Global Advanced Research Journal of Agricultural Science (ISSN: 2315-5094) Vol. 1(10) pp. 305-308, December, 2012
Available online http://garj.org/garjas/index.htm

Full Length Research Paper

Exploiting Microbial Diversity in Cocoa Ecosystems in Ghana to Control *Phytophthora* Pod Rot Disease

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Accepted 10 December, 2012

Black pod disease, caused by different *Phytophthora* species, is a major yield-limiting factor in cocoa production. Currently, there is no acceptable genetic resistance to *Phytophthora* in cocoa varieties and pesticides used are not always effective, bring serious risks to human health and environment, and lead to resistance in pathogen populations. Recent studies in Ghana and the Netherlands on biological control of *Phytophthora* diseases have led to the isolation of several antagonistic bacteria that significantly limit the growth and proliferation of different *Phytophthora* species. Among the antagonistic bacteria, biosurfactant-producing *Pseudomonas fluorescens* isolates gave promising results in eliminating zoospore populations and controlling late blight disease of tomato caused by *Phytophthora infestans*. The objective of this study was to further develop these findings into effective control measures to manage black pod disease of cacao. This paper reports on the distribution and diversity of *Pseudomonas* and *Phytophthora* species associated with cocoa in Ghana and also describes preliminary results on the biocontrol activities of selected *Pseudomonas* strains against *Phytophthora palmivora* and *Phytophthora megakarya*.

Keywords: Micobial diversity, Phytophthora, biocontrol, cocoa ecosystem

INTRODUCTION

Cocoa beans, the raw material for the chocolate industry, are the main agricultural product of most of the countries in West Africa. This subregion accounts for over 70% of the global cocoa production. Cocoa production in West Africa is hampered by a wide range of pests and diseases. Among the diseases, black pod caused by *Phytophthora palmivora* and *Phytophthora megakarya* causes more production losses globally than any other cocoa disease (Guest, 2007). *P. palmivora* and *P. megakarya* can affect every part of the cocoa plant and every stage of the plant's development. The infection process is initiated by motile zoospores, asexual spores released by *Phytophthora* under moist conditions. Zoospores are encased solely by a plasma membrane and produced in a vesicle or a sporangium, and when released they are responsible for the spread of the pathogen.

In Ghana, total loss of pods due to *Phytophthora* infection may occur when no control measures are taken (Opoku et al, 2000). Current disease management practices to control *Phytophthora* have been unsatisfactory. Cultural practices such as farm sanitation, weeding and removal of infected pods have proven

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relatively efficient in reducing the infection, but is arduous not often practised by farmers (Ndoumbè-Nkeng et al., 2004; Opoku et al., 2000). Chemical control with protectant sprays of copper-based fungicides, together with the systemic fungicide metalaxyl at 3- or 4-weekly intervals are rarely cost-effective for the majority of cocoa farmers (Opoku et al., 2007). Furthermore, frequent applications of high doses of pesticides bring serious risks to human health, lead to resistance development in pathogen populations, and have adverse effects on the environment. Moreover, in West-Africa no cocoa varieties genetically resistant to *Phytophthora* spp. have been found.

Biological control has been proposed as a potential supplementary strategy for black pod disease control. Fluorescent *Pseudomonas* species have been shown to be antagonistic against *Phytophthora infestans* on tomato (Tran et al. 2007) and *Phytophthora capsici* on black pepper (Tran et al., 2008; Kruijt et al., 2009). Hence, biosurfactant-producing *Pseudomonas* species may also represent a potential resource for the control of black pod disease. *Pseudomonas* species are found in many ecosystems, are metabolically versatile and are well adapted to plant-associated environments (Raaijmakers et al., 2002). Biosurfactants are surface-active metabolites that disrupt the membrane of *Phytophthora* zoospores, leading to quick elimination of zoospore populations (Souza et al., 2003; de Bruijn et al., 2007). However, despite the potential of biosurfactant-producing *Pseudomonas* species for controlling diseases caused by *Phytophthora* and other oomycete pathogens (Govers et al., 2009), their frequency and diversity in cocoa ecosystems have not been explored to date.

In this study, we determined the distribution and diversity of *Pseudomonas* and *Phytophthora* in the cocoa ecosystem in Ghana. The *Phytophthora* and *Pseudomonas* isolates from cocoa and other plant species growing in association with cocoa, were characterized by different phenotypic and genotypic methods. In-vitro assays were carried out to evaluate the effect of selected *Pseudomonas* strains on *P. palmivora* and *P. megakarya*.

