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Research Article
Applications of Biological Agents and Pruning Effectively Control Cocoa Pod Borer

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Abstract
Background and Objective: Cocoa pod borer (CPB) is the main pest of cacao. It can reduce cacao production by up to 80% in Indonesia particularly in Central Sulawesi. The aim of this study was to (a) Estimate the effects of *Beauveria bassiana* (*B. bassiana*) fungi, *Dolichoderus thoracicus* (*D. thoracicus*) cacao black ant and cacao pruning in controlling CPB at cacao plantation and (b) Evaluate the presence of *B. bassiana* in the soil environment after application. **Materials and Methods:** The study was conducted at one-hectare cacao farm covering 833 cacao trees with 3×4 m planting space. A completely randomized design was applied with five treatments: (1) *B. bassiana* fungi (BBF), (2) cacao black ant (CBA), (3) Pruning+*B. bassiana* (Pr+BBF), (4) Pruning+cacao black ant (Pr+CBA) and (5) Control (C). All treatments had 3 replicates. A10^6 mL^{-1} colony forming units of *B. bassiana* was used as initial inoculum to be propagated in the corn media. *B. bassiana* inoculum of 4 kg was mixed with 250 L of water and was applied each month during the research period. A one-way ANOVA was used to analyze the data followed by HSD test ($p<0.01$) to compare the mean. It measured the CPB infestation level, cocoa bean damage and cacao yield. **Results:** The application of both biocontrol agents was effective in reducing CPB infestation and cocoa bean damage leading to increased cacao yield compared to the single treatment of either biological agent or pruning. Interestingly after application, the *B. bassiana* was successfully detected at the soil top layer as soil fungi. The highest cacao production (2.14 t ha^{-1}) was obtained when biological agents were combined with pruning. **Conclusion:** This study has shown the effectiveness of *B. bassiana* and *D. thoracicus* when combined with pruning in controlling CPB and increasing cocoa yields.

Key words: Cacao, prune, *Conopomorpha cramerella*, *Beauveria bassiana*, *Dolichoderus thoracicus*

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

In Indonesia, the quality and production of cocoa (*Theobroma cacao* L.) have been decreasing over the last 10 years due to plant pests such as cocoa pod borer (CPB) *Conopomorpha cramerella* Snellen (Lepidoptera: Gracillariidae)[1,2]. It has been estimated to be responsible for upto 82% losses[3]. A total of 93% cacao national production was contributed by small holder farmers in Indonesia, of which, Central Sulawesi contribute 71%[4]. Chemical control against CPB by cacao farmers was not effective because CPB larvae are protected inside the cocoa pod. Imago of this pest also tends to escape from chemical pesticides applications due to inappropriateness between application time and daily activity of the pest. It also had been reported that impacts of using chemical pesticide in the cocoa industry have several detrimental effects on the environment and the food chain[5–7]. Ecosystem services of beneficial organisms, for example, natural enemies of pest and pollinators were threatened by chemical pesticide applications in cacao agroecosystem[8].

Effective biological technologies in controlling CPB have been developed into discoveries and applications. One of promising biological control is *B. bassiana*. The fungus *B. bassiana* (Balsamo) Vuillemin (Deuteromycotina: Hyphomycetes) is a soil-borne saprotrophic fungus found in the soil environment and known as an entomopathogenic fungus that has been widely used to control various types of plant insect pests and arthropods[9–12]. Thus entomopathogen was an effective tool that can be used in an integrated pest management to control scale insects[13]. *B. bassiana* has good prospects for controlling CPB because their spores produce beauvericin toxins that may kill pest insects[14]. Westwood *et al.*[15] reported that *B. bassiana* contains allergen Bb-Ald similar to aldehyde dehydrogenases that are considered as a major allergen. *B. bassiana* also can cause higher mortality of cocoa pod borer pupae (43.12%) compared to other entomopathogenic fungi (*Metarhizium anisopliae*)[16]. Another potential biological control was CBA, *D. thoracicus* (Hymenoptera: Formicidae) known as a natural enemy for the cocoa pod borer. This ant could protect cocoa pod from CPB infestation by interrupting the CPB from laying the eggs on pod surface. The population of CBA can be increased by augmentation of artificial nest made from bamboo and palm leaves[17] or plastic bag stuffed with dry cocoa leaves[18,19]. Subsequently, the artificial nest invited the CBA to cocoa trees and controlled the CPB. *D. thoracicus* can be maintained in suitable artificial nests for mass rearing[19,20]. The effectiveness of CBA as a predator of CPB had been reported as a promising biological control[18,21].

