INVESTIGATIONS INTO THE SEED-BORNE NATURE AND SEED TO SEEDLING TRANSMISSION OF *PHYTOPHTHORA* IN COCOA

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ABSTRACT

The study verified a report that Phytophthora spp., causing black pod disease of cocoa in Ghana, are seed-borne and systemically transmitted to seedlings. To demonstrate seed borne nature, seeds from healthy and diseased cocoa pods were assayed for Phytophthora spp. Seed to seedling transmission was studied by artificially inoculating visually healthy seeds with P. palmivora, incubating the seeds in humidified Petri dishes, sowing in plastic cups and biopsying parts of the emerging seedlings for the fungus. Uninoculated seeds were sown as controls. Mature seedlings from a farmer's nursery and seedlings from seeds of naturally diseased pods were also assayed. All 1,028 seeds from healthy pods were free of Phytophthora. In contrast, 63% of seeds from diseased pods harboured the fungus. Detection of Phytophthora from the various components of infected seeds was highest for the mucilage/testa (57% detection frequency,) followed by the embryo (38%) and cotyledon (37%). Phytophthora palmivora was detected in rhizosphere soils, roots, stem bases, undetached cotyledons and testas of undetached cotyledons of seedlings from artificially infected seeds. It was absent from the middle and upper portions of stems as well as from leaves of such seedlings. Similar results were obtained with naturally infected seeds. The fungus was neither detected in farmer seedlings nor in seedlings of uninoculated seeds. It is concluded that systemic transmission of Phytophthora from the cocoa seed to the leaves of seedlings is impossible.

Keywords: *Cocoa, Phytophthora,* spp., *seed to seedling transmission*

INTRODUCTION

Cocoa is a major export crop in Ghana. In 2002, the country produced 340,600 MT of cocoa bean with a foreign revenue value of US $463 m, representing 22.4 % of foreign revenue from both agricultural and non-agricultural sources (Anon, 2003). Currently, Ghana is second to La Côte d'Ivoire in cocoa production in Africa (Amoah, 2000).

Several fungal diseases such as *Phytophthora* pod rot and canker, seedling die-back, chupon blight, charcoal pod rot, warty pod, pink disease,

white thread and cushion gall (Akrofi, 2003) attack cocoa in Ghana. Of these, black pod disease is the most important. The disease affects the pods, beans, flower cushions and shoots. On pods the predominant symptom is a brownish/ black circular lesion on the husk, leading to blackening and rotting of the pod. On stems the symptoms appear as cankers (Lass, 1985; Opeke, 1987).

Dakwa (1987) and Luterbacher and Akrofi (1993) have reported two species of *Phytophthora viz. P. palmivora* and *P. megakarya* as the causal agents of black pod disease in Ghana. *Phytophthora megakarya* is of more recent occurrence in Ghana and the more destructive of the two pathogens (Dakwa, 1987). The less virulent *P. palmivora* causes yield losses of 4.9 – 19% (Dakwa, 1984) but with the more virulent *P. megakarya*, losses of 60 – 80% are possible (Dakwa, 1987) A recent survey in Ghana indicated that from 179 diseased cocoa plant parts collected from 171 farms in 20 districts of five cocoa growing regions, 59.9% of the 446 isolates were identified as *P. palmivora* and the remaining 40.4% as *P. megakarya* (Anonymous, 1995).

Plant debris, infected roots, leaves, stem cankers and soil are known to be sources of primary inoculum of *Phytophthora* spp. (Brassier *et al.*, 1981; Lass, 1985; Erwin and Ribeiro, 1996). Though the fungus may be present in seeds from diseased pods (Lass, 1985), seed to seedling transmission of *Phytophthora* in cocoa was not thought possible until Kumi *et al.* (1996) reported that in Ghana, the fungus is also present in seeds from symptomless pods on infected trees, and that systemic seed to seedling transmission of the fungus is possible through use of seeds from diseased as well as those from healthy pods. The authors suggested that the phenomenon possibly accounted for the widespread nature of black pod disease in Ghana. Seed infection by pathogenic fungi is undesirable because infected seeds often have reduced germination, poor seedling vigour and may transmit the fungus to the seedlings. (Chiarappa and Gambogi, 1986). Infected seeds also serve as sources of primary inoculum for introducing pathogens to previously disease-free areas. If seed to seedling transmission of *Phytophthora* is shown to be true in cocoa, control strategies for black pod may have to include pre-plant seed treatment.

