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Biological control of black pod disease of cocoa (*Theobroma cacao* L.) with *Bacillus amyloliquefaciens*, *Aspergillus* sp. and *Penicillium* sp. *in vitro* and in the field

Stephen Larbi-Koranteng[1*], Richard Tuyee Awuah[2] and Fredrick Kankam[3]

[1]Department of Crop and Soil Sciences, Faculty of Agriculture Education, University of Education, Winneba (UEW), College of Agriculture Education, Mampong Ashanti, Ghana.
[2]Department of Crop and Soil Sciences, Faculty of Agriculture, Kwame Nkrumah University of Science and Technology (KNUST), Kumasi, Ghana.
[3]Department of Agronomy, Faculty of Agriculture, University for Development Studies (UDS), Tamale, Ghana.

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Phytopathogenic fungi, *Phytophthora palmivora* and *Phytophthora megakarya* continue to be a major threat to cocoa production worldwide. To counter these drawbacks, producers rely heavily on agrochemicals leading to pathogen resistance and environmental hazards. There is also increasing demand by cocoa consumers for pesticide-free seeds. Therefore, biological control through the use of natural microbial antagonists is more rational and safer crop management option. The plant-associated *Bacillus amyloliquefaciens,* ESI was selected *in vitro*, among seven other *Bacillus* species as the most promising, using the zone of inhibition techniques. The *B. amyloliquefaciens* together with two other laboratory contaminants, *Aspergillus* and *Penicillium* spp. were used to control black pod disease of cocoa caused by *P. palmivora* and *P. megakarya* on detached cocoa pods and under field conditions. Even though all the eight bacterial isolates inhibited the black pod fungi *in vitro*, *B. amyloliquefaciens*, ESI inhibited *P. palmivora* with the highest inhibition zone of 21.21 mm and *P. megakarya* with 16.00 mm. The *Aspergillus* and *Penicillium* spp. also inhibited *P. palmivora* with an inhibition zone of 22.41 and 16.81 mm, respectively. Detached cocoa pod areas protected with broth suspensions of the three microbial antagonists and challenged with a zoospore suspension of *P. palmivora,* completely prevented black pod lesion development. Field pods sprayed with individual microbial broth suspensions and their mixtures and also challenged with a zoospore suspension inoculum, controlled black pod disease with percentage disease control ranging from 53.33-66.67% in the minor season and 40.00-66.67% in the major season. Results clearly show that these antagonists have the potential to be developed as biocontrol agents for the management of black pod disease of cocoa.

Key words: Biocontrol agents, pathogenic fungi, microbial antagonists, inoculum, *Bacillus amyloliquefaciens, Aspergillus* sp. *and Penicillium* sp.

INTRODUCTION

Phytopathogenic fungi are a threat to cocoa (*Theobroma cacao* L.) production worldwide as they are the major causes of crops losses. This has had a serious economic impact on cocoa production, particularly over the last few

decades as production intensifies. To counter these drawbacks, farmers have relied heavily on agrochemicals, basically copper-based fungicides. However, intensive and uncontrollable use of these chemicals has led to the emergence of pathogen resistance and severe negative environmental impacts. There is also increased demand from consumers for pesticide-free cocoa beans and willingness to pay a high premium for such organic products (EC, 2006; EFSA, 2009; 2012). Thus, biological control through the use of natural antagonists such as rhizosphere-associated bacteria as biocontrol agents and also stimulating plant growth has emerged as promising alternatives to chemical pesticides for more rational use and safe crop management (Lucy et al., 2004; Somers et al., 2004; Lugtenberg and Kamilova, 2009).

Antagonistic bacteria such as *Pseudomonas* spp., *Streptomyces* spp., and *Bacillus* spp. can synthesize a large array of antimicrobial compounds against fungi and favour the growth and defence response of the host (Walker et al., 2003; Ongena et al., 2005). *Bacillus* species stand out of these group as they permit an easy formulation and storage of the commercial products due to their ability to survive adverse environmental conditions of which *Bacillus amyloliquefaciens* is a typical example (Choudhary and Johri, 2009; Chen et al., 2009). Similarly, there are several reports of integration of biological control into control strategies against black pod (Phytophthora pod rot) disease using fungal antagonists. For example, Darmano (1994) and Adedeji et al. (2005) reported using *Trichoderma* species *in vivo* to control *Phytophthora* on cocoa pods. Adebola and Amadi (2011, 2012) reported using the fungi *Aspergillus* sp., *Paecilomyces* sp. and *Penicillium digitatum (*Pers.) Sacc. isolated from rhizosphere soils, to control black pod in the field.

These antagonists/agents provide beneficial protective effects by using different mechanisms of suppression and many of them are involved in mycoparasitism where the pathogen is directly attacked by a specific biocontrol agent that kills it or its propagules (Milgroom and Cortesi, 2004), antibiosis through the production of antifungal compounds including 2,4 DAPG, phenazine, pyrronitrin, iturin, surfactin bacillomycin D etc. (Raaijmakers et al*.,* 2002; Haas and Keel, 2003) and metabolite production such as lytic enzymes which can break down polymeric compounds including chitin, protein, cellulose, hemicelluloses and DNA (Anderson et al., 2004). Others are also involved in a competition for limited resources such as iron traces in the soil through production of siderophore (Loper and Buyer, 1991; Shahraki et al., 2009). They activate the defence systems in the host plant that triggers a systemic reaction that renders the host less susceptible to the subsequent infection (IRS) (Vallad and Goodman, 2004). They also colonize root directly and the surrounding soil layer (rhizosphere) providing direct protection from infection by the pathogen or influencing direct growth stimulation while the agents benefit from the nutrients secreted by the plant (Kamilova et al., 2006; Weller, 2007; Beneduzi et al., 2012).

