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Host Plant Resistance to *Phytophthora* Pod Rot in Cacao (*Theobroma cacao* L.): The Role of Epicuticular Wax on Pod and Leaf Surfaces

[1,2,3]D. Nyadanu, [2]R. Akromah, [3]B. Adomako, [2]C. Kwoseh, [3]H. Dzahini-Obiatey,
[3]S.T. Lowor, [3]A.Y. Akrofi and [3]M.K. Assuah
[1]Department of Crop and Soil Science, College of Agriculture Education,
University of Education, Winneba
[2]Crop and Soil Sciences Department, Faculty of Agriculture,
Kwame Nkrumah University of Science and Technology, Kumasi-Ghana
[3]Cocoa Research Institute of Ghana, P.O. Box, 8, Tafo-Akim

Abstract: Black pod disease caused by *P. palmivora* and *P. megakarya* in Ghana is one of the major causes of yield loss in cocoa. Several defense mechanisms are required to counter attack the pathogen. To investigate the role of epicuticular wax in resistance of cocoa to *Phytophthora* pod rot, 12 cocoa genotypes with and without epicuticular wax on pod were inoculated with *P. palmivora* and *P. megakarya*. The wax layers were removed by washing the surfaces of leaf and pod in chloroform for 30 sec. The level of resistance of cocoa genotypes was higher in leaves and pods with wax layer than in chloroform washed leaves and pods (wax removed). Cocoa genotypes with higher amount of wax were more resistant than cocoa genotypes with lesser amount of wax. These results suggest that epicuticular wax layer provide an extra defense against *Phytophthora* species. There was a significant difference in lesion number and lesion size of cocoa genotypes after removal of their waxes suggesting that other factors are also involved in the resistance of cocoa to *Phytophthora* species. Cocoa genotypes with higher amount of cuticular waxes on the surfaces of their leaves and pods retained smaller amount of water and take shorter time for moisture to evaporate from their surfaces than cocoa genotypes with lesser amount of wax. Water retention and time to drying of water varied significantly among the cocoa genotypes with intact wax layers. However, there were no significant differences among cocoa genotypes for moisture retention and time to drying of water from leaf and pod surfaces after washing leaves and pods in chloroform. This indicates hydrophobicity of epicuticular waxes on leaf and pod surfaces of cocoa. The implications of these findings in breeding for black pod disease resistance are discussed.

Key words: *Theobroma cacao* L., epicuticular wax, black pod disease, host plant resistance

INTRODUCTION

Cocoa is a major economic crop in Ghana. The popular saying "*Cocoa is Ghana, Ghana is Cocoa*" depicts the significance of cocoa production in Ghana (GCB, 2009). However, production of cocoa based on smallholder farmers, has been fluctuating around 681,000 and 740,000 over a decade (Research and Business Development (RBD) (2009). The stagnation of cocoa production in Ghana is due to outbreaks of pests and diseases of which the most important is black pod disease caused by *P. palmivora* and *P. megakarya* (ICCO, 2010). Adomako (2007) reported that yield loss in cocoa was mainly caused by black pod representing 64.1% of the total loss. Typical disease symptoms include brownish or black lesion on the pod husk, leading to blackening and rotting of the pod. The disease cycle is characterized by a parasitic phase occurring during the rainy season and a survival phase during the dry season (Gregory *et al*., 1984). In the latter phase, *Phytophthora* survive in apparently soil and on pod husks and leaf debris or roots of shade plants (Gregory, 1981; Opoku, 1994; Opoku *et al*., 2002). When the rainy season starts again, the sporangia germinates releasing motile zoospores of *Phytophthora* in free water and spread very rapidly and cause destruction of cocoa pods (Gregory *et al*., 1984).

