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SCOPE OF COMMERCIAL FORMULATIONS OF *BACILLUS THURINGIENSIS* BERLINER AS AN ALTERNATIVE TO METHYL BROMIDE AGAINST *TRIBOLIUM CASTANEUM* ADULTS

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Abstract

Montreal protocol has deprived the world from Methyl bromide which had been used as one of the most effective pesticide since many decades. Alternative fumigation methods are therefore urgently required to fill this gap in relation to the pest control especially the stored grain insect pest management. Investigations were therefore carried out to evaluate the potential role of *Bacillus thuringiensis* (Bt) as a natural pest control agent against the rust red flour beetle. Different formulations of the pathogens were tested for their efficacy against the pest. Results revealed that two out of the three commercial formulations used in the experiment i.e., Dipel ES and Bactospeine showed moderate results with 59.33 and 54.66 % mortality. Ecotech Pro was however found to be the most effective exhibiting 79.66 % mortality after 7 days of the treatment. The results further revealed that liquid formulations yielded comparatively better results as compared to the powder formulation. Mortality exhibited in case of Ecotech Pro was perhaps due to its active ingredient transconjugant Bt *kurstaki* x Bt *aizawi*, its potency as well as more ingestion of the Bt spores and toxins because of its liquid base. Liquid formulations not only enhanced the moisture contents but also made the grain more palatable for *T. castaneum* adults resulting in more mortality as compared to dry formulation.

Introduction

Extensive use of Methyl bromide is in practice for combating all sorts of grain infestations caused by insect and fungal pests since 1930s (Mueller, 1994). But destructive effects of Methyl bromide, CFCs, HCFCs and other ozone depleting substances (ODS) on the earth's stratospheric ozone layer, have convinced the global community to reduce and eliminate the production and use of these substances. Since, ozone layer has no boundaries; it needs to be protected by everyone on this planet. The Montreal Protocol is the first international environmental treaty, signed by 178 countries, to provide a plan to help solve this global problem. Methyl bromide (MB) is one of the most significant ozone depleting substances due to its bromine which is 45 times more reactive as compared to chlorine (Daniel *et al.*, 1999). That is why the chemical was added to the Montreal Protocol in (Anon., 1992). At the moment use and production of the fumigant has almost been scheduled to end in developed countries with effect from January 2005 and worldwide by 2015 (Anon., 2002). Phase out of this valuable disinfectant has created a lot of concerns as well as brainstorming among the scientists urging search for the safe alternatives. Although many chemical and non-chemical alternatives have been proposed and tried, yet each has limitations that prevent it from being a direct replacement for methyl bromide in all its current uses (Bell *et al.*, 1996).