Plant-associated *B. amyloliquefaciens* plays a vital role in the production of variety of secondary metabolites that are required in microbial antagonism (Chen et al., 2009) and enzymes like chitinase (Niazi et al., 2014), thus supporting disease suppression in plants. In the previous study, *B. amyloliquefaciens* was tested among several agricultural important fungal pathogens among which were *P. palmivora*, the causative organism of black pod disease of cocoa (Akrasi, 2005). Therefore, this experiment was conducted to evaluate the efficacy of application of a thermophilic biocontrol agent, *B. amyloliquefaciens*, from the soil and *Aspergillus* sp*. and Penicillium* sp. (laboratory contaminants with inhibitory effects) in managing black pod disease of cocoa caused by *P. palmivora and P. megakarya in vitro* and in field conditions.

MATERIALS AND METHODS

Laboratory screening of bacterial and fungal antagonists against *P. palmivora and P. megakarya*

Eight yam rhizobacterial isolates, namely; ESI (*B. amyloliquefaciens*), E7B8 (*B. velezensis*), E7B1 (*B. subtilis*), M7 (*B. subtilis*), M8 (*B. amyloliquefaciens*), M32 (*B. amyloliquefaciens*), K4 (*B. subtilis)* and M78 (*B. subtilis*) shown to possess antifungal activity (Koranteng and Awuah, 2011) were re-evaluated, using the zone of inhibition techniques against *P. palmivora* and *P. megakarya* for antagonistic activity due to their long storage in refrigeration at 4°C. For each of the bacterium, a 24 h-old single colony growing on Nutrient Agar (NA) was suspended in 10 ml distilled water in a 25 cc capped vials and shaken manually for 1 min. A 10 µl bacterial suspension was spotted at the centre of a Petri plate containing a mixture of green cacao mucilage agar (GCMA) prepared according to Awuah and Frimpong (2002) and nutrient agar (NA) (1:1 ratio). The bacterial spot was allowed to dry and the plate incubated upside down at 20±2°C in the dark for 24 h. Mycelial plugs (1 mm-diameter) from a 1-week-old culture of *P. palmivora* and *P. megakarya* growing on GCMA were separately placed, upside down, at four equidistant positions (25 mm) from the central rhizobacterial colony and the plates incubated for six days (Koranteng and Awuah, 2011). Four plates per treatment were maintained as replicates. Plates with only *P. palmivora* and *P. megakarya* served as a control. The experimental design was Complete Randomized Design (CRD). A zone of inhibition was determined by measuring lengths of inhibition zones, with a measuring rule, from the centre of the rhizobacterial colony to the edge of the *P. palmivora* and *P. megakarya* colonies. The lengths of the four zones per plates were then averaged.

In a similar experiment, an *Aspergillus* sp. and a *Penicillium* sp.

*Corresponding author. E-mail: larbikoranteng@yahoo.com.

obtained as laboratory contaminants and inhibitory to the *P. palmivora* were tested against the pathogen. *P. megakarya* was not included in this study because it performed like *P. palmivora* in the preliminary studies. Mycelia bit from a 14-day-old culture of each fungus growing on potato dextrose agar (PDA) was suspended in 10 ml distilled water in a 25 cc capped vial and shaken manually for 1 min. Ten microlitres of the suspension were spotted at the centre of a plate containing a mixture of GCMA and PDA (1:1 ratio). Four plates per treatment were established as replicates. The fungal spots were allowed to dry and plates incubated upside down at 28 ± 2°C in the dark for 48 h. Mycelial plugs (7-mm-diameter) from a 1-wk-old culture of *P. palmivora* were placed upside down at four equidistant positions (25 mm) from the central fungal colonies and the plates were incubated for six days. Plates with only *P. palmivora* served as a control. Zones of inhibition were determined as described before.

Assessment of black pod lesions on detached cocoa pods by bacterial and fungal antagonists

Seven-day-old potato broth cultures of antagonistic *Aspergillus* sp. and *Penicillium* sp. and a 14-day-old nutrient broth (NB; half strength) culture of *B. amyloliquefaciens* ESI, amended with 10% starch solution (as a sticker) were used as protectants on detached cocoa pods with uniform sizes against *P. palmivora* (Koranteng and Awuah, 2011). Cocoa pods (Hybrid: Amelonado x Amazon) were collected from the cocoa field of Kwame Nkrumah University of Science and Technology (KNUST), Kumasi-Ghana, washed with running tap water and left to dry on a laboratory bench. Fifty microlitres of each suspension of rhizobacterium, *Aspergillus* sp. and *Penicillium* sp. and their mixture were placed as protectant and spread to a size of one-centimetre diameter on the surfaces of pods and allowed to partially dry (four replicates pods per treatment). The treated pod areas were then inoculated with 10 µl of a zoospore suspension ($1x10^6$ zoospores/ml) from a 14-day-old culture of *P. palmivora*. Inoculated pods were placed in a humidified chamber constructed with transparent polyethylene wooden box and kept on a laboratory bench at room temperature (28 ± 2°C). Pods protected with Ridomil 72 plus (12% metalaxyl + 60% cuprous oxide) suspension and those treated with sterilized distilled water (SWD) were established as controls. After five days, the numbers of pods with black pod lesions were recorded. To demonstrate the persistence of the microbial antagonists on pods, those that did not show black pod lesions after five days were re-inoculated with a zoospore suspension of *P. palmivora* as before and observed for lesion development. The experiment was repeated.

