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Impact of *Bacillus subtilis* isolates on biochemical profile of *Spodoptera litura* (Fab.) larvae

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Abstract
A laboratory experiment was carried out during 2014-2015 to study the effect of toxins of *Bacillus subtilis* isolates on the biochemical profile of newly ecdysed 3rd instar *Spodoptera litura* larvae fed for 24 h on castor oil bean leaves treated with 1000 and 2000 ppm concentrations by leaf dip and larval dip methods at Tamil Nadu Agricultural University, Coimbatore. There was a severe reduction in total protein content of larvae when treated with the isolate KA3 (55.24 mg g^{-1}) followed by VB1 (56.90 mg g^{-1}) at 2000 ppm by leaf dip method and total lipid content after 48 h was decreased (11.38 mg g^{-1}, 12.33 mg g^{-1}) in larvae treated with isolate VB1 at 1000 and 2000 ppm by the leaf dip method. Isolate VB1 showed higher GOT (Glutamic-oxaloacetic Transaminase) enzyme activity (24.43 units ml^{-1}, 22.76 units ml^{-1}) at 2000 ppm.

Keywords: *Bacillus subtilis*, *Spodoptera litura* (Fabricius), Protein, Lipid, GOT activity

Introduction
In crop ecosystem, polyphagous insect pests are always more difficult for management than monophagous pests. *Spodoptera litura* Fabricius (Lepidoptera: Noctuidae), the tobacco caterpillar is one of the serious polyphagous insect pests, the larvae of which can defoliate more than hundred economically important crops [5, 17, 23]. For the control of this pest in the field, a number of insecticides belonging organochlorines, organophosphates, carbamates and pyrethroids proved effective but continuous and indiscriminate use resulted in development of resistance in *S. litura* larvae to these insecticides [22, 18, 1] including the new chemistry insecticides like lufenuron [25] in India [6, 18] and Pakistan [5]. Management of the pest has therefore become increasingly difficult all over the world. As a result of these efforts, increasing interest in the use of successful biological control strategies is rife. Biocontrol methods proved to be safer compared to chemical control of pest [5]. Bacterial biopesticides are the most common, cheaper and prime components for use in Integrated Pest Management Programmes (IPM) with high pathogenicity for target pests and safer to non-target organisms. Among commercially available products, Bacillus based bio pesticides make up over 90% of all bio pesticide sales worldwide [2]. Though many Bacillus spp are pathogenic to specific insects or diseases, their dual toxicity to both insects and diseases will be a boon for cultivators for simultaneous management of both insects and diseases in crop ecosystem. *Bacillus subtilis* one such bacterium largely employed for the biocontrol of the crop diseases, with isolated reports on insect also [11]. In the present investigation, the effect of bacterial toxins from *B.subtilis* on the biochemical profile *S. litura* larvae were studied, for its pathogenesis.

Materials and methods
Culture of insects and *B. subtilis* isolates
Initially, larvae of the test insect, *Spodoptera litura* Fab were collected from castor fields and cultured as per standard protocol [10]. Two *B. subtilis* isolates (VB1 and KA3) were obtained from the Department of Plant Pathology, TNAU, Coimbatore and maintained by standard subculturing and maintained at 27 ^{0}C in the form of slants in nutrient agar medium [11]. The bacterial isolates were used at 1000 ppm and 2000 ppm for bioassays.

Insect bioassays
Bioassays with the third instar larvae of *S. litura* were conducted in the laboratory with treated castor leaf discs and larval dip. Bioassays were conducted at ambient room temperature of 25-28 ^{0}C.

Leaf dip
Castor leaf discs (3g) were prepared and the discs were dipped in the two bacterial isolates (VB1 and KA3) at 1000 ppm and 2000 ppm separately for 30 seconds and shade dried. Two controls were also maintained with distilled water and methanol (10 µl/ 10ml) separately.
Third instar larvae of *S. litura* were pre starved for 30 minutes and then introduced into each castor leaf disc at 20 larvae per disc in bigger petridishes (15 cm dia) and allowed to feed for 24 hours. After 24 hours, untreated fresh leaf discs were provided for feeding. Three replicates were maintained for each treatment,

Larval dip
Third instar larvae of *S. litura* were dipped individually in the *B. subtilis* isolates at 1000 ppm and 2000 ppm for 30 seconds. Then the larvae were allowed to crawl on tissue paper in a petri dish to wipe off excess solution on their skin. These larvae were placed individually in plastic in containers (3.5×1.5 cm). These containers were places in crispers with wet towel paper to maintain humidity.
After 48 hours, the larvae were subjected to biochemical analysis.