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Candidate alternatives include some physical measures such as exploitation of low and high temperature, lowering the ambient humidity, entoleters, physical barriers, trapping, pheromones, food attractants, growth regulators, predators and parasites, space treatments with contact pesticides, residual treatment and environment friendly fumigants such as Phosphine, Sulfuryl fluoride, Carbonyl sulfide, Carbon dioxide, Carbon disulfide, Ethyl formate, Ethylene oxide, Hydrogen cyanide, Methyl iodide, Methyl isothiocyanate, Ozone, Sulphur dioxide, Ethyl or methyl formate and Acetaldehyde (Dobie, 1984; Annis & Waterford, 1996; Anon., 1998). Nevertheless, phosphine has been extensively used as a key alternative to the Methyl bromide in the grain industry to counter infestations of live insects and their eggs. Unfortunately, *T. castaneum* has shown resistance to this vital fumigant. Several cases of phosphine resistance and failure of fumigation have been reported from Indonesia, UK, India, Philippine, Australia and China (Pike, 1994; Pinniger, 1985; Rajendran, 1994; Sayaboc & Gibe, 1997, Nayak *et al*., 2000; Nayak *et al*., 2003). There is sufficient evidence in the literature that red flour beetle has shown resistance to some other groups of chemical insecticides too. On the other hand, this pest has been reported to cause both quantitative and qualitative damage to wheat and other grains. Quantitative damage is due to grain weight loss caused by insect feeding (Steffan, 1963; Golebiowska, 1969) whereas qualitative loss is due to product alterations such as loss of nutritional and aesthetic value, increased levels of rejects in the grain mass and loss of industrial (baking) characteristics. Among the qualitative losses, reduction in germination percentage is the most important as it has direct impact on lowering wheat yield (Gallo *et al.,* 1978; Vieira, 1983; Baier & Webster, 1992; Moino *et al*., 1998). Infestation caused by *T. castaneum* in wheat stored for 9 months reduced germination from 89% to 2% and increased visually damaged kernels from 9 to 39% (Edwards *et al*., 1991). Venkatrao *et al*., (1960) determined the positive correlation between insect numbers and fragment counts in wheat flour obtained from wheat infested by *T. castaneum*. Insect fragments in flour increased with time mainly due to the growth of immature stages of insects. Decreased loaf volume, compact and inelastic crumb, bitter taste, and off-flavours were observed in bread made from wheat flour infested by *T. castaneum* (Venkatrao *et al*., 1960; Smith *et al*., 1971; Edwards *et al*., 1991; Sanchez-Marinez *et al*., 1997). The quinone secretions of *T. castaneum* are very bitter and affect the baking and taste qualities of the products (Smith *et al*., 1971). While chemicals used for controlling this pest have also resulted in toxicity of the treated food grains ultimately creating some sanitary and phytosanitary problems. Biologists are therefore concentrating on controlling this pest with non-chemical alternatives including use of natural products, predators, parasitoids and microbes for the development of successful integrated pest management (IPM) approaches to meet the demands of HACCP (Hazard Analysis and Critical Control Points) (Quiniones, 1992; Copping & Menn, 2000). Luckily, a bacterium *Bacillus thuringiensis* Berliner has shown a promising role in controlling coleopteran as well as lepidopteron pests. The literature reveals that the pathogen was first used as an insecticide in an experiment during 1938 (Worthington, 1991). It took another 20 years to use *B. thuringiensis* as biopesticide on commercial scale in the United States for the first time in 1958 (Ghassemi *et al.,* 1981). Although bacterial toxins have proved to be effective weapons against many insects yet toxins of *B. thuringiensis* var. *kurstaki* have given optimistic results (Varma & Dubey, 1999). Much of the success lies in the ways in which it can be considered as similar to chemical insecticides. *B. thuringiensis*, a gram-positive bacteria, produces a proteinaceous parasporal crystalline inclusion during sporulation. This crystal structure is non toxic to insects. Upon ingestion by insect, this prototoxin, due to high pH in the stomach of the insects, cleaves the protein into smaller subunits called endotoxins. The activated toxins interact with the midgut epithelium causing a disruption in membrane integrity and ultimately leading to instant death. Insects that do not have a high pH in the gut are incapable of this reaction. Likewise mammals which have an acidic pH in the stomach are not capable of breaking the prototoxin down into smaller units (Knutti & Terwedow, 1987). There are currently 410 registered formulations of *B. thuringiensis* and 6 registered formulations of *B. lentimorbus* and *B. popillae* approved for use against insect pests. Conventional Bt products, which utilize naturally occurring Bt strains, account for approximately 90% of the world Microbial Pest Control Agent (MPCA) market. Most Bt products contain insecticidal crystal proteins (ICP) and viable spores, but in some Bt products, the spores are inactivated. Each year, around 13000 tonnes Bt pesticide is produced using aerobic fermentation technology (Anon., 1999). Dipel ES, Dipel 2X Bt subsp. *kurstaki* and Vectobac Bt subsp *israelensis* are the product of Abbot Laboratories, Bactospeine (Philips Duphar), Thuricide, Javelin and Teknar (Sandoz), Bathurin 82 and Moskitur (Slu1ovice), Coleoptera Bt *subsp. san diego* Trident (Sandoz), M-One (Mycogen) and Ecotech Pro is the product of Aventis **(**Taborsky, 1992; Thomson, 2005).

The present study focuses on use of some commercial formulations of *B. thuringiensis* against *T. castaneum* as an alternative to methyl bromide and other hazardous chemical insecticides.

Materials and Methods

Insect rearing: Adults of *T. castaneum* were collected from provincial reserve centers of the Punjab Food Department and were mass produced in glass jars (250 ml) covered with a piece of fine cloth. Insects were reared on a diet of wheat flour, wheat kernels and bean grains, respectively. The cultures were maintained in a controlled environment chamber under a 12/12 h light-dark photoperiod at 27 ± 2°C and 70 ± 5% r.h. All experiments were carried out in stored grain laboratory of the University of Arid Agriculture, Rawalpindi under the same environmental conditions used to maintain the cultures. Adult insects, two weeks after eclosion, were used for the experiments.