In the present study, we determined whether or not seeds from diseased and visually healthy cocoa pods in Ghana harbour *Phytophthora.* The role of such seeds in transmitting the fungus to seedlings was also studied.

MATERIALS AND METHODS

Pod collection and determination of seed borne nature of *Phytophthora*

From September 2001 to August 2002, mature, half ripe, cocoa pods mostly of the hybrid cocoa variety (Amazon x Amelonado) were collected from farms in the following districts of Ghana: Adansi East, Assin, Atwima, East Akim, Sekyere West, Tano, Upper Denkyira, Wassa Amenfi and Wassa West. Pods were also collected from the Plantations Crops farm of the Department of Crop Science, Kwame Nkrumah University of Science and Technology (K.N.U.S.T), Kumasi.

From each tree, two diseased pods and one visually healthy one were collected. In all, 100 diseased pods and 50 healthy pods from a total of 50 trees were collected. Pods were surface sterilized by wiping with absolute ethanol and seeds aseptically removed with forceps. Generally, each seed was cut into five pieces and all five pieces plated on a Green Cocoa Mucilage Agar (GCMA) plates (Awuah and Frimpong, 2002). Plates were incubated in the dark at 28±2 °C and emerging hyphae were observed microscopically after 3-4 days for presence of *Phytophthora* spp. With this direct plating method, 1028 seeds from healthy pods and 192 from diseased pods were biopsied. Component plating of some seeds was also attempted by aseptically dissecting the seeds into mucilage/testa, cotyledon and embryo

and plating each of the components separately on GCMA. Plates were incubated and presence of *Phytophthora* spp. from the various components determined as before. When seeds from diseased pods were component-plated, those from the symptom-free portions of the pods and those from the symptomatic portions were biopsied separately. Thus, 21 seeds from the symptom-free portions and 164 from the symptomatic portions of pods were assayed.

With both direct and component plating methods, a total of 1,405 seeds (1,028 from healthy pods and 377 from diseased pods) were examined.

Seed to seedling transmission of *Phytophthora palmivora*

Seeds from visually healthy cocoa pods were artificially inoculated with *P. palmivora* by washing off the mucilage under tap water, creating 4 mm diameter wounds with a cork borer at one lateral side of each seed and surface sterilizing the seeds in 10 % commercial bleach solution containing 12 % chlorine. Seeds were then dried in a laminar flow hood and 4-mm diameter plugs from 1-wk-old GCMA culture of *P. palmivora* (isolate T-1 from Trabuom in the Ashanti Region) placed into the wounds (1 mycelial plug/seed). The inocula were moistened with 0.5 µl drop of sterile distilled water. Ninety-two seeds were inoculated and placed in sterilized Petri dishes lined with moistened filter papers (5 seeds/dish) and incubated in the dark at 28 ±2° C. After 4 days, the success of the inoculation with *P. palmivora* was checked and found to be high through biopsy on GCMA of 42 randomly selected seeds. Forty-nine seeds with cork borer wounds but with only GCMA plugs in the wounds were used as uninoculated controls. Fifty inoculated and 25 uninoculated seeds were sown in sterilized soil in 200 ml plastic cups (one seed per cup). Components of emerging seedlings (leaves, stem, roots, undetached cotyledon and testa) were biopsied on GCMA for *P. palmivora* 1 wk after emergence and subsequently every 2 wk for 6 wk. Rhizosphere soils of the seedlings were also tested for the fungus. The experiment was repeated using seeds from pods with typical black pod symptoms. In all instances, seedlings which failed to emerge and those that were affected by post emergence damping-off were also biopsied. Four-month-old seedlings from a farmer's nursery at Apedwa in the Eastern Region of Ghana were similarly tested for *Phytophthora*.

