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A LOCAL *Bacillus thuringiensis*, SRBT1 WITH POTENTIAL FOR CONTROLLING *Metisa plana* (WLK).

Keywords: *Bacillus thuringiensis*, local isolate, delta-endotoxin, potency, *Metisa plana*.

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Most commercial products from Bacillus thuringiensis (B.t.) *were ineffective for controlling* Metisa plana *(Wlk.). The effectiveness of a* B.t. *product against an insect depends on its protoxin composition, and the presence of suitable receptor sites and factors activating the toxic crystals within the insect's midgut. The pH of the midgut must be suitable for solubilizing crystals prior to activation. The selection of a* B.t. *strain effective against the target pest is a practical approach. A* B.t. *strain containing the right toxin which binds to receptors sites present in the pest needs to be identified and selected by screening. This paper reports on the progress made in increasing the potency of a local strain of* B.t., *SRBT1, selected after a study of its toxicity.*

Alkaline and acidic compounds were added to SRBT1 to enhance the effect of its toxins. The addition of 0.5% tannic acid and 1.5% sodium borate showed a 4 fold increase in mortality of M. plana *at 4 days after treatment (DAT). A Three fold increase in mortality resulted from incorporation of 0.5% magnesium chloride and a two fold increase with 0.5% potassium carbonate and 0.5% sodium borate at 4 DAT. At 6 DAT, SRBT1 containing 1.5% sodium borate caused 94% mortality, as against 70% for the best commercial product tested, Florbac. One hundred percent mortality was observed at 9 DAT when 0.5% sodium borate, 0.5% and 1.5% tannic acid were added separately to*

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SRBT1. SRBT1 with no chemical additive produced 100% mortality at 11 DAT. SRBT1 with or without chemical additives, gave a marked reduction, 98 - 100%, in leaf area damaged (LAD) as compared with the control. This reduction is comparable to that produced by methamidophos 99.4%. Florbac gave only 59.7 % reduction in LAD. The reduction in LAD was more obvious when sodium borate and tannic acid were used. SRBT1 must harbour toxins suitable for the control of M. plana, because the effect on feeding behaviour is very pronounced. Determination of SRBT1 cry genes, cry proteins and exploitation of this isolate should be attempted.

INTRODUCTION

The bagworm, *Metisa plana* Wlk (Lepidoptera: Psychidae) has been a serious occasional defoliator of oil palm, *Elaeis guineensis* in Malaysia since 1956 (Wood, 1968). A total of 34 outbreaks affecting 11 900 hectares and 98 outbreaks affecting 37 102 hectares of oil palms were reported for 1975 - 1980 (Wood, 1982) and 1981-1985 (Basri *et al.*, 1988) respectively.

Various commercial products from *Bacillus thuringiensis* (*B.t.*) such as Bactospeine, Thuricide, BCBT, Florbac, Foray, Dipel, Biobit and CGA-BT were not sufficiently effective in the control of *M. plana* (Wlk.) (Mohd Basri *et al.*, 1994). In fact, the laboratory screening of these products against *M. plana* revealed that Florbac was the only effective one, giving 60% and 80% mortality of the second and fourth instars respectively after 7 days. In terms of feeding inhibition methamidophos, Thuricide, Dipel, and Bactospeine were very significantly more effective $p<0.001$, than Florbac. Methamidophos gave the largest reduction in leaf area damage (LAD), 99.4%, followed by Thuricide, Foray, Bactospeine and Florbac: 76.5%, 74.4%, 71.7% and 59.7% respectively (Mohd Basri *et al.*, 1994).