When, *P. palmivora* was the only pathogen causing black pod disease in Ghana, cocoa was produced virtually by observing good farm sanitation to economically manage the disease. However, with the advent of *P. megakarya*, the situation has changed, as cultural practices alone are not effective in controlling black pod

Corresponding Author: D. Nyadanu, Department of Crop and Soil Science, College of Agriculture Education, University of Education, Winneba

disease (Akrofi *et al*., 1997). Chemical control of the disease is expensive and unattractive from commercial and environmental points of view and is not always effective (Opoku *et al*., 2000).

The most practical and appropriate means to control cocoa black pod is by use of resistant or tolerant genotypes, supported by further measures of an integrated control system (Iwaro, 2000; Nyasse *et al*., 2007; Adomako, 2006). Although complete resistance has not been detected, differences in susceptibility among clones or among hybrids derived from crosses have been observed in various countries, including Ghana (Adomako, 2006, 2007; Lockwood *et al*., 2007; Nyadanu *et al*., 2009). Host plant resistance in cocoa is described as polygenic and additively inherited (Tan and Tan 1990; Adomako, 2007). Resistance to *Phytophthora* species even went further with the use of molecular markers (Crouzillat *et al*., 2000; Flament *et al*., 2001).

The effect of environmental factors, rainfall and humidity, on the infection of black pod disease is well documented (Efombagn *et al*., 2004; Lockwood, 1971; Dakwa, 1974). Pod setting of cocoa coincides with rainfall and humid weather (Lockwood, 1971). In selecting and breeding for black pod disease resistance, it is important to identify and involve host plant factors that make environmental factors unfavourable for the development of the disease. Jenks and Ashworth (1999) stated that, potentially, surface epicuticular waxes could impede the entry of fungal pathogens by providing physical barrier to penetration, via chemical signals that inhibit fungal development or by increasing the hydrophobicity of plant surfaces. Hydrophobic waxes on leaf surfaces form a water repellent surface and thereby facilitate evaporation of films of water on pod and leaf surfaces, in which pathogens may multiply and enter the leaf or pod interior using their own mobility (Cooper *et al*., 2001). Waxes also form part of the preformed plant defense system against biotic stresses such as insects, bacteria and fungi (Gulz *et al*., 1991; Rhee *et al*., 1998; Marcell and Beattie, 2002). In particular, their chemical makeup and abundance are known to affect resistance to fungus and insects (Kolattukudy, 1985). Little is known about the role of epicuticular wax on leaf and pod of cocoa on *Phytophthora* species infection. To elucidate the possible role of epicuticular wax on leaf and pod in resistance to black pod disease, waxes of cocoa genotypes varying in resistance were quantified and their effect on infection of *P. palmivora* and *P. megakarya* investigated. Also, the effects of waxes on wetness and duration of drying of moisture from cocoa leaf and pod surfaces were investigated.

MATERIALS AND METHODS

Plant materials: The experimental material consisted of a diverse array of 12 cocoa genotypes (Pa 7/808, Na 33, T60/887, T63/971, Imc 76, Pa 150, Sca 9, Imc 67, Imc 53, Sca 6, T85/799 and T79/501) maintained at the germplasm plot of Cocoa Research Institute of Ghana (CRIG). The experiments were conducted at CRIG during 2009-2011 seasons.

Pod and leaf surface wax load: Surface wax was extracted by dipping the distal end of pods up to the equator into chloroform for 30 S. The extract was transferred into a weighed flask (W1). The chloroform was evaporated using Rotary evaporator (Ratavapor BÜCHI, EL 131, made in Switzerland). The flask with the sample was placed in desiccators in a cool dry place for 24 h. The flask with the sample was weighed (W2). The extracted part of the pod (which turns brown) (Fig. 1) was cut into four pieces and after removing the inner tissue, their outlines were traced on brown paper. This was used to determine the pod area (A) cm^{-2} using a leaf area meter. Three pods were assessed per genotype per replication. Wax was extracted from both abaxial and adaxial surfaces of leaf by washing each surface at a time with chloroform. The area of the leaf was also determined using leaf area meter. Five leaves were assessed per genotype per replication.