***Bacillus thuringiensis* formulations used in the experiment:** Commercial Bt formulations used in the experiment are presented in Table 1. These included**,** Ecotech Pro 7.5 (24,000 international units [iu]/mg formulation, 3% [ai] *B. thuringiensis* subsp. *Kurstaki,* strain *EG 2348, transconjugant* Bt *kurstaki* x Bt *aizawi* [Ecogen); Bactospeine 16000 iuAK/mg 3% [ia] containing viable spores of *B. thuringiensis* subsp. *Kurstaki* [Biochem product] and Dipel ES (17,600 international units [iu]/mg formulation, 3.2% [ai] spores of *B. thuringiensis* subsp. *Kurstaki,* [Abbott Laboratories, North Chicago, IL]. Dipel is based on *B. thuringiensis* subsp. *kurstaki* HD-1 (17,600 iu mg^{-1} [wettable powder]), which produces Cry1Aa, Cry1Ab, Cry1Ac, Cry2A, and vegetative insecticidal protein (Schnepf *et al*., 1998). Ecotech pro and Dipel ES were used in liquid form at concentrations of 12000 iu/mg and 8800 iu/mg, respectively. Whereas, Bactospeine was used in powder form at concentrations of, 8000 iu/mg. The bio-insecticide were applied on Petri dish as well as mixed with grains used as diet in the medium for larvae.

Table 1. Commercial formulations of *B. thuringiensis* used in the experiment.

S.No.	Generic name & Active ingredient	Potency	Trade name	Formulation type	Source
1.	*Bacillus thuringiensis* subsp kurstaki strain EG 2348, transconjugant Bt *kurstaki* x Bt *aizawi*	24,000 iu/mg	Ecotech Pro 7.5	SC	Ecogen
2.	*Bacillus thuringiensis* subsp *kurstaki* strain SA-11	16000 iuAK/ mg	Bactospeine 20%	WP	Biochem product
3.	*Bacillus thuringiensis* subsp *kurstaki* HD-1	17,600 iu/mg	Dipel ES 3.2%	WP	Abbot laboratories

Table 2. Analysis of variance showing effect of different Bt formulations on mortality of *T. castaneum* adults for different exposure periods.

Source of variation	df	Sum of square	M.S.	F	P
Treatments	3	26832.7	8944.2	1114.79	0.000
Exposure time	6	21884.8	3647.5	454.61	0.000
Treatment x time	18	2869.0	159.4	18.86	0.000
Error	56	449.3	8.023		
Total	**83**	**52035.8**			

Bioassay: For carrying out bioassay, 30 uniform sized adults of *T. casatneum* (two weeks old) from each strain were taken from the jars and placed in the Petri plates already having grains and treated with different formulations of *B. thuringiensis*. For each treatment three replicates were used in completely randomized design. The Petri plates were placed in the incubator at 28^{o}C and 70% relative humidity (RH). The mortality data was recorded after 24, 48, 72, 96, 120 and 168 hours, respectively. Mortality in control was also noted to correct the mortality data according to Abbot's formula (Abbot, 1925).

Statistical procedures: Statistical analysis was carried out in multi-factorial completely randomized designs (CRD) in MSTATC package (Anon., 1999) and the means were compared by Duncan's Multiple Range tests at 95% level of confidence (Gomez & Gomez, 1984).

Results and Discussion

The mortality percentage of *T. castaneum* adults indicated statistically significant effect of Bt formulations used in the experiment. Exposure time also exerted significant effect on mortality of the pest. There was a positive interaction between the exposure periods and Bt formulations (Table 2).

Detailed analyses regarding performance of the individual treatments through Duncan's Multiple range Test at α=0.01 also indicated statistically significant variations among different treatments. Maximum mortality of 79.66% was observed in case of Ecotech Pro 7.5 treatment followed by Dipel ES and Bactospeine with 59.33 and 54.66%, respectively as compared to control which showed 20% mortality after 168 hours. Time of exposure to biocide significantly enhanced the pest mortality. In case of Ecotech Pro mortality was enhanced from 25.00 to 79.66% from 24 to 168 hours, respectively. Likewise mortality of the other two Bt formulations also increased from 2.00 to 54.66 and 8.66 to 59.33% vis-à-vis Bactospeine and Dipel ES, respectively (Table 3).

