Food Science and Applied Biotechnology
e-ISSN: 2603-3380

Journal home page: www.ijfsab.com
https://doi.org/10.30721/fsab2023.v6.i1

OPEN ACCESS

Research Article

Isolation and molecular characterization of *Bacillus thuringiensis* strains obtained from different habitats in Northwest Ethiopia

Molaligne Medfu Tarekegn[1], Mekash Teferra[2]

[1] *Department of Plant Science, College of Agriculture and Environmental Science, Debark University, Debark, Ethiopia*
[2] *Department of Agro Economics, College of Agriculture and Environmental Science, Debark University, Debark, Ethiopia*

Abstract

Bacillus thuringiensis (*Bt.*) is ubiquitous, a gram-positive and spore-forming bacterium found in natural habitats everywhere in the world. For this study, *Bt.* has been isolated and characterized using a variety of techniques. Twenty-one strains showed positive results for the four sets of primers including, cry1, cry2, cry3, and cry9 genes. The PCR amplified results cry1 (33.3%) were most abundant among the tested cry-type genes next to cry9 (25%), cry2 (16.6%), and cry3 (12.5%), respectively. Three strains did not amplify. Twenty-four *Bt.* isolates were tested for the bioassay with a third-instar diamondback moth. The mortality of this insect was not shown after treatment for 24h. However, after 48 and 72h showed 20-61% and 20-79% mortality, respectively. The four *Bt.* strains tested against diamondback moth larvae showed no insecticidal activity. Therefore, the isolates in this study were promising for bio-insecticidal properties for diamondback moth and plant pest control programs.

Keywords

Bacillus thuringiensis, *Cry* gene, bioassay, diamondback moth

Abbreviations

ANOVA – analysis of variance, *Bt.* – *Bacillus thuringiensis*

✉*Corresponding author: Molaligne Medfu Tarekegn, Department of Plant Science, College of Agriculture and Environmental Science, Debark University, Debark, Ethiopia, tel.: +251941751625;*
E-mail: medfu2013@gmail.com

Article history:
Received 21 September 2022
Reviewed 7 October 2022
Accepted 17 January 2023
Available on-line 17 March 2023

https://doi.org/10.30721/fsab2023.v6.i1.228

Introduction

Bacillus thuringiensis (*Bt*.) is a gram-positive, rod-shaped, spore-forming bacterium that produces endotoxin, an insecticidal crystal protein (Shishir et al. 2014). These bacteria develop one or two crystalline parasporal inclusions. All through spore germination; *Cry* as well as *Cyt* are encapsulated by the *cry* and *cyt* genes, in both (Schnepf et al. 1998). As a result, *Bt*. has a lot of potential for controlling insect pests from the lepidopteron, dipteran, and coleopteran orders (Khan et al. 2016). Several studies of *Bt*. and its toxicity against a variety of insect pests have recently surfaced (Domínguez-Arrizabalaga et al. 2020; Kamatham et al. 2021).

Many researchers have written about *Bt*. and its toxicity against numerous insect pests and disease vectors (Domínguez-Arrizabalaga et al. 2020). Based on their amino acid sequence homology, 74 classes of *Cry* proteins (*Cry*1 to *Cry*74) and three classes of *Cyt* proteins (*Cyt1*-*Cyt3*) have been shown to have unique binding to the receptor (Jurat-Fuentes and Crickmore 2017). The above particle proteins seem to be protoxins in the midguts of insects, where they are protease transformed in to one of relatively small harmful protein. Active toxins disrupt the epithelial cell membranes of target species, causing them to burst by osmotic lysis (Pardo-Lopez et al. 2013). Humans and other vertebrates are unaffected by these *cry* proteins. The use of synthetic chemical insecticides results from the rise of resistance to insect pests (Ahmad et al. 2008). As a result, ongoing research is underway to identify novel *Bt*. strains with a specific host repertoire or higher toxicity potential.

Industrial bio pesticides are still around the world, whereas conventional industrial insecticides apply in the world (Reyaz et al. 2017). As a result, these toxins' molecular components are about 80,000 times greater than organophosphates and 300 times bigger than synthetic pyroids (Khojand et al. 2013).

Its identification of innovative insecticidal proteins to increased toxic effects may result from the study of novel *Bt*. strains, this is going to critical in terms of offering solutions to deal with the rise of resistant pollinators. As the result, several novels *Bt*. strains must be isolated from different environmental conditions. Insecticidal crystal protein genes have been cloned in a variety of new ways (Khojand et al. 2013).

To classify *Bt*. strains, PCR was commonly utilized (Ganga et al. 2018). The above approach is extremely thoughtful to the quick finding as well as recognition of genetic sequences and simultaneous sampling of several *Bt*. strains help them to identify and forecast pest behaviour (Berón et al. 2005).

