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Utilization of Biological Control Agents against *Phytophthora palmivora* Causing Pod Rot Disease in Cacao

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Abstract
Cacao black pod rot, caused by *Phytophthora palmivora*, is an economically serious problem in all cacao-producing regions, leading to global yield loss and tree deaths. With the advent of Republic Act 10068, also known as the Organic Agriculture Act of 2010, the state is searching for and encouraging sustainable agriculture. Hence, this study was conducted to determine the antagonistic effects of the different biological control agents against *P. palmivora* and identify the most effective biological control agents against cacao pod rot. Conducted at a laboratory, the experiment was laid out in Complete Randomized Design (CRD) and replicated three times with five samples of cacao pods per replication. Treatments used were as follows; T1- Negative control, T2- *P. palmivora*, T3- Chemical control, T4- *Bacillus subtilis*, T5- *Bacillus amyloliquefaciens*, T6- *Trichoderma harzianum*. The *in vitro* test was done using a dual culture technique, while the *in vivo* test was done through a detached pod test. The dual culture test showed that *T. harzianum* reduced *P. palmivora* radial growth to 6.77mm with a percent growth inhibition of 70.54%. Moreover, the detached pod test recorded no disease incidence, severity, and incubation period in pods treated with *T. harzianum*. Whereas, the longer incubation period was noted in pods treated with chemical control at 4.27 days. Moreover, the highest degree of control was recorded in pods treated with *T. harzianum* (100%). These findings emphasized the potential of *T. harzianum* as a sustainable and effective solution for combating cacao black pod rot and promoting sustainable agriculture.

Keywords
Phytophthora palmivora, Biological control agents, *Bacillus subtilis*, *Bacillus amyloliquefaciens*, *Trichoderma harzianum*

INTRODUCTION

The cacao tree is scientifically known as *Theobroma cacao*. It is a small (13 to 26 ft) evergreen tree in the family Malvaceae [20] native to the deep tropical regions of Central and South America. Its seeds, cocoa beans, are used to make cocoa mass, cocoa powder, and chocolate. The fruit or cocoa pod is an ovoid shape, 15 to 30 cm long and 8 to 10 cm wide, ripening yellow to orange and weighing about 500 g when ripe. The pod contains 20 to 60 seeds, usually called "beans," embedded in a white pulp. The seeds are the main ingredients of chocolate, while the pulp is used in some countries to prepare refreshing juice, smoothies, jelly, and natal [32]. Each seed contains a significant amount of fat (40 to 50%), referred to as cocoa butter. Their most noted active constituent is theobromine, a compound similar to caffeine. Cacao is being cultivated on 12.10 million hectares worldwide [4], with the demand for cacao increasing up to 3 % per year [7].

In the Philippines, around 10,000 to 15,000 cacao farmers are producing cacao nationwide. Data from the Department of Agriculture-High Value Crops Development Program (DA-HVCDP) revealed that Mindanao contributes 90 percent of the country's total cacao production, and 80 percent of which comes from the Davao Region [8].

According to the Philippine Cacao Industry Roadmap [28], cacao may significantly contribute to poverty alleviation and inclusive growth through livelihood and job generation because cacao production only requires small monetary investment or start-up capital. This explains why 90% of the growers are of small farm holdings. Its diversified usage as food and non-food warrants a sustainable marketing opportunity. At present, Philippine cacao production stands at 10,000–12,000 metric tons from the 20,000–25,000 hectares of land planted with cacao per industry estimate.

However, cacao black pod rot is a particularly economically serious problem in all cacao-producing regions caused by *Phytophthora palmivora,* which causes global yield loss of 20-30% and tree deaths of 10% annually [19]. In Central and West Africa, cacao black pod disease is caused by two species of *Phytophthora*, *P. palmivora* and *P. megakarya*. If left unmanaged, it has the potential to result in crop losses ranging from 50 to 100%. Currently, multiple control measures are being developed and implemented in order to effectively manage the condition. Systemic fungicides are applied in regular intervals of 3 to 4 weeks. The sustainability of fungicide usage becomes problematic due to the persistence of soil-borne *Phytophthora* species, which can survive in soil and infected debris for extended periods ranging from months to several years [10, 29]. This pathogen is responsible for root infections and serves as a reservoir of inoculum, releasing zoospores that have the potential to infect other plant parts through the transfer of water splashes from the soil to the foliage. Furthermore, in addition to the aforementioned issues of exorbitant expenses, ecological degradation, and concerns regarding the quality of cocoa beans. It is recommended to implement cultural practices, such as appropriate spacing and pruning techniques, to optimize shading and aeration. These methods aim to minimize surface wetness and thereby diminish the occurrence of the disease. Regular and thorough harvesting, cleaning, and proper disposal of pod mummies/husks and sick pods have demonstrated some effectiveness in decreasing the presence of secondary inoculum. Nevertheless, these approaches exhibit a high degree of labor intensity, substantial financial costs, and economic feasibility only contingent upon the presence of elevated market prices for cocoa [10].

