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A New Formula (Bg Z1) For More Efficient Delivery Of *B. Thuringensis Var. Israelensis* Endotoxins Against Mosquito's Larvae

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The evaluation of the effects by which physical, chemical and biological agents (temperature, moisture, pH extremes, sunlight, proteolytic enzymes, bacteria, etc.) affect stability of the active substance of a biological insecticide, as the present one based on *B. thuringiensis israelensis* against mosquito's larvae, is a prerequisite to identify the appropriate mix of ingredients (antioxidants, radical scavengers, UV protectants, etc.) required to protect the active substance during field application. Furthermore it is necessary to study how to avoid not homogeneous application, or settling of the product in aquatic environment, that can leave large sensible areas not covered by the active substance and spoil the overall insecticidal effect displayed by the product. The final objective is to develop a formulate with appropriate characteristics and persistence time for large scale field applications. Actually a new product with these characteristics (named BG z1) has been developed by Bio & Geo S.r.l. by fermenting a *B. thuringiensis israelensis* H14 strain, recovering and drying the parasporal crystals and than formulating these crystals as emulsifiable liquid concentrate, with the addition of protective ingredients and surfactants to enable easy mixing with water for an application as a spray on large areas to fully exploit the insecticidal properties displayed even at ppm concentration.

1. Introduction

The insect control, especially those harmful to agriculture and public health, involves traditionally the use of chemicals that often can display negative drawbacks being toxics, not only to the target insects, but also to beneficial ones, as well as to animals and men. In addition, insects often develop resistance against these chemicals, after many successive applications, and this effect can have dramatic consequence in terms of increasing treatment doses and frequencies required, multiple pesticides, etc. and, at the end, in terms of heavy damage of environment.

So the alternative of biological insecticides, offered by modern biotechnologies, has been welcome and it is developing very much, well beyond expectations, particularly for the treatment of urban areas and/or for insects that can give rise to public health problems. This is the case of Dipteral insects (mosquitoes, etc.) that are able to transmit heavy dangerous illness such as Yellow Fever and Dengue (see particularly Aedes aegypti, etc.). The active substances, widely employed for the application against diptera larvae, are protein endotoxins produced by *Bacillus thuringiensis var israelensis* (Bti) as crystal parasporal inclusions. These proteins degrade quickly in nature and so they are not harmful to environment. But these advantageous elements produces the main inherent biopesticide disadvantages: the short persistence time in field conditions due to degradation effects by chemical, biological, physical and natural agents (temperature, moisture, pH, sunlight, bacteria, etc.). The present work is focused on these effects with the final objective to develop a new formulate with appropriate characteristics and persistence time.

2. Materials and Methods

2.1 Microbial strain

A strain of *Bacillus thuringiensis var. israelensis* (Bti) was isolated from soil samples and named z1. Microbiological characteristics and protein profiles of Bti z1 were identical to the reference strain, ATCC 35646 *Bacillus thuringiensis var. israelensis* serovar H14 (FIGURE 2).

2.2 Technical powder and formulate production

The production of experimental products has been developed by Bio & Geo S.r.l. in its laboratory and plants in Caserta (FIGURE 1), by fermenting the Bti z1 strain described above, recovering and drying the parasporal crystals (Technical Powder: TP) and than formulating these crystals as emulsifiable liquid concentrate (Formulate: FO), with the addition of protective ingredients and surfactants to enable easy mixing with water for an application as a spray on large areas.

FIGURE 1 Bio & Geo Plant facility in Caserta

2.3 Gel electrophoresis of active proteins

The protein profiles of the products obtained from Bti z1 fermentation has been obtained by standard SDS-PAGE gel electrophoresis (Lee et al. 2001): resuspending the spore-crystal mixture in the buffer (60mM Tris-HCl-(pH 6.8), 25% glycerol, 2% SDS, 5% 2-mercaptoethanol, 0.1% bromophenol blue), boiling it for 10 min, and subjecting it to SDS-10% polyacrylamide gel electrophoresis. SDS-PAGE was performed on a 10% separating gel with a 3% stacking gel. The gel was stained with 0.1% Coomassie brilliant blue (Sigma Co., USA).

To be noticed the two bands (130 kDa and 70 kDa, indicated by the arrows) characterizing the pesticidal crystal proteins of such Bti strains:

