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IN VITRO COMPATIBILITY BETWEEN CHEMICAL AND BIOLOGICAL PRODUCTS USED FOR SEED TREATMENT

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Abstract

The study reveals the possibility to use simultaneously biological and chemical control products as efficient and environmental friendly seed treatment for pest and disease control, which could provide a decrease of the chemical dose needed for plant protection. This phytosanitary strategy promotes microbial strain from Beauveria, Bacillus and Pseudomonas genera that can be used together with some chemical products for plant protection in order to decrease the amount of chemical product per unit area. Therefore, two chemical insecto-fungicides were selected based on the ecotoxicological risk assessment and legislation related to plant protection products (Regulation (EC) No 1107/2009 and Directive 2009/128/EC). The compatibility studies between the bio-control microorganisms and chemical pesticides were based on the microbial strains interaction with the active substance from the chemical insecticide and insecto-fungicides (imidacloprid 600g/l and mix of imidacloprid 460 g/l with thiram 176 g/l, respectively). The viability of the microbial strains was studied under laboratory conditions. The entomopathogenic fungi Beauveria bassiana (Bals.) Vuill. exhibit good compatibility with imidacloprid insecticide (600 g/L a.s. in comercial product), which does not affects the biological parameters of the beneficial microorganism. However, B. bassiana exhibits high sensitivity towards insecto-fungicide mixture, which inhibits completely the spore germination at the recommended concentrations. The results on the bacterial compatibility with the chemical phytosanitary products (at different concentration) did not inhibit completely the bacterial growth. The insecticide based on imidacloprid 600 g/l, did not affect the growth of any Bacillus biocontrol strains, when it was tested at 20% concentrations. Only the insecto-fungicides mixture, in 20% concentration, caused moderate growth inhibition (less than 5 mm) to some of the bacterial strains tested.

Key words: *pesticide, microbial compatibility.*

INTRODUCTION

The new plant protection products must fulfill increasingly higher efficacy with minimal environment impact in order to satisfy both farmers and consumers. A major requirement for every company producing chemical or biological pesticides is to ensure plant protection products for plant health without any environmental risks. Before pesticide registration, every plant protection product is thoroughly tested in greenhouses and in the field conditions in order to establish their efficacy, according to the Good Experimental Practices and OECD guidelines. They are also ecotoxicologically evaluated, according to Good Laboratory Practices and Regulation no. 440/2008, in order to establish their evolution in soil, water and air, and their effects on beneficial flora and fauna.

Ensuring food quality, human health and environmental protection are key considerations to develop the compatibility studies between chemical and biological pesticides. Compatible phytosanitary products are useful for the sustainable pest and disease management.

Given the importance of successful implementation of chemical pesticides with biological control agents in the Integrated Pest Management programs, there have been recently published results regarding the influence of common pesticides on the biological parameters of useful microorganisms (Rajanikanth et al., 2010; Ramazan-Asl. et al., 2010; Golshan et al., 2013). There were also developed *in vitro* testing protocols for compatibility evaluation between the entomopathogenic fungus *Beauveria bassiana* and different phytosanitary products (Da Silva and Neves, 2005). Results on the biocontrol

bacteria and their compatibility with several chemical pesticides, such as captan, thiram, mancozeb, carbendazim, nemacur and azoxystrobin, were also mentioned in several studies (Frances et al., 2002; Omar et al., 2006; Mohiddin and Khan, 2013; Ahila Devi and Prakasam, 2013).

MATERIALS AND METHODS

Microbial strains

Six bacterial biocontrol strains, *Bacillus subtilis* 98a and Us.a2, *Bacillus amyloliquefaciens* OS17 and BW, *Bacillus pumilus* OS15 and *Pseudomonas chlororaphis* ssp. *aurantiaca* Sal.c2 were used in this study. These strains were previously identified as biocontrol bacteria that could suppress different soil borne phytopathogenic fungi such as *Rhizoctonia solani* (Sicuia et al., 2012), *Fusarium oxysporum* (Constantinescu et al., 2010) *Sclerotium bataticola* (Dinu et al., 2012) or *Pythium debaryanum* (Constantinescu, 2000). Routinely, these strains were grown on Luria Bertani agar medium at 28ºC.