Ethiopia is one of the richest sources of genetic variability and biodiversity. However, there is a lack of information on the scientific reports of *Bt*. diversity from Ethiopia (Tenssay et al. 2009). Cabbage is damaged by diamondback moth insects. White cabbage byproduct dietary fiber concentrates (Ulardt et al. 2021). Therefore, the purpose of this study was to isolate, characterize, and bioassay several *Bt*. strains carrying the new *cry* gene from different locations in Northwest Gondar, Ethiopia. The findings of the current study may be beneficial for insect' bio-control programs in the future.

Materials and Methods

Sample collection. The study area was located in the northwest part of Ethiopia. Soil and chicken manure samples were collected from four different sites in northwest Ethiopia. All the soil samples (each ~ 8g) were taken from a depth of 2 to 5 cm of soil with a sterile spatula. The collected samples were stored at 4°C until use.

Reference strain. The Ethiopian Biodiversity Center (Ethiopia, Addis Ababa) provided *Bacillus thuringiensis* subsp. kurstaki HD1 is a positive influence.

Isolation of strains. During the study, the germination of spore-bearing bacteria present in samples was prohibited sodium acetate and non-spore bacteria were killed by heat treatment (Travers et al. 1987). Each sample weighted one gram was accurately weighted and added into tubes containing 10 mL of Luria and nutrient broth liquid medium with 0.25 molar sodium acetate mixed well and then placed on a rotary shaker incubator at 200 rpm for 4 h at 30°C. Then, put in the hot water base and the test tubes were kept at 8°C for 3 min. After that, the solution was serially diluted up to 10^{-5} times (10^{-1}, 10^{-2}, 10^{-5}), with sterile water 20 µL of each dilution sample (10^{-1}, 10^{-2}, 10^{-5}) supernatant was aseptically spread on nutrient agar plates aseptically and incubated at 30°C for 24 h (Al-Sahlany 2015).

***Bt.* isolation using the selective medium.** L-serine manufactured by SENOVA Technology Co., Ltd. in China. L-serine can prevent the growth of *Bacillus* speies, except for *Bt*. The M9 mimium medium containg 0.2 mM per liter of L-serine was used as a selective medium (Andrzejczak et al. 2008). This medium contains 33.7 mM Na_2HPO_4-$2H_2O$, 22 mM KH_2PO_4, 8.55 mM NaCl, 9.5 mM NH_4Cl, 1 mM $MgSO_4$, $CaCl_2$ 0.3 mM, 20g of glucose, 0.2 mM L-serine, and 0.25 M sodium acetate per litter at a pH of 7.2. After inoculation, the plates were incubated at 30°C for 24 to 48 h. The bacteria colonies were grown on a nutrient agar medium containing 0.25 M sodium acetate. The colonies obtained on nutrient agar medium were culture on a selective medium of L-serien for 48 h. The next step was to choose a white single colony and test the strains for the existence of protein crystals. Culture smears were prepared, heat-fixed, and painted with a Coomassie Brilliant Blue G250 stain solution of 0.133% in 50% acetic acid. After that, the smears were carefully cleaned in flowing tap water, blotted dry, and bright-field microscopically examined for the presence of crystalline inclusions. *Bt.* strains were chosen based on the existence of crystalline inclusions.

Molecular identification of *cry* gene screening in indigenous *Bt.* Strains. Four sets of universal primers were used in PCR with *Bt*. genomic DNA for *cry* gene screening. DNA extraction was performed using a DNA extraction kit (HIMEDIA™ Laboratories Pvt. Ltd Company, India) following the protocol for gram-positive bacteria provided by the manufacturer. PCR reactions with *cry*1, *cry*2, *cry*3, and *cry*9 primers were (synthesis of Eurofins Genomics India Company) in Table 1. The PCR activities for the *Cry* genes were performed in a total volume of 20 µL containing 5 µL 1X PCR buffer (containing 10 mM Tris-HCl, pH 8.0 at 25°C, 1.5 mM $MgCl_2$), 2 µL of bacterial genomic DNA, 20 pM.μL^{-1} each of the forward and reverse primers, 0.4 µL of Taq DNA polymerase, 1 µL of 200 µL Md NTPs, 1.5 µL of 3 mM $MgCl_2$, and 9 µL of sterile double distilled water in a final volume of 20 µL. Amplification was performed on a DNA thermal cycler with a step-cycle program package for 35 cycles and a cycle that included denaturation for 2 min at 94°C, followed by 1 minute at 95°C, annealing for 1 min at 48°C for *cry*1 and *cry*3, elongation for 5 min at 72°C, and final extension for 10 min at 72°C for *cry*1 and *cry*3. A single denaturation stage at 94°C for 5 min was followed by 25 amplification cycles, which included denaturation at 94°C for 1 min, annealing at 45°C for 1 min, elongation at 72°Cfor 2 min, and a final extension at 72°C for 10 min for the *cry*2 gene. A single denaturation stage at 94°C for 1 minute was followed by 35 amplification cycles that included denaturation at 94°C for 1 minute, annealing at 60°C for 1 min, elongation at 72°C for 1 min, and a final extension at 72°C for 10 min for the cry9 genes (Jain et al. 2012). At the end amplification 10 µL of each PCR product was taken to electrophoresed in 0.8%-1.5% agarose-EtBr with gel (Tris Borate ETDA electrohoreses buffer pH 8.0) at 100V for 40 min then the DNA bands were visualized using a gel documentation system (Carozzi et al. 1991).