With the advent of RA 10068, also known as the Organic Agriculture Act of 2010, the state is searching for and encouraging sustainable agriculture. Also, the heavy application of chemical fungicides would eventually lead to resistant pathogens and cause soil and water pollution. The more sustainable and environmentally friendly methods should be established and implemented, such as biological control and natural inputs.

Moreover, with the increasing awareness of environmental risks and the financial burdens associated with the use of chemical treatments for disease control, there is a rising shift towards the exploration of biological control as a more ecologically sustainable approach to managing plant diseases [9].

Hence, this study was conducted to evaluate the use and effectiveness of different biological control agents as an alternative control measure of black pod rot disease in cacao.

MATERIALS AND METHODS

This study was conducted at the Crop Science Laboratory of Davao de Oro State College, Compostela, Davao de Oro, Philippines. The experiment was laid out using a Complete Randomized Design (CRD) with six treatments and replicated three times (Table 1).

Table 1 Treatments and rates used in the experiment

Treatment No.	Treatment	Rate
T1	Negative Control (Uninoculated)	Untreated
T2	*P. palmivora*	Untreated
T3	Chemical Control (Trifloxystrobin and Tebuconazole)	0.75g/1L
T4	*Bacillus subtilis*	1.25g/1L
T5	*Bacillus amyloliquefaciens*	0.05g/1L
T6	*Trichoderma harzianum*	1.25g/1L

In Vitro Test for Antagonism

The Potato Dextrose Agar (PDA) medium was prepared following the standard procedure and sterilized in an autoclave at 121.6°C for 15 minutes. The sterilized PDA medium was dispensed to the petri dish by 20ml each dish.

Meanwhile, *P. palmivora* was isolated using a soil baiting technique where soil near the diseased plant infected by the pathogen was collected. Soil baiting with plant material susceptible to *P. palmivora* helped ensure the isolation of this specific pathogen. It provided a targeted approach for detecting the presence of this particular oomycete in soil.

For slide culture, it is a rapid method of preparing fungal growth for examination and identification. It permits *P. palmivora* to be studied visually in situ with as little disturbance. Through this technique, *P. palmivora* are identified by close examination of their morphology and characteristics. The *P. palmivora* grows directly on the slide with small cuttings of infected leaves. By doing this, the *P. palmivora* structures were directly viewed under a microscope (Biobase Model BMB-117M – Made from China). The distinct *P. palmivora* morphological characteristics, such as papillate sporangium, terminal chlamydospore, and intercalary chlamydospore, were observed.

Moreover, *B. subtilis* and *B. amyloliquefasciens* are commercially available and were purchased from a legitimate supplier. At the same time, *T. harzianum* was obtained from the Provincial Agriculturists Office of Davao de Oro, Philippines.

The assay for antagonism was performed on a PDA medium using the dual culture method [13]. Mycelial discs (3cm diameter) of seven-day-old *P. palmivora* and *T. harzianum* while three-day-old *B. subtilis* and *B. amyloliquefaciens* were placed on the same dish 1.5cm from each other. The mycelial disc of *P. palmivora* and *T. harzianum* were obtained from the prepared seven-day-old pure culture while the bacterial biocon (*B. subtilis* and *B. amyloliquefaciens*) were obtained from the prepared three-day-old pure culture. Meanwhile, the chemical control was prepared using the filter paper method at the recommended rate of the fungicide. The fungicide (Trifloxystrobin and Tebuconazole) used was a systemic broad-spectrum fungicide with protective and curative action that is locally available and widely used by the farmers in the locality [29].

$$PGI\ (\%) = \frac{\begin{array}{c}Radial\ Growth\ P.palmivora\ in\ the\\ positive\ control\ dish - Radial\ Growth\ of\ P.palmivora\ in\ the\\ treated\ dish\end{array}}{Radial\ Growth\ of\ P.palmivora\ in\ the\ positive\ control\ dish}\ x\ 100$$

The Percent Growth Inhibition (PGI) was categorized on a growth inhibition category (GIC) scale from 0 to 4, Where: 0= no growth inhibition: 1= 1-25% growth inhibition: 2 = 26-50% growth inhibition: and 4 = 76-100% growth inhibition.