The enthomopathogenic fungi used in the study was *Beauveria bassiana* Bals.(Vuill.) ICDPP#1 strain from the Enthomopathogenic Fungi Collection of the Research-Development Institute for Plant Protection, Bucharest - Department of Useful Organisms, also deposited in NCAIM, Budapesta, with the accession number (P) F001353. This strain was routinely grown on potato-dextrose-agar (PDA) medium, at 26ºC.

Chemical pesticides

Taking into consideration the legislation for placing plant protection products on the market and the sustainable use of pesticides, two chemical products for seed treatment were selected to be used in this study. These pesticides were imidacloprid 600 g/l, a systemic insecticide, and an insecto-fungicide mixture of imidacloprid 460 g/l with thiram 176 g/l.

Fungal compatibility evaluation with pesticides

Beauveria bassiana was multiplied on PDA medium using conidia from pure mature cultures. After 14 days of incubation at 25±1ºC, in the dark, the fungal material was suspended in sterile distilled water supplemented with 0.01% Tween 80 emulsifier.

The pesticides were tested in three concentrations: field recommended concentration (FR), 80% of FR and 120% of FR. These tested concentrations were noted as c1 = 120% FR, c2 = FR (using 800µl pesticide +200 µl pure water), c3 = 80% FR.

In order to test the influence on fungal viability, the pre-established concentrations were mixed in aqueous conidial suspensions and left to rest for one hour. After demarcation of three areas on the surface, disinfected microscope slides were placed in Petri dishes; humidity was maintained by filter paper moistened with distilled water. Each slide was covered with ~ 4 ml PDA medium (Sigma, Fluka) and one drop of the conidial suspension- test pesticide mix was placed in each demarcated area. Three repetitions were experienced for each concentration. Quantification of viability (germination %) was made after germination which was stopped by dripping a lactophenol-blue cotton solution after 18 hours of incubation (at 25±1º C, in darkness). Germination analysis was performed by light microscopy.

To express the percentage of germination, 200 germinated and non-germinated conidia were observed from three microscopic fields. Results were used to calculate the percentage of germination inhibition using the following formula:

$$\text{Inhibition of germination (\%)} = \\ = (G\%C - G\%var) / G\%C \times 100$$

where: G% C is the germination percentage in the control, and G%var is the germination percentage in experimental variants.

Vegetative growth and sporulation tests were assessed only for treatments with at least 60% conidia viability. The pesticides were individually tested for antimicrobial activity using the impregnated discs method.

PDA medium (Fluka) was poured into sterile Petri dishes. After solidification, 0.5 ml of fresh conidial suspension were inoculated and dispersed with a Drigalski spatula (Figure 1).

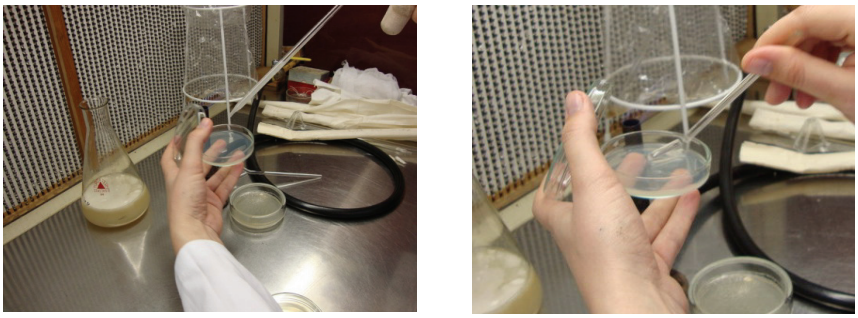


Figure 1. *Beauveria bassiana* test cultures preparation

Separately, sterile filter paper discs (6 mm Ø) were individually impregnated with 50µl of test pesticides and placed on the surface of the culture medium respecting the distance of 15 mm between disc and the edge of the plate and 30 mm between discs (Figure 2). Plates were incubated at 26°C.

