EXPERIMENTAL BIOLOGICAL PRODUCTS WITH LOW ENVIRONMENTAL HAZARD TOWARDS AQUATIC ORGANISMS USED IN POTATO CROP PROTECTION

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Abstract: Limiting the use of chemicals due to harmful effects on the environment and natural ecosystems has led to the successful development and application of eco-friendly products with biological active properties. This paper presents studies conducted under laboratory and experimental field conditions to determine the toxicity of some bioproducts based on *Bacillus subtilis* - RdB2 bacterial biomass and *Beauveria bassiana* fungal biomass towards fish, *Cyprinus carpio* and algae, *Selenastrum capricornutum.* Toxicity testing of selected products has been carried out on these two aquatic species, species for which plant protection legislation requires the assessment of the influence of plant protection products. At laboratory level, a procedure according to European guidelines has been used to determine the toxicity on fish, which establishes the methodology for determining the acute lethal toxicity of a substance, and for the determination of toxicity to algae, the Algal Growth Inhibition Test. The results highlighted that the two biological products tested are very selective to aquatic species and are unlikely to be toxic to them. Under field conditions, the experimental biological products were applied according to potato crop technology. The experimental field was located near Târlung River, Brasov County. At 30 and 45 days, respectively, after treatment, samples of water were taken and transferred to the laboratory in test vessels containing *C. carpio* fish and algae of the specie mentioned above. Analyses on the viability of the tested species were performed at 7 and 21 days and demonstrated that microbiological experimental products have low risk to aquatic organisms.

Key words: *biological products, Cyprinus carpio, Selenastrum capricornutum, environmental risk.*

INTRODUCTION

Water protection is an essential concern of the European Crop Protection Association (ECPA) and, implicitly, of the pesticide industry, materialized by developing projects that support the proper use of pesticides in a sustainable and productive agriculture. A promising alternative to the use of chemicals is represented by the use of biological products.

Microbiological control agents are eco-friendly and represent a potential substitute for the chemical pesticides. There are natural enemies for the pest populations with no harmful effect on human health and environment (Nicholson 2007). It is well established that biological control agents have complex mode of action and therefore, it is very difficult for a pest to develop resistance against them. The common BCA are viruses, bacteria, fungi and nematodes (Weeden at al., 2007; Uygun at al., 2010; Lacey and Kaya, 2007; Danismazoglu at al., 2013).

Biopesticides generally have several advantages compared to conventional pesticides (Lacey and Kaya, 2007). While chemical pesticides are responsible for extensive pollution of the environment, a serious health hazard due to the presence of their residues in food, development of resistance in targeted insect pest populations, a decrease in biodiversity, and outbreaks of secondary pests that are normally controlled by natural enemies, biopesticides, in contrast, are inherently less toxic to humans and the environment, do not leave harmful

residues, and are usually more specific to target pests. Often they affect only the target pest and closely related organisms, substantially reducing the impact on non-target species (Sporleder and Lacey, 2013). In this context, the use of biopesticides, may be an interesting alternative for crop protection as they are considered to be less harmful and environmentally safer (Dewhurst, 2001; Dayan et al., 2009, 2011; Cantrell et al., 2012; Seiber et al., 2014). Within the last few years, several studies highlighted the effectiveness of biopesticides for the control of different pests in various crops (Copping & Menn, 2000)

This study presents research regarding the ecotoxicological impact towards aquatic organisms of 2 bio-products based on biological active microbial biomass used for pest control in the potato crop.

MATERIALS AND METHODS

The biological material consisted in two bio-products based on biological active microbial biomass, (Figure 1) respectively:

- Biological active fungal biomass based on *B. bassiana*, also with entomopathogenic activity, isolated from a natural infection, on *Leptinotarsa decemlineata*. The original strain was maintained by successive cultivation passages and revirulented periodically on the origin insect. The inoculum was obtained from a 21 days pure culture by washing in 10 ml distilled water and Tween 80 (0.01%). For disaggregation of the mycelia conidia, the suspension was vortexed with glass beads (Ø 1.7-2.1mm, ROTH), at 2000 rpm. Afterwards, the suspension was filtered through cotton wadding, and the spores' concentration was adjusted to a titre of 10^8 conidia/ml using a hemocytometer for counting. For use in the field it is recommended a dose that will ensure 10^{11} conidia/ha.

