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Research Article

Role of gut bacteria associated with the chlorpyrifos resistant tobacco leaf eating caterpillar *Spodoptera litura* on the efficacy of entomopathogenic fungi *Beauveria bassiana* and *Poecilomyces* sp.

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ABSTRACT: Experiments were conducted to study the role of gut bacteria with insecticide-resistant, insecticide-susceptible and field-caught populations of the lepidopteran insect pest on tobacco, *Spodoptera litura* - Leaf Eating Caterpillar (LEC). The gut bacteria present in the larvae of LEC was studied in their developmental stages and were found to be significantly different. Results of the gut bacteria of LEC showed that the bacterial population from the chlorpyrifos-resistant larval gut was more varied with *Pseudomonas* sp., *Acinetobacter* sp., *Serratia* sp. and *Micrococcus*. The susceptible larvae of LEC were harboring *Acinetobacter* sp. and *Serratia* sp. and the field-caught population recorded *Serratia* and *Pseudomonas* sp. Siderophore producing *Pseudomonas* sp. showed antagonistic activity towards entomopathogenic fungi, *Beaveria bassiana* and *Paecilomyces* sp. whereas the chitinase producing bacteria promoted larval growth and development. The chitin produced by gut bacteria appears to contribute for host nutrition. Study reveals that the bacteria associated with the gut of *S. litura* is of present day interest to formulate a strategy for pest management programme in tobacco cultivation.

KEY WORDS: Chlorpyrifos, gut microflora, leaf eating caterpillar, *Spodoptera litura*, tobacco

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There are several reports on biodiversity of bacteria associated with insects (Broderick *et al.,* 2004; Xiang *et al.,* 2006). Association of bacteria with insects is of paramount importance for host nutrition, food digestion, antifungal toxin production, pheromone production, regulation of pH, synthesis of vitamins, temperature tolerance, resistance against parasitoid development, and detoxification of noxious compounds (Dillon and Dillon, 2004; Genta *et al.,* 2006). The development of resistance to insecticides is a bottle neck in insect pest management of crop plants (Sarfraz *et al.,* 2006). Insecticidal resistance development is mediated through enzymes like glutathione S-transferase and this aspect has been studied extensively by earlier workers. (Baek *et al.,* 2005). Tobacco (*Nicotiana tabacum* L.), an important commercial crop earning sizable foreign exchange and internal revenue, is susceptible to several pests and diseases both in nursery and field crop (Subhashini and Padmaja, 2009). Leaf is the end product of tobacco.

The Leaf Eating Caterpillar (LEC), *Spodoptera litura* a well-known insect pest that infests solanaceous crops, like tobacco, tomato, chillies and other crops worldwide, has developed resistance to almost all synthetic and microbial insecticides (Sarfraz *et al*., 2006). This is a serious pest both in nurseries and transplanted crop of tobacco. The young caterpillars gregariously feed on leaf tissues while the grown up caterpillars feed voraciously along the veins of leaves during night time. Iron is an essential nutrient for *in vivo* growth of bacteria. One mechanism that bacteria use to acquire iron from host insect is the production and uptake of the extracellular siderophores iron binding agents released into the environment in response to iron deficiency, which also act as antibiotic against invading microbial pathogens (Ciche *et al*., 2003). Most siderophores are phenolic (catechol) compounds and also known for their antifungal and antibacterial activity (Subhashini and Padmaja, 2009). Bacterial enzymes also play a role in host insect nutrition (Dillon and Dillon, 2004). The present investigation is to study the diversity of bacterial population in the guts of (i) chlorpyrifos (organophosophate insecticide) resistant, (ii) chlorpyrifos susceptible and (iii) field caught populations of LEC larvae. Apart from these, the beneficial effect of *Pseudomonas* sp. isolated from the insecticide resistant population and *Serratia* sp. isolated from the insecticide susceptible and field-caught populations, on the host was discussed.