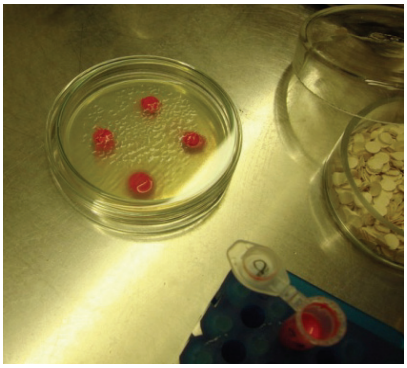


Figure 2. Testing pesticides towards *Beauveria bassiana* growth

Vegetative growth was evaluated at 10 days after fungal inoculation by measuring the inhibition area. The fungus sensitivity was assessed as follows: very sensitive if the inhibition zone diameter was greater than 2 mm, sensitive if the inhibition was between 2-4 mm and resistant when the inhibition zone was smaller than 2 mm or even absent.

To quantify the spores production, three discs per Petri dish containing sporulated mycelia were cut, using a glass tube (d = 7 mm). These were distributed in test tubes with 10 ml sterile distilled water and Tween 80 (0.01%), and then homogenized until conidia were separated completely from the surface of the media. The resulting suspension was appropriately diluted to be counted using a Burker chamber. Two readings (24 squares) for each repetition were performed and their average was used for statistical analysis.

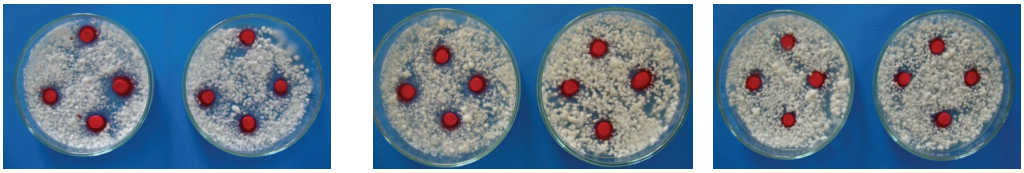
Bacterial compatibility evaluation with pesticides

The bacterial strains were multiplied in LB broth, and incubated for 48 hours at 28°C with 150 rpm rotary shaking. The bacterial cultures were than centrifuged at 3750 rpm for 20min at 10°C, in order to eliminate the supernatant and harvest the bacterial cells. The bacterial sediment was resuspended in sterile phosphate buffer, in order to prepare a suspension of 10^8 cfu/ml concentration. The pesticides were tested in four concentrations: 100%, 80%, 60% and 20% commercial pesticide. The compatibility evaluation between the chemicals and bacterial strains was studied *in vitro*. Petri dishes with LB-agar were inoculated with 100µl bacterial suspension, uniformly distributed on the surface of the medium using a Drigalski spatula. Subsequently, Whatmann paper plugs of 6 mm in diameter were moist with 50µl pesticide, at the mentioned concentration, and placed on the surface of bacteria inoculated plates. Control plates were similarly prepared, using sterile distilled water for paper plugs moistening. All plates were incubated at 28°C for 72h and than analyzed in order to evaluate the inhibition of bacterial growth. Bacterial strain sensitivity to pesticides was appreciated using the following index: 0 - for no bacterial growth, 1 - for inhibition zone greater than 5 mm, 2 - for inhibition zone less than 5mm, and 3 - when no inhibition hallo was present and the bacterial growth was not influenced by the pesticide.

