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Full Length Research Paper

Biological suppression of black pod lesion development on detached cocoa pods

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The study sought to suppress black pod lesion development on detached cocoa pods using yam rhizobacteria. Eight yam rhizobacterial isolates were initially screened on agar plates with the zone of inhibition technique against *Phytophthora palmivora,* causal agent of black pod disease of cocoa. All eight bacteria were antagonistic to *P. palmivora,* so one of the most promising isolates (isolate ESI) was further evaluated for black pod lesion suppression on detached cocoa pods. Areas on cocoa pods were treated with 50 l Nutrient Broth (NB) cultures of ESI. The treated pod areas (0.8 cm^2 approximately), on partial drying (10 min after applying the ESI culture broth), were inoculated with 10 l zoospore suspensions of *P. palmivora*. Kocide 101, NB and cell- free culture filtrate of ESI were similarly used as protectants. Stability of the ESI-NB culture on cocoa pods during a 72 h period was also assessed. Black pod lesions developed on all pods inoculated with *P. palmivora* without prior treatment with ESI, its filtrate and Kocide 101. No lesions developed when the ESI-NB culture, ESI cell- free culture filtrate and Kocide 101 were used as protectants. Generally, the ESI-NB culture was stable as a protectant during the 72 h test period. The rhizobacterium was frequently recovered from inoculation points on pods treated with its NB culture, suggesting persistence of the bacterium on detached cocoa pods. These results show that the ESI-NB culture and its filtrate can be exploited as biofungicides for use against black pod disease of cocoa.

Key words: *Phytophthora palmivora*, yam rhizobacterium, black pod disease of cocoa, biofungicide.

INTRODUCTION

Black pod disease is a major constraint to cocoa production in Ghana (Lass, 1985). In Ghana, the disease is known to be caused by *Phytophthora palmivora*, which occurs in all cocoa growing areas (Dakwa, 1974; Anon, 1995; Erwin and Ribiero, 1996) and *P. megakarya* Brassier and Griffin, which is known to be present in Brong Ahafo and Volta regions and certain parts of the Ashanti and Western regions (Anon, 1995; Dakwa, 1987; Akrofi, 2000). Yield losses due to *P. palmivora* in Ghana are estimated to be between 4.9-19% (Dakwa, 1984).

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The more virulent *P. megakarya* causes yield losses in Ghana ranging between 60-100% (Dakwa, 1987). Global yield loss due to the black pod disease is about 44% (Van der Vossen, 1999). An economic loss of £1540 million due to the disease had been reported (Evan and Prior, 1987).

Fungicides such as Kocide 101 (77 WP; 50% copper hydroxide) and Ridomil 72 plus (12% metalaxyl and 60% cuprous oxide) have been used effectively to control black pod disease in Ghana (Akrofi and Appiah, 1995). Cultural methods such as farm sanitation, shade reduction through pruning, removal and burying or burning of diseased pods and early harvesting are also effective control measures (Wood and Lass, 1985). However, intense chemical use in agriculture is

Table 1. *In vitro* inhibition of *Phytophthora palmivora* by some yam rhizobacteria.

Bacterial isolate	Length (mm) of inhibition zone [1]
ESI	21.50
E7B8	21.50
E71B	20.75
M-7	19.50
M-8	19.50
M-32	18.75
K-4	18.00
M-78	11.50
Control (Pp alone)	0.00
C V (%)	9.70
LSD (0.05)	2.21

[1]Values are mean lengths of inhibition zones from four replicate plates (four inhibition zones/plate); Pp=*P. palmivora.*

problematic (Hendelson et al., 1994; Pereira, 1985; Fulton, 1989) and cultural methods are often cumbersome. Other safer and user-friendlier methods for black pod disease management such as biological control could therefore be exploited to complement the use of chemical and cultural approaches.

Okasaibor (1968) reported *in vitro* inhibition of *P. palmivora* by *Botryodiplodia* (*Lasiodiplodia*) *theobromae* on agar plates. Similar studies by Odamtten and Clerk (1984) used *Aspergillus niger* and *Trichoderma viride* to inhibit *P. palmivora* on culture media. Gallindo et al. (1992) reported that isolates of the epiphytic bacterium, *Pseudomonas flourescens* was antagonistic towards *P. palmivora in vitro*. Even though some reports of *in vivo* inhibition of *P. palmivora* on cocoa pods with fungi exist (Okasaibor, 1968; Darmano, 1994; Adedeji et al., 2006), similar reports with bacteria are often rare though Attafuah (1966) reported that a bacterium, *Pseudomonas aeruginosa* suppressed *P. palmivora* on cocoa pods. It is, therefore, necessary to determine the utility of these rhizobacteria for the biological control of black pod disease of cocoa. In this regard, Akrasi (2005) screened some yam rhizobacteria against certain plant pathogenic fungi, including *P. palmivora,* and found that eight of them possessed antifungal properties.