Assessment of spread of bacterial and fungal antagonists on pod surfaces

Nutrient broth (NB) and sterilized distilled water suspensions of *B. amyloliquefaciens*, ESI was separately prepared by scooping a bit of 24 h-culture of the bacteria growing on NA into 10 ml sterile NB and 10 ml sterilized distilled water. Similarly, potato broth and water suspensions of the *Aspergillus* sp. and the *Penicillium* sp. were separately prepared by placing a mycelia bit of a 14-day-old PDA cultures of the fungi into 10 ml sterile potato broth and 10 ml water. 50 µl of each of the suspensions was placed and spread to a size of about 1 cm-diameter. Matured green cocoa pods (Hybrid: Amelonado x Amazon) were obtained from the cocoa field of KNUST and allowed to partially dry (four replicate pod per microorganism). Pods were then placed in a humidified chamber as before. They were biopsied for each microorganism by excising bits of cacao pod tissue from the centre of the inoculation point, one, two and three centimetres from the centre after 24 h. After 7 days they were placed on fresh NA and PDA plates to determine the presence or absence of the bacterium and the two fungi at those locations. Control pods treated with only nutrient broth and sterilized distilled water and potato dextrose broth for the bacteria and the fungi respectively were included in the study.

Field experiments

Experimental location, period and design

The experiment was conducted at the cocoa plantation of the University of Education, Winneba, Mampong campus. Black pod incidence in the field was generally low to moderate since the field is a University demonstration farm. The area lies in the Forest Savanna Transition zones of Ghana. It lies between latitude 7° 4' 0" North, 1° 24' 0" West (Meteorological Service Department, 2012). The area experiences a bimodal rainfall regime. The major rainy season begins from mid-March and ends in July. There is a short spell of rainfall in August. The minor season begins in September and ends in mid-March. The mean monthly rainfall of the area is about 91.2 mm and the mean daily temperature is about 30.5°C (Meteorological Service Department, 2012). The soil is classified by FAO/UNESCO legend as Chromic Luvisol and obtained from the voltaian sandstone of the Afram Plains (Asiamah, 1998). It belongs to the Savanna Orchrosol class and the Bediesi series which is well drained, friable and permeable (Asiamah, 1998). The soil is characterized by a moderate amount of organic matter and good water holding capacity. The planting distance for the cocoa in the plantation was 3m x 3m and the age of the trees was about 14 years.

The experiment was carried out in both minor (3rd October to 17th November, 2013) and major seasons (14th May to 30th June, 2014). For both seasons, the experiments ran for six weeks. Six rainfall periods, giving an average daily rainfall of 90 mm of rain, were recorded during the data collection period in the major season. In the minor season, however, no rainfall was recorded. The field study was established as a Randomized Complete Block Design (RCBD) with three blocks ((three plants per replicate (block); five pods per plant)). Blocks were 10 m apart. An average of 15 pods per treatment was used. A total of 45 pods were, thus, treated with each microbial suspension and their mixture. In all there were four treatments (180 pods) and two controls (90 pods).

Preparation of microbial suspension and field application

Microbial suspensions were prepared by growing the bacterial antagonist ESI, and the two fungi (*Aspergillus* sp. and the *Penicillium* sp.) on half strength nutrient broth and potato broth, respectively for 14 days. For each organism, four, 250 ml Erlenmeyer flasks each containing 100 ml of either nutrient broth (for the bacterium, ESI) or potato broth (for the fungal antagonists) were separately seeded with 10 µl of the microbial suspensions. A total of twelve flasks were established. After 14 days, the contents of the four flasks (each containing 100 ml of each microorganism) were pooled together to make up 400 ml and 10% (2.8 g of starch powder boiled in 100 ml SDW) added as a sticker. Healthy cocoa pods in the field with no sign of infection were individually sprayed, using a hand-held aerosol sprayer, with broth suspensions of ESI, *Aspergillus* sp. and *Penicillium sp.* and also with a mixture of the three microorganisms. The mixture was obtained by pooling together 100 ml each of the three-microbial suspension. Ridomil 72 plus (12% metalaxyl + 60% cuprous oxide) suspension used according to the manufacturer's instruction (1.3 g of powder in 400 ml of water) was similarly applied to pods as a positive control (standard reference product). In the minor season, Kocide 101 (77% cupric hydroxide) was used as a standard reference product

Table 1. *In vitro* inhibition of *P. palmivora* and *P. megakarya* by yam rhizobacterial isolates.

Rhizobacterial	Length (mm) of inhibition zone[2]		Percentage inhibition (%)[3]	
isolates[1]	*P. palmivora*	*P. megakarya*	*P. palmivora*	*P. megakarya*
ESI	21. 21	16. 00	84.84	64.00
M78	18. 95	11.00	75.80	44.00
K4	16.39	13.50	65.56	54.00
E7B8	15.77	13.62	63.08	54.48
E7B1	15.56	13.75	62.24	55.00
M7	14.79	8.75	59.16	35.00
M8	14.67	11.75	58.68	47.00
M32	14.65	9.00	58.60	36.00
Control	0.00	0.43	0.00	1.72
LSD (5%)	0.76	1.53		
CV(%)	3.00	9.70		

[1]Control = *P. palmivora* or *P. megakarya* alone. [2]Data was taken after seven days of incubation. Values are mean lengths of inhibition zone from three replicate plates (four inhibition zones/plates). [3]Percentage of inhibition was calculated from values of mean lengths of inhibition zones.

since infections were least during the period. Pods sprayed with a zoospore suspension of *P. palmivora* were also maintained as the negative control. Thus, the treatments consisted of pods protected with i) *B. amyloliquefaciens* (referred to as ESI) broth suspension, ii) *Aspergillus* sp. broth suspension, iii) *Penicillium* sp. broth suspension, iv) the mixture of broth suspensions of ESI, *Aspergillus* sp. and *Penicillium* sp., v) Ridomil 72 plus and vi) pods sprayed with a zoospore suspension of *P. palmivora.* The pods treated were left for 24 h to dry and challenged with a zoospore suspension (1×10^6 zoospore/ml) of *P. palmivora*. A zoospore suspension was applied, using a hand-held aerosol sprayer until runoff. Thus, in addition to natural field inoculum, an artificial inoculum was applied. Transparent polyethylene bags were tied around the pods to provide humid conditions for the *P. palmivora* to grow. They were removed after 48 h. Data collected for the six-week-period were, i) number of pods with black pod lesions, ii) number of pods without lesions (derived from (i)), iii) number of days to lesion development and size of the lesion. Percentage disease control for each treatment was calculated by using the formula (Koranteng and Awuah, 2011):

$$\text{Percentage disease control (\%)} = \left\{\frac{\text{Number of pods without lesion}}{\text{Total number of pods treated}}\right\} \times 100$$

