Screening of antibiotics to alter the *Plutella xylostella* L (Plutellidae: Lepidoptera) gut microbial diversity

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ABSTRACT

The diamondback moth, *Plutella xylostella* L (Plutellidae: Lepidoptera) is the most important pest of cruciferous crops in wide areas of the world. Larvae of *P xylostella* have rich microbial communities inhabiting in the gut and those bacteria contribute to the fitness. In the present investigations the effect of five different concentrations of eight antibiotics on gut bacterial diversity of *P xylostella* was studied. Bacterial culture (ISO-1) from gut homogenates was subjected to antibiotic screening tests. Cefixime (5 mg/ml) was the most effective antibiotic with the greatest inhibition zone (25 mm). The antibiotics were screened against the larvae of the insect. Higher mortality and reduced growth of larvae were recorded in case of larvae feeding on cefixime-treated leaves as compared to other treatments. Cefixime-exposed *P xylostella* was continuously reared in laboratory. Species diversity was significantly reduced in cefixime-exposed population. The cfu/ml of gut suspension was also reduced in cefixime-exposed population of the insect. The results indicated that bacterial symbionts play a crucial role in the successful development of the host.

Keywords: Antibiotics; *Plutella xylostella*; gut bacteria; cefixime

INTRODUCTION

The diamondback moth, *Plutella xylostella* (Linnaeus) (Lepidoptera: Plutellidae) is the major destructive pest on cruciferous crops such as cauliflower, cabbage and mustard. Most often growers resort to prophylactic and scheduled applications of chemical insecticides for the control of this pest. Consequently problems like resurgence, residues, replacement, destruction of non-target organisms and environmental pollution have been on the rise. *P xylostella* has developed the resistance to many available insecticides. To manage the pest population efficient biorational control measures are needed.

Knowledge about gut bacterial symbionts associated with insects is a key step to develop novel control strategies. Insect gut possesses a high range of microbial symbionts such as bacteria, fungi and viruses. Endosymbionts are involved in producing digestive enzymes, detoxifying enzymes, fitness and survival. Indiragandhi et al (2008a, 2008b) found that the bacteria isolated from *P xylostella* were involved in increased host consumption index, relative growth rate and inhibited phytopathogenic fungal growth. Gut bacteria are able to secrete antibiotics to suppress the growth of other invading microorganisms. Antibiotic insecticides such as jinggangmycin, abamectin and spinosyns are the biopesticides from microorganism metabolites of bacteria or fungi which have been used to control pest insects. These antibiotics have characteristics of high efficiency and low toxicity to plants, human and environment.

Antibiotics can be used for removing the gut symbiotic bacteria through feeding. To explore the gut

symbionts feeding with antibiotics is easily adaptable instead of sterile insect model or haemolymph transfer. According to Adams et al (1996) rifampicin and aureomycin antibiotics were used for curing the gut symbionts. Jung and Kim (2006) fed penicillin to *Spodoptera exigua* (Hubner) and Raymond et al (2009) reared *P xylostella* on artificial diet for clearing gut bacteria. Kafil et al (2013) reported that antibiotic feeding with artificial diet would eliminate the gut bacteria of Sunn pest, *Eurygaster integriceps*.

It is possible to clear the microbes by feeding antibiotics for a few generations. The literature is sparse on *P xylostella* gut floral reduction. Therefore some antibiotics were tested against *P xylostella* in the present study.

MATERIAL and METHODS

Collection and rearing of symbiotic *P xylostella*

The larvae of *P xylostella* were collected from cauliflower field at Odanchathiram, Tamil Nadu, brought to the laboratory and reared on cauliflower leaves at room temperature till pupation. Newly-emerged adults were transferred into wooden cages (30 x 30 x 45 cm) for mating and egg laying on mustard leaves. Adults were fed on cotton wool impregnated with 10 per cent honey solution. The rearing was continued for getting a steady supply of larvae for different experiments.

Antibiotic screening

Antibiotic screening was done by testing antibiotics against gut bacterial strain (ISO-1) and testing against the larvae of *P xylostella*. The antibiotic that gave the best results were tested against *P xylostella* for a few generations. Gut bacterial elimination was confirmed based on the cfu/ml gut suspension between antibiotic-exposed and non-exposed populations.