The specificity of *B. t.* depends mainly on the insecticidal crystal proteins (Milne *et al.*,1990; Lee *et al.*, 1996). For less susceptible insects, bacterial spores and other factors may play a role in insecticidal specificity (Wilson and Benoit, 1990; 1993). Spores cause septicaemia (Wilson and Benoit, 1993) or direct muscle and neurotoxic effects (Hickel and Fitch, 1990). Insect susceptibility is also dependent on the gut pH, proteases (Rajamohan *et al.*, 1995; Wilson and Benoit, 1993) and the presence and type of toxin-binding receptors (Chen *et al.*, 1995; Lee *et al.*, 1996; Lu *et al.*, 1994; Rajamohan *et al.*, 1995; Tang *et al.*, 1996). Extraction of the binding receptors of *M.plana* and their binding with the *B.t.* toxins are being studied. The main toxins of *B.t.* are a series of structurally related proteins present in the sporulated cultures as crystals (Lambert *et al.*, 1996; Lee *et al.*, 1996). These proteins are called the delta-endotoxins or insecticidal crystal proteins (Chilcott and Ellar, 1988; Whiteley and Schnepf, 1986) or cry proteins (Lee *et al.*, 1996). Different strains of *B.t.* produce different proteins, typically multiple proteins of molecular weights ranging from 27 to 140 KD (Lee *et al.*, 1996; Whiteley and Schnepf, 1986). Many strains of *B.t.*, in particular the important commercial species of *kurstaki* and *israelensis*, produce more than one protein (Lee *et al.*, 1996) which may exist as co-crystals or as independent crystal forms. (Li *et al.*, 1987). Many of the large protein crystals are held together by disulphide bonds which require high pH or reducing conditions to cleave them (Nickerson, 1980). These crystals are water insoluble. They dissolve in the alkaline insect gut to release proteins which may be processed proteolytically to yield the active toxins (Insell and Fitz-James, 1985; Rajamohan *et al.*, 1995; Wilson and Benoit, 1993). At pH $\leq$ 9 the crystals remain inactive (Insell and Fitz-James, 1985). Active toxins of *B.t.* exert their effect by binding to the midgut epithelium of insects (Chen *et al.*, 1995; Lu *et al.*, 1994; Wolferberger *et al.*, 1996) and disrupting the integrity of membranes (Schwab and Culver, 1990). The insects stop feeding and die of starvation. Gill *et al.* 1992 demonstrated the

mode of action of *B.t.* endotoxins.

The role of crystals and spores in the potency of *B.t.* strains is very much target-dependant and a subject of continuing controversy (Li *et al.*, 1987). The potency of *B.t.* spp. *entomocidus* and *aizawai* HD-133 against *Spodoptera littoralis* was enhanced by modifying its midgut condition by the addition of alkaline compounds, proteolytic activators, and some mildly toxic compounds (Salama *et al.*, 1984). Charles and Wallis (1964) reported an increase mortality of gypsy moth, *Porthetria dispar* when boric acid was added to the endotoxin of *B.t.* spp. *thuringiensis*. The experiment reported here were conducted with the objective of increasing the potency of a local *B.t.* carrying δ-endotoxin effective against *M. plana* by adding various chemicals.

MATERIALS AND METHODS

Local *B.t.* isolate – A local *B. t.*, SRBT1 was isolated from soil under oil palm using heat treatment (Chilcott and Wigley, 1992). One gram of soil was suspended in 10 ml of sterile distilled water shaken vigorously for two minutes. The suspension was heated at 60°C for 60 min in a water bath. Aliqouts of 0.1 ml from serial 10-fold dilutions of heat-treated samples were spread on nutrient agar plates and incubated at 30°C for five days.

Differential staining for *B.t.* – Bacterial colonies were smeared on microscope slide and stained using the method described by Chilcott and Wigley (1988). The slides were air dried and incubated at 100°C for 10 minutes prior to staining with naphthalene black 12B solution for two minutes. The slides were washed with tap water and immersed in Gurr's improved 1266 Giemsa stain for one minute. They were rinsed and dried before light-microscopic examination. The presence of crystals and spores were recorded.

Fermentation broth – Thirteen grams of nutrient broth were dissolved in one litre of deionized water and its pH was adjusted to 7.0 prior to autoclaving at 121°C for 15 min. When cooled the broth was filtered through Whatman No.1 paper and the pH of the filtrate was readjusted to 7.0 using 0.1M hydrochloric acid. Five hundred millilitres of 1% glucose solution were added to the broth prior to dispensing it into fifteen 250 ml Erlenmeyer flasks. These were plugged, autoclaved at 121°C for 20 min and cooled.

Fermentation of *B.t.* – An isolated colony of SRBT1 was inoculated into the nutrient broth and shaking-flask fermentation was carried out at 100 rpm and 30°C for 48 hours.

Harvesting – The fermented products were centrifuged at 10 000 g for three min. The pellet containing proteinaceous crystals and spores was rinsed twice with sterile distilled water.

Preparation of inocula – Colony-forming units (cfu) were counted using the haemocytometer. A suspension containing 1.5 x 10^6 cfu per ml of SRBT1 was used to prepare all the treatments except for the chemical controls and blanks, where milliQ water was used.

Treatments – Treatments in this trial were SRBT1 containing 0.5% (w/v) potassium carbonate (SRBT1+PC1), 1.5% (w/v) potassium carbonate (SRBT1+PC2), 0.5% (w/v) magnesium chloride (SRBT1+MC1), 1.5% (w/v) magnesium chloride (SRBT1+MC2), 0.5% (w/v) sodium borate (SRBT1+SB1), 1.5% (w/v) sodium borate (SRBT1+SB2), 0.5% (w/v) tannic acid (SRBT1+TA1), 1.5% (w/v) tannic acid (SRBT1+TA2), SRBT1 with no chemical (SRBT1), chemical control without SRBT1 (C-BT) and blank.

