Field Evaluation of Native *B. thuringiensis* Isolates Against Aphids (*Aphis fabae*)

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ABSTRACT

Objectives: The purpose of this study was to evaluate the aphicidal activity of native *Bacillus thuringiensis* (Bt) strains.

Methods: Soil samples of Provinces 2 and 3 of Nepal were collected randomly for isolation of Bt by acetate selection method. Bt were identified by observing insecticidal crystal proteins (ICPs) by Commassie Brilliant Blue (CBB) staining technique. Aphicidal activity of 12 *B. thuringiensis* isolates was evaluated by two processes. The preliminary screening was done by spraying the suspension containing the spore and ICPs mixture in *Phaseolus* species heavily infested with black aphids (*Aphis fabae*) in fields. The second process (selective bioassay) was done by counting the number of aphids (nymphs, instar, winged, wingless) before and after spraying 5ml of suspension containing the spore and ICPs mixture on the leaf or on the beans pods surface infested by Aphids. The mortality percentage of Aphids after treatment was calculated on the 4th day, by counting the live aphids and the result was recorded.

Results: Preliminary screening for aphicidal activity revealed that 4 isolates ML5(1), CW1(1), SN2(1) and MP2(1) producing spherical crystal protein, showed 100% mortality against nymphs, instar, winged and wingless Aphids. Isolates were effective in controlling the Aphid (*Aphis fabae*) within 4 days and the part of the plant that was sprayed becomes free of Aphids. Selective bioassay of native isolate MP3(3) was most effective in killing 95.83% of aphids followed by CW2(1), 85.71%, ML5(1), 77.34%, SN3(1), 72.72%, CW1(1), 70.21%.

Conclusion: This study revealed that indigenous *Bacillus thuringiensis* of Terai region of Nepal are effective in controlling Aphids.

Keywords: Aphicidal, ICPs, screening, *Bacillus thuringiensis*

INTRODUCTION

Insects are the major pests of crops. Among insect Aphids are plant lice, sap-sucking insects or phloem feeder, virus vector and enhancer of sooty mold. Aphids vary in their color but most popular ones are green, black, white and yellow. About 5000 species of aphids are known, all of which belong to Phylum- Arthropoda, Class- Insecta, Order- Hemiptera, Family- Aphididae, and Genus- *Aphis*. Around 400 species are serious pests for agricultural and forest plants as well as an annoyance for gardeners (Aphid 2020). Aphid infestation decline and causes extermination of major cash crop production in Nepal. Mustard Aphid declined rapeseed oil production in Chitwan (Kafle and Jaishi 2020). Similarly the cultivation of *vicia faba* in Bhaktapur municipality was declined due to poor efficiency of existing Aphid management practices (Subedi 2015).

Biopesticides appear to be ecologically safer then commercial chemical pesticides because they do not accumulate in the food chain. Biopesticides often are effective in very small quantities and often decompose

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quickly, thereby resulting in lower exposures and largely avoiding the pollution problems caused by conventional pesticides (Ammouneh et al. 2011). When used as a component of Integrated Pest Management (IPM) programs, biopesticides can greatly decrease the use of conventional pesticides, while crop yields remain high. Biopesticides falls in major three categories- microbial pesticides, plant incorporated protectants and biochemical pesticides (Çetinkaya 2002; Leahy et al. 2014; Damalas 2018). Biopesticide use at a global scale is increasing by almost 10% every year (Damalas, 2018). Various types of microorganism that can be used as biopesticides are bacteria, fungi and viruses. Almost 90% of the microbial biopesticides currently available in the market are derived from only one entomopathogenic bacterium i.e. *Bacillus thuringiensis* (Bt) (Leahy et al. 2014; Damalas 2018).

Bacillus thuringiensis is a bacterium known for producing protein crystals with pesticidal properties. *Bacillus thuringiensis* are Gram positive, rod shaped, spore forming, soil dwellers. During sporulation it produces insecticidal parasporal crystal proteins that are toxic against many insect pests. When orally ingested by insects, this crystal protein is solubilized in the mid gut of the insect and the insect dies within 6-12 hours (Schünemann, Knaak and Fiuza 2014). *Bacillus thuringiensis* biopesticide is called as Bt. Bt has been used commercially in the biological control of insect pests for the last 4 decades. These toxins are widely sought for controlling agricultural pests due to both their specificity and their applicability.

This study aims at isolating *Bacillus thuringiensis* from soil for controlling insect pest.

