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Biological Control of Early Blight on Potato Caused by *Alternaria Solani* by Some Bioagents

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***Abstract*—Early blight (*Alternaria solani*) is a potential disease of tomato that reduces its production globally both in conventional and tunnel cultivations. Due to variability in pathogenic isolates, prolonged active disease cycle phase and broad host range early blight is very difficult to manage. 8 microorganisms as a bioagent exhibiting inhibitory affects against *Alternaria solani,* were screened for their activity towards *A. solani* by a dual culture *in vitro* assay and in vivo (whole plant) test *in vitro* studies indicated that the microorganism's strains strongly inhibited the mycelial growth of the pathogen. The effect of microorganism's strains on the mycelial growth (mm) of the pathogen proved to be highest with *Trichoderma* sp. (0.55) followed by *Pseudomonas brassicacearum* (0.74) and *Pseudomonas jessenii* (0.81) on the high concentration (106 cells ml-1) compared to the control (2.30). On the other hand, *Bacillus mycoides* (2.14) in vivo studies 9 microorganism's strains were applied in two different application (foliar – soil) and two different varieties (Labella – Romano). The results showed significant reductions in the disease severity (%) with the treatment by *Trichoderma* sp (2%) followed by *Bacillus thuringiensis* (3%) and *Bacillus mycoides* (5%) compared with the control (46%) of Romano variety, while there were a less significant reductions in the disease severity (%) with the treatments compared with the control (16%) of *Labella* variety . The efficacy of antagonists to suppress the early blight disease varied in respect to the time and type of application.**

Keywords—Altrnaria solani, biocontrol, Eearly blight, microorganisms, potato

I. INTRODUCTION

Potatoes are one of the most important food and commodity crops in Russia and internationally. Potato plants are susceptible to a wide variety of diseases, including *Alternaria* diseases, which are globally ubiquitous. Synthetic pesticide control of the fungal pathogen *Alternaria solani* (the causal agent of *Alternaria* diseases) is not always efficacious, and there is a current trend to decrease the use of synthetic agrichemicals in crop production. If adequately understood, biological control of *Alternaria* diseases of potato could become a significant component of an integrated pest management system for this valuable crop. In the case of diseases caused by soil-borne plant pathogens, biological control (biocontrol) refers to either the introduction of organisms that are antagonistic to the pathogen or reduce its effects or to an increase in the density or activity of naturally occurring antagonistic organisms, resulting in a reduction of disease severity. Mechanisms of action which underpin biocontrol include either destruction of the pathogen directly (mechanisms include predation, mycoparasitism and/or production of antibiotic compounds), or excluding the pathogen through competition for resources, and the induction of host resistance [1].

The wide and indiscriminate use of chemical fungicides has been the cause of development of resistance among plant pathogens, leading to the occurrence of serious diseases. Due to this, there is an increasing interest to obtain alternative antimicrobial agents (biocontrol agents) for using in plant disease control systems.

Plant products of recognized antimicrobial spectrum could appear in food conservation systems as main antimicrobial compounds or as adjuvant to improve the action of other antimicrobial compounds. The development of such disease resistance to the pathogens and problems of environmental pollution due to excessive reliance on pesticides are the major causes today. Therefore, to avoid or minimize these problems.

Alternaria diseases appear usually as leaf spots and blights, but they may also cause damping – off of seedling, stem rots, and tuber and fruit rots. One of the most important diseases caused by *Alternaria* is the early blight of potato, early blight is a serious disease of tomato, it causes loss in yield is ranged from 5-78% [2]. In intensive agricultural production systems, protection against biological factors which adversely influence the efficiency of cultivation and the microbiological quality of crops as raw materials is of great significant. Owing to the emergence of sustainable agriculture, the protective methods available have been reassessed in the past decade.