The populations used in this studies were 1) chlorpyrifos susceptible lab population - continuously laboratory reared population for several generations; 2) chlorpyrifos nursery resistant population – population continuously exposed to chlorpyrifos in the tobacco nursery for several generations and 3) field population – populations collected from castor plants in waste lands. The larvae were reared in an acryl cage (30 · 30 · 30cm) with castor leaves and maintained at 25–28°C. Of the four larval stages the most destructive third and fourth instars of LEC population were selected to check whether the subsequent developmental stages are variable with their gut bacteria or not. Thirty third and fourth instars were selected from each population and starved for 24h. To isolate the gut bacteria, the starved larvae were surface disinfected with 70% ethanol for 60s followed by 5% Na OCl (60 s), then thoroughly rinsed with sterile distilled water twice to remove the disinfectant and the whole larva was individually homogenized with 1M phosphate buffer (pH 7.0). The bacterial isolates were recovered by dilution plating using three different media: Luria Bertani (LB), MacConkey (Mc) and Nutrient agar (NA). The plates containing the gut suspensions were incubated for 48h at 28ºC and observed every 24 h for colonies. The colonies were differentiated based on morphological characters, and a single representative isolate of each morphotype transferred to new plates. After five to six successive streaking, the culture purity was ascertained by examination under a light microscope. The purified strains were maintained in 50% glycerol.

The number of bacterial isolates from the insecticide resistant, susceptible, and field-caught populations of LEC larvae was 3, 4, and 3 in the LB media, 5, 6, and 5 in the Mc media, and 6, 9 and 5 in the NA media respectively. The isolates were initially Gram-stained and subjected to basic biochemical characterization, including oxidase, nitrate reduction, carbon (glucose, sucrose, lactose) utilization, H2S and gas production in a triple sugar iron agar (TSIA), IMViC reactions consisting of indole production in a tryptone broth, methyl red and Voges–Proskauer tests in an MR-VP broth and citrate utilization in a simmons citrate agar. The siderophore producing ability of the 46 gut bacterial isolates was ascertained on chrome azural S agar plates (Schwyn and Neilands, 1987). The presence of catechol-type siderophore was determined by mixing an equal volume of the culture supernatant with 5M HCl, a nitrite-molybdate reagent (10 g $NaNO_2$ and 10g $Na_2MoO_4.2H_2O$ in 100ml H_2O), and 1M NaOH, then maintaining at room temperature for five minutes. The development of a pink color indicated the production of a catechol (phenolate)-type siderophore. The mixtures were colorimetrically quantified at 500 nm (Arnow, 1937) and compared with a standard curve generated using catechol. The bacterial strains that were positive for siderophore synthesis were tested for their antagonistic potential against the entomopathogenic fungi *Beauveria bassiana*, *Paecilomyces* sp., and using the well- in-agar method (Santos *et al*., 2004). The fungal cultures used in this study were obtained from the CTRI, Crop Protection Division and maintained in potato dextrose agar slants. A suspension of 100 conidia ml^{-1} of each fungal culture was spread on potato dextrose agar plates, then a flamed cork borer 1 cm in diameter was used to remove agar plugs from the centre of the plate and the wells filled with 6 µl of the bacterial culture (10^8 CFU ml^{-1}). The antagonistic ability was determined after 5 days incubation at 30ºC based on the visibility of a clear halo zone around the bacterial colony. The inhibition of fungal growth was measured as the distance between the edge of the bacterial colony and the fungal mycelium.

The objective of this experiment was to determine whether the chitinase-producing gut bacteria associated with LEC larval gut facilitated the digestion of ingested food, thereby enhancing the growth and development of the host insect. The chitinolytic activity of the 46 bacterial isolates recovered was tested by streaking each isolate on a nutrient agar supplemented with 3% chitin. The plates were incubated at 30ºC until a clear zone was seen around the colony. Nine bacterial strains showing positive (clear zone in an opaque background) for chitinase production were selected to determine the chitinolytic activity. The chitinase-producing bacterial isolates were inoculated in 25 ml of a nutrient broth supplemented with colloidal chitin and incubated for 48 h at 30ºC (200 rpm). After harvesting the cells by centrifugation (5500 rpm at 4ºC for 20 min), the supernatant containing the crude enzyme was purified through a Millipore filter (45 µm). The enzyme analysis was then carried out by preparing an assay mixture consisting of 1 ml of the enzyme solution, 1 ml of 3% colloidal chitin, and 2 ml of a 1M McIlvaine buffer (pH 6.0). The reaction mixture was incubated at 35ºC for 25 min and the reaction terminated by placing in a water bath for 15 min and the addition of 2 ml of a 1 mM potassium ferricyanide reagent (Wiwat *et al*., 2000). The absorbance of the clear solution was measured at 420 nm (UV-spectrophotometer) and the activity calculated using a standard curve generated from known concentrations of N-acetyl glucosamine (NAG). One unit of activity was defined as the amount of enzyme that liberated 1 µM of NAG per minute.