Despite the importance of biological control, agricultural management practice may also contribute to controlling the CPB. The previous research tested the local cocoa resistance against CPB in Sulawesi. Pruning was included as a management practice treatment for successful controlling of CPB[22]. Integrated crop management, where pruning conducted to minimize the shade, also decreased the cocoa pod rot disease[12]. Both biological control and management practice potentially reduce CPB infestation at cocoa plantations. The evidence that biological control and pruning are associated in controlling CPB was weak and inconclusive. In this study, integrated the biological control and management practice by using *B. bassiana, D. thoracicus* and pruning in controlling CPB in smallholder cocoa farms.

MATERIALS AND METHODS

The field study was conducted at a one-hectare smallholder cocoa farm covering 833 cacao trees at a planting space of 3×4 m in Rahmat village, Palolo sub-district, Central Sulawesi-Indonesia.

The study was conducted in January, 2014-August, 2014. This is a multi-years research on a large scale and still running currently. The published which data were obtained are still relevant to the needs for controlling the cocoa pod borer in Indonesia. The cacao are mostly Criollo and Forastero cultivars and had been planted for 15 years. The field study was selected that had been untreated by pesticide and remained untreated through the whole period of the study. A completely randomized design was applied with 5 treatments: (1) *B. bassiana* fungi (BBF), (2) cacao black ant (CBA), (3) Pruning+*B. bassiana* (Pr+ BBF), (4) Pruning+cacao black ant (Pr+CBA) and (5) control (C). All treatments had 3 replicates.

The *B. bassiana* was propagated in corn media, which was massively produced by the Laboratory of Plant Protection of Tadulako University. A colony of 10^6 mL^{-1} *B. bassiana* was used as initial inoculum to be propagated in the corn powder media. *B. bassiana* inoculum of 4 kg was mixed with 250 L of water and was applied to the plantation each month during the research period. Cacao black ants were introduced into an artificial nest made of a bamboo stem (50 cm in length and 6.4 cm in diameter) where its hole was filled with dried coconut leaf and 10% honey[17]. Three artificial nests were placed each on a stem and primary and secondary branches of each cacao tree. The cacao branches were pruned as described by Anshary *et al.*[20] and Ho and Khoo[23] by removing cocoa primary branches that grow vertically over 3 m, as well as the secondary branches (40-50 cm).

Variables measured were CPB infestation level and percentage of bean damage. Both variables were recorded once per month for 6 months.

Subsequently, the data were analyzed using one-way ANOVA followed by honesty significant difference (HSD) test ($p<0.01$) to compare the mean[24]. Cocoa yield (Y) was estimated at the end of the observation following Siregar *et al.*[25] using a formula:

$$Y = P \times (Bb \times N)$$

Where:

P = Is a number of cacao pods
Bb = Is the weight of cocoa beans
N = Is a number of beans

Inorganic fertilizers were applied at the beginning of the research in a trench dug up circularly around the cacao tree. The trench was then back filled with soil to reduce fertilizer losses due to evaporation and erosion. The rates of the fertilizers were 150 kg ha^{-1} urea, 250 kg ha^{-1} TSP, 260 kg ha^{-1} KCl and 90 kg ha^{-1} kieserite. The occurrence of *B. bassiana* in soil environment was evaluated at three different layers i.e., 0-30, 31-50 and >50 cm by the insect bait method[26-28] modified by *C. cramerella* pupa (Lepidoptera: Gracillariidae). Ten soil samples of 100 g each were taken from each soil layer using a soil bor and each was placed in a 200 mL plastic cup. Therefore, there were 30 soil sample units. Ten pupae of *C. cramerella* were inserted into each plastic cup of 100 g soils and then incubated at a temperature of 27°C for 7 days. The number of *C. cramerella* used was 300 pupae (10×30 plastic cups filled with soil samples). After the incubation, dead *C. cramerella* pupae infected with *B. bassiana* were observed. The dead pupae were separated by immersing them in a 5% sodium hypochlorite solution, then the dead *C. cramerella* pupa was fed into PDA media in petri dishes. The observation of *B. bassiana* spore growth on the pupa was performed 1 week after the pupa was included in PDA.

RESULTS

The highest infestation level was recorded from the cocoa plot without treatment (control), which was significantly different with the other 4 treatments (ANOVA $F_{3,16} = 28,3$; $p<0.0001$). The lowest infestation was found in the integrated treatment (Pr+BBF) but it was not significantly with the other treatments. The results for the average percentages of CPB attack on the cocoa pods are presented in Table 1.

