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TESTING PATHOGENICITY OF WHITE GRUB, *Holotrichia serrata* (Fabricius) (COLEOPTERA: SCARABAEIDAE) FROM THE DARJEELING FOOTHILL TEA PLANTATIONS OF BENGAL

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ABSTRACT

Diseased grubs of *Holotrichia serrata* (Coleoptera) were collected form the tea gardens of Darjeeling foothills and three strains of entomopathogenic bacteria were isolated. Following standard protocols the strains were examined for their morphological, biochemical and physiological parameters and compared with the commercially available strain of *Bacillus thuringiensis* kurstaki (Btk). Isolates Hs01, Hs02 and Hs03 showed the characteristics of genus Bacillus with *viz.,* cell morphology, gram positivity, endospore production, facultative anaerobic, starch hydrolysis, catalase positivity, production of acid from glucose and motility etc. Biochemical characteristics of all strains showed marked difference among themselves and also with Btk. Further difference between the newly isolated strains and standard strain (Btk) was observed while analyzing crystal protein composition on SDS-polyacrylamide gel. Hs01 revealed two bands of 98. 55 and 19 kDa, while Hs02 had three distinct bands of 73, 56 and 15 kDa. Hs03 had two bands of 59 and 17 kDa, while Btk had a single band of 56 kDa. Thus, all the Bacillus strains Hs01-03 and the reference strain Btk differed in crystal protein composition.

Bioassay of these bacterial strains on early 2nd instar *H. serrata* revealed LC_{50} value of 323.7 µg ml^{-1}, 419.3 µg ml^{-1} and 572 µg ml^{-1} for Hs01, Hs02 and Hs03, respectively. In case of Btk the LC_{50} value was found to be 644 µg ml^{-1}. Therefore, considering the killing efficacy of these locally available Bacillus strains may be considered for development of additional biopesticide for management of the pest of interest.

(Key words: Bacillus strains Hs01, Hs02, Hs03, *Holotrichia serrata*, Tea, Darjeeling)

INTRODUCTION

Tea [*Camellia Sinensis* (L.) O. Kuntz] is second most favored beverage after water in the world (Martin, 2007). It is cultivated as a monoculture perennial plantation crop in different parts of the world spreading over 34 countries across Asia, Africa, Latin America, and Oceania to producing annual 3.22 million metric tons of made tea (Hazarika *et al.,* 2009). Naturally crops are attached by insect pest and for a perennially grown plantation crop having aggravated pest problem is often a common scenario (Adarsh *et al.,* 2002).

Similarly, tea plantation is also attacked by myriads of pests, at various stage of cultivation, in all seasons of the year and at every possible part of the plant (Hazarika *et al.,* 2009). White Grubs of *Holotrichia serrata* (Fabricius) (Coleoptera: Scarabaeidae) are one of such pest species which often cause considerable damage to the tea plantation. They also attack other plantation crops such as cashew nut, coconut, coffee, etc. and fruit crops with varying damage levels (Khan and Ghai, 1974; Veeresh, 1974, Chatterjee and Ghosh, 1995).

Severe attacks of white grubs of *H. serrata* are seen in the root of the nursery and young tea plants are more susceptible, especially during dry seasons when plants suffer from. The mature instars (4th and 5th) feed on the roots, whereas the adults feed on leaves thus defoliating the plants. Up to 70 per cent damage in several commercial crops due to white grub infestation (Yadava and Sharma, 1995). Several tactics have been adopted for the management of white grubs including cultural, mechanical, biological, chemical and integrated methods suggested by various workers (Kaunsale *et al.,* 1978). Adult collection and insecticidal applications are the major tactics of management followed against all white grub species (Veeresh, 1974; Raodeo *et al.,* 1976).

Synthetic pesticides are used to chiefly manage these pests by the use of leading to environmental contamination. Thus, efforts are being made to develop alternative pest management strategies by developing microbial pesticides.

