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RELATIONSHIPS OF *TRICHODERMA* SPP. QUANTITY IN SOIL TO REDUCING THE DAMPINGOFF IN TOBACCO SEEDLINGS

B. GVEROSKA
Scientific Tobacco Institute-Prilep, 7500 Prilep, Republic of Macedonia

Abstract

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The study was conducted to investigate the quantity of *Trichoderma* spp., its reducing effect on the damping off in tobacco seedling and find the most efficacious application way of biocontrol agent. Some variants of application of biocontrol agent and following applications were used. Variants with fungicide, alone or together with *Trichoderma*, as well as no sterilized soil and manure, were also included.

There is high population density of *Trichoderma* spp. when it is applicated by seed and treating the soil before sowing evens the soil and manure for mulching. These variants have the best results -the smallest intensity of the damping-off in seedlings. The level of a disease control in variants with a fungicide treatment and that by *Trichoderma* was equal or more effective in case of *Trichoderma*.

When the biocontrol agent is applicated after growing up the seedlings, there is a smaller amount of BCA than application before sowing. The investigations have shown that bigger *Trichoderma* spp. quantity has a positive effect on reducing disease intensity in tobacco seedlings caused by *R. solani*.

The model of BCA application with sowing (by seed and treating the soil or soil and manure for mulching) and several applications during the growing period are recommended for damping- off control in tobacco seedling.

*Key words***:** biological control, tobacco seedlings, damping off, *Trichoderma* spp*., Rhizoctonia solani,* quantity, reducingeffect

Abbreviations: CFU/g soil-colony-forming units in g absolute dry soil; BCA-Biocontrol agent

Introduction

Plant diseases play a direct role in the destruction of natural resources in agriculture. Fungal plant pathogens are among the most important factors that cause serious losses to agricultural products every year. In particular, soil-borne pathogens cause important losses, because fungi being the most aggressive (Benitez et al., 2004; Heydari and Pessarakli, 2010).

Excessive and lasting usage of chemicals causes resistance of pathogens, and it is not always effective (Benitez et al., 2004). They induce damage of the other, especially useful organisms (Lewis et al., 1998). Furthermore, it causes harmful consequences to human health and environmental security (Monte, 2001).Therefore, the scientific approach is focused to alternative methods of disease control.

According to Heydari and Pessarakli (2010), biological control of plant diseases including fungal pathogens has been considered a viable alternative method to chemical control. In plant pathology, the term biocontrol is concerning to the use of microbial antagonists to suppress diseases. Biological control agents are alternatives to synthetic pesticides due to their perceived increased level of safety and minimal environmental impact (Brimmer and Boland, 2003).

Biological control, the use of specific microorganisms that interfere with plant pathogens and pests is a nature-friendly, ecological approach to overcome the problems caused by standard chemical methods of plant protection (Chet et al., 2006).

Biological control offers an environmentally friendly approach to the management of plant disease and can be incorporated into cultural and physical controls and limited chem-

E-mail: bgveros@yahoo.com

ical 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)

Trichoderma spp. are free-living fungi that are common in soil and root ecosystems, beneficial to plants (Goes, 2002; Contreras-Cornejo et al., 2009).It is not known whether most of the benefits of *Trichoderma* occur because they directly attack and control disease-causing fungi, or because they have direct effects upon plants. Many recent findings suggest that plant development and biochemistry are strongly affected by *Trichoderma* strains (Chet et al., 2006). They are opportunistic, avirulent plant symbionts, as well as being parasites of other fungi. Colonizing the root, *Trichoderma spp.* have evolved numerous mechanisms for both attack of pathogens and enhancing plant and root growth (Harman, 2004, 2006; Benitez et al., 2004; Howell, 2003).

Trichoderma spp. is 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. These biocontol agents are effective, eco-friendly and cheap, who nullifying ill effects of chemicals (Palanna, 2007; Benitez et al., 2004).

