MICROBIOLOGICAL MEANS OF PLANT PROTECTION, SUSTAINABLE ALTERNATIVE AT CHEMICALS

MIJLOACE MICROBIOLOGICE DE PROTECTIA PLANTELOR, ALTERNATIVĂ DURABILĂ LA PRODUSELE CHIMICE

CONSTANTINESCU Florica[1], SICUIA Oana[1], DINU Sorina[1]
e-mail: cflori@yahoo.com

Abstract: *Large use of pesticides triggered several negative effects including harmful agents resistance to active ingredients. The researches focused on selection and formulation of some bacterial strains with beneficial qualities for crops and fungicides reduction in „damping –off" soil borne fungi control in vegetables. Selection of the strains was based on their antagonistic activity in vitro, capacity to produce enzimes, like cellulase, amylase and lactonase, swimming and swarming mobility and in vivo efficacy against targeted phytopathogens. The strains were formulated as retard microorganisms release granules and microemulsion. The results showed good efficacy of the bioproducts (65-90%) in controlling the diseases.*
Key words: biological control, soil borne fungi, useful microorganisms, biopesticides.

Rezumat: *Utilizarea pe scară largă a pesticidelor a determinat numeroase efecte negative, inclusiv apariţia unor agenţi de dăunare problemă. Cercetările efectuate au urmărit selecţia şi formularea unor tulpini bacteriene cu calităţi benefice pentru plantele cultivate şi reducerea utilizării fungicidelor în combaterea ciupercilor fitopatogene de sol care determină căderea răsadurilor de legume. Selecţia s-a realizat pe baza activităţii antagoniste in vitro, a capacităţii de a produce enzime, cum ar fi celulaza, amilaza şi lactonaza, a mobilităţii de migrare şi de agregare şi a eficacităţii de combatere a fitopatogenilor in vivo. Tulpinile au fost formulate sub formă de granule cu eliberare treptată a microorganismelor şi sub formă de microemulsie. Rezultatele au evidenţiat o eficacitate de combatere a fitopatogenilor studiati intre 65-90%.*
Cuvinte cheie: combatere biologică, ciuperci fitopatogene de sol, microorganisme utile, biopesticide.

INTRODUCTION

Intensive use of chemicals and the increasing number of treatments for crop plants diseases and pest control lead to occurrence of negative effects in ecosystems, like groundwater pollution and pathogens resistance. In addition, the price of pesticides and the demand of consumers for healthy food, free of toxicresidues, stimulated in the last decades the researches on alternative control means, such as biological control, enabling sustainable use of resources and reduction of chemicals use in agriculture (Cook, 1983).

[1] Research Development Institute for Plant Protection, Bucharest, Romania

Soil borne phytopathogenic fungi which cause seedlings "damping off" in favourable temperature (20-25^{0}C) and humidity (>70%) conditions, can generate important vegetable seedlings loss (40-50%) mainly in greenhouses but also in field conditions.

The aim of this study was to isolate, characterize and select bacterial strains with biological control qualities and to formulate the biomass as biopreparations for soil borne phytopathogenic fungi control in vegetable crops.

MATERIAL AND METHOD

Isolation and selection of microorganisms - Microorganisms isolation consisted in removal of 1 g rhizosphere from each vegetal sample followed by grinding it in 1 ml sterile distilled water and 100 µl from the suspension were distributed on Luria Bertani Agar medium in Petri dishes and incubated at 28^{0}C for 24 hours. Isolated colonies were purified by usual microbiological techniques on Nutrient Agar medium.

Microorganisms selection was based on (i) antagonistic activity *in vitro*, (ii) enzymes production as cellulase, amylase and lactonase, (iii) swimming and swarming motility, (iv) *in vivo* test of the microorganisms efficacy on controlling the studied phytopathogens.

The *in vitro* antagonistic activity of the strains was tested by double culture method. The phytopathogenic fungi *R. solani*, *P. debarianum* and *F. oxisporum f. sp. radicis-lycopersici* were grown on PDA (potato-dextrose-agar) and CDA (Czapek-Dox agar). Briefly, the test consisted in placing in the middle of the Petri dishes, with fresh PDA medium, qualibrated micelium discs (5 mm diameter) and at 2 cm on both sides the bacterial strains were streaked. The plates were incubated at 28^{0}C. Each variant had 3 repetitions. The plates were analized for fungal growth inhibition at 24, 48 and 72 hours.

