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# Bioremedy of Cotton Aphid (*Aphis gossypii* Glov.) (Hemiptera: Aphididae) by the Application of Different Fractions of Entomopathogenic Bacteria (*Xenorhabdus* Spp.)

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**EYI and AAN performed, analyzed and interpreted the aphids data regarding the biocontrol potential of bacteria. SF and SD supervised this research and helped in writing the manuscript.**

**Key words**
**Cotton aphid, EPB, Crude cell extract, Cell free Suspension, bacterial culture, methanol extract**

## ABSTRACT

Molecularly identified indigenous entomopathogenic bacteria (EPB) *Xenorhabdus* have been used to access their efficiency against cotton Aphid *Aphis gossypii* (Glov.). An efficient formulation of different parts of bacterial culture such as, cell-free supernatant, crude cell extract, bacterial culture and methanol extract in *in vitro* and green house condition have been exploited. The results have shown that different formulants of EPB produced significant effects on Aphid mortality, change in population density, population reduction and fecundity. Cell-free supernatant of new EPB species *Xenorhabdus steinernematis* n.sp. strain PAK. CB10 (KU097324) and the other *X. indica* PAK. S.B.56 (MF521953) resulted in both the highest mortality rate [(94.33±2.05, 100.00±0.00)%] @ 300µl/10ml at 30ºC and the lowest fecundity [(65.00±3.00) eggs/gravid female] in green house condition after 5 days of treatment. Crude cell extract of all bacterial fractions were found to be least effective as compared to cell-free supernatant, bacterial suspension and methanol extract suggesting that EPB has the potential to release its metabolites with insecticidal mode of actions in the surrounding culture media. New species proficiency was evaluated in comparison with other indigenous isolates and resulted in most efficient at 30ºC after 24h and proved to have good effectiveness as biological control agent and can be easily used instead of live bacteria in future formulations.

## INTRODUCTION

Aphids possibly are important crop pests globally (Blackman and Eastop, 2000). Due to small size, high reproductive potential, cryptic nature of feeding and ability to act as vectors of plant viruses, they have dominating value (Clark and Perry, 2002; Davis *et al*., 2005). Aphids like other phloem feeding insects, can exhibit a considerable degree of host specificity. (Hooks and Fereres, 2006; Rasmussen *et al*., 2008; Catangui *et al*., 2009).

Biological agents or biocontrol agents, for controlling agricultural pests have been widely used for several years. This is partly due to the strict governmental regulations for the use of hazardous chemicals, as these chemicals can remain prolong in the food chain (Bro-Rasmussen, 1996; Fareeha *et al*., 2017). During last two decades, a number of entomopathogenic bacteria (ENB) have gained considerable interest as biological control agents. Gram-negative bacteria *Xenorhabdus* spp. that belongs to the family Enterobacteriaceae and symbiotically associated with entomopathogenic nematodes of the family Steinernematidae (Boemare *et al*., 1993; Forst *et al*., 1997) has proved composite relation between these bacteria and their nematodes. Their association has justified the severe toxicity against many insect species (Akhurst, 1983; Herbert and Goodrich-Blair, 2007; Herbert *et al*., 2009). Nematodes release the symbiotic bacteria from their intestinal tracts into the insect host's heomocoel, as they infest their insect hosts, as a results of this action, death of insect hosts happened within 48 h, this is a resultant of the combined actions of bacterial propagation, septicemia, and toxins secretion (Bowen and Ensign, 1998; Sicard *et al*., 2004; Yang *et al*., 2012).

Both *Xenorhabdus* and *Photorhabdus* bacteria have been shown to grow successfully under laboratory conditions, and both cell suspensions and cell-free supernatants of these bacteria have been found to cause

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adverse effect and cause the death of several insect pests, such as desert locust *Schistocerca gregaria* (Forskål) (Mahar *et al*., 2004), red flour beetle *Tribolium castaneum* (Herbst) (Shrestha and Kim, 2010), wax moth *Galleria mellonella* (L.), beet armyworm *Spodoptera exigua* (Hübner), diamondback moth *Plutella xylostella* (L.), cotton leafworm moth *Spodoptera littoralis* (Boisduval) (Campos-Herrera *et al*., 2009) and black vine weevil *Otiorhynchus sulcatus* (Germar) (Mahar *et al*., 2008). Hence, these bacteria secrete their metabolic products that are toxic or immunosuppressive to the host insects cause the death of several important agricultural insect pests (Bowen *et al*., 2000; Chongchitmate *et al*., 2005; Grewal *et al*., 2005; Sharma *et al*., 2002; Mahar *et al*., 2004, 2008; Bode, 2009; Brivio *et al*., 2010). Effects of a protease inhibitor protein from *Xenorhabdus bovienii* on physiology of pea aphid (*Acyrthosiphon pisum*) was also determined (Jin *et al*., 2014).

