microbiology research

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Observations on the Potential of an Endophytic Fungus Associated with Cacao Leaves against *Phytophthora palmivora*

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Abstract: A study was conducted to test the pathogenicity of an endophytic fungus associated with asymptomatic cacao leaves, to determine its identity through cultural, morphological and molecular characterization, and to evaluate itsantagonistic ability vs. *Phytophthora palmivora* causing cacao black pod rot disease. Experiments were carried out under laboratory and shade house conditions. Homogeneity of variances and normal data distribution were determined using Bartlett's and Shapiro–Wilk tests, respectively. Inoculation of the endophyte in healthy cacao seedlings and pods at 5×10^5 to 1×10^6 conidia per mL by spraying resulted in asymptomatic infections. The endophyte was recovered from artificially inoculated tissues 14 and 26 days after inoculation (DAI) (UF18 seedlings), and at 10 (K9 seedlings) and 14 DAI from cacao pods. The endophyte was identified as *Colletotrichum siamense* based on its cultural, morphological and molecular characteristics. In vitroanti-pathogen assays showed that *C. siamense* had the potential to limit pathogen growth by antibiosis. At 3, 5 and 7 days after incubation period (DAIP), growth of the pathogen in co-cultivation with the endophyte measured 60.0, 70.0 and 71.0 mm, respectively, which wasconsiderably lower than the growth of the pathogen alone.

Keywords: biological control; black pod rot; *Colletotrichum siamense*; endophytic fungus; *Phytophthora palmivora*

Citation: Sadoral, J.P.; Cumagun, C.J.R. Observations on the Potential of an Endophytic Fungus Associated with Cacao Leaves against *Phytophthora palmivora*. *Microbiol. Res.* **2021**, *12*, 528–538. https://doi.org/10.3390/microbiolres12030037

Academic Editor: Valery M. Dembitsky

Received: 30 April 2021
Accepted: 15 June 2021
Published: 22 June 2021

1. Introduction

Cacao (*Theobroma cacao* L.) is an important crop cultivated worldwide, because of its properties as primary raw material in the production of chocolates and other by-products [1]. Like any other crop, cacao is affected by diseases that negatively influence its growth, yield and quality.Among them, black pod rot (BPR) caused by *Phytophthora palmivora* is considered the most devastating [2,3]. The pathogen attacks all plant parts at any growth stage, and can cause up to 20–30% pod losses [3]. One of the effective means to manage the disease is through chemical control [3,4]. However, despite its efficacy, long-term utilization of chemicals may yield adverse effects such as toxicity in plants and non-target organisms [5], contamination of soil and water systems and development of resistance among populations of *Phytophthora* [6]. Moreover, chemical control is not costeffective, particularly for small landholders who constitute the majority of cacao growers.

Because of the emerging negative impacts of the continuous usage of chemicals, biological control is considered as a viable component for disease management [7]. The strategy involves the use of microbial antagonists that suppress plant diseases [8]. Among the beneficial microorganisms, endophytes have potential to limit growth and development of pathogens. Endophytes inhabit plant organs and colonize the plant internal tissues without causing visible harm to their hosts [9]. Previous studies revealed the potential

of cacao foliar fungal endophytes by providing protection to seedlings and suppressing diseases such as leaf necrosis, leaf mortality, pod and frosty pod rots [10–12]. Aside from leaf tissues as the host of many endophytes, other aboveground plant parts, e.g., stems or branches, act as shelters of these microorganisms. Particularly, *Trichoderma martiale*, isolated from the sapwood of cacao trunk and branches, was found to suppress growth of *P. palmivora* causing black pod rot disease [13]. Culturable endophytic fungi belonging to the genera *Trichoderma*, *Pestalotiopsis*, *Curvularia*, *Tolypocladium* and *Fusarium*, recovered from stems and branches of cacao trees, also showed biocontrol effects against pathogens by decreasing disease severity when applied to cacao pods [14].

Although studies have been carried out to determine the antagonistic mechanisms of several endophytes, the processes underlying protection by endophytes, isolated from asymptomatic cacao leaves, from *P. palmivora* are not yet established. Considering the importance of biological control in disease management, it is therefore necessary to carry out research to explore the potential of biological control agents, particularly endophytes, to manage cacao diseases.Employing this approach will allow a more productive and sustainable production among smallholders, as it is costeffective, ecologicallysound and environmentallysafe.

This study was hence conducted to test the pathogenicity of an endophytic fungus associated with asymptomatic cacao leaves, to determine its identity through cultural, morphological and molecular characterization, and to evaluate its in vitroefficacy versus *P. palmivora* causing black pod rot disease.

2. Materials and Methods

2.1. Endophyte Isolation and Pathogenicity

Mature, fully expanded and healthy cacao leaves were collected in a smallholder farm in Davao del Norte, Philippines during March 2017. Isolation of endophytes was carried out following the consecutive immersion procedure: 60 s in 95% ethanol (EtOH), 3 min in 2.5% sodium hypochloride (NaClO) and 30 s in 95% EtOH [15], followed by rinsing in three changes of sterile distilled water (SDW).Cultures were maintained in potato dextrose agar (PDA) slants kept at 4–5 °C. Among isolated endophytes, *Colletotrichum* sp. Corda EF 39 was selected for the study. The isolate was similar to a dominant endophyte recovered from healthy cacao leaves and pods, able to limit the growth of *P. palmivora* [10–12,16]. A pathogenicity test was conducted using endophyte-free seedlings and healthy cacao pods. The inoculum was prepared by adding 7- to 14-day-old pure cultures to 10 mL SDW to dislodge conidia and mycelia. The fungal suspension was filtered using sterilized two-layer gauze cloth fixed at the aperture of a 250 mL conical Erlenmeyer flask. Conidia were calibrated at 5×10^5 to 1×10^6 per mL and sprayed on previously tagged 1st and 2nd leaves from the youngest leaf of 45- to 50-day-old cacao seedlings, using a plastic atomizer. Pods inoculated with the endophyte were placed on top of a wire screen laid above the moistened tissue paper inside a plastic container. Inoculated seedlings and pods were incubated and observed for development of disease symptoms. Colonization of the endophyte in planta was verified 14 and 26 (UF18 clone), or 10 and 20 days after inoculation (DAI) (K9 clone). For every scheduled date, one stage B leaf [17] was harvested from each of the five endophyte-inoculated and five non-inoculated seedlings.Developmental stage B of cacao leaf was selected as during this phase, the leaves are still soft, and thus, more receptive to infection. A total of 10 leaves (equivalent to 160 leaf segments) were seeded for each sampling schedule following current procedures [18]. Set-up was monitored daily for fungal growth. Colonization by the endophyte was evaluated seven days after seeding in culture plate. Re-isolation of endophyte from inoculated pods was carried out 14 DAI. Symbols such as + and − were used to indicate that the endophyte was recovered or not from the inoculated and uninoculated tissues.

