontrol agent was used in treating the soil before sowing as well as in treatment of seed (without the use of herbicide after sowing).

The disease is manifested with increased intensity in the second estimation (Table 4). However, the influence of the biocontrol agent can be observed, too. The highest intensity of attack was recorded in the check (nontreated variant). The absence of the use of fungicides, as well as nitrogen nutrition (which increases the susceptibility to the pathogen) contribute to susceptibility to pathogens and spread of infection.

Treatment with the biopreparation 15 days after sowing showed a poor effect in seedlings protection from the disease. Delayed treatment with Trianum P has negative effect on multiplication of *T. harzianum* T22 and on the expression of biocontrol mechanisms, resulting in highest percentage of infested area, which was even higher in Variant 2 than in the check. Gveroska (2013 a), comparing the *Trichoderma* models of applications prior to sowing and 15 days after sowing, determined the advantage of the first two modes over the third one. When the biocontrol agent was applied after seedlings had grown, there was a smaller amount of BCA than in application before sowing, as mentioned previously. Our results are in accordance with those of Izzati and Abdullah (2008), who reported that delayed time of application increased the index of disease severity.

Table 3. Influence of Trianum P on the intensity of damping off disease in seedbeds (Ist estimation)

Mark	Variant	Percentage of infested area			
		Replication			
		I	II	III	Mean value
Ø	Check (herbicide,saltpeter; without fungicide)	0,19	0,39	11,22	3,93
1	Sowing without herbicide; Treatment after 15 days	1,71	0	0	0,58
2	Sowing with herbicide; Treatment after 15 days	0,53	0,61	2,23	1,12
3	Sowing without herbicide; Pre-sowing soil treatment	0	0	0	0
4	Sowing with herbicide; Pre-sowing soil treatment	0	0,15	0	0,05
5	Sowing without herbicide; Seed treated with Trianum P	0	0	0	0
6	Sowing with herbicide; Seed treated with Trianum P	0	0,11	0	0,04

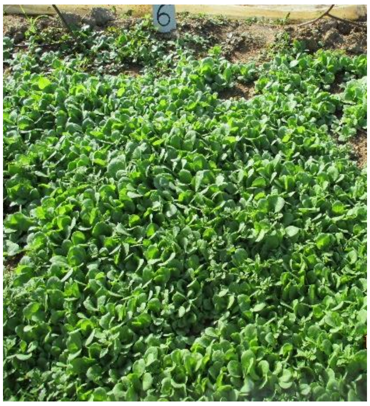
The variants where treatment began 15 days after sowing had one treatment less, which certainly had some influence on creating the conditions for growth and multiplication of the biocontrol agent population. In the other variants, the intensity of attack was lower as a result of the two additional treatments with biofungicide.

Table 4. Influence of Trianum P on the intensity of damping off disease in the seedbeds (2nd estimation)

Mark	Variant	Percentage of infested area			
		Replication			
		I	II	III	Mean value
Ø	Check (herbicide,saltpeter; without fungicide)	7,15	4,50	9,91	7,19
1	Sowing without herbicide; Treatment after 15 days	4,12	2,15	8,23	4,83
2	Sowing with herbicide; Treatment after 15 days	18,02	3,04	4,61	8,56
3	Sowing without herbicide; Pre-sowing soil treatment	6,42	1,46	0,59	2,82
4	Sowing with herbicide; Pre-sowing soil treatment	1,50	2,22	1,28	1,67
5	Sowing without herbicide; Seed treated with Trianum P	1,35	0,79	1,22	1,12
6	Sowing with herbicide; Seed treated with Trianum P	0,59	0,77	1,19	0,85

The lowest values for disease attack were found in the variants where seed was stored in suspension of the preparation and with two additional treatments. In the variant with treated seed and with herbicide use, the values were insignificant (Table 4). These results are confirmed by Gveroska (2013 a), who reported that application by seed has the best results in the control of damping off in seedlings. Majority of biocontrol products used against seedborne and soilborne fungal pathogens, including the causal agents of seed rot, damping off and root rot diseases, when it is used as seed treatment have been effective in protecting against fungal pathogens (Heydari and Pessarakli, 2010).

This situation is affected by the greater possibility of development and multiplication of *Trichoderma* population due to the possibility of seed colonization by following the growth of root system, stimulated by root exudates (Handelsman and Stabb, 1996; Harman 2000; Howell et al., 2000).



