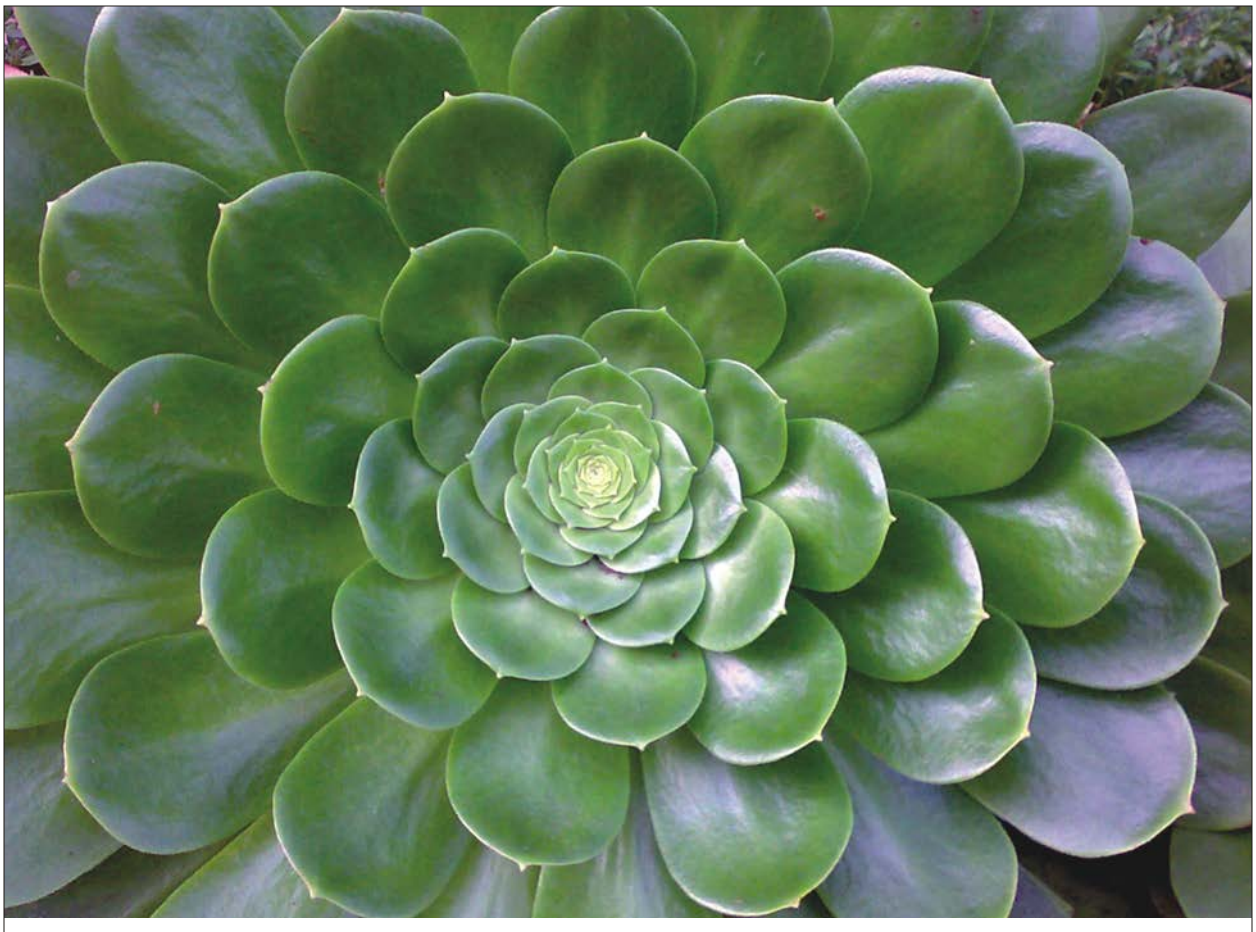

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Histological Mechanisms of Resistance to Black Pod Disease in Cacao (*Theobroma cacao* L.)

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ABSTRACT

Black pod disease caused by *Phytophthora palmivora* and *Phytophthora megakarya* is a serious constraint to cocoa (*Theobroma cacao* L.) yields in Ghana. To investigate the association between disease resistance and pod husk anatomical/histological traits, 8 cocoa genotypes were evaluated by leaf discs and detached pod inoculations and examination of anatomical factors. Width of vascular bundles, length of vascular bundles, epicarp thickness, distance between vascular bundles and epicarp, number of cells in epicarp and number of cells in mesocarp were negatively correlated with resistance to black pod disease. Number of vascular bundles and cell width were positively correlated with black pod disease resistance. Lower epicarp thickness and higher number of vascular bundles were observed in susceptible genotypes suggesting their porosity to *Phytophthora* species. The cells in epicarp and mesocarp were arranged more compactly in resistant than susceptible genotypes. Presence of extra thickness of phloem fiber and its gritty nature in resistance genotypes may act as strong mechanical barrier for penetration and absorption of sap from phloem. The cell walls of resistant genotypes stained deep red with phloroglucinol, a lignin specific stain, suggesting the presence of lignin. Principal components analysis showed that the first 2 components contribute to explain 98% of the total variation of anatomical traits. Number of cells in epicarp and number of vascular bundles were the major contributors to PC1 and PC2. The principal components, correlation coefficients, multiple and step-wise regressions indicated that number of vascular bundles, epicarp thickness, number of cells in epicarp and cell width were reliable histological traits and they could be used to screen and select for resistance to black pod disease of cocoa.

Key words: Black pod disease, *Theobroma cacao* L., resistance mechanisms, anatomical, cellular

INTRODUCTION

Black pod disease of cocoa (*Theobroma cacao* L.) caused by *Phytophthora* species, is a major problem for cocoa production in Ghana. The disease is characterized by the appearance of brownish or black lesion on the pod husk, leading to blackening and rotting of the pod. The disease can pass through the peduncles of the pod and affect the stem of the cocoa tree leading to development of

cankers. The cankers could cause young trees to wilt and die and the plants age prematurely (Okey *et al*., 1996). Reductions in yield and beans quality are the most striking aspects of the losses that are caused by the disease. Two species of the fungi, *P. palmivora* and *P. megakarya* (Opoku *et al*., 2007) have been reported as the agents of black pod in Ghana.