Herein, this study was aimed to determine the aphicidal activity of whole and in fractions of indigenous and a new EPB species *Xenorhabdus steinernematis* n.sp. strain PAK. CB10 (Erum *et al*., 2019) formulants against Cotton Aphid.

## MATERIALS AND METHODS

Cotton Aphids were obtained from the Pakistan Agriculture Research Council, Pakistan and were grown on a mixture of sawdust and soybean grain to establish a fresh spawn (Bussaman *et al*., 2009). A fresh aphid progeny in a glass bottle was used to maintain a pair of male and female at 28 °C for reproduction. This domestic bred was used for all experiments.

Entomopathogenic bacteria symbiotically associated with entomopathogenic nematodes were isolated from surface-sterilized infected *Galleria mellonela* larvae, (Table I). EPNs were obtained from culture stock of National Nematological Research Centre, University of Karachi maintained by Prof. Dr. Shahina Fayyaz and bacterial isolation method was followed, previously described by (Kaya and Stock, 1997).

*Culturing of EPB*

A nutrient bromothymol blue triphenyltetrazolium chloride agar (NBTA) medium, consisting of 37 g nutrient agar (Criterion, USA), 25 mg bromothymol blue powder (Lab-Chem, UK), 4 ml of 0.01 g/ml 2, 3, 5-triphenyltetrazolium chloride (Sigma-Aldrich, USA), in 1000 ml distilled water, was used to prepare culture plates for EPB (Lacey, 1997). The bacteria were spread onto NBTA plates, and the plates were sealed and incubated in the dark at 28 °C for 24h. These bacteria were found to form blue colonies on NBTA agar (indicated Phase I stage) (Stock *et al*., 1998; Grewal *et al*., 2005) and the colonies were selected and sub-cultured to acquire colonies with uniform characteristics. The selected colonies were individually grown in 25 ml of Luria Bertani (LB) broth (Sigma-Aldrich, USA) and placed in the incubator shaker (200 rpm/min) at 28 °C for 48h under complete darkness (Lacey, 1997). The concentration of whole bacterial cell suspension was determined by the plate-count technique (Klement *et al*., 1990) and adjusted to $1x10^4$ colony-forming units per ml (CFU/ml) using sterile 1 g/L peptone solution. To obtain cell-free supernatant, cell suspension was centrifuged at 2500×g and 4 °C for 5 min and filtered using a 0.22-µm filter. The resulting bacterial pellets was collected and used for preparing crude cell extract dilutions. A total of 100 ml of LB broth was added to 1 g (approximately $1×10^4$ cells) of bacterial pellets vortex for 1 min. these were then used as stock and for preparing further concentrations. Bacterial methanolic extract procedure was adapted from (Boszormenyi *et al*., 2009). For a single treatment 15.4 µg/ ml was examined for it efficeincy.

*Treatment preparation of EPB*

In order to determine the insecticidal potential of different EPB spp. strains, the bacterial culture (@ $10^4$ CFU/ml), bacterial cell free supernatant and crude cell extract (100,200,300µl/10ml) and methanol extract were tested in *in vitro* and *in vivo* against aphids.

Imidacloprid (50 µg/mL) (Product code: 00231, Brand: SKY SEEDS) was used as positive control. As negative controls, sterile cell-free LB was used for comparison with the whole culture, supernatant and bacterial residual treatments.

*Aphicidal activity of different* Xenorhabdus *species on mortality of cotton aphids*

Effect of different EPB were investigated using one hundred adult aphid female (1 d old) transferred to each (10-mm diameter × 10 inches in height) glass jars for each treatment. A total of 10ml ($10^4$ CFU/ml) of bacterial culture, cell-free supernatant and bacterial crude cell extract and methanol extract (100, 200, 300 µl/10ml) were then sprayed onto the aphids separately. The same volumes of LB broth and Imidacloprid (a commercial aphicide) at the concentration of 0.04% were used as negative and positive control groups, respectively (four replications/ treatment). All jars were covered with lids and placed in a temperature controlled chamber at 25, 30 and 35°C with 80% relative humidity in complete darkness. Aphids mortality was observed every 24 h for four consecutive days after treatment. The experiment was repeated twice.

