EDUCATUM JSMT Vol. 3 No. 1 (2016)
ISSN 2289-7070 / eISSN 2462-2451

Biosleeving with Epiphytic Bacteria as a Novel Approach to Oviposition Deterrence Against the Cocoa Pod Borer *Conopomorpha cramerella* (Snellen) (Lepidoptera: Gracillariidae)

Yusof Ibrahim[1] & Shari Fuddin Sha'ari[2]
[1*]Department of Agricultural Science, Faculty of Technical and Vocational Education,
Sultan Idris Education University, 35900 Tanjong Malim, Perak, MALAYSIA.
[2]Cocoa Research and Development Centre (MCB), KM 27, Simpang Jalan Tingkayu/Kunak, W.D.T. 175,
91207 Kunak, Sabah, MALAYSIA
[*] e-mail: yusofib@yahoo.com.my

Abstract

A great deal of information have been generated on the efficacy of bacterial entomopathogens against insect pests. However, relatively little information is available for the cocoa pod borer (CPB), *Conopomorpha cramerella* (Snellen). The pathogenicity of seven isolates as entomopathogen was evaluated against the CPB adult and their effects as oviposition deterrent in an ambient laboratory environment of 28±2ºC and 60-90% RH. A Sigma® hand atomizer was used to deliver an even volume median diameter (VMD) droplet spray of 75 µm of 1 x 10^6 c.f.u. mL^{-1}. The cells of all bacterial isolates tested could cause infection and inflicted mortality on the CPB adults. The isolates of *Pseudomonas aeruginosa* and *Serratia marcescens* significantly killed 93.33% of the CPB adults at the fourth day of exposure. The supernatant of *S. marcescens* also demonstrated significant kill of 86.66% of the CPB adults. The isolates *P. aeruginosa* and *S. marcescens* also significantly deterred the CPB moths from ovipositing activity, resulting in low mean number of eggs deposited per pod, 7.66 and 11.33 respectively, compared to the control. Subsequent exposures with supernatants reinforced the earlier findings whereby these supernatants significantly moderated oviposition activity of the CPB compared to the control. The integrated use of the bacterial cells and their supernatants as a biological barrier against oviposition activity on cocoa pods was discussed.

Keywords Cocoa, *Conopomorpha cramerella*, entomopathogen, oviposition deterrent, pathogenicity

INTRODUCTION

Cocoa, *Theobroma cacao*, an indigenous crop of South America was introduced into Malaysia through the Philippines as early as the 17th century. Koenig (1894) reported that in 1778 cocoa was grown in Malacca, and was first recorded in Sabah by Von Donop (1882) in his report on agriculture in British North Borneo. However, the first commercial planting started in 1950s in Jerangau, Trengganu and then followed by a plantation in Tawau, Sabah in 1955. From 1980 to late 1990s Malaysia experienced a rapid expansion and production of cocoa. The hectareage reached its peak in 1989 with areas planted in access of 414,200 hectares producing about 247,000 tons of dried cocoa beans. However, in the late 80s the cocoa pod borer (CPB), *Conopomorpha cramerella* (Snellen) had devastated about 5,000 Ha in the Tawau region, and within the following 2.5 years the CPB had established itself throughout Sabah and Peninsular Malaysia.

Beginning 1992, growers slowly shifted from cocoa to other alternative crops such as oil palm due to persistently low prices and severe outbreaks of the CPB. Malaysia's annual production of cocoa beans is 5000 tons from its present 20,543 hectares; the cocoa plantation area would be expanded and rehabilitated to 40,000 hectares within eight years' time at the rate of 2,000 hectares per year and producing an expected 60,000 tons of dried beans by the year 2020 [7]. Cocoa grinders in Malaysia are currently importing some 295,000 tons of cocoa beans annually from Indonesia, Ivory Coast and Ghana and have stabilized for the last three years, however, cocoa grinding is seen to go up by 20% by the year 2020 [15].