MATERIALS AND METHODS

Sample collection

Soil samples (50) were randomly collected from tropical regions of 2 provinces of Nepal (Province No: 2 Parsa, Bara, Rautahat, Sarlahi, Mahottari and Dhanusa and Province No: 3 Sindhuli, Chitwan and Makwanpur). Soil weighing about 10 grams was obtained from 5cm depth in a plastic bag (Soares-da-Silva et al. 2017). Collected samples were stored at 4°C before processing.

Isolation of *Bacillus thuringiensis*

Isolation was carried out using the acetate selection method as described by (Travers, Russells, Martin, Phyllis, and Reichelderfer, 1987). The Nutrient broth (NB) was acetated by using 0.25M sodium acetate for selective enrichment of *Bacillus thuringiensis*. To the sterile 9 ml enriched NB broth media 1gram of soil sample was added and incubated overnight at 35°C. After incubation the broth was heated at 100°C for 5 minutes as described by (Apaydin, Ozgur, Yenidunya, A. Fazil, Harsa, Sebnem and Gunes, 2005) with slight modification. Following heat treatment, the sample was serially diluted in dilution blank and the suspension was inoculated on Nutrient agar plate (NA) by spread plate technique and incubated at 35°C for 24 hours. The isolated colonies were further sub-cultured in Nutrient agar (NA) to obtain the pure culture.

Phenotypic characterization

Coomassie Brilliant Blue staining technique was performed to detect the presence of crystal protein production in the cells after incubation of culture for 72 hours in a Nutrient broth to distinguish from other *Bacillus* spp as described by Rampersad, Khan and Ammons (2002). Physio chemical characterization was done by biochemical tests like, Catalase test, Oxidase test, Indole test, Methyl Red (MR) test, Voges Proskauer (VP) test, Citrate test, Starch hydrolysis test, Gelatin hydrolysis test, beta-haemolysis test, motility test, sucrose, fructose, mannitol, lactose fermentation tests and lecithinase test in the respective biochemical test media and the result was recorded after 24 hours incubation. The identified isolates were preserved in nutrient agar media by adding 60% glycerol.

Formulation of bioinsecticide

Formulation of bioinsecticide was performed by submerged fermentation (SmF) /liquid fermentation (LF) technique as described by (Ralte, Nachimuthu, & Guruswami 2016) with slight modification by inoculating the isolates in a 250ml conical flask containing sterilized Luria-Bertani (LB) broth (Tryptone 10g/lt, NaCl 10g/lt and yeast extract 5g/lt). The conical flask was placed in a shaker water bath at 35^0C for 90hrs until the colony forming unit (cfu/ml) was greater than 10^9 and the bioactive ICPs (Insecticidal Crystal Proteins) was maximum when observed by light microscopy (Ammouneh et al. 2011). The formulated biopesticide was used to control the Aphids.

Rearing of aphids

For rearing of Aphid natural environment was used (Madant et al. 2016). Native host *Phaseolus* spp plant was planted in the garden. Using compost as a fertilizer the plant was allowed to grow till maturation producing

lots of flowers and pods and it was allowed to infest by aphids naturally (Abderrahmane 2015). The Aphid culture was maintained on *Phaseolus* spp plant during the study period. The plants were irrigated regularly and fertilized by using compost. No pesticides or any other chemicals were used during its growth (Photograph 2a).

Bioassay

The insecticidal property of the isolated *B. thuringiensis* against aphids was evaluated by two processes preliminary screening and selective bioassay.

Preliminary screening for aphicidal activity in the field

Preliminary screening was performed by foliar spraying the suspension containing the spore and the ICPs mixture (crude mixture) of randomly selected 4 isolates (ML5(1), CW1(1), SN2(1) and MP2(1)) (El-Kersh et al. 2016) in *Phaseolus* species heavily infested with black aphids (*Aphis fabae*) in field. The aphicidal activity was observed in nymphs, instars, wingless, and winged aphids. The mixture (spores and ICPs) was formulated as mentioned above. Crude suspension 50ml was used as bio insecticide without dilution and sprayed by use of manual sprayer in a heavily infested plant part (Abderrahmane 2015). The process was duplicated with each isolates. The sprayed part was observed for 4 days.

