PROTOCOL FOR WILD COCOA POD BORER (*CONOPOMORPHA CRAMERELLA* SNELLEN) COLLECTION METHOD: A STEP TOWARDS EFFICIENT INSECT MASS REARING FOR STERILE INSECT TECHNIQUE.

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ABSTRACT - *The cocoa pod borer (CPB), Conopomorpha cramerella, is a major insect pest of cocoa in Southeast Asian countries, causing significant yield losses and economic damage. Traditional control measures have shown limited success, prompting the exploration of alternative biological control methods such as the sterile insect technique (SIT). However, successful implementation of SIT requires an efficient insect collection method for laboratory mass rearing. This study presents a detailed protocol for CPB insect collection, adapted from previous methods, to support the development of an artificial diet for mass rearing at the Cocoa Biotechnology Research Centre (CBRC) in Malaysia. The protocol involves the active collection of infested cocoa pods from the field and subsequent incubation to obtain CPB pupae. The transformed pupae resulting adults can be used for various research purposes. The report demonstrated the efficacy of the collection method, ensuring a continuous supply of CPB for research activities with the mean percentage of larval survival to pupation of 100 %. The collected insects exhibited a 55.63% ± 23.76 emergence rate, yielding a consistent supply of healthy adults with a balanced sex ratio. The use of dry cocoa leaves as pupation substrate is highlighted as cost-effective and suitable for larval pupation. These findings contribute to sustainable cocoa pod borer research, aiding sterile insect technique investigations and mass-rearing systems. The method's potential extends to diverse studies on CPB and provides valuable insights for researchers studying CPB biology, pesticide effects, and cost-effective mass rearing systems, contributing to the development of sustainable pest control strategies in cocoa farming.*

***Keywords**:* cocoa pod borer, insect collection method, cocoa, mass rearing system, CPB.

INTRODUCTION

The cocoa pod borer (CPB), *Conopomorpha cramerella* (Snellen) (Lepidoptera: Gracillariidae), has been reported as the major insect pest of cocoa in Malaysia (Meriam, 2016) and other Southeast Asian countries including Indonesia, Philippines, and Papua New Guinea (Saleh, Armaniar and Ahmad, 2020, Amalin *et al.,* 2023, Ngim, Gende, and Crozier, J. 2016). The yield losses can be up to 93% with 99% infestation (Lim & Phua 1986) and if left uncontrolled could result in severe economic loss. Various control measures have been applied to address this problem with limited success such as the use of parasitoids or entomopathogens (Romana, *et al.,* 2009; Lim & Pan 1986) and black ants (See & Koo 1996) are inadequately efficient. Method of pod sleeving is effective in preventing the CPB females oviposit eggs on the cocoa pod's surface (Saripah & Azhar, 2007), however, this method is considered labour intensive to most of the cocoa farmers (Vanialingam *et al.,* 1981) and time-consuming (Wood and Chung, 1989). Proper agronomic practices are highly recommended (Saripah & Alias, 2016) to significantly reduce the pest populations, but only temporarily due to re-infestations from neighbouring farms (Lim, 1992). Pesticide is the most preferred control method by the cocoa growers, nevertheless, beside safety issue, this method is not cost-effective (Teh *et al.,* 2006) and with limited efficacy due to the pest's lifecycle where the larval stage is completely secured inside the cocoa pods without being exposed to the insecticides during the feeding time.

Sterile Insect Technique (SIT) is an alternative biological control measure that has been used successfully against lepidopteran pest species such as pink bollworm, codling moth, false codling moth, Australian painted apple moth, and cactus moth (Marec and Vreysen, 2019). However, for any successful SIT programme, an efficient, cost-effective mass-rearing system are necessary. To enable insect mass rearing system in laboratory, an efficient insect collection method from the field must be established which is a crucial requirements to maintain the harvested wild individuals. An efficient collection method would also ensure

adequate insect supply to support the process of developing artificial diet for mass-rearing system. There are two main insect collection method approaches commonly used for CPB collection. The first approach is active collecting involving harvesting infested cocoa pods from the field, bring back to the laboratory and collect insect adults emerged from pupa. The second approach is passive approach which involves the use of certain tools such as small plastic and aerial net trap.

This paper describes the detailed protocol of the CPB insect collection method used by the research team and proven to successfully provide wild CPB for the CPB artificial diet development project at the Cocoa Biotechnology Research Centre (CBRC) in Kota Kinabalu, Sabah, Malaysia (6º05'31.8"N 116º11'08.3"E). The protocol is an adapted method with slight modifications from the methods described by Niogret *et al*. (2019) and Azwan *et al.* (2006).