The control of CPB has largely relied on insecticides [22, 12, 20]. Approximately 15% of the potential crop is lost each year to CPB in Malaysia, and the nature of the pest makes it necessary to conduct spraying thoroughly and frequently [9]. As a result, continuous spraying has led to CPB developing resistance to the pyrethroids; high doses of fenvalerate of up to 2.68 times or deltamethrin of up to 4.45 times were required to kill CPB moths [11]. Therefore, there is a need to alternate the application of insecticides with other control measures including cultural practices such as frequent harvesting, canopy

pruning, bagging and sleeving, exploitation of natural enemies and confiscation of crops (Tay, 1987). However, these measures were costly and seemed inconsistent in efficacy. The use of pheromones, the black ants *Dolichoderus thoracicus* and the parasitoid *Trichogrammatoidea cojuangcoi* Nagarajah had been evaluated on laboratory scale [3]. The use of synthetic sex pheromone [4] was reported to have trapped large number of male moths compared to trap baited with virgin moths. Azhar (1986) suggested that the pheromone might permeate throughout the environment thus confusing the males and hence disrupting their mating behaviour. The trapping technique aimed at reducing the number of male moths in the wild such that mating was suppressed. However, the effect of mating disruption was found to be insufficient to realize a good control on field population due to insufficient concentration of pheromone in the air [1], hence the control was deemed a failure. The CPB is capable of inflicting losses of between 20-50% and occasionally reaching 100% if not controlled. Good husbandry practices such as pod sleeving with plastic bags have obtained limited control, and equally so by 'biocoater' sprays with tuber extract from the elephant yam *Amorphophallus muelleri* [23]. Efficient pest management is one of the factors that can help reduce economic injury as well as maintaining optimum level of productivity. Another tactical possibility is by establishing beneficial microbes, i.e. fungi and bacteria [13, 5]. The only successful CPB eradication was reported from Queensland in under three years of detection following an intensive eradication programme run under EPPRD [14].

The potential of bacterial antagonists against the CPB has yet to be explored. This study evaluates the potential of several selected bacterial isolates from the cocoa rhizosphere against the cocoa pod borer, *Conopomorpha cramerella* (Snellen) and its oviposition.

MATERIALS AND METHODS

Experimental Set-up

All experiments were carried out in the laboratories of the Malaysian Cocoa Board at Tawau, Sabah, Malaysia, conducted under an ambient laboratory environment of 28±2ºC and 60-90% RH.

Insect Culture

The CPB took almost one month to complete its life cycle. The culture was maintained systematically such that each life stage could be made available continuously. A heap of 150-200 infested pods was covered with clean dried cocoa leaves. The resident matured larvae would tunnel out and spin the cocoon to pupate on the leaves. These light green larvae or prepupae were removed from the cocoons before turning into obtect pupae three days later to be used for subsequent studies.

Pupae were collected daily and placed in plastic containers (23 x 12 x 16 cm) capped with a piece of muslin cloth. Upon emergence after 6-8 days of incubation, the moths were transferred to oviposition cages provided with 5% honey solution as food. Only three-day old adults were used in the study.

After sexed, a pair of adult was placed in a mating cage consisting of a transparent plastic container (23 x 12 x 16 cm) provisioned with 5% honey solution and allowed to mate for three nights. In another wire cage (10 cm diameter x 28 cm height) a freshly harvested clean cherelle (4-7 cm long) was hung and then covered with muslin cloth for oviposition. A clean cherelle was obtained by sleeving it with a transparent polyethylene bag for two weeks to prevent being infected by diseases or attacked by CPB or the mosquito bug *Helopeltis clavifer*. A pair of mated CPB adults was introduced into this oviposition cage provisioned with 5% honey solution and left overnight. Eggs oviposited on the cherelle were collected the following day.

Bacterial Growth Media and Bacterial Inocula

Seven bacterial isolates (Table 1) isolated from cocoa rhizosphere at different locations in Sabah as confirmed by Commonwealth Agricultural Bureau International (CABI) Bioscience were maintained by cultivating them on plates of nutrient agar and incubated in the dark at 26±2ºC and sealed with a masking film.