**Table I. Mortality rates of *cotton aphids* after treated with different fractions of EPB (crude cell extract, cell-free supernatant, bacterial culture and methanol extract) at different temperatures and durations.**

| Treatments | Bacterial | Mortality % | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Bacterial strains | fraction and concentration | 1 h | | | 24 h | | | 48 h | | |
| | | 25°C | 30°C | 35°C | 25°C | 30°C | 35°C | 25°C | 30°C | 35°C |
| Pak.S.B.50 | CFS | | | | | | | | | |
| | 100µl/10ml | 17.66± 0.57aB | 16.66± 0.94aA | 11.33± 1.69bA | 64.33± 1.69aC | 75.00± 2.44bC | 61.66± 2.49aC | 70.33± 1.69aC | 80.00± 4.65aC | 65.33± 3.26aC |
| | 200µl/10ml | 18.33± 0.47aB | 19.66± 1.69aB | 11.33± 1.88bA | 64.33± 4.49aC | 73.66± 3.29bC | 63.00± 1.63aC | 70.66± 2.16aC | 82.33± 6.32aC | 67.33± 4.21bC |
| | 300µl/10ml | 18.66± 0.47aB | 16.66± 1.24aA | 11.66± 0.47bA | 74.00± 6.37aC | 84.66± 3.68bC | 64.00± 1.63aC | 72.00± 0.81aC | 84.66± 4.21ac | 69.66± 2.33aC |
| | CCE | | | | | | | | | |
| | 100µl/10ml | 2.33± 0.47aA | 6.00± 2.16bA | 1.33± 0.47aA | 6.33± 2.49aB | 6.33± 2.05aA | 3.00± 0.81bA | 19.66± 1.69aB | 23.66± 2.26aB | 20.33± 1.05aB |
| | 200µl/10ml | 1.66± 0.47aA | 7.00± 1.41bA | 2.33± 0.47aA | 10.66± 2.49cB | 6.00± 2.16aA | 6.00± 2.16aA | 21.00± 2.49aB | 24.00± 4.21aB | 24.33± 2.35aB |
| | 300µl/10ml | 2.66± 0.47aA | 6.33± 0.94bA | 2.33± 1.24aA | 7.33± 2.49aB | 6.66± 1.69aA | 3.66± 1.24bA | 22.33± 2.35aB | 26.33± 2.36aB | 20.66± 1.24aB |
| | BC ($10^4$ CFU/ ml) | 17.66± 0.47aB | 16.66± 2.05aB | 14.66± 1.24aB | 61.33± 3.29aB | 68.00± 2.16aC | 62.00± 1.63aC | 86.33± 4.02aC | 84.22± 5.63aC | 85.33± 3.36aC |
| | ME | 16.66± 0.47aB | 13.33± 1.24aB | 13.00± 2.160aB | 63.33± 1.24aB | 72.00± 1.63bC | 64.33± 2.49aC | 78.22± 4.10aC | 88.66± 5.36bC | 85.33± 2.35bC |
| Pak.S.B.56 | CFS | | | | | | | | | |
| | 100µl/10ml | 15.00± 0.81aB | 17.33± 1.69aA | 12.00± 2.16bB | 88.66± 1.4aC | 96.00± 2.16bE | 68.33± 3.09cC | 100.00± 0.00aE | 100.00± 0.00aE | 100.00± 0.00aE |
| | 200µl/10ml | 21.00± 0.81aB | 21.66± 1.69aB | 15.00± 0.81bB | 85.66± 4.02aC | 100.00± 0.00bE | 65.00± 2.44cC | 100.00± 0.00aE | 100.00± 0.00aE | 100.00± 0.00aE |
| | 300µl/10ml | 21.66± 1.24B | 22.33± 1.24aB | 16.66± 0.47bB | 93.33± 3.39bE | 100.00± 0.00bE | 72.66± 2.63cC | 100.00± 0.00aE | 100.00± 0.00aE | 100.00± 0.00aE |
| | CCE | | | | | | | | | |
| | 100µl/10ml | 3.66± 0.94aA | 5.66± 0.47aA | 4.00± 1.41aA | 16.00± 0.81aA | 15.66± 3.29aB | 22.00± 1.41bB | 29.66± 2.62aB | 20.33± 2.15bB | 25.33± 2.26aB |
| | 200µl/10ml | 1.33± 0.47aA | 7.33± 1.24bA | 4.33± 1.69cA | 16.00± 2.16aA | 23.00± 2.16bB | 19.66± 3.68aB | 29.33± 3.68aB | 21.66± 1.25bB | 29.66± 2.45aB |
| | 300µl/10ml | 4.33± 0.94aA | 7.00± 0.81bA | 5.66± 1.24aA | 21.33± 1.69aA | 26.33± 1.88bB | 23.00± 1.41aB | 32.33± 1.69aB | 30.22± 3.36bB | 25.33± 4.26aB |
| | BC ($10^4$ CFU/ ml) | 15.33± 1.24aB | 19.33± 1.24bB | 13.33± 0.94aB | 81.66± 1.699aC | 95.00± 2.44bE | 68.33± 2.86aC | 100.00± 0.00aE | 100.00± 0.00aE | 89.66± 6.25bC |
| | ME | 15.00± 0.81aB | 23.33± 2.49bC | 15.66± 1.69aB | 89.66± 4.10bC | 100.00± 0.00aE | 72.00± 1.63bC | 100.00± 0.00aE | 100.00± 0.00aE | 85.66± 3.24bC |
