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Evaluation of the residual activity of newly developed aqueous formulation of novel biopesticide *Bacillus cereus* VCRC-641 through simulated field trial

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ABSTRACT: In the study a new aqueous formulation with the new mosquitocidal isolate of *Bacillus cereus* VCRC 641 was developed, and the residual activity was assessed from simulated field trials conducted in the natural environment. From laboratory bioassays, it was observed that the efficacy of aqueous formulation of *Bacillus cereus* was on par with lyophilized cell mass, as such. The LC_{50} values of formulated *B. cereus* against *Culex quinquefasciatus, Anopheles stephensi* and *Aedes aegypti* were 0.002, 0.009 and 0.008 mg L^{-1} respectively. Correspondingly, the LC_{50} values of lyophilized cell mass of *B. cereus* against these three mosquito species were 0.0019, 0.005 and 0.004 mg L^{-1} respectively. Finally, simulated field trial was carried out using formulated *B. cereus* and the residual efficacy against all three mosquito larval species revealed 100 per cent larval mortality up to seven days and 69 to 78 per cent mortality were up to 15 days. Thereafter, the mortality was declining gradually up to 21 days. It is concluded that the formulation of *B. cereus* may be used for mosquito control program. © 2023 Association for Advancement of Entomology

KEY WORDS: Bioassays, LC_{50}, *Culex quinquefasciatus, Anopheles stephensi. Aedes aegypti*

INTRODUCTION

Mosquito-borne diseases are a serious global public health concern. Dengue fever, filariasis, chikungunya, malaria, and other mosquito-borne diseases are producing high level of illnesses and death in many areas throughout the world (WHO, 2016). Many vector control programmes in many countries are focusing on adult mosquito control and larval management to combat these diseases. For nearly four decades, widespread use of chemical insecticides against vector mosquitoes for the management of malaria and other mosquito-borne diseases has resulted in the development of pesticide resistance in vector mosquitoes and environmental dangers (Mittal, 2003; Etang and Fondjo., 2006; Mohan and Ramaswamy, 2007; Becker *et al*., 2010; Raghavendra *et al*., 2011). Despite the widespread and long-term use of chemical pesticides, many diseases continue to be prevalent and can produce outbreaks (Mittal, 2003).

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As a result, alternative mosquito control strategies must be investigated urgently in order to reduce dependency on chemical insecticides. Chemical larvicides, which are famous to be eco-friendly, particular to target species, and ideally suited for community use, could be replaced by bacterial-derived biolarvicide (Mittal *et al.,* 1999). Some registered formulations of *Bti* and *B. sphaericus* have been established and experienced to kill mosquito larvae.

The effectiveness of *Bti* and *B. sphaericus* against the larvae of *Anophelines* and *Culicines* have been meticulously studied (Lacey, 2007). If *Bti* is free of biologically active substances other than insecticidal crystal proteins and non-*Bt* microorganisms, it is unlikely to be toxic to humans, other vertebrates, or non-target invertebrates (WHO, 2006). According to recent studies, the sequential evolution of the taxonomic organization and taxa richness of non-target populations of aquatic invertebrates are not significantly impacted by the long-standing application of *Bti* in coastal wetlands (Lagadic *et al*., 2013). In the present study, an aqueous formulation from *Bacillus cereus* VCRC 641 was developed and examined the toxic efficacy against the mosquito larvae of *Aedes aegypti* (Linnaeus), *Anopheles stephensi* Liston and *Culex quinquefasciatus* Say in the simulated field conditions.