Indiscriminate use of synthetic insecticides leads

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to high incident of pests in tea has led to the leading to problems such as termination of non-target organism including natural enemies of pests (Anonymous, 1994), human health hazards (Yaqub *et al.,* 2018; He *et al.,* 2020; Gurusubramanian *et al.,* 2008), enhanced environmental hazards like pollution of soil and ground water pollution (Chattopadhyay *et al.,* 2004). Long exposure to pesticides, resistant strains of insect emerge, requiring increased doses of insecticides and introduction of new insecticides. Different chemical pesticides (Organophosphates and synthetic pyrithorides) have been found to be less effective against these defoliators in recent times (Sannigrahi and Talukdar, 2003). Moreover, health-conscious consumers prefer organic tea to those of chemically managed conventional tea. Therefore, the future protection and production of tea appear to depend largely on non-conventional control methods. In many instance, alternative methods of insect management offer adequate levels of pest control and pose fewer hazards. One such alternative eco-friendly approach is the use of microbial insecticides that contain microorganisms or their by-products. Microbial insecticides are especially valuable because their toxicity to non-target animals and humans are extremely low. Compared to other commonly used insecticides, they are safe for both the pesticide user and consumers of treated crops (Mc Coy, 1987).

Microbial insecticides comprise of microscopic living organisms (Viruses, Bacteria, Fungi, Protozoan or Nematodes) or the toxins produced by these organisms. Hence, the need for exploring the entomopathological viruses and bacteria of these pests has become essential especially in organic farming where use of biopesticide instead of organosynthetic chemicals is inevitable. Thus, Integrated Pest Management in tea is greatly required (Barbora, 1994) in NE India. One of the eco-friendly approaches of pest control is the application of microbial pesticide and to conserve the local microbial bio-agents for future use. The proposed research contemplates to study these naturally occurring entomopathogenic bacteria in the lepidopteran and coleopteran pests of tea. Naturally occurring bacteria that infect and kill the pests will be surveyed, isolated, characterized and bioassayed for their efficacy so that, potential microbial pesticides may be developed out of these in future, and may be integrated in biocontrol and IPM programs of tea.

MATERIALS AND METHODS

Collection of dead larvae

Moribund and dead pest grubs were collected from natural populations occurring in tea gardens and those dying in laboratory reared population.

Isolation of entomopathogenic bacteria

For isolation of bacteria, procedure by Lacey and Brooks (1997) was followed. The infectivity was determined following Koch's postulates by infecting healthy first instar larvae with these isolated bacteria.

Morphological characteristics

Cell, spore and crystal protein shape and structure, colony texture, mobility was determined. *Bacillus thuringiensis* kurstaki (Btk) was used as control for comparative study which is used as biopesticides in many organic gardens.

Biochemical characteristics

Biochemical analyses like indole, Voges-proskour, methyl red, citrate utilization, esculin hydrolysis, lysine decarboxylase, ornithin decarboxylase, H_2S production, nitrate reduction, fermentation of different carbohydrates, urease tests were performed using biochemical testing kit (KB003) (Himedia) with *Btk* as reference.

Doubling time or Generation time

In this procedure, growth of the bacterial strain was determined by turbidimetric method (Cappuccino and Sherman, 1996).

SDS-PAGE profile (Qualitative) of crystal protein

Bacterial strains were grown in Luria Bertani medium at 37! without shaking. It was grown up to the phase of sporulation. The crystal was harvested in high pH buffer of sodium carbonate and 2-marcaptaethanol (Kranthi, 2005) with slight modification (2-marcaptaethanol was used instead of Dithiothreitol (DTT). This crystal protein was taken for SDS-PAGE analysis.

Qualitative (SDS-PAGE) analysis of whole body protein of the bacterium

The extracted protein was analyzed by SDS-PAGE after the method of (Costas, 1992). Gel was fixed in trichloroacetic acid and stained with Brilliant blue G-250.

Bioassay and determination of LC_{50} value and LT_{50} value

The efficacy of the entomopathogenic micro-organisms was determined by bioassay testing according to the procedure of (Unnamalai and Vaithilingam, 1995). The corrected mortality was calculated using Abbott's formula. Data were subjected to probit analysis (Finney, 1952) and median lethal concentration (LC_{50}) value was calculated from the regression equation. Median lethal time (LT_{50}) was also calculated simultaneously following the method of (Biever and Hostetter, 1971).\

RESULTS AND DISCUSSION

Fully grown grub is white with brownish head. Except the posterior part, the abdomen is wrinkly and transparent so that their intestinal content is visible from outside (Fig. 1a). The adult beetles are brown in colour and about 18 mm in length (Fig.1b). The typical putrefying and blackening symptoms in the grubs indicated bacterial infection (Fig. 1c). Since the pathogens (bacteria) regularly occur and naturally spread in the field populations, an investigative study involving characterization and evaluation of their pathogenicity was undertaken. These pathogenic bacteria, isolated from cadavers were compared with a reference, *Bacillus thuringiensis* kurstaki (Btk), the

commercially available and commonly used microbial pesticide.