A bacterial suspension was prepared by pipetting 2.5 ml sterilized distilled water into two-week old plate and the bacterial colony was scraped from the surface using a sterile scalpel. The suspension was kept in a universal bottle for subsequent studies. The bacterial supernatant containing proteinase, chitinase and lecithinase was extracted from the bacteria cell body and kept under 4ºC for subsequent studies.

Treatment cum Oviposition Cage

The cage consists of a hard transparent polyethylene cylinder (9cm diameter x 20 cm height) fitted to a glass Petri dish (9cm diameter) as a base and covered with a piece of muslin cloth equipped with a window. A moist cotton pad was placed at the base to provide a constant relative humidity. A fine sized mosquito net was over layered as a partition barrier.

Table 1 Bacterial antagonists as confirmed by CABI Bioscience

Isolate	Code	Source
Bacillus cereus	LKM/429	Kota Marudu, Sabah
Bacillus sphaericus	LKM/430	Tuaran, Sabah
Bacillus polymyxa	LKM/431	Tuaran, Sabah
Pseudomonas aeruginosa	LKM/432	Tuaran, Sabah
Bacillus macerans	LKM/436	Tenom, Sabah
Bacillus amyloliquefaciens	LKM/437	Tenom, Sabah
Serratia marcescens	LKM/433	Tuaran, Sabah

Tests for Pathogenicity

The pathogenicity of seven isolates of bacterial antagonists was studied for their effectiveness against adult CPBs and their oviposition in the laboratory. The original hosts for the bacterial antagonists used in this study are listed in Table 1.

Bacterial treatment against adults - Five CPB moths were introduced in the treatment cage and inoculated with 0.5 ml suspension of 1 x 10^6 c.f.u. mL^{-1} of the respective bacterial isolates as in Table 2 using a Sigma® hand atomizer capable of delivering an even volume median diameter (VMD) droplet spray of 75 µm. The cages were arranged in a completely randomised design. Each treatment was run at three replications with sterilised distilled water as the control. Mortality was recorded daily up to seven days of exposure.

Bacterial treatment against oviposition - Freshly harvested clean pods were sprayed to complete coverage, as a form of biosleeving, with 2.5 ml suspension of 1 x 10^6 c.f.u. of the respective bacterial isolates using a Sigma® hand atomizer. Each treatment was performed at three replications with sterilised distilled water as the control. The pods were hung in the oviposition cages and a pair of mated adults was introduced into each cage provided with 5% honey solution. The cages were arranged in a completely randomised design. Eggs deposited on the pods were recorded visually with the aid of a magnifying glass three days after exposure.

Bacterial supernatant against adults - Five moths were introduced in the treatment cage and inoculated with 0.5 ml of the respective bacterial supernatants using a Sigma® hand atomizer. The cages were arranged in a completely randomized design. Each treatment was performed at three replications with sterilized distilled water as the control. Mortality was recorded daily up to seven days of exposure.

Bacterial supernatant against oviposition - The two most effective bacterial isolates derived from the previous bacterial treatment against the adults were used. Freshly harvested clean pods were sprayed to complete coverage as biosleeves with 2.5 ml of either *P.aeruginosa* (LKM/432) or *S. marcescens* (LKM/433) supernatants. A total of 32 pods were used for each treatment with sterilized distilled water as the control. The pods were left hanging and a pair of mated adults was introduced into each oviposition cage provided with 5% honey solution. The cages were arranged in a completely randomized design. Eggs deposited on the pods were recorded visually with the aid of a magnifying glass three days after exposure.

Statistical Treatment of Data

The treatment means, i.e. percentages of adult mortality and mean number of eggs deposited were subjected to one-way analysis of variance (ANOVA) and compared by pairwise contrast applying Tukey's honestly significant difference (HSD) tested at 5% level of significance using the PROC GLM procedure (SAS Institute, 2001).

