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Mosquitocidal Potential of Native *Bacillus thuringiensis* Strain SY49-1 against Disease Vector, *Culex pipiens* (Diptera: Culicidae)

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Abstract. Mosquitoes are regarded as troublesome organisms worldwide due to their parasitic and pathogenic aspect causing malaria, yellow fever, dengue, West Nile and filariasis. *Bacillus thuringiensis* (*Bt*) products have effectively been used for decades in controlling the mosquito mediated diseases and also decreasing the chemical input into the environment. In the present study, biocontrol potential of previously characterized *Bt* SY49-1 strain was investigated on *Culex pipiens* larvae. Fourth instars of *C. pipiens* were subjected to spore/δ-endotoxins (50, 100, 250 and 500 µg mL^{-1}) of *Bt* SY49-1 strain, carrying *cry* genes active against Lepidoptera, Diptera, Coleoptera and Nematodes. The spore/δ-endotoxin mixture caused 100% mortality on the larvae at the dose of 500 µg mL^{-1}. PCR results indicated that *Bt* SY49-1 harbors Lepidopter-Dipter specific *cry2A* gene as well as Lepidopter specific *cry1Ab*, *cry1Aa/Ad*, *cry1C*, *cry9A*, *cry9C*, Lepidopter-Dipter-Coleopter specific *cry1B*, and Nematode specific *cry5* gene. Results indicated the potential usage of *Bt* SY49-1 in preventing the breeding of *C. pipiens* and the spread of diseases resulting therefrom.

INTRODUCTION

Mosquitoes, especially *Culex pipiens* (Diptera: Culicidae), are well-known species in temperate regions as well as in southern Europe/Mediterranean countries and vectors of medically important parasites and pathogens leading to serious health problems. They are regarded as a troublesome organisms worldwide (Peng *et al*., 1999; Jensen & Mehlhorn, 2009; Mehlhorn, 2011; Caminade *et al*., 2012; Benelli *et al*., 2014; Kioulos *et al*., 2014). Two forms of *Culex*, *pipiens* and *molestus*, are known to hybridize and exhibit intermediate feeding behavior between avian and mammalian hosts in U.S.A. and southern regions of Europe (Fonseca *et al*., 2004; Krida *et al*., 2015). In this respect controlling the vectors is a crucial step and thus insecticide-based inhibition methods with organophosphate based insecticides and insect growth regulators are commonly applied in controlling these pests of medical importance. However, these chemicals result in environmental harm and also mosquitoes can easily develop resistance against them over time (Sun *et al*., 2011; Lees *et al*., 2014; Benelli *et al*., 2014). Products of entomopathogen *Bacillus thuringiensis* (*Bt*) have effectively been used for decades for decreasing the chemical input into the environment and biologically controlling the *C. pipiens* mediated diseases (Federici *et al*., 2010; Visitsattapongse *et al*., 2014). *Bt* products have also been widely used against many lepidopteran, dipteran and coleopteran pests worldwide (Feitelson *et al*., 1992;

Schnepf *et al*., 1998; Yılmaz *et al*., 2012, 2013; Alper *et al*., 2016) and are highly advantageous due to their high specificity and environmental safety. Thus, they are considered as the most important biological products through limiting the use of chemicals (Carozzi *et al*., 1991; Schnepf *et al*., 1998). In the present study, mosquitocidal potential of previously characterized *Bt* SY49-1 strain was evaluated on fourth instar larvae of medically important disease vector *C. pipiens*.

MATERIALS AND METHODS

Activation of *Bt* SY49-1

Bt SY49-1 strain (Yılmaz *et al*., 2012) were activated from stock culture of Biological Control Laboratory at Erciyes University by incubating in 10 ml of LB broth medium at 30ºC and 200 rpm for overnight.

Spore/δ-endotoxin preparation and microscopy

Adequate amount of spore/δ-endotoxin from *Bt* SY49-1 was obtained through incubating in T3 medium (150 ml; 3 g triptone, 2 g triptose, 1.5 g yeast extract, 0.005 g $MnCl_2$, 6 g NaH_2PO_4, 7.1 g Na_2HPO_4) at 200 rpm and 30ºC for 7 days (Travers *et al*., 1987). The resulting suspension was centrifuged at 15.000xg and 4ºC for 10 min. Remaining pellet was washed twice with 20 mL sterile dH_2O and freeze dried. The mixture was visualized at 100x10 magnifications under microscope (Leica DM750, ICC HD50).

