International Journal of Applied Research 2016; 2(2): 20-23

ISSN Print: 2394-7500
ISSN Online: 2394-5869
Impact Factor: 5.2
IJAR 2016; 2(2): 20-23
www.allresearchjournal.com
Received: 18-12-2015
Accepted: 20-01-2016

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Activity of two indigenous *Bacillus thuringiensis* isolates on lepidopteran insect pest *Amsacta albistriga* (Arctiidae)

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Abstract
Two *Bacillus thuringiensis* isolates GM17 and GM21 were evaluated against lepidopteran insect pest *Amsacta albistriga.* These isolates produced various crystal inclusions with bipyramidal, spherical and cuboidal shape which confirmed by phase contrast microscopy. SDS-PAGE analysis revealed both isolates produced different Cry proteins in the range of 130 KDa to 50 KDa. Insect bioassay were carried out to determine LC_{50}against second instar larvae of *Amsacta albistriga*. LC_{50}value was found 0.055 $ngml^{-1}$ for *Btk-HD-1* while for *Bt* isolates GM17 and GM21, LC_{50}values were found 0.017 $ngml^{-1}$ and 0.027 $ngml^{-1}$ respectively. Phylogenetic analysis of *cry1Ac*gene sequence of GM17 isolate was found point mutation in domain II which can be used for domain swapping and *cry2* gene of GM21 to gene pyramiding strategies for insect resistance management.

Keywords: *Amsacta albistriga*, *Bacillus thuringiensis*, Insecticidal Cry protein, Red hairy caterpillar.

1. Introduction
Insect pests are major serious problem for high production of crops since many decades (Islam *et al*., 2014) [1]. Polyphagous key pests like *Helicoverpa armigera*, *Spodoptera lituralis* and *Pectinophora gossypiella* are spread worldwide and feed on different types of more than 170 plant species (Ranga Rao *et al.*, 1993) [2]. *Amsacta albistriga* (Family: Arctiidae) is one of the devastating pests found in fields of India and China. It feeds on ground nut, castor, cotton, cow pea etc (Bhatt *et al*., 2013) [3]. Larva feeds on leaves and fruits causing severe damage during complete larval life cycle and cause severe or total loss of production on agricultural important crops (Prabhu & Sudheer, 2008) [4]. Chemical pesticides have been widely used to control red hairy caterpillar from many decades. However, issues have been reported such as resistances development against chemical pesticide cypermethrin and environmental pollutions (Muthusamy & Shivakumar, 2015) [5]. Therefore, alternative approaches have been carried out by employing nuclear polyhedrosis virus (NPV) (Rabindra & Rajasekharan, 1996) [6] and entomopathogenic nematodes belong to steinernematidae and heterorhabiditidae families (Gaugler, 1981) [7] for biological control of *Amsacta albistriga*.
Bacillus thuringiensis is produce insecticidal Cry protein, β-exotoxin, zwittermicin, vegetative insecticidal protein, phospholipase, chitinase and various proteases (Heimpel & Angus, 1959; Patel& Ingle, 2011) [8, 9]. Cry toxins are very effective against wide range of insects belong different order lepidoptera, coleoptera, diptera. Thus, it one of the best biocontrol agent in agriculture, forestry and human disease vector control. However, certain drawbacks like single toxin unable to target more than one pest, inactivation on prolonged exposure of sunlight, failure to control sucking pests belongs to Homoptera, Hemiptera and Thysanoptera orders. (Catering *et al.*, 2015) [10] Thus, scientists promoted to find novel *Bt* strains, toxin with broad spectrum insect pest control and development of spore-crystal formulation to extend *Bt* toxin efficacy. Currently, Insect resistance development in lepidopteran insects like *Diamondback moth, Plutella xylostella* have been observed due to continues exposure of *Bt* in the field (Sayyed *et al.*, 2004) [11]. Its management by different genetic engineering approaches have been reported such as domain swapping between Cry1 and Cry3 protein against *Diabrotica virgifera* (Walters, *et al.* 2010) [12], deletion of alpha-helix of domain I from N terminal of toxin against *Spodoptera lituralis, Helicoverpa armigera, Agrotis ipsilon* (Mandal *et al*., 2007) [13], pink ball worm *(Pectinophora*

gossypiella) (Soberon *et al*., 2007) [14] Increase toxin solubility in insect gut of *Manduca sexta* by deletion of C-terminal of Cry1A (Benjamin *et al*., 2014) [15] Site directed mutagenesis in *cry1Aa* for change specificity lepideptran to dipteran (Liu, 2006) [16].

