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Trichoderma Harzianum in Tobacco Seedlings with the use of a Herbicide

Biljana Gveroska
Scientific Tobacco Institute-Prilep, Department of Tobacco Protection
From Diseases, Weeds And Pests, Prilep, Kicevski pat bb, Republic of Macedonia
Email: bgveros@yahoo.com

***Abstract* – *In vitro* effect of a herbicide on *Trichoderma harzianum* and *in vivo* influence on colony forming units / g soil and also, the interaction between its quantity and the root rot disease intensity caused by *Rhizoctonia solani* have been studied. Ohinol 50-SC (50% napropamide) was used in some doses and methods.**

Inhibition of dry biomass yield and radial growth increases proportionally of a dose.

The highest decreases of *T. harzianum* quantity was in 0.2 g/m^2 before +0.3 g/m^2 herbicide after sowing. The greater quantity reduce the disease intensity.

The most suitable method of *T. harzianum* application in tobacco seedlings is: herbicide in a dose of 0.5 g/m^2 after sowing and the seed kept in pure culture of a biocontrol agent.

***Keywords* – Biocontrol, *Rhizoctonia solani*, Tobacco, *Trichoderma harzaianum*.**

I. Introduction

Plant diseases cause a significant reducing of crop yield and income. The use of pesticides is conventional measurement in crop protection. But, long lasting use of chemicals causes a resistance in pathogen to them. At the same time, they cause harmful consequences to human health and environmental security.

Therefore, the use of biological control is an acceptable at ecological aspect and it is used as a contemporary plant disease control. Fungi of the genus *Trichoderma* have shown the biocontrol activity since1930 and today there are modern technologies for including them in biological control of various diseases. They are mostly applied against seed borne and soil borne fungal pathogens, including the causal agents of seed rot, damping off and root rot diseases [9]. The root rot disease caused by *Rhizoctonia solani* is the most important disease in tobacco seedlings. The biocontrol is a promising strategy, because application of *Trichoderma harzianum* before sowing and several times in a growing season of tobacco seedlings reduce the root rot disease caused by *R. solani* [4].

The full expression of the biocontrol must have seed with good germinability and free of contaminating weed seed [6]. Therefore, we must provide appropriate tillage practise including the control weeds through application of herbicides. But, herbicides also have another acompained effect in the plant community, i.e they influence on the number, composition and interaction between microorganisms [1], [11].

Reference [8] shows that disease suppression by biocontrol agents is the sustained manifestation of interactions between the plant, the pathogen, the biocontrol agent, and the physical environment. Therefore, whether a herbicide introduction into this system has an influence on the biocontrol agent and, in consequence, the biological control?

Because of a relatively poor data on this item, it is an attempt in understanding these relationships. Our aim is to study the influence of a herbicide used in tobacco seedling's production on *in vitro* development of *T. harzianum* and *in vivo* influence on its quantity in soil and furthermore, the interaction between its quantity and disease intensity.

II. Materials and Methods

The biocontrol agent *T. harzianum*(obtained from the root zone of rhizosphere, by a method of dilution) and the pathogenic fungus *R.solani*(isolated from infected seedlings)were grown on standard potato dexstroze agar (PDA).

The herbicide Ohinol 50-S (50% napropamide) as the most used herbicide in tobacco seedlings production was researched. Laboratory assay was taken with three doses: 0.2, 0.5 and 0.7 g/m^2. Five petri dishes (by each variant and control) were used. PDA without herbicide was the check. Trials were set up in three replications.

The herbicide was added in a sterilized and cooled (50°C) liquid PDA medium. 5 mm fragment of a pure culture of *T. harzianum* was placed in Petri dishes with 20 ml PDA. After incubation of 10 days at 25°C, dry mass of a target fungus was calculated [16].

For the herbicide's influence on the development of a biocontrol agent, a 5 mm fragment of a pure culture of *T. harzianum* was placed in a center of the solid medium with a herbicide. Diameter of the colonies was measured for 10 days during the incubation at 25°C.