The dual roles of antagonistic activity against plant pathogens and promotion of soil fertility make *Trichoderma* strains as an alternative to hazardous fumigants and fungicides (Monte, 2001). *T harzianum* strain T-22 is widely used for disease control instead of chemical fungicides because it is safer to use for growers, its disease control last longer -so it is less costly and root growth can be as well, or better than that achieved using pesticides (Harman, 2004). There are so many efforts to develop biocontrol systems that are used in commercial agriculture (Harman, 1996, 2000).

*Rhizoctonia solani*is a very common in most soils and has a wide range of host plants (Nunez, 2005). It is worldwide pathogen responsible for serious damage of many economically important agricultural and horticultural crops (Grosh, 2003). It causes the damping off in tobacco seedlings, which is a very destructive disease. Damages and losses are obvious because of seedling's importance for quality production.

It can considerably degrade the seedling production, especially in favorable conditions for a causing agent development and spreading of a disease. The available way of disease control is fungicidal treatment. Some active matters are effective at this pathogen (Csinos and Stephenson, 1998; Mocioni et al., 2003). However, there are only few preparates in case of tobacco production.

The use of biological control in case of the damping off in tobacco seedlings is a prospective method against crop rotation, soil conditioning, use of resistant cultivars and fungicide. Furtrhermore, it keeps an environment clean and healthy (Leach and Gaber, 1970; Gveroska and Ziberoski, 2011).

Trichoderma species are effective at *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 biolaboratory.Biological control effect of *Trichoderma* sp. on *R. solani*is confirmed by Leach and Gaber (1970), Lewis et al. (1998), Monte (2001), Goes (2002), Kucuk and M. Kivanc (2003), Harman (2000, 2004, 2006), Shalinni (2006), Osman (2011). *Trichoderma*harzianum has a good control of the damping off in tobacco seedlings (Gveroska and Ziberoski, 2011).

The development of feasible and efficient delivery system for the application of appropriate microorganisms to the ecosystem is an important component of biocontrol technology (Lewis et al., 1998). However, data about the appropriate time for applying the biocontrol agent is limited. Therefore, Izzati and Abdulah (2008) conducted a trial with various time of conidial suspension application. Handelsman and Stabb (1996) stated that colonization is essential for biocontrol and there is relationship between population size of the biocontrol agent and the degree of disease suppression.

According to Heydari and Pessarakli (2010), the one criterion for successful biocontrol is to determine factors of successful colonization and expression of biocontrol traits. Delivery system must ensure that biocontrol agent will grow well and achieve its purpose. Therefore, delivery and application processes must be developed for each crop (Harman, 1996). Therefore, we liked to determine how much a biocontrol agent retains in a soil, what colonization has in various ways of application, and how its quantity influences on the damping off in seedlings.

Thus, our aim was to estimate the quantitative presence of *Trichoderma* spp. and its influence in reducing the damping off in tobacco seedlings. Therefore, we will confirm the most efficacious way of application of biocontrol agent.

Materials and Methods

The pathogen fungus *Rhizoctonia solani* was isolated from infected plant material. The biocontrol agent *Trichoderma* sp. was obtained from rhizosphere, a root zone of tobacco plants, by dilution method. A standard medium – potato dexstroze agar (PDA) was used.

The investigations were taken in greenhouse, on tobacco seedlings of oriental type – variety P 23 (night pots per each

variant). A sterilized soil (non-sterilized soil in two variants) was used, and two replications were made. Included variants are:
T1 -Application of the biocontrol agent *Trichoderma* sp. before sowing.
T2 -*Trichoderma* sp.fifteen days after sowing (after the seedlings growing up).
T3 -Treatment of soil with *Trichoderma* sp.before sowing and use of Top M.
T4 – Only fungicide treatment.
T5 -Application of the biocontrol agent during the sowing- over the soil and manure for mulching.
T6 -Seed together with the biocontol agent.
T7 -Non sterilized soil treated with *Trichoderma* sp. before sowing
T8 -Application of the biocontrol agent during the sowing– over anon-sterilized soil and manure for mulching.
Ø- Check, only artificial inoculation with *Rhizoctonia solani*.

Additional application of the biocontrol agent in the proper variants was taken periodically, in 7-10-day intervals (Table 1). Seedlings are drenched with BCA suspension.