Insect homogenization
Each batch of larvae was mechanically homogenized in 10 volumes (w/v) of 0.1 M phosphate buffer, pH 7 for 2 min using a Teflon homogenizer surrounded with a jacket of crushed ice. The homogenates were then centrifuged at 4000 rpm for 30 min at 4 ^{0}C using cooling centrifuge. The resultant supernatant was used to determine the activities of glutamic-oxaloacetic tansaminase [24] as well as the amount of protein [19] and lipid [17] content.

Estimation of protein content
Reagents
1. 2 per cent Na2CO3 in 0.1 N NaOH
2. 1 per cent NaK tartarate in H2O
3. 0.5 per cent CuSO4.5H2O in H2O
4. 48 ml of 1, 1 ml of 2, 1 ml of 3
5. Folin phenol reagent – 1part Folin –Phenol (2N): 1 part water

Standard graph: Protein solutions of concentrations ranging from 10 to 100µg were prepared by pipetting 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100µl of BSA of working standard solution into a series of test tubes. The volume in all the test tubes was made upto 1.0ml with distilled water. A test tube with 1.0ml of distilled waterserved as blank. 1000 µl of solution 4 was added to each test tube, mixed well and incubated for 10 min. After incubation add 100 µl of solution 5 and mix it vigorously followed by incubation for 30 minutes. The purple colour developed was measured at 650nm using ELISA reader. A standard graph was drawn by plotting concentrations of protein along the X-axis and the readings for absorbance along the Y-axis.

Protein estimation in enzyme extract: 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 µl of enzyme extract were pipetted out into an ELISA plate to which 100 µl of solution v was added and allowed for colour development. The absorbance was read at 650 nm. Using the standard graph, the quantity of protein in the enzyme extract was calculated.

Estimation of total lipids
Reagents
Methanol: Chloroform (1:2) v/v, NaCl 3%.

Methodology
Larvae were starved till stomach contents were voided out. Known weight of larvae (10g) was macerated in a pestle and mortar with 1:2 mixture of methanol: chloroform. It was kept in a water bath for 5 min at 60 ^{0}C. The contents were centrifuged at 5000 rpm for 5 min. The supernatant was collected and partitioned with chloroform in a separating funnel. The lower layer was drained in a beaker and the extract was evaporated in a water bath. Final lipid content was estimated gravimetrically from the weight of residue retained in a beaker.

Estimation of Glutamic-oxaloacetic Transaminase (GOT)
Glutamaic-oxaloacetic transaminase (GOT) enzyme helps in the production of energy [7].It serves as a strategic link between the carbohydrates and protein metabolism and known to be altered during various physiological and pathological conditions [15]. For determination of GOT, 0.10 ml of enzyme source was taken and 0.50 ml of GOT substrate (aspartate) was added to it. Then 0.50 ml of 2, 4-dinitrophenyl hydrazine solution was added and the contents were left to stand for 15 min at room temperature. Then 5.0 ml of 0.4N NaOH was added and mixed well and allowed to stand at room temperature for another 20 min. The optical density was read at 505 nm after setting the blank. Standard curve was prepared by using oxaloacetic acid as working standard. The enzyme activity was expressed in Glutamic-oxaloacetic Transaminase activity units/ml.

Statistical analysis
Data were statistically analyzed after square root transformations for discrete values. For separating means, Least Significant Difference (LSD) test was adopted [16]. AGRES statistical package was utilized for analysis.