The weight of the wax load on pod/leaf surface was calculated using the formula:

Fig. 1: Cocoa pods turned brown after washing of surface with chloroform. a = chloroform washed pods, b = intact pods with waxes on surface

$$\text{Wax load } \mu g\ cm^{-2} = \frac{W2 - W1}{A} \times 1000000$$

where, W2 = Weight of sample+ flask, W1 = Weight of empty flask, A= Surface area of pod or leaf.

Effects of surface wax on *Phytophthora* species infection

Inoculum preparation: The isolates of *P. palmivora and P. megakarya* were grown on carrot agar medium and from a ten-day-old culture, a zoospore suspension was obtained by inundating each culture plate (9 cm diameter) with 10 mL sterile distilled water (chilled to 10°C), refrigerated for 25 min (5°C) and incubated in the dark at 25°C for 30 min. The zoospore concentration of the suspension was determined using a haemocytometer and adjusted to 200,000 mL^{-1}.

To assess effects of surface wax on *Phytophthora* infection, intact pod and chloroform washed (waxes removed) pod of the cocoa genotypes were inoculated with zoospores of *P. palmivora* and *P. megakarya* according to the methods of Iwaro *et al.* (1997). Inoculated pods were, arranged in a randomized complete block design and incubated at room temperature in moist plastic chamber boxes. Three replicates consisting of five pods each were assayed. After incubation for four days, the number of infection sites per inoculum site was counted. On the 7th day of incubation, sizes of the established lesions were traced on a transparent paper. The lesion sizes were determined from brown paper cutouts trimmed to the size of each lesion and were measured with a leaf area meter.

Wetability and duration of drying of moisture from leaf and pod surfaces: The patterns of leaf and pod wetability among 12 cocoa genotypes was assessed by misting leaves and pods and measuring the amount of water captured and the duration of time water remained on leaf and pod surfaces. Leaves and pods were sprayed until run off and the water was left to settle for approximately 2 min. Moisture was collected from the abaxial surfaces of individual leaves and from the surfaces of pods with filter paper fragments (mean mass 1162 mg) previously placed in 1.5 mL Eppendorf microfuge tubes (Eppendorf, Hamburg, Germany), oven dried and pre-weighed. After absorption of water from the leaf and pod surfaces was complete, the filter paper was placed back into the Eppendorf tube, resealed and reweighed to determine the amount of water absorbed off the leaf/pod surfaces. To measure how long leaves/pods retained surface moisture at room temperature, the same leaves and pods were misted with distilled water until runoff and then monitored for presence or absence of water every 5 min for the first 30 min. and every 15 min thereafter, until complete dryness. Five leaves and pods replicate from each of the 12 genotypes of cocoa were monitored in random order at room temperature. The leaf and pod surface area was measured using leaf area meter.

Statistical analysis: Data were subjected to analysis of variance (ANOVA) using GenStat® 11th version (GenStat, 2008). Significance of differences among the genotypes for each trait was tested by F-test. When the ANOVA showed significant genotypic differences, the significance of differences between the genotypic means was judged by least significant difference (LSD) at $p = 0.05$. The relationship between epicuticular wax on abaxial and adaxial surfaces of leaf were tested by regression analysis.