The realtive development of a biocontrol agent was estimated using an area of the colonies [19]:

$$RD = (A/B)\ X\ 100$$

where A= area of colony developed in PDA with active ingredieent and B = area of colony developed in PDA

Dry mass reduction in a liquid medium and inhibition of radial growth in the solid medium is presented as percentage of inhibition [17]:

$$\%\ \text{Inhibition} = [(X - Y)/\ X]\ \text{x}\ 100$$

where X= growth of control plate and Y = growth of a plate with a herbicide

Investigations of *T. harzianum* presence during the growing periodof tobacco seedlings were taken in greenhouse. Tobacco seedlings of oriental type – variety P 79 was used - night pots per each variant, in the two replications. The recommended dose of a herbicide Ohinol

50-S is 0.5 g/m^2 was used used in a two ways. *T. harzianum* was applicated before sowing and by seed together with a biocontrol agent.

Table 1 shows the variants and protocol of treatments.

Table 1: Variants and treatments (tobacco seedlings)

Variant	Treatment			
	(Sowing)	I	II	III
T1- Ohinol 50-S 0.2 g/m^2 before sowing, T, 0.3 g/m^2 after sowing	T		R + T	T
T2- Ohinol 50-S 0.2 g/m^2 before sowing, T, 0.3 g/m^2 after sowing	T	T	R + T	T
T3- T, Ohinol 50-S 0.5 g/m^2 after sowing	T		R + T	T
T4- T, Ohinol 50-S 0.5 g/m^2 after sowing	T	T	R + T	T
T5- Seed together with the T, Ohinol 50-S 0.5 g/m^2 after sowing			R + T	T
T6- Seed together with the T, Ohinol 50-S 0.5 g/m^2 after sowing		T	R + T	T
Ø1– Check 1, only application of T before sowing	T	T	R + T	T
Ø2– Check 2, only artificial inoculation with a pathogen			R	

T –*T. harzianum*.; R- *R. solani*

T. harzianum used in the sowing application was grown on a rice medium [17]. The seed (for the proper variants) was kept 72 hours before the sowing in this medium. Additional application of the biocontrol agent in the proper variants was taken periodically, in 10-15 day intervals, drenching with the BCA suspension.

Estimation of the colonyforming units in g soil (CFU/g soil) of *T. harzianum* was taking on the medium Rose Bengal Agar (RBA), using an antibiotic Tetracyclin.An average soil sample was taken from each variant and control. Serial dilution was made. One ml of the final(10^{-4}) dilution was pipeted into a Petri dish, then 20 ml of RBA medium was poured. Five replications per only variant and control were done and incubated at 28°C. The CFU/g soil was counted and recorded after incubation of ten days.

The initial presence of Trichoderma in the soil was estimated before the sowing.

Estimation of *T. harzianum* colony-forming units was made before the second, the third, the fourth application and the end of a vegetative period of tobacco seedlings.

Estimations of the intensity of a disease -percentage of an infected area was made after 15 days after inoculation, i.e. before the last application.

III. Results and Discussion

The herbicide Ohinol affects the dry biomass of the biocontrol agent*T. harzianum*. The yield ofdry biomass in variants with a herbicide ranges from 0.228 to 0.386 g, inversely of a dose.

Inhibition of the yield of dry biomass ranges from 14.98 in a dose of 0.2 g/m^2 to 49.78% at 0.7g/m^2. The percentage of inhibition in the recommended do seondry biomass of *T.harzianum* was only 22.47% (Table 2).

Table 2. The influence of a herbicide Ohinol on the dry mass yield of *T. harzianum*

Ohinol (dose)	0.2 g/m^2	0.5 g/m^2	0.7 g/m^2	Check Ø
Dry mass (g)	0.386	0.352	0.228	0.454
Inhibition of dry mass yield (%)	14.98	22.47	49.78	-

Therefore, the herbicide Ohinol 50-SC (50% napropamide) showed the inhibition of the drybiomassyieldof *T. harzianum* in a liquid medium inversely of a herbicide dose. The depressive or stimulative effect of chemical depends upon the kind and its concentration, possibly moderated by environmental conditions [11].

According to the impact of a herbicide Ohinol on the development of *T.harzianum*, the tested doses of herbicides affect the development of the fungus. Initial development at there commended dose 0.5 g/m^{2}does not greatly differ than reduced dose.

Development is the weakest in the case of an increased dose of herbicide. Even then, fulfillment of a Petry dish hason the 9th day, which is one day earlier than the other two doses (Table 3).