Cocoa growers still rely on the use of protective fungicides to control black pod. However, the use of fungicides over a number of decades has resulted in *Phytophthora* species having become resistant to many chemical compounds (Kebe *et al*., 2002; Hausbeck and Lamour, 2004). There is, therefore, a pressing need to develop new ways of controlling the disease. Moreover, the increasing concern for public health has motivated breeders to seek different strategies for the control of the disease. The development of new resistant cultivars appears to be the most eco-compatible way to control the disease (Cilas *et al*., 1998; Iwaro *et al*., 2005). Studies of the genetics of resistance to this disease are difficult because of the genetic variability of pathogen populations and the influence of environmental conditions (Van der Vossen, 1997). Host plant resistance to black pod disease is reported to be polygenic and additively inherited (Tan and Tan, 1990; Adomako, 2006). There is lack of complete resistance in cocoa genotypes against black pod but low to high levels of resistance have been reported (Iwaro *et al*., 2005; Adomako, 2007; Nyadanu *et al*., 2009).

Adult plant reactions in field tests have been the basis for evaluation of cocoa to pod rots caused by *Phytophthora* species. Non-uniform field inoculum distribution and micro-environmental effects often affect classification of genotypes in field tests. The low efficiency of field screening for disease reaction in breeding for resistance has emphasized the need for improved evaluation methods. Artificial inoculation of leaf discs and detached pods has been adopted (Nyasse *et al*., 1995; Iwaro *et al*., 1997, 2005), however little is known about factors contributing to resistance in cocoa.

Although, the mechanism of pathogen penetration and its interaction with host cellular and anatomical characters have been reported in other crops (Bisen and Channy, 1983; Shabana and Kumar 2003), there is limited information on the possibility that these traits provide a barrier or other type of physical protection against *Phytophthora* pod rot development in cocoa.

Histological studies of the host-pathogen interaction can help identify events that occur during the pathogenesis and ultimately lead to a better understanding of resistance mechanisms (Sillero and Rubiales, 2002; Vleeshouwers *et al*., 2000; Carisse *et al*., 2000). Groundnut (*Arachis hypogea*) cultivars resistant to *Cercosporidium personatum* possessed higher values for palisade index and thicker epidermis cum cuticle and palisade layers (Kaur *et al*., 1988). The cell walls in the epicarp and sclerenchymatous mesocarp were thicker and more lignified in the resistant than in the susceptible genotypes in peanut (Godoy *et al*., 1984). Sherwood (1996) found significant difference between resistant and susceptible genotypes for distance between parallel veins, proportion of large vascular bundles, or girding of vascular bundles.

Identification of histological factors associated with resistance to *Phytophthora* species could help breeders to develop cocoa genotypes with enhanced tolerance to black pod.

This study therefore sought to determine anatomical and cellular characters of cocoa pod husk and their relationship to resistance to *Phytophthora* pod rot.

MATERIALS AND METHODS

The experimental material consisted of a diverse array of 12 cocoa genotypes (Pa7/808, Na33, 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 2009/2010 and 2010/2011 seasons.

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}.

Leaf disc test: Leaf disc preparation and inoculation as described by Nyasse *et al.* (1995) and Tahi *et al.* (2006) was carried out. The flushes of leaves were tagged for the cocoa genotypes. The ages of the leaves for each treatment were established by following the growth of young flushes from bud break in the field. Leaves of good physiological condition (young lignified leaves) without insect attacks and of similar age and exposure to sunlight were collected. Sixteen leaf discs of 1.5 cm diameter from each genotype were made with a cork borer and replicated four times. Leaf discs were placed with their abaxial surface upwards on wetted plastic foam in five trays of 70 cm long, 60 cm wide and 15 cm high. Discs of the cocoa genotypes were randomly arranged in groups of 12 within each tray, giving 16×12 =192 discs per tray. The discs were inoculated and incubated at room temperature (25°C) in plastic trays and covered with another plastic tray in the laboratory to prevent direct sunlight until observations were made. Symptoms were scored 6 days after inoculation using a 0 to 5 point scale depending on the size of necrosis (0 = absence of symptoms, 1 = very small necrotic spots, 2 = larger number and size of necrotic spots, 3 = coalescence of brown spots into medium-sized, 4 = large uniform brown lesions and 5 = very large brown lesions, often expanding outside the area covered by the inoculum droplet) as described by Nyasse *et al.* (1995). The experiment was carried out twice.

Detached pod test: Detached pod test at penetration and post-penetration stages of infection as described by Iwaro *et al.* (1997) was carried out. The flowers of the cocoa genotypes were selfed (using hand pollination) so that their exact ages could be determined at the time of harvest.

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.

Field observations: Natural pod infections in the field were evaluated in 2009 and 2010 on individual crosses of the 6×4 factorial and 6×6 diallel mating designs. Pods infected by *Phytophthora* (Bp), rodent damaged pods (R) and healthy ripe pods (H) and other damages (OD) were counted each month after each harvesting round. The percentage of rotten pods (Bp) was estimated in relation to the total number of pods produced by the cross:

$$\text{Percentage black pod (\%BP)} = \frac{\text{Bp}}{\text{Bp} + \text{H} + \text{R} + \text{OD}} \times 100$$

Anatomical and cellular traits: Anatomical and cellular characteristics of pod husks of cocoa genotypes were compared for features that could be related to pod rot resistance. Pod samples were taken from each genotype to examine anatomical traits. To minimize variation due to pod age, pods of the same age were used to assess anatomical characters. The mid-section of the pod was used.

Three random pods of each genotype were cut cross-sectionally into pieces and placed in formalin-acetic acid-alcohol (FAA) for killing, fixing and storing pod tissues (Singh and Thind, 1987). Segments 2 cm in width and 2.5 cm long were excised, dehydrated in a butyl alcohol series and embedded in paraffin (Johansen, 1940; Sass, 1958). Sections 10-15 μm thick were cut with a rotary microtome, mounted on slides and stained according to a fast green and safranin schedule (Johansen, 1940; Sass, 1958). Also, slides prepared from the same paraffin blocks were examined for lignin deposition by adding a saturated aqueous solution of a lignin specific stain, phloroglucinol, in 20% HCl (Jensen, 1962). Pod section preparation was as described except that the blocks were placed in a freezer for approximately 2 h prior to cutting to ease sectioning. Two 5 min baths in distilled water were added to the dehydration series. The slides were immediately examined with the light microscope after staining for lignin. Lignin distribution was examined in three pods of each cultivar.

Specimens were examined at various magnifications of 100 and 400x under a Leitz Ortholux compound microscope following published procedures (Duvedi and Singh, 1985).Observations under the microscope were photographed. Measurements taken on pods of each genotype included the width of a vascular bundle, the distance between adjacent vascular bundles, epicarp thickness, the number of mesocarp cells per mm^2 and the width of the cells. An index representing μm of cells/mm^2 was calculated by multiplying the average number of cells by the average cell width.