| Pak.S.B. 65 | CFS | | | | | | | | | |
| | 100µl/10ml | 11.33± 0.94aC | 14.00± 1.63bB | 16.00± 1.63bB | 36.33± 2.86aB | 26.33± 1.24aB | 16.66± 0.81bB | 52.33± 3.22aC | 64.33± 2.65bC | 54.33± 2.68aC |
| | 200µl/10ml | 13.33± 1.69aC | 13.33± 1.69aB | 13.00± 2.16aB | 37.33± 2.44aA | 23.66± 2.05bB | 23.00± 2.86bB | 57.66± 6.34aC | 68.66± 4.23bC | 54.66± 5.36aC |
| | 300µl/10ml | 12.00± 1.41aC | 13.66± 1.24aB | 14.00± 2.44aB | 41.66± 1.24aB | 29.33± 2.49bB | 26.66± 1.69bB | 58.66± 5.64aC | 72.00± 5.21bC | 57.33± 7.12aC |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | CCE | | | | | | | | | |
| | 100µl/10ml | 1.66± 0.47aA | 2.66± 0.94aA | 2.00± 0.81aA | 4.00± 0.81aA | 10.33± 0.47bA | 12.66± 1.69bB | 21.33±2. 36aB | 23.66± 3.26aB | 20.66± 2.56aB |
| | 200µl/10ml | 2.00± 0.00aA | 0.66± 0.47aA | 3.66± 0.47aA | 6.33± 0.94aA | 13.33± 1.69bA | 14.00± 1.63bB | 24.33± 2.35aB | 27.66± 2.34aB | 22.33± 4.26aB |
| | 300µl/10ml | 2.33± 1.24aA | 2.66± 0.94aA | 5.00± 1.41aA | 7.33± 1.69aA | 12.66± 0.94bA | 17.33± 1.69cB | 28.66± 4.21bB | 29.66± 4.21bB | 25.33± 2.15aB |
| | BC ($10^4$ CFU/ ml) | 10.33± 1.24aC | 15.00± 0.81bB | 11.33± 0.94aB | 21.66± 2.86aB | 22.00± 1.63aB | 19.00± 1.63aB | 55.66± 6.21aC | 59.22± 2.35bC | 57.66± 5.36aC |
| | ME | 8.66± 0.47aC | 11.00± 2.16bB | 15.33± 1.24cB | 32.66± 2.05aB | 23.33± 1.69bB | 18.00± 0.81cB | 60.33± 5.21aC | 65.33± 4.21bC | 62.33± 4.21aC |
| Pak.C.B.10 | CFS | | | | | | | | | |
| | 100µl/10ml | 21.33± 1.41aB | 22.00± 1.41aB | 16.33± 1.69bB | 81.33± 2.62aC | 94.00± 2.94aE | 71.66± 2.86aC | 100.00± 0.00aE | 100.00± 0.00aE | 100.00± 0.00aE |
| | 200µl/10ml | 26.00± 0.81Ba | 22.66± 1.69aB | 21.33± 0.81bB | 84.33± 3.68aC | 94.33± 2.05aE | 75.66± 1.69bC | 100.00± 0.00aE | 100.00± 0.00aE | 100.00± 0.00aE |
| | 300µl/10ml | 31.00± 1.41aB | 35.00± 2.94aC | 21.66± 0.94bB | 84.66± 1.69aC | 100.00± 0.00bE | 84.33± 3.68cC | 100.00± 0.00aE | 100.00± 0.00aE | 100.00± 0.00aE |
| | CCE | | | | | | | | | |
| | 100µl/10ml | 4.00± 1.24aA | 5.33± 1.24aA | 2.66± 0.94aA | 12.00± 1.63bA | 15.66± 1.69aA | 16.66± 1.24aB | 35.66± 2.31aB | 45.33± 4.21cB | 50.33± 2.15cB |
| | 200µl/10ml | 4.66± 0.47aA | 6.00± 2.16aA | 5.66± 1.24aA | 14.66± 0.94bA | 20.33± 1.24aA | 17.00± 1.63aB | 39.33± 1.06aB | 55.33± 3.65bC | 54.66± 6.36bC |
| | 300µl/10ml | 6.00± 0.81aA | 5.66± 0.94aA | 6.00± 2.16aA | 20.00± 1.63aB | 23.66± 2.62aA | 15.66± 2.49bB | 40.66± 2.20bB | 60.00± 4.21bC | 59.33± 6.45bC |
| | BC ($10^4$ CFU/ ml) | 28.66± 1.24aB | 29.33± 1.24aB | 21.66± 1.69bB | 77.33± 1.69aC | 90.33± 6.94bE | 91.00± 0.81bE | 100.00± 0.00aE | 100.00± 0.00aE | 100.00± 0.00aE |
| | ME | 27.33± 1.69aB | 26.66± 2.05aB | 15.66± 1.69bC | 76.33± 2.62aC | 94.00± 1.41bE | 96.33± 0.94aE | 100.00± 0.00aE | 100.00± 0.00aE | 100.00± 0.00aE |
| Imidacloprid | 50µg/ml | 25.00± 0.81aB | 28.66± 2.05aB | 23.33± 1.24aB | 100.00± 0.00aE | 100.00± 0.00aE | 100.00± 0.00aE | --- | --- | --- |
| Luria Bertani Broth | | 0.00± 0.00cD | 0.00± 0.00 cD | 0.00± 0.00 cD | 0.00± 0.00cD | 0.00± 0.00 aD | 0.00± 0.00 aD | 0.00± 0.00aD | 0.00± 0.00aD | 0.00± 0.00aD |