Morphological characteristics

All the morphological characteristics such as vegetative body structure, spore-shape, motility, colony texture, of the isolated bacteria (Hs01, Hs02 and Hs03) were found to be similar to that of *Bacillus thuringiensis* kurstaki (Btk) (Fig. 2).The isolated strains showed characteristics of genus *Bacillus* such as rod shaped vegetative body, endospore formation, gram positivity, facultative anaerobic nature, catalase positivity, acid production from glucose and motility (Sneath, 1986).

Biochemical characteristics

Biochemical characteristics of Hs01 strain showed positive reaction in lysine decarboxylase, ornithin decarboxylase, urease, Voges-Proskaur, oxidase tests, esculin hydorlysis and in utilization of malonate, citrate, trehalose and glucose. It showed difference with *Btk* in ONPG, urease, nitrate and esculin hydrolysis tests. In utilization tests it showed difference in citrate, malonate, arabinose, xylose, cellobiose, mellibiose, saccharose and lactose. Strain Hs02 showed positive reaction in lysine decarboxylase, ornithin decarboxylase,Voges-Proskaur, urease tests, nitrate reduction, esculin hydrolysis and in utilization of trehalose and glucose. It showed difference with *Btk* in ONPG, urease esculin and nitrate tests and in utilization of arabinose, xylose, cellobiose, melibiose, saccharose and lactose. On the other hand Hs03 strain showed positive reaction in lysine decarboxylase, ornithin decarboxylase, urease, nitrate reduction, H_2S production, and in utilization of citrate, malonate, rhamnose melibiose, and glucose. It showed difference with *Btk* in ONPG, urease, H_2S production and in utilization of citrate, malonate, arabinose, xylose, rhamnose, cellobiose, saccharose and trehalose.

Growth phase or determination of doubling time

The doubling time was 40 min in case of Hs 01, 36 min in case of Hs 02, 39 min in case of Hs 03, and 42 min in case of *Btk*.

SDS-PAGE of crystal protein

When composition of crystal protein was analyzed by SDS-PAGE, crystals of Hs01 showed three major protein bands having the molecular weight 98 kDa, 55 kDa and 19 kDa which in case of *Btk* was 56 kDa protein band. So, a narrow difference in banding pattern was found between Hs01 and *Btk* . In Hs02 three protein bands having molecular weight 73 kDa, 56 kDa and 15 kDa were present. In Hs03 two major protein bands 59 kDa and 17 kDa were found which were absent in all the other three strains including *Btk*. (Fig 3).

Qualitative (SDS-PAGE) analysis of whole cell protein of the bacterium

When protein composition of whole body of bacteria was analyzed by SDS-PAGE, protein of Hs01 showed four major protein bands having the molecular weight 99 kDa, 76 kDa, 53 kDa and 37 kDa which in case of *Btk* were 84 kDa, 51 kDa and 34 kDa. Hs02 had three prominent bands of 77 kDa, 57 kDa and 39 kDa. Hs03 also had three major bands having molecular weight 71 kDa, 51 kDa and 37 kDa. All the three strains showed difference in their banding pattern and also differed from *Btk*. (Fig. 4)

Bioassay

The LC_{50} value of Hs01 was found to be 323.75 μg^{-1} with fiducial lower limit of 219 μg^{-1} and upper limit of 478 μg^{-1}. The LT_{50} values were found to be 5.83 days for 1000 μg^{-1}, 7.5 days for 750 μg^{-1} and 9.6 days for 500 μg^{-1} concentrations.

In case of Hs02 strain the LC_{50} value was found to be 419.34μg^{-1} with fiducial lower limit 298 μg^{-1} and upper limit 589 μg^{-1}. The LT_{50} values were 7.5 days for 1000 μg^{-1}, 8.5 days for 750 μg^{-1}, 8.6 days for 500 μg^{-1} concentrations.

In case of Hs03 the LC_{50} value was found to be 572 μg^{-1} with fiducial lower limits of 384μg^{-1} and upper limit of 849 μg^{-1}. The LT_{50} values were 8 days for 1000 μg^{-1}, 9.67 days for 750 μg^{-1} concentrations.