2.2. Phytophthora Isolation and Pathogenicity

Phytophthora palmivora was obtained from cacao pod rot specimens collected from the clonal garden of the University of Southern Mindanao Agricultural Research Center (USMARC), Kabacan, Cotabato, Philippines. Pods showing typical disease symptoms were collected and processed in the laboratory. Specimens were washed in running tap water and surface sterilized in 10% commercial bleach for 5 min, rinsed in three changes of SDW and blotted dry. Isolation was carried out by excising ~2 mm^2 sections from the edge of an actively growing lesion. The cut sections were seeded in sterile plastic plates (90 mm × 15 mm) containing V8 juice agar (V8JA) medium (3 g calcium carbonate, 17 g agar, 200 mL V8 juice and 1 L SDW) amended with 2–3 drops of ampicillin (500 mg diluted in 5 mL of SDW) to suppress bacterial growth. Seeded plates were properly sealed and incubated in an inverted position at room temperature for 2–3 days. A segment of fungal growth emerging from the seeded tissues was transferred to a V8JA medium culture plate for purification and maintenance of the isolates. Pathogenicity tests were carried out by pricking the surface-sterilized cacao pods with a needle. Later, a mycelial disc (12 mm^2) from a 7-day-old pure culture of the pathogen was placed with 30 µL of SDW in each inoculation point for treated and control pods, respectively. Following pathogen incubation, the disease development was observed 2–3 DAI.

2.3. Cultural, Morphological and Molecular Characterization

Agar discs (12 mm^2) obtained from a 7-day-old pure culture of *Colletotrichum* sp. Corda EF 39 were placed individually at the center of V8JA plates. The cultures were incubated at room temperature in an inverted position. Three days later, growth diameter was measured and colony morphology parameters such as shape, color, texture, density and zonation were measured. Morphological characterization was carried out on a small agar block (6 mm^2) in the middle of a sterile glass slide. The mycelium of a 7-day-old endophyte culture was placed individually in one corner of the block and covered with a sterile cover slip, and incubated with moistened tissue paper for 5–7 days. Size, shape and color of the conidia and appressoria were observed under a light microscope (Olympus CX41RF).

For molecular identification, genomic DNA of the endophyte was extracted utilizing the Qiagen Extraction Kit (DNeasy® Plant Mini Kit) following the manufacturer protocol. The extracted DNA was used as a PCR templateusing primers (forward) ITS1 (TCCGTAGGTGAACCTGCGG-3′) and (reverse) ITS 4 (5′-TCCTCCGCTTATTGATATGC-3′) [19] for amplification of the ITS regions. PCR was carried out in a total volume of 50 µL reactionmixture containing 4 µL of genomic DNA (~50 ng), 10 µL of 5 × MyTaq Reaction Buffer, 1 µL of 20 µM of each primer (forward and reverse) and 1 µL of MyTaq DNA polymerase, with water (ddH_2O) up to 50 µL. Amplifications were performed in a thermal cycler with cycling conditions: 95 °C for 1 min for initial denaturation, followed by 35 cycles at 95 °C for 15 s for denaturation, 55 °C for 15 s for annealing and extension at 72 °C for 10 s, followed by a final extension at 72 °C for 5 min. The PCR product was separated by gel electrophoresis using 1% agarose gel stained with ethidium bromide (EtBr).Loading buffer (bromophenol blue and xylene cyanol FF) at 2 µL was added to 2 µL PCR products before wells loading. DNA fragment separation wasvisualized using ultraviolet illumination. The PCR products were sent to 1st BASE Laboratories (Malaysia) for purification and sequencing. The sequence similarity of the isolates was determined using the NCBI Basic Local Alignment Search Tool (BLAST). The sequence of the ITS region of the endophyte was compared with 20 accessions of *C. siamense* sequences previously deposited in GenBank. Sequences of other reference isolates such as *C. acutatum*, *C. dematium* and *C. gloeosporioides* were also obtained from the same source. Alignment was carried out by Muscle alignment program using MEGA version 10. Phylogenetic tree was constructed using neighbor-joining (NJ) method. Test of phylogeny was performed utilizing thebootstrap method at 1000 replications.

2.4. Anti-Pathogen Assay

2.4.1. Dual Culture Test

Mycelial discs (12 mm^2) from a 7-day-old pure culture of *P. palmivora* were excised and paired individually with *Colletotrichum* sp. Corda EF 39 opposite to each other at a distance of 40 mm apart. *Trichoderma harzianum* was included as a standard check treatment. Pathogen seeded alone on plated V8JA medium served as a control. The growth diameter of both pathogen and endophyte was measured 3, 5 and 7 days after incubation. The percent inhibition of radial growth (PIRG) was evaluated using the formula:

$$\text{PIRG} = \frac{\text{R1} - \text{R2}}{\text{R1}} \times 100$$

where R1 = diameter (mm) of the pathogen colony in the control; R2 = diameter (mm) of the pathogen in the endophyte-tested plate.

