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Resistance of three different populations of mulberry silkworm (*Bombyx mori* L.) to *Bacillus thuringiensis*

Resistenz von drei verschiedenen Populationen des Seidenspinners (*Bombyx mori* L.) gegen *Bacillus thuringensis*

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Summary

The relative resistance levels of three different populations of mulberry silkworm, *Bombyx mori* L. to *Bacillus thuringiensis* (Bt) has been studied. All three populations (two Australian and one Indonesian) were observed for similar characteristics including 3rd instar larval mortality at 24, 48, 72 and 96 h after treatment (HAT), LD_{50} ratio and probit mortality. Among the Australian two populations, the QuBill (yellow coloured, oval shaped cocoon) population showed higher larval mortality to Bt toxicity compared to the QuBite (white coloured, oval shaped cocoon) population. When all the populations were compared, the Insab (Indonesian population with white coloured, peanut shaped cocoon) showed lower larval mortality and highest LD_{50} ratio up to 48 HAT. The Insab population also showed a 24 h longer incubation/latent period prior to the start of mortality.

Key words: *Bombyx mori; Bacillus thuringiensis;* Bt treatment; leaf disc bioassay; LD_{50}

Zusammenfassung

Das relative Resistenzniveau von drei verschiedenen Populationen des Seidenspinners *Bombyx mori* L. gegen *Bacillus thuringiensis* (Bt) wurde untersucht. Bei allen drei Populationen (zwei australische und eine indonesische) erfolgte die Überprüfung auf ähnliche Merkmale wie die Mortalität des dritten Larvenstadiums 24, 48, 72 und 96 h nach Behandlung (HAT), LD_{50}-Verhältnis und Probitmortalität. Die australische Population QuBill (gelbfarbige, ovale Kokons) zeigte eine höhere Larvensterblichkeit durch das Bt-Toxin im Vergleich zur QuBite-Population (weiße, ovale Kokons). Beim Vergleich aller Populationen wies die Population Insab (aus Indonesien; weiße, erdnussförmige Kokons) die niedrigste Larvensterblichkeit und das höchste LD_{50}-Verhältnis bis 48 HAT auf. Bei der Insab-Population dauerte die Inkubations- bzw. latente Zeit 24 h länger bevor die Mortalität einsetzte.

Stichwörter: *Bombyx mori; Bacillus thuringiensis;* Bt-Behandlung; Blattscheibentest; LD_{50}

1 Introduction

Bacillus thuringiensis (Bt) is a Gram-positive spore-forming bacterium characterized by the formation of parasporal inclusions during sporulation (Aronson et al. 1986; Ohba 1996). After being ingested by the lepidopteran larvae, the parasporal inclusions are dissolved in larval midgut juice and release protoxins. The activated toxins interact with the larval epithelial membrane and induce pore formation in the membrane, which ultimately leads to insect death (Gill et al. 1992). Bt is considered to be the causative agent for a special type of "flacherie" disease of silkworms, known as "Sotto". Larvae affected by Bt lose their appetite, undergo convulsions and their bodies become stretched and cracked. The cadavers gradually become brown to black-brown and finally when rotting they turn black (Aruga 1994).

Because of their efficacy against insects, commercial preparations of different varieties of Bt have been sold and used as biopesticides against crop pests world-wide for over half a century. However, growing public concern surrounding Bt use has sparked world-wide debate over current policies (Jayaraman 1991). According to Ohba (1996), Bt was commonly (96 %) present in mulberry plants especially on the leaves in Japan, however, did not explain how Bt lodges themselves on the leaves. van Driesche and Bellows (1996) reported that in India, fear over a potential epizootic, or microbial pathogen out-break, inspired a governmental ban on the use of Bt in silkworm/mulberry-growing areas, despite the nation's moves to reduce the use of traditional chemical pesticides. Pramanik and Somchoudhury (2001) during their study on the adverse effects of *Bacillus thuringiensis* var. *kurstaki* against different larval instars of the silkworm, *Bombyx mori* reported that it is highly lethal to larvae, causing over 50 % mortality of 4th instar larvae at a low concentration of 0.01 % of Bt. Inagaki et al. (1992) found that *B. mori* larvae were 2,500-fold more susceptible to the Bt var. *kurstaki* infection than *Spodoptera litura* larvae. They found rapid growth of Bt in the hemolymph of *B. mori*. Hassan et al. (1989) compared the effects of sub-lethal doses of Bt (var. *kurstaki*) on third instar *B. mori* larvae and found that Bt significantly reduced the fecundity and fertility of surviving females.