RESULTS

Variation of quantity of cocoa epicuticular wax on leaf and pod: There were significant variations in the amount of wax on the surfaces of leaf and pods of cocoa. Table 1 presents the amount of cuticular wax on abaxial and adaxial surfaces of leaf and on the pod of cocoa. Amount of wax on the abaxial surface of leaf of the cocoa genotypes varied largely between 5.06±0.39 and 29.27±0.23 with an average value of 19.82 and a coefficient of variation (CV) of 6.70. On the adaxial surface, the amount of wax varied between 3.64±0.54 and 24.35±0.91 with an average of 15.72 and a coefficient of variation (CV) of 11.9. The amount of cuticular wax on the surface of pod of the cocoa genotypes varied largely between 15.18±1.39 and 55.54±2.15 with an average of 37.58 and a coefficient of variation of 8.50. Significantly higher cuticular waxes were produced on the abaxial and adaxial surfaces of leaf and on pod of T60/887, Pa150, Sca6 and Pa7/808 than on the leaf and pod of the other genotypes. Epicuticular waxes were significantly fewer on the leaf and pod of Imc53, Na33 and T63/971. Comparatively, there were higher amount of cuticular wax on the surface of pod than on the surface of leaf. Also, the amount of epicuticular wax on the abaxial surface of leaf was relatively higher than on the adaxial surface.

Figure 1 and 2 shows lesion numbers on the surface of intact pods and pods washed in chloroform (waxes removed) and inoculated with *P. palmivora* and *P. megakarya,* respectively. Significantly higher lesion numbers were observed on pods washed in chloroform than pods without waxes washed. Figures 3 and 4 shows lesion sizes on the surface

Table 1: Amount of epicuticular wax load on leaf and pod surfaces of cocoa genotypes

	Leaf surfaces		Pod surface
Genotypes	Abaxial wax±SE (μg cm^{-2})	Adaxial wax±SE (μg cm^{-2})	Pod wax±SE (μg cm^{-2})
Pa7/808	28.01±0.46^{a}	20.10±1.55^{b}	38.28±1.05^{b}
Na33	9.23±0.80^{e}	6.10±0.99^{d}	28.06±1.44^{d}
T60/887	29.27±0.23^{a}	24.35±0.91^{a}	55.54±2.15^{a}
T63/971	7.97±1.02^{e}	4.51±2.16^{d}	18.14±0.70^{d}
Imc 76	25.53±0.77^{b}	19.77±0.34^{b}	46.28±1.87^{b}
Pa150	29.09±0.69^{a}	21.71±0.36^{ab}	48.33±2.16^{ab}
Sca9	17.76±0.83^{d}	13.72±1.53^{c}	23.54±1.06^{c}
Imc 67	21.14±1.47^{c}	20.20±0.90^{b}	41.75±4.17^{b}
Imc 53	5.06±0.39^{f}	3.64±0.54^{d}	15.18±1.39^{d}
Sca 6	28.25±0.53^{a}	23.59±0.27^{a}	53.84±1.76^{a}
T85/799	18.50±0.76^{d}	15.44±0.61^{c}	41.03±0.85^{c}
T79/501	18.04±0.77^{d}	15.52±0.81^{c}	41.00±2.11^{c}
LSD	2.24	3.16	5.41
SE	1.32	1.86	3.19
CV%	6.70	11.9	8.50

Mean value with different alphabets with in a column are significantly different at $p<0.05$

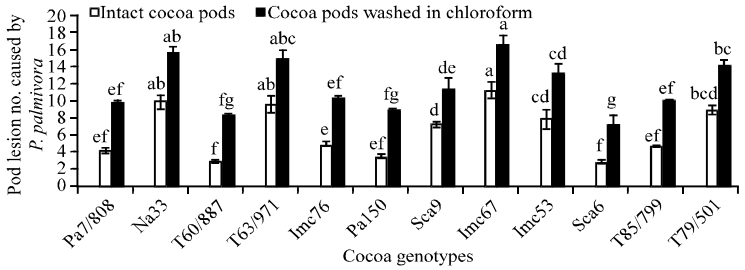

Fig. 2: Lesion number on intact and chloroform washed cocoa pods after inoculation with *P. palmivora*. Bar indicates standard error. Different letters indicate significant differences between means at the level of $p<0.05$ (Tukey's test)