Based on the chitinase producing activity, representative strains of *Serratia* sp. from the susceptible populations were selected, to test their effect on the nutritional indices of their respective host insects as a consequence of chitinase

production. A supernatant containing the crude enzyme chitinase was prepared by growing the bacterial strain in a nutrient media amended with chitin and incubated at 30ºC for 48 h. The cells were harvested by centrifugation (5500 rpm for 20 min) and the supernatant containing the crude enzyme purified through a Millipore filter (45 µm) and used for the bioassay. The cell suspension was prepared by washing the cell pellet twice, followed by suspension in sterile distilled water. The test solutions (treatment) for the bioassay were as follows: (i) 1 ml of the cell suspension + 1 ml of a 5M phosphate buffer, (ii) 1 ml of the cell suspension + one ml of the filtered sterilized crude enzyme, (iii) 2 ml of the crude enzyme, and (iv) 2 ml of the buffer per 5 mm leaf disc (Table 6). Third instar larvae from the susceptible population placed on castor leaf discs (5 cm dia) coated with the test solutions. Each treatment was replicated five times with 10 larvae per replication. The experiment was conducted over 3 days and observations made every 24 h. The consumption, growth rate, and post-ingestive food utilization efficiencies, including the consumption index (CI) = E/TA, relative growth rate (RGR) = P/TA, approximate digestibility (AD) = 100(E-F)/E, efficiency of the conversion of the ingested food (ECI) = 100 P/E, efficiency of the conversion of the digested food (ECD) = 100 P/(E)F), were all calculated gravimetrically on a dry weight basis (Nathan *et al*., 2005), where A = the mean dry weight of the larvae during the experimental time period (T), E = the dry weight of the food eaten, F = the dry weight of the feces produced, and P = the dry weight gain of the larvae. The data were statistically analyzed by an analysis of variance (ANOVA). The highest number of bacteria (log11.74 CFU ml^{-1} of gut suspension) was recorded from the resistant larval gut in Nutrient Agar media, while the smallest number of bacteria (log 7.68 CFU ml^{-1} of gut suspension) was from the field caught larvae in the Luria Bertani media.

Table 1. Gut bacterial population of *Spodoptera litura* in different growth media

Larva of *spodoptera litura*	Gut bacterial population (log CFU ml)[1] gut suspension)					
	Luria Bertani		MacConkey		Nutrient Agar	
	Third instar	Fourth instar	Third instar	Fourth instar	Third instar	Fourth instar
Insecticie resistant population	10.070	10.700	10.890	10.770	11.740	11.220
Susceptible laboratory population	08.830	09.080	09.820	10.130	10.730	10.000
Field population	07.680	08.300	10.000	09.480	10.230	10.750
S.Em±	00.163	00.095	00.317	00.315	0.056	00.116
CD at 5%	00.637	00.372	NS	NS	0.221	00.457
CV%	03.170	01.750	05.360	05.390	00.890	01.890

Irrespective of the growth stage and media, a significant difference was found in the bacterial populations from the three different LEC populations. The isolates recovered in the present study were mostly Gram negative rod or cocci. As expected, the oxidase-positive strain *Pseudomonas* that showed fluorescence in KB media was positive to nitrate reduction, urease, and H_2S production, yet negative to a VP test (Table 2).