Isolation and screening of *Bt* strains were carried out worldwide in order to find out different potent toxins or toxins harbouring broad insecticidal activity. The aim of present study to determination of potency of lab isolates to red hairy caterpillar.

2. Material & Methods

2.1 Enrichmentof *Btk-HD1* and native isolates

Standard strain *Btk-HD1* obtained from BGSC, Ohio, USA. GM17, GM21 were lab isolated from central Gujarat soils. Cells were grown in 100 ml of sporulation medium namely GYS (in gram%: 0.1 g glucose; 0.2 g yeast extract powder; 0.2 g $NH_4(SO_4)_2$; 0.006 g $MnSO_4$; 0.04 g $MgSO_47H_2O$; 0.008 g $CaCl_2$; 0.5 g K_2HPO_4) till sporulation (48-72 h) of bacteria at 30 °C on an orbit Incubator Shaker at 180 RPM.

2.2 Phase contrast microscopy analysis

Loopful of sporulated culture of *Btk HD-1*, GM17, GM21 were heat fixed on cleaned glass slide subsequently stained with 0.5 % crystal violet for 5 min, washed slide in water, kept for air dry. Dark field phase contrast microscopy was performed to evaluate shape of crystal inclusions synthesised by isolates.

2.3 SDS-PAGE analysis

Sporulated culture of *Btk HD-1,* GM17 and GM21 were used for SDS-PAGE analysis. Two ml aliquot was taken of fully sporulated cultures, centrifuged at 10,000 RPM for 10 min, pellets taken and washed two times with Tris 10mM, EDTA 1mM, PMSF 1mM buffer subsequently with 0.85 % saline. Finally, pellet was solubilised by suspended in 100 µL of 0.1M NaOH and incubate at RT for 5 min. Immediately, 20uL of 6X SDS-PAGE loading dye was added and mixed thoroughly and boiled in water bath for 10 min. An aliquot of 20 µL sample was loaded onto 10% SDS- PAGE.

2.4 Insect bioassay

Larvae of *A. albistriga* were reared in laboratory by feed freshly sterile castor leaves with control environment condition (28 ± 1 °C, 70 to 60% RH, and 12h L: D regimes) till bioassay performed. Sporulated cultures of *Btk-HD1*, GM17 and GM21 were centrifuged (10000 RPM, 10 min), washed three times with 0.85% saline. Cry protein was estimated by Bradford method and prepared insecticidal dose by serial dilution in range 3.0 μgml^{-1} to 0.00003 μgml^{-1} to. An aliquot of 250 µL in 0.1% Tween 80 was spread on five cm^2 castor leaf piece. The leaf piece was further cut in to 1cm ^{2} leaf discs and a single piece was fed to each larvae of *Amsacta albistriga*. Thirty larva were used for each dose and experiment performed with two replications. Insect larvae were fed with 0.1% Tween -80 used as negative control (Dulmage, 1981) [17]. Mortality was recorded till 7th day of experiment. Probit analysis was performed using SPSS software program 1.5 version.

3. Results and Discussion

3.1 Phase contrast microscopy analysis

Bt isolates GM17 and GM21 produced bipyramidal, spherical and cuboidal shaped crystals respectively (Fig.1). The morphology, shape and size of parasporal inclusions vary among different *Bt* strains. It includes bipyramidal, cuboidals, amorphous, flate and square crystals, small spherical crystals by (Ramparasad and Ammons,(2005) [18]. Bravo(1998) [19] *et al.* reported *cry1* gene encoding bipyramidal crystal more frequently present cry gene in *Bacillus thuringiensis* and insecticidal to lepidopteran group while spherical shape of inclusion body very rare found but possess broad spectrum insecticidal activity.