Inoculation technique – Oil palm leaflets from frond number 17 were cut into segments each with an approximate area of 32 cm^2, the segments were washed with 1% Teepol solution, surface sterilized with 75% ethanol and rinsed repeatedly in milliQ water. They were then air dried in laminar flow and inoculated by the brushing method. The inoculum was mixed throughly using magnetic stirrer and evenly brushed on to three leaflets.

Test insect – Newly moulted first generation fifth instars of *M. plana* originated from Teluk

Merbau Estate, Sepang were reared using indoor culture method (Mohd Basri and Kevan, 1995). Their mean weight was 15.32 mg with the range of 9.4 - 24.2 mg. Five larvae were placed on each treated leaflet. There were thus fifteen insects per treatment.

Incubation – The experimental packages were incubated in a controlled environment room for two weeks at a constant temperature of 27±1°C, a relative humidity of 35-50% and a photoperiod of 12:12 hr (light:darkness).

Recording – Larval feeding behaviour, mortality and LAD were recorded daily for two weeks. The total LAD was measured using a Delta-T leaf area meter.

Midgut pH – The midguts were dissected from ten larvae from the second to the seventh instar, turned inside-out and streaked on separate whole-range Whatman pH indicator papers. The pH was determined by comparing the colour change to the profile provided.

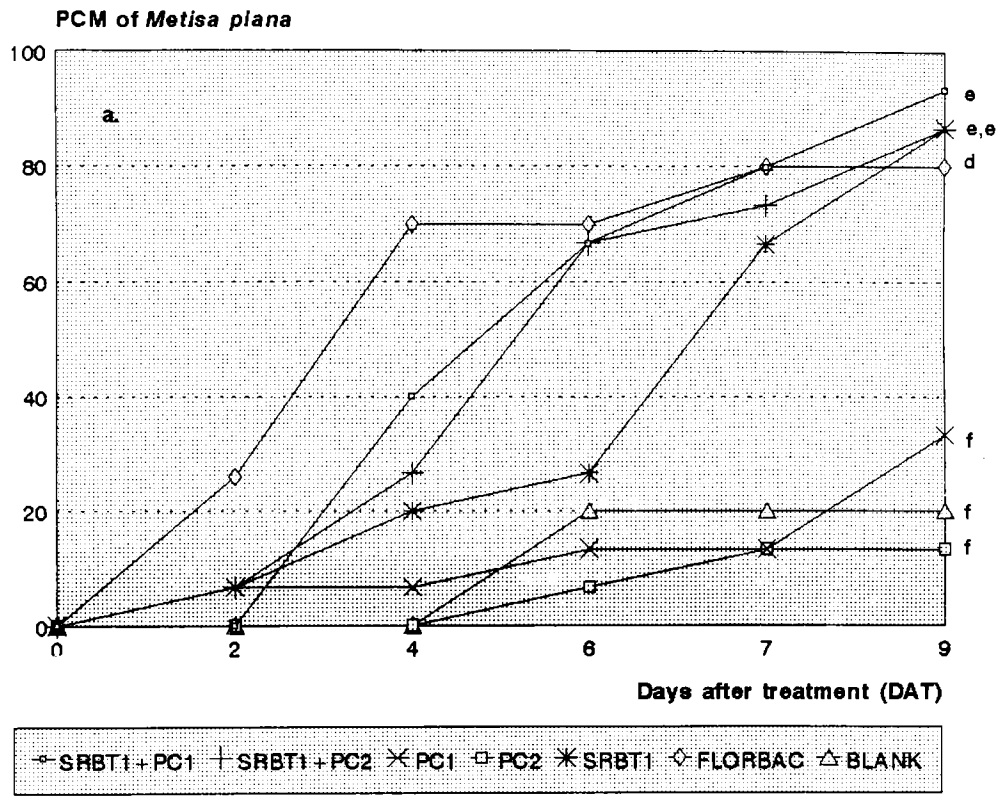
RESULTS

The percentage cumulative mortality (PCM) of *M. plana* was significantly increased when it was subjected to chemically treated SRBT1, as seen in *Figures 1* and *2*. The PCM of *M. plana* was not significantly affected by the chemicals alone, since it was comparable to that of the blank (*Figures 1* and *2*). SRBT1 by itself had a lag period of six days, as seen in *Figure 1* and *2*, where the gradient slopes up very gently before the 6th day. With the addition of potassium carbonate, magnesium chloride (*Figure 1*), sodium borate or tannic acid (*Figure 2*) the mortality of *M. plana* increased sharply, and was particularly obvious at 2 to 4 DAT. At 4 DAT, a two fold increment was observed with 0.5% potassium carbonate (*Figure 1a*), 0.5% sodium borate (*Figure 2a*), a three fold increment with 0.5% magnesium chloride (*Figure 1b*) and a four fold increment exceeding the PCM with Florbac with 0.5% tannic acid (*Figure 2b*) and 1.5% sodium borate (*Figure 2a*). The PCM of SRBT1 increased sharply at 6 DAT, while the PCM for most chemically treated SRBT1 was higher and still increasing, *Figures 1* and *2*.