The establishment of a new sericulture industry in Australia might face a number of disease problems including Bt, which might turn up either from mulberry leaf sources or from the use of Bt pesticides in nearby areas. These organisms may therefore be a major constraint in silkworm disease management, because it is difficult to exclude pathogens from the silkworm rearing environment (Balavenkatasubbaiah et al. 1999). Thus, it is important to study the effects of Bt on different Australian and imported silkworm races and to develop disease resistant silkworm races.

The present investigation was carried out to study the *in vivo* lethal effects of Bt var. *kurstaki* against three different races of *B. mori* larvae under laboratory conditions.

2 Materials and methods

Three silkworm populations, two Australian origin and one of Indonesian origin, coded as QuBite, QuBill and Insab, respectively, were used for the present research study. The experiment was set in a completely Randomized Block Design with three replications. After hatching from the eggs, neonates were brushed and reared up to second moulting on fresh leaves of mulberry *(Morus alba)*. One-day-old 3rd instar larvae of uniform size were selected from all three races and used for the experiment. All insect rearing and experiments were done under the following laboratory condition at the School of Agronomy and Horticulture, University of Queensland: temperature 25 ± 2 °C, relative humidity 75 ± 2 % and a light : dark ratio, 16 : 8 h.

Dipel®, a commercial preparation of *Bacillus thuringiensis* var *kurstaki*, was used as a source of insecticide (Aurther Yates and Co. Ltd, Australia, active constituents: 4320 international units of potency per mg of *B. thuringiensis* var. *kurstaki*). The preparation of stock solution was done by dissolving 66.7 mg of Dipel® in 100 ml of distilled water (strength: 2881.44 IU/ml). All further dilutions were made from this stock. All mixtures were used at once for insect bioassays or stored for shorter period of time (less than 12 h) in refrigerator (4 °C) before using.

Fresh leaves were collected from the mulberry trees in the field. Before using the leaves, they were washed and dried to remove any dust or foreign materials and checked for arthropod infestation. With the help of a sterile cork-borer, mulberry leaves were cut as discs of 4.50 cm diameter and 15.90 cm^2 area.

Tests for Bt toxicity by leaf disc bioassay was conducted according to the method described by Sen et al. (1999) with some modifications. Immediately after the 2nd moulting, newly emerged (moulted) 3rd instar larvae of a similar size were collected from the rearing trays. The stock solution of Bt insecticide were prepared by dissolving 66.7 mg of Dipel® in 100 ml distilled water, so that 150 µl of stock solution contains 100 µg of Dipel® (432 IU of Bt). Lower concentrations (80, 70, 60, 50 and 40 µg/150 µl solution) were obtained. The stock solution was diluted with the appropriate amount of distilled water, so that 150 µl insecticide solution (would apply either 80, 70, 60, 50 or 40 µg of Dipel®, equivalent to 345, 302, 259, 216 or 172 IU of Bt) was applied to the under-surface of the leaf disc using a micro-applicator. The solution was smeared evenly with the help of a flat-tipped glass-rod and then allowed to air-dry under vacuum cupboard conditions. The control leaf discs were treated with 150 µl distilled water only and smeared and air-dried in the same way. After drying, each leaf disc was transferred to a 250 ml plastic cup, whose bottom was already layered with a filter paper. Ten newly moulted 3rd instar silkworm larvae were released on each leaf disc and allowed to feed for next 24 h. Three leaf discs were used for each concentration, including the control, each disc as a replication. All plastic cups were kept at 25 ± 2 °C temperature, 75 ± 2 % relative humidity and using light : dark ratio of 16 : 8 h. At the end of 24 h, the larval mortality was counted in each cup. After removing the dead, all surviving larvae were transferred to new plastic containers and fed with untreated fresh mulberry leaves. Larval mortalities were also recorded at 48, 72 and 96 h after treatment (HAT), and all dead larvae were removed from the boxes on a regular basis. For the statistical analysis, the original mortality data were transformed into arcsin √ percentage values and then analyzed using ANOVA and Duncan's multiple range test (Duncan, 1951).