SDS-PAGE analysis

The medium containing spore/δ-endotoxin was centrifuged at 4ºC and 15.000xg for 10 min and resolubilized in 12 mL of 20 mM Tris-HCl (pH 7.5). The pellet was solubilized in 5 ml of 20 mM Tris-Cl (pH 7.5) containing 50 µl lysosyme (10 mg/mL) and incubated for 15 min at 30ºC. Subsequently it was centrifuged at 4ºC and 15.000xg for 10 min. The pellet was resuspended in 2 ml of 20 mM Tris-HCl (pH 7.5) and used for SDS-PAGE analysis. Suspension containing spore/δ-endotoxin mixture was mixed in equal amount of sample buffer (4 mL of 10% SDS, 2 mL glycerol, 1.2 mL of 1M tris (pH 6.8), 0.01% bromophenol blue, 10 mL b-mercaptoethanol, 2.8 mL dH_2O) and kept at 95-100ºC for 5-10 min. Electrophoresis was carried out in 12% separating gel with a 5% stacking gel and stained with Coomassie brilliant blue R250 according to the method of Sambrook *et al*. (1989). Fermentas SM0661 marker was used as protein weight.

Culex pipiens culture

Larvae of the *C. pipiens*, kindly supplied by Dr. Mehmet Fatih ŞİMŞEK from Department of Biology at Adnan Menderes University, were reared in cube shaped veil cages. Rearing conditions were set up at 27±1ºC, 65±5% relative humidity and 14L:10D h photoperiod. Larvae were fed with flake fish food up to pupation. Adults were transferred into cages including plastic containers (10 cm diameter x 5 cm) and supplied with 10% sugar solution for oviposition. The oviposited eggs were transferred to larvae rearing containers (40x25x10 cm) with fresh water.

Biological activity

The insecticidal activity of spore/δ-endotoxins of *Bt* SY49-1 was tested on 10 fourth instar larvae as three replicates by daily supplying with flake fish food. Trials were performed in sterilized glass bottles (300 mL) containing 25 mL water with 50, 100, 250 and 500 µg mL^{-1} spore/δ-endotoxin concentration. Sterilized tap water was used as control. Results were recorded for ten days by removing dead larvae. Data were subjected to analysis of variance (ANOVA) and means were separated at the 5% significance level by using the Tukey HSD posthoc test. Total mortality was corrected using Abbott's formula. The LC_{50} values were estimated using probit analysis with 95% confidential limit.

Statistical analysis

Data were subjected to analysis of variance (ANOVA) using SPSS 10.0 for Windows. Means were separated at the 5% significance level by using Tukey Honest Significant Differences post hoc-test for each group.

RESULTS

Microscopy analysis

Microscopic analysis indicated the presence of bipyramidal (130-140 kDa), dipteran active cuboidal (65-70 kDa), and spherical (130 kDa) proteins in the suspension (Figure 1). These types of Cry proteins have specific toxicity against pest insects belonging to Diptera, Lepidoptera and Coleoptera.

SDS-PAGE analysis

SDS-PAGE (Figure 2) analysis revealed the proteins of *Bt* SY49-1 strain as dipteran active Cry2 (~65 kDa), lepideopteran active Cry1 (~140 kDa) and Cry9 (~130 kDa), and nematode active Cry5 (~78 kDa) as known from the literature (Crickmore, 2000; Khyami-Horani *et al.*, 2003; Arrieta *et al.*, 2004; Azizoglu *et al.*, 2015). The result is supported by PCR analysis revealing that the *Bt* SY49-1 harbors Lepidopter-Dipter specific *cry2Aa* (Azizoğlu, 2014) as well as Lepideopteran specific *cry1Ab* (Azizoğlu, 2014; Azizoglu *et al.*, 2016), *cry1Aa/Ad*, *cry1C*, *cry9A* and *cry9C* (Yılmaz, 2010; Yılmaz *et al.*, 2012), Lepidopter-Dipter-Coleopter specific *cry1B* (Yılmaz, 2010; Yılmaz *et al.*, 2012), and Nematode specific *cry5* (Yılmaz, 2010; Yılmaz *et al.*, 2012) genes.

Bioassay

Spore/δ-endotoxin mixture of *Bt* SY49-1 was tested to control fourth instars larvae of *C. pipiens* in 25 mL water under controlled conditions. LC_{50} value was used for assessing the mosquitocidal activity of spore/δ-endotoxin mixture. Efficacy of 100 µg mL^{-1} was slightly higher than control, but the greatest larval mortality was observed at 500 µg mL^{-1} during 10 days ($F=20.921$; $df=4$; $P \leq 0.0001$) (Figure 3). LC_{50} of *Bt* SY49-1 was estimated as 285 µg of spore-crystal per mL of water.

