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Variation and inheritance of peroxidase activity and phenol and saccharide content in cacao in relation to susceptibility to black pod disease

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Abstract

The relationship between susceptibility to black pod disease and activity of peroxidases in crude extracts and soluble phenols and saccharides contents was studied in the pod cortex and in the seeds of three cacao clones: SNK413 (lowly susceptible), SNK10 (highly susceptible) and ICS95 (mildly susceptible) and in the F1 (SNK413 × SNK10) and the F'1 (SNK10 × SNK413) progeny. Phenol content and peroxidase activity in seeds increased as the pods matured; they were not the same in the proximal, middle and distal parts within the same pod at maturity. This variation could be correlated to the stage of development of the seeds. Total soluble saccharides and ketohexoses in the pod cortex did not vary significantly from one clone to another and could not be related to the susceptibility of the pods. Nevertheless, their contents were 2 to 4 times less in the F1 and F'1 progeny. Total soluble phenols, flavanol and hydroxycinnamic derivatives in the pod cortex were higher in the SNK413 clone. Hydroxycinnamic derivatives were not detected in the SNK10 clone and this character was transmitted to the progeny when SNK10 was male (F1 progeny). Phenolic cornpounds decreased in the F1 and F'1 progeny. These results suggest that phenolic compounds and peroxidase activity could be correlated to the susceptibility of cacao clones to black pod disease and that crossing two clones of different susceptibility produces hybrids with lower phenols and saccharides contents which may be responsible for their poor tolerance to black pod disease.

Key words: clones, F1 progeny, *Phytophthora megakarya*, *Theobroma cacao*

Introduction

Black pod disease caused by *Phytophthora megakarya* is responsible for about 50 % loss in cocoa production in Cameroon. Clones cultivated in Cameroon differ in their

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Abbreviations: D.M. - dry mass; F.M. - fresh mass; POD - peroxidase activity; SPOD - specific peroxidase activity; SPh - total soluble phenols.

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susceptibility to *P. megakarya*. Clones referred to as highly susceptible are those where the rate of development of the fungus is so high that pods rot before maturity.

An abundant scientific literature has so far been published on the involvement of phenols and peroxidase (EC 1.11.1.7) isozymes during plant defence against parasites (for reviews see Van Loon 1986, Barz *et al.* 1990). During pathogen infection, carbohydrates are not only substrates for the pathogen but may also affect certain metabolic processes capable of producing active substances like phenolic compounds and proteins associated with resistance. In cacao, studies in this field are scarce. Blaha and Paris (1987) found that total soluble phenols (SPh) accumulate following infection more promptly in less susceptible than in highly susceptible clones. This suggests that the accumulation of phenolic compounds delays evolution of symptoms and colonization of the pods. Nevertheless, a strict relationship between the biochemical constituents of cacao pods and their level of susceptibility to black pod disease is yet to be established. To the best of our knowledge, no results have been reported on the inheritance pattern of peroxidase activity (POD), phenols and saccharides in *Theobroma cacao*. In the present work, the possible relationship between POD and saccharides and phenols (SPh, hydroxycinnamic and flavanol derivatives) contents in seeds and pod cortex and the susceptibility of the pods to *Phytophthora megakarya* was studied in three clones of different susceptibility and F1 progeny.

Materials and methods

Plant material: Healthy cacao pods were collected in the field genebank within the Institute of Agronomic Research Station at Barombikan (Cameroon). Clones studied differ in their susceptibility to *Phytophthora megakarya* (Blaha and Letode 1976): SNK413 (low susceptibility), ICS95 (mild susceptibility), SNK10 (high susceptibility) and the hybrids F1 (SNK413 × SNK10) and F'1 (SNK10 × SNK413) where the female is indicated first in the cross. Pods were referred to as infant, young or mature when they were about 45, 75 or 105 d old, respectively. Experiments were carried on the pod cortex and on the seeds. Where stated, peroxidase activity and phenol content were estimated separately in the proximal, middle and distal parts of the same pod.