In case of *Btk* the LC_{50} value was found to be 644 $\mu g^{-1}$with fiducial limits of 471 μg^{-1} (lower limit) and 880 μg^{-1} (upper limit). LT_{50} values recorded were 8.6 days for 1000 μg^{-1}, 9.67 days for 750 μg^{-1} concentrations (Table 1).

The strains of bacteria isolated from *Holotrichia serrata* showed typical characteristics of *Bacillus thuringiensis* especially in their vegetative body structure and crystal production. As crystals are the typical distinguishing characteristics of *Bt* (Heimpel and Angus,1958; Bai *et al.,* 2002) the new strains were identified as *Bt* strains on the basis of the spore structure and crystal formation (Brussock and Currier, 1990). However, on the basis of biochemical testing and generation time the *Bacillus* strains showed a significant difference with *Bacillus thuringiensis* kurstaki. Even the crystal protein profiles on PAGE and the whole body protein profiles were found to be different for these new strains from that of *Btk*.

In case of bioassay, the new natural strains Hs01 of *Bacillus* was found to be highly pathogenic compared to *Btk*. As the LT_{50} values of the new strains were shorter than the commercially used *Btk*, and the LC_{50} values of two of the strains much lower, these appeared to have better killing efficacy.

As no naturally occurring *Bacillus* strains has so far been reported from white grubs of *H. serrata* of sub Himalayan tea plantation, the newly isolated strains were designated as, *Bacillus* sp. Hs01, *Bacillus* sp. Hs02, and *Bacillus* sp. Hs03 and are being reported as new to science.

Report of development of insect resistance to *Btk* has stimulated new research to find additional *Bt* strains and other microbes that have specific activity spectrum against certain insect pests (McGaughey, 1985; Salama and Abdel-Razek, 2000; Bai *et al.,* 2002; Monnerat *et al.*, 2007). So, these newly reported strains of *Bacillus*, Hs01, Hs02, and Hs03 with their appreciable entomopathogenicity appeared to be promising as future biopesticide.

Fig. 1 a) Advance instar grub; b) Adult *H. serrata* beetle; c) Bacterial infected grubs

