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Study of endospore forming bacteria in rhizosphere soil having insecticidal activity against *Helicoverpa armigera*: Microbial cells based bio-insecticide

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Abstract
Helicoverpa armigera feed on a wide range of many important cultivated crops hence has become an economic important insect pest. It damages the entire crop by boring the pod or fruits of crops hence named as pod borer as it bores the chick pea pod, cotton and other crops so commonly known as gram pod borer. Pod borer is the most polyphagous and cosmopolitan pest species. In this present work endospore forming bacteria were isolated by sodium acetate and heat shock methods. These isolates were then screened to study the presence of endospores and crystal structures within the bacterial cells with the help of malachite green and CBB staining respectively. The positive strains were selected for cell based insecticidal test where we feed the entire bacteria cells to pod borer to detect their insecticidal properties. All of the potent insecticidal bacteria were gram positive rod shaped bacteria and other biochemical properties resembling with *Bacillus spp.* as partial identification results. As we got four potent insecticidal bacteria out of 17 isolates of endospore forming bacteria that were named as D_1, D2, D_3 and D_4. These results are showing that it is possible to get insecticidal strains naturally present in soil samples, which we can isolate to produce microbial cell based bio-control as an eco-friendly and cost effective bioinsecticide.

Keywords: *Helicoverpa armigera*, gram pod borer, bio-insecticide, bio-control of insect, microbes based insecticide.

Introduction
The worldwide annual estimation of the damage caused by *H. armigera* (pod borer) is around US$2 billion hence the pod borer is listed as an A2 quarantine pest by EPPO. In the Middle East, pod borer is a major insect pest of legumes crops like chick pea, peas and beans, maize (corn), cotton, tomatoes and other solanaceous crops. Productivity of gram crop is strongly affected by pod borer *Helicoverpa armigera*, which damages 90-95% crop during favorable weather conditions due to its high fecundity, migratory and polyphagus nature, resistance against insecticides. The yield loss of 400Kg/ha by pod-borer was resulted after 30-40% average damage of pods during favourable weather conditions and the damage reached upto 90-95% (Dhaliwal *et al.*2010) [3].
Attacking chick pea, they bore into these parts, leaving large, round holes. Older larvae often enter the plant tissue with the anterior part of their bodies only. Young instars, however, may disappear completely inside, so they are sometimes not discovered before the product (e.g. tomatoes) is processed. The developing cobs, larvae penetrate mainly through the "silk" and feed on the seeds. In all cases, the economic value of the crops, for commercial or for industrial use, is much reduced. So chemical pesticides were in use to control the pod borers. Organochlorides, pyrethroids, carbamates and organophosphates were formerly in use, but the pest has developed considerable resistance to them. One approach is to rotate these chemicals every few weeks during the season. But these chemicals cause toxic effect to the soil and water and are the major source of pollution as well.
Need to control these insect pest with the help of eco-friendly biological means. Microorganisms are the goof source of metabolites which may have different activities including insecticidal activity. This work is based on the isolation and investigation of insecticidal soil born bacteria as a bio-control against *Helicoverpa armigera* for the protection of food crops. The objectives of present works are to isolates the bacteria from rhizospheric soil with the help of sodium acetate and heat sock method and investigate for the insecticidal activities in all isolates followed by staining bases characterization.

Material and Method

Isolation of endospore forming soil bacteria

The soil samples were collected from the different divisions of eastern Uttar Pradesh including (Gorakhpur division, Varanasi division, Mirzapur division, Basti division, Azamgarh division).

To isolate the insecticidal bacteria, we followed the sodium acetate and heat shock method so that only endospore forming bacteria can be isolated from the soil sample and we followed the serial dilution method as well (El-kersh *et al.*, 2012) [6].

Screening for endospore forming isolates

After isolation all isolates were examined to form endospores under light microscopy with the help of endospore staining by Malachite green stain. The culture having high number of endospores was selected for insecticidal activity test.