MATERIALS AND METHODS

Isolation of Bacteria: The gut content of fresh water fishes have been collected and about 1gram of sample was mixed with 9 ml of sterile water and processed by standard serial dilution method and then kept in a water bath (Technico serological water bath, Made in India) at 80ºC for 30 minutes in order to kill the non-spore formers (Radhika *et al*., 2011). After cooling, 50µl of the sample was streaked on LB media agar plates and incubated at 37^0C overnight. Single pure colony from the culture plate was taken and inoculated into 10 ml of Luria Bertani broth and incubated overnight in an Orbitek Incubator shaker (Scigenics Biotech LT4676, Made in India) at 250 rpm. 10µl of this culture was inoculated into 100ml of LB broth in a 500ml Erlenmeyer flask and incubated for 72 hours at 250 rpm in an orbital shaker. Further, the culture was harvested and centrifuged at 10,000 rpm for 15 minutes in a Hitachi high speed refrigerated centrifuge (CR22III, Made in Japan). The bacterial cell mass was collected and kept in deep freezer (-80°C) overnight and freeze-dried in a lyophilizer Lark Innovative (Penguin classic, Made in India). The lyophilized bacterial powder was preserved in a refrigerator until further use.

Preparation of aqueous formulation: Aqueous formulation of *B. cereus* VCRC 641 was prepared (5%) by combination of Congo red (0.025%), sodium benzoate (0.5%), calcium chloride (4%) and sodium alginate (1%). Composition is - *B. cereus* VCRC 641 lyophilized cell powder 5g, Congo red 0.025g, sodium benzoate 0.5g, calcium chloride 4g, sodium alginate 1g and water 100ml. The formulated product was stored at room temperature (4ºC) until further use.

Laboratory bioassay: Toxicity bioassays were conducted against three mosquito species of laboratory reared late third instars (*Cx. quinquefasciatus, An. stephensi* and *Ae. aegypti*). The toxicity test was performed in disposable wax-coated paper cups using a homogenous stock solution of the liquid formulation. Serial dilutions were made by dissolving appropriate known volume of formulation (7 different doses) in 100 ml of chlorine-free tap water and 25 late third instar larvae of each mosquito species were added for bioassay (WHO, 1985).

Simulated field trials: *Bacillus cereus* VCRC 641, 5 per cent aqueous formulation was developed and potency of the formulation evaluated for perceiving the residual activity of the strain. Laboratory reared (Unit of Mosquito Rearing and colonization, Vector Control Research Centre, Puducherry) late third instar larvae were used for the simulated field trial. Cement tanks with a capacity of 75 litres (*i.e.,* Diameter of outer surface 60cm, height 47.5cm) were used to study the *B. cereus* VCRC 641 aqueous formulation and test the residual efficacy. The tanks were filled with 50L of water and covered with a mosquito net. To

avoid direct exposure to rain and sunshine, the cement tanks used in the simulated field trial were placed under shaded roof shelter.

The primary goal of simulated field investigation was to test and determine the residual efficacy of the *B. cereus* VCRC 641 formulation in the field condition. 50 numbers of late third instar larvae of each species were introduced in each experimental and control cement tanks. *B. cereus* was tested at 3 dosages from the stock solution of 5mg $10ml^{-1}$ ($625\mu l\ 50L^{-1}$, $625\mu l\ 50L^{-1}$ and $1.25ml\ 50L^{-1}$) against *Ae. aegypti, Cu. quinquefasciatus* and *An. stephensi* respectively. Dosage of formulation for simulated field trial was calculated based on the LC_{90} values of the isolate. Three replicates were kept for each dosage in each experiment for all the three species. The experiments were repeated three times at different time intervals. Larvae were counted and removed from the cement tank every day after treatment; mortality of larvae was recorded for the assessment of residual activity. After recording the mortality values, fresh alive larvae of each species were introduced in alternative days.

RESULTS AND DISCUSSION

Formulation of *Bacillus cereus:* In the present study, a new aqueous formulation was standardized from the lyophilized cell mass and toxicity assay was tested against laboratory reared mosquito larval species (*An. stephensi, Cx. quinquefasciatus,* and *Ae. aegypti*). It was observed that there was no significant variation in the toxicity levels (at LC_{50} and LC_{90}) of cell mass and the formulation of *B. cereus* (Table 1).