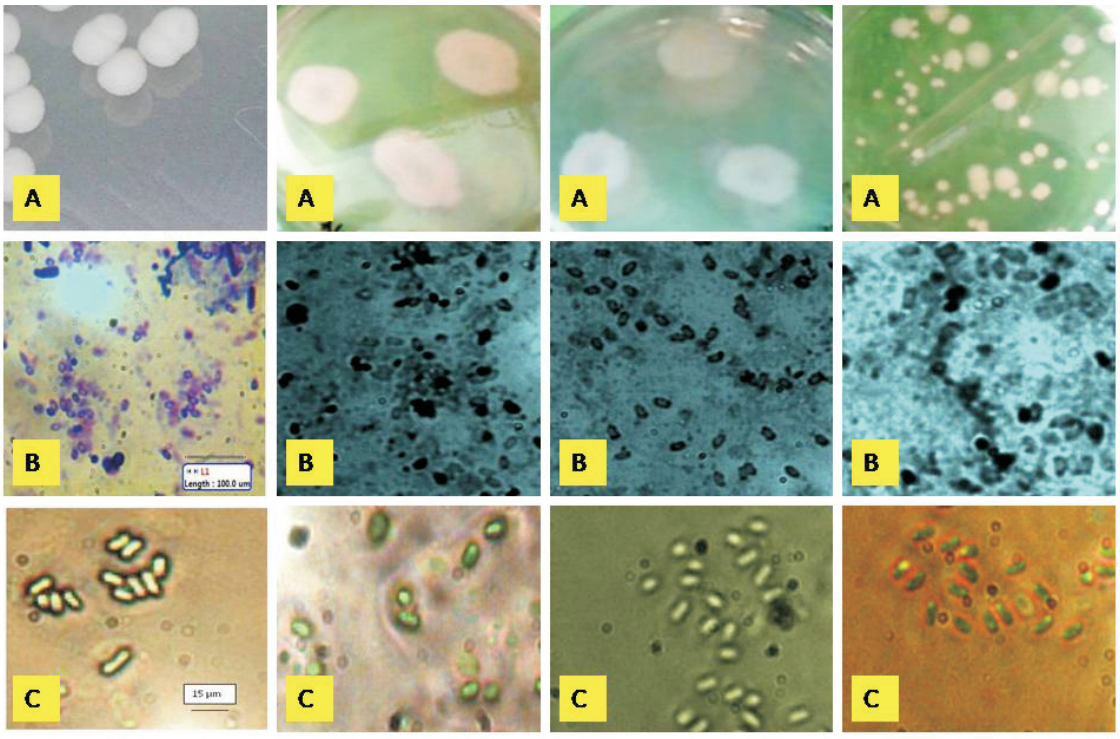

Fig. 2 A) Colony morphology of *Btk*, Hs01, Hs02, Hs03; B) Gram stain of bacterial spores of *Btk*, Hs01, Hs02, Hs03; C) Phase contrast microphotograph of spore and crystals of *Btk*, Hs01, Hs02, Hs03

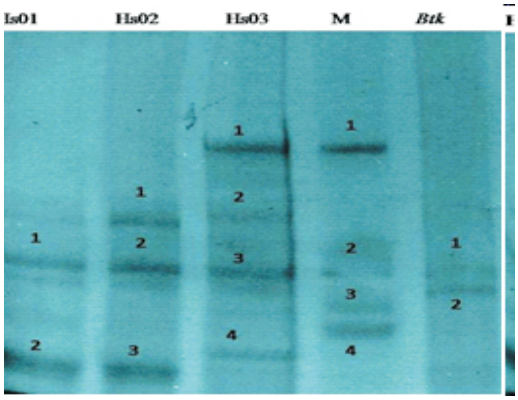

Fig. 3. SDS-PAGE of crystal proteins of three isolates of *Bacillus* from *H. serrata* (Hs01, Hs02, Hs03) and *Btk* (reference) [Hs 01: 69 kDa, 17 kDa; Hs 02: 73, 65 kDa, 15 kDa; Hs 03: 98 kDa, 72 kDa, 66 kDa, 21 kDa, M; 97.4 kDa, 66 kDa, 43 kDa, 29 kDa; *Btk*: 62 kDa, 54 kDa]

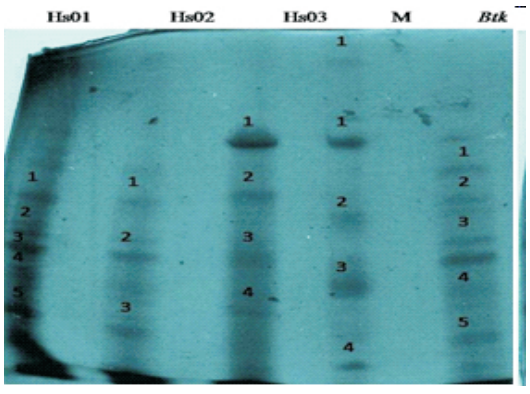

Fig.4. SDS-PAGE of whole cell proteins of three isolates of *Bacillus* from *H. serrata* (Hs01, Hs02, Hs03) and *Btk* (reference) [Hs 01: 71 kDa, 67 kDa, 51 kDa, 47 kDa, 37 kDa; Hs 02: 77, 57 kDa, 40 kDa; Hs 03: 119 kDa, 76 kDa, 53 kDa, 40 kDa, M; 203 kDa, 97.4 kDa, 66 kDa, 43 kDa, 29 kDa; *Btk*: 84 kDa, 75 kDa, 58 kDa, 54 kDa, 34 kDa]

Table 1. Bioassay of three *Bacillus* isolated from white grubs of *H. serrata* (Hs01, Hs02, Hs03) and *Btk*

Isolates	LC_{50}	Fiducial limits	Regression	Chi square	LT_{50}
Hs01	323.7	219-478	Y= 2.510x+1.22	0.30337	5.83 days for 1000 µg ml^{-1}, 7.5 days for 750 µg ml^{-1}, 9.67 days for 500 µg ml^{-1}
Hs02	419.3	298-589	Y= 2.622x+1.41	0.24282	7.5 days for 1000 µg ml^{-1}, 8.5 days for 750 µg ml^{-1}, 8.67 days for 500 µg ml^{-1}
Hs03	572	384-849	Y= 2.757x+1.20	0.56588	8 days for 1000 µg ml^{-1}, 9.67 days for 750 µg ml^{-1}
Btk	644	471-880	Y= 2.808x+1.59	3.49731	8.67 days for 1000 µg ml^{-1}, 9.67 days for 750 µg ml^{-1}

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