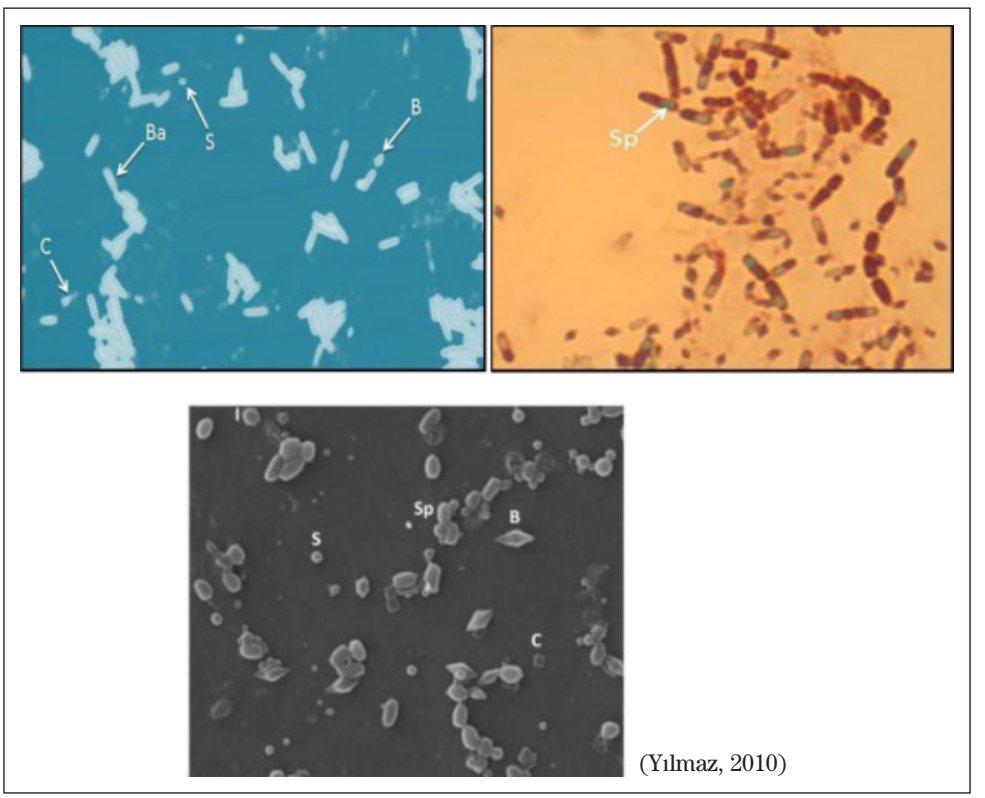

Figure 1. Microscopy image of *Bt* SY49-1, Bipyramidal (B); cuboidal (C); spherical (S); Bacterium (Ba) and Spore (Sp).

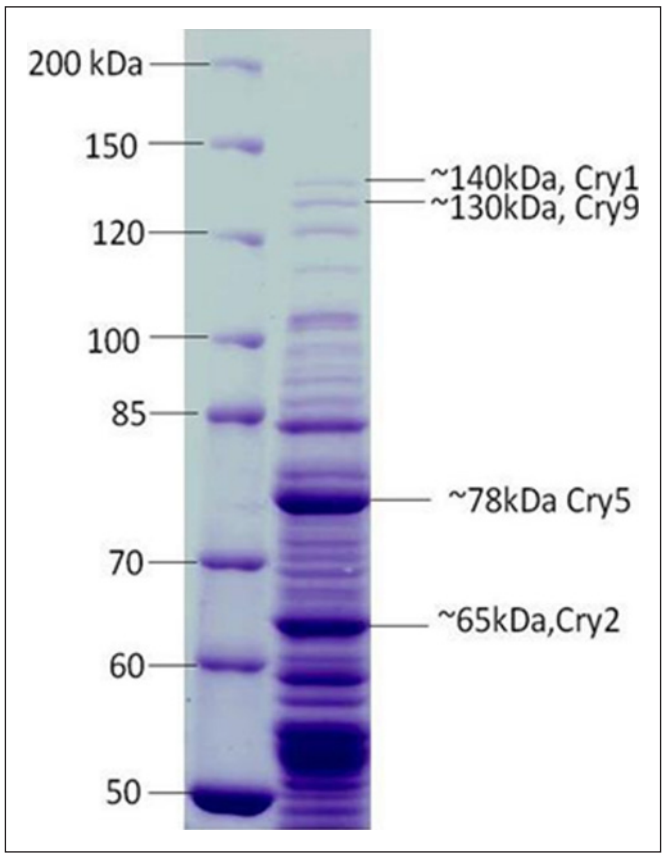

Figure 2. SDS-PAGE (12%) analysis of *Bt* SY49-1 spore/δ-endotoxin mixture.