RESULTS

Sixty percent of seeds from diseased pods biopsied by direct plating were positive for *Phytophthora* spp. and 14% were positive for other fungi mainly *Lasiodiplodia theobromae* (Table 1). Only 21% of such seeds were free of fungi. All 1,028 seeds from corresponding healthy pods biopsied by direct plating were free of *Phytophthora* spp. and as high as 94% of them did not yield any fungi. Only 6% of seeds from healthy pods were positive for other fungi, which included *Fusarium, Aspergillus, Penicillium, Rhizopus, Curvularia* and *Collectotrichum* spp.

When seeds were obtained closer (up to about 4 cm) to the symptomatic portion of diseased pods, 57%, 37% and 38%, respectively, of testa/mucilage, cotyledons and embryos were positive for *Phytophthora* (Table 2). However, with seeds obtained further away (> 4 cm) from the symptomatic portions of pods, only 17% of testa/mucilage and none of the other seed components tested positive for *Phytophthora* (Table 2).

Only 16 of the 50 artificially infected seeds sown emerged and survived. The rest either died or emerged from the soil but were attacked by post emergence damping-off. *Phytophthora palmivora* was consistently associated with the dead seeds and seedlings affected by post emergence damping-off. Of the 16 surviving seedlings, *P. palmivora* was detected in 37% of the roots, 37% of stem bases, 44% of undetached cotyledons and 31% of testas of undetached

Table 1: Occurrence of *Phytophthora* in cocoa seeds from healthy and diseased pods

District	Diseased pods[1]			Healthy pods[1]		
	Phytophthora	Other fungi	uninfected seeds	*Phytophthora*	Other fungi	uninfected seeds
Kumasi	6/18 (33)	12/18 (67)	0/18 (0)	0/20 (0)	10/20 (50)	10/20 (50)
Atwima	38/40 (95)	0/40 (0)	2/40 (5)	0/52 (0)	7/52 (13)	48/52 (92)
Sekyere West	7/12 (58)	4/12 (33)	1/12 (8)	0/120 (0)	21/120 (18)	99/120 (83)
Adansi East	4/8 (50)	2/8 (25)	2/8 (25)	0/100 (0)	7/100 (7)	93/100(93)
Assin	2/10 (20)	4/10 (40)	3/10 (30)	0/120 (0)	1/120 (1)	119/120 (99)
Wassa Amenfi	¾ (75)	0/4 (0)	1/4 (25)	0/30 (0)	3/30 (10)	27/30 (90)
Upper Denkyira	0/4 (0)	2/4 (50)	2/4 (50)	0/40 (0)	2/40 (5)	38/40 (95)
Wassa West	3/8 (38)	2/8 (25)	3/8 (38)	0/66 (0)	6/66 (9)	60/66 (91)
East Akim	22/32 (69)	1/32 (3)	9/32 (28)	0/270 (0)	6/270 (2)	264/270 (98)
Tano	35/56 (63)	0/56 (0)	18/56 (32)	0/210 (0)	4/210 (2)	206/210 (98)
TOTAL	120/192 (63)	27/192 (14)	41/192 (21)	0/1028 (0)	67/1028 (6)	964/1028 (94)

[1]*Number of seeds positive for Phytophthora or other fungi / total number of seeds assayed. Percent values are in parenthesis. For diseased pods, Lasidiplodia theobromae dominated the other fungi. For healthy pods, the other fungi were Fusarium, Aspergillus, Penicillium, Rhizopus, Curvularia and Collectotrichum* spp.

Table 2: Occurrence of *Phytophthora* and *L. theobromae* in components of seeds from diseased cocoa pods

Seed component	Seeds closer to symptomatic portion of pod			Seeds further away from symptomatic portion of pod		
	Phytophthora[1]	*L. theobromae*[1]	Uninfected seed components[2]	*Phy-tophthora*[1]	*L. theobro-mae*[1]	Uninfected seed components[2]
Testa/ mucilage	93/164 (57)	19/164 (12)	25/164 (15)	2/21 (17)	3/21 (25)	16/21 (76)
Cotyledon	61/164 (37)	11/164 (7)	62/164 (38)	0/21 (0)	0/21 (0)	19/21 (90)
Embryo	63/164 (38)	8/164 (5)	70/164 (43)	0/21 (0)	0/21 (0)	20/21 (95)