Simulated field trials: The residual activity of formulated *Bacillus cereus* VCRC 641 against three major mosquito larval species using cement tanks. The percentage mortality was recorded till 20^{th} day of each experiment. From the findings of residual activity of *B. cereus* against *Cu. quinquefasciatus, An. stephensi* and *Ae. aegypti* revealed that end of first week (7 days) all species showed 100 per cent mortality, on the second week (14 days) it was shown 75, 78 and 69 percent respectively. Subsequently, the mortality was declining gradually up to 21 days (Fig. 1). Indicating that the new formulation of *B. cereus* was efficient in mosquito control.

Due to its proven safety for both the environment and human health, *B. thuringiensis* (Bt) has been used in bio pesticide formulations on a large scale for the past 40 years. Increased manufacturing and formulation costs generally impede the widespread use of *Bt*. Complementary local media have progressively replaced expensive synthetic media, but the actual barrier is the cost of the formulation. Since formulation affects cost, shelf life, ease of use, and field efficacy, it serves as a crucial bridge between manufacturing and use. UV light, rain, pH, temperature, and foliage physiology are all environmental factors that reduce the efficacy of Bt formulations. To overcome the negative environmental consequences, various formulations – solid and liquid – have been developed based on application target and practicality (Brar *et al*., 2006).

The new formulated *B. cereus* toxicity assay in laboratory (bioassays) revealed that the LC_{50} and LC_{90} values for *Cu. quinquefasciatus*, *Ae. aegypti* and *An. stephensi* were 0.002 and 0.007 mg L^{-1}, 0.008 and 0.0180 mg L^{-1} and 0.009 and 0.019 mg L^{-1} respectively. The results from simulated field trials revealed that the aqueous formulation was on par with lyophilized cell mass toxicity assay and pronounced effect on mosquito larvae. Gunasekaran *et al.* (2004) reported Teknar HP-D, an enhanced biolarvicide formulation of *Bti,* effectiveness in the field against *Cx. quinquefasciatus*. The formulation's toxicity was tested in the laboratory on larvivorous fish, water bugs. Teknar HP-D was evaluated in the ground at three suggested doses, 1, 1.5, and 2 L ha^{-1}, in five different habitats. In the lab, *Ae. aegypti* was the most susceptible to the *Bti* toxin. Up to day 6 post-treatment in drains, all three doses resulted in a >80 per cent reduction, implying that a weekly application at the lowermost level would be required for long-term management. The formulation's residual activity lasted longer in unused wells, resulting in a >80 per cent drop in pupal recruitment

Table 1. LC50 and LC90 values of aqueous formulation of *B. cereus* VCRC 641 cell pellet

Larval species	LC_{50} (mg L^{-1}) (UCL-LCL)	LC_{90} (mg L^{-1}) (UCL-LCL)	χ^2	p-value
Aedes aegypti	**0.0089** (0.011-0.007)	0.018 (0.02-0.014)	80.55	<0.05
Anopheles stephensi	**0.009** (0.01-0.006)	0.019 (0.035-0.013)	94.14	< 0.05
Culex quinquefasciatus	**0.002** (0.003-0.002)	0.007 (0.01-0.003)	89.65	<0.05