The pure culture suspension of biocontrol agent was prepared and taken (one Petri dish in 100ml distilled water per pot) in the corresponding variants. Pathogen suspension for inoculation was prepared at the same way.

In a variant with a seed, it was stored 48 hours over the pure culture of the agent.

A foliar treatment with a fungicide Top M 0.1% (the most-used fungicide in our seedling's production) was taken at the corresponding variants.

The situation was investigated daily. Two estimations of intensity of attack -percentage of an infected area, for each replication, were taken.

The estimation of the colony-forming units (CFU/g soil) of *Trichoderma* spp. 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 of the soil samples was made until 10^{-4}dilution. One ml of the final (10^{-4}) dilution was pipeted into a Petri dish, then 20 ml of RBA medium was poured, gently shaken and left to cool. Five replications per only variant and control were done and incubated at 28^0C. The CFU/g soil was counted and recorded after five-day incubation.

Results

The smallest intensity of a disease attack had variants T6 and T2- 2.78 and 3.89%. However, there is not appearance of a disease in variants T1 and T5 (Table 2).

Table 1
Treatments of variants

Variant	Date of treatment			
	(sowing) 18.05 29.08	6.06. 15.09	17.06 28.09	27.06 12.10
T1- before sowing	T	T	R + T	T
T2- after sowing		T	R + T	T
T3- before sowing, Top M, T	T		R + Top M	T
T4- Top M			R + Top M	
T5- soil and manure for mulching	T	T	R + T	T
T6- seed with biocontrol agent		T	R +T	T
T7- non-sterilized soil before sowing	T	T	R +T	T
T8- non-sterilized soil and manure for mulching	T	T	R +T	T
Ø Check			R	

T –*Trichoderma* sp.
R-*R. solani*

Table 2
The influence of *Trichoderma* spp.quantity on intensity of attack (first replication)

Variant	% of infected area		*Trichoderma* spp. CFU/g soil (x 10^4)
	1st estimation	2rd estimation	
T1- before sowing	-	+	37.6
T2- after sowing	3.89	5.00	31.80
T3- before sowing, Top M, T	5.56	6.44	12.67
T4- Top M	6.11	6.67	0.00
T5- soil and manure for mulching	-	+	31.60
T6- seed with biocontrol agent	2.78	5.22	42.80
T7- non-sterilized soil before sowing	5.56	8.89	19.30
T8- non-sterilized soil and manure for mulching	7.22	10.56	8.60
Ø Check	12.77	21.67	0.00

- no appearance
+ a weak appearance

There is a disease appearance in these variants and only a weak increasing of damping off in the others in a second rating. The percentage of an infected area is almost no changed in variants with a fungicide treatment; meanwhile the percentage of an infected area in check is double increased.

There is an obvious distinction between damaged area in a check and variants with application of biocontrol agent before sowing and by seed - T1 and T6 (Figures 1 and 2).

The highest intensity of disease attack had variants where the biocontrol agent is applied by use of non-sterilized soil and manure -T7 and T8 (Figure 3).

Comparation between *Trichoderma* application before sowing and by seed (T1 and T6), and fifteen days after sowing (T2) shows the advantage of the first two mentioned ways of application (Figure 4).

There is a big difference in a *Trichoderma* quantity among a check and variants with the biocontrol agent, as well as an infected area (Table 2, Figure 5).

The CFU count of *Trichoderma* spp. is the highest in a variant T6-42.8 x 10^4colonies in gramme soil (Figure 6). The highest CFU also, have variants T1-37.6 x 10^4 and T5-31.6 x 10^4CFU/g soil. However, it seems that treatment of manure for mulching with a biocontrol agent does not have a big influence on a *Trichoderma* spp. quantity than application over a soil only (Table 2).

Besides big colonies, there is small quantity of *Trichoderma* spp. in variants with a non-sterilized soil and manure (Figure 7). The same situation there is in a variant with application of biocontrol agent before sowing and fifteen days after sowing (Figure 5).

Application of the *Trichoderma* sp. in a fungicide treatment has an influence on its quantity (Figure 8).