- Biological active bacterial biomass based on *B. safensis* Rd.b2 with fungicide activity. Rd b2 strain was selected based on its antagonistic activity against phyto-pathogen agents and it was investigated regarding the effect towards aqueous organisms.

Bacterial bioproduct based on *B. safensis* Rd.b2 designed for potato crop protection and plant growth stimulation, was developed as an aqueous suspension. For inoculum preparation, Rd.b2 strain was cultivated on solid Luria-Bertani medium, by successive passage technique, in order to obtain pure isolated colonies. The bacterial cell concentration of the aqueous suspension was quantified with a spectrophotometer, at 600 nm wave length. The bacterial inoculum was amended with carboxymethylcellulose 2%, a thickening and adhesion agent which facilitate the attachment of bacteria to the substrate during application of treatments.

For bacterial bioproduct use in agricultural practice it is recommended an application dose between 200 and 270l/ha of water. For field treatments, the *Bacillus* sp. bacterial bioproduct is prepared at a concentration of 10^8 cfu/ml inoculum.

Figure 1. Biological products

In order to establish the toxicity of the selected bioproducts, the studies were performed first under laboratory conditions on fish, specie *C. carpio* and on algae specie *S. capricornutum*, using two specific technical procedures, property of the Ecotoxicology

Laboratory from Research-Development Institute for Plant Protection Bucharest (RDIPP), procedures in compliance with OECD guideline referring to chemical substances' testing.

First it was investigated the toxicity of the two bioproducts on *C. carpio* using a procedure which establishes the acute lethal toxicity of a substance.

The fish were procured from Nucet Research Development Station for Pisciculture. They belonged to a single batch, corresponded in terms of health, being approximately the same age and no visible malformations. During the acclimatization period, the fish were fed on a daily basis with standardized feed, with a 24-hour break before testing. The adjustment period consisted in 7 days, during which the fish were maintained into reconstituted water, at an adequate temperature for the tested specie, with 12-16 lighting hours per day. The oxygen concentration was at least 80% of the saturation value in the air and the pH of the water 6.0-8.5. Fish that had atypical swimming behavior, respectively disorderly movements or rising to the surface of the water, were isolated from the batch and not used for testing. These, as well as surviving fish at the end of the test period were euthanized according to a specific procedure and disposed of in accordance with specific procedures.

Exposure conditions: The fish were exposed to the test bioproducts for 96 hours; for each test concentration, including for the control, 7 fish were used: the volume of water was 20 l / aquarium, the other conditions were provided according to the above mentioned requirements. During 96 hours, they were not given food.

The fish were exposed to the water-solubilized test substance in a series of concentrations over a period of 96 hours. The mortality was recorded at 24, 48, 72 and 96 hours and the median lethal concentration causing 50% death of the fish group was calculated. The first observations were recorded 2-4 hours after the treatment and then at 24, 48, 72 and 96 hours. The individuals were considered dead if stimulating the caudal peduncle induced no reaction and no breathing movements were visible.

In order to establish the impact of the bioproducts the test organisms (*C. carpio*) were exposed to a dose of 10^8 cfu/ml for *B. safensis* Rdb2 and $2{,}2x10^5$/ml fungi suspension for *B. bassiana*. The bioproducts were applied diluted taking into account the fact that the concentrations present in the surface and phreatic waters are considerably lower than the ones' used in production.