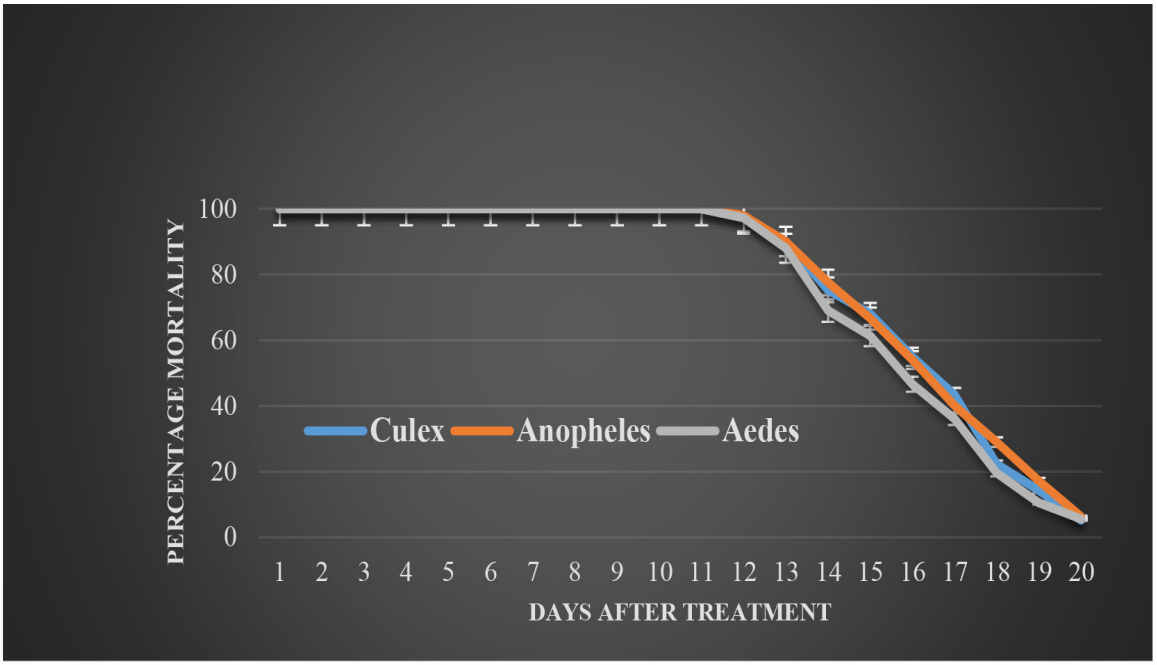

Fig. 1 Average value of simulated field trial using *B. cereus* VCRC 641formulation against *Culex quinquefasciatus*, *Anopheles stephensi* and *Aedes aegypti*

for 17 days after treatment (Gunasekaran *et al*., 2004).

For evaluating the efficacy and residual activity of *B. cereus* aqueous formulation simulated field experiment revealed that, *B. cereus* efficacy was long lasting upto 20 days. The residual activity of *B. cereus* against *Cx. quinquefasciatus, An. stephensi* and *Ae. aegypti* revealed that, end of the first week all species showed 100 per cent mortality, on the second week its showing 75, 78 and 69 per cent respectively. Residual efficacy of *B. cereus* against all three larval species showed 100 per cent mortality up to 11thday. Field application of *B. cereus*, after 24 hours of exposure to *B. cereus* VCRC B540 in the laboratory, doses of 0.0047 and 0.037 mg l^{-1} resulted in 50 and 95 percent mortalities, respectively, and in the field, the necessary concentration was 0.047 grams per square meter. The target mosquito larvae (*Cx. quinquefasciatus*) studied were particularly sensitive to the *B. cereus* VCRC B540 liquid formulation (various places in Puducherry, India), with the early instars being the most sensitive. This study confirms 17 days of residual activity (100 to 80% reduction) of *B. cereus* VCRC B540 like *B. sphaericus*, which is better than residual activity of *Bti* demonstrated earlier (Margalit and Dean, 1985; Mani *et al*., 2018). Uragayala *et al*. (2018)

described, in natural habitats in Bengaluru, India, the effectiveness and residual activity of a novel formulation of Bti over larvae of *Ae. aegypti*, *An. stephensi* and *Cx. quinquefasciatus*. In Phase III tests, Bactivec SC at 1 ml/50 litre dosage produced 10-17 days' effectiveness (>80% reduction in pupae) in fresh water environments examined, whereas 0.5 ml/50 litre dosage produced residual activity from 7 to 14 days against *Ae. aegypti* and *An. stephensi*. In Phase III, efficacy against *Cx. quinquefasciatus* could be measured for 4-7 days in polluted water environments. It is concluded that the formulation of *B. cereus* may be used for mosquito control program.

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