MATERIALS AND METHODS

Wild CPB Source

Wild CPB were harvested from infested-cocoa pods collected from Madai Cocoa Research and Development Centre (MCRDC), Kunak, Sabah (4º47'1.887"N 117º58'3.804"E). The sampling plot area is about 3 hectares (with approximately 3000 adult cocoa trees), maintained with standard agricultural practice management without pesticide spraying. Pod sleeving was used to control CPB infestation in the plot area. However, due to the laborious nature of pod sleeving method, there are bound to be unsleeved cocoa pods and infested with the CPB.

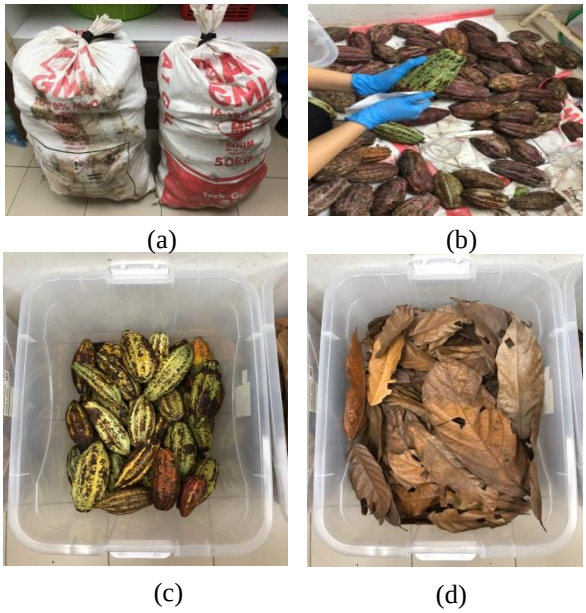
Infested Cocoa Pods Collection

The infested-cocoa pods were collected by selecting infested pods in the harvested cocoa pods heap (if during harvest time) or selectively harvesting pods on the cocoa trees during any other season. Infested cocoa pods were identified as described by Beevor *et al.* (1986) which is premature yellowing of cocoa pod husk (Figure 1).

The collected infested cocoa pods were put inside plastic bags and closed neatly using a rope on the same day of collection. The infested cocoa pods were then sent to laboratory at CBRC using land transportation which usually take about twelve hours period.

Upon arriving at CBRC laboratory, the infested cocoa pods were unpacked, cleansed by spraying with 70% ethanol and wiped with multi-fold tissue (Scott), arranged and fully covered with layers of dry cocoa leaves inside boxes (Figure 2) in the incubation room equipped with ventilation fans. The dry cocoa leaves used for covering the infested cocoa pods were fallen cocoa leaves under cocoa trees which were collected a day before the anticipated arrival of the infested cocoa pods. Collected dry cocoa leaves were kept inside thick plastic bags such as the standard laboratory biohazard autoclavable plastic (500 x 750 mm) bags until use (Figure 3).

Figure 1: Cocoa pod with CPB infestation characteristic. Premature uneven yellowing and when cut off, frass on placenta indicating infestation by the CPB larvae.



(a) (b)

(c) (d)

Figure 2: (a) Newly arrived CPB-infested cocoa pods. (b) Cleaning of the unpacked cocoa pods. (c) Heap of infested cocoa pods inside the plastic box. (d) Heap of infested cocoa pods covered with layers of dry cocoa leaves.

Figure 3: Dry cocoa leaves kept in biohazard plastic to be used as CPB larvae pupation substrate.

Figure 4: The adult emergence cage.

CPB Pupae Collection

The boxes of infested cocoa pods were inspected for the presence of CPB pupae after two days of incubation and twice weekly thereafter until no more new CPB pupae were obtained, usually after two weeks of incubation.

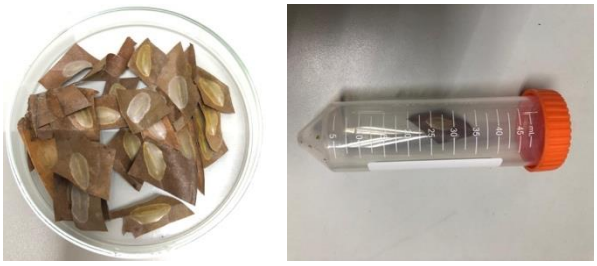
The pupae inside cocoons were then collected by cutting the cocoa leaves (approximately 2 cm x 1.5 cm) area where the cocoon was located. For pupae found on the cocoa pod's surface, the pupae were removed carefully from their cocoon by making a small hole on the cocoon, using a scalpel blade (size 10).