The different treatments containing SRBT1 were equally effective, with the exception of SRBT1 in sodium borate which gave a significantly better effect ($p = 0.05$) at 7 DAT (*Table 1*). SRBT1 with 1.5% sodium borate gave 95% mortality at 6 DAT, being superior to Florbac which gave 70% (*Figure 2*). One hundred per cent mortalities were observed at 9 DAT with 0.5% sodium borate, 0.5% tannic acid and 1.5% tannic acid, as compared with 80% with Florbac (*Figure 2*). Chemically treated SRBT1 and SRBT1 by itself were significantly more effective than chemical control (C-BT) and blank (*Table 1*). Sodium borate produced a highly significant increase in the potency of SRBT1 ($p < 0.001\%$) as compared with the chemical control. Potassium carbonate and tannic acid increase the potency ($p < 0.01$) as compared with (C-BT) and blank. Magnesium chloride increases the potency at $p < 0.01$ and $p < 0.05$ as compared with C-BT and blank respectively.

Table 2 shows the leaf area damage (LAD) cause by *M. plana* after being subjected to different treatments. The chemical and the blank resulted in 17.03 and 28.84cm^2 LAD, respectively. Treatments containing SRBT1 successfully reduced the LAD from 28.84 to a range of 0 - 2.8 cm^2. Addition of chemicals such as 0.5% magnesium chloride, 0.5% sodium borate, 1.5% sodium borate, 0.5% tannic acid and 1.5% tannic acid resulted in a drastic impact arising from interaction between toxin, bagworm and chemicals. The larvae hardly fed on the leaflet at all after SRBT1 treatments. The LAD was < 0.68 cm^2. The activity of SRBT1 toxins with chemicals or otherwise, was well expressed in terms of feeding inhibition as compared to blank and chemical control (C-BT) *(Table 2)*. The feeding inhibition is believed to be due to paralysis of gut walls of *M. plana* by the cry proteins of SRBT1.

Statistical analysis of leaf damage at 14 DAT indicated that treatments with SRBT1 were highly effective against *M. plana*, significant at $p<0.001$. The percentage reduction in LAD caused by various SRBT1 treatments and by Florbac and methamidophos are shown in *Figure 3*. With or without chemical additives, SRBT1 is comparable to