Mass Production of all isolates

All of the endospore positive strains were selected for the large-scale production for insecticidal test. To proceed with this, 1000 ml flasks were filled with 250 ml of Luria-Bertani broth and autoclaved properly at 121◦C for 15 mins at 15 psi. Each flask was inoculated by different cultures and it was for 72 hours incubation at 37◦C on shaker incubator. After 72 hrs cell were harvested by centrifugation at 10,000 rpm for 10 mins. Now the pellet was separated from liquid supernatant for insecticidal test.

Insecticidal test

Castor leaves were surface sterilized with autoclaved distilled water and let the water evaporate well. The pellet of bacterial culture was dissolved in 15 ml autoclaved water and coat the caster leaves well with this solution. Put 10 larvea in each plate containing castor leaves coated with the bacterial cells and maintained the record of mortality with respect to time.

Partial Identification of isolated cultures:

Select the culture which could cause the mortality in pod borer and then CBB staining was performed to study the presence of crystal and gram staining was performed to know if the cultures are gram positive or negative.

Results

All 17 isolated cultures were endospore forming soil bacteria and 4 out of 17 showed positive results as insecticidal bacteria and they killed the *Helicoverpa*. These all four strains were showing crystal structure on CBB staining and they are rod shaped gram positive bacteria also showed all characters of *Bacillus spp.*

Malachite green staining

Bacillus spp. is well known to produce endospores. Sporulation process is induced by adverse environmental conditions indicating the end of their life cycle. This process is governed by many factors - such as extreme unfavorable environment like heat, desiccation, and ultraviolet radiations and unavailability of nutrients, carbon and nitrogen sources. Endospores are metabolically dormant and they are resistant to desiccation, heat, radiation, pH extremes and toxic chemicals. Many important by-products (solvents, antibiotics, enzymes, insecticides, etc.) are produced by spore-formering bacteria. And we are looking for the insecticidal bacteria so selected only endospore forming *Bacillus spp.* Malachite-green, a low cost stain is commonly used for staining spores, which specifically stains endospores greenish-blue, thus fast visual screening for endospores and crystals. In the present study, malachite-green was used as the primary stain for staining the endospores; the vegetative cells were pinkish upon counter staining with safranin and crystals also darkly stained with safranin. Hamouda *et al*. (2002) stained endospores of Bt at different stages of germination with malachite green and safranin, and found that with malachite-green, spores were stained greenish-blue and vegetative cells took pink colouration by safranin. And this was followed by CBB staining (Jisha 2014) [9]

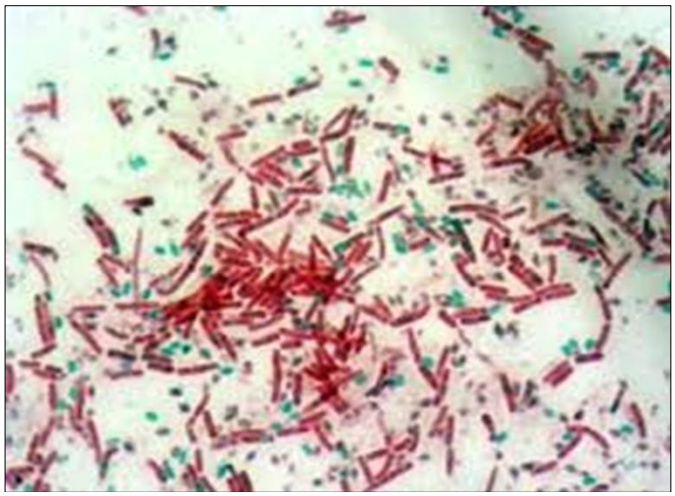

Fig 1: Endospore of *Bt.*

CBB straining

The crystal structures were observed by CBB staining and only positive strains were selected for insecticidal activity (Neethu 2015).