Cellulase activity was determined by the breakdown of the substrate carboxy-methyl cellulose (CMC). For this, the strains were grown on medium suplimented with 1% CMC and incubated at 28^{0}C for 5 days. Cellulase activity was revealed by floading the plates with 0,3% Congo Red for 30 minutes, subsequently rinsed with tap water and the dye was fixed by incubation with a 10% acetic acid solution for 15 min. at 28^{0}C.

Amylase production was cheked by streaking the bacterial strains on Nutrient Agar medium + 0,4% soluble starch. Plates were incubated at 28ºC for 48 -72 hours, after that were flooded with iodine solution in potassium iodine. Iodine will react with starch, and form a complex colored in dark blue. Clear areas around bacterial growth after adding iodine solution will indicate the decomposition of starch in the medium and therefore the production of amylase.

For the lactonase production test, the bacterial strains were inoculated in 2 ml of Luria Bertani (LB) broth medium containing 5 µM of C6-hexanoyl homoserine lactone (C6-HHL) and grown overnight at 28^{0}C and 150 rpm. As a negative control (to see if the media could cause lactolysis) the same media without bacteria was also incubated under these conditions. After 12 hours, Petri dishes with LBA medium containing 50 µg/ml kanamycin was overlaid with *Chromobacterium violaceum* CV026 (McClean *et al*., 1997). Wells were punctured into the plate (5 mm ø) and filled with a 100 µl of bacterial culture. The plates were incubated at 28^{0}C and scored for the presence or absence of purple halos. Absence of purple halos indicates that all of the C6-HHL was degraded.

Swimming and swarming motility of the strains was tested on LB agar medium containing 0,3% (swimming) or 0,5% (swarming) agar. Each plate was

toothpick inoculated and scored for swimming and swarming motility after 18 h incubation at 28^0C. A negative control, a non-motile derivative of *Pseudomonas putida* strain PCL1760 (Validov et. al., 2007) was used to check the results.

Conditioning of the selected bacterial strains – Two types of biomass formulation was experimented: sodium alginate beads and microemulsion.

For the ***granular*** formulation the following recipe was used: broth bacterial culture, saline phosfate buffer (PBS), sodium alginate 2%, calcium chloride 2% and sterile saline solution 0,8%.

The strains were refreshed on LBA medium by ooze epuization technique. After 24 hours incubation at 28^0C single colonies from each strain were used to inoculate 100 ml LB broth / Erlenmayer flask. The liquid cultures were incubated for 48 hours at 28^0C and 150 rpm. In order to separate the biomass from the media the cultures were spin at 3000xg for 20 min. at 10^0C. The pellet was washed with PBS and resuspended to a final concentration of 10^8 cfu/ml. Twenty ml of 10^8 cfu/ml bacterial suspension was mixed with 2% sodium alginate and homogenized for 15 min. follow by dripping it in 2% $CaCl_2$ solution.

For the ***microemulsion*** formulation the following recipe was used: spores biomass from the Gram positive strains, sucrose, Soprofor FL, tristirilphenol phosphate ethoxylated neutralized with triethanolamine, emulsifier CL3, C_{12-14} etoxylated with 3 moles of ethylenoxid; poliethilenglycol 400, carboximetilcellulose 5%, sodium benzoate. In a first stage the organic phase was prepared by mixing 30g solvent, 9,6 g Soprofor FL and 2,4 g emulsifier CL3. In the same time, the aqueous solution was prepared by mixing 10 g of bacterial biomass, 5 g sucrose, 0,2 g sodium benzoate, 3 g poliethilenglycol, 5 g carboximetilcellulose 5% and ~ 80% of the amount of water required. The microemulsion was achieved by adding the organic phase gradually over the aqueous phase and continue stirring. This resulted in 100 ml microemulsion biopreparation.

In vivo test of the biopreparations – The biopreparations were tested in *in vivo*, in growth chamber conditions, in order to establish the efficacy in *R. solani*, *P. debarianum* and *F. oxisporum f. sp. radicis-lycopersici* control in tomato and cucumber crops.