The degree of antagonism was evaluated using a rating scale [20]: 1—antagonist completely overgrew the pathogen and covered the entire medium surface; 2—antagonist overgrew the pathogen by at least two-thirds of the medium surface; 3—antagonist and the pathogen each colonized one-half of the medium surface and no organism appeared to dominate the other; 4—pathogen overgrew the antagonist by at least two-thirds of the medium surface and appeared to withstand invasion by the antagonist; and 5—the pathogen completely overgrew and occupied the entire medium surface.

The antagonistic activity of the endophyte was also described through visual assessment [21]: >75% PIRG = very high antagonistic activity; 61–75% PIRG = high antagonistic activity; 51–60% PIRG = moderate antagonistic activity; <50% PIRG = low antagonistic activity.

2.4.2. Test for Antibiosis

Assay was carried out by placing the mycelial disc (12 mm^2) obtained from a 7-day-old pure culture of *P. palmivora* (USMARC-Psp) at the center of a plastic plate previously amended with 15 mL of V8JA.Thereafter, the bottom plate seeded with the pathogen was inverted over the bottom plate of *Colletotrichum* sp. Corda EF 39. For the control and standard check treatments, the bottom plates with pathogen were inverted over the bottom plates seeded with agar disc and *T. harzianum* (standard check), respectively. The overlapping edges of the inverted plates were sealed properly. Seeded plates were incubated at room temperature with the pathogen on top. Colony diameter of the pathogen was measured 3, 5 and 7 days after incubation.

2.4.3. Statistical Design and Data Analyses

In vitroassays were laid out in a completely randomized design (CRD). Bartlett's and Shapiro–Wilk tests were carried out to determine homogeneity of variance and normal distribution of data. Afterwards, analysis of variance was performed. Treatment means were compared using the least significant difference (LSD) test at 5% level of significance.

3. Results

3.1. Pathogenicity Test

Colletotrichum sp. Corda EF 39 was identified as *C. siamense* based on its cultural, morphological and molecular characteristics. Several days after EF 39 introduction by spraying, no disease manifestation was observed among the inoculated leaf samples of UF18 seedlings. At 14 and 26 DAI, the endophyte was recovered from a number of randomly selected endophyte-inoculated leaves (Table 1). The fungus was also re-isolated from another cacao clone (K9) tested, without showing disease manifestations, 10 DAI. However, its colonization did not progress overtime. Furthermore, *C. siamense* was not recovered from any of theuninoculated cacao leaves sampled. However, absence and low colonization frequency of the endophyte were scored in a number of inoculated leaf samples, confirming earlier findings, for *Colletotrichum*-like strains [22]. Pods inoculated

with *C. siamense* remained healthy and their appearance was comparable to that of those uninoculated (Figure 1).

Table 1. Re-isolation of *C. siamense* from inoculated cacao leaves by spraying method at different sampling times (days after inoculation, DAI).

Leaf Sample	UF18 Clone		K9 Clone	
	14 DAI	26 DAI	10 DAI	20 DAI
Inoculated				
1	+	+	+	−
2	−	−	−	−
3	−	−	+	−
4	+	+	−	−
5	−	+	+	−
Control				
1	−	−	−	−
2	−	−	−	−
3	−	−	−	−
4	−	−	−	−
5	−	−	−	−

+ Endophyte re-isolated from inoculated cacao leaves. − Endophyte not re-isolated from inoculated and uninoculated cacao leaves.

Figure 1. *C. siamense* inoculated (**A**) and uninoculated cacao pods (**B**) showing asymptomatic infection, 14 days after inoculation (DAI).

3.2. Cultural, Morphological and Molecular Identification

Colony growth of the endophyte grown in V8JA medium was uniform and circular, with a white color (both plate sides). The fungus had a felty texture, medium density at the center while sparse at the margin and was without zonation (Figure 2). Three days after incubation at room temperature, growth diameter measured 54.0 mm (n = 5). The conidia were unicellular or one-celled, hyaline and with fusiform shape. Conidial size ranged from 10.4 to 17.9 (L) × 2.6 to 5.5 µm (W) with a mean of 13.0 ± 1.1 (SD) × 3.6 ± 0.6 µm (n = 50). The color of the appressoria was light brown to brown with sizes ranging from 6.9 to 13.9 (L) × 5.2 to 8.8 µm (W) with a mean of 9.6 ± 1.3 × 6.3 ± 0.8 µm (n = 15). The endophyte did not produce setae in all the slide cultures examined. Based on the

sequence of ITS regions, the endophyte exactly matched *C. siamense* with 100% sequence similarity. A total of 64 accessions deposited in the GenBank nucleotide database of *C. siamense* was found, similar to the *Colletotrichum* sp. Corda EF 39 amplified sequence (Supplementary Materials Table S1) from asymptomatic cacao leaves. The phylogenetic tree showed that the endophyte sequence grouped with those of *C. siamense* deposited in GenBank (Figure 3). On the other hand, the endophyte was distant from other species of *Colletotrichum* as reference isolates, such as *C. acutatum*, *C. dematium* and *C. gloeosporioides*.

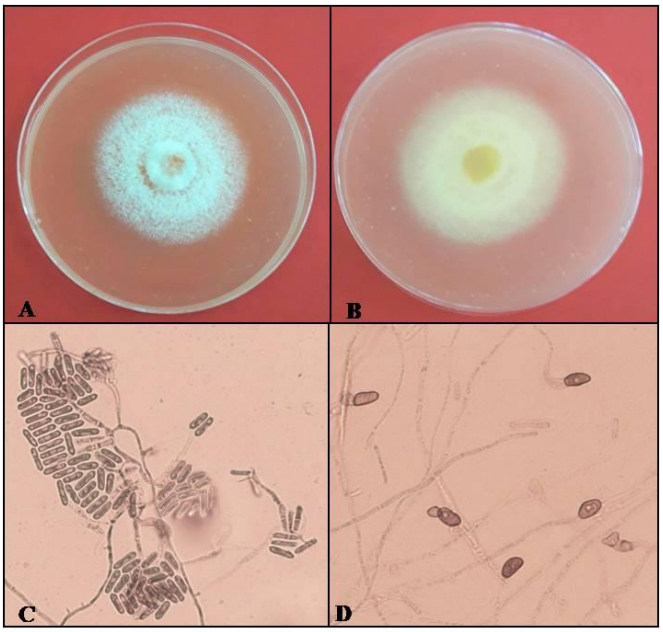

Figure 2. Colony growth of 3-day-old *C. siamense* in V8JA medium, obverse (**A**) and reverse (**B**); unicellular and hyaline conidia (**C**) and brown to brownish appressoria (**D**). Scale bars = 20 µm.