For the recovery of microbial antagonists from pod surfaces, three additional pods per treatment were sprayed with microbial broth suspensions and biopsied (Koranteng and Awuah, 2011) after 24 h of protection onto NA (for ESI) or Chloramphenicol amended PDA (CPDA) (for *Aspergillus* sp. and *Penicillium* sp.). The CPDA was prepared by incorporating 100 mg of Chloramphenicol powder into 100 ml PDA before autoclaving. To recover ESI, pieces of pod tissue without surface sterilization were plated directly on ½ strength NA and incubated on laboratory bench at room temperature (28 ± 2°C) for 72 h. For fungal antagonists (*Aspergillus* sp. and *Penicillium* sp.), pieces of pod tissue were excised from pod surfaces, surface sterilized with 10% commercial bleach for two minutes and plated on CPDA and incubated as before. The *Aspergillus* and the *Penicillium* spp. were incubated as before for seven days and compared morphologically and microscopically with the existing cultures. Pods which did not develop lesion of the black pod from the protected treatments were similarly biopsied for the antagonists after six weeks. Pods that developed lesions were also biopsied for *P. palmivora* by excising tissues (1 cm from the edge of lesions), surface sterilizing them and plating on GCMA and observing after seven days.

Statistical analysis

Data were transformed where necessary before analysis. Data on percentage disease control were arcsine transformed. Analysis of variance (ANOVA) was performed on the data, using GenStat statistical package (2008). When ANOVA indicated a significant ($P\leq0.05$), the treatment effects were further separated using Least Significant Difference (LSD) test.

RESULTS

Antagonistic activity of biocontrol agents against *P. palmivora* and *P. megakarya*

All eight rhizobacterial isolates screened showed some level of activity towards both *P. palmivora* and *P. megakarya* after 12 years of refrigerated storage (Table 1). The average inhibition zone sizes associated with the rhizobacteria ranged from 14.65 to 21.21 mm and 8.75 to 16.00 mm, respectively, for *P. palmivora* and *P. megarkaya* (Table 1). Of the rhizobacteria, isolate ESI was most effective, giving inhibition zone sizes of 21.21 mm (*P. palmivora*) and 16.00 mm (*P. megakarya*) (Table 1; Figure 1). Isolates ESI, M78, K4, E7B8 and E7B1 continued to be the most effective, producing inhibition zone sizes significantly different ($P<0.05$) from each other. The least anti- Phytophthoral activity was exhibited by M7, M8 and M32 for both *P. palmivora* and *P. megakarya* (Table 1).

When the rhizobacteria were re-tested against *P. palmivora* in comparison with the two fungi *viz*., the *Aspergillus* and *Penicillium* spp., all eight rhizobacteria

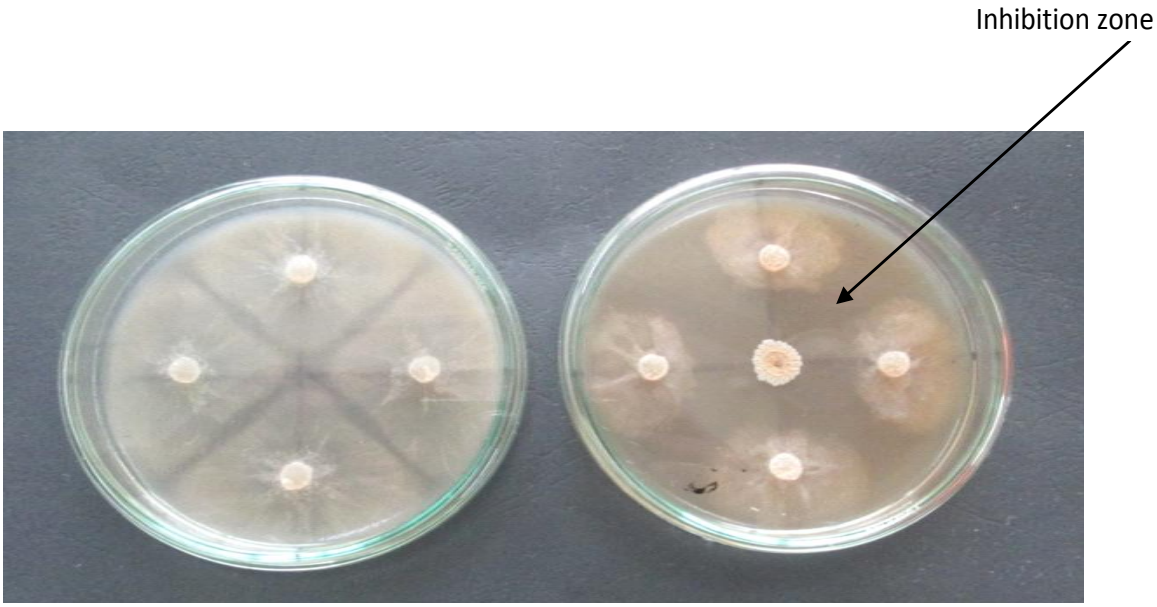

Figure 1. Inhibition of *P. palmivora* on an agar plate by the centrally placed rhizobacterium, ESI (right plate). Control plate without ESI is on the left.

Table 2. Antagonistic activity of the rhizobacteria, *Aspergillus* sp. and *Penicillium* sp. against *P. palmivora*.