The importance of using environment-friendly and food hygienically-safe plant-protecting methods, and plant protecting agents of biological origin, has been greatly emphasized. Although agro-chemicals are the most effective and immediate solution to most disease problems, might accelerate degradation of ecosystems and induce pathogens resistance. Moreover, persistent residues will causehealth problems to human beings. Biological control using microorganisms to suppress plant disease, offers a powerful alternative to the use of synthetic chemicals [3, 4].

The aim of this study was to identify strains of beneficial microorganisms with the capacity to suppress *Alternaria* diseases of potato, and generate information regarding their physical limitations and mode(s) of action. Failing the identification of such organisms.

II. EXPERIMENTAL

Isolation of microorganisms for biocontrol:

Samples were collected from the soil and Potato plant tissues. Soils were collected in August 2016 from fields in the three main potato producing regions of Saratov, Russia, Soil was stored at 8°C in plastic bins prior to experiments. Tubers were also collected from the same sites. Suspended soil samples were also each serially diluted to 1×10-5 g/mL in water agar [5]. Isolations from Potato plant tissues were same sites in Saratov. All tissue samples were each separately macerated in a few drops of sterile water and portions of non-heat treated macerated tissue were transferred to TSM for selection of Trichoderma spp. A sterile loop was used to spread macerated tissue fluid from each heat-treated sample onto nutrient agar, and from each non-heat treated sample onto Kings Medium 'B'.

In vitro bioassays.

Dual culture test was used to investigate the effect of 8 microorganisms on the inhibition of mycelial growth and spore germination of the pathogen *(Alternaria solani*). Two cylindrical pieces (0.9 mm) of agar colonized by the pathogens were placed on two edges of a Petri dish. Bacterial colonies of three-day old culture were streaked between the pathogen disks, one day before, one day after, or at the same time of pathogen culturing. To evaluate the effective concentration of microorganisms, 250 µL of 104, 105, 106, and 107 cells mL-1 of cells were distributed on culture medium. After one day of incubation in darkness at 21°C, one disk of individual pathogen was placed in the center of the plates.

The effect of microorganisms on spore germination was studied according to the method described by [6]. A drop of each isolate was deposited on dried clean glass slides as a film. A drop of the spore suspension of the pathogen was spread over this film. Control treatment was prepared as a film of sterilized distilled water. Percentage of spore germination was determined microscopically using 400 folds magnification.

In vivo bioassays.

For each microorganism tested for suppression of disease, the relationship between Optical Density (OD) of broth culture and concentration of colony forming units (CFUs) was determined prior to greenhouse assay initiation. The CFU concentration of the re-suspension was determined by plating aliquots of the serial dilutions onto NA, with subsequent colony counting once colonies formed, after incubation (25°C, darkness). The calculated CFU concentration for each dilution step was plotted against the recorded OD value, and linear regression was used to produce the formula describing the relationship between CFU concentration and OD. This was:

$x = (y+a)/b$, where x = CFU/ mL, y = OD and a and b are constants supplied by the regression.

Culture broths (100 mL) were prepared to be ready for the day of assay initiation. A 2 mL aliquot of each 100 mL of culture broth was centrifuged, the supernatant removed, and the bacterial pellet re-suspended in 2 mL of PPS and diluted 10-fold in PPS prior to OD reading. The pre-determined equation and OD value were then used to calculate CFU concentration of the broth culture, and the required volume dispensed, centrifuged, supernatant removed and pellet re-suspended in 50 mL of the selected application solution. For screen assays 1, 3 and 4, the bacterial application solution was 1:1 (v/v) LB:PPS. For screen 5, 6 and 7, PPS was used. A 10-fold serial dilution of the bacterial application solution was also made in PPS and aliquots plated onto NA so that colony counting after incubation (25°C, darkness) could be used to determine actual CFU concentrations

III. RESULTS AND DISCUSSION

1. Influence of microorganisms on myclial growth of *Alternaria solani*

1.1. Influence of application time of microorganisms on myclial growth *Alternaria solani*

To evaluate the efficacy of microorganisms against *A. solani in vitro*, dual culture test was used. Microorganism's colonies were streaked between two agar pieces colonized with the pathogen one day before or one day after or at the same time of pathogen presence. The results of application time of microorganism's strains on pathogen growth have been summarized in Table I.

