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APPLICATION OF *BACILLUS SUBTILLIS* CH13 IN THE CONTROL OF DAMPING OFF DISEASE IN TOBACCO SEEDLINGS

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ABSTRACT

Bacillus subtillis Ch13 is an active ammonifying bacteria who plays a main role in nitrogen cycling. But, it has multifunctional properties that, beside the stimulating, has the fungicidal effect on plants, too. It is a base of a microbiological product Exstrasol which is used for examination. Modern tobacco production follows the ecological principles of sustainable agricultural production and supports the use of environment-friendly products, especially the use of biological products in the control of tobacco pathogens. The aim of this research is to determine the possibilities for application of this microbiological product in protection of tobacco seedlings from damping off disease. Investigations were performed in biological laboratory by artificial inoculation with the diseasecausing agents - *Rhizoctonia solani* and *Pythium debarianum*. Investigations of disease were also made in conditions of natural infection. It was determined that the application of *B. subtillis* Ch13 reduces the intensity of disease attack. The percentage of infected area is lower in the variant where treatment is included. The best results in artificial inoculation with *R. solani* are obtained when soil treated with 1% is planted with seed treated with 10% Extrasol. In *P. debarianum*, the best result was achieved with seed treated with 10 and 20% Extrasol. The best results in natural infection are achieved in seed treated with 10% Extrasol. Additional treatment of soil when the seed was treated with this product had a negative effect. The microbiological product Extrasol has good prospects for its use in tobacco control from damping off disease.

INTRODUCTION

The long-term use of pesticides leads to inefficiency due to the occurrence of resistance among pathogens, but it also causes various undesirable effects on the environment. Therefore, sustainable agricultural production in line with modern principles addresses the use of ecologically friendly products. To protect tobacco seedlings from damping off disease, a number of chemicals have been applied for several years and negative effects are still observed. Contemporary protection of tobacco, however, follows ecological principles and seeks for increasing use of environmentally friendly fungicides. World supply of such products is large and various biological products of different kind are recommended, such as antagonists, ferments, plant extracts, microorganisms with specific activity, etc. Special attention is given to rhizospheric bacteria as plant growth promoters and as biocontrol agents against plant pathogens. According to Alizadeh *et al*. (2013), rhizobacteria are ideal biocontrol agents since they inhabit the rhizosphere and provide front-line defense against attack by pathogens. Pathogens encounter antagonism from rhizobacteria before and during infection of the root. Some authors are cited who specified the biocontrol mechanisms, such as: synthesis of plant hormones, production of antibiotics, low- molecular weight metabolites such as hydrogen cyanide with antifungal activity, production of numerous enzymes with leasing activity on fungal cells, etc. *Bacillus subtilis* is an active rhizosphere resident. As an amonification bacteria it plays a main role in nitrogen cycling, but it is also characterized by other properties, which explain its wide application. It produces a wide range of antibiotics. It is also applied as a fungicide in seeds of vegetable crops, soybeans, cotton, flowers and ornamental plants (Probiotics, 2015).

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Bacillus subtilis strain QST 713 is widely used in integral control of many pathogens, as a main active ingredient of the biofungicide Serenade® (Edgecomb and Manker, 2006). The authors describe its multiple mode of action against pathogens: it is a barrier to the pathogens and competes with them, it creates fungal metabolites-lipopeptides and degrades the spores and mycelium of phytopathogenic fungi, etc. Junaid *et al*. (2013) reported two strains of *B. subtilis* as biocontrol agents against the pathogen *Rhizoctonia*, which are involved in commercial products. Exstrasol is a microbial product that contains multifunctional active rhizobacteria, based mainly on *Bacillus subtillis* Ch13. According to Chebotar *et al*. (2013), the effects of Exstrasolduring vegetation are based on the fact that the suspension of microorganisms and their products affects the regulation of vital functions and protective-adaptive reactions of the plant. Rhizobacterium *Bacillus subtilis* Ch13 has important role in plant supply with nitrogen matters and increases the absorption coefficient. In the same time, it also feeds on root exudates. Actually, its rapid development makes it competitive in relation to other microorganisms, including the phytopathogenic ones. It synthesizes substances that prevent the development of phytopathogenic fungi. The multifunctional action of this bacterium enablesthe stimulating and fungicidal effect of Exstrasol (SRTI,1998; Bisolbi-inter, 2015). The above data initiated the purpose of this investigation - to determine the impact of this product on the intensity of damping off disease in tobacco seedlings and the possibility of its application in protection of tobacco.

MATERIALS AND METHODS

The soil was treated with 2 *l*/ha Exstrasol, by using 50 ml of water / 0,3 m^2 for each variant, in three replications with 0,1 m^2. Further treatments were applied by spraying the seedlings with 1% solution of the product (100 ml for an area of 0,3 m^2). Tobacco seed was treated with 10% and 20% solution of the preparation before sowing. Seed of each variant (0,5 g/m^2) was soaked in 1,5ml of the appropriate concentration and kept 24 hours at room temperature before sowing.