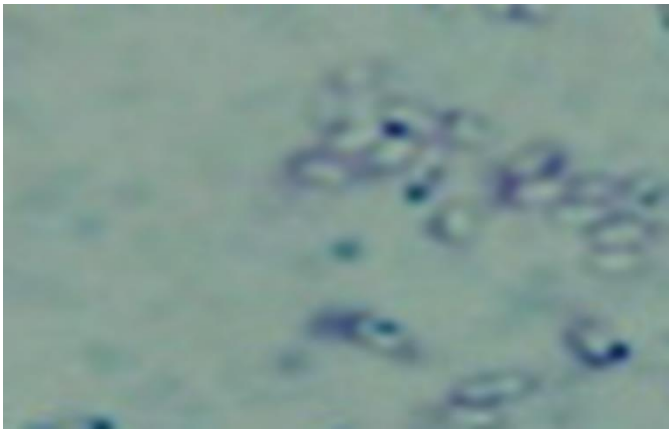

Fig 2: Crystal Present in Bacterial cell

Insecticidal bacteria

Four cultures showed insecticidal activity and they all were gram positive bacteria, hopefully they all are *bacillus spp.* In the year 1911, *Bacillus thuringiensis* (*Bt.*) was first described by Berliner, this was followed by isolation of a Bacillus species from the Mediterranean flour moth, *Anagasta kuehniella*, and named it after the province Thuringia in Germany where the infected moth was found. Although it was not the very first isolation. In fact in 1901, a Japanese biologist, Ishiwata Shigetane discovered a previously undescribed bacterium as the causative agent of a disease afflicting the silkworms. In the year 1956, T. Angus demonstrated that the crystalline protein inclusions formed in the course of sporulation were responsible for the insecticidal

action of Bt. Bt δ-endotoxins are globular protein molecules that accumulate as protoxins in crystalline form during late stage of the sporulation. Protoxins are liberated in the midgut following solubilization; and then they are cleaved off at C-terminal part to release ~66 kDa active N-terminal toxic molecule. The protoxin contains well-conserved cysteine residues (as many as 16 in Cry1Ac), which helps in bridging the protoxin molecules through intermolecular disulphide bonds and thereby crystal formation. Currently, 3-dimensional protein structures have been determined for three Bt toxins through X-ray crystallography. So the isolated cultures may or may not be *Bt*.

Fig 3: Laeves coated with insecticidal cultures

Four strains were selected for insecticidal assay as follows

Table 1: Larvicidal activity of 2a strains on larvae of Pod borer

Time (hrs)	0	0.5	3	9	12	18	24	32	48
L	10	10	9	9	7	5	4	2	1
X	0	0	1	1	3	5	6	8	9
Mortality (%)	0	0	10	10	30	50	60	80	90

Table 2: Larvicidal activity of 2h strains on larvae of Pod borer

Time (hrs)	0	0.5	3	9	12	18	24	32	48
L	10	10	8	6	6	6	6	3	0
X	0	0	2	4	4	4	4	7	8
Mortality (%)	0	0	20	40	40	40	40	70	80

Table 3: Larvicidal activity of 4b strains on larvae of Pod borer

Time (hrs)	0	0.5	3	9	12	18	24	32	48
L	10	8	7	4	4	3	3	2	2
X	0	2	3	6	6	7	7	8	8
Mortality (%)	0	20	30	60	60	70	70	80	80

Table 4: Larvicidal activity of 4c strains on larvae of Pod borer

Time (hrs)	0	0.5	3	9	12	18	24	32	48
L	10	10	9	7	4	3	0	0	0
X	0	0	1	3	6	7	10	10	10
Mortality (%)	0	0	10	30	60	70	100	100	100

Table 5: Larvicidal activity of Reference strains (*Bt.* MTCC) on larvae of Pod borer

Time (hrs)	0	0.5	3	9	12	18	24	32	
L	10	9	9	7	7	5	4	4	1
X	0	1	1	3	3	5	6	6	9
Mortality (%)	0	0	0	30	30	50	60	60	90

Table 6: Larvicidal activity of Control (sterilized distilled water) on larvae of Pod borer

Control experiment									
Time (hrs)	0	0.5	3	9	12	18	24	32	48
L	10	10	10	10	8	8	8	7	7
X	0	0	0	0	2	2	2	3	3
Mortality (%)	0	0	0	20	20	20	20	30	30