Extraction and measurement of soluble saccharides: Fresh material was chopped and immediately extracted twice under reflux with 80 % ethanol and then 4 times with 70 % ethanol (Singh *et al.* 1990). Ethanolic extracts of each sample were combined and their proteins removed by basic lead acetate. The extracts containing saccharides were concentrated by evaporating ethanol at 50 °C under vacuum. The total free saccharides were determined using the anthron method (Yemm and Willis 1954): 1 cm^3 of the extract was incubated with 5 cm^3 anthron solution (0.12 g anthron in 100 cm^3 6.5M H_2SO_4) at 90 °C for 10 min. The absorbance of green product was measured at 630 nm (*Beckman DU 68* spectrophotometer). Glucose equivalents were calculated from a standard curve obtained with pure analytical grade glucose. Ketohexoses were determined using resorcinol-HCl procedure (Ashwell 1957) with

some modifications. Incubation mixture (10 cm^3 final volume) containing 0.01 % resorcinol, 0.03 % thiourea, 9.3 M HCl and 2 cm^3 of the extract was incubated for 10 min at 80 °C. The mixture was cooled for 5 min in tap water and the absorbance recorded immediately at 520 nm. Fructose equivalents were calculated from a standard curve obtained with pure analytical grade fructose.

Extraction and measurement of soluble phenols: Soluble phenols were extracted according to the method described by Bastide (1987) with some modifications. 1 g of lyophilised tissue was extracted twice with 50 cm^3 of 70 % methanol at room temperature for 45 min with constant shaking (*IKA-Vibrax-UXR* shaker, type *Vx5, IKA Werk*, Germany). The methanolic extracts of the same sample were combined, filtered with *Whatman no. 1* paper and the methanol evaporated at 40 °C under vacuum using a rotary evaporator (*Heidolph model 2000, Bidblock Scientific Ltd.*, Germany). The aqueous phase obtained was adjusted to 70 cm^3 with redistilled water and the pigments were removed by adding 50 cm^3 of 40 % $(NH_4)_2SO_4$, 1.4 cm^3 of 85 % H_3PO_4 and 50 cm^3 of petroleum ether. This process was repeated thrice. The ether phases were discarded and the aqueous phases of the same sample combined and extracted 4 times with 50 cm^3 ethyl acetate. The aqueous phases were discarded and the organic phases of the same sample were combined, dried by addition of 5 g $MgSO_4$ and filtered after 5 min with *Whatman no. 1* paper. The salt residue was discarded and the clear organic phase was dried at 40 °C under vacuum using a rotary evaporator. The dried extract of soluble phenolic compounds was dissolved in 2 cm^3 of pure methanol.

Total soluble phenols (SPh) were estimated with the Folin-Ciocalteu reagent at 725 nm. For phenol analysis, the purified phenol extracts were diluted 800 times and the absorbance screened between 200 and 400 nm in a *Beckman DU-68* spectrophotometer supplied with a *Quant I Beckman* software and connected to a *P.C Tandon* printer. Standard curves (Villeneuve *et al.* 1989) were obtained with pure analytical grade catechin for flavanol compounds (maximum absorbance around 280 nm) and with pure analytical grade ferulic acid for hydroxycinnamic compounds (maximum absorbance around 320 nm).

Soluble peroxidase assay: Crude enzyme extracts were prepared according to Thorpe *et al.* (1978). 3 g of tissue were ground in liquid nitrogen and homogenized with 9 cm^3 phosphate buffer (Sorensen, 0.06 M, pH 6.1) at 2 °C. The macerate was centrifuged at 18 000 *g* for 20 min and the supernatant was used for enzyme determination. Guaiacol-peroxidase activity (incubation mixture: 7.8 cm^3 phosphate buffer, pH 6.1 + 1 cm^3 H_2O_2 + 1 cm^3 1 % guaiacol + 0.2 cm^3 enzyme) was measured at 25 °C by recording absorbance at 420 nm after 5 min.

Protein concentrations were determined by the Coomassie Blue dye binding method of Bradford (1976) using bovine serum albumin as standard.

Results

Soluble saccharides: Total soluble saccharides content did not vary significantly in the three clones, SNK413, SNK10 and ICS95. It dropped to 15.1 and 10.9 mg g^{-1}(f.m.) in the F1 and F'1 progeny, respectively. Ketohexoses displayed the same tendency: about 10 mg g^{-1}(f.m). in the three clones, 6.3 and 2.7 mg g^{-1}(f.m.) in the F1 and F'1 progeny, respectively (Table 1).