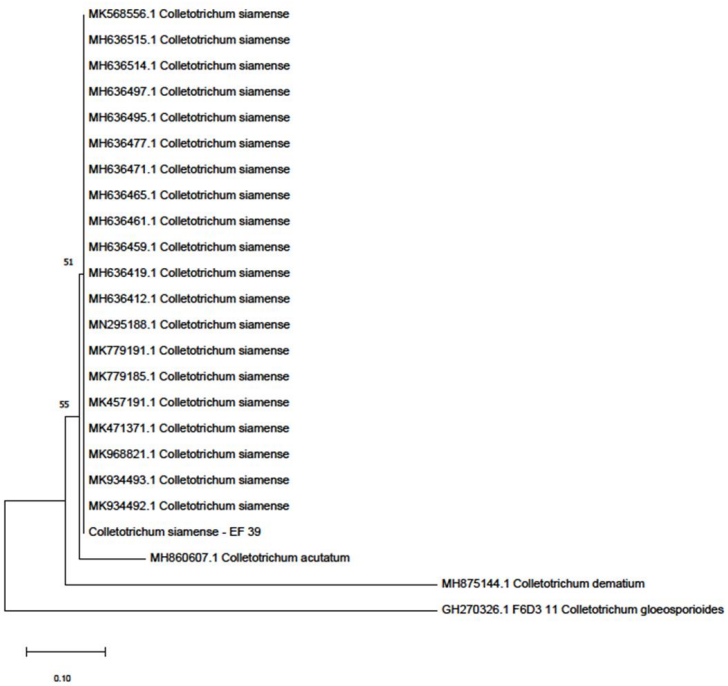

Figure 3. Phylogenetic analysis of ITS sequence of *C. siamense* and reference isolates.

3.3. Anti-Pathogen Assay

3.3.1. Competition

At 7 DAI, *C. siamense* showed low antagonistic activity against the pathogen, as shown by a low mean PIRG significantly different from the standard check treatment, *T. harzianum*, which obtained a moderate mean PIRG value (Table 2). Based on the scale [20], *C. siamense* exhibited a low degree of antagonism by colonizing only one-half of the medium surface. *Trichodermaharzianum*, on the other hand, manifested its ability as a substrate competitor by completely overgrowing the pathogen with a mean score of 1. Along with other mechanisms of action, competition for space and nutrients is one of the most common antagonistic interactions occurring between a biological control agent (BCA) and a pathogen. In such interactions, the BCA and the pathogen compete forlimited available nutrient resources in soil and on living plant surfaces [8]. Subsequently, deprivation of space and nutrients by BCA leads to failure of the pathogen to grow and establish a parasitic relationship with the host. However, in this in vitrotest, endophytic *C. siamense* did not outcompete *P. palmivora* for space and nutrient resources.

Table 2. Degree of antagonism of *C. siamense* in co-cultivation with *P. palmivora*, 7 days after incubation period (DAIP).

Treatment	PIRG [1]	Bell's Rating [2]	Antagonistic Activity [3]
C. siamense	22.0b	3	Low
T. harzianum (Standard check)	51.0a	1	Moderate

[1] Means with the same letter were not significantly different at 5% level of significance using LSD. Means of 15 replications. [2] Degree of antagonism [20]: 1—antagonist completely overgrew the pathogen and covered the entire medium surface; 2—antagonist overgrew the pathogen by at least two-thirds of the medium surface; 3—antagonist and the pathogen each colonized one-half of the medium surface and no organism appeared to dominate the other; 4—pathogen overgrew the antagonist by at least two-thirds of the medium surface and appeared to withstand invasion by the antagonist; and 5—the pathogen completely overgrew and occupied the entire medium surface. [3] Visual assessment on antagonistic activity of the endophyte [21]: >75% PIRG = very high antagonistic activity; 61–75% PIRG = high antagonistic activity; 51–60% PIRG = moderate antagonistic activity; <50% PIRG = low antagonistic activity.

3.3.2. Antibiosis

Colletotrichum siamense exhibited an antagonistic potential by restricting the growth of *P. palmivora* in culture plate. Growth and development of the pathogen when grown together with the endophyte was inhibited, exhibiting a lower mean diameter of mycelial growth at 3, 5 and 7 days after incubation (Figure 4). The values were significantly lower than the control treatment in which the pathogen obtained its maximum growth after 7 days of incubation. *T. harzianum* as a standard check treatment, however, manifested its potential as a toxic metabolite producer by inhibiting the growth of the pathogen at 3, 5 and 7 days after incubation.

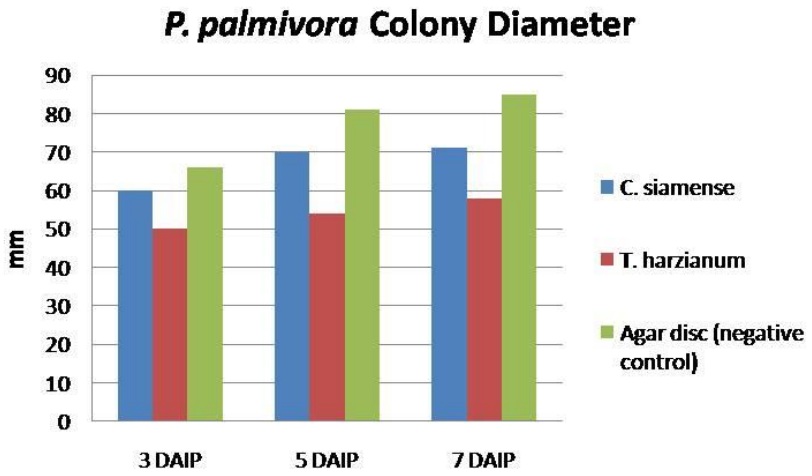

Figure 4. Mycelial growth (mm) of *P. palmivora* as affected by *C. siamense* at 3, 5 and 7 days after inoculation (DAIP).