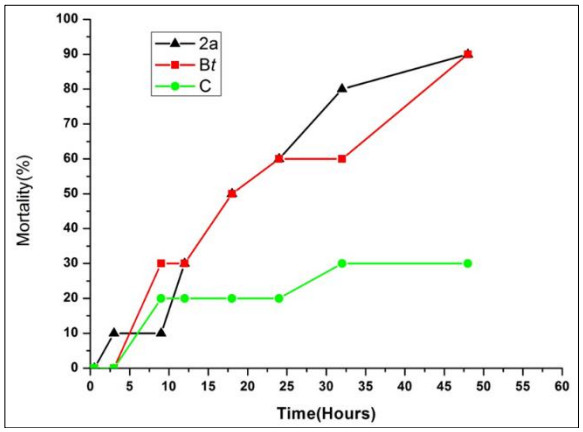

Fig 3.1: Larvicidal activity of 2a strains on larvae of Pod borer

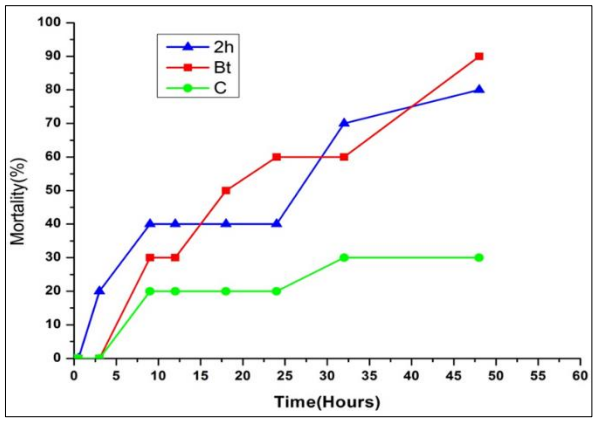

Fig3.2: Larvicidal activity of 2h strains on larvae of Pod borer

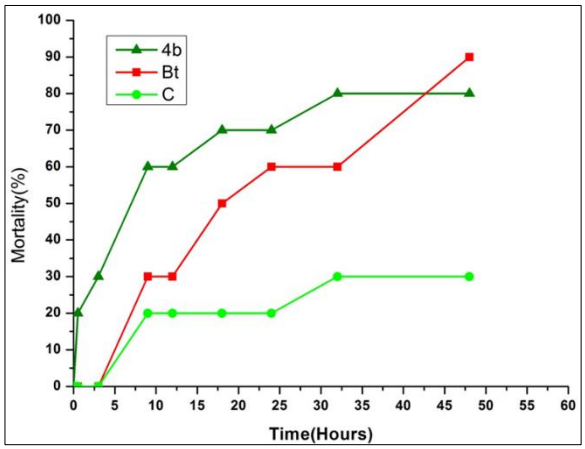

Fig 3.3: Larvicidal activity of 4b strains on larvae of Pod borer

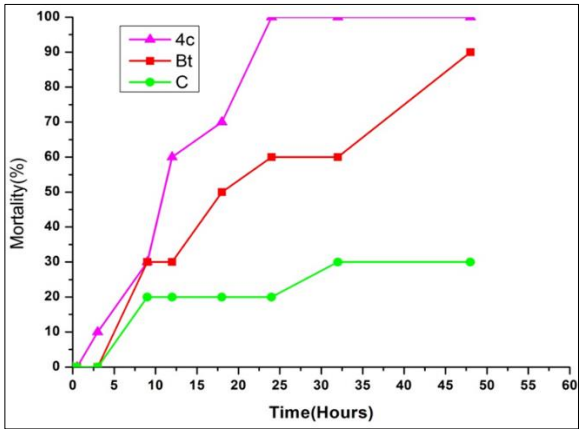

Fig 3.4: Larvicidal activity of 4c strains on larvae of Pod borer

Conclusion and future aspects

We could isolate four *bacillus spp.* which were causing insecticidal activities to the pod borer. These bacteria can be used for the bio formulation of bio insecticides, with the help of microbial cells. In this work the entire cell was used as a insecticidal product. That means cells itself can cause death to the larvae, no need to isolate the specific protein and purify it. Protein isolation and purification is a costly process and a developing country needs cost effective technique. Growing bacterial cells on large scale and feed them as insecticidal bio control is the best alternative for chemical pesticide.

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