There is a significant increase of disease intensity in the check than in the first replication (Table 3). However, a value of a damaged area in the other variants is less by that of a check. When the biocontrol agent is applicated before sowing, and by seed stored in a pure culture of *Trichoderma* sp. (T1 and T6), the intensity of attack is weak. There are not symptoms of a disease in a variant T5 (Table 3).

At the second estimation - there is only a weak increase of the intensity in all variants where the application of *Trichoderma* sp. is used and an appearance of a disease in a variant T5.

There is an expected situation in the variants with a fungicide treatment, with a little advantage for this with an additional treatment with *Trichoderma* sp.

The value of a damaged area is the biggest in the two variants with application of a biocontrol agent in a non-sterilized soil and manure, as well as at the first replication.

Two variants with application of a biocontrol agent in anon-sterilized soil and manure (T7 and T8) have a small quantity of *Trichoderma* spp. as well as in the first replication (only 5.80 and 7.80 CFU /g soil) (Table 3).

There is the highest quantity of *Trichoderma* spp. in T6 - 45.80x 10^4CFU /g soil. This value is also the biggest in variants with good situation with damping off (T1 and T5) - 29.80 and 31.80CFU /g soil respectively).

Discussion

Nearly all crops propagated from seed loss due to seedling diseases. Seedling disease may appear at different stages of the young plant's growth. It may appear as a seed rot or seedling decay before the seedling emerges (pre-emergence damping off), seedling decay after the seedling emerges (post - emergence damping) or seedling root rot (rot pruning) (Nunez, 2005).

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, i.e.,

Table 3
The influence of *Trichoderma* spp.quantity on intensity of attack (second replication)

Variant	% of infected area		*Trichoderma* spp. CFU/g soil (x 10^4)
	1st estimation	2rd estimation	
T1- before sowing	+	4.44	29.80
T2- after sowing	9.44	11.11	19.00
T3- before sowing, Top M, T	+	+	13.60
T4- Top M	8.89	9.44	0.00
T5- soil and manure for mulching	-	5.56	31.80
T6- seed with biocontrol agent	2.77	5.83	45.80
T7- non-sterilized soil before sowing	13.88	18.36	5.80
T8- non-sterilized soil and manure for mulching	10.71	13.57	7.80
Ø Check	41.0	57.78	0.00

+ a weak appearance

Fig. 1. Intensity of damping off in the check (Ø) and T 1(1)

Fig. 2. Intensity of damping off in the check (Ø) and T 6 (6)

Fig. 3. Intensity of damping off in the check (Ø) and T 7 (7)

Fig. 4. Intensity of damping off in variants T 1 (1), T 2 (2) and T 6 (6)

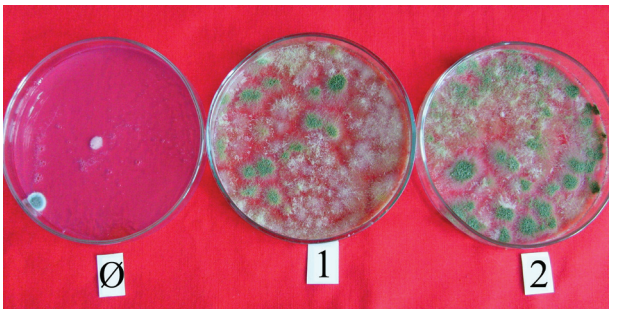

Fig. 5. Quantity of *Trichoderma* spp. in the check (Ø), variant T 1 (1) and T 2 (2)

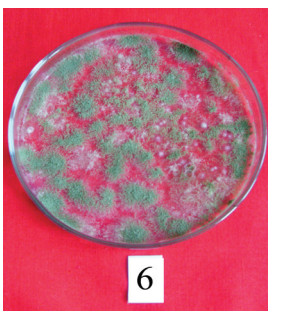

Fig. 6. Quantity of *Trichoderma* spp. in the variant T6 (6)