Statistical analysis: Data were subjected to analysis of variance (ANOVA) using GenStat® 11th version. 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 $p \leq 0.05$. Simple correlation, multiple and step-wise regression was performed to understand the relationship between the anatomical traits and resistance to black pod disease.

RESULTS

Relative susceptibility of cocoa genotypes to *Phytophthora* species: Reaction of the 12 cocoa genotypes used in this study to *Phytophthora palmivora* and *P. megakarya* is presented in Table 1. Significant differences ($p<0.001$) in mean severity values were detected among the genotypes in the inoculation tests Pod Lesion Number (PLN), Pod Lesion Size (PLS), Leaf Disc Score (LDS). The PLN on pods among the genotypes varies from 2.93 for Sca 6 to 15.23 for Imc 67. Significantly smaller PLS were recorded on Sca 6, T60/887 and Pa 150 than for the other genotypes. PLS on Imc76 and Sca 9 were moderate; but significantly larger sizes were produced on the rest of the genotypes. Disease severity scores of LDS varied from 1.57 for Sca 6 to 4.24 for T63/971. The lesion number, lesion sizes and leaf disc scores increased when *P. megakarya* was used as the inoculum.

Variation in anatomical and cellular traits of cocoa genotypes differing in black pod disease resistance: The thickness of epicarp in cocoa pod varied significantly ($p<0.001$) among the cocoa genotypes. The epicarp thickness of Pa 150, T60/887 and Pa7/808 was significantly higher than the rest of the cocoa genotypes. The epicarp thickness was significantly thinner in Na33 and T63/971 (Table 2). There was significant differences ($p<0.001$) among the cocoa genotypes in the number of vascular bundles in the cocoa pod husk. The number of vascular bundles was significantly higher in T63/971 and Na33. Significantly fewer vascular bundles were

Table 1: The parental mean values for pod lesion number, pod lesion size and leaf disc scores of the 12 cocoa varieties after inoculation with *P. palmivora* and *P. megakarya*

Parents	PLN(Pp)	PLN(Pm)	PLS(Pp)	PLS(Pm)	LDS(Pp)	LDS(Pm)
Pa7/808	4.23±0.30^{ef}	7.60±0.84^{fg}	31.30±2.59^{d}	49.60±2.85^{de}	1.44±0.04^{c}	2.10±0.05^{c}
Na33	9.83±0.89^{ab}	13.70±0.70^{ab}	62.30±3.07^{b}	76.40±4.45^{b}	2.96±0.05^{ab}	3.10±0.05^{abc}
T60/887	3.00±0.10^{f}	6.00±0.87^{g}	25.80±1.55^{d}	33.30±3.14^{f}	1.58±0.06^{c}	2.00±0.05^{c}
T63/971	9.67±0.97^{abc}	13.20±0.67^{bc}	78.30±3.81^{a}	103.30±8.46^{a}	2.89±0.05^{ab}	4.24±0.05^{a}
Imc 76	4.97±0.24^{e}	8.67±0.43^{ef}	40.80±2.33^{c}	58.10±3.67^{cd}	2.05±0.05^{abc}	2.57±0.04^{bc}
Pa 150	3.53±0.18^{ef}	7.23±0.45^{fg}	28.70±1.68^{d}	39.50±1.47^{ef}	1.62±0.05^{c}	2.09±0.04^{c}
Sca 9	7.37±0.29^{d}	10.53±1.05^{de}	42.30±2.89^{c}	51.90±3.14^{d}	1.95±0.04^{bc}	2.51±0.04^{c}
Imc 67	11.23±0.93^{a}	15.23±0.87^{a}	61.18±1.04^{b}	67.20±1.05^{bc}	3.25±0.05^{a}	3.81±0.06^{ab}
Imc 53	8.03±1.05^{cd}	11.73±0.68^{cd}	61.14±1.48^{b}	67.30±1.32^{bc}	2.08±0.05^{abc}	2.91±0.05^{bc}
Sca 6	2.93±0.29^{f}	5.97±1.41^{g}	26.53±0.67^{d}	30.00±0.22^{f}	1.57±0.06^{c}	1.91±0.05^{c}
T85/799	4.70±0.06^{ef}	8.40±0.31^{f}	23.97±2.26^{d}	34.60±2.03^{f}	1.72±0.05^{bc}	2.42±0.04^{c}
T79/501	8.90±0.50^{bcd}	12.60±0.70^{bc}	65.24±6.41^{b}	76.9±6.32^{b}	2.27±0.05^{abc}	2.94±0.04^{bc}
LSD	1.78	1.86	8.76	11.81	0.14	0.15
SE	1.06	1.20	5.17	6.98	0.90	0.91

PLN: Pod lesion number, PLS: Pod lesion size, LDS: Leaf disc score, Pp: *Phytophthora palmivora*, Pm: *Phytophthora megakarya*, Different letters indicate significant differences between means at the level of $p< 0.05$

Table 2: Variation of NVB, WVB, LVB, EPT and MDBVB among cocoa genotypes

Genotypes	NVB	WVB	LVB	EPT	MDBVB
Pa7/808	23.33±1.20^{c}	4.37±1.52	5.20±1.27	3.03±0.18^{ab}	6.40±0.515^{a}
Na33	39.00±1.16^{b}	3.57±0.80	4.50±0.74	2.10±0.15^{c}	5.47±0.65^{c}
T60/887	21.00±1.53^{cd}	4.30±0.72	5.50±1.02	3.17±0.09^{ab}	6.37±0.49^{a}
T63/971	44.33±3.28^{a}	3.53±0.66	4.53±0.71	1.67±0.23^{c}	4.03±0.89^{c}
Imc 76	34.33±1.45^{b}	3.60±0.85	4.57±1.07	2.90±0.12^{ab}	4.63±1.24^{b}
Pa 150	16.67±1.45^{d}	4.53±0.54	5.20±0.67	3.27±0.09^{a}	6.43±0.71^{a}
T85/799	25.33±2.03^{c}	4.13±0.94	5.33±0.58	2.93±0.18^{ab}	5.30±0.84^{b}
T79/501	24.67±1.86^{c}	3.53±0.78	4.27±0.78	2.73±0.19^{b}	5.53±0.78^{bc}
LSD	5.16	ns	ns	0.51	2.51
SE	2.94	1.56	1.55	0.29	1.43