Figure 1 shows the development of *T.harzianum* with different doses of herbicide, on the third day of incubation (when actually calculated the percentage of inhibition).

The relative growth of *T. harzianum*at the smallest dose of herbicide is 87.10% and decreases with increasing dose. However, with 0.5 g/m^2 its development reaches 60.36%.

The percentage of inhibition growth of *T. harzianum* by the herbicide is only 12.91% in a dose of 0.2 g/m^2. Inhibition is increasing in the dose of 0.5g/m^2 (39,63%). Even at the dose 0.7g/m^2, percentage of inhibition reaches 47.18% i.e., it does not exceeded 50%. (Fig. 1 and 2).

Table 3. The influence of a herbicide Ohinol on the *T. harzianum* development

Ohinol	(dose)	Diameter of a colony (mm)									
		day									
		1	2	3	4	5	6	7	8	9	10
	0.2 g /m^2	25.75	61.50	95.80	99.60	103,70	104.90	105.30	105.30	108,00	110.00
	0.5 g /m^2	23.55	46.00	74.50	81.70	91.80	103.40	106.10	107.40	108.70	110.00

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	0.7 g /m^2	19.90	37.10	58.10	70.50	83.10	95.35	102.70	105.00	110.00	110.00
Check Ø		32.00	74.20	110.00							

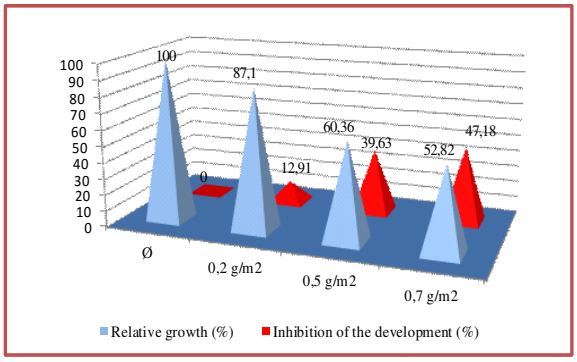

Fig.1. The influence of a herbicide on *T. harzianum*- solid medium

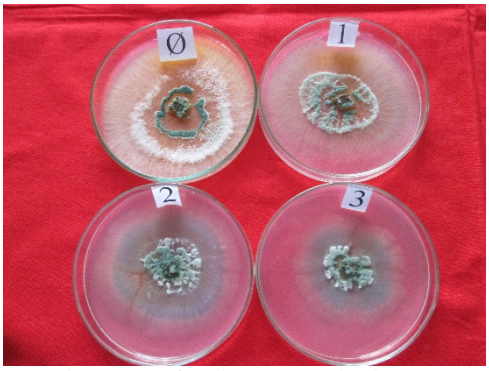

Fig.2. Development of *T. harzianum* in the presence of a herbicide

Some herbicides resulted with no mycelial growth of Trichoderma, only in Paraquat 20 W/W in which the growth is almost 50% lees than in control [10].

Afalon 450 SC (45% linuron) and Racer 250 WP (25% fluorochloride) in the dose of 100 ppm of a.i. had a powerful fungistatic activity and their Trichoderma spp. colony growth inhibition reached 67-89%. But, the evaluation of individual biotic effect for Trichoderma spp. growing (when exposed to herbicides) against phytopathogen R. solani had a degree +6 and +7 on a scale in which +8 was the highest degree - Trichoderma fungus colony totally inhibiting the pathogen development [3].

Examining the influence of a herbicide on the quantitative presence of *T. harzianum*, there was some decreasing of quantity. CFU / g soil in control 1 (Ø1) is 6.25 x 10^4. Control 2 (Ø2) has the smallest value – 0.45 x 10^4. This number is almost the same as in the initial presence of *Trichoderma* (Table 4).

Among variants, the lowest number have those with the use of the herbicide 0,2 g/m^2 before +0,3 g/m^2 after sowing (T1 and T2), i.e. they have the largest decreasing in the number of CFU / g soil.

The obtained results of CFU/g soil showed some decreasing in the quantity of T. harzianumafter the herbicide application. These results are in agreement with [1] who found that herbicide treatments at recommended rates resulted in lower fungal counts compared to the control soil. But, *T. harzianum* was the most resistant to herbicides between the all fungi studied [3].