Isolation of *P xylostella* gut bacteria

Of the four larval stages the most destructive third instar of *P xylostella* was used for isolating the gut bacteria. For this 9 third instars were selected and starved for 24 h. The starved larvae were surface disinfected with 70 per cent ethanol for 60 sec and then thoroughly rinsed with sterile distilled water to remove the disinfectant. The dissected larval gut was homogenized with 0.1 M phosphate buffer (pH 7.0) and the bacterial isolates were recovered by 10^{-4}, 10^{-5} and 10^{-6} dilution planting in NA media (de Vries et al 2004). The plates containing gut suspensions were incubated for 48 h at room temperature and surveyed every 24 h for new colonies. Each dilution was replicated three times. Colonies were differentiated based on their size, colour and morphology and a single representative isolate of each morphotype was transferred to new plates. After five to six successive streakings the culture purity was ascertained by a light microscope. Purified strains were maintained in 50 per cent glycerol at -80°C. For the experimental purpose the bacteria were revived in a nutrient broth containing (g^{-1}) beef extract 3.0 and peptone 5.0. These grown bacteria were used for subsequent antibiotic screening test.

Screening of antibiotics with bacterial isolates

Only one bacterial strain (ISO-1) was isolated from the gut of *P xylostella*. Antibiotic screening was studied against the ISO-1. The antibiotic screening test was carried out in vitro as per Visotto et al (2009). Eight antibiotics viz amoxicillin, cephalexin, doxycycline, cefixime, cefpodoxime, ciprofloxacin, levofloxacin and tetracycline were used to compare their efficacies of bacterial elimination and their toxicities to *P xylostella* larvae. Five gradient concentrations (1, 2, 3, 4 and 5 mg/ml) of each antibiotic were tested. NA medium amended with the gut bacterial suspension of ISO-1 was poured into the Perti dish. Paper disc was immersed into the antibiotic solutions for a minute according to respective treatments. The antibiotic discs were placed above the medium which was containing the gut bacterial suspension of ISO-1. The culture plates were incubated at 28°C and inhibition zones were measured.

Screening of antibiotics with *P xylostella* larvae

Eight antibiotics viz amoxicillin, cephalexin, doxycycline, cefixime, cefpodoxime, ciprofloxacin, levofloxacin and tetracycline were used to compare their efficacies of bacterial elimination and their toxicities of *P xylostella* larvae. Eight gradient concentrations (1, 2, 3, 4 and 5 mg/ml) of each antibiotic were tested. Cabbage leaves were first washed with distilled water; leaf disc was prepared and dipped in antibiotic solutions for two hour. The leaf disc was placed slanting for about 2 min over a blotting paper in a tray to drain excess solution and then flattened to dry the test solution for about 30 min. Second instar larvae starved for 2 h were placed in the small plastic cups (bioassay containers). Each replication contained 30 larvae. The larvae were allowed to feed treated leaf discs. Larval and pupal mortality and abnormality

of adults was calculated. Leaf disc was replaced everyday till the larvae entered in pupation. The experiment was laid out in completely randomized design (CRD) with water as control and replicated thrice.

After the antibiotic screening, best antibiotics were selected; *P xylostella* larvae were exposed to the best antibiotics through leaf dip method. Antibiotic-exposed *P xylostella* larvae were reared on cauliflower leaves for further generations as per the method described above.

Confirmation of gut symbiont elimination

Gut suspension (cfu/ml) was calculated to find out bacterial load present in the gut of *P xylostella* before (symbiotic *P xylostella*) and after exposure of antibiotic (aposymbiotic *P xylostella*). Gut suspension (cfu/ml) was calculated based on the universal protocol.

RESULTS

Effect of gut bacterial isolate on different antibiotics

Based on the antibiotic screening tests all eight tested antibiotics were separated into six groups according to the inhibition zone they produced viz 0.0-5.0 cm (first group), 5.0-10.0 cm (second group), 10.0-15.0 cm (third group), 15.0-20.0 cm (fourth group), 20.0-25.0 cm (fifth group) and >25 cm (sixth group) represented as highly resistant, moderately resistant, slightly resistant, intermediate, susceptible and highly susceptible respectively (Table 1).