Under laboratory conditions it was also determined the impact of these two bioproducts towards algae specie *S. capricornutum*, using the Algae Growth Inhibition Assay was performed. Unicellular green algae species are recommended for products testing, due to their fast growing capacity, so that relatively short trials can establish the effects of a chemical substance on several generations. The procedure is used in the Ecotoxicological Testing Laboratory of the RDIPP to determine the effects of substances on algal growth using as reference document SR EN ISO / CEI 17025: 2005 - General Requirements for the Competence of Testing and Calibration Laboratories and the OECD Guidelines for Testing of chemical products no. 201 /23.03.2006.

The procedure consists in exposing the test organism to various concentrations of the test substance for 72 hours. The system's response consists in reducing the growth of algal culture and is evaluated according to the concentration of the test substance compared to an untreated control. In order to have a complete system response to tested product as much possible, algae culture exposed to the tested products is maintained in a nutrient-enhancing environment to ensure adequate growth under continuous lighting conditions.

Growth and inhibition of growth are quantified and recorded by counting the cells at the beginning of the test and then at 24, 48 and 72 hours using Thoma blade. For the test to be valid, the growth factor on the untreated control should be at least 16, after 72 hours.

Algae have been grown using specific growth medium according to OECD guidelines with the following components:

Table 1. Algae growth medium

Stock solution	Nutrients	Stock concentration
Stock solutions 1 Macronutrients	NH_4Cl	1,5 g/l
	$MgCL_2.6H_2O$	1,2 g/l
	$CaCl_2.2H_2O$	1,8 g/l
	$MgSO_4.7H_2O$	1,5 g/l
	KH_2PO_4	0,16 g/l
Stock solution 2 –iron	$FeCl_3. 6H_2O$	64 mg/l
	$Na_2EDTA. 2H_2O$	100 mg/l
Stock solutions 3	H_3BO_3	185 mg/l
	$MnCl_2. 4H_2O$	415 mg/l
	$ZnCl_2$	3 mg/l
	$CoCl_2. 6H_2O$	1.5 mg/l
	$CuCl_2. 2H_2O$	0.01 mg/l
	$Na_2MoO_4. 2H_2O$	7 mg/l
Stock solutions 4	$NaHCO_3$	50 mg/l

The growth medium was inoculated with *S. capricornutum* sp. and incubated under controlled light and temperature conditions, respectively 21-24^0 C, ±2^0 C and continuous light between 4440 and 8880 lux. For each tested concentrations and control variant were performed 3 replications. The initial cells' concentration in test cultures was 10^4 cfu/ml.

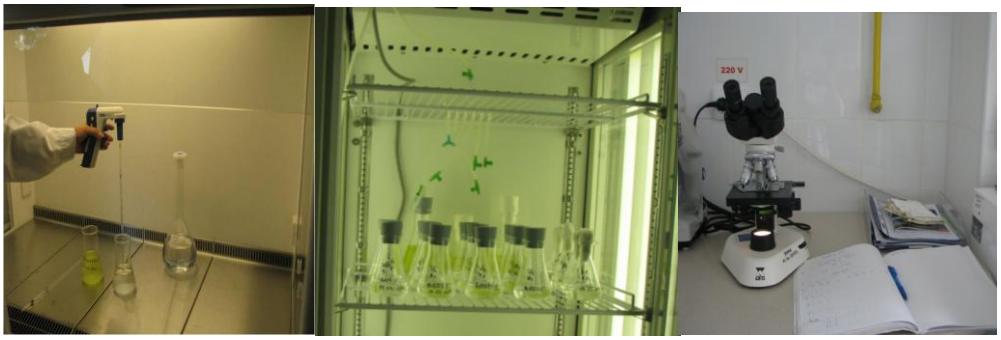

Figure 2. Laboratory aspects

In order to determine the relation between concentration and effect it was used the areas comparison method. To establish the impact of the biologic compound on algae growth, a concentration of 10 thousand algal cells were exposed to various concentrations of tested substance.