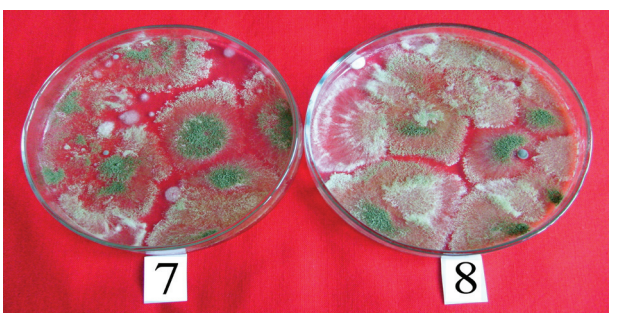

Fig. 7. Quantity of *Trichoderma* spp. in variants T 7 (7) and T 8 (8)

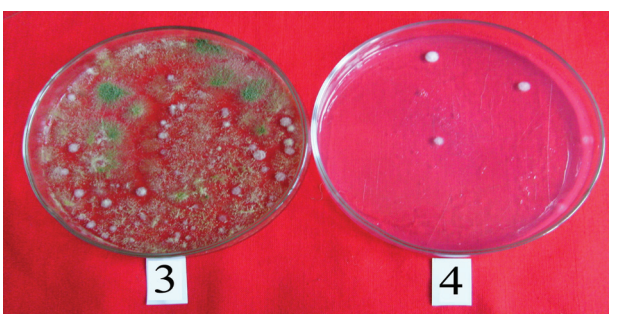

Fig. 8. Quantity of *Trichoderma* spp. in variants T 3 (3) and T 4 (4)

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 does not cause economic loss (Hollaway, 2008).

Since that, the chemical control is not always effective. Therefore, biological control is an acceptableand effective means of disease management, since microbial organisms can control resistant pests and reduces 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 soilborne pathogens, since the rhizosphere is available for combined biocontrol mechanisms and the other interactions that contribute to general suppression (Howell, 2003; Heydari and Pessarakli, 2010).We use the *Trichoderma* isolate from tobacco rhizosphere, because, the best method for obtain a potential biocontrol agent mightbe one where it is isolated from area where it is expected to function in disease control and because of similarity of conditions where it is growing (Howell, 2003). Al-Mahareeq (2005) found that between 44 isolates, which reduced the *Rhizoctonia* damping-off on bean, local *Trichoderma* isolates reduced disease index more than foreign isolates.

Biocontrol abilities of *Trichoderma* strains are strongly confirmed (Harman, 2000, 2004, 2006). However, there is not general solution and biocontrol system must be developed for each crop (Harman, 1996). In order to have more effective biological control strategies in the future, it was critical to carry out more research studies on some aspects of biocontrol, including development of an appropriate time of application for the current crop, in this case, tobacco.

Application technology cans have a significant impact on the efficacy of BCA. Targeted delivery, deposition and coverage of the infection court are essential for good disease control (Walters, 2009). Successful application of biological control strategies requires more knowledge – intensive management. Various methods include application directly to the infection court at a high population level to swamp the pathogen, application at one place but at lower populations, which then multiply and spread to other plant parts and give protection against pathogens and one time or occasional application that maintains pathogen populations below threshold levels (Heydari and Pessarakli, 2010).

According to Grosh et al. (2003), the effect of biocontrol strains needs to be tested in different experiments in dependence on their application frequency and density. Therefore, we have researched models of application and frequency andinfluence on the quantity of *Trichoderma* spp. and, lastly, on a disease intensity.

It was estimated the high population density of *Trichoderma* spp. when it is applied by seed and treating the soil before sowing, even the soil and manure for mulching. These application ways have the best results to reduce the damping off in seedlings.Accordingly, that, a majority of biocontrol products is applied against seed borne and soil borne fungal pathogens, including the causal agents of seed rot, damping-off and root rot diseases. When it is used as seed treatment, these products have been effective in protecting several major crops such as wheat, rice, corn, sugar beet and cotton against fungal pathogens (Heydari and Pessarakli, 2010).

Seed treatment is an attractive delivery system for either fungal or bacterial bioprotectans. Thus, seed treatment systems that will enhance efficacy of biological agents are needed (Harman and Taylor, 1988). It is a logical target, which ensures seed protection and perhaps increased vigour from planted seeds (Harman, 2000). 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 (Harman, 1996). 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).