Antagonists	Inhibition zone (mm)[1]	Percentage of inhibition (%)[2]
Aspergillus sp.	22.41	89.64
ESI	19.00	76.00
M32	17.00	68.00
Penicillium sp.	16.81	67.24
M7	16.75	67.00
E7B8	16.12	64.48
M8	16.00	64.00
K4	15.62	62.48
M78	15.62	62.48
Control	0.00	0.00
LSD (5%)	1.50	
CV (%)	6.70	

Control is *P. palmivora* alone, [1]Values are mean lengths of inhibition zones from four replicate plates, [2]Percentage of inhibition was calculated from values of mean lengths of inhibition zones

together with the two fungi were antagonistic (Table 2). The *Aspergillus* sp. which was being tested for the first time, proved to be the most efficacious of the ten antagonists with a significantly highest ($P< 0.05$) inhibition zone width of 22.41 mm, representing 89.64% inhibition (Table 2 and Figure 2). The *Penicillium* sp. which was also being tested for the first time, also exhibited 67.24% inhibition of *P. palmivora* (Table 2; Figure 3). Both rhizobacteria isolate M78 and K4 had the least inhibition zone width of 15.62 mm each representing 62.48% respectively.

Black pod lesion suppression on detached cocoa pods with bacterial and fungal antagonists

When inoculation sites on detached cocoa pods were treated with suspensions of the rhizobacterium, ESI, the *Aspergillus* sp., the *Penicillium* sp., as well as a mixture of the three microorganisms, and inoculated with a zoospores of *P. palmivora*, black pod lesions were completely suppressed (Table 3 and Figure 4). Similar results were obtained when Ridomil plus, the fungicide traditionally used in controlling of black pod disease was

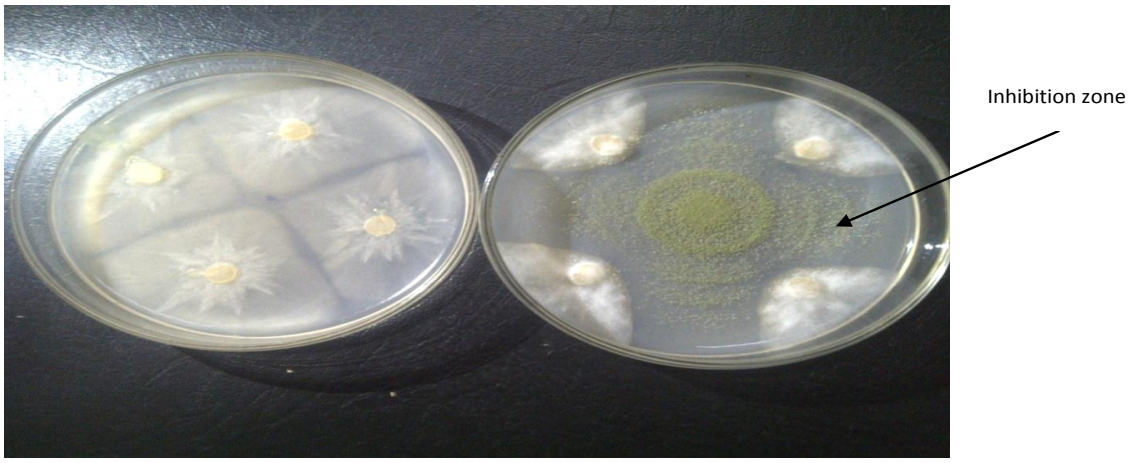

Figure 2. Inhibition of *P. palmivora* on an agar plate by a centrally placed *Aspergillus* sp. (right plate). Control Plate without *Aspergillus* is on the left.

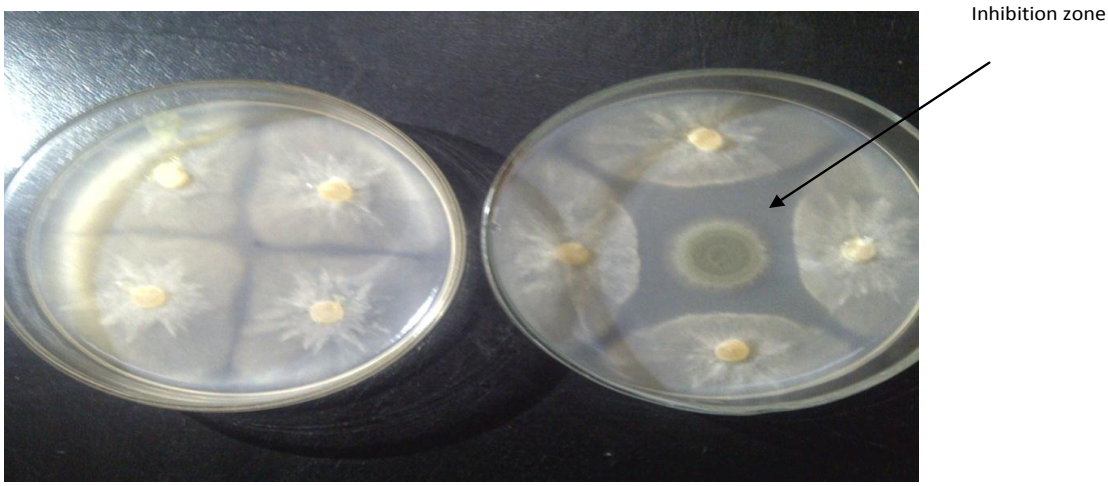

Figure 3. Inhibition of *P. palmivora* on an agar plate by a centrally placed *Penicillium* sp. (right plate).Control plate of *P. palmivora* without the *Penicillium* sp. is on the left.

Table 3. Percentage infection of black pod lesion development on detached cacao pods protected with microbial antagonists and challenged with a zoospore suspension of *P. palmivora* after five days of incubation.