Genotypes	MDVBEP	NCE	NCM	CWD	CIE	CIM
Pa7/808	5.50±0.64^{a}	46.33±3.18^{b}	32.33±2.03^{a}	6.87±0.35^{c}	319.0±30.43	221.3±12.65^{a}
Na33	2.40±0.26^{b}	27.33±2.40^{e}	17.00±2.65^{cd}	9.50±0.40^{a}	258.3±15.86	162.2±28.99^{c}
T60/887	5.63±0.59^{a}	54.00±2.65^{a}	33.00±3.22^{a}	5.73±0.32^{c}	310.2±27.65	187.2±8.60^{b}
T63/971	1.07±0.24^{b}	25.33±2.03^{e}	12.33±1.45^{d}	10.53±0.55^{a}	268.1±30.44	130.8±19.15^{d}
Imc 76	5.17±0.78^{a}	41.00±2.08^{b}	27.33±1.76^{ab}	8.23±0.18^{b}	338.3±24.14	224.7±13.11^{a}
Pa 150	5.30±0.55^{a}	58.00±2.65^{a}	32.67±3.53^{a}	5.77±0.32^{c}	332.8±5.74	186.2±11.20^{b}
T85/799	5.30±0.84^{a}	43.00±2.31^{b}	21.00±2.08^{bc}	6.10±0.46^{c}	261.9±22.29	128.9±19.57^{d}
T79/501	5.37±0.48^{a}	28.33±2.03^{c}	17.33±2.03^{cd}	9.47±0.43^{a}	269.9±31.52	163.6±17.73^{c}
LSD	1.51	7.32	6.36	1.18	ns	12.48
SE	0.86	4.18	3.63	0.67	44.20	28.99

NVB: No. of vascular bundles, WVB: Width of vascular bundles, LVB: Length of vascular bundles, EPT: Epicarp thickness, MDBVB: Mean distance between adjacent vascular bundles, MDVBEP: Mean distance between vascular bundles and epicarp, NCE: No. of cells in epicarp, NCM: No. of cells in mesocarp, CWD: Cell width, CIE: Cell index in epicarp, CIM: Cell index in mesocarp, Different letters indicate significant differences between means at the level of $p<0.05$, ns: Non significant

observed in Pa 150 and T60/887. The length and width of vascular bundles were not significantly different among the cocoa genotypes (Table 2). Distance between vascular bundles and the distance

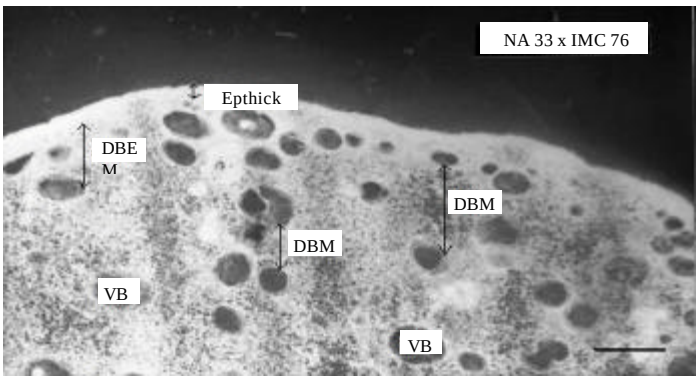

Plate 1: Transverse section of pod husk of black pod susceptible cross Na33 x Imc 76 with large No. of vascular bundles

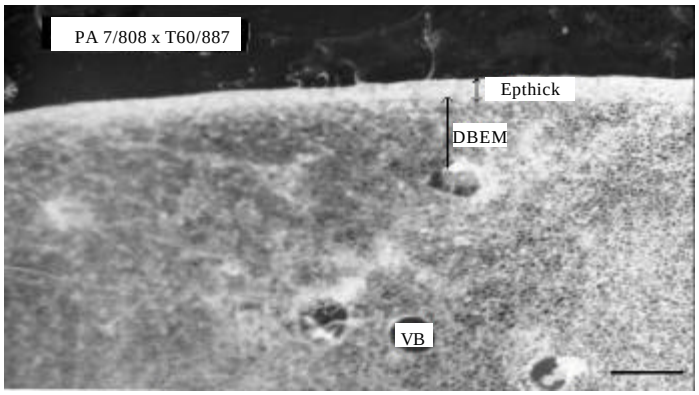

Plate 2: Transverse section of pod husk of black pod resistant crosses PA7/808 x T60/887 with fewer numbers of vascular bundles, Epthick: Epicarp thickness, DBEM: Distance between epicarp and vascular bundles in mesocarp, VB: Vascular bundles

between vascular bundles and epicarp was significantly different ($p<0.001$) among cocoa genotypes. The mean distance between vascular bundles and distance between vascular bundles and epicarp was significantly wider in Pa 150, T60/887 and Pa7/808 than the rest of the cocoa genotypes. The distance was closer in T63/971, Imc 76 and Na33. The number of cells in epicarp and mesocarp was significantly different among the cocoa genotypes. The number of cells was significantly higher in Pa 150 and T60/887 in both epicarp and mesocarp than the rest of the cocoa genotypes. Significantly less number of cells was observed in T63/971, Na33 and T79/501 (Table 2).

The width of the cells was significantly different among the cocoa genotypes. The width of cells was significantly larger in T63/971, T79/501, Na33 and Imc 76 (Table 2). There was no significant difference in cell index among the cocoa genotypes in the epicarp of the cocoa pod husk. However, significant differences were observed among the cocoa genotypes for cell index in the mesocarp of the cocoa pod husk. The cell index in the mesocarp was significantly higher in Imc 76, Pa7/808, Pa 150 and T60/887 than in the rest of the cocoa genotypes (Table 2).

Plates 1 and 2 show anatomical differences in a highly susceptible cross Na33 x Imc 76 and a resistant cross Pa7/808 x T60/887, respectively.

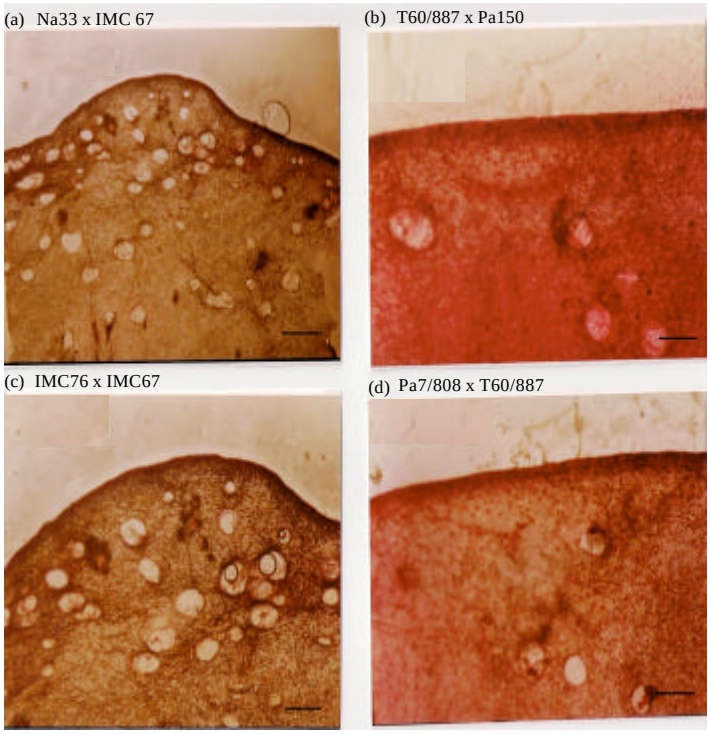

Plate 3(a-d): Comparison of phloroglucinol stained pod husk of resistant (b) T60/887 x Pa 150, (d) Pa7/808 x T60/887 and susceptible (a) Na33 x Imc 67, (c) Imc 76 x Imc 67 cocoa genotypes, The deep red colour of the resistant crosses indicates presence of lignin