Antibiotics cephalexin, doxycycline and levofloxacin were placed in high resistance group that had the least activity against gut bacteria. Ciprofloxacin 5 mg/ml had a 19.75 mm inhibition zone which was placed in the intermediate group. Cefixime and tetracycline had good inhibition zone of above 20 mm. On comparing the cefixime and tetracycline only former produced a very good inhibition zone against gut bacteria in the in vitro assay (Fig 1).

Effects on the growth of the *P xylostella* larvae

Effects of eight antibiotics on *P xylostella* larvae are shown in Table 2. All antibiotics reduced the performance of larvae. With the increase of antibiotic concentration the larval mortality increased. Highest larval mortality occurred in cefixime (0.88) followed by tetracycline (0.80) and the least was recorded in ciprofloxacin (0.20). Pupal mortality was at par in cefixime (0.66), ciprofloxacin and amoxicillin (0.64) and tetracycline (0.14) showed least pupal mortality.

Due to antibiotic exposure pre-pupae could not pupate and develop successfully. Antibiotics-fed *P*

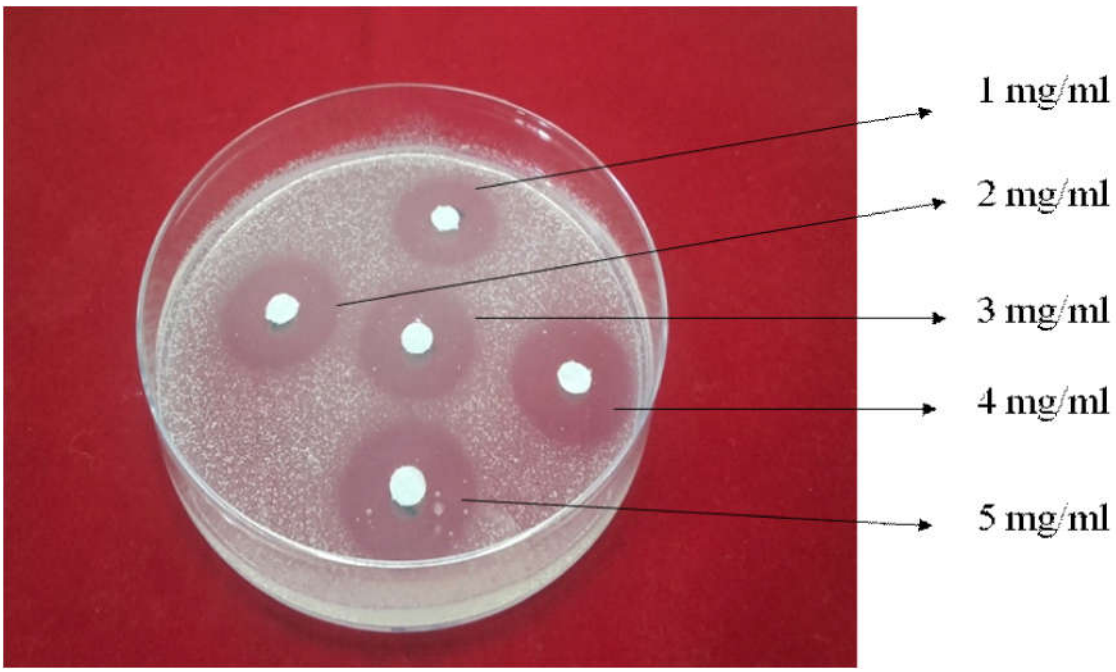

Fig 1. Inhibition zone produced by cefixime different concentration

Table 1. Antibiotics, their concentrations used in the antibiotics screening tests and their effect on gut bacteria of ISO-1

Antibiotic	Concentration (mg/ml)	Inhibition zone (mm)	Bacterial response to antibiotic
Amoxicillin	1	4	Highly resistant
	2	4.25	Highly resistant
	3	6.575	Moderately resistant
	4	5.75	Moderately resistant
	5	3	Highly resistant
Cephalexin	1	1	Highly resistant
	2	4.5	Highly resistant
	3	2.25	Highly resistant
	4	4	Highly resistant
	5	3.5	Highly resistant
Doxycycline	1	2.5	Highly resistant
	2	3.75	Highly resistant
	3	4.25	Highly resistant
	4	4.25	Highly resistant
	5	9.675	Highly resistant
Cefixime	1	6.75	Moderately resistant
	2	8.925	Moderately resistant
	3	14.725	Slightly resistant
	4	14.75	Slightly resistant
	5	28.5	Highly susceptible
Cefpodoxime	1	3.5	Highly resistant
	2	3	Highly resistant
	3	4.7	Highly resistant
	4	6.25	Moderately resistant
	5	5.75	Moderately resistant
Ciprofloxacin	1	3.3	Highly resistant
	2	6.5	Moderately resistant
	3	11	Slightly resistant
	4	4.5	Highly resistant
	5	19.75	Intermediate
levoflaxin	1	3	Highly resistant
	2	1.25	Highly resistant
	3	1.25	Highly resistant
	4	4.5	Highly resistant
	5	0.5	Highly resistant
Tetracycline	1	7.75	Moderately resistant
	2	9.5	Moderately resistant
	3	16.75	Intermediate
	4	22	susceptible
	5	14	Slightly resistant
Untreated control	0	0	Highly resistant