Bt concentration – larval mortality relationships were calculated using probit analysis (Finney 1971) with a $\log_{10}$ transformation of concentrations of Bt. Results (LD_{50}) were expressed as micrograms of Dipel® per leaf disc (µg/disc). Two LD_{50}s (Median Lethal dose of Dipel®) were considered to be significantly different ($P < 0.05$) if their 95 % fiducial limits did not overlap; slopes were similarly considered to be significantly different if their standard errors (S. E.) did not overlap.

3 Results

The preliminary investigations on the relative resistance levels of the three different populations of *Bombyx mori* against different doses of *Bacillus thuringiensis* var. *kurstaki* had been carried out. The results from the three populations are given in Tables 1A, 1B and 1C.

The effects of different Bt doses against 'QuBite' population (Table 1A) showed that all doses of Bt possessed lethal capabilities against this silkworm population. The mortality rate was found to be Bt dose and time dependent. The highest larval mortalities (53.33 %, 66.67 % and 70.00 % at 24, 48 and 72 HAT, respectively) were observed at the highest Bt dose (80 µg Dipel®/disc). However, no additional mortality was observed at 96 HAT. The 'QuBill' population (Table 1B) had a higher mortality rate (63.33 %, 86.67 % and 86.67 % at 24, 48 and 72 HAT, respectively) at 80 µg Dipel®/disc dose, than the 'QuBite' population. No further larval mortality was observed at 96 HAT. The 'Insab' population (Table 1C) showed that at 24 h after treatment (HAT), most of the Bt doses did not cause any mortality to this population. Though the highest Bt dose (80 µg/disc) showed some insect mortality at this stage, it was negligible (3.33 %) in relation to the insect mortality caused by Bt in the other two populations. However, at 48 HAT and 72 HAT, a significant number of Insab population (53.33 % and 76.67 %, respectively) of *B. mori* larvae died due to the lethal effects of Bt. Similar to the results in the other two populations, no additional mortality was observed at 96 HAT. The 'QuBite' population showed most resistance to the highest Bt dose at 72 HAT (larval mortality 70.00 %), followed by the 'Insab' (76.67 %), whereas the 'QuBill' population showed the highest susceptibility to Bt attack at the same time (larval mortality 86.67 %).

The probit analysis, estimate of LD_{50} and their 95 % fiducial limits for *Bombyx mori* larval mortality are presented in Table 2. Comparison of LD_{50}'s on the basis of the probit analyses at 48 h after treatment (48 HAT) showed that the 'QuBill' was the most susceptible (LD_{50} 38.29 µg Dipel®/disc) of the three populations tested, followed by 'QuBite' (LD_{50} 55.10 µg/disc). However, at 72 HAT, the susceptibility sequence changed to the 'QuBill' (LD_{50} 33.93 µg/disc) followed by 'Insab' (LD_{50} 45.73 µg/disc), when the 'QuBite' appeared as most resistant to Bt attack (LD_{50} 48.96 µg/disc).