Contrary of these results, in the greenhouse investigations with non-sterile soil inoculated with *T. harzianum* and some post-emergent herbicides, no detrimental effect of CFU/g soil of this biocontrol agent was determined [14].

The greatest number of *T. harzianum*was in those where the seed was kept 72 hours before sowing in the culture of *T. harzianum* and a herbicide was applied at a dosage of 0.5 g/m^2 after sowing (T5 and T6). Decreasing the quantity in variant T6 is only - about 5% compared to the control.

In this case, root colonization is not inhibited by chemical treatments [18]. These findings support presented results.

In variants with the same treatment with a herbicide, beside decreasing, CFU/g soil of *T. harzianum* is almost the equal (Table 4).

Table 4. The influence of a herbicide Ohinol50-S on the quantity of *T. harzianum*

Variants	Ø1	Ø2	T1	T2	T3	T4	T5	T6
Estimation	Colony forming units (x 10 ^{4})							
1	**6.25**	**0.45**	**2.33**	1.18	**4.83**	4.55	5.73	**5.88**
2	**11.75**	**0.40**	4.25	**6.00**	15.75	**1.,25**	18.50	**22.50**
3	**16.50**	**0.60**	4.75	**6.50**	16.00	**19.00**	20.50	**23.50**
4	**17.00**	**0.55**	5.00	**6.75**	17.50	**20.00**	22.25	**25.75**

At the second estimation, this situation is exceeded. The quantity is growing up and reaches or overgrows the value of the control. The greatest quantity has variants T5 and T6. The smallest value, over again, is estimated in variants T1 and T2.

This can be explained with the accommodation period needed for some microbial population to the new conditions, such a stress caused by the herbicide [11]. *Trichoderma* have a strong reproductive capacity and an ability to survive under very unfavorable conditions [7].

Further more, between variants with the same way of the herbicide application (T1 and T2, T3 and T4, T5 and T6) the number is greater when the second application is practiced during development (Fig. 3and 4).

Number of colony forming units, on the other hand, when comparing the more numerous variants, is greatest in the variant T6 compared with T2 and T4 (Fig5).

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Fig.3. *T. harzianum* presence at Ohinol (0.2g/m^2 + 0.3 g/m^{2}after sowing)

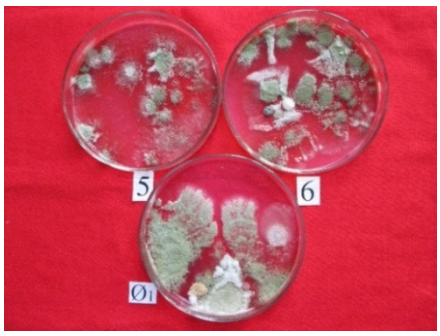

Fig.4. *T. harzianum* presence at Ohinol (0.5 g/m^{2}after sowing) and the seed keptin the biocontrol agent's culture

Fig.5 The quantitative presence of *T. harzianum* in the variants T2, T4 and T6

Population of *T.harzianum,* regardless of its first value inindividual variants increases during the development of tobacco seedlings (Table 4).

Looking the quantity in the third estimation, we can conclude the same state - the number of colony forming units is higher in variants T2, T4 and T6, i.e. those who had got a biocontrol agent during the intensive development of tobacco seedlings.

The increasing of *Trichoderma* quantity follows an experimental period, i.e., until the end of vegetation. These results are in agreements with that of [1], who reported that fungal counts reached a peak in the fourth week after treatment. *Penicillium sp.* and *Trichoderma sp.* were the most frequently isolated fungi from herbicide treated soils. It is also known that genus *Trichoderma* belongs to the herbicide-decomposing microorganisms, which also support these findings.

The high population density of a biocontrol agent at the last estimationhaveanadvantagein the transplantation of tobaccoseedlings, because of its influence on theroot growthandthewholeplant.The young plants are also protected in a first stage of field vegetation. It is reported that pepper seedlings produced in greenhouse with T22 better survived transplanting into the infested field than seedling that were not inoculated [18].

According to data for the disease intensity, the percentage of an infected area is the largest in Ø 2. All variants (including Ø 1), where the biocontrol agent is applied have a lower value (Fig. 6).