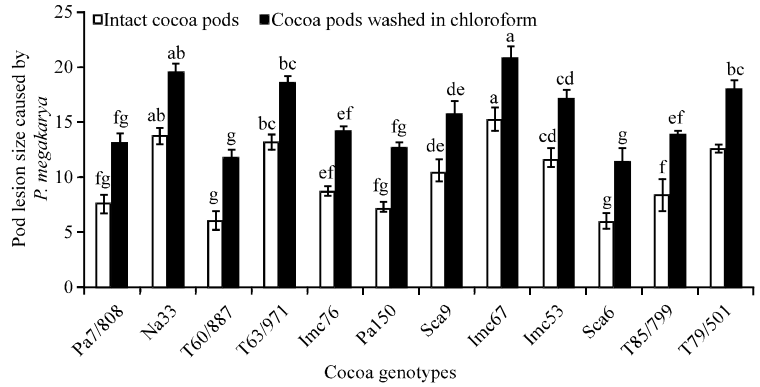

Fig. 3: Lesion number on intact and chloroform washed cocoa pods after inoculation with *P. megakarya*. Bar indicates standard error. Different letters indicate significant differences between means at the level of $p<0.05$ (Tukey's test)

of intact pods and pods washed in chloroform and inoculated with *P. palmivora* and *P. megakarya*, respectively. Significantly larger lesion sizes were observed on pods washed in chloroform than on pods with wax.

The cocoa genotypes were significantly different in lesion numbers and sizes after removal of wax from the surfaces of pods. Significantly fewer and smaller lesion numbers and sizes were observed in T60/887, Sca6, Pa150, T85/799 and Pa7/808 (Fig. 2-5).

Relationship between epicuticular wax on the surface of leaf and surface of pod: Figure 6 and 7 shows the relationship between epicuticular wax on abaxial and adaxial surfaces of leaf and epicuticular wax on pod surface. There was significant positive regression

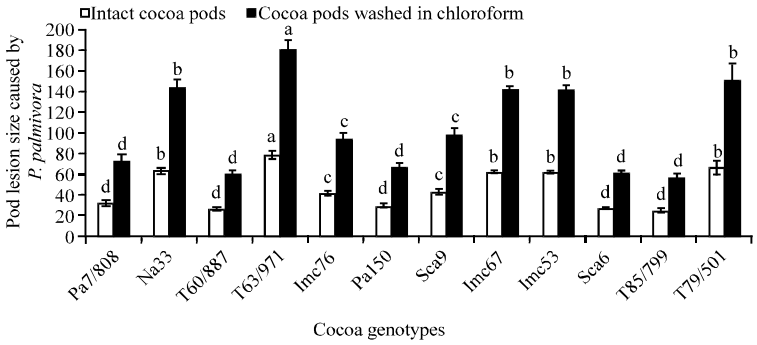

Fig. 4: Lesion size on intact and chloroform washed cocoa pods after inoculation with *P. palmivora*. Bar indicates standard error. Different letters indicate significant differences between means at the level of $p<0.05$ (Tukey's test)

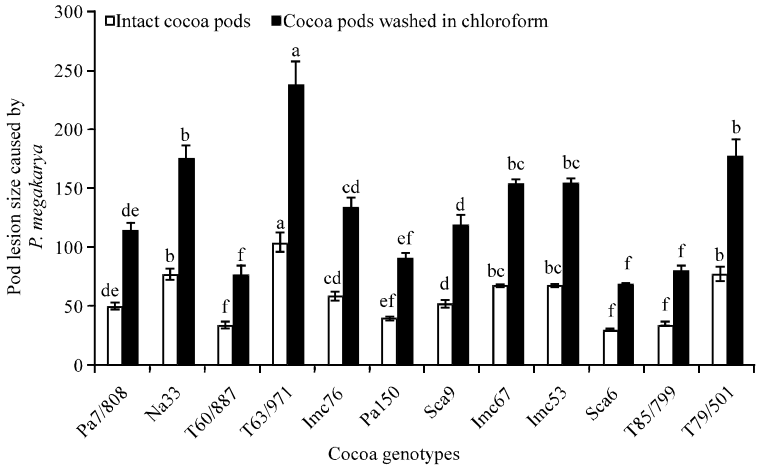

Fig. 5: Lesion size on intact and chloroform washed cocoa pods after inoculation with *P. megakarya*. Bar indicates standard error. Different letters indicate significant differences between means at the level of $p<0.05$ (Tukey's test)