[1]*Number of seed components positive for either Phytophthora or L. theobromae/total number of seed components plated. Percent values are in parenthesis.*
[2]*Number of seed components free from infection/total seed components plated. Percent values are in parenthesis.*

Table 3: Detection of *Phytophthora palmivora* in parts of cocoa seedlings and seedling rhizosphere soils[1]

Week of Emergence	leaves	cotyledon	testa	stems[2]	root	rhizosphere soil
1	0/7 (0/7)	3/7 (0/7)	3/7 (0/7)	1/7 (0/7)	1/7 (0/7)	2/7 (0/7)
2	0/7 (0/4)	2/7 (0/4)	0/7 (0/4)	3/7 (0/4)	3/7 (0/4)	4/7 (0/4)
5	0/1 (0/2)	1/1 (0/2)	1/1 (0/2)	1/1 (0/2)	1/1 (0/2)	1/1 (0/2)
7	0/1 (0/3)	1/1 (0/3)	1/1 (0/3)	1/1 (0/3)	1/1 (0/3)	1/1 (0/3)
Total	0/16 (0/16)	7/16 (0/16)	5/16 (0/16)	6/16 (0/16)	6/16 (0/16)	8/16 (0/16)

[1]*Number of seedling part positive for P. palmivora/total number of plants plated. Data in parenthesis are for uninoculated seeds.*
[2] *P. palmivora was detected only from the portion of the stem in contact with the soil line.*

cotyledons and 50% of seedling rhizosphere soils (Table 3). The fungus was neither detected in the middle and upper portions of stems nor in the leaves of such seedlings. All 25 uninoculated control seeds sown emerged and survived. When 16 of such seedlings were biopsied, none yielded *P. palmivora* (Table 3). Biopsy of 4- mn- old cocoa seedlings from a farmer's nursery also revealed absence of *Phytophthora* from the plants. Thirty-five of 69 seeds from naturally diseased pods germinated when sown, but 25 emerged. Of 22 emerged seedlings that were assayed; *Phytophthora* was detected in four of the rhizosphere soil samples (18%), four root samples (18%) and five stem bases (23%). Three out of 13 undetached cotyledons (23%) also tested positive for the fungus. *Phytophthora* was not detected in the middle and upper portions of stems as well as in the leaves. The fungus was also frequently associated with damped-off seedlings.

DISCUSSION

The success of this study, to a large extent, depended on accurate detection of *Phytophthora* spp. in cocoa plant parts. This was possible by using GCMA for culturing the plant parts. On this medium, *Phytophthora* spp., if associated with a plated tissue, can be detected by microscopic examination of mycelia as early as 3 days after incubation due to abundant production on the medium of sporangia and chlamydospores. Awuah and Frimpong (2002) alluded to the benefits of using GCMA in culturing *Phytophthora palmivora* as including abundant sporulation of the fungus, clear definition of fungal colony margins from the underside of plates and the slightly acidic reaction of the medium which inhibits bacterial contamination of cultures.

To some extent, our results agree with those of Kumi *et al.* (1996) who also reported a high percentage (50%) of association of *Phytophthora* with seeds from diseased pods. In our study *Phytophthora* was detected in 63% of such seeds. This is not surprising since *Phytophthora* can grow from the pod husk into the pod, infecting its contents (Lass, 1985). Occurrence of *Phytophthora* in seeds of diseased cocoa pods has also been reported by other investigators (Opeke and Gorenz, 1974; Lass, 1985; Akrofi and Opoku, 2003). Our results, however, contradict those of Kumi *et al.* (1996) in that *Phytophthora* was not detected in any of the 1,028 seeds from healthy cocoa pods collected from trees bearing diseased pods. This is expected since the mucilage surrounding seed of clean pods is sterile (Wood, 1985). Data by Akrofi and Opoku (2003) also indicate that seeds from pods without black pod symptoms are free of *Phytophthora*. It is noteworthy that in the present study, as high as 94% of seeds from healthy pods were free of fungi. *Fusarium, Penicillium, Aspergillus, Rhizopus, Curvularia* and *Colletotrichum* spp. present on a few of such seeds possibly were contaminants picked up during the biopsy.