With regard to the effect of application, Heydari and Pessarakli, (2010) stated that application of biocontrol agent to soil at the site of seed placement is one of the most- used procedures for achieve successful control. Furthermore, inoculation of *T. harzianum*to the soil one month before sowing reduced the level of *R. solani* on *Phaseolus vulgaris* beans (Bazgir and Okhovat, 1996, loc. Cit. Osman et al., 2011). Because,when living microorganisms are introduced, they may augment natural beneficial populations and reduce the damage caused by target pathogens (Heydari and Pessarakli, 2010). Our statement that application of BCA over a soil ensures a good BCA population level is in accordance with this.

Papavizas et al. (1982) found that aqueous suspensions of conidia or dry preparations of some *Trichoderma harzianum* biotypes, added at 4.4 $x10^5$ cfu/g soil significantly suppressed *Rhizoctonia solani* damping-off cotton and radish. They have also found that surviving varies from 16 to 22 weeks depending on the isolate. The minimal effective amount of *Trichoderma* found by Chet and Baker (1980) to be around 1x 10^6cfu/g soil.

According to Lewis et al. (1998), application of the biocontrol fungus before a pathogen is introduced into the ecosystem might be an appropriate approach. As Nunez (2005) stated, the damping-off may appear from seed to young seedling. Therefore, application of Trichoderma before, or a meantime with sowing (as in this researching) is of importance because

if the pre-emergence occurs, it dies before it can emerge. The biocontrol agent who can persist in a soil ensures the control at every stage at which disease can appear.

In our investigations, we have found that seeds treated with *Trichoderma* have germinated earlier 3-4 days. This can be explained by the ability of *Trichoderma* to inhibit minor pathogens in the rhizosphere, which might induce seed rots and preemergence damping off. Al-Mahareeq (2005) also found that been seeds, which were planted in *Trichoderma* treated soils germinated earlier by four days than those planted in non-treated soils in addition to better emergence rate.

If species of the genus *Trichoderma* is applied directly in the soil or by seed, they grow up simultaneously by the root system of treated plants (Harman, 2000;l 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 when shoots emerged and photosynthesis was initiated (Handelsman and Stabb, 1996). Therefore, when the biocontrol agent is applied after growing up the seedlings, there was a smaller amount of BCA than application before sowing, as we have stated.

The amount of colonies in the variants with non-sterilized soil is so smaller than in sterilized soil, although *Trichoderma* spp. survives in semiarid soil with low organic matter and even exposure to chemical contamination and increases its quantity (E&GA, 2010). It seems there are disturbances in interactions among the living organisms in this ecosystem, because, the biological control may be considered a positive result arising from different specific and non-specific interactions (Heydari and Pessarakli, 2010).According to Handelsman and Stabb (1966), disease suppression by biocontrol agent is the sustained manifestation of interactions among the plant, the pathogen, the biocontrol agent, the microbial community on and around the plant and the physical environment. In this case, they resulted worse.

The level of damping-off control in variants with a fungicide treatment and that by *Trichoderma* was equal or more effective in case of *Trichoderma*. Maketon et al. (2008) noted similar results, where treatment with *T. harzianum* AP-001 $2x10^8$ cfu/g applied three days after inoculation showed better results than *B. subtilis* or chemical treatment against tobacco damping-off caused of *Pythium aphaniedermatum*.

The same situation has a variant with *Trichoderma* treatment with fungicide and fungicide alone. The chemical provides good short-term seed protection, and the biocontrol fungus provide long-term root protection (Harman, 1996). Furthermore, a fungicide may enhance the ability of biocontrol agents to reduce plant diseases (Heydari and Pessaraklii, 2010). It could be related to a higher competitiveness of fungicide-resistant isolates in the presences of fungicide, due to a weakening of pathogens and other saprophytic fungi in this specific ecological niche. Therefore, a fungicide-resistant isolates of *Trichoderma* can be combined either simultaneously or subsequently with appropriate fungicide for more efficient control of plant diseases (Wojtkowiack-Gębarowska and Pietr, 2006). The best yield was in integrated T-22 + maneb + metalaxyl program Harman (2000). It seems there is a possibility of the biocontrol on tobacco in conjunction with chemical fungicides. In our case, it is needed to investigate the influence of the certain fungicide on the quantity of *Trichoderma* spp.