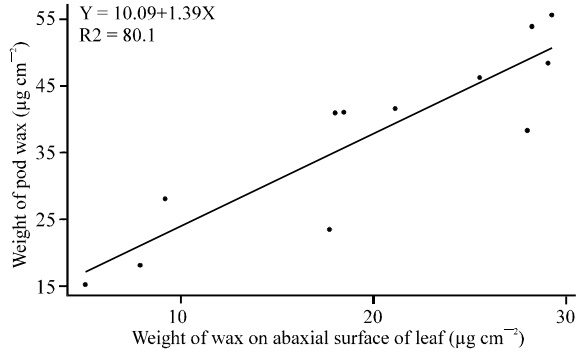

Fig. 6: Relationship between weight of epicuticular waxes on abaxial surface of leaf and surface of pod

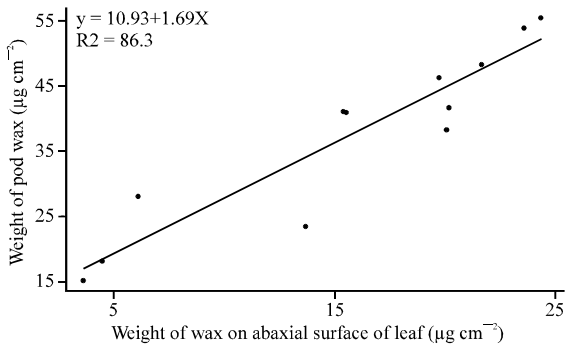

Fig. 7: Relationship between weight of epicuticular waxes on adaxial surface of leaf and pod surface

between epicuticular wax on abaxial and adaxial surfaces of leaf and epicuticular wax on cocoa pod surface, $R^2 = 80.1$ ($p<0.001$) and $R^2 = 86.3$ ($p<0.001$), respectively.

Effects of surface wax on moisture retention and dryness of moisture on pod surface: Figures 8 and 9 shows relationship between retention of moisture and time to