Applying the microorganisms at different times resulted in significant reduction of the mycelial growth compared to untreated culture media. The effect was more pronounced in application of microorganism's strains before the pathogen culture and reduced after grown the mycelia of the pathogen on the media with no significant difference between the two strains in mycelia growth inhibition. The pathogen culture media treated by *Pseudomonas jessenii*, *Trichoderma* sp., Bacillus thuringiensis resulted in a significant reduction in the myclial growth compared to untreated culture media.

TABLE I. INFLUENCE OF APPLICATION TIME OF MICROORGANISMS ON PATHOGEN MYCELIAL GROWTH USING DUAL CULTURE TEST

Application	Application time (day) *	Pathogen mycelial growth (mm)
Flavobacterium sp.	Before	2.13 a
	After	1.45 b
	Same	1.66 b
Pseudomonas mohnii	Before	1.24 c
	After	2.32 a
	Same	1.88 b
Pseudomonas jessenii	Before	0.24 d
	After	0.70 d
	Same	0.48
Trichoderma sp.	Before	0.48 d
	After	1.22 c
	Same	1.04 c
Endospore bacterium	Before	1.67 b
	After	2.22 a
	Same	2.09 a
Bacillus thuringiensis	Before	0.64 d
	After	1.34 b
	Same	1.29 c
Bacillus mycoides	Before	0.98 c
	After	2.11 a
	Same	1.55 b
Pseudomonas brassicacearum	Before	2.46 a
	After	1.07 c
	Same	1.48 b
Control (water)	-	2.80 a

*Application time: placing the bacterial colonies one day before, after, or at the same time of pathogen culture. (Line for individual microorganism marked with a common letter do not differ statistically using Duncan`s Multiple Range Test at P≤ 0.05; n=4).

1.2. Influence of inoculum density of microorganisms on myclial growth *of* Alternaria *solanion*

The affectivity of microorganism's strains against the mycelium growth of the pathogen was investigated using different concentrations ranged between 104-106 cells mL^{-1}. Generally, the microorganism's strains strongly inhibited the mycelial growth of the pathogen (Table II).

Increasing high concentrations of microorganism's strains intensified this inhibition. The effect of microorganism's strains on the growth of the pathogen proved to be highest with *Trichoderma* sp. followed by *Pseudomonas brassicacearum* and *Pseudomonas jessenii*. On the other hand, *Bacillus mycoides* had a week effect in inhibition the mycelium growth of the pathogen.

1.3. Influence of microorganisms on spore germination of *Alternaria solani*

The efficacy of microorganisms against spore germination of *Alternaria solani* on glass surfaces was studied according to the method described by [6]. The results showed that the inhibitory effect varied according to the microorganism and the strongest effect was against spore germination of *Bacillus thuringiensis* followed by *Trichoderma* sp. and *Pseudomonas jessenii* in descending order (Fig. 1).

In comparing with the control, the reduction was not significant against the pathogen with the treatment by Endospore bacterium, was approximately 30% inhibition of spore germination.

TABLE II. INFLUENCE OF MICROORGANISMS ON MYCLIAL GROWTH OF *ALTERNARIA SOLANI* ON DEPENDING ON DIFFERENT CONCENTARATIONS OF MICROORGANISMS (CELLS mL^{-1}) APPLIED ONE DAY BEFORE PLACING THE PATHOGEN DISK