Table 1. Larval mortality in three different silkworm populations due to the application of different Bt doses on leaf discs

Population and Bt conc. (µg/disc)	No. of treated insects	Average larval mortality percentage (%) ± SE at			
		24 HAT	48 HAT	72 HAT	96 HAT
A. QuBite:					
80	30	53.33 ± 9.81 a	66.67 ± 2.72 a	70.00 ± 0.0 a	70.00 ± 0.0 a
70	30	43.33 ± 7.20 ab	56.67 ± 5.44 ab	66.67 ± 7.20 a	66.67 ± 7.20 a
60	30	40.00 ± 4.71 ab	50.00 ± 4.71 ab	60.00 ± 4.71 ab	60.00 ± 4.71 ab
50	30	36.67 ± 7.20 ab	46.67 ± 2.72 b	46.67 ± 2.72 b	46.67 ± 2.72 b
40	30	26.67 ± 2.72 b	40.00 ± 4.71 b	43.33 ± 2.72 b	43.33 ± 2.72 b
Control	30	0	0	0	0
B. QuBill:					
80	30	63.33 ± 2.72 a	86.67 ± 2.72 a	86.67 ± 2.72 a	86.67 ± 2.72 a
70	30	60.00 ± 16.33 ab	80.00 ± 12.47 a	83.33 ± 9.81 a	83.33 ± 9.81 a
60	30	60.00 ± 4.71 ab	76.67 ± 2.72 ab	76.67 ± 2.72 ab	76.67 ± 2.72 ab
50	30	33.33 ± 5.44 b	63.33 ± 2.72 ab	66.67 ± 5.44 ab	66.67 ± 5.44 ab
40	30	33.33 ± 5.44 b	53.33 ± 9.81 b	60.00 ± 4.71 b	60.00 ± 4.71 b
Control	30	0	0	0	0
C. Insab:					
80	30	3.33 ± 2.72 a	53.33 ± 9.81 a	76.67 ± 2.72 a	76.67 ± 2.72 a
70	30	0 a	46.67 ± 11.86 ab	66.67 ± 5.44 ab	66.67 ± 5.44 ab
60	30	0 a	36.67 ± 5.44 ab	63.33 ± 2.72 ab	63.33 ± 2.72 ab
50	30	0 a	26.67 ± 7.20 bc	50.00 ± 9.43 b	50.00 ± 9.43 b
40	30	0 a	16.67 ± 9.81 c	46.67 ± 7.20 b	46.67 ± 7.20 b
Control	30	0	0	0	0

* Population means within a column followed by the same letter are not significantly different at the 5 % level (Duncan's Multiple Range Test)

Table 2. Probit Analysis for lethality of different Bt concentrations on three populations of mulberry silkworm, *Bombyx mori*

Silkworm race	No. of larvae	LD_{50} (µg/disc)	X^2 value	95 % fiducial limit	Slope ± S. E.
QuBite:					
24 HAT	150	77.43 a	0.24	55.21–108.64	2.11 ± 0.99
48 HAT	150	55.10 a	0.34	23.11–77.75	2.11 ± 0.98
72 HAT	150	48.96 a	0.32	25.34–59.04	2.52 ± 0.99
96 HAT	150	48.96 a	0.32	25.34–59.04	2.52 ± 0.99
QuBill:					
24 HAT	150	58.34 a	1.97	47.44–71.60	3.00 ± 0.99
48 HAT	150	38.29 b	0.17	20.12–46.28	3.40 ± 1.06
72 HAT	150	33.93 b	0.13	9.73–43.52	2.97 ± 1.07
96 HAT	150	33.93 b	0.13	9.73–43.52	2.97 ± 1.07
Insab:					
24 HAT	150	N/A	N/A	N/A	N/A
48 HAT	150	74.94 a	0.01	64.84–109.03	3.53 ± 1.05
72 HAT	150	45.73 b	0.49	22.55–54.82	2.67 ± 1.00
96 HAT	150	45.73 b	0.49	22.55–54.82	2.67 ± 1.00

* Values followed by the same letter within a column are not significantly different at the 0.05 level ($P \leq 0.05$).