Treatment[1]	Pods with lesion/Total pods treated	Percentage infection (%)
ESI + Pp	0/4	0
Asp + Pp	0/4	0
Pen + Pp	0/4	0
Mixture of ESI, Asp & Pen + Pp	0/4	0
Ridomil Plus + Pp	0/4	0
SDW + Pp (control)	4/4	100

[1]ESI +Pp = pods treated with ESI suspension and challenged with a zoospore suspension of *P. palmivora*. Asp+Pp = pods treated with *Aspergillus* sp. and challenged with a zoospore suspension of *P. palmivora*. Pen+ PP = pods treated with *Penicillium* sp. and challenged with a zoospore suspension of *P. palmivora*. Mixture + Pp = pods treated with the mixture of ESI, *Aspergillus* and *Penicillium* spp. and challenged with a zoospore suspension of *P. palmivora*. Ridomil plus+Pp = pods treated with Ridomil plus and challenged with a zoospore suspension of *P. palmivora*. SDW+Pp = pods treated with sterilized distilled water and challenged with a zoospore suspension of *P. palmivora.*

Figure 4. Detached cacao pods treated with the microbial antagonists and challenged, after 24 h with *P. palmivora*. Pods were treated wth the following; (A) ESI bacterial suspension; (B) *Aspergillus* suspension (ASP); (C) *Penicillium* suspension (PEN); (D) Mixture of ESI, ASP and PEN; (E) Ridomil plus (positive control); (F) Sterilized distilled water (SDW) (negative control). The Picture was taken five days after inoculation. Reddish-brown spot on the Ridomil-treated pod is the residue colour of the fungicide.

Table 4. Percentage infection of the black pod on detached cacao pods previously protected with microbial antagonists and without lesion development re-challenged with *P. palmivora*.

Treatment	Pods with lesion/total pods treated	Percentage of infection (%)
ESI+ Pp	0/4	0
Asp + Pp	0/4	0
Pen + Pp	0/4	0
Mixture of ESI, Asp & Pen + Pp	0/4	0
Ridomil Plus + Pp	0/4	0
SDW + Pp (control)	4/4	100

ESI +Pp = pods treated with ESI suspension and challenged with a zoospore suspension of *P. palmivora* after 5 days. Asp + Pp = pods treated with *Aspergillus* sp. and challenged with a zoospore suspension of *P. palmivora* after 5 days. Pen + PP = pods treated with *Penicillium* sp. and challenged with a zoospore suspension of *P. palmivora* after 5 days. Mixture + Pp = pods treated with the mixture of ESI, *Aspergillus* and *Penicillium* spp. and challenged with a zoospore suspension of *P. palmivora* after 5 days. Ridomil plus + Pp = pods treated with Ridomil plus and challenged with a zoospore suspension of *P. palmivora* after 5 days. SDW + Pp = pods treated with sterilized distilled water and challenged with a zoospore suspension of *P. palmivora* after 5 days.

used. However, with unprotected controls of pods, black pod lesions were apparent on the pods' surfaces.

Persistence and spread of microbial protectants on cacao pods

After five days of application of the microbial antagonists to pods, the antagonists continued to persist on pods and inhibited black pod lesions when the previous inoculation sites, which did not show any lesion development, were re-challenged with *P. palmivora* (Table 4 and Figure 5). When pod tissue segments were excised from the centre, one, two and three centimetres away from protected pod surfaces and biopsied on NA (ESI) and CPDA (*Aspergillus* sp. and *Penicillium* sp.), ESI was found to be restricted within one centimetre of point of the application after five days; while the *Aspergillus* sp. spread beyond three centimetres away from the centre. The *Penicillium* sp. moved slightly beyond one centimetre from the center of the application on the pod surface (Table 5). The ripening of the pods in this figure (Figure 5) is as a result of the same pods used in Figure 4 and the complete rotting of the control pod on the extreme right is also as a result of

Figure 5. Cacao pods previously protected with microbial antagonists in Figure 4 and without lesion development re-challenged with *P. palmivora*. Pods were treated with the following; (A) ESI bacterial suspension; (B) *Aspergillus* suspension; (C) *Penicillium* suspension; (D) Mixture of ESI, *Aspergillus* and *Penicillium* suspensions; (E) Ridomil plus (positive control); F) Sterilized distilled water (SWD). The Picture was taken eight days after inoculation. Reddish-blown spot on Ridomil plus-treated pod is the residual colour of Ridomil plus.

Table 5. Distance of detection of ESI, *Aspergillus* sp. and *Penicillium* sp. on detached cacao pods surfaces five days after application.

Treatment	Bioassay of tissues from protected pods onto fresh NA and PDA media			
	Distance of detection from the central point of the application on cacao pods (cm)			
	Centre	1	2	3
H_2O susp of ESI	+	-	-	-
NB susp of ESI	+	-	-	-
H_2O susp of *Aspergilus* sp.	+	+	+	+
PDB susp of *Aspergillus* sp.	+	+	+	+
H_20 susp of *Penicillium* sp.	+	+	-	-
PDB susp of *Penicillium* sp.	+	+	-	-

+ = presence of ESI, *Aspergillus* or *Penicillium* spp, - = absence of ESI, *Aspergillus* or *Penicillium* spp.

initial infection obtained in Figure 4.

Field control of black pod disease of cocoa with microbial antagonists

Field application of broth suspensions of the microbial antagonists (*Bacillus amyloliquefaciens*, ESI, *Aspergillus* sp. and *Penicillium* sp.) and their mixtures (*B. amyloliquefaciens,* ESI + *Aspergillus* sp. + *Penicillium* sp.) on cocoa pods in the minor season resulted in the three antagonists showing some level of protection against black pod disease. Together, the three microbial antagonists gave between 53-60% protection. A mixture of the three microbial antagonists, however, gave 67% protection of pods. These were significantly different ($P< 0.05$) from those associated with pods treated with Ridomil plus (positive control) and pods without treatment (*P. palmivora* as negative control) (Table 6).