Detached Pod Test

The source of cacao pods with the variety of "UF-18" was procured at Terec cacao farm P-1 Cabinuangan, New Bataan, Davao de Oro, Philippines.

For the Detached Pod Test (DPT), matured unripe pods of UF18 with similar size at four (4) months old were used as test samples. Pods were harvested with care and kept in clean sacks. The harvested cacao pods were washed thoroughly with tap water and blotted dry. Afterward, it was surface sterilized with 10% sodium hypochlorite.

Treatments were calibrated to determine the amount of solution to be applied to the cacao pods. Proper tagging of the sample pods was also properly done. Cacao pods were wounded using a cork borer (6mm diameter) before treatment application. The chemical and biocon treatments were prepared using the recommended rate and applied to the cacao pods with 0.5ml treatment solution on the wounded part per pod and air-dried for ten minutes as a preventive control.

A 14-day-old *P. palmivora* culture was used for the inoculation. It was standardized using a cork borer (10mm diameter), and the mycelial discs were then inoculated to the wounded cacao pods [21]. Cacao pods were incubated in a humid chamber using clean plastic cellophane for seven days at 28 to 32^{o}C.

Incidence of cacao pod rot was recorded by observing it daily for its symptom development, such as necrotic lesions with brown or black color, which eventually enlarged to cover the whole pod. This was taken from 15 cacao sample pods. It was computed using the formula below:

$$Percent\ Disease\ Incidence\ (\%DI) = \frac{Number\ of\ Infected\ fruits}{Number\ of\ Fruits\ evaluated}\ x\ 100$$

The disease severity was evaluated based on five sample pods per treatment per replication. The percentage of disease severity was determined following the damage rating scale of Akrofi [1].

Table 2 Cacao black pod rot severity rating scale

Score	Infected Portion	Percentage Infection	Inference
0	None	0%	Healthy
1	1/5	20%	Not Severe
2	2/5	40%	Mildly Severe
3	3/5	60%	Averagely Severe
4	4/5	80%	Severe
5	All	100%	Extremely Severe

Based on the rating of the severity of cacao pod lesion manifest by *P. palmivora*, the disease index for each of the symptoms was computed using the formula below:

$$Disease\ Severity\ Index\ (DSI) = \frac{\sum(Number\ on\ scale\ x\ Number\ of\ pods\ in\ that\ scale)}{\sum(Number\ of\ treated\ pods)}$$

The degree of effectiveness of the treatments was based on computed percent degree of control (%DC) using the following arbitrary scale shown in Table 3.

Table 3 Degree of effectiveness of treatments

Disease Index (%)	Degree of Effectiveness
1-20$^{\%}$	Not Effective
21-40%	Less Effective
41-60%	Moderately Effective (ME)
61-80%	Effective
81-100%	Very Effective

The degree of control was computed using the formula below:

$$Percent\ of\ degree\ of\ control = \frac{\%\ Disease\ Incidence\ untreated - \%\ Disease\ Incidence\ treated}{\%\ Disease\ Incidence\ untreated}\ x\ 100$$

The data from the *in vitro* test for antagonism and detached pod test (DPT) were analyzed using the Analysis of Variance (ANOVA) following Completely Randomized Design (CRD). The differences among treatment means were computed using Tukey's HSD when the variance was significant. Photo documentation was taken to support the experiment.

RESULTS AND DISCUSSION

Growth Response of *P. palmivora* to Different Biological Control Agents

The different biological control agents (BCAs) were tested against *P. palmivora* using the dual culture method. Radial growth and percent inhibition of *P. palmivora* were recorded. Results showed that all three BCAs tested in this study exhibited antagonistic activities against *P. palmivora*, the pathogen of cacao pod rot. All of the test antagonists considerably hindered the radial growth of the pathogen under the conditions of this study. The *T. harzianum* shows the shortest radial growth of *P. palmivora* with 6.77mm, followed by *Bacillus subtilis* with 10.33mm, then the chemical control with 18.77mm. Extensive radial growth of *P. palmivora* was noted against *B. amyloliquefaciens* with 21.20mm (Table 4). The results implied the potential biocontrol of *T. harzianum*, for it was able to inhibit the growth of *P. palmivora* and subsequently reduce its radial growth due to its modes of action such as competition and antagonism with the pathogen, production of certain compounds against pathogenic fungi and parasitism on pathogenic fungi [26]. Also, in the study of Sarria et al. [30], *T. harzianum* showed other mechanisms to reduce the growth of *P. palmivora*, such as mycoparasitism, competition, and production of antifungal compounds and metabolites.