Figures 5-7. Variants in seedbeds test

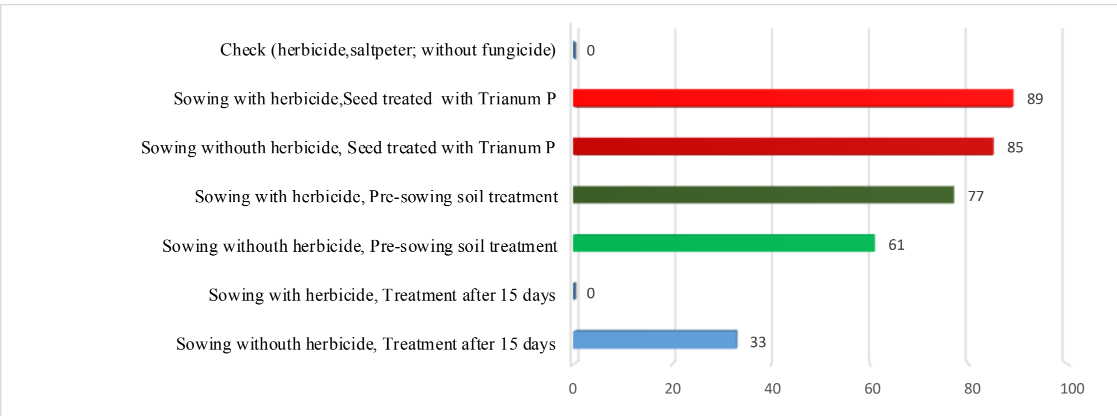
The values for disease intensity were also lower in variants where the preparation was applied in soil, compared to the check and the variants treated after 15 days (Table 4, Figures 5-7).

However, among variants 5 and 6 and 3 and 4, smaller intensity of damping off disease was observed in variants 4 and 6 that were treated with herbicide.

T. harzianum is multiplied in the soil as a natural environment where the possibility of herbicide influence is lower because it is bound to the organic matter from the fertilizer. Such results for herbicide effect on the population of *T. harzianum* are in agreement with previous studies (Gveroska, 2014 a), according to which the small decrease of the population caused by herbicide application is compensated or even increased during the additional treatments.

According to investigations, T.harzianum T22 has fungicidal effect on the causing agent of damping off disease on tobacco seedlings. This is in agreement with the results of Chacon et al. (2006) and Devaki et al. (2008), who reported antagonistic action of *T. harzianum* on *Pythium* sp. It also has biocontrolling effect on *Rhizoctonia solani*, as confirmed by Monte (2001), Harman (2000, 2004, 2006), Chet et al. (2006), Wilson et al. (2008) and Gveroska (2013 b, 2014 b).

Therefore, *T. harzianum T22 (the basic component of* the biopreparation *Trianum P) is effective in reducing the damping off disease. Its* effectiveness depending on the application mode is presented in Graph 2.



Graph. 2 Effectiveness of variants depending on the mode of application

CONCLUSIONS

* The application of *T. harzianum* T22 as the basic component of the biofungicide Trianum P confirmed the previous *in vitro* and *in vivo* studies on the impact of this biocontrol agent on the control of pathogenic fungi on tobacco.
* *T. harzianum* T22 is a biocontrol agent that can be used in the control of causing agents of the dampig off disease in tobacco seedling.
* In the biolaboratory tests, the best results in controlling the disease were obtained by treatment of seeds with suspension of the preparation and two additional treatments.
* Soil treatment showed good results, but still weaker compared to field conditions.
* The treatment of infested soil showed no satisfactory results in prevention of the infection, which is understandable knowing the principle of action of this preparation.
* In field investigations, the highest effectiveness in the control of the disease was recorded in variants with treated seed, with and without herbicide application.
* Pre-sowing soil treatment can be, to a certain extent, an alternative to the treated seed.
* The herbicide application did not show any adverse effects in these variants.
* Delayed treatment gives the worst results. In variants where the first treatment was made 15 days after sowing, the lowest fungicidal effect was found both in biolaboratory and in field condition
* The best model of application is to treat the seed with the preparation (storage in suspension) before sowing, with minimum two additional treatments of the seedling.
* Trianum P biofungicide confirmed its fungicidal effect. It has a good perspective in the sustainable tobacco production.

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