Table 1. Total soluble saccharides [mg(glucose equivalent) g^{-1}(f.m.)], ketohexose [mg(fructose equivalent g^{-1}(f.m.)], protein content (μg g^{-1}(f.m.)] and specific peroxidase activity, SPOD, [ΔA_{420} μg^{-1}(protein) s^{-1}] × 10^{-2})] in the cortex of mature pods of 5 genotypes of *Theobroma cacao* L.. Data are means ± SE of 10 experiments from 10 different pods of each genotype.

Genotypes	Soluble saccharides	Ketohexoses	Proteins	SPOD
SNK413	31.76 ± 2.28	11.94 ± 1.16	90.35 ± 8.79	0.36 ± 0.02
ICS95	34.36 ± 0.51	14.60 ± 0.16	84.36 ± 9.36	0.56 ± 0.04
SNK10	29.66 ± 1.59	9.71 ± 0.50	69.50 ± 8.83	0.63 ± 0.04
SNK413 × SNK10	15.05 ± 0.30	6.31 ± 0.73	75.75 ± 7.24	0.26 ± 0.01
SNK10 × SNK413	10.87 ± 0.66	2.66 ± 0.33	89.38 ± 7.82	2.30 ± 0.06

Soluble phenols: SPh in the seeds increased as the pods matured (Table 2). Seeds from different parts within the same pod at maturity did not contain the same amount of soluble phenols. In the SNK413 clone SPh was higher in the middle part [150 mg g^{-1}(d.m.)] than in the proximal and the distal parts [about 120 mg g^{-1}(d.m.)]. In the SNK10 clone, SPh was 96, 105 and 120 mg g^{-1}(d.m.) in the proximal, middle and distal parts, respectively (Table 2).

Analysis of the pod cortex of the five genotypes showed that the SNK413 clone contained the highest amount of SPh [75 mg g^{-1}(d.m.)], while ICS95 and SNK10 contained 41 and 35 mg g^{-1}(d.m.), respectively. SPh in the F1 progeny was intermediary [54 mg g^{-1}(d.m.)] between the two parents (Table 3). It was almost the

Table 2. Total soluble phenols [mg(chlorogenic acid equivalent) g^{-1}(d.m.)], soluble peroxidase activity, POD [× 10^{-4} ΔA_{420} mg^{-1}(f.m.) s^{-1}] and total soluble proteins [μg mg^{-1}(f.m.)] in seeds of cacao clones SNK413 and SNK10 at different stages of pod development and in seeds from the different parts of the same pod at maturity. Otherwise as in Table 1.

Clone	SNK413			SNK10		
	infant	young	mature	infant	young	mature
Phenols	1.80 ± 0.40	35.90 ± 3.90	71.40 ± 5.00	1.20 ± 0.10	45.20 ± 1.50	53.10 ± 2.80
POD	0.15 ± 0.01	2.50 ± 0.02	6.63 ± 0.04	0.12 ± 0.01	2.47 ± 0.02	5.76 ± 0.03
	proximal	middle	distal	proximal	middle	distal
Phenols	121.00 ± 6.00	150.00 ± 11.00	119.00 ± 11.00	96.00 ± 5.00	105.00 ± 9.00	120.00 ± 9.00
Proteins	6.70 ± 0.50	8.20 ± 1.30	12.00 ± 0.50	3.00 ± 0.20	3.00 ± 0.60	3.10 ± 0.01
POD	3.70 ± 0.02	3.56 ± 0.03	4.63 ± 0.01	1.56 ± 0.01	3.03 ± 0.03	4.90 ± 0.04

Table 3. Total soluble phenols [mg(chlorogenic acid equivalent) g^{-1}(d.m.)], hydroxycinnamic [μg(ferulic acid equivalent) g^{-1}(d.m.)] and flavanol [μg(catechin equivalent) g^{-1}(d.m.)] derivatives in the cortex of mature pods of five genotypes of *Theobroma cacao* L. Otherwise as in Table 1.