Bioassay for toxicity evaluation of *Bt.* isolates

Spore-endotoxin preparation. Spore-endotoxin preparation of *Bt.* isolates followed the protocol given by Carozzi et al. (1991). To ensure complete lysis of living cells, *Bt*. isolates were grown in nutrient broth media for 7 d. According to light microscopy, the bulk of the population was in the shape of spores-crystals at the end of this incubation.

After being washed twice with pure filtered water, basic samples of spores and toxic crystals (spores and parasporal crystal proteins) were centrifuged at 10,000 rpm for 15 min. The pellets were then dried in a freeze dryer (at a temperature of 50°C) before being tested for biological activity. Toxic sample supernatants were autoclaved for 10 min at 121°C before being used in toxicity tests.

Insect rearing. Larvae of the diamondback moth were collected from the cabbage production field in the irrigated farmland around Gondar Town. Diamondback moth larvae were reared in groups of ten on a wheat germ-based artificial diet, with each group receiving 20 mL of artificial diet (Htwe et al. 2009).

Molecular Biotechnology Laboratory, Department of Biotechnology, University of Gondar, Ethiopia, held the diet and the insects in an environmental chamber at 27°C, 50% relative humidity, and a photoperiod of 16:8 (light/dark, h).

Table 1. Primer sequences for different *cry* genes

Genes selected	Sequence (5'-3')	Product size, bp	Annealing temperature, °C	Reference
Cry1	TGTAGAAGAGGAAGTCTATCCA TATCGGTTTCTGGGAAGTA	272-290	48	(Ceron et al. 1995)
Cry2	TAAAGAAAGTGGGGAGTCTT AACTCCATCGTTATTTGTAG	1556-1523	45	(Sauka et al. 2005)
Cry3	TTAACCGTTTTCGCAGAGA TCCGCACTTCTATGTGTCCAAG	652-733	48	(Ceron et al. 1995)
Cry9	CGGTGTTACTATTAGCGAGGGCGG GTTTGAGCCGCTTCACAGCAATCC	351-354	72	(Ben-Dov et al. 1997)

Bioassay. The diamondback moth larvae's susceptibility to *Cry1Ac* and other *Cry* toxins was determined using a diet overlay assaying system defined by (Zhao et al. 2002). The 3rd instar larvae of the diamondback moth were used for the bioassay. It was applied by taking an aliquot of 0.1 mL of a *Cry* toxin *Bt.* lysate in a diet containing 5 g of wheat germ artificial diet placed in a Petri dish. Six 3rd instar larvae were placed in each assaying petri dish. Each isolate's toxicity was tested three times. A photoperiod of 16:8 (light/dark, h) was used to incubate the Petri dish at room temperature. After three days of feeding the *cry*-toxin-treated diet to the larvae for 24, 48, and 72 h, the toxicity of *Cry* toxins on the larvae was assessed.

Statistical data analysis. The data were recorded for the percentages of *Bt*. isolates obtained from different sample sources, sites, *Bt*. indexes and, molecular patterns of *Cry* genes taken for the study. The bioassay for the mortality of the insect pest was done by using Statistical Package for SAS software. The evaluation of treatments was performed using analysis of variance (ANOVA), and Duncan's multiple ranges tests were used to detect a large variation between treatment means at $p \leq 0.05$.

Results and Discussion

Characterization of *Bt.* Morphology. *Bt*. strains have been found in a variety of environments around the world; including soil microflora and marine habitats (Travers et al. 1987; Unalmis et al. 2018). *Bt.* was used in 32% of the 503 soil samples obtained around Ethiopia's 16 agroecological zones (Tenssay et al. 2009). 24 *Bt.* strains were isolated from soil and chicken manure samples collected from various locations in North Gondar, Ethiopia, for this research. Similar findings were reported by several researchers, (Reyaz et al. 2017) isolated a total of 68 *Bt.* strains of 159 soil samples were collected from the Himalayan valley of Kashmir. Similarly, Shishir et al. (2014) isolated 317 *Bt.* strains from 231 samples collected around Bangladesh's 26 districts, including six separate regions. The most popular approach to classify *Bt.* is to look for crystalline inclusions.