In vivo test of the sodium alginate granular biopreparations against *Fusarium oxysporum* f. sp. *radicis lycopersici* in tomato crop: *Fusarium oxysporum* f. sp. *radicis lycopersici* (*Forl)* inoculum, strain ZUM 2407 (IPO-DLO, Wageningen) was refreshed on PDA medium (Difco). After 4-5 days grown at 28^0C, the mycelium was cutted in small pieces and used to inoculate 200 ml Czapek-Dox medium follow by incubation at 28^0C and 150 rpm. for 4-5 days. The spores were separated from mycelium by filtration through a sterile cloth.

Tomato seeds (Heintz 2274 variety) were sown in sterile soil (universal peat "FLORIMO") sterilized by irradiation at 25Kgrey, infected with *Forl* at 2 x 10^6 spores/ kg soil. Each variat had 3 repetitions and a total of 30 plants. The growth chamber was setted at 21–24^0C and 70% relative humidity.

The experiment consisted in 7 variants as follow: V1-OS17, V2-OS15, V3-Usa2, V4-Salc2, V6- chemical control TOPSIN®500sc 0,25%, V7- negative control (infected, nontreated) and V8– positive control.

The biopreparations were distributed in soil at sowing. After 4 weeks, the roots and the crown of each tomato plant was analized for tipical crown and root rot symptoms and the attack degree and the treatments efficacy was calculated.

In vivo test of the biopreparations formulated as microemulsion against *R. solani* – DSM 63002 and *P. debarianum* DSM 62946 in tomato and cucumber crops: The fungal inoculum was obtained on double sterilized oat seeds (1 atm., 20 min.) distributed in Roux plates. These were uniformelly inoculated with fresh mycelium and

incubated for 5 days at 27^{0}C. Tomato (Heintz 2274 variety) and cucumber (Cornichon variety) sterile seeds were treated by immersion for 15-20 min. in the microemulsions.

Universal peat "FLORIMO" soil, sterilized by irradiation, was inoculated with the pathogenic fungi at 1:9 (v/v). The experiment had 7 variants, similar to those decribed above. After 4 weeks, the experiments were assessed on the efficacy of the applied biological treatments.

RESULTS AND DISCUSSIONS

Of all vegetal samples, from wild and cultivated plants, 74 bacterial isolates from rhizosphere were subjected to selection in order to obtain biopreparations useful for soil borne phytopathogenic fungi control. Of these, 5 strains with biological control agents' qualities were selected for formulation. The selected strains are presented in table 1.

Table 1

Taxonomy and the provenience of the selected bacterial strains

Isolate code	Taxonomy (BIOLOG system)	Provenience/Strain characteristics
Us.a2	*Bacillus subtilis*	Garlic rhizosphere, antagonistic activity *in vitro* against several phytopatogenic soil borne fungi.
98a	*Bacillus subtilis*	Wheat rhizosphere, antagonistic activity *in vitro* against several phytopatogenic soil borne fungi.
OS.17	*Bacillus subtilis*	Onion rhizosphere, antagonistic activity *in vitro* against several phytopatogenic soil borne fungi.
OS.15	*Bacillus subtilis*	Onion rhizosphere, antagonistic activity *in vitro* against several phytopatogenic soil borne fungi.
salc2	*Pseudomonas chlororaphis*	Lettuce rhizosphere, antagonistic activity *in vitro* against several phytopatogenic soil borne fungi.

Of the 5 bacterial strains, the highest inhibition zone *in vitro* against *Forl*, which cause the foot and root rot of tomato plants, were achieved in the variant of *B. subtilis* Usa2 and *P. chlororaphis* salc2. Strain Usa2 induced the highest inhibition zones (9 mm) against *R. solani* being followed by the strains OS.17 and salc2 (7 mm). Against *P. debarianum* the most significant inhibition zone was noticed in the *P. chlororaphis* salc2 variant (7 mm).