Artificial inoculation

Rhizoctonia solani and Pythium debarianum - pathogenic fungi that cause the damping off disease on tobacco seedlings were isolated from infected seedlings on standard medium PDA. The pure cultures were used for inoculation (two Petri dishes with 300 ml of water for each variant -0,3 m^2). The soil was treated just before sowing (the first treatment). The second as well as inoculation with the disease agents (along with treatment of variants 5,8 and 11) were carried out every 15 days. Assessment of the disease attack (percentage of infected area) was after fifteen days from inoculation.

Natural infection

Next year was planned to carry out artificial inoculation, too. However, disease outbreak was recorded in two trials prior to inoculation. Therefore, assessment was made of the infected area during natural infection. Soil and seedlimgs from the respective variants were treated with the same rate and in the same way as the previous year. The infected area was assessed 7-10 days after the disease outbreak. Variants where the second treatment was to take place at the time of inoculation (variants 5, 8 and 11) have not been taken for analysis of this year. The percentage of infected area for both types of investigations was assessed for the three replications in each variant and average values were calculated.

The variants and treatments are presented in Table 1.

RESULTS AND DISCUSSION

Artificial inoculation

In artificial inoculation with the pathogen *R.solani*, the highest intensity of disease attack was found in the inoculated checkvariant (Table 2). In variants 3 and 4, the infected area was less in the soil which was treated twice with Exstrasol. Treatment with this preparation during inoculation (variants 5, 8 and 11) had no effect - the infected area was the same as in the untreated variants. Treatment of seeds with 10% and 20% Exstrasol (variants 6 and 9) gave good results - no occurrence of disease. These results confirm the suggestion of Chetobar *et al*. (2013) that seed treatment with Exstrasol results in colonization of the seeds with beneficial microflora. When the treated seed is sown, these bacteria rapidly multiply and inhabit the plant rhizosphere. They also synthesize substances that inhibit the pathogens growth and stimulate the growth of beneficial microorganisms. In variants 7 and 8, i.e. 10 and 11 where both soil and seed were treated, there was occurrence of disease, with values similar to other variants (Table 2). When inoculation was made with *P. debarianum*, the intensity of attack in the check variant is higher than by inoculation with *R. solani* (Table 3).It is interesting to note that in this case (two soil treatments with Extrasol), the percentage of infected area was higher than with only one treatment (variants 3 and 4). The best results were obtained in the treatment of seed with 10% Exstrasol (as in seed and soil treatment with 10% Exstrasol). No symptoms of disease were also observed inseed treatment with 20% Exstrasol, which is not the case with the additional treatments with this preparationin variants 10 and 11 (Table 3).

Natural infection

In a natural outbreak, the highest intensity of damping off was observed in both checks (3,1 and 3,3%). Lower intensity of the disease attack was observed in adequately treated variants (Table 4). This confirms the results obtained in the previous investigations. Similar data were presented by the Edgecomb and Manker (2006), who concluded that preventive application of *B. subtillis* provides good protection against many pathogens. Treatments (one or two treatments with 1% Extarsol) led to a reduction of the intensity of attack compared to the check. Two treatments of seedlings with 1% solution of Extrasol gave better results than only one treatment (Table 4,5).The best time for sprayingis the early stages of plant development, duringthe colonization of plant surface by bacteria, which prevents the spread of pathogens and stimulates the plant growth. Plants should be treated once or twice during the growing season, depending on weather and technical conditions and the estimated costs (Chebotar *et al*., 2013).

Table 1. Variants and treatments

N0. Variant	Sowing (Itreatment)	IItreatment	Inoculation	Inoculation (+Exstrasol)
1	Ø Check			
2	Ø Check		+	
3	Soil treatment with Exstrasol		+	
4	Soil treatment with Exstrasol	+	+	
5	Soil treatment with Exstrasol	+		+
6	Seed treated with Exstrasol (10% solution)		+	
7	Soil treatment with Exstrasol + seed with Extrasol (10% solution)		+	
8	Soil treatment with Exstrasol + seed with Extrasol (10% solution)			+
9	Seed treated with Exstrasol (20% solution)		+	
10	Soil treatment with Exstrasol + seed with Extrasol (20% solution)		+	
11	Soil treatment with Exstrasol + seed with Extrasol (20% solution)			+

Table 2. Percentage ofarea infected with damping off disease –artificial inoculation with *R.solani*

Variant No.	Replication			$\bar{X}$
	I	II	III	
1	4,3	0,6	2,4	2,4
2	8,7	9,0	4,2	7,3
3	2,1	5,3	5,0	4,1
4	9,0	0,9	-	1,3
5	1,2	0,6	2,0	1,3
6	-	-	-	-
7	2,0	0,9	1,2	1,4
8	1,6	-	2,4	1,3
9	-	-	-	-
10	-	1,6	3,0	1,5
11	1,5	0,6	2,4	1,5

Table 3. Percentage of area infected with damping off disease – artificial inoculation with *P.debarianum*

Variant No.	Replication			$\bar{X}$
	I	II	III	
1	3,2	4,8	6,4	4,8
2	7,7	11,0	9,9	9,5
3	0,6	1,8	3,2	1,9
4	3,0	9,0	2,4	4,8
5	4,2	7,0	0,6	3,9
6	-	-	-	-
7	-	-	-	-
8	0,6	0,9	-	0,5
9	-	-	-	-
10	0,9	0,9	1,2	1,0
11	0,9	1,2	1,2	1,1