The objectives of this study were to re-test the eight yam rhizobacterial isolates reported by Akrasi (2005) as a means of verifying their antagonistic activity towards *P. palmivora* and use the most effective rhizobacterium as well as its cell-free culture filtrate to suppress black pod lesion development on detached cocoa pods.

MATERIAL AND METHODS

Source of *P. palmivora*

The *P. palmivora* isolate, CRIG-2, used was originally obtained from the Cocoa Research Institute of Ghana (CRIG), Tafo in the Eastern Region. It was maintained on Green Cocoa Mucilage Agar (GCMA) (Awuah and Frimpong, 2002) in a refrigerator until needed.

In vitro screening of rhizobacteria against *P. palmivora*

Eight yam rhizobacterial isolates (Table 1) previously shown to possess antifungal activity (Akrasi, 2005) were tested against *P. palmivora* for antagonistic activity using a modification of the zone of inhibition technique (Axelrood et al., 1998). The bacterial isolates were obtained from yam rhizosphere soils (Akrasi, 2005) and constitute part of a refrigerated microbial collection at the Plant Pathology Laboratory, KNUST. For each bacterium, a 24 h- old single colony growing on Nutrient Agar (NA) was suspended in 10 ml sterile distilled water in a 25 cc capped vial and shaken manually. Ten microlitres bacterial suspensions were spotted at the centres of plates containing a mixture of GCMA and NA (1:1). The bacterial spots were allowed to dry and plates incubated upside down at 28 ± 2ºC in the dark for 24 h. Mycelial plugs (7-mm-diameter) from a 1-week-old culture of *P. palmivora* (CRIG-2) were placed upside down at four equidistant positions (25 mm) from the central rhizobacterial colony and the plates incubated as indicated previously for 6 days. Four replicate plates per rhizobacterium were established. Plates with only *P. palmivora* served as controls. A clear zone of inhibition around the test rhizobacterium after 6 days suggested antagonism. The extent of inhibition was determined by measuring the widths of inhibition zones from the mid-point of the centrally growing rhizobacterial colony to the margin of the *P. palmivora* colony. The average width of inhibition zone per plate was then calculated. The data was analyzed by Analysis of Variance (ANOVA) and differences among means compared with the Least Significant Differences (LSD) test (P 0.05).

Test of efficacy of the rhizobacterium, ESI on detached cocoa pods

The rhizobacterial isolate, ESI was further evaluated on detached cocoa pods because of its high degree of antagonism, *in vitro*, against *P. palmivora*. Fourteen-day-old NB cultures of ESI were prepared by placing 10 l of a water suspension of ESI into each of four 25 ml capped vials containing 10 ml NB. The vials were incubated in the dark for 14 days and their contents pooled together and divided into two portions. One portion was centrifuged at 1000 rpm for 1 min. and the crude supernatant decanted and autoclaved (121°C, 20 min.) to obtain a sterile filtrate. Fifty microlitre aliquots of the ESI-NB culture were applied, with an Eppendof pipette, on the lateral sides of four washed, dry, mature, green cocoa (Hybrid: Amelonado x Amazon) pods (one application per pod) as protectants. The protectants were then spread with the tip of the pipette to a size of approximately 0.8 cm^2 and allowed to partially dry (10 min after applying the broth culture). The ESI cell-free culture filtrate was similarly used. The protected pod areas were inoculated with 10 l zoospore suspensions (10^6 zoospore/ ml) of CRIG-2. The zoospore suspension was prepared by flooding a 14-day-old plate culture of CRIG-2 with 10 ml of sterile, cold, distilled water, placing the flooded culture in a refrigerator for 45 min and incubating the culture at 28±2°C for 30 min. Inoculated pods were placed in a humidified transparent polyethylene chamber with wooden frames (1.5 × 0.6× 0.3 m) at room temperature for 7 days and the number of pods with black pod lesions noted. Pods similarly protected with 2% Kocide 101 suspension or with prior treatment with NB served as controls. Another set of pods treated with only the *P. palmivora* zoospore suspensions was maintained. The experiment was repeated once.