Lignified areas were readily visible under the microscope when pod husks of cocoa genotypes were stained with phloroglucinol and provided a sharp contrast between the black pod resistant and susceptible genotypes. Plate 3 compares the sharp contrast between phloroglucinol stained pod husk of resistant and susceptible cocoa genotypes.

The secondary walls of inner bundle sheath cells and fibres seem to be more in resistant genotypes.

Relationship between anatomical traits and resistance to black pod disease in cocoa: The correlation coefficients of number of vascular bundles, epicarp thickness, mean distance between vascular bundles and epicarp, number of cells in epicarp, number of cells in mesocarp and cell width were significant and negative ($p<0.05$) for leaf disc score ($r = 0.86$-0.96) (Table 3). The correlation coefficients of width of vascular bundles, length of vascular bundles, epicarp thickness, number of cells in epicarp, number of cells in mesocarp and cell width were significant and negative ($p<0.05$) for pod lesion number ($r = 0.85$-0.96) (Table 3).

Correlation coefficients of length of vascular bundles, epicarp thickness, number of cells in epicarp, number of cells in mesocarp and cell width were significant and negative ($p<0.05$) for pod lesion size ($r = 0.85$-0.97) (Table 3). The correlation coefficients of number of vascular bundles and cell width were positive. Correlation coefficient of number of vascular bundles, length of vascular bundles, epicarp thickness, mean distance between vascular bundles, mean distance between vascular bundles and epicarp, number of cells in epicarp and number of cells in mesocarp were

significant and negative with mean natural field infection in 6×4 factorial and 6×6 diallel designs (Table 3). Correlation coefficients of cell index in epicarp and cell index in mesocarp were negative but non-significant with pod lesion number, pod lesion size and leaf disc scores caused by *P. palmivora* and *P. megakarya* (Table 3).

Multiple linear regression analysis indicated that anatomical and cellular traits explained 94.9% of the total variation in PLN(Pm), 96.0% of the variation in PLS(Pm) and 97.8% of the variation in LDS(Pm). Stepwise regression analysis indicated that number of vascular bundles, epicarp thickness, number of cells in epicarp and cell width accounted for 89.94% of total variation in PLN(Pm), 92.07% of the variation in PLS(Pm) and 83.07% of the variation in LDS(Pm) (Table 3).

Character association: Most of the anatomical factors were not significantly correlated with each other. However, there was significant and negative correlation between number of vascular bundles and epicarp thickness, number of vascular bundles and mean distance between vascular bundles, number of vascular bundles and mean distance between vascular bundles and epicarp, width of vascular and number of cells in epicarp, width of vascular bundles, epicarp thickness and

Table 3: Correlations of anatomical factors with resistance to *P. palmivora* and *P. megakarya*

	Resistance anatomical and cellular traits										
Components	X1	X2	X3	X4	X5	X6	X7	X8	X9	X10	X11
PLN(Pp)	0.76	-0.85*	-0.85	-0.89**	-0.61	-0.78	0.91**	-0.84	0.94***	-0.73	-0.52
PLN(Pm)	0.75	-0.85*	-0.87*	-0.87*	-0.62	-0.76	-0.96***	-0.92**	0.93**	-0.73	-0.52
PLS(Pp)	0.77	-0.84	-0.88**	-0.86*	-0.66	-0.79	-0.91**	-0.84	0.97***	-0.56	-0.41
PLS(Pm)	0.82	-0.82	-0.85*	-0.91**	-0.71	-0.82	-0.89**	-0.82	0.98***	-0.69	-0.36
LDS(Pp)	0.87**	-0.85*	-0.79	-0.93**	-0.69	-0.88**	-0.88**	-0.87*	0.91**	-0.63	-0.49
LDS(Pm)	0.86*	-0.78	-0.71	-0.96***	-0.83	-0.89**	-0.86*	-0.89**	0.89**	-0.61	-0.59
MFI+	0.89**	-0.64	-0.78	-0.88*	-0.91**	-0.67	-0.90**	-0.89**	0.73	-0.51	-0.40
MFI*	0.86*	-0.73	-0.47	-0.71	-0.61	-0.86*	-0.94**	-0.57	0.81	-0.71	-0.58