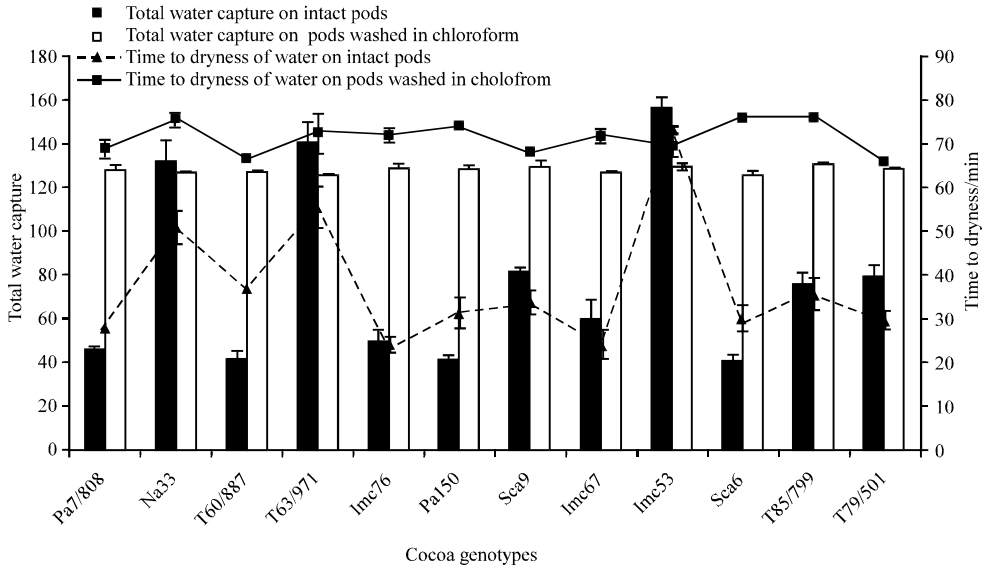

Fig. 8: Total water capture (mg) and time to dryness of water (min) from intact and chloroform washed abaxial surfaces of leaf of cocoa

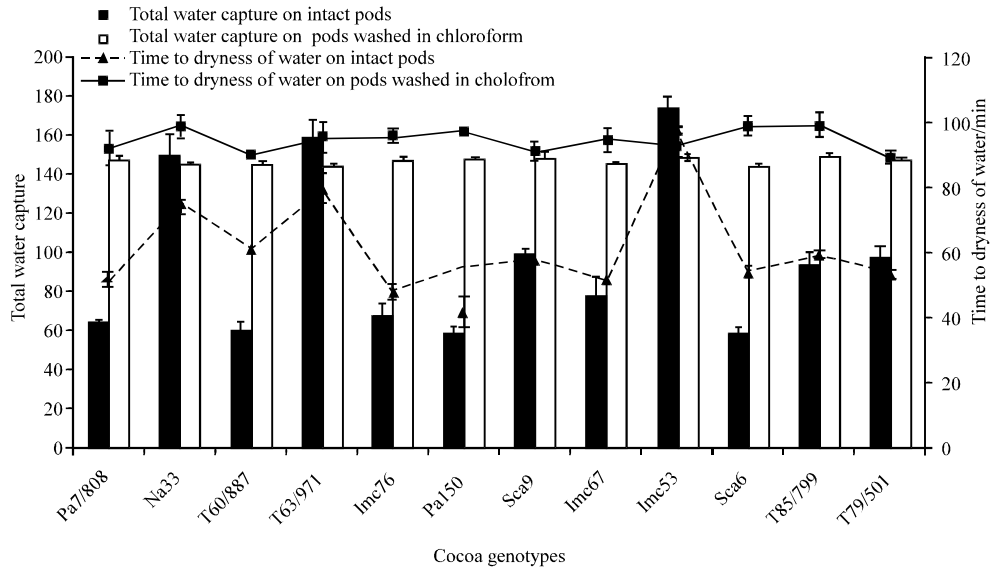

Fig. 9: Total water capture (mg) and time to dryness of water (min) from intact and chloroform washed surfaces of cocoa pod

dryness of moisture from intact and chloroform washed leaf and pod surfaces of the cocoa genotypes, respectively. The figures revealed that moisture retention and time to drying of water increase after washing wax from the surface of leaf and pod. Water capture and time to drying of water varied significantly among the cocoa genotypes with intact waxes on leaf and pod. Cocoa genotypes with high amount of cuticular waxes on the surfaces of their leaves and pods captured smaller amount of water and time taken for moisture to dry from their surfaces was shorter. There were no significant differences among cocoa genotypes for moisture retention and time to drying of water from the surfaces of leaf and pod washed in chloroform. The amount of moisture captured and time taken to dry was higher in leaf and pod washed in chloroform than cocoa leaf and pod with intact waxes on their surfaces (Fig. 8, 9).