For the high-throughput examination of stained bacterial colonies for the presence of crystals and the detection of small crystals, microscopy is more useful than phase-contrast microscopy (Rabha et al., 2017).

Both of the *Bt.* isolates tested positive for gram-positive bacteria and formed endospores Fig. 1.

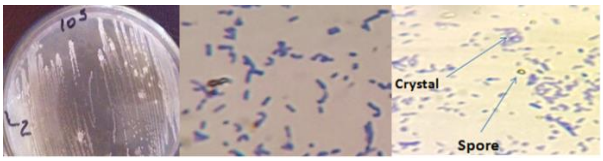

Figure 1. Screening of medium containing L-serine, gram staining, and Coomassie brilliant blue respectively

Similarly, different crystal morphologies were observed. The bipyramidal crystals type of morphology was found at the maximum frequency, followed by spherical, irregular pointed and cubical crystal types. The crystalline inclusions differed in form among the 24 *Bt*. strains in Fig. 2.

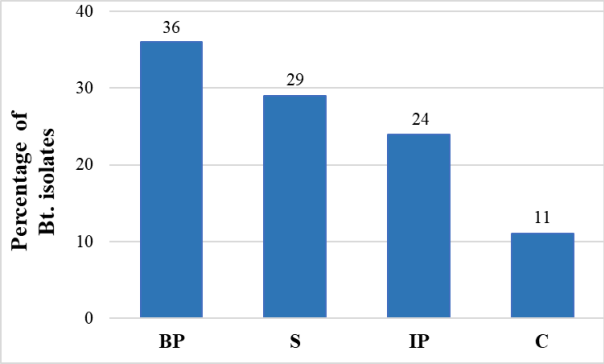

Figure 2. The crystal proteins of *Bt*. isolates
Note: BP – bipyramidal, S – spherical, IP – irregular pointed and C – cuboidal

Shishir et al. (2014) published a related discovery, identifying *Bt.* isolates based on hemolytic behaviour and the existence of parasporal crystal proteins. Five different forms of parasporal crystal proteins, including circular, bipyramidal, triangular pointing, cuboidal, and irregularly shaped crystal proteins, were found among the isolates, formation of irregular white colonies on a pink background. This shows how diverse the local *Bt.* isolates are (Unalmis et al. 2018) isolated *Bt*-like colonies and used Gram staining, spore staining, and crystal staining to identify them. Bright-field microscopy revealed the existence of crystalline inclusions in 28 of 60 bacterial isolates, identifying them as *Bt.* A total of 107 *Bt*. strains were isolated from soil and chicken manure samples obtained in Northwest Ethiopia, by *Bt*. indexes of 0.7 and 0.57, respectively, Table 2.

The 0.86 *Bt*. indexes in their Bangladesh samples were recorded in previous studies (Shishir et al. 2014) various values of the *Bt*. the index has been published in several studies recently, ranging from 0.028 to 0.075 in India (Reyaz et al. 2017). The *Bt.* index, according to may be influenced by biotic environmental factors such as the vegetal top, the type of insect commonly found in the region, or soil microorganisms, as well as abiotic factors such as nutrient availability, texture, pH, temperature, and humidity (Lobo et al. 2018).

Table 2. *Bacillus thuringiensis* isolated

Sample source	TNSC	TNCO	TNIBT	*Bt.* Index
Soil	4	20	16	0.8*
Chicken manure	4	14	8	0.57*
Total	8	34	24	0.7*

Note: TNSC – Total number of samples collected; TNCO – Total number of colonies obtained; TNIBT – Total number of isolates for *Bt.*

* *Bt.* an index is the ratio of the total number of isolates for *Bt*. per the total number of colonies obtained from samples (soil or chicken manure)

They avoid contrasting their findings to those of other scholars because of these considerations. In our study, we observed some differences in the frequency of isolation of *Bt.* from one sample site to another. However, it cannot be said which specific factors influenced the distribution in different habitats. Hence, further examination of the association of the Physico-chemical properties of the habitats of the sample sites with the abundance and distribution of *Bt.* might be required.

***Cry* gene checking using PCR through native *Bt.* Isolates.** The existence of describing the related gene in *Bt.* isolates were confirmed by amplification of the predicted size of PCR products using four sets of universal primers. *Cry1* (33.3%) was the most common *cry*-type gene in the local *Bt.* strains, with all of them carrying the gene, led by *cry9* (25%), *cry*2 (16.6%), and *cry*3 (16.6%) (12.5%) in Table 3.