xylostella larvae showed malformation but in some treatments malformation was not exhibited. Mean malformed adult emergence in ciprofloxacin, cefpodoxime, cefixime and tetracycline was 1.00. Cefixime was most toxic antibiotic as it showed high larval and pupal mortality.

On the basis of larval bioassay and screening based on the bacterial isolate, cefixime 5 mg/ml was selected for eliminating the gut symbionts. Cefixime-exposed *P xylostella* population was maintained in the laboratory.

Effects of antibiotics on gut bacterial diversity

Gut bacterial load varied between antibiotic-exposed and non-exposed *P xylostella* (Table 3). *P xylostella* gut contained 139 cfu/ml at 10^{-4} dilution and it was reduced after the antibiotic exposure which contained only 53.33 cfu/ml. Reduction in the gut bacteria showed the elimination of gut bacteria.

Table 2. The influence of antibiotics on *P xylostella* population

Antibiotic	Concentration (mg/ml)	Larval mortality (Mean ± SE)	Pupal mortality (Mean ± SE)	Abnormality rate of adult (Mean ± SE)
Amoxicillin	1	0.30 ± 0.22e	0.46 ± 0.23efg	0.75 ± 0.22b
	2	0.42 ± 0.37d	0.47 ± 0.30efg	0.67 ± 0.18c
	3	0.30 ± 0.18e	0.49 ± 0.20ef	0.25 ± 0.06d
	4	0.21 ± 0.17f	0.60 ± 0.35bcd	0.00 ± 0.00e
	5	0.50 ± 0.23cd	0.64 ± 0.20bc	0.00 ± 0.00e
Cephalexin	1	0.45 ± 0.51d	0.30 ± 0.15jk	0.00 ± 0.00e
	2	0.53 ± 0.55cd	0.33 ± 0.21ij	0.00 ± 0.00e
	3	0.41 ± 0.24d	0.44 ± 0.18fg	0.67 ± 0.12c
	4	0.45 ± 0.42d	0.31 ± 0.18jk	0.33 ± 0.08d
	5	0.46 ± 0.42d	0.47 ± 0.15efg	1.00 ± 0.09c
Doxycycline	1	0.50 ± 0.36cd	0.16 ± 0.08m	0.50 ± 0.14c
	2	0.30 ± 0.55e	0.41 ± 0.27fgh	0.50 ± 0.09d
	3	0.24 ± 0.42f	0.53 ± 0.22de	0.00 ± 0.00e
	4	0.30 ± 0.67e	0.16 ± 0.09m	0.00 ± 0.00e
	5	0.64 ± 1.05bc	0.41 ± 0.21fgh	0.00 ± 0.00e
Cefixime	1	0.40 ± 0.29d	0.30 ± 0.15jk	0.51 ± 0.14c
	2	0.50 ± 0.46cd	0.36 ± 0.23hij	1.00 ± 0.28b
	3	0.53 ±0.42cd	0.44 ± 0.18fg	0.00 ± 0.00e
	4	0.64 ± 0.67b	0.46 ± 0.09efg	1.00 ± 0.08d
	5	0.88 ± 0.58a	0.66 ± 0.21ab	1.00 ±0.14c
Cefpodoxime	1	0.30 ± 0.64c	0.46 ± 0.29efg	0.00 ± 0.00e
	2	0.36 ± 0.48e	0.17 ± 0.07lm	0.00 ± 0.00e
	3	0.31 ± 0.67e	0.16 ± 0.09m	0.00 ±0.00e
	4	0.43 ± 0.42d	0.40 ± 0.13ghi	0.00 ± 0.00e
	5	0.41 ± 0.21d	0.40 ± 0.20ghi	1.00 ± 0.29a
Ciprofloxacin	1	0.20 ± 0.37f	0.46 ± 0.29efg	0.67 ± 0.18c
	2	0.24 ± 0.24f	0.63 ± 0.26bc	1.00 ± 0.12c
	3	0.40 ± 0.34d	0.64 ± 0.38bc	0.00 ± 0.00e
	4	0.53 ± 0.71cd	0.63 ± 0.23a	0.00 ± 0.00e
	5	0.54 ± 0.81cd	0.57 ± 0.29cd	0.00 ± 0.00e
levoflaxin	1	0.50 ± 0.46cd	0.29 ± 0.18jk	0.67 ± 0.18c
	2	0.51 ± 0.30cd	0.46 ± 0.19efg	0.50 ± 0.06d
	3	0.64 ± 0.59bc	0.49 ± 0.29ef	0.56 ± 0.08d
	4	0.69 ± 0.32bc	0.60 ± 0.19bcd	0.00 ± 0.00e
	5	0.57 ± 0.28c	0.31 ± 0.16jk	0.75 ± 0.22b
Tetracycline	1	0.50 ± 0.46c	0.24 ± 0.16 kl	0.50 ± 0.18c
	2	0.60 ± 0.36b	0.40 ± 0.17ghi	0.50 ± 0.06d
	3	0.67 ± 0.59ab	0.41 ± 0.24fgh	0.00 ± 0.00e
	4	0.80 ± 0.36ab	0.46 ± 0.14dfg	0.00 ± 0.00e
	5	0.37 ± 0.33g	0.14 ± 0.07m	0.00 ± 0.00e
Untreated control	0	0.17 ± 0.22g	0.36 ± 0.23d	0.00 ± 0.00e