Selective bioassay

The second process was done by counting the number of aphids (nymphs, instar, winged, wingless) present on the leaf or on the beans/ pods surface before and after spraying. The spray suspension for selective bioassay was prepared by centrifuging the fermented broth at 5000rpm for 10minutes and the sediment was serially diluted to obtain 10^9 cfu /ml in saline solution (to remove soluble exotoxins, bacterial cultures were centrifuged at 5000rmp) (Heidari and Zeinali, 2019). The 10^9 cfu/ml concentration of biopesticides of 12 isolates of 5ml was spread onto the infested leaves and beans pods surface containing the counted aphids, the mortality percentage was calculated by counting the live aphids on the 4thdays and the result was recorded (Lobo *et al.* 2018). The infested leaves were kept fresh till the 4th day by spraying water. Mortality was calculated by using the following formula.

$$Mortality\ P\% = \frac{Total\ No.of\ Aphids - No.of\ live\ Aphids}{Total\ No.of\ Aphids} \times 100\%$$

RESULTS

***Bacillus thuringiensis* isolation and identification**

From the randomly collected 50 soil samples, 84 isolates of *Bacillus thuringiensis* (Bt) were obtained. The colony morphology revealed that 7 different types of colony morphologies were produced by the Bt strains Table 1. Fried egg type of colony was present in all the soil samples collected Photograph 1. The light microscopic morphology revealed that they were Gram positive, spore producing and their vegetative size varied. Presence of ICPs and their shapes was confirmed by Coomassie Brilliant Blue staining technique by light microscopy. Five different crystal morphology were observed: Amorphous (17.85%), Rod (53.57%), Spherical (10.71%), Ovoid (8.3%) and Cap headed (9.5%) Fig 1.

Table 1: Types of Colony Morphology and the Number of Isolates

S.N.	Sample size	Type of colony Morphology	Colony code	No. of isolates	Percentage
1		White, raised wavy (fried egg type)	A	50	100%
2		White, flat, irregular	B	23	46%
3		Yellow, raised, smooth	C	1	2%
4	50	White, raised, round, smooth, mucoid	D	1	2%
5		Shiny(watery type),raised, round	E	1	2%
6		White rhizoid type	F	1	2%
7		White membraneouse slightly raised center	G	7	14%
	Total			**84**	

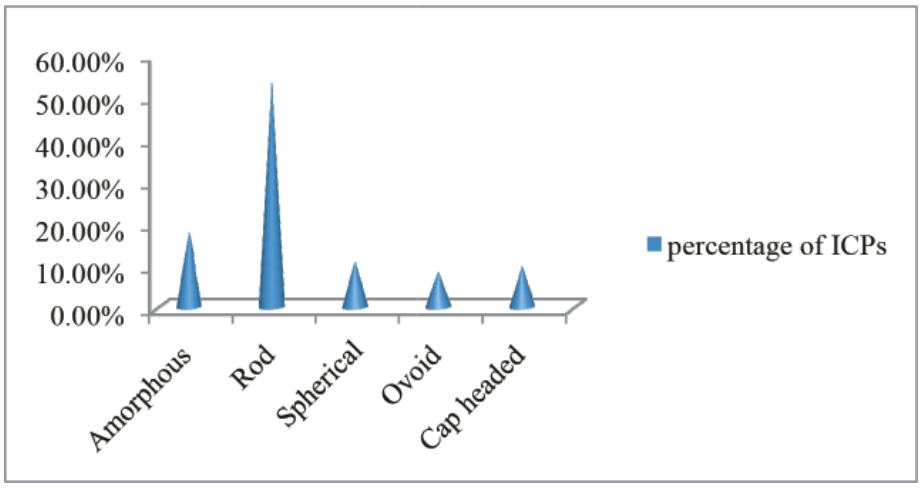

Figure 1: Types of ICPs and their frequency.

Photograph 1: Isolates obtained from the sample ML5(1) by spread plate technique. Only 2 colony morphology were observed in this sample (creamy white fried egg & flat pale yellow).

The dominant type of ICPs was rod shaped. Based on the biochemical characteristics, all the isolates were positive for Catalase, Oxidase, Starch hydrolysis, Gelatin hydrolysis, beta - hemolysis, lecithinase and sucrose, fructose, mannitol, and lactose fermentation tests but showed variable reaction in Indole, MR, VP, Motility and Citrate tests.

Bioassay

Preliminary screening: Preliminary screening for aphicidal activity of the 4 isolates ML5(1), CW1(1), SN2(1) and MP2(1) of native *Bacillus thuringiensis* (Bt) revealed 100% mortality, against nymphs, instar, winged and wingless aphids. Isolates were effective in controlling the aphid within 4 days the plant part that was sprayed became free of aphids Photograph 2. These four isolates produced spherical shaped crystal protein.