Table 3. Abundance of *cry*1, *cry*2, *cry*3, and *cry*9 genes of the *Bt.* isolates among the study samples

S. No	Sample's type	*Cry* gene frequence	% of Isolates tested by PCR
1	Chicken manure and soil	8	33.3%
2	Chicken manure and soil	2	16.6%
3	Chicken manure and soil	3	12.5%
4	Chicken manure and soil	9	25%
5	Chicken manure and soil	3	Did not react with four primer
	1, 2, 3 and 9	Soil	15 62.5%
		Chicken manure	6 25%
Total		**21**	**87.5%**

Similarly, the frequency of genes shown was by the frequency stated by several workers among the tested *cry* related gene, *Cry*1 (83.33 %) genes were the most plentiful, followed by *cry*2 (38%), *cry*4 (27.77%), and *cry*3 (16.6%), respectively (Salama et al. 2015). *Cry*-type genes were found 8 *Bt.* isolates (IS1-IS8), according to Jain et al. (2012). based on this, *cry1* type gene was the majority common in indigenous isolates, with all strains containing these genes, come after by *vip3A* (87.5%), *cry2* (75%), *cry9* (62%), *cry3* (50%), *cry11* (37.5%), *cry*7–8 (37.5%), *cry*5, 12, 14, 21 (25%), *cyt*1 (25%), *cry*4 (12.5%), and *cyt*2 (12.5%), as found by PCR. As a result, these findings demonstrate how geographic regions influence the diversity of *cry* gene content in *Bt.* strains.

*Cry*1, *cry*2, and *cry*9 encode Lepidoptera-specific toxins; *cry2* encodes the Diptera-specific toxins, and *cry*3 encodes Coleopteran-specific toxins, according to the findings. As a consequence, 18 isolates having *cry*1, *cry*2, and *cry*9 are at least theoretically toxic to Lepidoptera. Four isolates having *cry*2 were theoretically toxic to Dipteran order. Three isolates having *cry*3 were theoretically toxic to the Coleopteran order. Furthermore, 74 *cry* protein classes (*Cry*1 to *Cry*74) and three *cyt* protein classes (*Cyt*-*Cyt3*) have been shown to have unique binding to the receptor based on amino acid sequence homology (Jurat-Fuentes and Crickmore 2017). In previous studies, various geographical regions of the world recorded differing numbers of isolates generating no PCR commodity (ranging from 14% to 40%) (Lobo et al. 2018). All of these findings point to the existence of a large number of isolates around the world that contain novel and unexplained crystalline protein genes. The findings demonstrated that a PCR-based approach could be used to conduct a systemic, large-scale sampling of *Bt.* isolates to identify cry genes and characterize their toxicity. The existence of the above-mentioned *cry*-type genes and the distribution of *Bt.* strains were confirmed by amplification of the predicted size of the PCR products in the four primers. As a result, *cry* gene identification has significantly improved thanks to the use of PCR. This approach, however, is restricted to members of previously identified gene families and necessitates the use of a large number of primers.

The mortality test of the diamondback moth. Assessment of larval mortalities was ex-pressed as the percentage mortality of total larvae within each treatment and in the control after 3 d. The amount of 0.1 mg.mL^{-1} of the cell pellet (spore endotoxin) from each isolate was used for the bioassay test to detect the most toxic isolates. Water was used as a control. The dead larvae appeared to be dark brown to black and the swelling shrivelled experiments are shown in Fig. 3.

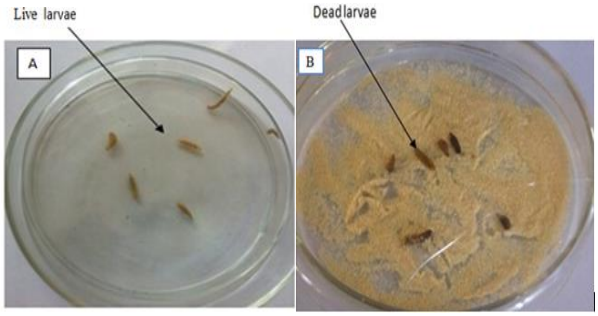

Figure 3.
(A) Live larvae of the diamondback moth; (B) The difference in size between the diamondback moth and an artificial diet supplemented with a spore-endotoxin mixture of *Bt*. isolate

From the 24 isolates used to screen bioactivity against the 3rd instar larvae of the diamondback moth, all 24 isolates caused significant mortality of the third instar diamondback moth, under laboratory conditions after 3 d of treatment compared with the sample treated with water control.

There was a significant difference in the percentage larval mortality efficacies among the 24 isolates and times (h) interval after treatment (isolates: p = 0.0001; h: p = 0.0001), whereas the interaction of isolates and time (h) (p = 0.0002). Without including the water control in the analyses, efficacies among the 24 isolates at 24, 48 and 72 h were significantly different (hours: p = 0.0001).