4. Discussion

Previous studies reported the association of endophytic fungi with healthy tissues of several plant species, and cacao was known to harbor numerous endophytic fungi in leaves [12,18,23,24], pods [12,25], branches or stems [13,14,22,25,26] without causing any apparent symptoms. Along with those endophytes inhabiting asymptomatic tissues of cacao, members of the genus *Colletotrichum* were frequently recovered [11,12,23,24,26], and their potential to limit pathogen damage was ascertained. For instance, *Colletotrichum* sp. from healthy cacao leaves protected the crop from leaf necrosis and mortality caused by *P. palmivora* [11]. Moreover, *C. gloeosporioides* from healthy leaf and pod tissues of cacao was also proven to have an inhibitory effect against the pathogen causing one of the most economically important diseases of cacao, pod rot [12]. Mechanisms underlying the defense and resistance conferred by *Colletotrichum* endophytes against pathogens causing damage in cacao include competition, antibiosis [11,12] and upregulation of host defensive pathways [10].

Data indicate that survival and recovery of *Colletotrichum* species, after being introduced artificially into cacao seedlings, can be considerably difficult, likely due to abiotic factors, particularly high temperature. Temperature, along with other environmental factors, could affect the growth rate, development and survival of microorganisms, particularly biological control agents [27–29]. During the course of the experiment, an average temperature of 36 °C was recorded inside the plastic shade house where endophyte-inoculated cacao seedlings were raised and maintained. The optimum temperature for growth of *C. siamense* is 28 °C [30], while *C. tropicale* and *C. ignotium* grow best at 25–30 and 25 °C, respectively [23]. The existing environmental temperature during and after endophyte inoculation in cacao seedlings exceeded the preferred condition required by the endophyte for successful colonization and survival.

Consistent with previous findings, we confirm the association of *C. siamense* with mature, healthy and asymptomatic cacao leaves from the sampled smallholder farms. *C. siamense* was also reported from healthy tissues of tropical grass species such as dwarf napier (*Pennisetum purpureum*) and lemon grass (*Cymbopogon citratus*) [31]; asymptomatic leaf and bud of cacao; leaves of jackfruit (*Artocarpusheterophyllus*), pedalai (*Artocarpussericicarpus*), japanese plum (*Eriobotrya japonica*), common fig (*Ficuscarica*) and rosemary (*Rosmarinus officinalis*); and leaves, petiole and stem of robusta coffee (*Coffeacanephora*) in the Northern Territory, Australia [24]. It was also described as being among the antagonistic fungi isolated from cacao leaf, stem and fruit tissues in Indonesia [32]. Contrary to earlier findings, reports also emerged pertaining to *C. siamense* as a pathogen causing disease in a number of plant species, including chilli [33,34], strawberry [35,36], lychee [37] and persimmon [38]. The fungus was also recovered from leaf lesions of coffee, black pepper and *Mentha* sp. [24]. Although consistently isolated from healthy and asymptomatic host plant tissues, the likelihood of endophytes becoming pathogens or a saprophytes cannot be discounted. The literature revealed that the usual relationship that exists between an endophyte and its host plant is mutual [39]. Moreover, research findings showed that an endophyte may switch its lifestyle or ecological strategy from being endophytic into necrotrophic [40,41] or saprotrophic [42]. Some endophytic fungi are closely related to plant pathogens [43], and based on maximum-likelihood analysis with ancestral mapping, fungal endophytes can switch several times between endophytic and necrotrophic lifestyles. In the case of *Colletotrichum*, many species show a partial or unlimited endophytic lifestyle in a wide range of hosts [34]. However, a few species of *Colletotrichum* also manifested a shift in their lifestyles from being endophytic into necrotrophic, such as *C. coccodes*; isolated as an endophyte of potato and other crop species such as cabbage, chrysanthemum, cress, lettuce and white mustard [44], it was also reported as a pathogen causing anthracnose disease in chilli [45]. In this study, *C. siamense* was associated with healthy and asymptomatic leaf tissues of cacao, but is a known pathogen in other host plant species. This further means that the endophyte is not host specific and does not have a specialized relationship with its host plant. Although isolated as an endophyte, *C. siamense* may have the ability to switch

into a necrotrophic lifestyle; therefore, caution should be considered to avoid the possible negative impacts it could cause ina given crop, improperly used.

The outcome of the anti-pathogen assays imply that, among the different mechanisms of biological control tested, antibiosis is the form of interaction exhibited by *C. siamense.* This further means that the endophyte has a direct effect on the pathogen through the production of either antibiotics, volatile substances or enzymes, which resulted in the inhibition of growth and further development of the pathogen. This finding conforms to a number of studies carried out in the past highlighting the potential of endophytic fungi, specifically *Colletotrichum* species, as producers of toxic metabolites, enzymes and even hormones. *Colletotrichum* species known for producing inhibitory compounds were recovered from a wide range of host plant species, including medicinal plants (*Artemisia annua*, *Artemisia mongolica*, *Vitex negundo*), tea (*Camellia sinensis*), black pepper (*Piper nigrum*), flowers (*Rafflesiacantleyi*) and many others [46–50].