After developing the experimental model under laboratory conditions, there was conducted a field trial to assess the impact of the biological products towards the same aquatic species. The experimental fields (Figure 3) were located in Brasov County, near Tarlung river, an area favorable for potato crop. The presence of nearby water shadows (irrigation channels, ponds), ensured that the bioproducts used for potato crop protection will reach into these waters.

The experimental model consisted in randomized blocks with 6 variants and 4 replications. Potato Panam variety, sewed at the end of March, is characterized by high

productivity and resistance to the attack of *Phytophtora infestans*. In order to establish the influence of phytosanitary treatments on the aquatic organisms studied, respectively C. *carpio* and *S. capricornutum*, the biological experimental products were applied according to the crop maintenance technology, and to the development cycle of specific pest agents within potato crop such as *Ph. infestans*, *Alternaria solani, Leptinotarsa decemlineata*, etc.

Figure 3. Potato experimental plot

The impact of bioproducts towards the aquatic organisms from the waters nearby the treated experimental potato plots was determined under laboratory conditions by analyzing water samples taken at 30 and 60 days after treatment. Samples were taken in plastic containers of 20 liters, 100 liters for the two specified intervals. In the ecotoxicology laboratory, the water samples were distributed in special vessels, in which were placed the model aquatic species, namely *C. carpio*, *S. capricornutum* (figure 4) and *Daphnia magna*, a key species to characterize the influence of plant protection products on aquatic ecosystems. Observations have been done regarding fish behavior, algal growth rate and crustacean mobility.

Figure 4. Aspects from laboratory experiments

RESULTS AND DISCUSSIONS

In order to protect the aquatic ecosystem, it is recommended to use primarily pesticides that are not classified as dangerous for the aquatic environment, the use of the most efficient application techniques, and low diversion equipment, the use of mitigation measures to reduce the risk of external pollution caused by spray drift, drainage and leakage. These include the establishment of buffer zones of adequate size to protect aquatic organisms and protection areas for groundwater or surface water sources.

Concentrations of the tested bioproducts were calculated based on the recommended use dose per hectare, taking into account that if the potato crop is located less than 10 m from a water luster, there is the possibility of leaching the product into surface water. Thus, the concentrations used were 10 times lower. These concentrations have been established to demonstrate that the products used do not have a negative impact on the aquatic fauna and flora.

The development of the experimental model and the toxicity testing of the bioproducts on *C. carpio* specie conducted into the following results from table 2:

Table 2. The impact of bioproducts on *Cyprinus carpio*

Variant	Dose	Observation after :							
		24 h		48 h		72 h		96 h	
		alive	dead	alive	dead	alive	dead	alive	dead
Bacillus safensis Rd.b2	10^8 CFU/ml inoculum	7	0	7	0	7	0	7	0
Beauveria bassiana	$2,2x10^5$/ml fungal suspension	7	0	7	0	7	0	7	0
Untreated control	-	7	0	7	0	7	0	7	0

Regarding the toxicity of the biological products towards *S. capricornutum* specie, the analysis performed established that none of the products exerted toxicity on algae. After 24, 48 and 72 hours from the treatment it was quantified the increase of the algal cell number, the growth factor ranging from 16, in treated variants to 18, for the control variant.

After conducting the experimental model under field conditions, it was determined after treatment that the two bioproducts used for potato crop protection were highly efficient in controlling the main pest agents. Analysis regarding the viability of the test species exposed to water samples taken from the field nearby, performed at 7 and 21 days established that the experimental bioproducts have a low impact towards aquatic organisms.

CONCLUSIONS

From the conducted researches it can be concluded that the experimental bioproducts based on bacterial biomass of *B. safensis* Rd.b2 and fungal biomass of *B. bassiana* have shown to be very selective towards the *C. carpio and S. capricornutum*, and also highly efficient for the potato crop pest control.

It was determined that the experimental bioproducts tested can be recommended in potato crop pest control schemes due to their very good selectivity, by applying risk management measures for aquatic organisms and proper distances from water lusts and surface waters, ensuring by this, protection of the environment, as well as healthy crops.

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