Data are expressed as mean ± standard deviation (SD). Means within the same rows followed by the same lower case letters are not significantly different ($P<0.05$) as compared by LSD test; Means within the same columns followed by the same upper case letters are not significantly different ($P<0.05$) as compared by LSD test. CFS, Cell free supernatant; CCE, Crude cell extract; BC, Bacterial culture; ME, Methanol extract.

Mortality rate of Cotton Aphids was calculated using Abbot's formula (Abbott, 1925), which compares living aphids in each treatment (At) with living aphids in each control (Ac):

$$Mortality\ (\%)=(1-At/Ac)\times 100$$

*Greenhouse experiment*

Single seed of cotton var. (TH-120/05 (Ghotki-1)) was sown in 10 ×10 inches earthen pots. After one month, pots were transferred in greenhouse net cage, when plants became infected with aphid as they reached the economic threshold level (ETL) i.e., 5-7 aphids per leaf (Afshari *et al*., 2009). All treatments were applied same as for *in vitro* experiment @ 50ml /plant by using handheld sprayer. Observation on three leaves /plant were randomly made from the top, middle and bottom (Kaushik *et al*., 1994).

*Aphicidal activity of different* Xenorhabdus *species on fecundity of cotton aphids*

Al living pregnant females were excised using sterile needle and number of eggs/gravid female recorded under stereomicroscope.

*Statistical analysis*

Mortality percentage of aphids in *in vitro* experiment was subjected to one way analysis of variance (ANOVA),

SAS Institute, Cary, NC, USA] Significant differences between means of the treatment were determined using the least significant difference (LSD) test at P≤0.05. Green house experiment was designed in RCBD with 3 replicates for all treatments. Data were recorded after 1, 24 and 48h of treatment. Change in population density (CPD%) and Population Reduction percentage (PR%) in relation to untreated were calculated by (Henderson Tilton's formula, 1955).

$$CPD\ \% = (Xi\text{-}X)/X \times 100$$

X=mean number of alive pest before treatment

Xi= mean number of alive pest after treatment

$$PR\% = \frac{Ta \times Cb}{Tb \times Ca} - 1 \times 100$$

Where: Ta is Number of total pests after treatment, Tb is Number of total pests before treatment, Ca is number of pests in control after the time of treatment, Cb is number of pests in control before the time of treatment.

The data were subjected to ANOVA and LSD by using SPSS software.