In particular, *Colletotrichum* sp., isolated from healthy stems of annual wormwood (*Artemisia annua*), was reported to produce novel bioactive metabolites which showed a fungistatic ability against pathogens causing diseases in crops, such as *Gaeumannomycesgraminis* var. *tritici*, *Rhizoctoniacerealis*, *Helminthosporium sativum* and *Phytophthora capsici* [46]. The endophytic fungus was also found to produce extracellular hydrolytic enzymes, specifically chitinase and protease, and an antimicrobial metabolite, colletotric acid, which were proven to have inhibitory effects against several plant pathogens [48,51]. Thus, previous findings show that species of *Colletotrichum* with an endophytic lifestyle are potential sources of metabolites that could effectively restrict the growth and development of plant pathogens. In this present study, *C. siamense* was isolated from healthy and asymptomatic cacao leaves, and to our knowledge, this is the first report on the recovery of such a fungus as an endophyte of cacao in the Philippines. No reports, so far, indicate that *C. siamense* is a pathogen in cacao. However, a recent study in Puerto Rico disclosed that *C. siamense* and *C. tropicale* caused pod rot of cacao [52], although they were previously reported as endophytes inhabiting healthy tissues [23,24], and are potential biological control agents against cacao diseases [10,16].

5. Conclusions

Biological control is a viable approach in plant disease management. *C. siamense* associated with asymptomatic cacao leaves inhibited the growth of *P. palmivora* by antibiosis. Our observations on the potential of *C. siamense* as a possible toxic metabolite producer imply that such an organism can be considered as a management option for cacao black pod rot. Thus, further investigation must be conducted to identify the inhibitory compounds produce by the endophyte, and determine the efficacy level of those compounds and appropriate methods for application.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/microbiolres12030037/s1, Table S1: Molecular identity of *Colletotrichum* sp. Corda EF 39 based on Internal Transcribed Spacer (ITS) sequence.

Author Contributions: Conceptualization, J.P.S. and C.J.R.C.; methodology, J.P.S. and C.J.R.C.; formal analysis, J.P.S. and C.J.R.C.; investigation, J.P.S. and C.J.R.C.; writing—original draft preparation, J.P.S.; writing—review and editing, C.J.R.C.; supervision, C.J.R.C. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Commission on Higher Education under the K to 12 Transition Program Scholarship in the Philippines.