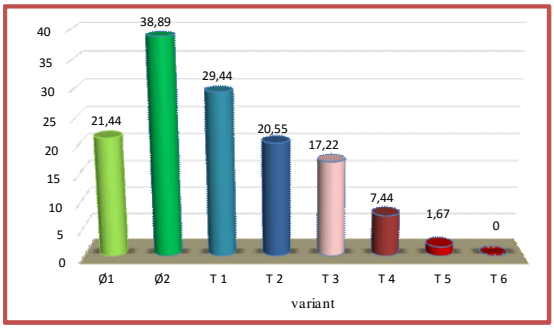

Fig.6. Infected area (%)

Trichoderma has success in reducing intensity of the root rot disease even the presence of a herbicide. The greatest advantage of *Trichoderma* treatments to plants occurs when they are under biotic and abiotic stress. It is also resistant to conditions resulting in oxidative damage such as application of fungicides or application of some herbicides[12].

There is data on enhanced inhibitory effect of these biocontrol fungi in a presence of some ions. It suggests that some elements participate in processes of antagonistic substances formation [3].

Between individual variants with the same mode of herbicides application, the percent age of infected areais lower in those where the quantity of *T.harzianum* in gsoilis greater. For example T4 compare to T3 (Fig 7) as well as T2 with T1 and T6 with T5.

These results are in agreement with [18] who stated that high concentration of antagonist in the inoculum is reguired for effective suppression of the pathogen. There are data for some examples of significant relationships between population size of the biocontrol agent and the degree of disease suppression [8].

The lowest intensity of disease have variants where the bio control agent is applied with the seed-T5. It has a very low occurrence of infection, while in T6 has no disease appearance (Fig. 8).

These results are in agreement with [2],who found that application of *T. lignorum* as a seed coating or wheat bran preparation, greatly reduced the number of bean seeds infested by *R.solani,* and the percentage of healthy seeds reached 92%.

When the biocontrol agent is added as a seed treatment, it colonizes a root surfaceand can persist at useful numbers up to 18 months after application [5], [6]. There is abundant and constant quantity of root exudations from

the root which follows the root development [8]. These substances may induce proliferation of *T. harzianum.*

The influence of the quantity of a biocontrol agent *T.harzianum* on the intensity of the root rot disease in tobacco seedlings is noticeable, also in a situation with the two controls i.e. intensity of disease is much greater in Ø 2 where no application of *T.harzianum* (Fig. 9).

Fig.7. The infected area in the variants T3 and T4

Fig.8 The infected area in the variants T2, T4 and T6

Fig.9. The intensity of a disease in between Ø 1and Ø 2

Effective microorganisms must remain active against target pathogens during periods favorable for plant infection [9]. Therefore, application during the growing period (which ensure a good BCA population level and a low disease intensity) is justifiable. Thus, the proper method of herbicide use and application of a biocontrol agent, can successfully impact on its quantity increasing.

IV. Conclusion

The presented results are a contribution to understanding the effect of a commonly used herbicide in tobacco. In vitro researching showed that herbicide Ohinol 50-SC influence the yield of a dry biomassas well as radial development of a biocontrol agent *T.harzianum*, but they are not strongly affected.

There is some decreasing of the number ofcolony forming units in g soil. But the quantity increased over a vegetative period. The greater quantity of *T.harzianum* take an advantage in tobacco seedling's protection from the root rot disease in tobacco seedlings i.e. the lower percentage of infected area is determined in variants with a larger number of colony forming units.

The way of the proper application of a biocontrol agent *T. harzianum* ensures to utilize its usefull features in a conventionaly practice using this herbicide.

The presented results showed that application of *T.harzianum*in tobacco seedlings in the use of a herbicide Ohinol 50-SC (50% napropamide) is possible. The most suitable methodofapplication is-herbicide used at there commended dose 0.5g/m^2 after sowing and the seed kept in culture of a biocontrol agent. The application during the development of tobacco seedlings is also of particular importance.

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AUTHOR'S PROFILE

Dr. B. Gveroska
Doctor of Agriculture Science, Scientific Tobacco Institute-Prilep, St. Kliment Ohridski University – Bitola, Republic of Macedonia, 2005.
She works on tobacco diseases.
Her research work is focused on Biocontrol, especially application of biocontrol agents in tobaco growing practice. She had published papers in International Journals.