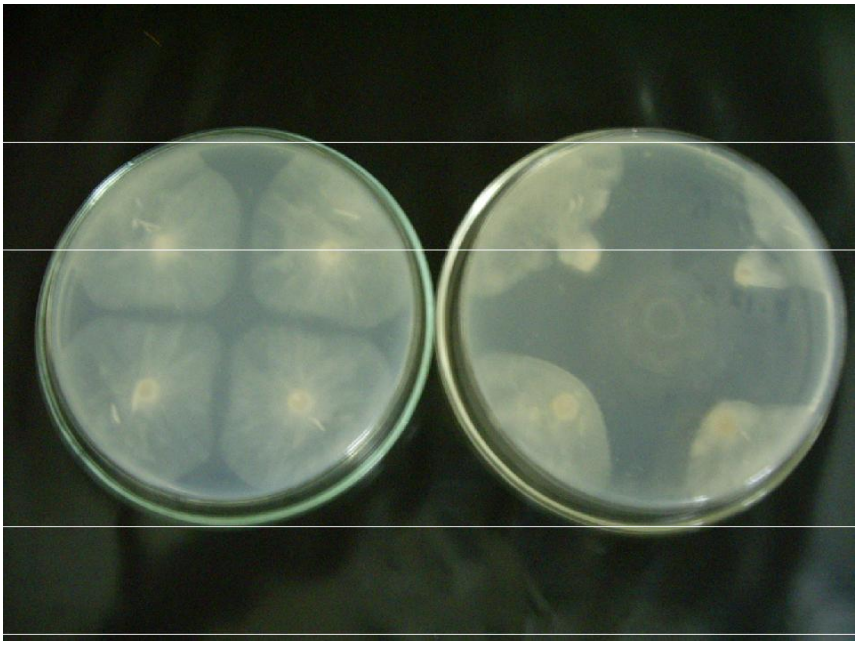

Figure 1. Inhibition of *P. palmivora* on an agar plate by the yam rhizobacterium with ESI (right plate), and without ESI (left plate).

Stability of the ESI broth culture on cocoa pods

ESI-NB culture was applied and spread on the lateral sides of three mature green cocoa pods of uniform sizes and kept in humidified transparent polyethylene incubation chamber as indicated previously. The treated pod areas were inoculated with 10 l zoospore suspensions of *P. palmivora* at 0, 24, 48 and 72 h intervals. Pods inoculated with only zoospore suspensions of *P. palmivora* without prior treatment with ESI-NB culture were maintained as controls. Inoculated pods were incubated as indicated previously and the number of pods with black pod lesions recorded 7 days after inoculation. The experiment was also repeated once.

Recovery of ESI and *P. palmivora* from infection courts

To recover *P. palmivora* from infection courts, tissue bits were excised from the infection courts (within 1 cm radius) on treated pods, surface sterilized with l0% commercial bleach for 2 min and plated on GCMA. Plates were incubated on a laboratory bench at room temperature for 4-6 days. To recover ESI, tissue bits without surface sterilization were plated directly on half strength NA and incubated as stated above for 72 h. For each organism, twenty-one tissue pieces were biopsied and the frequency of detection of *P. palmivora* and the ESI recorded.

RESULTS

All eight yam rhizobacterial isolates tested showed some level of activity towards *P. palmivora* with inhibition zone widths ranging between 11.50 to 21.50 mm (Table 1). The isolates ESI, E7B8 and E71B were the most effective and produced inhibition zone widths that were not significantly different ($P< 0.05$) from each other. The least anti- *Phytophthora* activity was exhibited by isolate M-78. Figure 1 (right plate) shows *P. palmivora* being inhibited on an agar plate by the rhizobacterium, ESI.

All four detached pods inoculated with *P. palmivora* developed typical lesions of black pod disease after 7 days (Table 2, Figure 2). Pods treated with only NB and inoculated with *P. palmivora* also showed typical black pod lesions. The rhizobacterium ESI did not cause any disease on cocoa pods.

Generally, pods receiving prior treatment with the ESI-NB culture, ESI cell-free culture filtrate and Kocide 101 before inoculation with *P. palmivora* did not have any black pod lesions (Table 2). The ESI broth culture maintained fungicidal activity 72 h after application to cocoa pod (Table 3).

Frequency of isolation of *P. palmivora* from inoculated points where only the fungus was used was 100%. The rhizobacterium was also recovered from all inoculation points on pods treated with its broth culture but did not cause disease on the pods. Similar results were obtained when the experiment was repeated.