Acknowledgments: The authors would like to express their gratitude to the University of Southern Mindanao Agricultural Research Center (USMARC) in Kabacan, Cotabato, Philippines through its former director, Romulo L. Cena, for allowing the researcher to perform the experiment in his laboratory.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. International Cocoa Organization (ICCO). Available online: https://www.icco.org (accessed on 25 January 2018).
2. McMahon, P.; Purwantara, A. Phytophthora on Cocoa. In *Diversity and Management of Phytophthora in Southeast Asia*; Drenth, A., Guest, D.D., Eds.; ACIAR: Canberra, Australia, 2004; pp. 104–115.
3. Guest, D. Black pod: Diverse pathogens with a global impact on cocoa yield. *Phytopathology* **2007**, *97*, 1650–1653. [CrossRef]
4. Guest, D.; Anderson, R.D.; Foard, H.J.; Phillips, D.; Worboys, S.; Middleton, R.M. Long-term control of Phytophthora diseases of cocoa using trunk-injected phosphonate. *Plant Pathol.* **1994**, *43*, 479–492. [CrossRef]
5. Drenth, A.; Guest, D. Phytophthora in the tropics. In *Diversity and Management of Phytophthora in Southeast Asia*; Drenth, A., Guest, D.A., Eds.; ACIAR: Canberra, Australia, 2004; pp. 30–41.
6. Cohen, Y.; Coffey, M.D. Systemic fungicides and the control of Oomycetes. *Ann. Rev. Phytopathol.* **1986**, *24*, 311–338. [CrossRef]
7. Kamil, M.J.A.; Fuddin, S.S.; Bong, C.L. Biological control of black pod disease on cocoa in Malaysia. In *Diversity and Management of Phytophthora in Southeast Asia*; Drenth, A., Guest, D.D., Eds.; ACIAR: Canberra, Australia, 2004; pp. 174–177.
8. Pal, K.K.; McSpadden Gardener, B. Biological Control of Plant Pathogens. The Plant Health Instructor. Available online: https://www.apsnet.org (accessed on 20 February 2020).
9. Petrini, O. Fungal endophytes of tree leaves. In *Microbial Ecology of Leaves*; Andrews, J.H., Hirano, S.S., Eds.; Springer: New York, NY, USA, 1991; pp. 179–197.
10. Christian, N.; Herre, E.A.; Mejia, L.C.; Clay, K. Exposure to the leaf litter microbiome of healthy adults protects seedlings from pathogen damage. *Proc. R. Soc. B* **2017**, 20170641. [CrossRef]
11. Arnold, A.E.; Mejia, L.C.; Kylo, D.; Rojas, E.I.; Maynard, Z.; Robbins, N.; Herre, E.A. Fungal endophytes limit pathogen damage in a tropical tree. *Proc. Natl. Acad. Sci. USA* **2003**, *100*, 15649–15654. [CrossRef]
12. Mejia, L.C.; Rojas, E.I.; Maynard, Z.; Bael, S.V.; Arnold, A.E.; Hebbar, P.; Samuels, G.J.; Robbins, N.; Herre, E.A. Endophytic fungi as biocontrol agents of *Theobroma cacao* pathogens. *Biol. Control* **2008**, *46*, 4–14. [CrossRef]
13. Hanada, R.E.; Souza, T.J.; Pomella, A.W.V.; Hebbar, K.P.; Pereira, J.O.; Ismaiel, A.; Samuels, G.J. *Trichoderma martiale* sp. nov., a new endophyte from sapwood of *Theobroma cacao* with a potential for biological control. *Biol. Control* **2008**, *46*, 4–14. [CrossRef]
14. Hanada, R.E.; Pomella, A.W.V.; Costa, H.S.; Bezerra, J.L.; Loguercio, L.L.; Pereira, J.O. Endophytic fungal diversity in *Theobroma cacao* (cacao) and *T. grandiflorum* (cupuacu) trees and their potential for growth promotion and biocontrol of black-pod disease. *Fungal Biol.* **2010**, *114*, 901–910. [CrossRef] [PubMed]
15. Johnson, G.I.; Mead, A.J.; Cooke, A.W.; Dean, J.R. Mango stem end rot pathogens—Fruit infection by endophytic colonisation of the inflorescence and pedicel. *Ann. Appl. Biol.* **1992**, *120*, 225–234. [CrossRef]
16. Mejia, L.C.; Herre, E.A.; Sparks, J.P.; Winter, K.; Garcia, M.N.; Van Bael, S.A.; Stitt, J.; Shi, Z.; Zhang, Y.; Guiltinan, M.J.; et al. Pervasive effects of an endophytic fungus on host genetic and phenotypic expression in a tropical tree. *Front. Microbiol.* **2014**, *5*, 479. [CrossRef] [PubMed]
17. Mejia, L.C.; Guiltinan, M.J.; Shi, Z.; Landherr, L.; Maximova, S.N. Expression of designed antimicrobial peptides in *Theobroma cacao* L. trees reduces leaf necrosis caused by *Phytophthora* spp. In *Small Wonders; Peptides for Disease Control*; Rajasekan, K., Cary, J.W., Jaynes, J.M., Montesinos, E., Eds.; American Chemical Society: Washington, DC, USA, 2012; pp. 379–395.
18. Arnold, A.E.; Herre, E.A. Canopy cover and leaf age affect colonization by tropical fungal endophytes: Ecological pattern and process in *Theobroma cacao* (Malvaceae). *Mycologia* **2003**, *95*, 388–398. [CrossRef]
19. White, T.J.; Bruns, T.; Lee, S.; Taylor, J. Amplification and direct sequencing of fungal ribosomal RNA genes for phylo-genetics. *PCR Protoc.* **1990**, 315–322. [CrossRef]
20. Bell, D.K.; Wells, D.H.; Markham, R.C. In vitro antagonism of *Trichoderma* species against six fungal plant pathogens. *Phytopathology* **1982**, *72*, 379–382. [CrossRef]
21. Soytong, K. Identification of Species of Chaetomium in the Philippines and Screening for Their Biocontrol Properties against Seedborne Fungi of Rice. Ph.D. Thesis, University of the Philippines Los Baños, Laguna, Philippines, 1988.
22. Asman, A.; Amin, A.; Abdullah, T. Endophytic fungi associated with cacao branch and their potential for biocontrol vascular streak dieback disease on cacao seedling. *IOP Conf. Ser. Earth Environ. Sci.* **2018**, *157*, 012039. [CrossRef]
23. Rojas, E.I.; Rehner, S.A.; Samuels, G.J.; Van Bael, S.A.; Herre, E.A.; Cannon, P.; Chen, R.; Pang, J.; Wang, R.; Zhang, Y.; et al. *Colletotrichum gloeosporioides* s.l. associated with *Theobroma cacao* and other plants in Panama: Multilocus phylogenies distinguish host-associated pathogens from asymptomatic endophytes. *Mycologia* **2010**, *102*, 1318–1338. [CrossRef]
24. James, R.S.; Ray, J.; Tan, Y.P.; Shivas, R.G. *Colletotrichum siamense*, *C. theobromicola* and *C. queenslandicum* from several plant species and the identification of *C. asianum* in the Northern Territory, Australia. *Australas. Plant Dis. Notes* **2014**, *9*, 138. [CrossRef]
25. Crozier, J.; Thomas, S.E.; Aime, M.C.; Evans, H.C.; Holmes, K.A. Molecular characterization of fungal endophytic morpho species isolated from stems and pods of *Theobroma cacao*. *Plant Pathol.* **2006**, *55*, 783–791. [CrossRef]
26. Rubini, M.R.; Silva-Ribeiro, R.T.; Pomella, A.W.V.; Maki, C.S.; Araujo, W.L.; Dos Santos, D.R.; Azevedo, J.L. Diversity of endophytic fungal community of cacao (*Theobroma cacao* L.) and biological control of *Crinipellis perniciosa*, causal agent of witches' broom disease. *Int. J. Biol. Sci.* **2005**, *1*, 24–33. [CrossRef] [PubMed]
27. Hashizume, Y.; Fukuda, K.; Sahashi, N. Effects of summer temperature on fungal endophyte assemblages in Japanese beech (*Fagus crenata*) leaves in pure beech stands. *Botany* **2010**, *88*, 266–274. [CrossRef]
28. Kiewnick, S. Effect of temperature on growth, germination, germ tube extension and survival of *Paecilomyceslilaci nus* strain 251. *Biocontrol Sci. Technol.* **2006**, *16*, 535–546. [CrossRef]