In the major season, the three microbial antagonists, as well as their mixture similarly protected the pods against black pod disease (40-67% protection). The values obtained were significantly different ($P< 0.05$) from the unprotected control treatment (Table 7). Lesions, when

Table 6. Percentage disease control of broth cultures of the three microbial antagonists on lesion development by *P. palmivora* on field pods (Minor season)[1].

Treatment[2]	Number of pods without lesions/Number treated	Percent disease control (%)[3]
ESI + *P. palmivora*	8/15	53.33 (0.82)
Aspergillus sp. + *P. palmivora*	9/15	60.00 (0.89)
Penicillium sp. + *P. palmivora*	9/15	60.00 (0.89)
Mixture +*P. palmivora*	10/15	66.67 (0.97)
Kocide 101+ *P. palmivora* (positive control)	6/15	40.00 (0.68)
P. palmivora (negative control)	5/15	33.33 (0.62)
LSD (5%)		0.11
CV (%)		7.40

[1]Values are total of pods from three replicates blocks; five pods per block, [2]Mixture consists of ESI, *Aspergillus* and *Penicillium* spp. [3]Values in parenthesis are arcsine transformed values.

Table 7. Percentage disease control of broth cultures of the three microbial antagonists on lesion development by *P. palmivora* on field pods (Major season)[1].

Treatment[2]	Number of pod without lesions/Number treated	Days to onset of lesion	Lesion size development rate (mm/day)[3]	Percent disease control (%)[4]
ESI + *P. palmivora*	6/15	8.00	15.80	40.00 (0.68)
Aspergillus sp. + *P. palmivora*	8/15	8.33	15.20	53.33 (0.82)
Penicillium sp. + *P. palmivora*	10/15	7.00	14.60	66.67 (0.97)
Mixture + *P. palmivora*	9/15	8.00	11.00	60.00 (0.89)
Ridomil Plus + *P. palmivora* (+ control)	14/15	6.33	7.30	93.33 (1.42)
P. palmivora (- control)	1/15	3.67	17.10	6.67 (0.16)
LSD (5%)		1.98	1.74	0.62
CV (%)		15.8	7.10	40.5

[1]Values are total of pods from three replicates blocks; five pods per block.[2]Mixture consists of the three microbial antagonists (ESI, *Aspergillus* and *Penicillium* spp.)[3] Over seven day period. [4]Values in parenthesis are arcsine transformed values.

they occurred, developed much slower on pods where the microbial antagonists and the fungicide Ridomil plus were used. Lesion development rates of 11.00-15.80 mm/day obtained for the microbial antagonists were significantly lower ($P<0.05$) than 17.00 mm/day measured for the unprotected control treatment (Table 7). The lowest lesion development was obtained with the Ridomil plus treatment. It took between seven to eight days for the pods to become infected using the microbial antagonists and their mixture but as early as 3.67 days when no protectant was used (Table 7). On pods that received Ridomil plus, lesions appeared at day six which was significantly ($P < 0.05$) comparable to the values associated with the microbial antagonists.

Recovery of microbial antagonists from field pod surfaces

The three microbial antagonists were recovered from pod surfaces 24 h after their application (Table 8). However, they could not be recovered from the surfaces of the pod after six weeks. It was also observed that there were other microorganisms such as *Collectorichum* sp. and some unidentified bacterial species present on pod surfaces. *P. palmivora* was also re-isolated from lesions.

DISCUSSION

The eight rhizobacterial isolates used in this study were among the isolates originally obtained from the rhizosphere of yam (*Dioscorea* sp.) at different locations in the Ashanti region of Ghana. They were screened against 22 fungi from four Phyla, including *P. palmivora*, the black pod pathogen of cocoa (Akrasi, 2005; Awuah and Akrasi, 2012). Since the bacteria had been initially isolated and kept under refrigerated storage (5°C; dark incubation) with occasional sub-culturing as far back as 2001 (Akrasi, 2005), there was the need to periodically find out whether they were still viable. In 2008, the bacteria were re-tested and confirmed to be viable (Koranteng and Awuah, 2011).

Table 8. Recovery (Presence or absence) of microbial antagonists and the pathogen from field pods.

Microorganism[1]	Presence or absence of microorganisms on field pods[2]	
	24 h	After 6 week
ESI	+	-
Aspergillus sp.	+	-
Penicillium sp.	+	-
Mixture	+	-
Others (*Colletotrichum* sp., bacteria)	+	+
P. palmivora	NA	+

[1]Mixture of the three microbial antagonists; *P. palmivora* was re-isolated after 7 days, [2] + = presence; - = absence; NA= not applicable.

The inhibition of *P. palmivora* and *P. megakarya* by the bacteria, in the current study, suggests that they are stable and still effective against the black pod pathogens which emphasize their potential for biological control.

The result obtained from the laboratory experiment or *in vitro* study revealed that the rhizobacterium, *B. amyloliquefaciens*, isolate ESI, selected from the eight for further studies together with *Aspergillus* sp. and *Penicillium* sp. showed strong inhibitory effects on both pathogens of black pod *in vitro*. Broth cultures of the bacterium and the fungi, applied as spots on detached cocoa pods and challenged with a zoospore suspension of *P. palmivora,* inhibited black pod lesion development on the pods. The bacterium could not, however, spread on pod surfaces beyond the point of application (1 cm) after seven days of incubation. The bacterium, however, persisted at the points of application on pods surfaces due to its endurance to adverse environmental conditions. The two fungi spread on pod surfaces after the spot application to about three centimetres from the point of the application after seven days, even though the *Aspergillus* sp. was faster in its spread than the *Penicillium* sp. This development is expected since bacteria require a rich medium for growth, establishment and spread (Agrios, 2005). The surface of the cocoa pod does not offer such suitable conditions (Sutikno, 1997). Fungi, nevertheless, can grow and establish in poor media, provided there is a minimal requirement for carbon (Agrios, 2005). This might be the case with the exudates secreted by the pods which enabled the fungi to spread on the pod surfaces. The ability of an antagonist to proliferate within a short time duration of favourable environmental conditions before it controls the pathogen is desirable as this will enhance biocontrol efficacy (Robert, 1990; Campbell, 1998; Janisiewicz, 1998). Bhavani and Abraham (2005) evaluated seven selected epiphytic fungi and five bacteria from cocoa pods for the control of Phytophthora pod rot on detached cacao pods. *Penicillium digitatum* and *Aspergillus repens* have been used against *P. palmivora* as protectants on detached cacao pods (Adebola and Amadi, 2012).