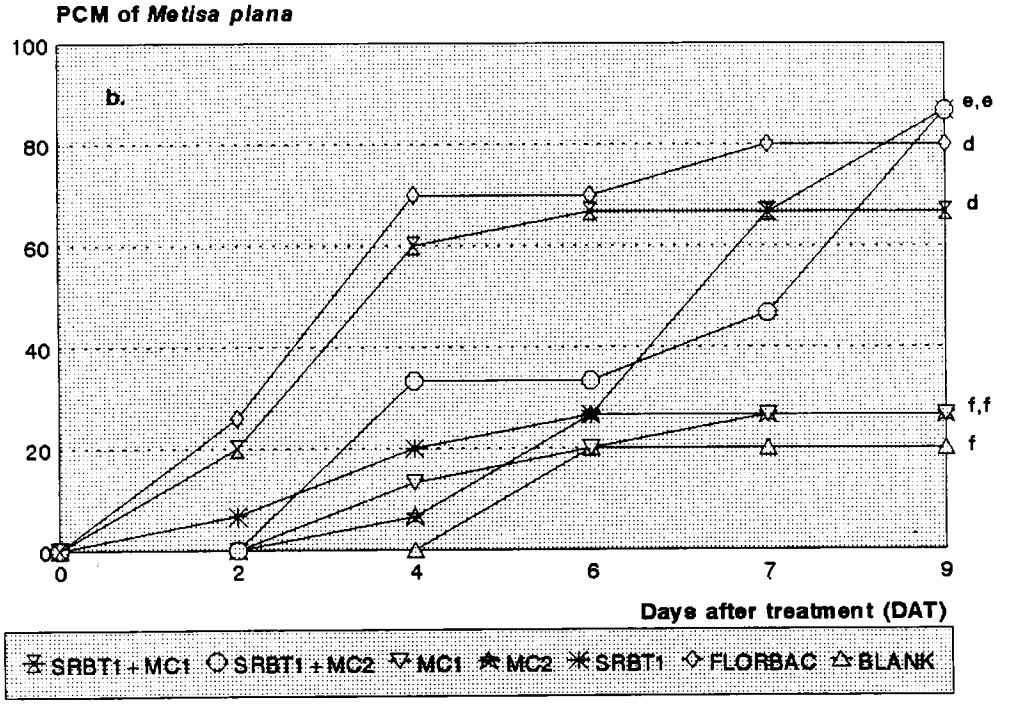
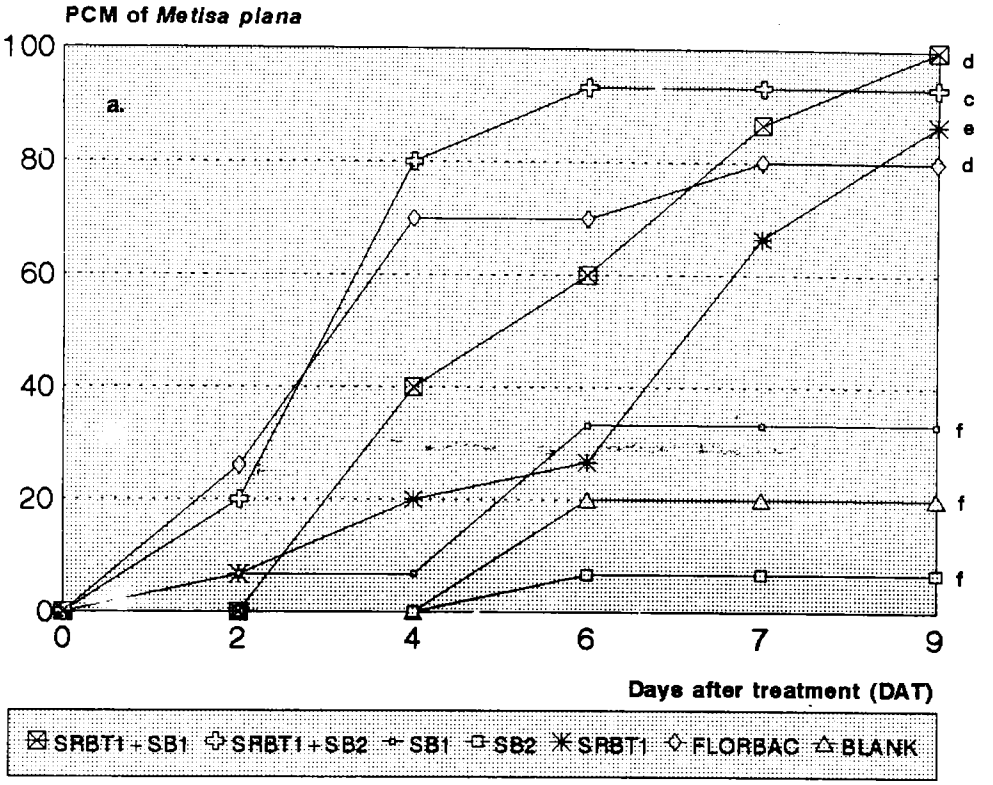


Figure 1. Percentage cumulative mortality (PCM) of fifth instar of M. plana *treated with SRBT1 and chemicals*
SRBT1 = Local *B.T.* isolate, PC1= 0.5% potassium carbonate, PC2 = 1.5% potassium carbonate, MC1 = 0.5% magnesium chloride, MC2=1.5% magnesium chloride, PCM with the same letters are not significantly different at P=0.05.