29. Elad, Y.; Kirshner, B. Survival in the phylloplane of an introduced biocontrol agent (*Trichoderma harzianum*) and populations of the plant pathogen *Botrytis cinerea* as modified by abiotic conditions. *Phytoparasitica* **1993**, *21*, 303–313. [CrossRef]
30. Prihastuti, H.; Cai, L.; Chen, H.; McKenzie, E.H.C.; Hyde, K.D. Characterization of Colletotrichum species associated with coffee berries in Northern Thailand. *Fungal Div.* **2009**, *39*, 89–109.
31. Manamgoda, D.S.; Udayanga, D.; Cai, L.; Chukeatirote, E.; Hyde, K.D. Endophytic *Colletotrichum* from tropical grasses with a new species *C. endophytica*. *Fungal Div.* **2013**, *61*, 107–115. [CrossRef]
32. Sudarma, I.M.; Suniti, N.W.; Darmiati, N.N. Endophytic fungi with potential as biological control agents for black pod rot disease (*P. palmivora* Butler). *Int. J. Curr. Res. Biosci. Plant Biol.* **2018**, *5*, 41–48. [CrossRef]
33. Sharma, G.; Shenoy, B.D. *Colletotrichum fructicola* and *C. siamense* are involved in chilli anthracnose in India. *Arch. Phytopathol. Plant Prot.* **2014**, *47*, 1179–1194. [CrossRef]
34. De Silva, D.D.; Crous, P.W.; Ades, P.K.; Hyde, K.D.; Taylor, P.W.J. Life styles of *Colletotrichum* species and implications for plant biosecurity. *Fungal Biol. Rev.* **2017**, *31*, 155–168. [CrossRef]
35. Capobiango, N.P.; Pinho, D.B.; Zambolim, O.L.; Lopes, U.P. Anthracnose on Strawberry Fruits Caused by *Colletotrichum siamense* in Brazil. The American Phytopathological Society (APS) Publication. Available online: https://www.apsnet.org (accessed on 20 February 2020).
36. Chung, P.C.; Wu, H.Y.; Ariyawansa, H.A.; Tzean, S.S.; Chung, C.L. First Report of Anthracnose Crown Rot of Strawberry Caused by *Colletotrichum siamense* in Taiwan. The American Phytopathological Society (APS) Publications. Available online: https://apsjournals.apsnet.org (accessed on 25 February 2020).
37. Ni, H.F.; Huang, C.W.; Wu, C.J.; Yang, H.R.; Lin, C.Y.; Chang, J.Y.; Chang, J.W. First report of pepper spot disease of lychee caused by *Colletotrichum siamense* in Taiwan. *J. Plant Pathol.* **2017**, *99*, 808.
38. Chang, T.; Hassan, Q.; Jeon, J.Y.; Shin, J.S.; Oh, N.K.; Lim, T.H. First Report of Anthracnose of Persimmon (*Diospyros kaki* L. f.) Caused by *Colletotrichum siamense* in Korea. The American Phytopathological Society (APS) Publication. Available online: https://apsjournals.apsnet.org/ (accessed on 25 February 2020).
39. Faeth, S.H. Are endophytic fungi defensive plant mutualists? *OIKOS* **2002**, *98*, 25–36. [CrossRef]
40. Alvarez-Loayza, P.; White, J.F., Jr.; Torres, M.S.; Balslev, H.; Kristiansen, T.; Svenning, J.C.; Gil, N. Light converts endosymbiotic fungus to pathogen, influencing seedling survival and niche space filling of a common tropical tree, *Iriartea deltoidea*. *PLoS ONE* **2011**, *6*, e16386. [CrossRef] [PubMed]
41. Photita, W.; Lumyong, S.; Lumyong, P.; McKenzie, E.H.C.; Hyde, K.D. Are some endophytes of *Musa acuminata* latent pathogens? *Fungal Div.* **2004**, *16*, 131–140.
42. Promputtha, I.; Lumyong, S.; Dhanasekaran, V.; McKenzie, E.H.C.; Hyde, K.D.; Jeewon, R. A phylogenetic evaluation of whether endophytes become saprotrophs at host senescence. *Microb. Ecol.* **2007**, *53*, 579–590. [CrossRef]
43. Delaye, L.; Garcia-Guzman, G.; Heil, M. Endophytes versus biotrophic and necrotrophic pathogens–are fungal lifestyles evolutionarily stable traits? *Fungal Div.* **2013**, *60*, 125–135. [CrossRef]
44. Chesters, C.G.C.; Hornby, D. Studies on *Colletotrichum coccodes*: II. Alternative host tests and tomato fruit inoculations using a typical root isolate. *T. Brit. Mycol. Soc.* **1965**, *48*, 583–594. [CrossRef]
45. Sharma, P.N.; Katoch, A.; Sharma, P.; Sharma, S.K.; Sharma, O.P. First report on association of *Colletotrichum coccodes* with anthracnose in India (Abstract). *Am. Phytopathol. Soc.* **2011**, *95*, 584.
46. Lu, H.; Zou, W.X.; Meng, J.C.; Hu, J.; Tan, R.X. New bioactive metabolites produced by *Colletotrichum* sp., an endophytic fungus in *Artemisia annua*. *Plant Sci.* **2000**, *151*, 67–73. [CrossRef]
47. Ezhil Arivudainambi, U.S.; Anand, T.D.; Shanmugaiah, V.; Karunakaran, C.; Rajendran, A. Novel bioactive metabolites producing endophytic fungus *Colletotrichum gloeosporioides* against multidrug-resistant *Staphylococcus aureus*. *FEMS Immunol. Med. Microbiol.* **2011**, *61*, 340–345. [CrossRef]
48. Rabha, A.J.; Naglot, A.; Sharma, G.D.; Gogo, H.K.; Veer, V. In vitro evaluation of antagonism of endophytic *Colletotrichum gloeosporioides* against potent fungal pathogens of *Camellia sinensis*. *Indian J. Microbiol.* **2014**, *54*, 302–309. [CrossRef]
49. Munasinghe, M.V.K.; Kumar, N.S.; Jayasinghe, L.; Fujimoto, Y. Indole-3-acetic acid production by *Colletotrichum siamense*, an endophytic fungus from *Piper nigrum* leaves. *J. Biol. Act. Prod. Nat.* **2017**, *7*, 475–479.
50. Refaei, J.; Jones, E.B.G.; Sakayaroj, J.; Santhanam, J. Endophytic fungi from *Rafflesia cantleyi*: Species diversity and antimicrobial activity. *Mycosphere* **2011**, *2*, 429–447.
51. Zou, X.; Meng, J.C.; Lu, H.; Chen, G.X.; Shi, G.X.; Zhang, T.Y.; Tan, R.X. Metabolites of *Colletotrichum gloeosporioides*, an endophytic fungus in *Artemisia mongolica*. *J. Nat. Prod.* **2000**, *63*, 1529–1530. [CrossRef]
52. Serrato-Diaz, L.M.; Mariño, Y.A.; Guadalupe, I.; Bayman, P.; Goenaga, R. *Colletotrichum siamense* Causing Cacao Pod Rot and First Report of *C. tropicale* Causing Cacao Pod Rot in Puerto Rico. The American Phytopathological Society (APS) Publications. Available online: https://apsjournal.apsnet.org (accessed on 25 February 2020).