X1: Number of vascular bundles, X2: Width of vascular bundles, X3: Length of vascular bundles, X4: Epicarp thickness, X5: Mean distance between adjacent vascular bundles, X6: Mean distance between vascular bundles and epicarp, X7: Number of cells in epicarp, X8: Number of cells in mesocarp, X9: Cell width, X10: Cell index in epicarp, X11: Cell index in mesocarp. MFI+: Mean field infection of factorial crosses, MFI*: Mean field infection of diallel crosses. Multiple linear regression equation; lesion numbers on pod caused by *P. palmivora*; PLN(Pp) = $2.97^{*}-0.05X_1^{**}-4.27X_2+2.70X_3-14.4X_4^{*}+3.67X_5+2.34X_6+0.61X_7^{**}-1.53X_8-0.20X_9+0.03X_{10}^{***}+0.13X_{11}$ (R^2 = 92.8%, R^2 (adj.) = 80.3%). Stepwise regression equation; PLN(Pp) = $3.069^{**}-0.033X_1^{*}-0.20X_4^{**}-0.08X_7^{*}+0.99X_9^{**}$ (R^2 = 91.01%, R^2(adj.) =85.87%). Multiple linear regression equation; lesion numbers on pod caused by *P. megakarya*; PLN(Pm) = $-27.6^{**}-0.117X_1^{*}-6.64X_2+8.13X_3^{*}-13.6X_4^{**}+4.77X_5+2.12X_6+0.96X_7^{*}-2.62X_8+0.23X_9^{**}+0.03X_{10}+0.24X_{11}$ (R^2 = 94.9%, R^2(adj.)=81.2%). Stepwise regression equation; PLN(Pm) = $23.95^{**}-0.04X_1^{*}-2.0X_4^{**}-0.26X_7^{*}$ (R^2 = 89.94, R^2(adj.)=84.20). Multiple linear regression equation; lesion sizes on pod caused by P. palmivora; PLS(Pp) = $4.97^{*}+1.74X_1^{**}+47.5X_2-101X_3+28.3X_4^{**}-17.7X_5-2.05X_6-7.77X_7^{*}+21.2X_8+5.60X_9^{**}+0.006X_{10}-2.34X_{11}^{*}$ (R^2 = 95.8%, R^2(adj.)=76.7%). Stepwise regression equation; PLN(Pp) = $11.60^{**}-0.03X_1^{*}+4X_4^{**}-0.65X_7^{*}+6.2X_9^{**}$ (R^2 = 90.28, R^2 (adj.) =84.73). Multiple linear regression equation; lesion sizes on pod caused by *P. megakarya*; PLS(Pm) = $1016^{***}+2.35X_1^{**}+104X_2-174X_3+71.6X_4^{**}-49.9X_5-11.4X_6-16.5X_7^{**}+39.6X_8-3.29X_9^{**}-0.09X_{10}^{*}-3.94X_{11}$ (R^2 = 96.0%, R^2 (adj.) = 78.2%). Stepwise regression equation PLS(Pm) = $8.43^{**}-0.04X_1^{*}-0.2X_4^{**}-0.11X_7^{*}+0.97X_9^{**}$ (R^2= 92.07%, R^2 (adj.) = 87.55%). Multiple linear regression equation; leaf disc scores caused by *P. palmivora*; LDS(Pp) = $-5.07^{*}-0.013X_1^{**}-2.19X_2+1.72X_3-1.11X_4^{**}+0.93X_5-0.04X_6+0.18X_7-0.45X_8+0.06X_9^{*}+0.008X_{10}+0.04X_{11}^{**}$ (R^2= 90.5%, R^2(adj.)=47.8%). Stepwise regression equation; LDS(Pp) = $1.966^{**}-0.006X_1^{*}-0.31X_4^{**}-0.005X_7^{*}+0.16X_9^{**}$ (R^2 = 75.89, R^2 (adj.)= 62.12). Multiple linear regression equation; leaf disc scores caused by *P. megakarya;* LDS(Pm) = $25.1^{**}+0.02X_1^{*}+1.70X_2-3.06X_3+0.63X_4^{**}-1.21X_5-0.23X_6-0.30X_7^{*}+0.73X_8-0.112X_9^{**}-0.0005X_{10}-0.07X_{11}$ (R^2 = 97.8%, R^2(adj.)=87.9%). Stepwise regression equation LDS(Pm) = $3.81^{**}+0.001X_1^{*}-0.47X_4^{**}-0.016X_7^{**}+0.09X_9^{***}$ (R^2 = 83.07%, R^2(adj.)= 73.40). *,**,***= correlation and regression coefficients were significant at p = 0.05, p<0.05, p<0.001, respectively

Table 4: Pearson's correlation among the anatomical characters of cocoa genotypes

	NVB	WVB	LVB	EPT	MDBVB	MDVBEP	NCE	NCM	CWD	CIE
NVB	1									
WVB	-0.81	1								
LVB	-0.66	0.91**	1							
EPT	-0.92**	0.75	0.65	1						
MDBVB	-0.86*	0.80	0.63	0.75	1					
MDVBEP	-0.87**	0.59	0.51	0.96***	0.66	1				
NCE	-0.81	0.91**	0.85*	0.87*	0.72	0.71	1			
NCM	-0.76	0.82	0.72	0.87*	0.76	0.74	0.93**	1		
CWD	0.84	-0.92**	-0.92**	-0.88**	-0.73	-0.76	-0.95***	-0.84	1	
CIE	-0.46	0.52	0.36	0.66	0.38	0.54	0.73	0.85*	-0.52	1
CIM	-0.34	0.30	0.14	0.56	0.42	0.53	0.48	0.75	-0.31	0.86*

*: Significant at p= 0.05, **: Significant at p< 0.05, ***: Significant at p<0.001. EPT: Epicarp thickness, LVB: Length of vascular bundles, MDBVB: Mean distance between vascular bundles, MDVBEP: Mean distance between vascular bundles and epicarp, NCE: No. of cells in Epicarp, NCM: Number of cells in mesocarp, NVB: Number of vascular bundles, CWD: Width of cells, WVB: Width of vascular bundles. CIM: Cell index in mesocarp, CIE: Cell index in epicarp, X: Anatomical and cellular factors

Table 5: Principal components analysis of anatomical factors of 12 cocoa genotypes

Anatomical character	PC1	PC2	PC3	PC4	PC5
NVB	-0.51	0.84	0.06	0.12	-0.06
EPTHICK	0.03	-0.02	-0.03	0.19	-0.02
WIDVB	0.02	0.00	0.03	-0.14	-0.13
LVB	0.02	0.01	0.06	-0.06	-0.33
MDVBEP	0.09	-0.12	-0.14	0.83	0.01
MDVB	0.04	-0.05	-0.08	-0.43	-0.35
NCELLEP	0.72	0.38	0.55	0.01	0.15
NCELLMES	0.46	0.36	-0.79	-0.06	-0.05
WDCELLS	-0.11	-0.003	-0.15	-0.22	0.85
Proportion of variance	0.90	0.08	0.02	0.002	0.001

EPTHICK: Epicarp thickness, LVB: Length of vascular bundles, MDVB: Mean distance between vascular bundles, MDVBEP: Mean distance between vascular bundles and epicarp, NCELLEP: No. of cells in epicarp, NCELLMES: No. of cells in mesocarp, NVB: No. of vascular bundles, WDCELLS: Width of cells, WIDVB: Width of vascular bundles. PC1, PC2, PC3, PC4 and PC5: Principal components 1,2,3,4 and 5, respectively

cell width, number of cells in epicarp and cell width. There was significant and positive correlation between number of cells in mesocarp and cell index in epicarp and cell index in epicarp and cell index in mesocarp (Table 4).

Principal components analysis: Principal component analysis (PCA) shows that the first two components contribute to explain 90.08 (PC1 = 90, PC2 = 0.08) of the total variation of anatomical traits in cocoa pod husk. Number of cells in epicarp was the major contributor of total variation of anatomical characters in PC1. In PC2, number of vascular bundles was the major contributor to total variation (Table 5). It is apparent that number of cells in epicarp defined PC1 and number of vascular bundles defined PC 2. Graphic representation of the principal components analysis (Fig. 1) shows the 8 cocoa genotypes separated according to their anatomical characteristics.