The probit regression lines for the effects of Bt on three silkworm populations showed a clear linear relationship between larval probit mortality rate (Y) and log Bt concentrations (X) (Fig. 1A and 1B). Because the larvae were treated with more lethal agents at higher concentrations, the slopes of the probit lines were steeper as concentration increased. At 48 HAT (Fig. 1A), the regression lines were Y = – 3.981 + 2.108 X for the 'QuBite' population, Y = – 5.299 + 3.00 X for the 'QuBill' population and Y = – 6.623 + 3.533 X for the 'Insab' population. Comparing all three regression lines, the steeper line was found in the 'QuBill' population. At the 72 HAT (Fig. 1B), the regression lines was Y = – 4.265 + 2.524 X for the 'QuBite' population, Y = – 4.541 + 2.967 X for the 'QuBill' population and Y = – 4.433 + 2.670 X for the 'Insab' population were calculated. Comparing all three regression lines, the steeper line was found in the 'QuBill' population.

4 Discussion

Inagaki et al. (1992) found that *B. mori* larvae were highly susceptible to the Bt var. *kurstaki* infection than *Spodoptera litura* larvae. Our current results are in agreement with the findings of the above authors, as we observed higher larval mortalities (60–86 %) of *B. mori* at very low doses of Bt (172–345 IU of Bt).

Jayanthi and Padmavathamma (1997) reported that larval mortality rates of *B. mori* was increased at higher concentrations of *Bacillus thuringiensis* (Bt), but decreased at lower Bt concentrations. We fully agree with the above findings, as we also observed the significant decreases in larval mortality at lower doses of Bt. Hassan et al. (1989) reported that when *B. mori* larvae were treated with sub-lethal doses of Bt var. *kurstaki,* surviving females showed significantly reduced fecundity and fertility. Jayanthi and Padmavathamma (1997) also reported the longer incubation period at lower concentrations of Bt. They found that the higher concentrations of Bt were associated with reduced pupation, higher pupal mortality, and lower emergence of normal adult moths. During our experiments, we observed similar situations, where treated larvae took longer periods than the untreated larvae to become pupae (data were not shown). We also observed that the surviving females from the treated silkworm population laid less numbers of eggs than the untreated control females (data were not shown).

Mohan et al. (1997) reported that commercial formulations of Bt var. *kurstaki* (BtK) are being extensively used by farmers in India for the control of insect pests on vegetables. The endospores of BtK persist in soil and are infectious to the mulberry silkworm *B. mori*. van Driesche and Bellows (1996) previously reported the Indian government's decision on the use of Bt in silkworm/mulberry-growing areas, despite the nation's moves to reduce the use of traditional chemical pesticides. In Australia, large amount of Bt pesticides are in use for controlling different crop and vegetable pests. Therefore, the chance of unintended contamination of *B. mori* by Bt is obvious. Therefore, the development of a Bt-resistant *B. mori* population will be a positive and realistic step toward the establishment of a new sericulture industry in Australian environment.

The preliminary investigations on the lethality of different doses of Bt against three silkworm populations showed that the 'QuBite' population had highest resistance capability in relation to the other two test populations. However, among the three, the 'Insab' population showed a unique characteristic to be slower to kill (die) by the Bt attack for first 24 h after treatment. Therefore, this special characteristic might be a very important defence mechanism for sericulture industry. The study confirmed that among the two Australian silkworm populations, the 'QuBite' population has more promising future in relation to the bacterial (Bt) infection, which might come from natural Bt infestation on mulberry leaves or from Bt biopesticide used in neighbouring crop fields.