CPB Adult Collection

The pupae were then transferred to the adult emergence and mating room and placed inside an adult emergence fine breathable mesh cage for good aeration (Dimensions (LxWxH): 40 cm x 30 cm x 40 cm) (Figure 4). Newly emerged adults were fed with a 10% honey and tap water solution, using a cotton ball as a feeding medium. The cotton ball was soaked in the feeding solutions and squeezed before being introduced into the cage.

Sexing CPB

Data on the sex ratio and age of the individual pupae were collected on live CPB adults. Pupae selected for the sexing study were harvested from dry cocoa leaf cuttings (Figure 5(a)) and placed in individual vials (50 ml centrifuge cone tube) (Figure 5(b)). Sexing examination was done by looking at the external genitalia of the live adults according to the method described by Bradley (1986). The male genitalia was distinguished by a darker, broader caudal segment and the presence of a hair pencil, whereas the female genitalia were distinguished by the hairy anal papillae of the ovipositor (Figure 6). The sex of the CPB adults was determined under a Stereo Microscope (Integrated CMOS microscope camera Leica IC90 E, Leica, Singapore).



(a) (b)

Figure 5: (a) Pieces of cocoa leaves with pupa inside the cocoon. (b) A 50ml centrifuge tube used as a temporary cage for individual pupa selected for sexing study.

(a) (b)

Figure 6: (a) Male CPB. (b) Female CPB.

Parameters to be evaluated

Data on the number of infested cocoa pods per consignment, larva survival, pupation, adult emergence, malformation, and sex ratio.

RESULTS AND DISCUSSIONS

Infested Cocoa Pods Collection

There was no available source of CPB near the laboratory so the CPB samples had to be outsourced from the MCRDC. Based on the result obtained in this study, despite the long twelve hours travel by land to the laboratory, the larvae inside the infested cocoa pods were not affected negatively. Table 1 shows the average number of cocoa pods collected, pupa, adult, sex ratio, and malformation for the month collection period of March to July 2022. According to Table 1, with the average number of 166 pods per consignment, it provides us 156 adults. This number is adequate to supply CPB eggs for laboratory testing for two weeks. By using this collection method, we could obtain an adequate continuous supply of CPB that aid the project activities implemented in a timely manner.

CPB Larva Survival

Pre-pupa larvae were observed to start exiting the pods the day after arrival at the laboratory. Thus, upon arrival at CBRC, the infested cocoa pods must be unpacked and packed in boxes with dry cocoa leaves layers soonest possible to ensure that before pre-pupa larvae start exiting the pods, the pupation substrates (dry cocoa leaves) were all ready.

Covering infested cocoa pods with layers of dry cocoa leaves was observed to be the most suitable way of providing substrate for larvae pupation. Azwan *et al*. (2006) previously described using newspapers as a pupation substrate. However, based on experience, the newspapers would absorb moisture from the infested cocoa pods and become wet and thus not favourable as pupation sites. Additionally, wet newspapers would encourage the mould growth which eventually hasten the decaying of cocoa pods before all the larvae exited from the pods. Using dry cocoa leaves is also more cost-effective and economical as no extra cost is needed to acquire newspapers/papers.

In the natural environment, the CPB larvae that survived in the cocoa pods tunnelled out from the cocoa pod, spun down to the ground, and spun a cocoon on the leaf litter and pupate. Azhar and Long (1996) reported that the mean proportions of egg survival to emergence are less than 10%, while larval survival to pupation is only 4.1 ± 0.5%. In contrast, this study found that the mean percentage of larval survival to pupation is 100%. This result indicates that this collection method is highly efficient in providing adequate insect supplies for the laboratory insect-rearing development for CPB.

Pupal Survival

Table 1 shows the total number of cocoa pods collected and the total number of pupa, and adult CPB sexing results. Out of the 1165 infested cocoa pods, 2349 pupa were collected but only 1097 successfully emerged as healthy (with no malformed condition) adult CPB. Based on this data, the pupal survival to adult success rate was computed as 55.63 ± 23.76 (Figure 6) which is slightly lower than the pupal survival success rate reported by Niogret *et al*., (2019) which was 66.2 ± 15.5% of the pupae collected.

Sex Ratio and Adult Malformation

Healthy adult CPB sexing shown that out of 1097, 551 adult CPB were female and 546 male which came up to 1:1 adult CPB sex ratio. The adult CPB sex ratio observed at emergence in this study is consistent with the findings reported by Niogret *et al.*(2019). Nevertheless, this ratio is notably deviates from the field's male-biased sex ratio which was 1.2:0.8 (male:female) as reported by Niogret *et al.*, (2019). No malformed adult was observed during the collection period March to July 2022. This indicates that, the insect material is genetically good to start the laboratory colony.