Table 4 Radial growth (mm) and percent growth inhibition of *P. palmivora* as affected by different biological control agents

Treatments	Radial Growth of *P. palmivora* (mm)[1]	Growth Inhibition (%)[1]	Growth Inhibition Category[2]
P.palmivora	22.97[d]	-	-
Chemical control	18.77[c]	18.30[c]	1
Bacillus subtilis	10.33[b]	55.01[b]	3
Bacillus amyloliquefaciens	21.20[cd]	7.71[c]	1
Trichoderma harzianum	6.77[a]	70.54[a]	3
CV (%)	**6.67**	**13.31**	

[1]*Means with the same letter superscript are not significantly different at 1% level by Tukey's HSD.*
[2]*Growth Inhibition Category (GIC) scale: - = not applicable 0= no growth inhibition; 1= 1-25% growth inhibition; 2 = 26-50% growth inhibition; 3= 51-75 growth inhibition; and 4 = 76-100% growth inhibition*

Moreover, the percent growth inhibition of *P. palmivora* followed the same trend, such in radial growth where the highest inhibition was noted in *T. harzianum* with 70.54%, followed by *B. subtilis* with 55.01% inhibition next is chemical control with 18.30% growth inhibition. Lastly, lesser inhibition of *P. palmivora* was recorded in *B. amyloliquefaciens* with 7.71%.

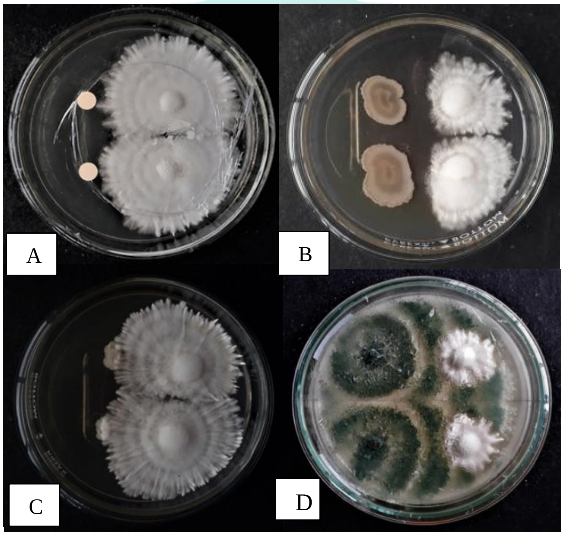

Fig. 1 Growth response of *Phytophthora palmivora* (Right) to biological control agents (Left); A.) Chemical control; B.) *Bacillus subtilis*; C.) *Bacillus amyloliquefaciens;* D.) *Trichoderma harzianum*

According to Fatima et al. [12], *T. harzianum* was found to be an effective inhibitor reduction in the radial growth of *Phytophthora infestans* with an inhibition rate of (85%). Based on Moayedi and Ghalamfarsa's [24] report, the isolation of mycoparasitic *Trichoderma* can suppress *Phytophthora* root rot. Pal and McSpadden [27] reported that the application of *T. harzianum* produces potential antibiotics and other chemicals that are harmful to pathogens and inhibit their growth. It produces gliotoxin, which inhibits *Rhizoctonia solani*, a soil-borne pathogen that causes root rot in plants. Moreover, according to Zeidan [33], *T. harzianum* can control several soil-borne fungal pathogens.