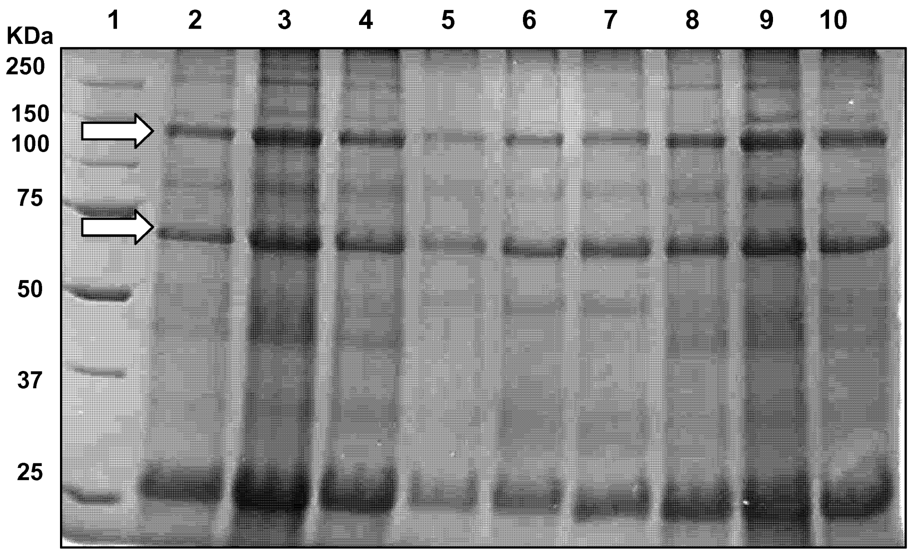

FIGURE 2 Gel electrophoresis of Bti active proteins in the technical powder obtained from z1 strain fermentation (at different charges: lanes 2 (20 mg/ml); 3 (40 mg/ml) and 4 (30 mg/ml)), from ATCC strain fermentation (lanes 8 (20 mg/ml); 9 (40 mg/ml) and 10 (30 mg/ml)) and in the formulate BGz1 (lanes 5 (100 mg/ml); 6 (200 mg/ml) and 7 (300 mg/ml)).

2.4 Bioassay

Efficacy of a technical powder or a formulation is generally determined by measurement against a standard formulation and expressed in terms of International Toxic Units (ITU/mg) (de Barjac 1983). Potency is calculated by:

LC50 of the standard

----------------------- x potency of the standard = Potency of the sample (ITU/mg)

LC50 of the sample

Where LC50 is the lethal concentration of that substance that produced a 50% mortality frequency in standard conditions.

2.5 Large scale field tests
The tests have been carried out in two drainage canals in Metaponto plain (Basilicata Region - South of Italy). The test product was sprayed on the infested area and samples of water were collected before and after the treatment to measure the variation of concentration of mosquito diptera larvae (microscopic observation) versus time. Multiple samples are taken at each time to get statistically sound results (average and % Abbott test values are shown at the point 3). The results were compared with untreated controls on the same infested drainage canals

FIGURE 3 Large scale field test site

3. Results and Discussion

3.1 Laboratory Tests
Preliminary laboratory tests have been carried out to evaluate direct intrinsic activity and stability properties of the active substance as function of basic parameters, such as dosage, temperature, pH, etc. These studies make the basis to understand the actual performance of the bio-insecticides, such as the formulate BG z1, although indirect effects and interaction of environmental factors determine the final overall efficacy. These indirect effects have been studied in the second part of the work (3.2 Large scale field tests).

DOSAGE AND CONCENTRATION

The larvicidal activity of technical powder and of the formulate BGz1 measured in the laboratory is shown in figure 4 in terms of average mortality frequency of Culex pipiens larvae as function of the concentration of the tested substance supplied. Actually the activity appears to be very high: it is enough a very low concentration (fraction of ppm) for causing a lethal effect. Typical lethal concentrations LC50 (for 50% mortality) are 0.08 mg/l for technical powder (activity about 3,000 ITU/mg) and 0.32 mg/l for the formulate (activity about 750 ITU/mg). The ratio of these two activities correspond to the “dilution ratio” of the active substance during the formulation process (addition of 3 parts of ingredients to 1 part of technical powder). The actual ratio of activity displayed in field conditions by TP and FO is obviously totally different and makes the reason for the required formulation process.

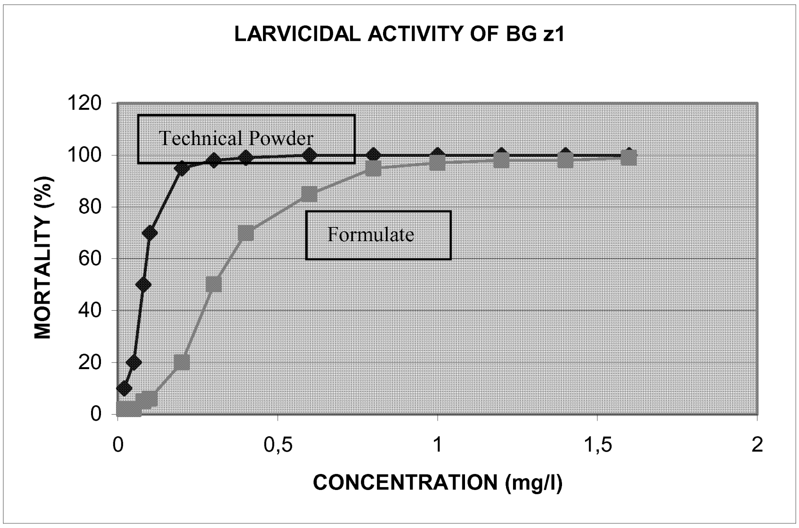

FIGURE 4 Larvicidal activity of technical powder and BGz1 formulate

TEMPERATURE

In general terms temperature is a very significant factor in the decomposition – deactivation processes of the active substance. In the present case it was possible to demonstrate that the active substance, being a protein crystal, is quite stable at ambient temperature, both as technical dry powder and as a formulate, at least during the storage phase of the product (less than 10% decay of the activity for year). The situation becomes much more intriguing during the application phase when temperature effects combine with other degradation effects and, even more important, with activity and feeding behaviour of the insects. For example the reduced activity of Bt observed at low temperature has been described as indirect consequence of the reduced activity and amount of feeding by target larvae, rather than by any other direct effect (Walker 1995).