Application	Concentration Cells mL-1	pathogen mycelial growth (mm)
Flavobacterium sp.	10^4	1.23 c
	10^5	1.51 b
	10^6	1.22 c
Pseudomonas mohnii	10^4	1.88 b
	10^5	0.97 c
	10^6	1.03 c
Pseudomonas jessenii	10^4	1.46 b
	10^5	1.78 b
	10^6	0.81 d
Trichoderma sp.	10^4	1.45 b
	10^5	0.97 c
	10^6	0.55 d
Endospore bacterium	10^4	2.06 a
	10^5	1.14 c
	10^6	0.85 d
Bacillus thuringiensis	10^4	1.19 c
	10^5	1.17 c
	10^6	0.91 c
Bacillus mycoides	10^4	2.14 a
	10^5	1.78 b
	10^6	1.98 a
Pseudomonas brassicacearum	10^4	1.98 a
	10^5	1.06 c
	10^6	0.74 d
Control (water)	-	2.30 a

Column for individual pathogen marked with a common letter do not differ statistically using Duncan`s Multiple Range Test at P≤ 0.05; n=4..

The use of beneficial microorganisms in agriculture and other distorted ecosystems can help to protect crops against phytopathogens. It is known that microorganisms associated with plants can promote their growth and development, e.g. due to the growth inhibition of phytopathogenic microorganism. Isolation of new antagonistic strains is necessary to improve biological control methods and restrain plant diseases [7, 8].

The *in vitro* antibiosis test showed that the microorganism's strains presented direct activity against the pathogen *A. solani*, inhibiting their growth. These results confirmed that the antagonists produce some type of toxic substance with antimicrobial effect against the pathogen, causing the antibiosis phenomenon. Possibly, these substances are bioactive compounds derived from lipopeptides of the surfactin, iturin and fengycin families, frequently reported as toxic to pathogens [9, 10].

The iturins and fengycins exhibit a strong antifungal activity and are inhibitory for the growth of a wide range of pathogens. Bacterial bio-control agents belonging to the genera *Agrobacterium*, *Bacillus*, *Pseudomonas*, and *Streptomyces*, have been found by observing zones of inhibition in Petri plates [11].

That several bacteria such as B. subtilis (IK-83, IK-92 and IK159), B. cereus-GC subgroup A (IK-34) and B. amyloliquefaciens (IK-104) were very effective against A. solani *in vitro* tests [12, 13].

2. Influence of different applications of microorganism's strains on disease severity

To identify the activity of microorganism's strains under greenhouse conditions two different applications (foliar – soil) were applied on two potato varieties (Romano – Labella) before and after inoculation of the pathogens. The results showed significant reductions in disease severity when the agents were applied prior to inoculation (protective effect).On Romano variety; the soil application was more significant than the foliar application in the treatment with *Trichoderma* sp., while foliar application was more significant than the soil application in the treatment with *Bacillus thuringiensis.*

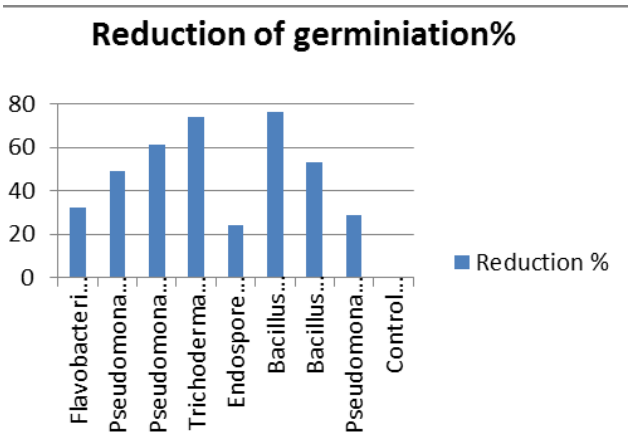

Fig. 1. Inhibitory effects of microorganisms on spore germination of *Alternaria solani* on glass surface.

The suppression was more pronounced with Bacillus mycoides, no significant reduction on the disease severity in the treatment with *Flavobacterium* sp. and *Endospore* bacterium compared with the untreated plants. On the other hand the resistance variety (Labella) had a low disease severity in the untreated plants compared with the susceptible variety (Romano). The efficacy of antagonists to suppress the early blight disease varied in respect to the time and type of application (Table III).