(A) (B)

Photograph 2: Preliminary screening of insecticidal property of native isolates (a) heavily infested *Phaseolus* plant before spraying (b) *Phaseolus* plant free of *Aphis fabae* on 4th day.

Selective bioassay: The mortality percentage of aphids after treatment (spraying 5ml of diluted pellets with 109cfu/ml with 10^9cfu/ml of the suspension containing spores and the ICPs) on the leaf and pods surface was calculated on the 4th day as shown in Table: 2.

Table 2: Effectiveness of each Bt isolates in terms of Aphid Mortality percentage

SN	Isolates	Types of crystal	Mortality %
1	P1(1)	Spherical	56.00
2	CW3(1)	Spherical	48.14
3	CW1(1)	Spherical	70.21
4	SN3(1)	Spherical	72.72
5	ML5(1)	Spherical	77.34
6	CW2(1)	Spherical	85.71
7	MP3(3)	Short rods	95.83
8	SN3(3)	Short rods	43.80
9	CW3(3)	Short rods	67.64
10	D2(2)	Long rod	39.65
11	CW2(2)	Long rod	43.90
12	D3(2)	Long rod	42.15

Native isolate MP3(3) was most effective in killing 95.83% of aphids followed by CW2(1), 85.71%, ML5(1) 77.34%, SN3(1) 72.72%, CW1(1) 70.21%. In an average 40%, mortality rate was observed in other isolates.

DISCUSSION

All the 50 soil samples processed for the isolation of Bt showed the presence of Bt strain and a total of 84 Bt isolates were obtained. The isolates were confirmed as Bt by detecting the crystal protein by Coomassie brilliant blue staining (CBBS) technique. Coomassie brilliant blue R-250 (CBB) is a popular dye used for detection of proteins. Crystal protein of Bt stained by CBB appear as dark blue color and the spore remain unstained and the vegetative cell took up the light blue stain as describe by (Rampersad et al. 2002). In this study, the numbers of isolates are greater than the number of soil samples processed for isolation Table 1. These findings differ from earlier reports by (Ralte, Nachimuthu and Guruswami, 2016) who isolated 29 Bt isolates from 55 soil samples. This may be due to focusing on only one type of colony morphology either to fried egg or white glossy or rough creamy etc, neglecting other types of colony morphology which may also harbor crystal protein producing gene. In this research, Bt with diverse type of colony morphology was observed, and produced ICPs. The isolates produced 7 different types of colony morphology indicating the presence of

different strains of Bt (Table 1). The dominant colony type was fried egg, which was present in all the 50 soil samples (frequency 100%), followed by flat white type of colony (frequency 46%). In this research, enrichment was performed by acetate selection method, followed by isolation in NA (composition) as described by (Travers, Russells, Martin, Phyllis, and Reichelderfer, 1987). On an average, only 3 to 4 different colony morphologies developed in the agar media by spread plate technique (Photograph 1). All of which demonstrated the presence of ICPs by CBB staining. In case of others research the isolation technique as well as the media used is different, and they focus on particular colony morphology so the isolates are less in numbers. Other colony morphology, colony codes C, D, E, F and G are less commonly isolated during this research. This may be due to, the isolates have gained the plasmid that carry cry gene from the prevalent strains and appear as positive Bt strain (Table 1). As the ICPs producing gene is located in the plasmid and in the chromosome (Reyaz et al. 2013). The plasmid might have been transmitted to the same type of strain by conjugation or the selection process, temperature or media used may have been favorable for the growth of such types of isolates. Diversity, distribution and abundance of *cry* gene types are dependent on the geographical area where *B. thuringiensis* strains were collected as well as the cultural condition provided may enhance in the isolation of organism with different ICPs producing isolates (Tuba, 2002). Even though the colony morphology remained same, some isolates produced different shapes of ICPs indicating that different *cry* gene may present and expressed in a particular environment. The plasmid may harbor different types of genes in a bacterial cell or different plasmid harbors different genes. More than one plasmid is present in Bt. The plasmids may contain different *cry* gene responsible for producing different types of crystal protein (Rangeshwaran et al. 2014).