Table 3. Comparative performance of different *Bacillus thuringiensis* formulations against mean mortality of *T. castaneum* adults at 28°C and 70 ± 5% r.h.

B.T. Formulations	Mortality (%) at different exposure times (hours)						
	24	48	72	96	120	144	168
Ecotech Pro 7.5	25.00 **a**	40.66**a**	57.66 **a**	64.00 **a**	71.33 **a**	75.00 **a**	79.66 **a**
Bactospeine 20%	2.00 **c**	5.00 **c**	6.66 **c**	17.33 **c**	17.66 **c**	22.33 **c**	54.66 **b**
Dipel ES 3.2%	8.66 **b**	15.33 **b**	25.66 **b**	35.66 **b**	46.00 **b**	54.33 **b**	59.33 **b**
Control	0.00**c**	0.00 **c**	3.33 **c**	5.00 **d**	11.00 **d**	15.33 **c**	20.00 **c**

It is further evident from the above Table that none of the Bt formulations exhibited 100% control of the pest. Besides, mortality percentages of *T. castaneum* recorded for Bactospeine and that of control were found statistically similar at 24, 48, 72 and 144 hours. However, performance of Bactospeine statistically equaled to that of Dipel ES at 168 hours exposure time. The highest performance of Ecotech Pro may primarily be attributed to its potency i.e., 12000 iu/mg which was greater as compared to the other two formulations Dipel ES and Bactospeine having potencies of 8,800 and 8,000 i.u/mg, respectively. The second possible reason for the highest performance of Ecotech pro may be the characteristics of its active ingredient i.e., the strain EG 2348 which is reportedly a hybrid of subsp Bt *kurstaki* and Bt *aizawi* (Ahmedani *et al*., 2007). The type of formulation might be the tertiary reason because Ecotech Pro and Dipel ES used in the experiment was liquid based as compared to the Bactospeine which was used in powder form. The liquid formulation might have made the grain tender and more palatable due to moisture contents. The findings are in line with the work of Donovan *et al*., (2002) who found that *B. thuringiensis* strains contain novel crystal proteins which exhibit insecticidal activity against coleopteran insects including red flour beetle larvae (*T. castaneum*) and Japanese beetle larvae (*Popillia japonica*. Other scientists have also revealed significant effect of *B. thuringiensis* against *T. castaneum* (Abdel Razek & Salama, 1999). An investigation into the histopathology of two isolates of *B. thuringiensis* (subsp. *indiana* and subsp. *morrisoni*), in the larval tissues of *T. castaneum* and *Plodia interpunctella* (Hubner) revealed that most changes caused by ingestion of the pathogens were localized in the midgut of the insects. Other effects were observed in muscles and tracheoles. In *T. castaneum*, larvae were treated with a lethal dose of 5000 μg of B.t. subsp. *morrisoni/g* of grains. Changes appeared in the regenerative cells, where cytoplasmic vacuolization and pycnotic nuclei were observed. The tracheal cells showed relaxation of the taenideal cuticular lining and disintegration of mitochondria and chromatin clumping granules around the nuclear membrane 48h after treatment. Seventy-two hours after treatment, the tracheal and circular muscle fibers showed complete relaxation. The two varieties of Bt. that were collected from grain dust in Egypt, had potential as control agents for stored-product moths and beetles. The toxic action differed in the two tested insect species (Abdel-Razek *et al*., 2002). Three years later, Donovan *et al*., (2005) found that a diet containing spores and δ-endotoxin of Bt isolates B- 21365, B-21366, and B-21367 was active against the larvae of other species of Coleoptera, including the red flour beetle, *T. castaneum* and the Japanese beetle, *Popillia japonica*.

Keeping findings of the present investigations and work of previous workers in view, it may be concluded that *B. thuringiensis* has a great potential to prove as an alternative to methyl bromide for controlling coleopterous pests including *T. castaneum*. It can even be used in combination with synthetic insecticides wherein some synergistic effect has been reported (Saleem *et al*., 1995, 1996). There is a dire need to search and test more and more isolates for tracing out the best to achieve maximum control of this pest.

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