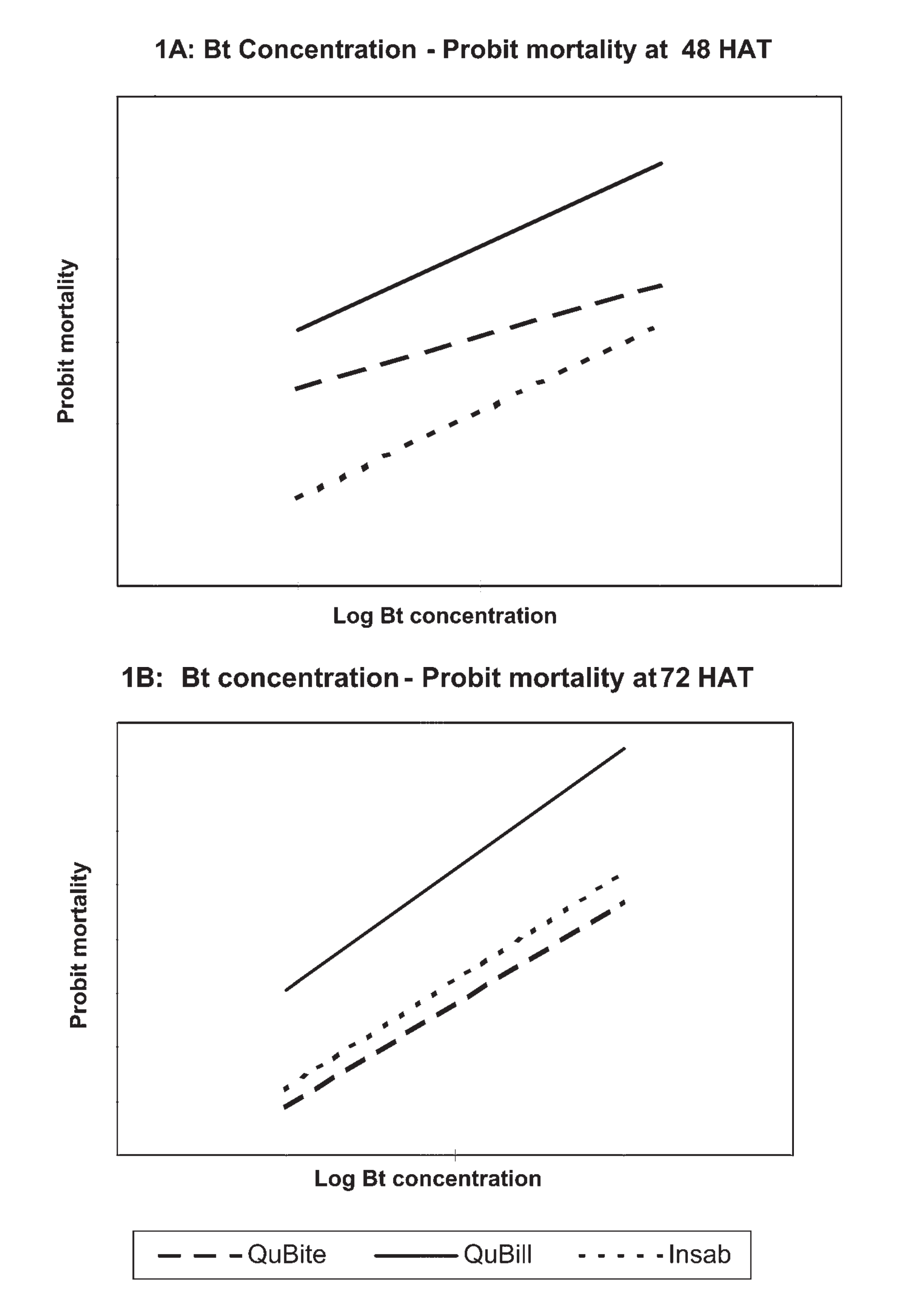

Figure 1. Bt concentration – Probit mortality in different silkworm populations at 48 and 72 h after Bt treatment

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