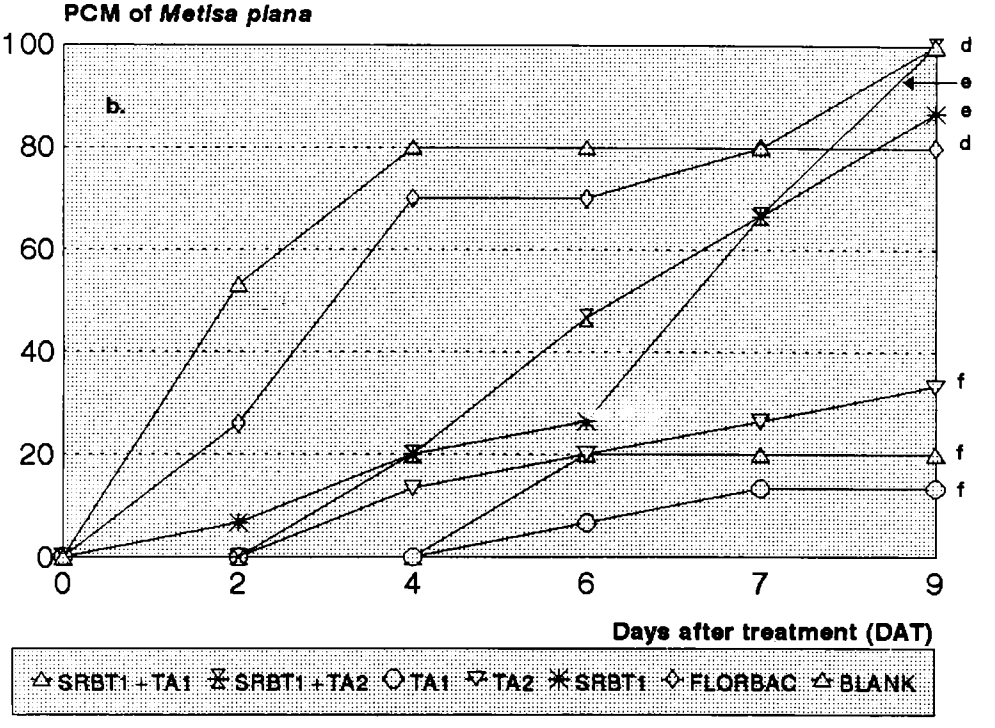


Figure 2. Percentage cumulative mortality (PCM) of fifth instar of M. plana *treated with SRBT1 and chemicals*
SRBT1 = Local B.T. isolate, SB1= 0.5% sodium borate, SB2 = 1.5% sodium borate, TA1 = 0.5% tannic acid, TA2=1.5% tannic acid, PCM with the same letters are not significantly different at P=0.05.

TABLE 1. ANALYSIS OF VARIANCE OF MEAN PCM OBSERVED WITH VARIOUS CHEMICALS AT 7 DAT

Chemicals#	Mean PCM	Difference from the various controls		
		SRBT1	C-BT	Blank
Sodium borate	90.00	23.40*	90.00***	70.00**
Potassium carbonate	76.65	10.05ns	76.65**	56.65**
Tannic acid	73.35	6.75ns	73.35**	53.35**
Magnesium chloride	56.70	9.90ns	56.70**	36.70*

= Chemicals plus SRBT1, *,**,*** = significant at p value of 0.05, 0.01 and 0.001 respectively, ns= no significant difference or equally effective as chemically treated SRBT1. $LSD_{0.05}$= 11.17, $LSD_{0.01}$ = 41.97 and $LSD_{0.001}$= 78.52.

TABLE 2. LEAF AREA DAMAGE CAUSED BY CHEMICALLY TREATED SRBT1

Treatments	LAD# (cm^2)
Blank	28.84
C-BT	17.03
1.5% Potassium carbonate + SRBT1	2.80***
1.5% Magnesium chloride + SRBT1	2.22***
SRBT1	1.74***
0.5% Potassium carbonate + SRBT1	1.44***
0.5% Tannic acid + SRBT1	0.68***
1.5% Tannic acid + SRBT1	0.55***
0.5% Sodium borate + SRBT1	0.03***
1.5% Sodium borate + SRBT1	0.03***
0.5% Magnesium chloride + SRBT1	0.00***

= total leaf area damage caused by 15 larvae at 14 DAT.
*** = significantly reduced LAD at $p < 0.001$.

methamidophos (Mohd Basri *et al.*, 1994) in giving a 90% reduction in LAD. SRBT1 with 0.5% magnesium chloride gave 100% reduction in LAD. SRBT1 with 0.5% and 1.5% sodium borate gave 99.9% reduction in LAD. SRBT1 with 1.5% tannic acid, SRBT1 with 0.5% tannic acid and the chemical control gave 98%, 97.5% and 38.8% respectively.

The pH profile of *M. plana* midgut is as shown in *Table 3*. The midgut of second instars was found to be slightly acidic, with a pH of 6. An increment of about 0.5 pH unit was noted for every increment of larval stage. The biggest or seventh instar *M.plana*