MATERIALS AND METHODS

Isolation and characterisation of *Phytophthora*

From July and October, 2008, samples of *Phytophthora* infected pods and rootlets of food crops and other plants growing in association with cocoa were collected from 22 districts in 6 cocoa growing regions of Ghana. The number of districts surveyed per region was proportional to the output. *Phytophthora* was isolated from randomly selected infected cocoa pods by direct plating on V8 medium (200 ml Campbell Vegetable juice (V8), 800 ml sterile distilled water, 2.5 gmL^{-1} $CaCO_3$) amended with pimaricin (10 mgL^{-1}), vancomycin (100 mgL^{-1}) and pentachlonitrobenzene (PCNB) (10 mgL^{-1}). Rootlets of food crops and other plants growing in association with cocoa were cut into pieces (approx. 5 mm in length), surface sterilized, inserted into green cocoa pods and incubated in a humid chamber. Typical *Phytophthora* lesions produced on the pods from the inserted root pieces were aseptically cut, plated on the selective V8 medium and incubated at 28^0C. After 4 days of incubation, colony morphology of each isolate was recorded.

Mycelium for DNA extraction was grown in 10% clarified V8 medium at 28^0C. After 5 days, mycelium was harvested, blotted dry on filter paper and freeze dried. DNA was extracted using a fastprep protocol. DNA extraction buffer (0.2 M Tris, pH 8.5; 0.25 M NaCl; 25 mM EDTA; and 2% sodium dodecyl sulfate) and 3-mm glass beads were added to frozen mycelium and the mixture was ground in a FastPrep instrument. One phenol/chloroform and three chloroform/isoamylalcohol extractions were performed, followed by RNAse treatment and DNA precipitation. The extracted DNA was dissolved in $T_{10}E_1$ buffer (10 mM Tris; 1 mM EDTA, pH 8.0) and stored at -20^0C. Polymerase chain reaction (PCR) amplification of the ITS region of the template DNA was performed using primers A2 (ACTTTCCACGTGAACCGTTTCAA) and I2 (GATATCAGGTCCAATTGAGATGC). PCR was undertaken in 50 µL volumes consisting of 27.8 µL MQ water, 5 µL 10X PCR buffer (10 mM Tris HCl, pH 8.3; 50 mM KCl; 0.15 mM $MgCl_2$), 5 µL of 2 mM dNTPs, 1 µL (10 pmol) each of primers A2 and I2, 0.2 µL Supertaq and 10 µL template DNA. Cycling conditions were 94^0C for 5 min, followed by 30 cycles of 94^0C for 30 sec, 62^0C for 30 sec and 72^0C for 1 min. After the cycling reactions, there was a final step of 72^0C for 5 min. The PCR products (700 to 800 base pair fragments) of the ITS1 and ITS2 region were purified and sequenced.

Isolation and characterization of *Pseudomonas* bacteria

Green pods (approximately 4-months old) from *Theobroma cacao* from various locations and uncultivated cocoa types, *Theobroma speciosum, Theobroma bicolor, Theobroma grandilifolium, Theobroma microcarpum* and *Herrania* spp., assembled at Tafo (Ghana) were used to isolate biosurfactant-producing *Pseudomonas* species. Each pod was washed in 20 ml sterile distilled water (SDW) and serial dilutions of the suspension were plated on *Pseudomonas* semi-selective medium (PSA) (Simon and Ridge, 1974). After incubation for 48 hrs at 25^0C, morphologically different *Pseudomonas* colonies were selected, purified on PSA and subsequently tested in triplicate for biosurfactant production by the drop-collapse assay (de Souza et al., 2003).

Pseudomonas isolates which tested positive for biosurfactant production were characterized phenotypically based on colony morphology on PSA and genotypically by BOX-PCR. The following four biosurfactant-producing *Pseudomonas* isolates from the collection maintained at the Laboratory of Phytopathology, Wageningen University were included as reference: *P.*

Table1. Morphological classification of *Phytophthora* isolates obtained from cocoa and other plant species in six cocoa growing regions in Ghana.

Region	No. of districts surveyed	*Phytophthora* isolates	*P. palmivora*	*P. megakarya*
Western	8	67	50	17
Ashanti	4	23	9	14
Brong Ahafo	3	28	9	19
Eastern	3	33	18	15
Volta	2	14	2	12
Central	2	18	18	0
Total	22	183	106	77

fluorescens SS101, *P. fluorescens* SBW25, *P. fluorescens* Pf-5 and *P. putida* 267.

BOX-PCR was carried out as described by Rademaker et al., (1997). Bacterial suspensions were prepared from freshly grown colonies. A small colony was suspended in 50 µl sterile demineralised water with a sterile toothpick. One µl of the bacterial suspension was used as template in 25 µl BOX-PCR reactions. BOX-PCR fragments were separated on a 25-cm-long 1.0% agarose gel at 40 volts for 16h in 1x TAE buffer containing ethidium bromide (20 ng ml^{-1}).