In vitro screening of microbes as candidates for biological control of plant pathogenic *Alternaria solani* is commonly performed in a hierarchical manner. Because each isolate possessed both growth promotion and anti-fungal characteristics; it was difficult to determine which mechanism was the more important for biological control.

The present selection of isolates for in vivo screening based on potential plant growth promotion, antibiotic/inhibitory compound production, physical competition with *Alternaria solani*, with some overlap between these categories, allows for future analysis of which of these factors, or combination of factors, is the most reliable indicator of an isolate's capacity to reduce the severity of *Alternaria* diseases of potato. *Trichoderma* strains are always associated with plant roots and root ecosystems.

Some authors have defined *Trichoderma* strains as plant symbiont opportunistic avirulent organisms, able to colonize plant roots by mechanisms similar to those of mycorrhizal fungi and to produce compounds that stimulate growth and plant defense mechanisms [14].

The *Trichoderma* isolates induced protection of tomato plants against *A. solani* from 30.69 to 95.23% [15]. *T. asperellum* T-203 conferred 80% protection in cucumber seedlings to *P. syringae* pv. Lachrymans when applied to roots 5 days before inoculation with the leaf pathogen, and induced on leaves the production of antifungal compounds [16].

IV. CONCLUSION

This study confirms *in vitro* and *in vivo.* We have isolated 8 micoorganisms that differ in their antagonistic activity against *Alternaria solani*. *In vitro Trichoderma* sp., *Bacillus thuringiensis* and *Pseudomonas* jessenii have shown strong reducing the growth of *A. solani*. While Trichoderma sp. and *Bacillus thuringiensis* were able to protect potato plants from *A. solani in vivo*.

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TABLE III. INFLUENCE OF DIFFERENT APPLICATION (FOLIAR - SOIL) FOLIAR APPLICATION BY MICROORGANISMS STRAINS ON DISEASE SEVERITY OF EARLY BLIGHT 7 DAYS POST INOCULATION OF POTATO PLANTS. THE PRODUCTS WERE APPLIED BEFORE OR POST PATHOGEN INOCULATION

Application	**Applied concn (spores or CFU /g)**	**Application time (day) ***	**Disease severity (%)**			
			Foliar application		***Soil application***	
			Romano	***Labella***	***Romano***	***Labella***
Flavobacterium sp.	1.31×10^6	Before	22 bc	22 bc	16 cd	15 cd
		After	38 ab	21 bc	24 bc	16 cd
Pseudomonas mohnii	1.63×10^6	Before	19 cd	11 d	22 bc	8 de
		After	15 cd	9 d	27 ab	12 d
Pseudomonas jessenii	2.03×10^6	Before	9 d	16 cd	12 d	15 cd
		After	17 cd	17 cd	17 cd	17 cd
Trichoderma sp.	1.55×10^5	Before	9 d	2 f	2 f	3 f
		After	13 d	5 e	6 e	6 e
Endospore bacterium	1.00×10^6	Before	31 b	17 cd	19 cd	16 cd
		After	16 cd	25 bc	24 bc	21 bc
Bacillus thuringiensis	3.08×10^6	Before	3 f	3 f	10 d	2 f
		After	9 d	3 f	16 cd	2 f
Bacillus mycoides	1.74×10^5	Before	5 e	8 de	6 e	6 e
		After	12 d	3 f	11 d	2 f
Pseudomonas brassicacearum	2.11×10^5	Before	11 d	9 d	6 e	9 d
		After	18 cd	15 cd	14 d	16 cd
Control (water)	-	-	46 a	16 cd	46 a	16 cd

Line for individual microorganism marked with a common letter do not differ statistically using Duncan`s Multiple Range Test at $P\leq 0.05$; n=4).

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