RESULTS AND DISCUSSION

Bacterial Pathogenicity and Supernatant Efficacy Against Adults

All bacterial isolates could infect and was able to inflict mortality on the adults (Table 2). The isolates *Serratia marcescens* and *Pseudomonas aeruginosa* significantly killed 93.33% of the adults by the fourth day of exposure, followed by *Bacillus amyloliquefaciens* (73.0%), *B. polymyxa* (66.66%), *B. sphaericus* (53.33%), *B. macerans* (46.66%) and *B. cereus* (46.66%). In contrast, the effect by their supernatants was generally lower, except for *S. marcescens* which was significantly lethal by inflicting 86.66% kill against the adults by the third day of exposure. Both the bacteria have the potential of hindering the moths from laying eggs on the pod surface which was protected by a biofilm layer of the bacteria. Many pathogenic bacteria are known to produce an outer layer that is comprised of hydrated polysaccharides to minimise desiccation and to serve as a nutrient source. It also acts as a preventing mask or in the present case functions as a biological barrier. The slimy layer also can act as an adhesive, binding the bacterial cells together. At a colony level, these layers produce a polysaccharide biofilm within which the bacterial cells are impregnated [6].

The cells and supernatant of *S. marcescens* demonstrated the most encouraging effects by significantly killing 93.33% and 86.66% of the adults respectively (Table 2). The colonies with pinkish pigment appeared on the CPB cadavers one week after infection. *Serratia marcescens* cells generally are non-pathogenic when present in the digestive tract but multiply rapidly in the hemocoel and then become highly pathogenic, thus killing the host as was reported with the boll weevil *Anthonomus grandis* causing death within 1-3 days after exposure [17]. Bacteria infect insects mostly orally through the mouth and digestive tract and less common through eggs, integument or trachea. Bacteria very rarely enter an insect by means of parasitoids and predators [18].

Table 2 Effects of selected bacterial cells and their supernatant against CPB adults and their oviposition

Isolate	Code	Mean percent mortality		Mean egg count
		Cell	Supernatant	
Pseudomonas aeruginosa	LKM/432	93.33 a	30.00 bc	7.66 a
Serratia marcescens	LKM/433	93.33 a	86.66 a	11.33 ab
Bacillus amyloliquefaciens	LKM/437	73.00 a	20.00 bc	19.33 b
Bacillus polymyxa	LKM/431	66.66 ab	23.33 bc	18.88 b
Bacillus sphaericus	LKM/430	53.33 ab	46.66 b	18.00 b
Bacillus macerans	LKM/436	46.66 ab	23.33 bc	15.33 ab
Bacillus cereus	LKM/429	46.66 ab	6.66 c	17.66 b
Control		0 b	0 b	21.66 b

Means within columns followed by the same letter are not significantly different at p=0.05 according to Tukey's HSD.

Bacterial Pathogenicity and Supernatant Efficacy Against Oviposition

Table 2 shows a significantly lower mean egg count of *S. marcescens* (11.33) and *P. aeruginosa* (7.66) isolates recorded by the fourth day of exposure compared to the control (21.66). It is presumed that the bacterial spray had formed a thin film layer enveloping the cocoa pod surface and had deterred the CPB from laying eggs. In the subsequent experiment where only the above two isolates and their supernatants were used, the results reinforced the earlier findings whereby the bacteria and their supernatants

ISSN 2289-7070 / eISSN 2462-2451

significantly reduced oviposition activity of the CPB compared to the control (Table 3). Apparently, the biofilm layer of the bacteria and their supernatants functioned as a biological barrier to deter oviposition. It is obvious that the supernatant is easier to handle and more stable during storage thus would be a better candidate for a more extensive application in a field trial provided the problem of desiccation is solved.