Table 4. Intensity of damping off disease (Ist trial)

No.	Replication			$\bar{X}$
	I	II	III	
1	3,0	3,6	2,8	3,1
2	10.0	-	-	3.3
3	2.5	1.2	2.4	2.0
4	4.0	2.4	-	2.1
5	0.2	3.9	-	1.4
6	1,2	3,9	-	1,7
7	4,5	3,0	-	2,5
8	5,4	2,4	-	2,6

Table 5. Intensity of damping off disease (IInd trial)

No.	Replication			$\bar{X}$
	I	II	III	
1	12.0	6.4	40.0	19.5
2	40.0	37.5	27.0	34.8
3	14,0	7,0	8,2	9,7
4	12,0	1,6	1,6	5,1
5	11,2	4,0	3,6	0,6
6	3.0	10.4	4.8	6.1
7	2,7	2,0	0,9	1,9
8	1,5	8,4	19,6	9,8

The lowest intensity of the disease among variants was found in variant 5 - seed treated with 10% Exstrasol (1,4%) (Figure 1 and 2). In the second trial, the disease intensity was significantly higher than in the first trial, both in the checks and in the treated variants (Table 5). The best results were observed in variant 5, where the seed was treated with 10% Extrasol.

Figure 1. Damping off in variant with double treatment by 1% Exstrasol

Figure 2. Damping off in variant with seeds treated by 10% Exstrasol

Figure 3. Damping off in variant with seed treatment by 10% and soil treatment by 1% Exstrasol

Data presented in Table 4 and 5 show that the treatment of both soil and seed resulted in significantly higher disease intensity compared to the variants with seed treatment only (Figure 3).

Ahmadzadeh *et al*. (2004) reported that certain *Bacillus* species have the ability to inhibit bacterial and fungal root rot diseases in many crops. Such results were obtained in our investigations of damping off disease in seedlings. Our results on the impact of Extrasol on the intensity of damping off disease both in artificial inoculation and during natural infection are consistent with those of SRTI (1998). In their investigation, they came to a conclusion that two weeks after treatment of tobacco seedlings with Extrasol, the development of black root rot had stopped and seedlings obtained a color typical for the investigated variety. This product proved effective in blue mold control in field conditions. According to these trials, tobacco treatment with 2*l*/ha Extrasol improves the formation of root and tobacco assortment and reduces the infection from fungal and bacterial diseases both in seedlings and intransplanted tobacco. According to the investigation, in seedlings already treated with Extrasol, additional treatment will lead to poorer results and to occurrence of disease with even higher intensity. There fromit can be concluded that this phenomenon was influenced by the presence of amonification bacteria and the amount of nitrogen matters which the plant receives during treatments. Plant susceptibility to diseases largely depends on the optimal amounts of nitrogen. Also, various forms of nitrogen have different impact on plant and on its response to pathogens. Some diseases (foliar, wilting or root rot) are more serious in the presence of ammoniacal nitrogen, while others in nitrate nitrogen (Henn, 2004) According to Katan (2009), damping off disease in beans caused by *R. solani* is significantly more severe in plants receiving NH4 form of nitrogen than in plants receiving NO_3 (Katan, 2009). These results explain and confirm our findings on damping off disease in conditions of increased population of ammonification bacteria and the received form of nitrogen during their activity. Henn (2004) reported that organic matters allow better supply and retention of water, which sometimes increases their skofpathogens infection. Excess nitrogen promotes thinner and weaker cell walls, increased plant density, poor microclimate and low light conditions and it also delays maturity of plant tissues and therefore increases the risk of disease infection and development. The imbalanced N:K ratio affects both yield and disease resistance (Anonymous, 2015). There is an interaction between nitrogen and other nutrients. The uptake of phosphate is favoured by NH_4 nutrition, in comparison with NO_3 as the N source (Katan, 2009). A large number of data on the use of rhizobacteria as biocontrol agents refer to the increased uptake of phosphorus, which consequently makes the plants more vigorous and more resistant to pathogen invasion (Lioussanne, 2010). Yet, it is not due only to the P status. The effect of bacteria as biocontrol agents depends on the bacterial/fungal species ratio, availability of soil nutrients and other environmental conditions (Barea *et al*., 2005).

Conclusion

Application of *Bacillus subtillis* Ch13 has a reducing effect on the severity of damping off disease. In all variants where the

microbiological product Exstrasol was applied, the percentage of infected area was lower compared to that in the check. The best results are obtained in treatment of seeds with 10% and 20% solution of Exstrasol. Additional treatment of soil in variants where the seed have already been treated with the product has a negative effect and can increase the intensity of the disease. *Bacillus subtillis* Ch13 can be used in the control of damping off disease in tobacco seedlings, by choosing the appropriate mode of application. The most acceptable mode of application is the treatment of seed with 10% solution of a microbiological product Exstrasol. The microbiological preparation Exstrasol has good prospects in the tobacco seedlings protection.

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