RESULTS AND DISCUSSIONS

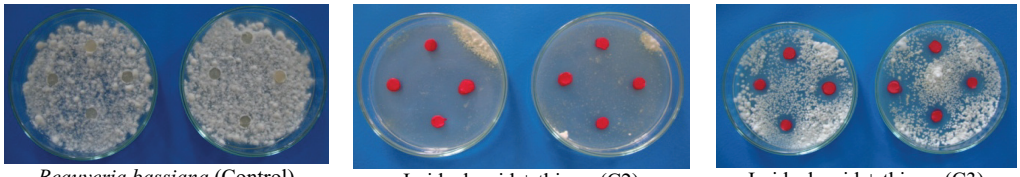
In vitro compatibility between Beauveria bassiana entomopathogenic fungi and two chemical pesticides used as seed treatments

The tested pesticides did not significantly inhibit the conidial germination of *Beauveria bassiana* in any of the concentrations used (Figures 3 and 4). Comparing with the control, the differences were very small (Table 1).



Imidacloprid (C1) Imidacloprid (C2) Imidacloprid (C3)

Figure 3. *Beauveria bassiana* vegetative growth in treatment variants with imidacloprid insecticide (10 days after inoculation)



Beauveria bassiana (Control) Imidacloprid + thiram (C2) Imidacloprid + thiram (C3)

Figure 4. *Beauveria bassiana* vegetative growth in treatment variants with insecto-fungicide (10 days after inoculation)

Table 1. Pesticides influence on *Beauveria bassiana* biological parameters

Pesticide	Tested conc.	Fungal spore germination		Vegetative growth reduction		Fungal sporulation	
		Average %	% of inhibition	Average (cm)	Sensitivity	Average % (conidia x 10^8)	% of inhibition
Insecto-fungicide mixture of imidacloprid 460g/l + thiram 176g/l	C1	93.65±1.80	5	-	Very sensitive	-	-
	C2	92.80±3.27	6	-	Very sensitive	-	-
	C3	92.10±2.95	6	1.38±0.08	Sensitive	6.6±0.9	83
Imidacloprid 600g/l insecticide	C1	94.63±0.45	4	1.40±0.04	Sensitive	32.61±4.3	18
	C2	95.25±0.25	3	1.04±0.11	Sensitive	33±1.8	17
	C3	97.65±1.86	1	0.91±0.03	Sensitive	35.8±1.7	10
Control		98.78±0.54	0	0	0	40±0.03	0

Regarding the insecto-fungicide treatment, the concentration tested were not directly related to the percentage of germination, the difference between them being statistically insignificant ($p = 0.29$). When the imidacloprin insecticide was tested, the last concentration, C3, revealed a similar value as in the control.

Although the fungal germination was not so significantly influenced, the vegetative growth in the presence of insecto-fungicide treatment proved to be completely inhibited at C1 and C2 concentrations, demonstrating a fungistatic effect. The sporulation percent was reduced with 83% when the insecto-fungicide was used at C3 concentration. *Beauveria bassiana*

showed sensitivity also to imidacloprid treatments, at all three concentrations.

In vitro compatibility between several biocontrol bacterial strains and two chemical pesticides used for seeds treatment

The results revealed that none of the pesticides tested inhibited totally the bacterial growth at the tested concentration (Table 2).

In case of *Pseudomonas chlororaphis* ssp. *aurantiaca* Sal.c2 strain, there was no inhibition of bacterial growth at any of the tested pesticides concentrations (Figure 5). Likewise, in the presence of imidacloprid insecticide, at 20% concentration, all tested bacterial strains were able to grow without any inhibition (Figure 6).