Table 3 Effect of no choice treatment of selected bacterial isolates against CPB adults

Isolate	Mean egg count
Pseudomonas aeruginosa	
Cells	2.63 a
Supernatant	3.78 a
Serratia marcescens.	
Cells	5.69 a
Supernatants	3.44 a
Control	18.69 b

Mean followed by the same letter are not significantly different at p=0.05 according to Tukey's HSD performed on SQRT (x + 1) transformation for normality.

In a recent report pertaining to the use of entomopathogenic fungi, isolates of *Beauveria bassiana* and *Metarhizium anisopliae* had been demonstrated as successful biocontrol agents against the CPB adults [8]. This could lead to a more interesting phenomenon whereby the combined use of entomopathogenic fungal and bacterial pathogens as a microbial control approach could be capitalized on their synergistic effects; the fungi targeting on the adults and the bacteria as a biosleeving agent to hinder CPB oviposition. Provided that there is no interference effect, this dual-pathogen approach could combined infect the CPB. It is the general understanding that entomopathogenic fungi are considered safe on mammals as they cannot grow at the homothermic temperature. The pathogenicity of combined pathogens could be greater than a single pathogen as suggested by Tanada and Kaya (1993). Potentially, both these entomopathogenic fungi and epiphytic bacteria could either replace or complement chemical insecticides in an integrated management programme for a better control approach against the CPB.

CONCLUSION

The potential use of the bacterial isolates *S. marcescens* and *P. aeruginosa* as insect pathogens and as a deterrent or a barrier to disrupt CPB oviposition was apparent. It seemed that by completely covering the cocoa pod, the bacterial solution was able to disrupt or deter the CPB from ovipositing. This phenomenon could be introduced as a new fascinating concept termed as biosleeving. A field experiment could be pursued such that many other factors should be considered such as spraying in the late afternoon so as to benefit the bacterial pathogens.

ACKNOWLEDGEMENT

Thanks are extended to the Director General of Malaysian Cocoa Board and the staffs of the Plant Pathology Laboratory for the kind permission and assistance to use their laboratory facilities.

REFERENCES

[1] Alias, A., Sadao,W. & Tay, E.B. (2004). Efficacy of mating disruption using synthetic sex pheromone for the managment of cocoa pod borer Conopomorpha cramerella Snellen (Lepidoptera: Gracillariidae). *Malaysian Cocoa Journal*, 1: 46-52.

[2] Azhar, I. (1986). Ancaman pengorek buah koko (*Conopomorpha cramerella*) terhadap industri koko di Malaysia. II Teknik-teknik Kawalan. *Teknologi Koko-Kelapa*, 2:61-69.

[3] Azhar, I., Alias, A. & Meriam, M.Y. (2000). Research on the management of cocoa pod borer in Malaysia. In *Proceeding INCOPED 3rd International Seminar on Cocoa Pests and Diseases*, 16-17 October, 2000. Kota Kinabalu: Malaysian Cocoa Board.

[4] Beever, P.S., Day, R.K. & Mumford, J.D. (1986). Female sex pheromone of cocoa pod borer moth, *Acrocercops cramerella*: Identification and field evaluation. In Cocoa and Coconuts: Progress and Outlook (E. Pushparajah & P.S. Chew, eds.), pp. 287-292. Kuala Lumpur: Incorporated Society of Planters.

[5] Bong, C.L., Ruslan, A., Norlela, H., Shari Fuddin, S., Alias, A. & Markos, A. (1999). *Beauveria bassiana* as a potential biocontrol agent of the cocoa pod borer *Conopomorpha cramerella*. In *Biocontrol In The Tropics* (W.H. Loke *et al*., eds.), pp. 147-150. London: CAB International.

[6] Boucias, D.G. & Pendland, J.C. (1998). *Principles of Insect Pathology*. Alphen: Kluwer Academic Publisher.

[7] Hamdan, I. (2013). Malaysia to increase cocoa production. www.namnewsnetwork.org/v3/read.phy?id=MTg0OTk5 (Retrieved August, 2013).