DISCUSSION

Of the eight rhizobacterial isolates screened, three proved to be very effective against *P. palmivora*. These three bacteria were also among the most promising candidates screened by Akrasi (2005) against *A. niger, Curvularia lunata, Fusarium solani, Rhizopus stolonifer*

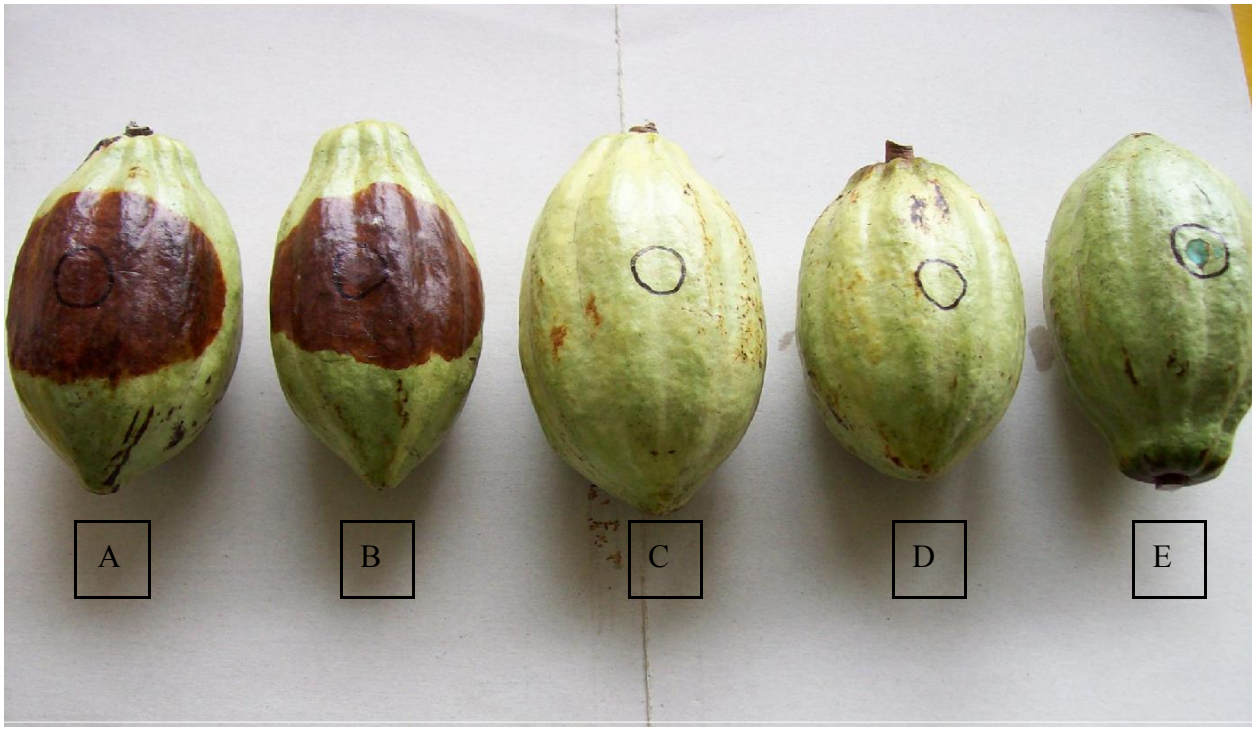

Figure 2. Effect of broth culture and cell- free culture filtrate of the rhizobacterium, ESI on black pod lesion development on detached cocoa pods. A, pod inoculated with *P. palmivora* zoospore suspension; B, pod inoculated with *P. palmivora* and treated with nutrient broth; C, pod inoculated with *P. palmivora* and treated with ESI broth; D, pod inoculated with *P. palmivora* and treated with ESI cell-free culture filtrate; E, pod inoculated with *P. palmivora* and treated with Kocide 101. The black circles show where the protectants were applied.

Table 2. Inhibition of black pod lesion development with broth culture and cell- free culture filtrate of the yam rhizobacterium, ESI.

Pod treatment	No. of pods with lesion / no. of pods treated [1]	
	Experiment 1	Experiment 2
P. palmivora alone	4/4	4/4
P. palmivora + ESI broth culture	0/4	0/4
P. palmivora + ESI culture filtrate	0/4	0/4
P. palmivora + Kocide 101	1/4	0/4
P. palmivora + nutrient broth	4/4	4/4

[1] Data was taken 7 days after inoculation with *P. palmivora*.

Table 3. Black pod incidence on cocoa pods previously treated with ESI broth culture [1].