In general, all treatments significantly decreased the cocoa bean damages compared to control (ANOVA $F_{3,16} = 8,22$ $p<0.01$). The BBF and CBA combined with pruning practices were the most effective in the management of CPB black pod, subsequently protected the cocoa bean. The application of *B. bassiana* and pruning (Pr+BBF) was the best treatment that minimize the damage of beans (9.51%). The treatment, however, did not differ significantly from the other treatments except with control (Fig. 1). The highest estimated cocoa yield was obtained in the treatment of *B. bassiana* with pruning (Pr+BBF). Combining pruning with either *D. thoracicus* or *B. bassiana* resulted in a higher cocoa yields compared to the other treatments (Table 2).

The presence of *B. bassiana* fungi in soil samples from different layers in the cocoa plantation is depicted in Table 3. *B. bassiana* that infected the C. *cramerella* insects was found only in the top soil layer samples and they do not exist at the soil layers deeper than 30 cm.

Morphological characteristics of *B. bassiana* attacking *C. cramerella* pupa: *B. bassiana* colonies inoculated on the PDA medium and incubated at 27°C for 14 days formed a white-like layer of flour (Fig. 2a-d). The *C. cramerella* pupa infected with *B. bassiana* was characterized by symptoms like the fungus with a colony of white hyphae growing on the integument (thorax and abdomen) of the pupa (Fig. 2d). The phenotypic character of the colony growth was apical and

Table 1: Percentage of pod damaged by CPB in control, *B. bassiana* (BBF), cacao black ant (CBA), pruning with *B. bassiana* (Pr+BBF) and pruning with cacao black ant (Pr+CBA) treatments

	Observation time (months)					
Treatments	I	II	III	IV	V	Mean (%)
Control	15.30	19.66	20.22	23.15	25.42	20.75[a*]
BBF	12.43	12.85	12.66	10.44	10.25	11.73[b]
CBA	11.66	11.55	11.35	10.55	10.65	11.15[b]
Pr+BBF	10.25	10.45	9.85	9.15	7.25	9.39[b]
Pr+CBA	11.33	11.56	10.45	10.25	10.65	10.85[b]

*Number followed by the same letter in the same column are not significantly different. HSD: 3.69 ($p<0.01$), Data are mean (n = 10+SD)

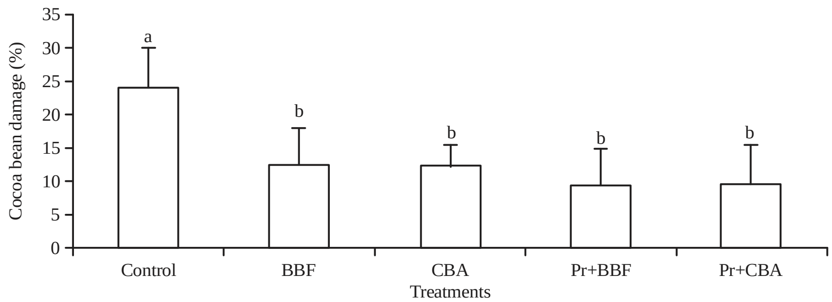

Fig. 1: Percentage of bean damaged by CPB in control, *B. bassiana* (BBF), cacao black ant (CBA), prune with *B. bassiana* (Pr+BBF) and prune with cacao black ant (Pr+CBA) treatments

Data are mean (n = 10+SD). The number followed by the same letter in the same column are not significantly different

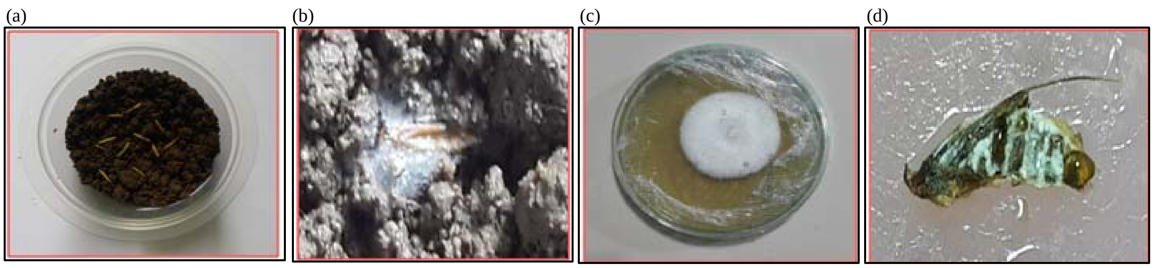

Fig. 2(a-d): Insect bait method (a) *C. cramerella* pupa under soil, (b) Pupa infected by *B. bassiana* on soil, (c) *B. bassiana* colony and (d) *C. cramerella* pupa infected by *B. bassiana*