TABLE 3. THE MIDGUT PH FOR THE VARIOUS LARVAL INSTARS OF *M.plana.*

Larval instars	pH Range
2nd	6
3rd	6-7
4th	7
5th	7-8
6th	8-9
7th	9

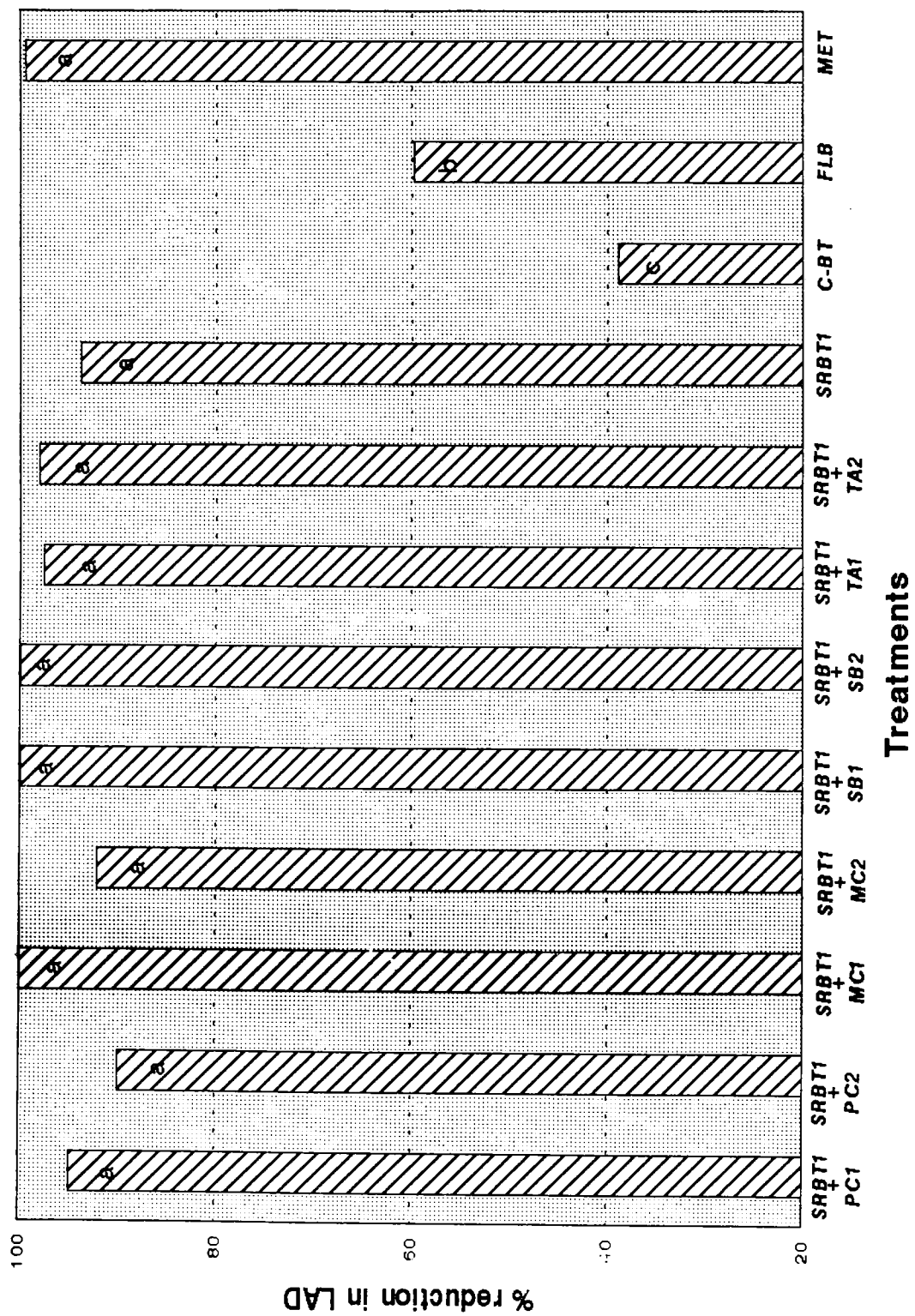


Figure 3. Percentage reduction in LAD caused by various treatments

SRBT1 = Local B.T. isolate, PC1= 0.5% potassium chloride, PC2 = 1.5% potassium chloride, MC1 = 0.5% magnesium chloride, MC2=1.5% magnesium chloride, SB1= 0.5% sodium borate, SB2=1.5% sodium borate, TA1=0.5% tannic acid, TA2=1.5% tannic acid, C-BT= chemical control, FLB=Florbac, MET= Methamidophos. Histograms for % reduction in LAD labelled with the same letters are not significantly different at P=0.05

had the most alkaline midgut pH, of 9. The fifth larval instar used in the experiment reported here has a midgut pH varying from 7 to 8, which is not suitable for dissolution of *B.t.* proteinaceous crystals to form the soluble protoxin and activation of *B.t.* spores.

DISCUSSIONS

The mortality of *M. plana* caused by the local SRBT1, has a lag period of 6 days: PCM was then 25% (*Figure 1* and *2*). This slow kill could be due to unfavourable midgut pH of fifth larval instars of *M. plana* which, as noted earlier, ranges from 7 to 8 (*Table 3*). Spores of *B.t.* which are not activated by the midgut pH, will show a delay in resuming vegetative growth (Wilson and Benoit, 1990; 1993). A more alkaline pH and the addition of a reductant have been shown to activate spores (Narayanan *et al.*, 1976; Rajamohan *et al.*, 1995; Wilson and Benoit, 1993).