On the other hand, *B. subtilis* is potentially useful as a biological control agent and produces various biologically active compounds with a broad spectrum of activities toward phytopathogens [31]. The strains of bacteria *Bacillus* have been widely used against many economically important plant pathogenic fungi, possess a resistant spore stage, produce several kinds of antifungal compounds, and have shown significant inhibitory activity against *Fusarium moniliforme* and *Colletotrichum gloeosporioides* [17]. Thus, through their various mechanisms against the pathogen, this might be one of the reasons that *T. harzianum* and *B. subtilis* got the highest growth inhibition of *P. palmivora.*

Detached Pod Test

The detached pod test was conducted to validate the result of the *in vitro* test. The same biological control agents were used in this test, such as *B. subtilis*, *B. amyloliquefaciens*, and *T. harzianum.* Different BCAs were applied on four-month-old UF18 cacao pods prior to the inoculation of *P. palmivora*. The data, such as disease incidence, incubation period, and severity of infection, were evaluated after seven days of *P. palmivora* inoculation. Based on the results, cacao pods treated with *T. harzianum* recorded no disease incidence (0%), followed by *B. amyloliquefaciens* and *B. subtilis* with 40% and 13.33% disease incidence, respectively. The cacao pods treated with a fungicide (chemical control) had a disease incidence of 86.67%, while the untreated cacao pods inoculated with *P. palmivora* got 100% disease incidence (Table 5). The efficacy of biocontrol agents was effectively established using the detached pod test, which demonstrated the potential of *T. harzianum* as a highly effective solution with a disease incidence of 0%.

Table 5 Summary of the data gathered on detached pod test inoculated with *P. palmivora*

Treatments	Disease Incidence (%)**/1	Incubation Period (days)**	Disease Severity on cacao pods**
Negative Control	0.00^{a}	0.00^{c}	0.00^{a}
P. palmivora	100.00^{d}	3.73^{a}	1.00^{c}
Chemical control	86.67^{cd}	4.27^{a}	0.87^{c}
Bacillus subtilis	13.33^{ab}	1.27^{c}	0.20^{ab}
Bacillus amyloliquefaciens	40.00^{bc}	2.40^{ab}	0.40^{b}
Trichoderma harzianum	0.00^{a}	0.00^{c}	0.00^{a}
CV (%)	**24.59**	**40.38**	**34.49**

[1] Data were Square root transformed prior to analysis of variance.

***Means with the same letter superscript are not significantly different at 1% level by Tukey's HSD*

Moreover, the symptom appearance of black pod rot on inoculated cacao pods treated with *B. amyloliquefaciens* is 2.40 days, followed by *B. subtilis* with 1.27 days, while no symptom appearance on cacao treated with *T. harzianum.* Meanwhile, cacao pods treated with chemical control got the longest symptom appearance at 4.27 days, followed by the untreated and inoculated cacao pods at 3.73 days. The recent research conducted by Liu et al. [22] further reinforces this by highlighting *Trichoderma*'s ability to achieve a 0% disease incidence, underscoring its exceptional biocontrol potential. These mechanisms encompass mycoparasitism, antibiosis, and competition for resources, ultimately enabling *Trichoderma* to outcompete and suppress the growth of oomycete pathogens like *P. palmivora* [6, 11]. Moreover, *Trichoderma* species are renowned for their capacity to colonize plant roots and induce systemic resistance, thereby enhancing their biocontrol capabilities [18]. The consistency of *Trichoderma*'s effectiveness across recent studies underscores its potential as a sustainable and environmentally friendly solution for disease management in cacao production.