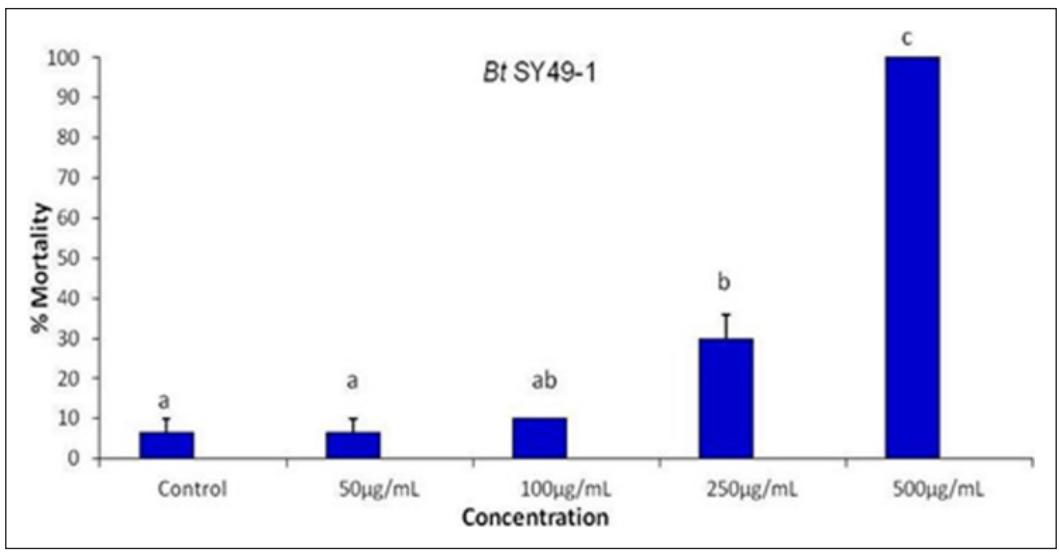

Figure 3. Toxicity of *Bt* SY49-1 on the larvae of *C. pipiens*.

DISCUSSION

The efficacy of *Bt* formulations is well known and have been used for years in controlling pest insects of economic importance (Roh *et al*., 2007; Gonza´lez-Cabrera *et al*., 2011). Researchers reported that it is possible to reduce the impact imposed by pests to very low levels using the *Bt* based formulates without introduction of chemicals into the environment. *cry2*, *cry4*, *cry11* and *cyt* gene carrying *Bt* isolates exhibit insecticidal activity against dipteran species (Yamagiwa *et al*., 2001; Kamauchi *et al*., 2003, Ben-Dov, 2014). Cry2Aa protein specifically targets *C. pipiens*, *Aedes aegypti* and *Anopheles gambiae*, a potential mosquito vector of yellow fever and malaria, respectively (Mcneil & Dean 2011). Besides, it is expressed by many researchers that the Cry toxin combinations with spore mixtures was more effective than individual toxins (Crickmore *et al*., 1995; Poncet *et al*., 1995; Hughes *et al*., 2005; Beltrao *et al*., 2007; Lopez Pazos *et al*., 2009; Azizoğlu, 2014). Although estimated LC_{50} value for *Aedes aegypti* was reported to be 0.12, 1.35 and 13.01 mg L^{-1} for Cry4Ba, Cry11Aa and Cry4Aa individual toxins, respectively, this value decreased to 0.013 mg L^{-1} when *Bti* spore/δ-endotoxin mixtures was applied to the same pest larvae (Beltrao *et al*., 2007). Similarly, our previous study indicated that individual Cry2A toxin exhibited lower toxicity when compared with spore/δ-endotoxin mixture of *Bt* SY49-1 on *C. pipiens* (Unpublished data). On the other hand, in a study carried out by Ben-Dov (2014), *cry4Aa* δ-endotoxins exhibited higher toxicity compared to spore-crystal mixture on the larvae of *Culex* but not on *Anopheles* and *Aedes*. In the current study, the spore/δ-endotoxin mixture used is relatively high, but more detailed studies are required for determining the optimal concentration both in controlled and natural surroundings for efficient and long lived control of *C. pipiens*. As a conclusion, results obtained from the laboratory treatments with the inoculation of *Bt* SY49-1 spore and parasporal bodies have proved its potential usability in preventing the proliferation of *C. pipiens* and the spread of diseases resulting therefrom.

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