In many cases of good performance of a biocontrol agent *in vitro*, such an agent failed when introduced to the field. This might be due to the dynamic nature of environmental conditions in the field which makes it difficult for biocontrol agents to adapt. Thus, the three microbial antagonists were evaluated in the field. The field trial therefore showed that when the broth suspensions of the bacterium and the fungi were used as “one-off” application on cocoa pods and challenged with a zoospore suspension of *P. palmivora* in the field in both minor and major seasons, black pod disease suppression was achieved to some extent with respect to percentage disease control, lesion size and days before the start of the infection. Successful biocontrol against black pod disease of cocoa in the field has also been achieved with *Trichoderma* sp. (Darmano, 1994; Adedeji et al., 2005), *Aspergillus* sp. and *Penicillium* sp. (Adebola and Amadi, 2011, 2012). However, fewer *Bacillus* spp. have been reported as antagonists to cocoa. *B. cereus* and *B. subtilis* (Odigie and Ikotun, 1982), *Bacillus* spp. (Adejumo, 2005), and *Bacillus pumillus* (Melnick et al., 2008) are a few examples of *Bacillus* spp. reported to have been used as antagonists to control black pod disease.

This study used “one-off” application of the microbial antagonists on field pods which were then challenged with a zoospore suspension of *P. palmivora*. Thus, protection obtained with the microbial antagonists resulted from this initial “one-off” application. Since the plot used is a demonstration farm, there were generally low black pod incidence. It is obvious that if the microbial antagonists were washed-off, pods surfaces, as could not have been solely the case since there was no rains during the minor season, re-infection of the pods would be low. This experiment, therefore, needs to be repeated in hot spot areas with repeated application of the microbial antagonists.

Adedeji et al. (2010) used different cocoa farms as blocks in their study with *Trichoderma* sp. in the field control of black pod disease of cocoa. In such a situation, the inoculum levels could differ among the plots in the blocks since the blocks (farms) were far apart from each other and the protected pods were likely to have variable disease pressures. The method adopted in the current

study for field screening of the antagonists against black pod disease also had its lapse in the sense that the field used had a low disease pressure and the pods had to be artificially inoculated with *P. palmivora* a zoospores in "one-off" manner to augment the disease pressure. In the proposed repeat study, it is suggested that three to five trees close to each other in a black pod endemic farm be selected to represent the blocks and five pods on each tree selected to represent the plots to receive the various microbial antagonists and other treatments. This arrangement will have an advantage over the experiment of Adedeji et al. (2010) since the variation of inoculum level per blocks will be minimized because the pods would be exposed to natural infection and variability in disease pressures on the pods would be low. Repeated applications of the microbial antagonists to pods rather than the "one-off" application would be used as done in fungicide trials.

Even though the microbial suspensions generally offered some protection individually, their mixture offered better protection in both seasons. This result is important because the three microbial antagonists complemented each other. According to Guetsky et al. (2002), the application of a mixture of biocontrol agents enhances control efficacy by reducing inconsistency and variability among the individual biocontrol agents. Other authors have also corroborated this assertion, indicating that the use of more than one biocontrol agent that operates by different mechanisms to control one or more pathogens may be a way to reduce the variability among biocontrol agents (Raupach and Kloepper, 1998; Whipps, 2001; Jetiyanon and Kloepper, 2002). According to these authors, soil suppressiveness of plant pathogens occurring in crop fields may be due to naturally existing mixtures of microbial antagonists rather than high populations of a single antagonist. Therefore, the application of a mixture of biocontrol agents would be more similar to the natural situation in the field and permit a broader spectrum of biocontrol activity with improved efficacy and reliability of control. These results clearly show that these antagonists have the potential to be developed as biocontrol agents for the management of black pod disease of cocoa.

Conclusion

The cocoa industry worldwide is bedeviled with pathogenic fungi mainly; *P. palmivora* and *P. megakarya* which cause major crop losses. Besides the environmental effects of the heavy application of copper-based fungicides, this also leads to the emergence of pathogen resistance. Recently, consumers demand for pesticides free cocoa beans has also increased and as a result of their willingness to pay a high premium for such organic products (EC, 2006; EFSA, 2009, 2012). Therefore, it has become imperative the use of alternative control measure that will reduce the dependency of such agrochemicals. The study demonstrated that all eight rhizobacteria used showed the potential of inhibiting *P. palmivora* and *P. megakarya in vitro* and in the field with *B. amyloliquefaciens (ESI),* as well as the two fungi, *Aspergillus* and *Penicillium* spp. as most potent. Again, the rhizobacteria and the two fungi, as well as their mixtures, protected detached cocoa pods from infection by a zoospore suspension of *P. palmivora*. The field study also showed that intact cocoa pods protected with broth suspension of antagonists and their mixtures and challenged with an inoculum of *P. palmivora* were generally adequately protected from black pod infections in both minor and major seasons. The current study has clearly demonstrated that these antagonists have the potential to be developed as biocontrol agents for the management of black pod disease of cocoa.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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