The results indicated that most larvae did not die within 24 h: it is found that 20% - 70% of the larvae died within 48 h and >80 % at 72 h after treatment. The mortality response of third-instar larvae of the diamondback moth to 24 isolates and time interaction indicated that mortality ranged values varied greatly. The interaction of isolates for 72 h showed a mortality response of 70% to 80%: whereas isolates like GS3, LS11, LS14, LA16, CS18, and CA23 showed mortality responses that ranged between 50 and 70%, isolates GA1, GA5, DA8, GA4, DA9, LS12, LS15, LA17, CS21, GS22,

and LS24 also showed a mortality response between 0% and 50%, isolates likes DS2, DS7, LS13, DS6, DS10, CS19, and GS20 showed a mortality response at a concentration of 0.1 mg.mL^{-1}. No larval mortality was observed with DS6, DS10, CS19, and GS20 from the samples in this study were not toxic to the insects tested against diamondback moth larvae and the control throughout the observation period, as shown in Fig. 4 and Table 4 (Goudar et al. 2012; Topagi et al. 2018) performed a bioassay with 44 strains against diamondback moth third instar larvae and found that total mortality ranged from 17 to 100% after 72 h of exposure.

Table 4. ANOVA was used to determine the larval toxicity of BT against the diamondback moth

Source	DF	Mean Square	F – Value	Pr > F
Isolates	23	0.23740	5.99	< 0.0001
Time	2	6.51437	164.47	< 0.0001
Isolates* time	46	0.08742	2.21	0.0002

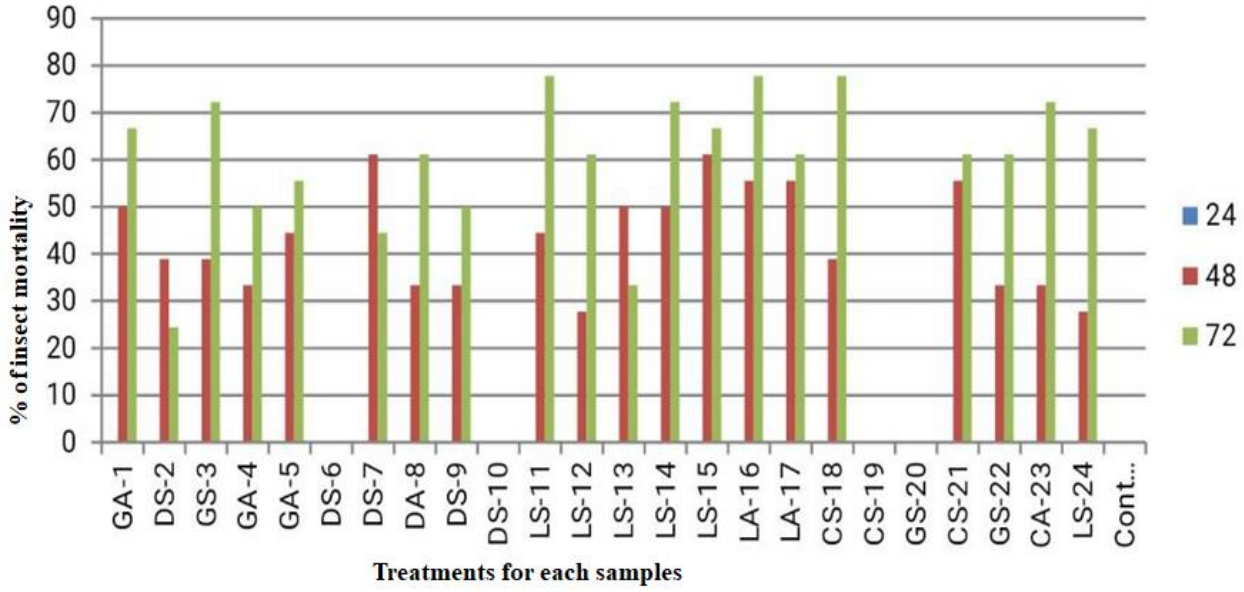

Figure 4. The death rates response of third-instar diamondback moth larvae to 24 isolates and in 24, 48, and 72 h interactions revealed that mortality varied

Note: The color percentage of insect mortality was blue after 24 h, red after 48 h, and light green after 72 h. **CA** – Chiliga chicken manure samples, **CS** – Chiliga soil samples, **DA** – Dembia chicken manure samples, **DS** – Dembia soil samples, **GA** – Gondar Zuria chicken manure samples, **GS** – Gondar Zuria soil samples, **LA** – Lay Armacho chicken manure samples, **LS** – Lay Armacho soil samples.

Conclusions

In this study, the genetic variability was clear among different isolates, thus causing variation in their *cry*-type gene to present great diversity concerning their crystalline protein content and insecticidal activity, even in the isolates from the same sample. This study discovered that 21 isolates out of 24 amplified with the four primer pairs tested, and three of them were *Bt.*, making them potential candidates for diamondback moth control. The highly active, *Bt*. isolates LS11, LA16, and CS18 tested in diamondback moths showed promise for novel insecticide or bio pesticide preparations. For future prospects, experimental studies will be used to create genetically engineered pest-resistant plants and will lead to new avenues in the field of integrated pest control for sustainable crop production.