According to Insell and Fitz-James (1985) the toxic crystal is stable at pH below 9. However, the 128-KD crystals undergo proteolysis at the alkaline pH of the midgut of susceptible insect larvae to release the active toxin, which in turn destroy gut epithelial cells (Armstrong *et al.*, 1985; Rajamohan *et al.*, 1995). Complete dissolution of the *B.t.* crystals takes place at pH 10.5 (Insell and Fitz-James, 1985). Most Lepidoptera are susceptible to *B. t.* because the pH in their midgut is usually high, 9-10.5 (Nickerson, 1980). The bagworm, *M. plana* is best controlled when the larvae are at the early larval instar (Ramlah Ali *et al.*, 1993; Mohd Basri *et al.*, 1994). However, the midgut pH of the early instars is unfavourable for control using *B. t.* Recent studies in this area show that the solubility of *B.t.* protoxins depends more on their compositions. For example, the midgut pH of Coleopteran is mostly 6.8, the Cry IIIA toxin recrystallized in this condition, but still causes mortality when forced feeding was conducted (Dean, 1995).

Addition of chemicals to SRBT1 reduced the lag period and resulted in 2 to 4-fold increase in mortality on the second to fourth days after treatment. The largest increase in potency was observed at 4 DAT for 0.5% tannic acid and 1.5% sodium borate (*Figure 2).* Chemical enhancement on the potency of *B.t.* (Dipel) against the rice moth *Corcyra cephalonica*, has been documented (El-moursy *et al.*, 1993). Salama *et al.* (1984) also showed that the potency of *B. thuringiensis* var. *entomocidus* and *aizawai* against *Spodoptera littoralis* (Lep:Noctuidae) can be enhanced by chemical additives. In our study, the addition of 0.5% tannic acid and 1.5% sodium borate (*Figure 2*) shortened the LT_{80} from 8 days to 4 days. It is essential to obtain reasonably high mortality at early as possible, in order to avoid crop loss. In nature the action of *B.t.* on the target pest is specific and favourable to the survival of desirable parasitoids, i.e. after the crystals are activated, the pest remain alive (Mac Intosh *et al.*, 1990) and so able to accommodate the parasitoids but it suffers from anorexia and thereby loses its ability to damage the crop (Bai *et al.*, 1993).

The addition of chemicals to SRBT1 did not significantly affect the feeding behaviour of *M. plana* (*Table 2* and *Figure 3*). Although slightly better results were obtained with both tannic acid and sodium borate as compared with other SRBT1 treatments, the differences were not significant. It is clear that all treatments with SRBT1 led to anorexic behaviour in *M. plana* as compared with the chemical control (C-BT) and the blank at $p<0.001$. For field control the slow production of lethal and anorexic effect by SRBT1 is useful for the survival of predators and parasitoids in integrated pest management (IPM) and for prevention of crop loss respectively. Some additives affect only the mortality (Charles and Wallis, 1964): thus the addition of boric acid to endotoxin of *B.t.* ssp. *thuringiensis* resulted in increase mortality. Other treatments enhanced septicaemia, e.g. through the modification of the condition in the midgut of *S. littoralis* by the addition of alkaline compounds, proteolytic activators and mildly toxic compounds (Salama *et al.*, 1984). The midgut components of Lepidoptera killed by *B.t.* have been partially identified as:- i) buffers rendering the midgut fluid alkaline (Wilson and Benoit, 1993), ii) protease (Tojo and Aizawa, 1983), iii) ascorbic acid or reductant (Narayanan *et al.*, 1976; Wilson and Benoit, 1990). These components have roles in the dissolution of proteinaceous crystals and

activation of spores and protoxins (Tojo and Aizawa, 1983).

CONCLUSION

The SRBT1 described in this paper could be included as a component of integrated management of *M. plana*. Its slow lethal and highly anorexic effect on *M. plana* make it a potential useful biological agent. The anorexic effect prevents the characteristic damage to oil palm leaves caused by this insect. The slow lethal effect could be favourable for the survival of useful predators and parasitoids. The mortality of *M. plana* caused by SRBT1 has a lag period of 6 DAT, the probable reason being the unfavourable midgut pH that ranges from 6 to 9. At this pH SRBT1 spores and crystals remain inactive. Subsequently, the low pH slows down septicaemia. The addition of certain chemicals, particularly 0.5% tannic acid and 1.5% sodium borate increased the mortality from 20% to 80% at 4 DAT. This reduces the lag in mortality, so making SRBT1 superior to Florbac, the most effective commercial *B.t.* tested earlier. SRBT1 without any chemical additive is still a good component of integrated pest management for bagworm. It gave a highly significant reduction in LAD of 94% at $p < 0.001$ compared with the chemical control and the blank. This reduction in LAD was comparable to that produced by methamidophos, which was 99 per cent. Determination of *cry* genes and Cry proteins of SRBT1 is an immediate avenue of research.

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