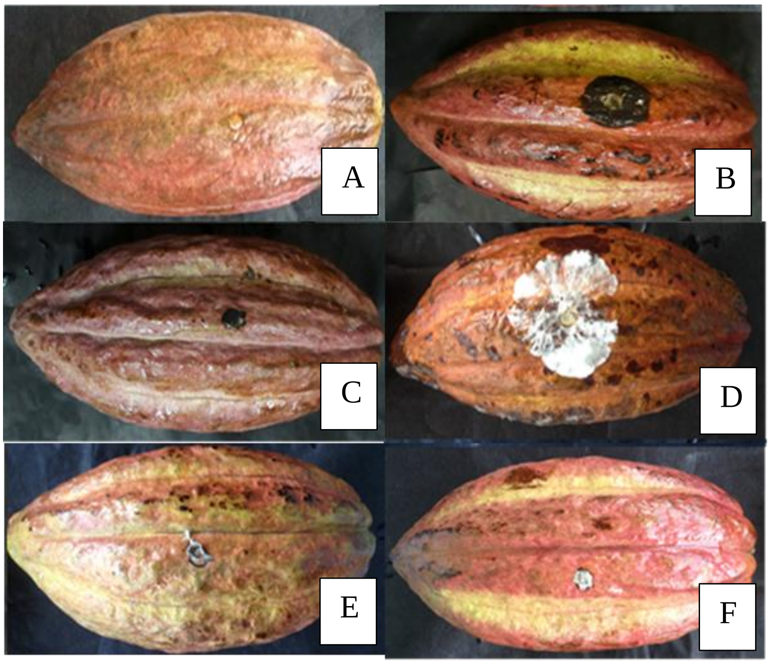

Fig. 2 Cacao pods treated with different biological control agents after seven days of inoculation: A.) Sterilized Distilled Water (SDW); B.) *Phytophthora palmivora*; C.) Chemical control; D.) *Bacillus subtilis*; E.) *Bacillus amyloliquefaciens*; F.) *Trichoderma harzianum*

Degree of Control

Results showed that *T. harzianum* got the rating of very effective due to zero disease incidence recorded, and *B. subtilis* with a rating of very effective with 13.33% disease incidence, followed by *B. amyloliquefaciens* with a moderately effective rating with 40% disease incidence. Whereas chemical control was rated as not effective due to a high pod rot incidence of 86.67%. However, the highest disease incidence was noted on positive control (inoculated and untreated) with 100% disease incidence (Table 6).

Table 6 Percentage of disease incidence and degree of control of black pod rot on cacao treated with different biological control agents[1]

Treatments	Disease Incidence**/[1]	Degree of control	Degree of effectiveness
Sterilize Distilled Water (SDW)	0.00^{a}	-	-
P. palmivora	100.00^{d}	-	-
Chemical control	86.67^{cd}	13.33^{b}	Not Effective
Bacillus subtilis	13.33^{ab}	86.67^{a}	Very Effective
Bacillus amyloliquefaciens	40.00^{bc}	60.00^{a}	Moderately Effective
Trichoderma harzianum	0.00^{a}	100.00^{a}	Very Effective
CV %=	**24.59**		

[1] *Data subjected to ANOVA were Square root transformed.*

** *Means with the same letter superscript are not significantly different at 1% level by Tukey's HSD.*

Based on the result, it is evident that *T. harzianum* is very effective among the different biological control agents in managing black pod rot on cacao pods. According to Claydon et al. [5], *T. harzianum* was able to reduce disease incidence of the pathogen. Meanwhile, based on the report of Mpika et al. [25], *T. harzianum* was tested, and the inhibitor effect of the *P. palmivora* agent of brown rot cacao pods of 83.33%. The result implied that *T. harzianum* is a potential antagonist against *P. palmivora,* causing black pod rot of cacao [15]. *Trichoderma* employs various mechanisms that reduce the incidence of plant diseases. These mechanisms encompass nutrient and space competition, synthesis of antifungal metabolites, mycoparasitism, production of lytic enzymes that degrade cell walls of fungal plant pathogens, and the induction of plant resistance [3, 14]. In the study of Mahadevi et al. [23], *T. harziaum* showed potential biocontrol against *Phytophthora* root rot disease in Papaya caused by *P. palmivora*

Meanwhile, *B. subtilis* has both a direct and indirect biocontrol mechanism for suppressing pathogen-caused disease. The direct mechanism involves the synthesis of numerous secondary metabolites, hormones, cell wall-degrading enzymes, and antioxidants, all of which aid the plant's defense against pathogen attack. The indirect mechanism includes plant growth stimulation and the induction of acquired systemic resistance [16]. In the study by Ashwini and Srividya [2], *B. subtilis* showed a 65% reduction in disease incidence caused by *Colletotrichum gloeosporioides* and *Phytophthora capsici.*

CONCLUSION

Different biological control agents were tested against cacao black pod rot disease. The biological control agents used in the study were *B. subtilis*, *B. amyloliquefaciens,* and *T. harzianum.* The dual culture test showed that *T. harzianum* reduced *P. palmivora* radial growth to 6.77mm with a percent growth inhibition of 70.54%. Moreover, the detached pod test recorded no disease incidence (0%), severity, and incubation period in pods treated with *T. harzianum*. Whereas, the longer incubation period was noted in chemical control of 4.27 days. Moreover, the highest degree of control was recorded in pods treated with *T. harzianum* (100.00%).

Therefore, based on the result of the study, the application of *T. harzianum* can potentially control the cacao black pod rot caused by *P. palmivora* in both dual culture and detached pod tests. It is recommended to further study the application of *T. harzianum* on the cacao field for validation of the result.

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DECLARATION OF CONFLICT

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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