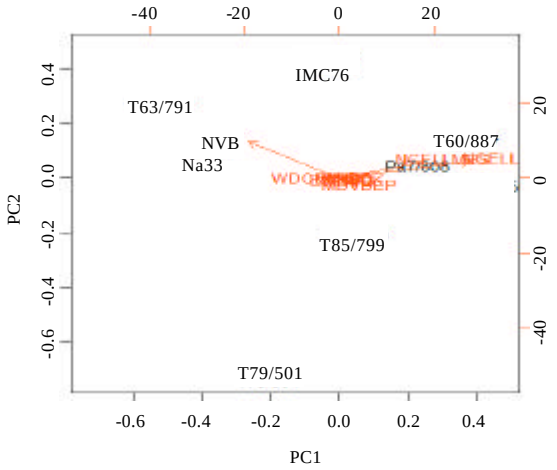

Fig. 1: A bi-plot of the principal component analysis on eight cocoa genotypes and nine anatomical factors of pod husk of cocoa as variables. EPTHICK: Epicarp thickness, LVB: Length of vascular bundles, MDVB: Mean distance between vascular bundles, MDVBEP: Mean distance between vascular bundles and epicarp, NCELLEP: No. of cells in epicarp, NCELLMES: No. of cells in mesocarp, NVB: No. of vascular bundles, WDCELLS: Width of cells, WIDVB: Width of vascular bundles

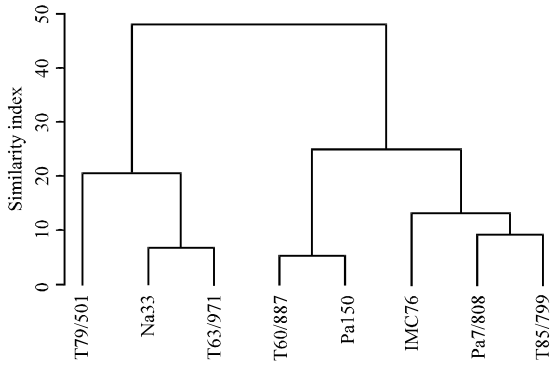

Fig. 2: Dendrogram of 8 cocoa genotypes based on principal components 1 and 2 of the anatomical factors evaluated in pod husk of cocoa

Genotypes Imc 76, T79/501 and T85/799 are clearly classified as different from the rest. The PC1 which discriminates genotypes according to number of cells in epicarp classify Pa7/808, T60/887 and Pa 150 as different because of higher number of cells in the epicarp of these genotypes. Likewise, PC2 allows separation of genotypes T63/971 and Na33 from others because these genotypes have high number of vascular bundles. The angle between number of cells in epicarp and number of cells in mesocarp are very acute suggesting a close relationship between them (Fig. 1).

Cluster analysis (Fig. 2, 3) based on PC1 and PC2 and the original values, respectively, shows the variability among the 8 cocoa genotypes based on the anatomical traits. Two major clusters were evident with their subgroups distinctively separating the black pod resistant and black pod

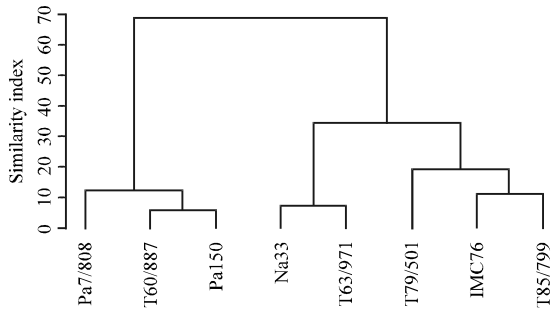

Fig. 3: Dendrogram of 8 cocoa genotypes based on original data of the anatomical factors evaluated in pod husk of cocoa

susceptible genotypes. Pa 150 and T60/887 were sub-clustered and Na33 and T63/971 were also sub-clustered (Fig. 2, 3). The grouping of genotypes based on the PCA was similar to the original data suggesting the true representativeness of the PCA.

DISCUSSION

The study shows considerable genetic variability among cocoa genotypes for PLN, PLS and LDS caused by *P. palmivora* and *P. megakarya*. The differential response of cocoa genotypes further suggested that resistance to black pod disease was under genetic control and should therefore be liable to genetic improvement. Ample genetic variability for black pod resistance has also been reported in cocoa by Iwaro *et al.* (1997), Nyadanu *et al.* (2009), Nyasse *et al.* (2002) and Tahi *et al.* (2006).

Lesion numbers and lesion sizes reflect host-pathogen interactions after penetration succeeds or fails. In the present study, the comparative anatomy of black pod resistant and susceptible clones and progenies were investigated, to know the possible anatomical basis of resistance of cocoa against *Phytophthora* species. Epicarp thickness revealed significant difference among resistant and susceptible group of genotypes. Thickness of resistant genotypes was higher compared to susceptible genotypes. This indicates varying amount of different tissues making up the internal pod anatomy. In susceptible genotypes, pod epicarp thickness was less indicating lesser amount of tissue in turn lower number of cells making up the internal pod anatomy. The thickness and toughness of the outer wall of epidermal cells are apparently important factors in the resistance of some plants to certain pathogens (Agrios, 2005). Interestingly, the mean distance of resistant genotypes for distance between vascular bundles and epicarp was higher as compared to susceptible genotypes. Therefore, it is possible that large number of cells and greater distance between vascular bundles and epicarp in resistant genotypes increase the compactness of internal anatomy and the distance and energy required by *Phytophthora* to reach the phloem and xylem for nutrients and water. This is in accordance to the hypothesis put forth by Blaich *et al.* (1984) and Eibach (1994) that resistance to *Botrytis cinerea* infection in grape berries increases with increasing surface compactness. Sarig *et al.* (1998) found that skin thickness of grape berry was correlated with resistance but suggested that fungal invasion was related more to cell density in the skin than to its absolute thickness. They observed gibberellin applications enlarged cells and caused an increase in the total thickness of epidermal and hypodermal cells, which increased the incidence of decay. Conversely, cytokine applications increased cell division, which resulted in decreased decay incidence. Prudet *et al.* (1992) reached similar conclusions.