Hours after pod treatment with ESI	No of pods with lesions/No of pods treated [2]	
	Experiment 1	Experiment 2
0	0/3 (3/3)	0/3 (3/3)
24	0/3 (2/3)	1/3 (3/3)
48	0/3 (3/3)	0/3 (3/3)
72	1/3 (3/3)	0/3 (3/3)

[1] Treated pod areas were inoculated with a zoospore suspension of *P. Palmivora* at 0, 24, 48 and 72 h intervals. For each treatment period, data was taken 7 days after inoculation. [2] Values in parentheses are for pods inoculated with *P. palmivora* without prior treatment with brothculture.

and an *Aspergillus* sp. The current study, therefore, validates Akrasi (2005) and further shows that the rhizobacteria from yam possess broad-spectrum antifungal properties. Thus, the three yam rhizobacteria viz. isolates ESI, E7B8 and E71B have potential for development into broad-spectrum biofungicides.

The nature of the *in vitro* inhibition of *P. palmivora* by the rhizobacteria tested suggests secretion of antifungal metabolite(s) and indicates that the cell-free culture filtrate of the producing bacteria might also be fungitoxic. This phenomenon was observed in the current study where the cell-free culture filtrate of ESI completely inhibited *P. palmivora* on detached cocoa pods. Effectiveness of cell-free culture filtrate against other fungi has been reported. For example Pfender et al. (1993) reported that cell-free culture filtrate of a rhizobacterium, *P. fluorescens* strain Pf-5 was inhibitory to *Pyrenophora tritici-repentis.* Studies by Siddique et al. (2003) with *P. aureginosa* strain IE6 and *Pochonia chlamidospora* also showed that both broth cultures and cell-free culture filtrates of the two microorganisms were inhibitory to the fungi tested. Similar findings were reported by Islam et al. (2005) with *Lysobacter* sp. against *Aplanomyces cochlioides*, Ongena et al. (2005) with *Bacillus subtilis* against *Pythium ultimum* and Ajit et al. (2006) with *P. fluorescens* against *Fusarium oxyporium* f. sp. *dianthi*.

Although Attafuah (1966) showed that *P. aeruginosa* was able to suppress *P. palmivora* on cocoa pods; he used a broth culture and not the cell-free culture filtrate of the bacterium. Our findings regarding the effectiveness of cell-free culture filtrate of the rhizobacterium, ESI against a fungal pathogen of cocoa is, therefore, a novelty.

Since the ESI broth culture, its cell-free culture filtrate and the fungicide, Kocide 101 were equally effective against *P. palmivora* on detached cocoa pods, it suggests the highly potent nature of the fungicidal principle secreted by ESI. Previously, Akrasi et al. (2006) compared cell-free culture filtrate of ESI with the fungicides Topsin M and Ridomil 72 plus and reported that they were similar in their fungicidal effects against *A. niger*. In several studies where a cell-free culture filtrate has been compared with a synthetic fungicide, the results have shown both the cell-free culture filtrate and the synthetic fungicide to be equally effective (Kupper et al., 2003; Slininger et al., 2003; Kishore et al., 2005).

The rhizobacterium ESI did not cause any disease on cocoa pods which is desirable since a potential antimicrobial agent should not only be shown to suppress the target pathogen but should also be found to have no negative effects on the plant.

Akrasi (2005) recovered the rhizobacterium ESI from infection courts of yam which had been treated with the NB culture of the bacterium. Persistence of ESI on cocoa pods is now demonstrated for the first time in Ghana, under laboratory conditions, with the current study. However, it is yet to be established whether or not ESI can persist, spread and produce its antifungal metabolite on cocoa pods under field conditions. When tested and it does, it would be a very positive step in the biological control of black pod disease of cocoa. This is because an effective biological agent needs to rapidly establish, persist and overcome the pathogen under field conditions

Previous work on the thermostability of ESI filtrate on yam tubers by Akrasi (2005) suggested that the fungicidal principle in the ESI filtrate would be stable on cocoa pods. This has been validated by the present study in which the broth culture of ESI exhibited fungicidal activity towards *P. palmivora* on detached cocoa pods during the 3-day test period. Two things, however, remain unknown. First we are not certain if the active fungicidal principle in the ESI-NB culture will maintain stability and be effective after 3 days. This needs to be studied. Also requiring a critical study is the application of the ESI –NB culture on intact cocoa pods in the field and inoculating such pods with *P. palmivora.* Results of such studies will help determine the utility of the ESI broth culture as a protectant of cocoa pods against *P. palmivora.*

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