## RESULTS

*Effects mortality of cotton aphids*

Different formulants of *EPB* culture were found to induce mortality of cotton aphids at different levels (Table I). For all the bacterial treatments, the percentages of aphids mortality reached a maximum after 48h post-treatment and remained non- significantly unchanged afterward. Among all EPB, cell free supernatant of *Xenorhabdus indica* S.B.56 and *Xenorhabdus steinernematis*. C.B. 10 @ 300µl/10ml have shown best results (100.00±0.00) after 24h at 30ºC. *X. indica* S.B. 50 was found to be relative less efficient in performance and caused mortality of aphids up to (84.66±3.68) % after 24h @ 300µl/10ml, which was significantly different from other concentrations of same treatment as well as other treatments. Maximum morality caused by bacterial culture was (95.00±2.44, 94.00±1.41) % of *Xenorhabdus indica* S.B.56 and *Xenorhabdus steinernematis* C.B. 10, respectively after 24h at 30 ºC which is non-significantly different form the results of methanol extract of same species after 24h at 30 ºC (100.00±0.00, 94.00±1.41), respectively. Crude cell extract was found to be least effective and has shown significantly lower results than cell free suspension, bacterial culture and methanol extract of all studied EPB species as well as positive control (Imidacloprid). No dead aphids were observed after application with cell free LB broth.

*Effects on different* Xenorhabdus *species on change in population density and population reduction*

After 3 days of treatment, cell free suspension, bacterial culture and methanol extract of all EPB showed a maximum change in population density % (CPD %) along with population reduction % (PR %) of *A. gossypii* as compared to crude cell extract and negative control. Among all EPB species cell free suspension of *Xenorhabdus steinernematis* C.B. 10 @ 300µl/10ml have given significantly high results for CPD% and PR% (93.27%, 97.23%), respectively, followed by *Xenorhabdus indica* S.B.56 (78.76%, 74.56%). Bacterial suspension and methanol extract of *Xenorhabdus steinernematis* C.B. 10 and *Xenorhabdus indica* S.B.56 were rated second in their efficacy for CPD% i.e. (87.59%, 70.21%) and PR% (95.23%, 87.59%) of bacterial culture, CPD% (85.67%, 84.65%) and PR% (96.48%, 76.56%) for methanol extract. *X. stokiae* Pak. S.B. 65 was found to be least effective and have shown significantly reduced CPD% and PR% for all the fractions after 3 days of treatment. An overall performance of all the bacterial fractions (Fig. 1) represents that the *Xenorhabdus steinernematis* C.B. 10 @ 300µl/10ml was the highly effective one against the aphid, bacterial suspension and methanol extract.

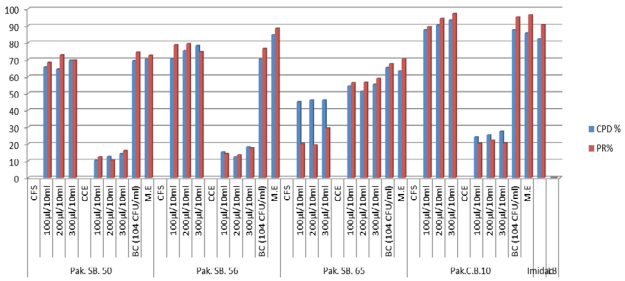


Fig.1. Change in Population density (%) and Population Reduction (%) of *Aphis gossypii* by using EPB under greenhouse condition.

*Effects on fecundity of aphids*

After 5 days of treatment, the reproduction of Cotton Aphids was found to decrease significantly after the exposure of all fractions of different EPB (Table II). Cell-free supernatant of *Xenorhabdus indica* S.B.56 *X. indica* S.B.50 and *Xenorhabdus steinernematis* C.B. 10 have shown significantly reduced aphids fecundity, at 25 C and 30 C as compared to 35 C. (Table III). Bacterial culture and methanol extracts of isolates S.B. 56 and C.B.10 have shown significant and equal to CCE results at 25 C and 30 C. Later on, CCE reduced the number of eggs/female to a significantly level lower than bacterial culture after 2 days. In contrast, crude cell extract of all EPB species showed least effect on aphids fecundity at all temperatures. Also, no aphids were found to survive after Imidacloprid application; hence, no fecundity was recorded. Even in

negative control, (LB broth) fecundity of aphid was seems to be under the influence of temperature in which 25 C and 30C favors the relative high fecundity results.