Table 2. *In vitro* compatibility between the biocontrol bacteria and the tested pesticides

Biocontrol bacterial strain	Imidacloprid 600g/l				Imidacloprid 460g/l + thiram 176g/l			
	Bacterial growth at different pesticide concentrations (%)							
	100	***80***	***60***	***20***	***100***	***80***	***60***	***20***
Pseudomonas chlororaphis ssp. *aurantiaca* Sal.c2	3	3	3	3	3	3	3	3
Bacillus subtilis Us.a2	2	2	2	3	1	1	2	2
Bacillus subtilis 98a	2	2	2	3	2	2	2	2
Bacillus pumilus OS15	2	2	2	3	1	1	1	2
Bacillus amyloliquefaciens OS17	2	2	2	3	1	2	2	2
Bacillus amyloliquefaciens BW	2	2	3	3	1	2	2	2

were: 1 = inhibition hallo greater than 5 mm; 2 = inhibiton hallo less than 5mm; 3 = no inhibition hallo, uninfluenced bacterial growth.

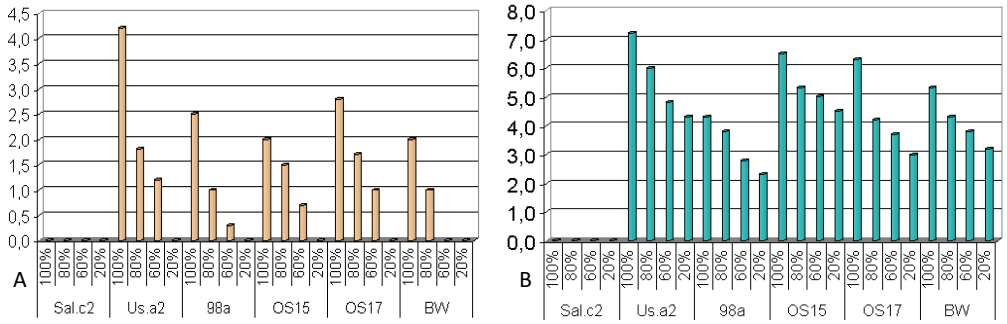


Figure 5. Pesticides influence on the bacterial growth inhibition.
A) Imidacloprid 600g/l and B) Imidacloprid 460g/l + thiram 176g/l.
Ordinate – average of the growth inhibition hallo (mm), Abscissa – bacterial strains and pesticide concentrations

Figure 6. Bacterial growth in the presence of imidacloprid insecticide

Results showed that the imidacloprid insecticide was less toxic to the microbial strains analyzed compared to the insecto-fungicide mixture. However, the biocontrol activity of these microbial strains in pest and disease management, when used in combination with chemical pesticides, allow lowering the recommended dose of chemicals. This hypothesis should resolve the compatibility problems, taking into consideration that the

pesticide applied in low doses did not visibly affect the microbial strains. On the other hand, such phytosanitary strategy that promotes biocontrol microbial strains used together with lower doses of chemical pesticides are one of the most promising strategies for plant protection in the Integrated Pest Management programs.

CONCLUSIONS

Pest biocontrol products based on *Beauveria bassiana* could be used together with chemical insecticides based on imidacloprid, since this chemical does not affect the biological parameters of the entomopathogenic fungus.

Beauveria bassiana entomopathogenic fungi exhibit very high sensitivity towards insecto-fungicide mixture of imidacloprid 460 g/l and thiram 176 g/l at the concentration recommended for use, this pesticide completely inhibit fungal spore germination.

The biocontrol bacterial strain *Pseudomonas chlororaphis* ssp. *aurantiaca* Sal.c2 could be used together with any of the mentioned pesticides, since the tested concentration of chemical did not affect the bacterial development.

The imidacloprid 600 g/l insecticide used at 20% concentration did not inhibit the biocontrol *Bacillus* sp. strains used in this study.

The pesticide mixture of imidacloprid 460 g/l and thiram 176 g/l, tested at 20% concentration, determined a moderate bacterial inhibition (with less than 5mm hallo around the chemical spots).

Phytosanitary strategy based on combined biocontrol and chemical treatments are promising strategies for plant protection within the Integrated Pest Management programs.

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