Table 1: Data collected during the five-month collection period for parameters evaluation. The table presents the total number of five months and monthly average collection of cocoa pods, pupae, adult, female, male, and malformation. The collection period was from March to July 2022.

Parameter	Total per five month	Average per month
Cocoa pods	1165	166.43 ± 35.60
Pupae	2349	335.57 ± 264.57
Adult	1097	156.71 ± 105.54
Female	551	78.71 ± 51.67
Male	546	78 ± 54.20
Malformation	0	0.00 ± 0.00

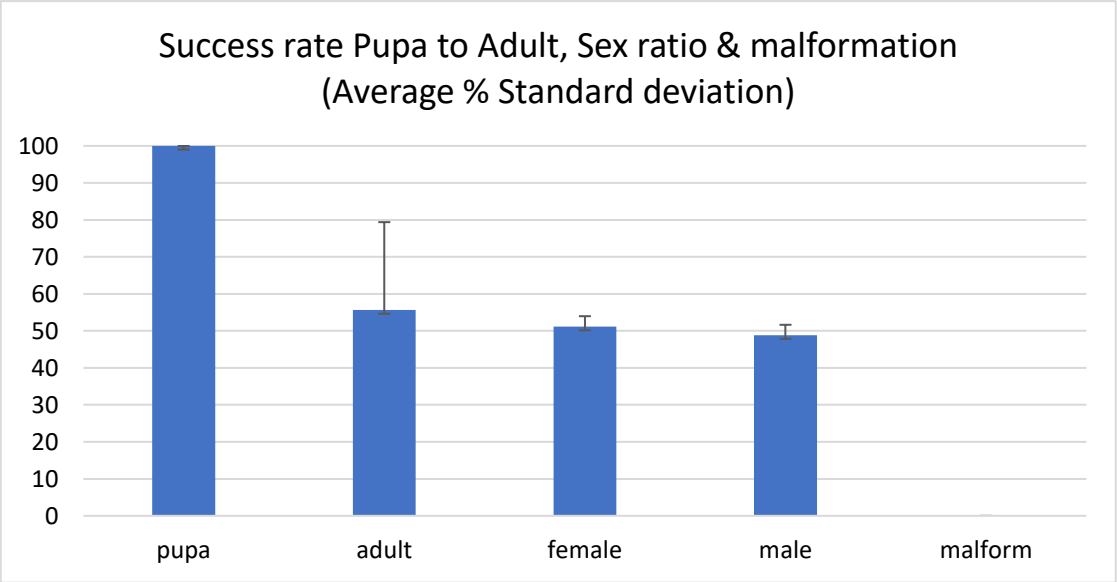


Figure 7: Development stage success rate, sex ratio, and malformation of CPB from 1165 infested cocoa pods collected from March to July 2022 at Madai Cocoa Research and Development Centre in Madai. The graph presents the success percentage of pupation (100.00 ± 0.00), adult emergence (55.63 ± 23.76), females (51.16 ± 2.81), males (48.84 ± 2.81), and malformation events (0.00 ± 0.00).

CONCLUSION

In this study, although the CPB adult emergence is slightly lower which is only 55.63 ± 23.76, the pupation rate is 100 %. This indicates that the heavier the infestation where the infested cocoa pods were collected, the higher the number of adult insects that could be obtained. However, with the current infestation condition where the cocoa pods were collected with the 55% adult emergence rate, it adequately provided insect supply for the CPB artificial diet development. In addition, the insect quality (no malformation) is good and the sex ratio is 1:1. This indicates that the insect material is genetically good and provides an adequate balance of males and females to start the laboratory colony to facilitate the CPB artificial diet development.

The insect collection method reported in this paper is important to support the development of an artificial diet for the mass rearing of CPB and explore the sterile insect technique (SIT) in controlling the CPB. The collected CPB from the suggested method able to be used in studying the lifespan cycle and ecological behaviour of CPB in a particular area. The CPB collection method is also applicable in most experiments and studies that required wild CPB such as the study of pesticide effects on different stages of the CPB lifecycle and cost-effective CPB mass-rearing system.

In conclusion, this study indicates that this collection method is efficient in providing adequate insect supplies for the laboratory insect-rearing development for CPB and other fields that require adult CPB. However, to obtain similar results, the CPB infestation where the infested cocoa pods are collected must be at the similar infestation density to the infestation described here.

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