3.2 SDS-PAGE analysis

SDS-PAGE analysis of lab isolates found different Cry proteins in range of 130-120KDa, 100-90 KDa, 80-60 KDa and 50-45 KDa while standard strain *Btk HD-1* showed ~130 KDa of Cry1A and 65 KDa of Cry2A toxin (Figure:2a). The presence of different Cry proteins in single *Bt* stain reported earlier. Ben-Dov *et al*., 1997 [20] reported presence of different cry genes like *cry1, cry3, cry7* and *cry8* in *Bacillus thuringiensis* isolate. In current study, molecular characterization of GM-17 and GM21 isolates by PCR analysis were revealed presence of *cry1, cry3, cry7, cry 8, cry11, cyt* and *cry2* respectively. RFLP analysis of *cry2* of GM21 isolate was shown unknown cry2 respectively. (Patel K.D. *et al*., 2011); (Patel K.D, 2012) [21, 22].

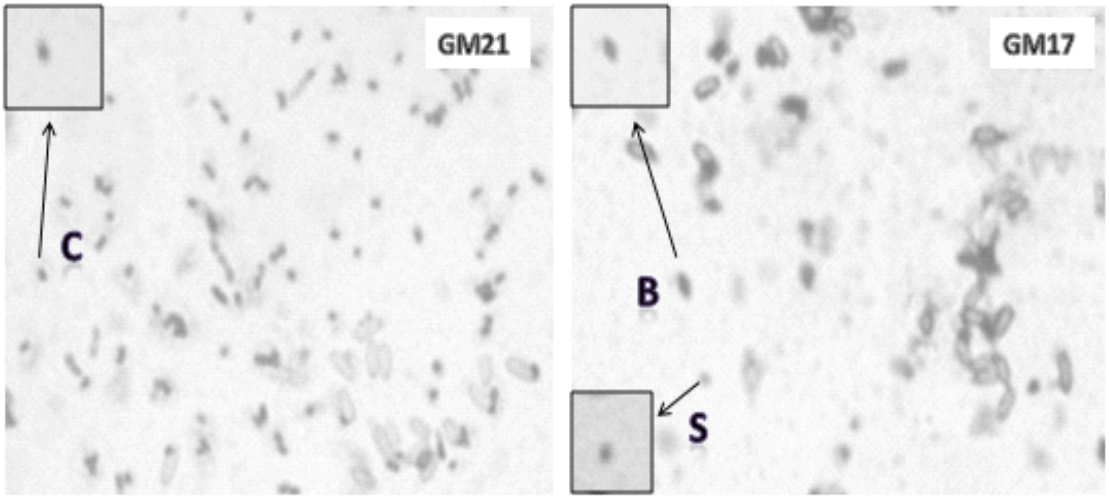
3.3 Insect Bioassay analysis

Insect bioassay was performed against *Amsacta albistriga*. Effect of spore-crystal complex of *Bt* was noticed after 24h on *A. albistriga*. Major symptoms were observed such as larval growth inhibition, sluggishness and color change of healthy larvae from light orange to blackish and ultimately to death due to septicemia (Figure: 2 b). LC_{50} value was calculated 0.055 $ngml^{-1}$ of standard strain *Btk HD-1* to *A. albistriga* while lab isolates GM-17 and GM-21 were shown comparative 0.017 $ngml^{-1}$ and 0.027 $ngml^{-1}$. Thus, it concluded toxicity of lab isolates GM17 and GM 21 around 3.2 and 2.0 fold higher than standard references strain *Btk HD-1*. Multiple sequence alignment by CLUSTER ω of partial translated cry1Ac gene sequence (NCBI: Accession: EU 906916) of GM17 observed point mutation at 312 position where Gly replace to Glu in domain II region of cry1Ac compared to standard sequence *cry1Ac*. Thus, GM17 isolate was more potent compared to *Btk HD-1* due to possibility of higher affinity binding of Cry toxin to insect gut receptors.