Distance between adjacent vascular bundles varied significantly among the resistant and susceptible genotypes. This distance indicates the porosity of the pod husk. In susceptible genotypes, mean distance was less indicating presence of more vascular bundles compared to resistant genotypes which had higher space with compactness of internal pod husk anatomy. Thus greater number of cells with low porosity may add toughness to internal pod anatomy of resistant genotypes. Compared to susceptible genotypes, low free space adds succulence to internal pod anatomy. This finding agrees with the results of Sherwood and Berg (1991) who reported that regression indicated genotypes with small leaf spot size do not have increased numbers of large vascular bundles, small distance between vascular bundles or small amounts of constitutive lignin. Because susceptibility to black pod disease among the cocoa genotypes was related directly to the number of vascular bundles and inversely to the distance between vascular bundles, it followed that the differences between small lesion size and large lesion size would be related to the number of vascular bundles that the fungi penetrated. The small vascular bundles would be easier to penetrate than the large vascular bundles. The phloem and xylem tissues are closely linked to the vascular bundles. As observed, the number of small and large vascular bundles was more in susceptible genotypes than in resistant genotypes indicating availability of more amounts of nutrients and water in the pod husk of susceptible genotypes. Nyadanu *et al.* (2011) reported moisture content of pod husk to be positively correlated with infection of *P. palmivora* in cocoa. Xylem vessels seem to be involved more directly in the resistance and susceptibility to vascular diseases. For example, xylem vessel diameter and the proportion of large vessels were strongly correlated with the susceptibility of elm to Dutch elm disease caused by the fungus *Ophiostoma novo-ulni* (Agrios, 2005). Parenchyma cells are important cells of pod where metabolic activities take place. Significant difference was observed for the width of parenchyma cells around vascular bundle sheath between resistant and susceptible genotypes. For successful infection of pod, *Phytophthora* has to pass its infection peg invariably through intracellular or intercellular to reach the phloem. Agrios (2005) has reported that most fungi species penetrate intercellularly supported by the action of hydrolytic enzymes particularly pectinase which dissolves cell wall. Thus presence of thick layer of parenchyma cells may act as barrier for fungi haustoria penetration but during this process, fungi may invariably damage more number of parenchyma cells which may lead to change in chemical nature of pod by way of triggering host defence mechanism. Freytag and Hardham (1991) stated that cell walls swell and thicken in response to several pathogens by producing what appears to be cellulosic phenolic substances that are cross-linked and further increases its resistance to penetration. Localized application of hemicellulase (a mixture of wall-degrading enzymes) to small scratches in the cuticle of cowpea epidermal cells results, sequentially, in the localized extracellular generation of H_2O_2, accumulation of phenolic compounds and finally cell wall protein cross-linking (Mellersh *et al.*, 2002). Phloem fibre is one of the most important parts of vascular bundle and its main function is to provide mechanical strength against collapse and avoid phloem damage by invaders. In the present study, resistant genotypes had significantly higher thickness of phloem fibre compared to susceptible genotypes. Phloem fibre is made up of sclerenchymatous tissue which is dead and lignified. Presence of extra thickness of phloem fiber and its gritty nature in resistance genotypes may act as strong mechanical barrier for penetration and absorption of sap from phloem. The cell walls of resistant genotypes stained deep red with safranin, suggesting the presence of lignin. Lignified areas were readily visible through the microscope when stained with phloroglucinol and provided a sharp contrast between the black

pod resistant and susceptible genotypes. Lignin has been demonstrated to be important mechanisms of resistance to fungal penetration (Bell, 1981; Chang *et al.*, 1980; Vance *et al.*, 1980; Hammerschmidt and Kuc, 1982).

The index representing μm of cells/mm of pod husk made quantification of the observations easier and provided a better numerical characterization than either measurement separately. Analysis of the index in the epicarp and mesocarp per unit area suggested that there are more cells in the epicarp than in the mesocarp and demonstrated numerically that the cell arrangement of black pod resistant genotypes were more compact and had less intercellular space than those of susceptible genotypes.

The significant and positive correlation between number of vascular bundles and cell width and pod lesion size, pod lesion number and leaf disc score suggests that as number of vascular bundles and cell width increase, infection of cocoa genotypes by *Phytophthora* species also increase.

The significant and negative correlation between epicarp thickness, mean distance between vascular bundles and epicarp, number of cells in epicarp, number of cells in mesocarp and cell width and pod lesion size, pod lesion number and leaf disc score suggests that as these factors increases, infection of cocoa genotypes by *Phytophthora* species decreases. Although the correlation between mean distance between vascular bundles, cell index in the epicarp and mesocarp and pod lesion size, pod lesion number and leaf disc score was not significant, the negative relationship suggests that as these factors increases, infection of cocoa genotypes by *Phytophthora* species decreases.

Selection for a specific character is known to result in correlated response in certain other character (Falconer, 1960). Generally plant breeders make selection for one or two attributes at a time and then it becomes important to know the effect on other characters. Simple Pearson correlations indicate broadly the type of association that exists between various attributes. Most of the anatomical factors were not significantly correlated with each other suggesting that they should be selected independently. However, the significant and positive correlation between width of vascular bundles and length of vascular bundles, width of vascular bundles and number of cells in epicarp, length of vascular bundles and number of cells in epicarp, epicarp thickness and mean distance between vascular bundles and epicarp, number of cells in mesocarp and cell index in epicarp and cell index in epicarp and cell index in mesocarp suggest that these factors could be simultaneously improved without any compensatory negative effects.

Conversely, the significant and negative correlation between number of vascular bundles and epicarp thickness, number of vascular bundles and mean distance between vascular bundles and epicarp, number of vascular bundles and mean distance between vascular bundles, width of vascular bundles and cell width, length of vascular bundles and cell width, epicarp thickness and cell width and number of cells in epicarp and cell width suggests that these factors cannot be improved simultaneously.

The PCA showed that number of cells in epicarp and number of vascular bundles was the major contributors of variation in the anatomical traits. These factors exhibited non-significant negative association between themselves. Therefore improvement in any of the characters in general would certainly bring compensatory negative effects in the improvement of the other. However, attention could be focused on number of cells in epicarp, which defined PC1, the major contributor to the total variation of anatomical traits.

The clustering of resistant genotypes differently from the susceptible genotypes indicates that the resistant genotypes are similar in relation to the anatomical structure of their pod husk.

The principal components, correlation coefficients, multiple and step-wise regressions indicated that number of vascular bundles, epicarp thickness, number of cells in epicarp and cell width are reliable histological traits and these could be used as marker traits to screen and select for resistance to black pod disease of cocoa.

CONCLUSIONS

This study provides new information on the associations between differential genotypic responses to black pod disease, based on variation in pod husk anatomical characters. The findings indicate that number of vascular bundles, width of vascular bundles, length of vascular bundles, epicarp thickness, distance between vascular bundles and epicarp, number of cells in epicarp, number of cells in mesocarp and cell width were associated with resistance to black pod disease. The higher relationship of epicarp thickness and number of cells in epicarp on resistance to black pod disease suggests that they could form pragmatic anatomical criteria for selecting resistance to black pod disease in cocoa.

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