Randomly selected 4 isolates (ML5(1), CW1(1), MP2(1) and SN2(1)) producing spherical shaped ICPs were used for screening aphicidal activity by preliminary bioassay against nymphs, instar, winged and wingless Aphids by spraying about 50ml mixture containing endospore count $>10^9$ cfu/ml and ICPs, on a heavily infested part of the plant (Photograph 2) within 4 days cent percent mortality was observed on *Aphis fabae*. The result was consistent with (Palma et al. 2014) cry protein (cry2A, cry3A, cry11A) of Bt show toxicity against hemiptera. So four isolates producing spherical ICPs harbors either of these cry protein producing genes and thus are able to show 100% mortality against *Aphis fabae*. In this research, the concentration of ICPs was not determined but presence of excess ICPs i.e the crystal shapes was observed by light microscopy and the spore count present in the broth was 10^9cfu/ml as determined by endospores count method in NA. Aphids take up the nutrients for their survival by suckling the sap from the plants. When the aphicidal mixture (spore and ICPs) is present in higher dose on the surface of the leaves and on stems, the mixture is also absorbed by the plant tissues. While suckling the nutrients, the Aphids consumes the aphicidal mixture along with the nutrients causing toxicity to Aphids. According to (Elatti et al. 2010) toxins should be circulated throughout the phloem to control suckling insects like Aphids., Thus it ensures effective control of aphids. Thus the study suggests high dose and excess volume of aphicidal concentration (biomass, Bt spore and the ICPs) must be used to control Aphid pests in the field. In this bioassay, bacterial culture suspension as a whole was used as an insecticides, whole of the bacterial culture suspensions may contain in addition to cry and cyt protein other secreted toxins like vegetative insecticidal proteins (Vip) and secreted insecticidal proteins (Sip)(Schnepf et al. 1998) that are secreted into the medium and have insecticidal properties against aphids. The part of phaseolus plant which was heavily infested by Aphids was observed to have dried out after their mortality. The sprayed biopesticide mixture was sucked by the aphids, after ingestion of the of mixture, Aphids got paralyzed, were not able to move to other parts of plants for feeding, so the infested part dried out and the aphids were turned to black powder on the surface of infected plant part. The infested plant part dried out because of excess sucking of sap by the paralyzed aphid. According to the observation of this study, crude extract, mixture of proteins (cry, cyt, Vip, sip) present in the suspension kills aphids within 4 days but spraying only once does not make the plants free of aphids for the whole season. So repeated spraying in the infested parts should be carried out. For effective control of Aphids Bt gene should be incorporated in the plant so the plant itself can synthesize the ICPs to control the Aphids it would be much better to incorporate a cry gene showing abroad spectrum of activity towards insect pests. Selective bioassay performed by using 12 native isolates showed a different mortality rate (Table

2). The most effective native isolate MP3(3) showed 95.83% of aphicidal activity followed by CW2(1), 85.71%. In an average, 12 isolates were effective in controlling the aphids. Based on the toxicity test, the type of *cry* gene present in the isolates may be the *cry1I, cry2, cry3, cry10* and c*ry11*. According to Ibrahim et al. (2010) based on the insecticidal toxicity test the Bt are categorized as Group 1—lepidopteran (*cry1, cry9* and *cry15*); Group 2—lepidopteran and dipteran (*cry2*); Group 3—coleopteran (*cry3, cry7* and *cry8*); Group 4—dipteran (*cry4, cry10, cry11, cry16, cry17, cry19* and *cry20*); Group 5—lepidopteran and coleopteran (*cry1I*); and Group 6—nematodes (*cry6*). The *cry1I, cry2, cry3, cry10* and *cry11* toxins (73–82 kDa) are unique because they appear to be natural truncations of the larger Cry1 and Cry4 proteins (130–140 kDa). *Aphis fabae* belong to Order Hemiptera, the native isolates showing insecticidal activity towards this Aphids may contain *cry1I, cry2, cry3, cry10* and *cry11* or other types of genes.

Less effectiveness by selective bioassay may be due to the absence of other exotoxins like (Vip), (Sip) which are removed by centrifugation. So only the ICPs or cry proteins is less effective in killing the Aphids.

Based on the morphology of ICPs, the indigenous Bt stains can be related to the type of *cry* gene present in it. Spherical and ovoid related to *cry1*or *cry3* or *cry8* or *cry9*,(Tuba 2002; Zonthansanga et al. 2016). Rod shaped ICPs may be related to rectangular type, that relates to *cry1* gene (Tuba, 2002). The reference strain used during the study *Bacillus thuringiensis* var *Kurstaki*, serotype 3a, 3b, 3c, Strain DOR Bt-1 also produced spherical type of crystal protein (Cry1).

CONCLUSION

This study reveals that indigenous *Bacillus thuringiensis* isolated from soil of Terai region of Nepal exists in variable diversity. The native Bt isolates with spherical and rod shaped ICPs are effective in controlling Aphids.

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