If a pod was slightly blackened, *Phytophthora* could not be detected in seeds far away from the symptomatic portion. Such seeds generally appear wholesome with high germination percentage. This is desirable as such seeds may be salvaged and used either for planting or processing. These results again contradict those of Kumi *et al.* (1996) who reported a high recovery frequency of *Phytophthora* from symptomless portions of cocoa pods. The authors indicated that the fungus infected 98% of embryos and 4% of seed coats of such seeds. It is not readily understood how the embryo can harbour the fungus in high levels while the testa, which, together with the cotyledon, must be traversed by *Phytophthora* before infecting the embryo, harbours the fungus in low levels.

Phytophthora could not be systemically transmitted from the seed to the leaves in the present study again contrary to the report by Kumi *et al.* (1996). The fungus was only detected in the roots, stem bases of seedlings at the soil line, testas of undetached cotyledons and undetached

cotyledons when artificially infected seeds were sown. It was also detected in seedling rhizosphere soils. The fungus could also not be systemically transmitted to the middle and upper stem sections as well as the leaves of seedlings when seeds naturally infected by *Phytophthora* were sown. Many of such seedlings could not survive even after emergence due to death by *Phytophthora*.

Detection of *Phytophthora* in the testa and undetached cotyledons of seedlings was expected since the seeds used harboured the fungus in those components. When such infected seeds are sown, they would release inocula of *Phytophthora* into the soil from where they can infect the emerging roots and the portion of the seedling in contact with the soil line. This would constitute seed to seedling transmission (Maude, 1996) but in a non-systemic manner (Neergaard, 1977). Transmission in such a local, non-systemic manner is similar to what has been described for tomato stem rot where the causal organism, *Didymella lycopersici* is seed-borne and contaminates the soil when seeds are sown. From the soil the pathogen causes stem rot near the soil surface (Neergaard, 1977). Using artificially infected cocoa seeds, Akrofi and Opoku (2003), however, could not transmit *Phytophthora* to seedlings in any manner. Seeds from symptom-free pods do not harbour *Phytophthora* let alone transmit the fungus to the resulting seedling as reported (Kumi *et al.* 1996).

It has not been possible in the present study to grow seedlings from infected seeds to the transplanting stage and assay them for *Phytophthora*. This is because most of the infected seeds, when sown, are killed by *Phytophthora* and do not emerge (seed decay/pre-emergence damping-off) and some of the few that emerge become affected by post-emergence damping-off also due to *Phytophthora*. A similar situation has been reported by Chant (1957). Thus, farmers would most likely not use seeds from a pod at an advanced stage of symptom development, as germination would be poor. Indeed, farmers in Ghana plant seeds from visually clean pods, which do not harbour *Phytophthora*. Therefore, seed transmission of *Phytophthora* even to the roots and stem base as noticed in the present study would be rare at the farm level. This was found to be the case when mature seedlings from a farmer's nursery were examined. Thus, seed to seedling transmission in cocoa cannot explain the widespread nature of black pod in Ghana as hypothesized by Kumi *et al.* (1996).

If soil infested with *Phytophthora* is used in raising seedlings, the roots of the seedlings can become infected (Erwin and Ribeiro, 1996; Opoku and Wheeler, 1998) and if the seedlings can grow to maturity and are planted in the field, they would spread the inoculum of *Phytophthora* to pathogen-free soils.

In conclusion, *Phytophthora* may be associated with seeds from diseased pods but not those from healthy ones. We obtained no evidence of systemic transmission of the fungus from the seed to the leaves, though non-systemic infection of the roots and the stem base has been demonstrated using infected seeds. This means of transmission is unlikely to occur at the farm level because farmers in Ghana do not use seeds from diseased but rather those from clean pods for planting. Seeds from such pods do not harbour *Phytophthora* and, therefore, do not transmit the fungus. The implication of this is that the widespread nature of black pod disease in Ghana cannot be attributed to seed to seedling transmission.

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