Result and discussion
There was a decrease in total protein content of larvae when treated with the isolate KA3 at 2000 ppm (55.24 mg g^{-1}, 62.59 mg g$^{-1)}$ by leaf dip and larval dip methods and in the isolate VB1 maximum reduction in protein content was 56.90 mg g^{-1}, 60.17 mg g^{-1} by leaf dip and larval dip methods. There was a reduction in protein content of *S. litura* with the treatment of Bt [11]. Treatment of *S. litura* larvae by the bacterial formulation Dipel ®-2x resulted in negative changes in the total protein content and that this might be due to bacterial toxins which led to inhibition of protein synthesis by forming a protein complex [13].
Total lipid content after 48 h was decreased (11.38 mg g^{-1}, 15.87 mg g^{-1}) in larvae treated with isolate VB1 by leaf dip and larval dip methods and the treatment with the isolate KA3 at 2000 ppm by leaf dip and larval dip method recorded the maximum reduction of 12.98 mg g^{-1} and 14.89 mg g^{-1} respectively. The controls showed higher total lipid content of 19.27 mg g^{-1} with methanol and 18.48 mg g^{-1} with water respectively. The lower lipid content in larvae could be possibly due to blocked food ingestion, and the fat reserves might have been utilized for the maintenance during larval period. Infected larvae may produce enzymes that utilize lipids in efforts to remove the invading organisms [8]. There was a sharp decrease in lipid content of *S.littoralis* larvae treated with *B. thuringiensis* var. *kurstaki* [2]. *B. thuringiensis*

var. *kurstaki* also caused a significant reduction in the lipid content of *S. littoralis* larvae [3].

Treatment with the isolate VB1 showed higher GOT enzyme activity (24.43 units' ml^{-1}, 22.76 units' ml^{-1}) at 2000 ppm by leaf dip and the larval dip method and the maximum GOT activity (21.31 units' ml^{-1}, 22.08 units' ml^{-1}) was in isolate KA3 at 2000 ppm by leaf dip and larval dip method respectively. Among them, isolate VB1 showed higher enzyme activity (24.43 units' ml^{-1}, 22.76 units' ml^{-1}) at 2000 ppm using leaf dip than the others. The lowest (18.34 units' ml^{-1}) enzyme activity was found in control with water followed by control with methanol (19.76 units' ml^{-1}). Glutamic-oxaloacetic transaminase (GOT) enzyme is known to be altered during various physiological and pathological conditions [15]. Diet with high level of bio insecticides significantly increased the activities of transaminases [8]. In the present study, also the activity was marginally higher in larvae treated with the isolate VB1 at 2000 ppm by the leaf dip method.

Table 1: Effect of *B. subtilis* isolates on some biochemical profile of *S. litura* larvae *in vitro*.

Isolate/Treatment		Dose of toxin (ppm)	Protein content (mg g^{-1})	Lipid content (mg g^{-1})	GOT* (units ml^{-1})
			After 48 h	After 48 h	After 48 h
VB1	Leaf dip	1000	60.48 (7.78)^{de}	12.33 (3.51)^{b}	19.67 (4.44)^{e}
		2000	56.90 (7.54)^{b}	11.38 (3.37)^{a}	24.43 (4.94)^{a}
	Larval dip	1000	64.23 (8.01)^{f}	17.46 (4.18)^{h}	22.45 (4.74)^{bc}
		2000	60.17 (7.76)^{cd}	15.87 (3.98)^{f}	22.76 (4.77)^{b}
KA3	Leaf dip	1000	59.10 (7.69)^{bc}	13.76 (3.71)^{d}	20.87 (4.57)^{d}
		2000	55.24 (7.43)^{a}	12.98 (3.60)^{c}	21.31 (4.62)^{d}
	Larval dip	1000	66.57 (8.16)^{g}	16.45 (4.06)^{g}	21.98 (4.69)^{c}
		2000	62.59 (7.91)^{ef}	14.89 (3.86)^{e}	22.08 (4.70)^{c}
Control (water)			86.23 (9.29)^{i}	18.48 (4.30)^{i}	18.34 (4.28)^{f}
Control (Methanol)			78.40 (8.85)^{h}	19.27 (4.39)^{j}	19.76 (4.45)^{e}
SE (d)			0.05	0.029	0.032
CD (P = 0.05)			0.12	0.059	0.068

*GOT- Glutamic Oxaloacetic Transaminase

Values are mean of three replications

Figures in the parentheses are square root transformed values

Figures with same superscripts are not significantly different (LSD, P=0.05)

Conclusion

Among the two *Bacillus subtilis* isolates, KA3 isolate at 2000 ppm through leaf dip assay showed less protein and lipid contents. However GOT activity did not show any marked variation among both isolates by both leaf dip and larval dip methods, though it was marginally higher in VB1 at 2000 ppm.

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