Acknowledgement

The authors greatly acknowledge the Department of Biotechnology, the University of Gondar for providing the necessary laboratory facilities for conducting the study. The authors are also thank you to Dr Deepak Kumar Verma for primer processing from India and Mr Eyob for their technical support during the study.

References

Ahmad M., Sayyed A.H., Saleem M.A., Ahmad M. Evidence for field evolved resistance to newer insecticides in *Spodoptera litura* (*Lepidoptera: Noctuidae*) from Pakistan. *Crop Protection*, 2008, 27(10): 1367-1372. https://doi.org/10.1016/j.cropro.2008.05.003

Andrzejczak S., Lonc E.L. Selective isolation of *Bacillus thuringiensis* from soil by use of L-serine as minimal medium supplement. *Polish Journal of Microbiology*, 2008, 57(4): 333-335. Available at: http://www.pjmonline.org/wp-content/uploads/2015/12/vol5742008333.pdf

Ben-Dov E., Zaritsky A., Dahan E., Barak Z.E., Sinai R., Manasherob R., Khamraev A., Troitskaya E., Dubitsky A., Berezina N., Margalith Y. Extended screening by PCR for seven cry-group genes from field-collected strains of *Bacillus thuringiensis*. *Applied and Environmental Microbiology*, 1997, 63(12): 4883-4890. https://doi.org/10.1128/aem.63.12.4883-4890.1997

Berón C.M., Curatti L., Salerno G.L. New strategy for identification of novel cry-type genes from *Bacillus thuringiensis* strains. *Applied and Environmental Microbiology*, 2005, 71(2): 761-765. https://doi.org/10.1128/AEM.71.2.761-765.2005

Carozzi N.B., Kramer V.C., Warren G.W., Evola S., Koziel M.G. Prediction of insecticidal activity of *Bacillus thuringiensis* strains by polymerase chain reaction product profiles. *Applied and Environmental Microbiology*, 1991, 57(11): 3057-3061. https://doi.org/10.1128/aem.57.11.3057-3061.1991

Ceron J., Ortíz A., Quintero R., Güereca L., Bravo A. Specific PCR primers directed to identify cryI and cryIII genes within a *Bacillus thuringiensis* strain collection. *Applied and Environmental Microbiology*, 1995, 61(11): 3826-3831. https://doi.org/10.1128/aem.61.11.3826-3831.1995

Domínguez-Arrizabalaga M., Villanueva M., Escriche B., Ancín-Azpilicueta C., Caballero P. Insecticidal activity of *Bacillus thuringiensis* proteins against coleopteran pests. *Toxins*, 2020, 12(7): 430. https://doi.org/10.3390/toxins12070430

Goudar G., Alagawadi A.R., Krishnaraj P.U., Goud K.B. Characterization of *Bacillus thuringiensis* isolates of Western Ghats and their insecticidal activity against diamond back moth (*Plutella xylostella* L.). *Karnataka Journal of Agricultural Sciences*, 2012, 25(2): 99-202.

Htwe A.N., Takasu K., Takagi M. Laboratory rearing of the diamondback moth *Plutella xylostella* (L.). *Journal of the Faculty of Agriculture, Kyushu University*, 2009, 54(1): 147-151. Available at: https://catalog.lib.kyushu-u.ac.jp/opac_download_md/14051/p147.pdf

Jain D., Kachhwaha S., Jain R., Kothari S.L. PCR based detection of cry genes in indigenous strains of *Bacillus thuringiensis* isolated from the soils of Rajasthan. *Indian Journal of Biotechnology*, 2012, 11(1): 491-494. Available at: http://nopr.niscpr.res.in/bitstream/123456789/15692/1/IJBT%2011%284%29%20491-494.pdf

Ganga G.C., Arjya C., Khadka Y., Dhamala S. Diversity of Insecticidal Crystal proteins (ICPs) of indigenous *Bacillus thuringiensis* strains. *Tribhuvan University Journal of Microbiology*, 2018, 5(2108): 11-18. https://doi.org/10.3126/tujm.v5i0.22296

Jurat-Fuentes J.L., Crickmore N. Specificity determinants for Cry insecticidal proteins: Insights from their mode of action. *Journal of Invertebrate Pathology*, 2017, 142(1): 5-10. https://doi.org/10.1016/j.jip.2016.07.018

Kamatham S., Munagapati S., Manikanta K.N., Vulchi R., Chadipiralla K., Indla S.H., Allam U.S. Recent advances in engineering crop plants for resistance to insect pests. *Egyptian Journal of Biological Pest Control*, 2021, 31(1): 1-4. https://doi.org/10.1186/s41938-021-00465-8