pH EXTREMES

In general terms pH of the product can affect its stability and activity in many ways, but in the present case it was possible to demonstrate a quite good stability of the formulate, both during the storage phase of the product and during the application, unless very low (less than 4) or high (more than 8) pH environment is present. Actually alkaline components can break down the active proteins fairly quickly (pH higher than 10); for this reason buffering chemicals could be included in pesticide formulations if pH extreme environment is expected in the area of application. But the normal pathway for converting microbial protoxins to the biologically active toxins, after ingestion by susceptible insect larvae, should not be obstructed by formulation ingredients.

PHOTODECOMPOSITION

Natural sunlight, especially the ultraviolet (UV) portion of the spectrum (UV-B, UV-A), has been demonstrated to be responsible for inactivation of microbial insecticides

(Behle et al., 1997) and the specific damage to Bt insecticidal proteins appeared to be related to the damage of the tryptophan residues of the Bt protoxin by solar radiation in the 300-380 nm range (Pusztai et al. , 1991). A recent publication (Wan et al., 2007) suggests the utilization of melanin as photoprotective ingredient in bioinsecticide applications. These aspects have been taken into account in BGz1 formulation that has been designed to protect Bt protein crystals from UV damage, as indicated by laboratory experiments, that demonstrated no inactivation effect, at least in the lab. experimental conditions.

SETTLING CHARACTERISTICS

Bio-insecticides are normally applied directly to water for the control of mosquitos and blackflies. Rapid sedimentation of protein crystals, and the consequent removal of active substance from the surface where larvae normally feed, is than an important limitation on the efficacy of such applications. The present formula BG z1, being based on the addition of lipid and surfactants, have been designed to prolong residence time of Bt protein crystals at the surface of water, as indicated by laboratory experiments that demonstrated stable, homogeneous emulsion formed by mixing BG z1 with water.

3.2 Large Scale Field Tests

Final large scale field tests have been carried out to evaluate direct, indirect and overall actual performance of the formulate BG z1 in the presence of environmental factors.

Two main tests have been carried out in two drainage canal trials in Metaponto plain. The test product was compared with an untreated control, spraying BGz1 formulate on two mosquito diptera larvae infested drainage canals.

In general terms, the activity displayed by BGz1 product can be judged very high in both trials (almost complete disinfestations in very short time (1- 4 hours) even at low dosage (1 kg/ha), but a quite large difference is observed in the two trials: the first one being much more successful, with higher efficacy values.

	TEST 1			TEST 2		
	BGz1 treated area	CONTROL area	Efficacy	BGz1 treated area	CONTROL area	Efficacy
	Average (n/l)	Average (n/l)	(% test Abbott)	Average (n/l)	Average (n/l)	(% test Abbott)
Time = 0	54	54		47	47	
1 h	11	76	85.5%	18.5	36.0	48.6%
4 h	3	86	96.5%	10.7	23.5	54.5%
24 h	2	91	97.8%	10.5	23.3	54.8%

It is quite interesting to notice that these differences are not due to basic parameters (product, dosage, etc. are the same) but to a number of indirect factors playing a role in the test 2: such as the larger amount of vegetation present, the lower temperature, the physiological state of the larvae at the time of treatment (October 2007).

The effect of low temperature and of the declining activity of the larvae has already discussed above (Walker 1995). The effect of the amount of vegetation present is understandable too, in terms of larger area to be covered and of presence of vegetation exudates; in any case this effect has been noticed by other researchers too (unsuccessful control of Culex peus in a primary oxidation pond with a dense cover of water hyacinth and reduced effectiveness of Bti against Odagmia ornate in the presence of dense vegetation: Mata et al. 1986).

4. Conclusions

The present investigation stressed the need to protecting the active substance of biological insecticides during the application phase (Bt formulations frequently have half-lives of up to 10 days, while unformulated Bt may have a half-life of only a few hours: Dent, 1993). For this reason a new formula (BG z1) for more efficient delivery of Bti endotoxins against mosquito's larvae has been developed. Samples of this new product have been prepared by Bio&Geo and tested at large scale field level, in collaboration with Metapontum Agrobios. Very positive results have been obtained in terms of stability, activity, persistence in field conditions and environmental protection. Registration of this new product is in progress by Italian Authorities.

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