Table 2

Biological characteristics of the selected bacterial strains

Isolate code	*In vitro* antagonistic activity after 48 hours incubation at 28^{0}C (mm)			Strains motility		Enzymes production		
	*Forl**	*Rs**	*Pdb**	swimming	swarming	cellulase	amylase	lactonase
Us.a2	8	9	6	+	+	+	+	+
98a	5	5	6	+	+	+	+	+
OS.17	5	7	5	+	+	+	+	+
OS.15	6	6	5	+	+	+	+	+
salc2	8	7	7	+	-	-	+	-

Legend: *- *Forl= Fusarium oxysporum* f. sp. *radicis-lycopersici*; *Rs= Rhizoctonia solani* ; *Pdb= Pythium debarianum*

Selected *B. subtilis* strains showed both swimming and swarming motility and produced cellulase, amylase and lactonase (table 2).

Table 3

Granular biopreparations efficacy against *F.oxysporum* f. sp. *radicis lycopersici* in tomato crop

Variants	Efficacy % (ABOTT)
V1- Usa2	71
V2- 98a	57
V3- OS.15	75
V4- OS.17	79
V5- salc2	71
V6- Chemical control- Topsin 500SC 0,25%	96
V7- Negative control - *Forl*	0
V8- Positive control	100

In vivo test of the granular biopreparations against *F.oxysporum* f. sp. *radicis lycopersici* in tomato crop, highlighted the variant of OS.17 biopreparation were it was registered the highest disease control efficacy (79%). This was followed by the variant treated with the OS.15 biopreparation (75%) (table 3).

Table 4

Efficacy of the microemulsions biopreparations against *R. solani* in tomato and cucumber crops

Variants	Efficacy % (ABOTT)	
	tomato	cucumber
V1- Usa2	76	80
V2- 98a	60	65
V3- OS.15	81	78
V4- OS.17	76	85
V5- salc2	68	54
V6- Chemical control- Topsin 500SC 0,25%	96	98
V7- Negative control – *R. solani*	0	0
V8- Positive control	100	100

From the microemulsion biopreparations, against *R. solani* in tomato and cucumber crops, the variant treated with the biopreparation based on *B. subtilis* OS.15 showed the highest efficacy (81%) in tomato crop and in cucumber crop the highest efficacy was obtained in the variant treated with the biopreparation based on *B. subtilis* OS.17 (85%) followed by Usa2 (80%) (table 4).

The test of microemulsion biopreparations against *P. debarianum* highlighted the variant treated with the *P. chlororaphis* salc2 microemulsion (75%) in tomato, and in cucumber the variant treated with the *B. subtilis* OS.15 microemulsion (70%) (table 5).

Table 5

Efficacy of the microemulsions biopreparations against *P. debarianum* in tomato and cucumber crops

Variants	Efficacy % (ABOTT)	
	tomato	cucumber
V1- Usa2	65	33
V2- 98a	54	37
V3- OS.15	68	70
V4- OS.17	48	41
V5- salc2	75	63
V6- Chemical control- Topsin 500SC 0,25%	98	96
V7- Negative control – *P. debarianum*	0	0
V8- Positive control	100	100

CONCLUSIONS

1. The 5 bacterial strains selected on their biological traits, belonged to *B. subtilis* and *P. chlororaphis* species.

2. Granular and microemulsion biopreparations significantly protected the tomato and cucumber plants against *F.oxysporum* f. sp. *radicis lycopersici*, *R. solani* and *P. debarianum*, in some experimental variants being insignificant differences between the chemical control and the biological treatments.

3. Useful microorganisms included in the two types of formulations preserved their biological qualities during the *in vivo* trials, which indicate the possibility of their use in ecological agricultural systems.

REFERENCES

1. **Cook R., Baker K.F., 1983 -** *The Nature and Practice of Biological Control of Plant Pahtogens,* American Phytopathological Society, St. Paul, Minnesota
2. **McClean K.H., Winson M.K., Fish L. et al., 1997** - *Quorum sensing and Chromobacterium violaceum: exploitation of violacein production and inhibition for the detection of N-acyl homoserine lactones*, Microbiology, vol. 143, p. 3703–3711.
3. **Validov S., Kamilova F., Lugtenberg B., 2009** - *Pseudomonas putida strain PCL1760 controls tomato foot and root rot in stonewool under industrial conditions in a certified greenhouse*, Biological Control, vol. 48, Issue 1, p. 6-11.