[8] Ibrahim, Y.B. & Shari Fuddin, S. (2014). Potential of selected isolates of entomopathogenic fungi in causing lethal infection against various developmental stages of the cocoa pod borer, *Conopomorpha cramerella* (Snellen) (Lepidoptera: Gracillariidae). *International Journal of Agriculture Innovations and Research*, 3(1): 885-891.

[9] Johney, K.V., Teo, H.T. & Ng, K.Y. (1986). Evaluation of synthetic pyrethroid for the control of cocoa pod borer *Conopomorpha cramerella* (Snellen) (Lepidoptera: Gracillaridae). In *Proceedings Second International Conference on Plant Protection in the Tropics*, pp 390-394 (extended Abstract). Kuala Lumpur: Malaysian Plant Protection Society (MAPPS).

[10] Koenig, J.C. (1894). Journal of a voyage from India to Siam and Malacca in 1779. *Journal of Straits British Royal Asiatic Society*, 26: 104.

[11] Lee, C.T. (1996). Insecticide resistance in cocoa pod borer, *Conopomorpha cramerella* Snellen and the strategy to prevent its development. *The Planter*, 72: 593-599.

[12] Lim, G.T. (1992). Biology, ecology and control of cocoa pod borer *Conopomorpha cramerella* (Snellen). In *Cocoa Pest and Disease management in Southeast Asia and Australasia* (P.J. Keane and C.A.J. Putter, eds.) pp 85-100. Rome: FAO of the United Nation.

[13] Lim, G.T., Tay, E.B., Pang, T.C. & Pan, K.Y. (1982). The biology of cocoa pod borer *Acrocercops cramerella* Snellen and its control in Sabah, Malaysia. In *Proceedings First International Conference on Plant Protection in the Tropics.* (W.H. Loke *et al*., eds.), pp 257-287. Kuala Lumpur: Malaysian Plant Protection Society (MAPPS).

[14] Plant Health Australia. (2014). Cocoa pod borer eradicated from Australia. www.planthealthaustralia.com.au/cocoa-pos-borer-eradicated-fro-australia/ (Retrieved June, 2014).

[15] Raghu, A. (2014). Malaysia's annual cocoa grindings seen up 20% by 2020. www.reuters.com/article/2013/10/07/cocoa-malaysia-idUSL4000HTOEL (Retrieved June, 2014).

[16] SAS Institute (2001). *SAS Stat User's Guide*, Version 8.1. Cary, North Carolina, USA.

[17] Slatten, B.H. & Larson, A.D. (1967). Mechanism of pathogenicity of *Serratia marcescens*. I. Virulence for the adult boll weevil. *Journal of Invertebrate Pathology*, 9: 78-81.

[18] Tanada, Y. & Kaya, H.K. (1993). *Insect Pathology*. Berkeley: University of California Berkeley, USA.

[19] Tay, E.B. (1987). Control of cocoa pod borer – The Sabah experience. In *Proceedings Symposium Management of Cocoa Pod Borer*, pp 7-17. Kuala Lumpur: Malaysian Cocoa Board.

[20] Tay, E.B. & Bong, C.L. (1992). Management of cocoa pests and diseases in Malaysia in the year 2000. In *Pest Management and the Environment in 2000* (S.A. Kadir and H.S. Barlow, eds.) pp 231-249. Kuala Lumpur: CAB International.

[21] Von Donop, L.B. (1882). Report on the Agricultural Prospects of British North Borneo.

[22] Wood, B.J. (1987). Prospects on the management of the cocoa pod borer in Malaysia. In *Proceedings Symposium Management of Cocoa Pod Borer* (P.A.C. Ooi *et al*., eds.) pp 139-165. Kuala Lumpur: Malaysian Plant Protection Society (MAPPS).

[23] World Cocoa Foundation. (2014). Cocoa Pod Borer Programs: Effectiveness of Bio-coater Spraying in Controlling Cocoa Pod Borer. www.worldcocoafoundation.org/cocoa-pod-borer-research-programs/ (Retrieved June, 2014).