Table 2: Estimated cocoa yield sat each treatment

Treatments	Pod number (P)	Bean number	Average number of bean/pod (N)	Average number of bean/100 g	Average bean weight (g) (Bb)	Estimated production (t ha^{-1}*)
Control	50	2.440	48.79	120.56	0.83	1.68
BBF	50	2.438	48.76	120.75	0.82	1.66
CBA	50	2.331	46.62	100.90	0.99	1.92
Pr+BBF	50	2.375	47.50	92.56	1.08	2.14
Pr+CBA	50	2.341	46.82	95.62	1.04	2.03

*Y: P(Bb×N), where P is number of cacao pods, Bb is weight of cocoa beans, N is number of beans

Table 3: Detecting the *B. bassiana* at soil layers after application

Layers	Depth (cm)	Occurrence of Fungi	Annotation
Top	0- 5	+	found *B. bassiana* fungus
	16-30	+	found *B. bassiana* fungus
Middle	31-40	-	not found *B. bassiana* fungus
	41-50	-	not found *B. bassiana* fungus
Bottom	>50	-	not found *B. bassiana* fungus

grows in a circular direction. These characteristics are similar to other *B. bassiana* isolates reared in the same PDA media.

DISCUSSION

This study demonstrated that both *B. bassiana* and *D. thoracicus* effectively controlled the CPB when they are combined with pruning. Prior studies have reported the importance of these biological agents in controlling CPB. Amin *et al.*[29] had figured out the effectiveness of entomopathogenic fungi in controlling the CPB. Multiple techniques in applying the *B. bassiana* were recommended by McKinnon *et al.*[30]. Increasing population of *D. thoracicus* on cocoa has significantly decreased the CPB[17,31]. This study results in Table 1 and 2 indicate the successful of the CPB control method when *B. bassiana* and *D. thoracicus* are applied with pruning.

Present results show that the damage on cocoa pods decreased with application of *B. bassiana* and *D. thoracicus* when combined with pruning. The application of *B. bassiana* in cacao plantation is not only for controlling the CPB but also can provide a benefit since they act as endophytic fungi[32]. *B. bassiana* produce beauvericin, a potential agent for pesticides, which is a cyclic hexadepsipeptide[33]. *B. bassiana* has a wide host range with several pathogenic strategies, including many bacterial-like toxins[34]. Yustina and Pu'u[35] reported that the *B. bassiana* may cause mortality of CPB larvae up to 100% in 5-6 days after application. It was assumed that high density of spores allowed more contact between conidia and larval body. Germination of *B. bassiana* has been recorded as a virulence factor[36]. Controlling *Aphis craccivora* (Koch) using *B. Bassiana* in peanut might increase yield to1.72 t ha^{-1}[37].

The application of *B. bassiana* (4 g/10 L of water) reduced CPB and increased number of healthy pods[38]. Using of *B. bassiana* against CPB was more successful on the adult stage and consistently inflicted high mortality than the pupae or the eggs, suggesting that application of entomopathogenic fungi would be best in adults stage[16].

The effectiveness of this entomopathogen in the field depends on some factors, e.g., environment and entomopathogenic fungus virulence. The main environmental factors are ultra violet, rainfall, humidity and temperature[39]. The entomopathogenic factor may include the concentration of the spores inoculum that was applied either by foliar sprays or soil drenches[14]. Similarly, *D. thoracicus* also appears to have significant results in controlling CPB in this study. This study results are in agreement with those of previous studies[18,20,21] in which CBA was found as potent agents against CPB. However, the effectiveness of CBA for pest control in a cocoa plantation strongly depends on the CBA population. The higher the population density of CBA the more effective in controlling CPB infestation[21]. A significant result in controlling the CPB by CBA has been reported by Saripah[18] when sufficient populations of CBA and *Cataenococcus hispidus* or cocoa mealy bug (CM) are available in the cocoa tree. Anshary and Pasaru[17] reported that *D. thoracicus* reduced the damage of cocoa beans by 2.43%. In contrast, in the absence of D. *thoracicus* as the cocoa bean damages might be up to 27.79%. Results showed a less bean damage (0.39%) by the presence of CBA and the damage increase by 16.53% without CBA. Saripah[18] revealed that the effectiveness of CBA in controlling CPB almost similar with insecticides and suggesting to distribute CBA population using artificial nests spread evenly on each cacao tree and broken nests should be replaced regularly for better results of CPB control. Availability of cocoa mealy bug as the food source for CBA strongly influence the development of CBA of the cacao plantation. Accordingly augmentation of those useful insects to cacao tree frequently is also needed to enhance the performances of CBA against CPB. The application of biological agents and pruning which reducing the damage of cocoa beans (Table 2), indicates that the necessity of an integrated management for controlling CPB at the cocoa plantation. Although there is no clear information that has been reported concerning the mechanism of pruning in controlling CPB, changing microclimate condition of the cocoa tree may lead to a situation not in favor to CPB. Because the dark or shaded condition is a preferred site for the imago of CPB to perch, so good pruning will indirectly protect the cacao tree from the infestation of this pest[22]. Successful agricultural management practice reported that plant pruning can help farmers in controlling plant pests[40]. Pruning as a strategy for removing branches and stems of large trees will contribute to the cacao architecture in order to provide an appropriate microclimate condition to the plant and to control plant pests and pathogens and also to ensure the cocoa bean quality[41]. However, heavy pruning may reduce CBA and CM population[18]. Therefore, maintenance of suitable condition, especially favorable environment is important to ensure sustaining of CBA and CM in the cocoa plantation.