De Maagd *et al*., 1996 and Sakai *et al*., 2001 [23, 24] reported domain swapping of Cry toxin could one of the successful approach to insect resistance management. From current study, Domain II of *cry1Ac* of GM17 isolate could be exchange with different *cry* genes in order to overcome insect resistance. Karlova *et al.* (2005) [25] reported hybrid toxin constructed by domain I & II of Cry1Ca and domain III of Cry1Ac enhanced toxicity 115 times more than Cry1Ca to *H. virescens*. Also, Naimov *et al.* (2001) [26] reported domain I-II of cry 1Ia and domain III of cry1Ba led to change specificity of hybrid toxin towards different order of coleoptera. Thus, present study might be further extend to domain swapping between domain I and II of GM17 to other cry genes in order to insect resistance management in lepidopteran order. Gene pyramiding technique have been demonstrated with Cry1Ac & Cry2Aa by (Maqbool *et al.* 1999) [27] to delay insect resistance. Jianhua Gao. *et al*., 2011 [28] proved transgenic rice bearing fusion protein Cry1Ab and Cry9Aa could significant resist to Oriental armyworm and Asian corn borer. Therefore, Cry2 of GM21 isolate could be

efficiently fused with other Cry toxins in order to combat insect resistance in lepidoptera.

4. Acknowledgement
Jigar V. Shah is thankful to University Grant Commission, New Delhi, India for the fellowship.



Note: B: Bipyramidal, S: spherical, C: cuboidal

Fig 1: Phase contrast Microscopy of GM 21 and GM 17

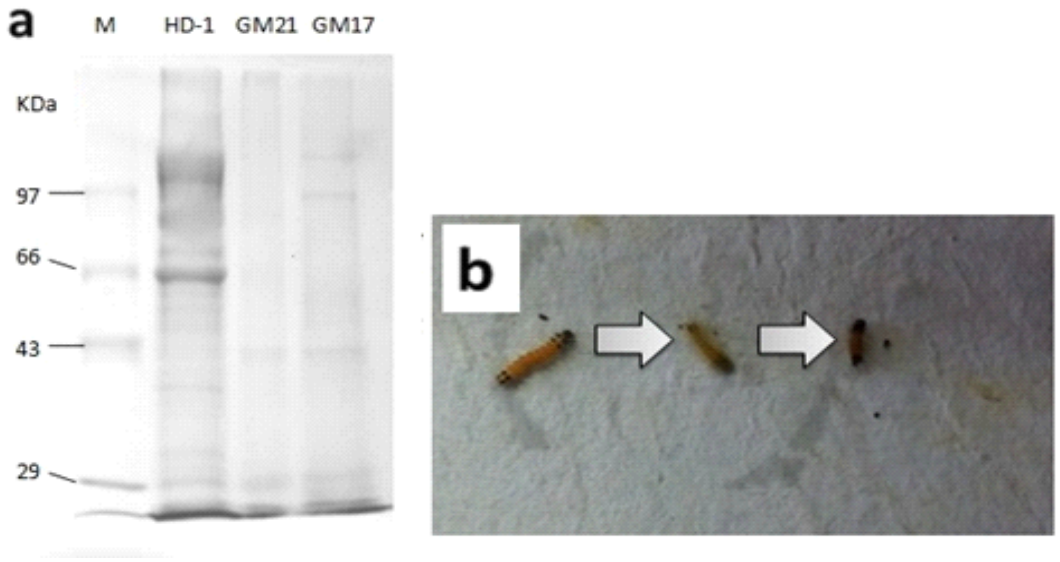


Fig 2: (a) SDS-PAGE analysis of *Btk HD-1* and isolates (b): Bt toxin treated larvae color change from light orange to blackish

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