**Table II. Fecundity of *cotton aphids* after treated with different fractions of EPB (crude cell extract, cell-free supernatant, bacterial culture and methanol extract) at different temperatures in *in vitro* condition.**

| Bacterial strains | Bacterial fraction and Conc. | Fecundity (eggs/female) | | |
|---|---|---|---|---|
| | | 25°C | 30°C | 35°C |
| Pak.S.B.50 | CFS | | | |
| | 100µl/10ml | 65.00± 5.00aA | 70.65± 6.59aA | 100.66± 8.61aB |
| | 200µl/10ml | 65.00± 7.34aA | 75.33± 5.64aA | 90.33± 7.45aB |
| | 300µl/10ml | 70.33± 6.25aA | 75.66± 6.59aA | 89.66± 6.58aB |
| | CCE | | | |
| | 100µl/10ml | 200.00± 10.28aC | 200.33± 14.59aC | 220.33± 20.51aC |
| | 200µl/10ml | 185.33± 12.36aC | 225.66± 18.69bC | 225.66± 13.45aC |
| | 300µl/10ml | 105.00± 12.86aB | 130.22± 16.47aB | 230.33± 11.36bC |
| | BC ($10^4$ CFU/ml) | 115.00± 14.95aB | 140.66± 13.72cB | 100.66± 8.64aB |
| | ME | 150.66± 17.26aB | 100.66± 11.26cB | 95.221± 6.48aB |
| Pak.S.B.56 | CFS | | | |
| | 100µl/10ml | 70.66± 7.58AA | 75.33± 7.56aA | 85.33± 7.69aB |
| | 200µl/10ml | 68.33± 4.67aA | 82.66± 6.54aA | 90.66± 6.59aB |
| | 300µl/10ml | 65.33± 5.69aA | 89.33± 7.54aA | 85.66± 7.68aB |
| | CCE | | | |
| | 100µl/10ml | 155.33± 10.47aB | 158.33± 14.12aB | 120.33± 7.63aB |
| | 200µl/10ml | 150.66± 17.56aB | 194.33± 11.45aC | 150.68± 13.64aB |
| | 300µl/10ml | 149.66± 13.26aB | 146.33± 14.16aB | 155.33± 13.45aB |
| | BC ($10^4$ CFU/ml) | 85.66± 9.48aA | 100.66± 9.45aB | 92.33± 7.15aA |
| | M.E | 100.00± 14.57aB | 115.33± 8.43aB | 90.66± 6.14aA |
| Pak.S.B. 65 | CFS | | | |
| | 100µl/10ml | 190.33± 18.69aC | 200.66± 15.46aC | 160.33± 9.48aB |
| | 200µl/10ml | 200.66± 22.69aC | 220.33± 14.26aC | 220.66± 18.15aC |
| | 300µl/10ml | 200.33± 17.61aC | 250.66± 18.45bC | 268.33± 16.59bC |
| | CCE | | | |
| | 100µl/10ml | 210.33± 15.24aC | 250.33± 17.16bC | 235.66± 12.58bC |
| | 200µl/10ml | 225.33± 24.26aC | 260.33± 10.26bC | 249.33± 17.26bC |
| | 300µl/10ml | 200.33± 12.69aC | 265.66± 14.26bC | 275.26± 15.34bC |
| | BC ($10^4$ CFU/ml) | 185.66± 13.64aC | 245.33± 11.26bC | 200.33± 14.23bC |
| | M.E | 175.33± 15.45aC | 260.66± 14.23bC | 210.33± 14.23bC |
| Pak.C.B.10 | CFS | | | |
| | 100µl/10ml | 80.33± 7.45aA | 95.66± 6.45aA | 120.33± 11.14aB |
| | 200µl/10ml | 95.66± 7.56aA | 99.33± 9.15aA | 122.66± 10.25bB |
| | 300µl/10ml | 60.26± 4.65aA | 80.33± 5.64aA | 125.33± 8.25bB |
| | CCE | | | |
| | 100µl/10ml | 160.23± 6.65aA | 135.33± 17.56bB | 165.33± 7.62bB |
| | 200µl/10ml | 154.33± 7.56aA | 145.33± 12.41bB | 153.66± 6.54bB |
| | 300µl/10ml | 150.66± 6.12aA | 159.33± 16.25bB | 144.66± 10.26bB |
| | BC ($10^4$ CFU/ml) | 80.66± 7.65aA | 70.66± 4.36bA | 110.66± 15.26bB |
| | M.E | 75.66± 6.54aA | 79.33± 7.16aA | 125.66± 10.32bB |
| Imidaclo-prid | 50µg/ml | 0.00± 0.00cD | 0.00± 0.00cD | 0.00± 0.00cD |
| Luria Bertani Broth | | 336.33± 18.76dF | 355.33± 20.56dF | 300.66± 22.36dE |

Data are expressed as mean±SD. Means within the same column followed by the same letters are not significantly different ($P$<0.05) as compared by LSD test. CFS, Cell free supernatant; CCE, Crude cell extract; BC, Bacterial culture; ME,Methanol extract.