Khan M., Paul B., Ahmad W., Paul S., Aggarwal C., Khan Z., Akhtar M. Potential of *Bacillus thuringiensis* in the Management of Pernicious Lepidopteran Pests. In: *Plant, Soil and Microbes* (K. Hakeem, M. Akhtar Eds). Springer, Cham. 2016, pp. 277-301, Print ISBN: 978-3-319-29572-5, eBook ISBN: 978-3-319-29573-2. https://doi.org/10.1007/978-3-319-29573-2_13

Khojand S., Keshavarzi M., Zargari K., Abdolahi H., Rouzbeh F. Presence of multiple cry genes in *Bacillus thuringiensis* isolated from dead cotton bollworm *Heliothis armigera*. *Journal of Agricultural Science and Technology (Iran)*, 2013, 15(6): 1285-1292. Available at: https://www.sid.ir/paper/622069/en

Lobo K.D., Soares-da-Silva J., Silva M.C., Tadei W.P., Polanczyk R.A., Pinheiro V.C. Isolation and molecular characterization of *Bacillus thuringiensis* found in soils of the Cerrado region of Brazil, and their toxicity to Aedes aegypti larvae. *Revista Brasileira de Entomologia*, 2018, 62(1): 5-12.

https://doi.org/10.1016/j.rbe.2017.11.004

Pardo-Lopez L., Soberon M., Bravo A. *Bacillus thuringiensis* insecticidal three-domain Cry toxins: mode of action, insect resistance and consequences for crop protection. *FEMS Microbiology Reviews*, 2013, 37(1): 3-22. https://doi.org/10.1111/j.1574-6976.2012.00341.x

Rabha M., Sharma S., Acharjee S., Sarmah B.K. Isolation and characterization of *Bacillus thuringiensis* strains native to assam soil of North East India. *3 Biotech*, 2017, 7(5): 1-9. https://doi.org/10.1007/s13205-017-0935-y

Reyaz A.L., Gunapriya L., Indra Arulselvi P. Molecular characterization of indigenous *Bacillus thuringiensis* strains isolated from Kashmir valley. *3 Biotech*, 2017, 7(2017): 143. https://doi.org/10.1007/s13205-017-0756-z

Salama H.S., Abd El-Ghany N.M., Saker M.M. Diversity of *Bacillus thuringiensis* isolates from Egyptian soils as shown by molecular characterization. *Journal of Genetic Engineering and Biotechnology*, 2015, 13(2): 101-109. https://doi.org/10.1016/j.jgeb.2015.10.001

Sauka D.H., Cozzi J.G., Benintende G.B. Screening of cry2 genes in *Bacillus thuringiensis* isolates from Argentina. *Antonie Van Leeuwenhoek*, 2005, 88(2): 163-165.https://doi.org/10.1007/s10482-005-3368-2

Schnepf E., Crickmore N., Van Rie J., Lereclus D., Baum J., Feitelson J., Zeigler D.R., Dean D. *Bacillus thuringiensis* and its pesticidal crystal proteins. *Microbiology and Molecular Biology Reviews*, 1998, 62(3): 775-806. https://doi.org/10.1128/mmbr.62.3.775-806.1998

Shishir A., Roy A., Islam N., Rahman A., Khan S.N, Hoq M.M. Abundance and diversity of *Bacillus thuringiensis* in Bangladesh and their cry genes profile. *Frontiers in Environmental Science*, 2014, 2(6): 20. https://doi.org/10.3389/fenvs.2014.00020

Tenssay Z.W., Ashenafi M., Eiler A., Bertilson S. Isolation and characterization of *Bacillus thuringiensis* from soils in contrasting agroecological zones of Ethiopia. *SINET: Ethiopian Journal of Science*, 2009, 32(2): 117-128. https://doi.org/10.4314/sinet.v32i2.68863

Topagi S.C., Bhanu K.R.M., Ashok Kumar C.T. Mass trapping technique using pheromones: A standalone method for management of diamondback moth, *Plutella xylostella* (Linnaeus) (Plutellidae: Lepidoptera) in Cabbage. *International Journal of Applied Science and Engineering*, 2018, 15(3): 211-232. Available at: https://gigvvy.com/journals/ijase/articles/ijase-201812-15-3-211.pdf

Song Y., Liu L., Shen H., You J., Luo Y. Effect of sodium alginate-based edible coating containing different anti-oxidants on quality and shelf life of refrigerated bream (*Megalobrama amblycephala*). *Food Control*, 2011, 22(3-4): 608-615. https://doi.org/10.1016/j.foodcont.2010.10.012

Volpe M.G., Siano F., Paolucci M., Sacco A., Sorrentino A., Malinconico M., Varricchio E. Active edible coating effectiveness in shelf-life enhancement of trout (*Oncorhynchus mykiss*) fillets. *LWT - Food Science and Technology*, 2015, 60(1): 615-622. https://doi.org/10.1016/j.lwt.2014.08.048