Data presented as Mean ± SE of the mean, Mean ± SE followed by different letters differed significantly with each other at P <5

Table 3. The microbial abundance of symbiotic and aposymbiotic *P xylostella*

Type	Bacterial count (log CFU/ml) at different dilutions		
	10^{-4}	10^{-5}	10^{-6}
Symbiotic *P xylostella* gut	139 ± 20.38	70.4 ± 13.6	17.7 ± 1.76
Aposymbiotic *P xylostella* gut	53.33 ± 2.67	43.7 ± 2.33	12.0 ± 1.53

Data are represented as Mean of (CFU/ml) of gut suspension ± Standard error of the mean

DISCUSSION

The results showed that the culturable gut bacteria could be significantly reduced but not completely when larvae were treated with oral antibiotics. With the increase of the concentration of antibiotics the efficacy of the antibiotics was much increased in cefixime group. All antibiotics caused harm to *P xylostella* larvae as shown by higher larval mortality, pupal mortality and malformation of adults. It showed that antibiotics may directly affect the gut cells of the larva and indirectly affect the normal growth and development. Antibiotics killed most of the gut bacteria including beneficial microorganisms, broke the intestinal micro-ecological balance and made gut flora disorder.

Antibiotic screening test showed that tested antibiotics fell into six groups as per the inhibition zone they produced. Among them cefixime yielded the largest inhibition zone.

Cefixime was orally administrated to reduce the abundance of symbiotic bacteria in the gut. Cefixime is a broad-spectrum antibiotic used to treat a variety of infections like urinary tract infections, ear infections and lung infections caused by bacteria. The bactericidal action of cefixime is due to the inhibition of cell wall synthesis. It binds to one of the penicillin-binding proteins (PBPs) which inhibits the final transpeptidation step of the peptidoglycan synthesis in the bacterial cell wall thus inhibiting biosynthesis and arresting cell wall assembly resulting in bacterial cell death.

Similar observations were made by Indiragandhi (2011) who reported that larvae feeding on antibiotics failed to pupate. Results of this study indicated that, as in several other hemipteran taxa, aposymbiotic larvae produced by antibiotic administration exhibited retarded growth and increased mortality suggesting that the symbionts play a crucial role for the host insects. Understanding the interrelations among gut bacterial flora the effects of antibiotics on the diversity of symbionts in the gut and the development of antibiotic therapy will be useful for integrated pest management of *P xylostella* in the future.

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