DISCUSSIONS

The first barriers encountered by plant pathogens during infection are generally cuticle and cell wall (Agrios, 2005). In this work, significant variations were observed among cocoa genotypes for the amount of cuticular wax on both abaxial and adaxial surfaces of leaf and on the surface of cocoa pods. Lesion numbers on pods, lesion sizes on pods and leaf disc scores caused by *P. palmivora* and *P. megakarya* decreased with an increase in the amount of wax on the surface of the cocoa genotypes. This suggests that epicuticular wax layer restricts *Phytophthora* infection in cocoa. Fungal penetration into intercellular spaces could be favoured in genotypes or pod or leaf sides with lower quantities of surface waxes, while higher wax quantities observed in other genotypes could reduce rates and number of fungi invading the mesophyll or pod husk. This agrees with the findings of Zinsou *et al.* (2006) who reported surface waxes as a mechanism of resistance against *Xanthomonas* blight in cassava. Resistance to *Botrytis cinerea* increased with an increase in the amount of wax on the surface of berries (Gabler *et al.*, 2003).

There were significant differences among the cocoa genotypes in lesion number and lesion sizes after removal of waxes from the surface of cocoa pod. This suggests that other factors are involved in the resistance of cocoa pods to *Phytophthora* species. Omokolo *et al.* (2002) reported phenols, amino acid and carbohydrates as major biochemical factors in the resistance of cocoa to *Phytophthora* species.

The significant positive relationship between epicuticular waxes on abaxial and adaxial surfaces of leaf and epicuticular wax on pod surface suggests that leaf and pod surfaces could be similar in structure. This could offer theoretical explanation to the use of foliar resistance to *Phytophthora* species to predict pod resistance as reported by Nyass *et al.* (1995), Nyadanu *et al.* (2009) and Tahi *et al.* (2007).

The smaller amount of water captured and the shorter time to dryness of water in intact leaf and pod surfaces as compared to chloroform washed surfaces suggests hydrophobicity property of waxes on the surfaces of leaf and pod of cocoa. Jenks and Ashworth (1999) reported that potentially, these surface waxes could impede the entry of fungal pathogens by providing physical barrier to penetration, via chemical signals that inhibit fungal development, or by increasing the hydrophobicity of plant surfaces which results in less water retention, thereby, removing moisture required for spore germination. The persistence of leaf surface moisture, a condition critical for the development of most fungal pathogens (Jones, 1986), plays a key role in the epidemiology of fungal diseases. Many pathogens require extended periods in free water for spore germination, germ tube growth and host penetration (Everts and Lacy, 1990; Wadia and Butler, 1994; Vloutoglou *et al.*, 1996; Gilles *et al.*, 2000). A waxy surface tends to repel water, leading to the formation of droplets which evaporate from a leaf surface more rapidly than a film of water (Grammatikopoulos and Manetas, 1994). Cooper *et al.* (2001) stated that the abaxial leaf surface of cassava is non-wettable and seems unlikely as route of entry for *Xanthomonas axonopodis* pv *monihotis*. Studies of relative humidity in West Africa (Wood, 1974) have shown that long periods during which the atmospheric humidity is at saturation point are necessary for the rapid spread of black pod disease in cocoa. Duniway (1979) reported that, sporangia of *Phytophthora* species must be in contact with liquid water to germinate either directly by the growth of germ tubes or indirectly by the release of zoospores. Therefore, cocoa genotypes with high amounts of epicuticular wax on pod shed off moisture rapidly and have dry surfaces which are unfavourable for infection of *Phytophthora* species.

The epicuticular waxes of several plants contain fungistatic compounds and acts as a blockage to leaching of nutrients from the host (Inyang *et al.*, 1999; Alcerito *et al.*, 2002). Future studies need to take chemical composition in cocoa pod and leaf surface wax into account in order to elucidate potential chemical compounds in waxes which has fungistatic effects on *Phytophthora* species infection in cocoa.

CONCLUSION

Improving the load of cuticular wax on the surfaces of cocoa pods in cocoa breeding programmes could help reduce the impact of black pod disease on yield of cocoa genotypes. More research of the interaction between *Phytophthora* and the epicuticular wax layer in cocoa has to be done in order to understand more about this possible mechanism of resistance. Nevertheless, breeding for resistance using this interesting trait could help in the battle against this important pathogen.

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