The successful applications of *B. Bassiana* in controlling *C. cramerella* in the cacao plantation could be detected through the soil below the plantation. This study shows that *B. bassiana* are capable of infecting the pupae of *C. cramerella* within the soil. However, it can only effectively infect the pupae that are introduced to the soil sample taken from the upper soil layer and not in the soil layer deeper than 30 cm (Table 3). It is likely that high organic matter in the upper soil layer provides a favorable environment for *B. bassiana*. This is in line with Tkaczuk *et al.*[42] who found that the number of *B. bassiana* stay high in field soil seven days after application and amid larval drop. They also found that *B. bassiana* infected more insect larvae than another fungus such as *Isaria fumosorosea*. Sanchez-Pena *et al.*[43] used *Tenebrio molitor* (L.) (Coleoptera: Tenebrionidae) larvae as bait in the insect bait method to a mass entomopathogenic fungi from four nearby soil habitats and found that the fungi collected was highly dominated by *Beauveria bassiana* (Balsamo) Vuillemin (Hypocreales: Cordycipitaceae) compared to the other fungi including *Metarhizium anisopliae* (Metschnikoff) Sorokin (Hypocreales: Clavicipitaceae) and *Isaria* (*Paecilomyces*) sp. (Hypocreales: Cordycipitaceae).

The advancement of *B. bassiana* as an effective biological control for insect pest might be assessed through its biodiversity and pathogenicity[44]. In previous research, isolation, identification and evaluation of *B. bassiana* pathogenicity in soil had been successfully done[45]. *B. bassiana* as well as other important pathogenic fungi such as *Metarhizium* spp., *Paecilomyces* spp. and *Lecanicillium* spp., exist in soils as their natural habitat[46,47]. *B. bassiana* is pathogenic to different insect species causing white muscardine diseases.

B. bassiana adapt to unfavorable soil condition or low host densities either as saprophytic mycelia or conidia. *B. bassiana* infect an insect host through three stages; adhesion, germination and penetration. High hydrophobicity of the conidia leads to easily adhesion to the host cuticle[48]. The hydrophobicity is due to the unique interwoven of the conidia outer layer which also functions as protection for the conidia from external factors[49]. Inglis *et al.*[49] found that numerous conidia of *B. bassiana* penetrating crop canopies are dropped on the soil surface and remain in the soil afterward. They also found that aerial application of *B. bassiana* conidia to vegetated roadsides may control ovipositing grasshoppers and emerging nymphs. This study points out the importance of *B. bassiana* occurrence and others fungi as reservoirs of natural enemies for biological control purposes on cacao plantation.

CONCLUSION

The results of our study have shown the effectiveness of *B. bassiana* and *D. thoracicus* when combined with pruning, subsequently resulting in a higher cocoa yield compared to the control treatment. Moreover combining either *B. bassiana* or *D. thoracicus* with pruning tends to increase cocoa yields. The *B. bassiana* also was successfully detected from soil top layer after application. It indicates that this fungus is a promising biological agent for CPB. The results suggest an integrate of biological and cultural control is potent for controlling CPB and increase cocoa yields.

SIGNIFICANT STATEMENTS

This study discovers the effectiveness of the combination of application entomopathogenic fungi of *B. bassiana* and ant predator of *D. thoracicus* as, biological control agents and pruning as a farming management practice in controlling *C. cramerella.* Therefore, this study makes a major contribution to the research in controlling the *C. cramerella* as the main cacao pest by demonstrating application biological control and farming management practice. Interestingly, both of the pest controlling methods only showed a significant result when they were applied simultaneously.

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