## DISCUSSION

Different formulants of EPB culture, particularly crude cell extract and methanol extract, demonstrated harmful effects on Cotton Aphids survival and fecundity.

**Table III. Details of symbiotic bacteria used for controlling cotton aphids.**

| Strains | Bacterial symbionts | EPN | Accession No. | Authority* |
|---|---|---|---|---|
| Pak.S.B.50 | Xenorhabdus indica | *S. abbasi* | MF498486 | Shahina F. and Salma J. |
| Pak.S.B.56 | Xenorhabdus indica | *S. pakistanense* | MF521953 | Shahina F. and Salma J. |
| Pak.S.B. 65 | Xenorhabdus stockiae | *S. siamkayai* | MF521964 | Shahina F. and Salma J. |
| Pak.C.B. 10 | Xenorhabdus steinernematis | *S. maqbooli* | KU097324 | Shahina F. and Salma J. |

* Accession No. authorized by these persons.

The high mortality rate caused by crude cell extract, bacterial culture and mewthanol extract of EPB suggest that toxic metabolites can transmit horizontally (most likely by direct contact) that is between infected aphids that come in contact with uninfected ones. Moreover, the decrease of fecundity of aphids caused by EPB proves that infection indicates vertical transmission of toxins effect between reproductive females and their offspring.

There are some reports of other microorganisms that have produced similar effects like aphids, such as *Microsporidium phytoseiuli* against *Phytoseiulus persimilis* Athias-Henriot and *Wolbachia* bacteria against *Tetranychus urticae* (Koch) (Bjørnson and Keddie, 2001; Vala *et al*., 2004). Cell-free supernatant of EPB in this study have shown to induce higher aphid M% and lower CPD%, PR% and fecundity than the other factions of bacteria. This may suggest that metabolites with insecticidal properties that were produced by EPB are more likely to be secreted to culture supernatant. Mahar *et al*. (2005) also found that *X. nematophila* cell-free metabolites required 4 days to kill 95% *G. mellonella* larvae whereas cell suspension needed up to 6 daya to induce 93% mortality. There are several reports indicated that *Xenorhabdus* sp. could produce and secrete several secondary metabolites with effective bioactivities such as benzylideneacetone (antibacterial compound), iodinine, phenethylamides, indole derivatives, xenorhabdins, xenorxides, and xenocoumacins (antibiotics), and primary metabolites, such as alkaline protease (Morgan *et al*., 2001; Caldas *et al*., 2002; Ji *et al*., 2004; Mohamed, 2007; Bode, 2009), whereby all of which are optional to play roles as insecticidal and immunosuppressive compounds. On the contrary, crude cell extract had least effect on aphid mortality and fecundity. This is probably due to the loss of important metabolites of bacterial crude cell extract during the withdrawal process, or they have never been there. Secondly, difference in the results of different EPB attributed the fact that different species of EPB have different protein density. It has previously determined that protein encoding density of different species of *Xenorhabdus* varied considerably (I1 Hwankim *et al*., 2017) The results clearly demonstrates that some toxic proteins are present in the crude cell extract which persist there after centrifugation and responsible for mortality but it was found to be least effective than other fractions of EPB. However, temperature dependency is also a valid factor affecting biological processes. The mean generation times, of *A. gossypii*, ranged from 17.43 (15°C) to 5.63 days (30°C) (Zamani *et al*., 2006). Below 25°C, this time significantly increased (Takalloozadeh, 2010).

## CONCLUSIONS

In conclusion, it has been depicted that different parts of *X. indica* isolate SB50 and *Xenorhabdus steinernematis* C.B.10 culture produced varying effects on Cotton Aphids mortality and fecundity. As *X. indica* and *Xenorhabdus steinernematis* C.B.10 cell-free supernatant was found to be the most effective than its surfactant, this may advocate that *X. indica* isolate SB.50 and *Xenorhabdus steinernematis* C.B. 10 are more likely to secrete their metabolites with aphicidal activities to the adjacent culture media. This is measured to be imperative for future formulation of *X. indica* isolate SB.50 and *Xenorhabdus steinernematis* C.B. 10 as an environmental friendly biological control agent. More work is under process to formulate the supernatant of these bacteria and their effects under field conditions.

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*Statement of conflict of interests*

The authors declare that they have no competing interests.

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