In-vitro antagonism assay

Selected *Pseudomonas* isolates, each from one BOX-PCR group, were randomly selected and evaluated in-vitro for their ability to inhibit mycelial growth of *P. palmivora* (GhCR15) and *P. megakarya* (GhBAR24). *P. fluorescens* SS101 (strain 60) was included as a reference *Pseudomonas* isolate, and control plates contained only *Phytophthora*. A 7-mm-diameter agar plug of a 4-day-old *Phytophthora* culture was inoculated in the centre of 1/5th strength PDA media (4.8 g potato dextrose broth, 12 g agar, pH 7.0) in 9-cm diameter petri dish. Three µl of *Pseudomonas* suspension (10^9 cfu ml^{-1}) was spot-inoculated 2.5 cm from the *Phytophthora* plug at three points equidistant from each other and from the mycelial plug. There were three replicates per *Pseudomonas* isolate. Percentage inhibition of radial growth (PIRG) by the *Pseudomonas* strains was calculated after *Phytophthora* in the control plates had covered almost the entire plate using the formula:

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

where R1 is the diameter of *Phytophthora* growth in the control plate and R2, the diameter of *Phytophthora* in test plate.

Zoospore lysis by *Pseudomonas* isolates

The effect of selected *Pseudomonas* isolates on zoospores of *Phytophthora* was studied. Zoospores were obtained by flooding 5-day-old V8 juice agar plates (9-cm diameter) of *P. palmivora* (isolate GhCR15) and *P. megakarya* (isolate GhBAR24) with 20 ml of sterile distilled water. The flooded plate was kept at 4^0C for 30 min and then transferred to 30^0C for another 30 min. The zoospore suspension was filtered to remove mycelial fragments and sporangia, and adjusted to a concentration of 2 x 10^5 zoospores per ml. Lysis of zoospores was tested by placing 3 µl of the zoospore suspension under the microscope together with 3 µl of *Pseudomonas* suspensions (10^9 cfu ml^{-1}). Zoospore motility, encystment and disintegration were observed microscopically for up to 3 min.

RESULTS AND DISCUSSION

One hundred and eighty three successful *Phytophthora* isolations were made. Based on colony morphology, 106 (57.9%) of the 183 isolates had petalloid or stellate-striate patterns and were classified as *P. palmivora* (Table 1). Seventy seven isolates (42.1%) had a cotton wool pattern typical for *P. megakarya* (Table 1). Within each category, however, there was a range of morphology types. Out of the 183 isolates, 103 (56.28%) were from cocoa pods, and the remaining 80 (34.72%) from other plant species. Preliminary analysis of the ITS1 and ITS2 sequences confirmed the morphological classification and further showed no genotypic differences within the *P. palmivora* or *P. megakarya* groups. These results suggest that *P. palmivora* and *P. megakarya* are the predominant species of *Phytophthora* in the cocoa regions surveyed in this study. Furthermore, plants other than cocoa harbour both *Phytophthora* species and may represent a refuge for both pathogen species.

From a total of 1260 randomly selected bacteria isolated from cocoa pods, only 62 (~ 5%) were positive for biosurfactant production based on the drop collapse assay.

Preliminary analysis by BOX-PCR showed that these isolates clustered in 14 genotypic groups and three singletons; each BOX-PCR group consisted of at least two isolates. The isolates were numbered randomly from 1to 64.

Results of the in vitro assays with *P. palmivora* and *P. megakarya* showed that *Pseudomonas* isolate 55 was the most effective in inhibiting mycelial growth of *P. palmivora* (PIRG of 50.5) while *Pseudomonas* isolate 4 was the most effective in inhibiting growth of *P. megakarya* (PIRG of 62.9). *Pseudomonas* isolates 17, 21, 31 and 64 did not show any inhibitory activity against either of the two *Phytophthora* species.

Pseudomonas isolates 3, 4, 16, 24 and 52 caused lysis of zoospores of both *Phytophthora* species. The time from cessation of motility to lysis of zoospores ranged from 47 to 125 seconds. Reference strain *P. fluorescens* SS101 caused cessation of zoospore motility, zoospore encystment and disintegration within 33 seconds. Since mycelium and zoospores play major roles in black pod disease epidemics, several of the isolated indigenous *Pseudomonas* strains may have potential for disease control. The efficacy of these bacterial isolates to control black pod disease is currently being investigated in small-scale bioassays as well as in field trials in Ghana.

CONCLUSIONS

The results of the survey conducted in the six cocoa growing regions show that *P. palmivora* and *P. megakarya* are the predominant species present on cocoa farms in Ghana. The ability of several indigenous *Pseudomonas* isolates obtained from cocoa pods to inhibit mycelial growth and lyse zoospores holds promise for further development of a biocontrol strategy in black pod disease management.

ACKNOWLEDGEMENTS

The authors wish to acknowledge assistance in sampling by staff of the Mycology Section of the Plant Pathology Division of CRIG. This research was part of a sandwich PhD study at the University of Wageningen, Wageningen, The Netherlands co-funded by the Nuffic Fellowship of the Netherlands Government and the Government of Ghana. This paper is published with the permission of the Executive Director, Cocoa Research Institute of Ghana.

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