Table 2. Morphological and biochemical characteristics of gut bacteria of *Spodoptera litura*

Characteristics	*Pseudomonas*	*Serratia*	*Acinetobacter*	*Micrococcus*
Colony morphology	Smooth circular	Irregular circular	Mucoid	Circular
Color	Dull white	Dull white	White	Pure white
Gram stain	-ve	-ve	-ve	+ve
Motility	Motile	Motile	Non Motile	Motile
Shape	Rods	Rods	Cocoid to ovoid	Cocoid to ovoid
Fluorescence on KB	+	-	-	-
Oxidase test	+	-	-	-
Starch hydrolysis	-	-	-	-
Sugar fermentation	K/K	A/K	A/K	A/K
Nitrate reduction	+	+	+	+
Urease	+	+	-	+
Vogus Prosker	-	+	+	+
H_2S production	+	+	+	+
Gas production	-	+	+	-

The application of a CAS universal siderophore assay resulted in the development of a deep orange color in both a plate and colorimetric assay, confirming the production of one or more iron chelating molecules by the gut bacteria from the three different LEC populations. Among the 46 gut bacterial isolates tested, 3 *Pseudomonas*, 6 Acinetobacter, and 14 *Serratia* were positive for this trait. However, *Micrococcus* was negative to siderophore production. In the CAS plate assay, *Pseudomonas* sp. showed highest (3 cm) orange halo zone. Further the assay revealed that the siderophores produced by *Serratia* sp. and *Acinetobacter* sp. were catechol type. Gut bacterial antagonism is a significant component of the host insect defense mechanism against invading pathogens. Among 23 siderophore producing bacterial strains, *Pseudomonas* sp., was able to inhibit the growth of the entomopathogenic fungi *B. bassiana* and *Paecilomyces* sp. Maximum inhibition was observed in the case of *B. bassiana* (5.53 cm) as compared to *Paecilomyces* sp. (3.90 cm).

Table 3. Antifungal activity of chlorpyrifos resistant *Spodoptera litura* larval gut bacteria *Pseudomonas* sp. against different entomopathogens

Entomopathogen	Inhibition zone diameter (cm)
Beauveria bassiana-1	5.530
Beauveria bassiana-2	5.270
Paecilomyces sp. - 1	3.900
Paecilomyces sp. – 2	3.300
Control *(B. bassiana)*	2.290
Control *(Paecilomyces* sp*)*	2.050
S.Em ±	0.189
CD (*P*=5%)	0.657
CV %	7.300

The bacterial genera reported in the present study were previously in the guts of different Lepidopteran families (Broderick *et al.,* 2004; Xiang *et al.,* 2006). The present study revealed that the field population of LEC larvae harbored single *Serratia* sp., and the occurance of the same had already been reported in other insect orders, including Orthoptera, Thysanoptera, and Hemiptera (Kikuchi *et al.,* 2005). Association of dominant bacteria may eliminate or prevent the colonization of other competitive microorganisms, probably by siderophore synthesis, which sequesters and pre-empts the available iron, an essential growth factor for microorganisms in an insect gut environment (Genta *et al.,* 2006).

While there have been a few studies related to the gut microbiota of Lepidoptera, these studies have only dealt with the isolation of the microbial flora (Broderick *et al.,* 2004; Xiang *et al.,* 2006). However, in the present study, the beneficial effect of the gut bacteria were revealed, siderophore producing *Pseudomonas* sp. was able to inhibit the growth of entomopathogenic fungi. Similar findings were also reported by Indira Gandhi *et al.* (2007), where the siderophore-producing gut bacteria *Pantoea agglomerans* was found to inhibit the growth and spore germination of *Metarhizium anisopliae* in diamond back moth guts. Present study revealed that the antagonistic ability of *Pseudomonas* sp. against entomopathogenic fungi could contribute to the host protection. The characteristic yellow-green pigment pyoverdine, a well-known siderophore of *Pseudomonas*, has already been extensively studied for its antagonism against various pathogenic fungi in tobacco (Subhashini and Padmaja, 2009). However, catechol produced by the gut bacteria might serve as growth substrate and maintain the composition and population of the host insect gut bacteria rather than act as antimicrobial agent (Dillon & Charnley, 2002). Thus results revealed that the bacteria in the different LEC populations differed significantly may be due to the selection pressure created by insecticide chlorpyrifos. Resistance to pathogenic fungi and enhanced insect growth may be accomplished by gut bacteria through the production of siderophore and chitinase. Thus, further analysis of the different populations of LEC gut bacteria may pave the way for new ways to exploit bacterial association with *S. litura* in pest management.

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