Our model of BCA application (application with sowing and several applications during the growing period) in tobacco achieves the *Trichoderma* spp. population to remainin the current eco-system and enables to express its mechanisms. An effective antagonist must become established in plant ecosystems and remain active in target pathogens during periods favorable for plant infection. Monthly application of *T. harzianum* could provide a second step in protection by reducing the spread of disease or other methods of inoculum dissemination (Heydari and Peskarli, 2010).

For the optimization of biological control in pathosystem, Wilson et al. (2008) suggest that higher amount of the biocontrol agent need to be placed close to the plant. The higher amount or constant presence of biocontrol agent has more possibilities to hinder it, act as a barrier and involve its mechanisms. Biological control is complex, and its use varies with the kind of biocontrol agent, pathogen and host plant involved in the interaction. It is the culmination of a number of different mechanisms working synergistically to achieve disease control (Howell, 2003). Continuous presence of rhizosphere-competitive isolates of *Trichoderma* spp. on the root make longelife induced resistance of plants, which exist by weeks or months (Harman, 2000, 2004).

In general, results show that the biggest quantity of *Trichoderma* spp. (when it is applied by seed, treating of a soil and soil and manure for mulching) has a good reducing effect on the root rot disease in tobacco. They are in agreement with Aziz et al. (1997), who found that application of *Trichoderma lignorum* as a seed coating ($8x10^6$ conidia/seed) or wheat bran preparation ($1x10^6$ cfu/g) at a rate of 20 g/kg soil, greatly reduced the number of bean seeds infested by *Rhizoctonia solani,* and the percentage of healthy seeds reached 92%. Additionally, in agreement with Harman and Taylor (1988), who reported that the greater activity of *Trichoderma* strains on seed was reflected in numbers of colony-forming units of *Trichoderma* in soil at the termination of the experiments? Soil planted with no treated cucumber seeds contained less than 100 cfu/g while soil with seeds treated with *Trichoderma* alone contained about 10^4cfu/g. Handelsman and Stabb (1996) have given some examples of significant relationships

between population size of the biocontrol agent and the degree of disease suppression.

As a confirmation of the reported findings, there are data of Izzati and Abdullah (2008) that disease severity index increased with delaying BCA application time. There were the biggest cfu in a variant of the earliest way of application. They estimated maximum cfu/g of *T. harzianum*– 7.67 x10^4.

The use of antagonistic microorganisms to *R. solani* applied as treatment of seeds or soil, have been demonstrated to control a variety of cultures in the greenhouse and field studies (Goes, 2002). Biocontrol agent can both reduce pesticide use in the greenhouse by limiting root-attacking diseases and protect transplants in the field by its ability to colonize roots (Harman, 1996). Therefore, a bigger quantity of *Trichoderma* spp. take an advantage in tobacco seedling's protection and this is a good base for successful tobacco production.

Conclusions

We have stated the biggest quantity of *Trichoderma* spp. when seed applies it and treating the soil before sowing, even the soil and manure for mulching. There is the smallest intensity of the damping off in these variants, also. Therefore, application of *Trichoderma* with sowing is of importance because the biocontrol agent can persist in a soil and ensure the control at every stage in which disease can appear.

When the biocontrol agent is applied after growing up the seedlings, there is a smaller amount of BCA than application before sowing. In addition, the amount of colonies in the variants with non-sterilized soil is so smaller than in sterilized soil.

The level of damping-off control in variants with a fungicide treatment and that by *Trichoderma* was equal or more effective in case of *Trichoderma*.The same situation has a variant with *Trichoderma* treatment with fungicide and fungicide alone.

The investigations have shown that bigger *Trichoderma* spp. quantity has a positive effect on reducing disease intensity in tobacco seedlings caused by *R. solani*. So, we have to ensure the most efficient way for the BCA quantity establishment and give protection against pathogens in a proper time.

The model of *Trichoderma* spp. application with sowing (by seed and treating the soil or soil and manure for mulching) and several applications during the growing period are recommended for damping-off control in tobacco seedlings.

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