Genotype	Phenols	Flavanol derivatives	Hydroxycinnamic derivatives
SNK413	75 ± 5	49 ± 4	3.4 ± 0.3
ICS95	35 ± 3	22 ± 2	1.8 ± 0.2
SNK10	41 ± 3	22 ± 3	0
SNK413 × SNK10	54 ± 5	41 ± 3	0
SNK10 × SNK413	38 ± 2	19 ± 3	1.7 ± 0.1

same [38 mg g^{-1}(d.m.)] in the F'1 as in the female parent (SNK10). Phenols of the flavanol group were always higher than those of the hydroxycinnamic group (Fig. 1). The highest content in flavanols was in the SNK413 clone, followed by the F1 progeny; the amounts in the three other genotypes were comparable. Phenols of the hydroxycinnamic group were higher in the SNK413 clone than in the ICS95 clone and the F'1 progeny. Phenols of this group were not detected in the SNK10 clone nor in the F1 progeny.

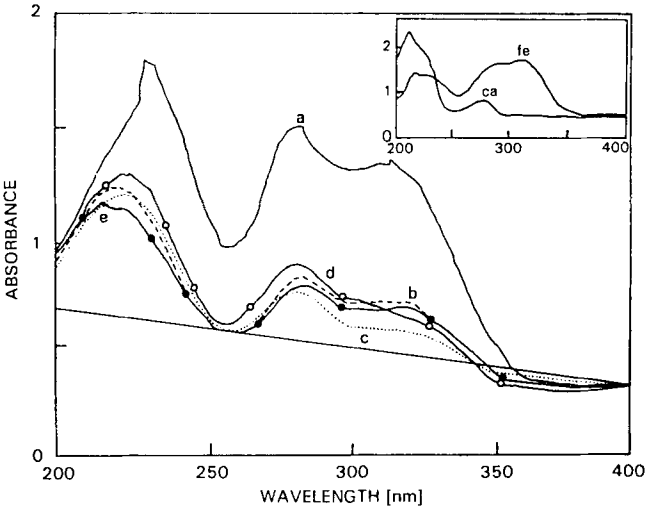


Fig. 1. Absorption spectra in U.V. of hydroxycinnamic and ferulic compounds in the cortex of mature pods of 5 genotypes of *Theobroma cacao* L.. *a* - SNK413; *b* - ICS95; *c* - SNK10; *d* - SNK413 × SNK10; *e* - SNK10 × SNK413. Inset shows the absorption spectra of pure catechin (*ca*) and ferulic acid (*fe*).

Protein content and peroxidase activity: The soluble protein content was different in the seeds from different parts of the same pod (Table 2). In the SK413 clone it was 6.7, 8.2 and 12.0 μg g^{-1}(f.m.) in the proximal, middle and distal parts, respectively. In the SNK10 clone, the soluble protein content was uniform in the different parts. In the cortex (Table 1), the highest protein content was in the

SNK413 clone and in the F'1 progeny, followed by the ICS95, F1 progeny and SNK10 clone.

POD increased in the seeds during pod development in the SNK413 and SNK10 clones (Table 2). At the mature stage, POD was different in the seeds from different parts of the same pod. SPOD in the cortex of mature pods of the five genotypes was from the highest to the lowest in the F'1 progeny, the SNK10, the ICS95, the SNK413 and the Fl progeny (Table 1).

Discussion

Comparison of total soluble saccharide and ketohexose contents in the cortex of the clones SNK413 (low susceptibility), ICS95 (mild susceptibility) and SNK10 (high susceptibility) did not vary significantly and could not be correlated to the susceptibility of these clones to black pod disease. Hwang (1983) reported the same results in apple fruits in relation to their susceptibility to *Botryosphaeria ribis*. Pastil *et al.* (1985) found that the level of total saccharides was 2 to 3 times higher in tolerant than in susceptible clones of *Sorghum* to charcoal rot. Total soluble saccharides and ketohexoses were 2 to 4 times lower in the F1 (SNK413 × SNK10) and F'1 (SNK10 × SNK413) progeny than in the parents. They were 50 % higher in the F1 than in the F'1. If saccharides in healthy cacao pods did not vary significantly from one clone to another, this does not however, exclude a possible variation during infection.

POD, protein and SPh in the seeds increased as the pods developed and at maturity, they were not the same in seeds within the same pod. In fact, during the development of the pods, seeds do not mature at the same rate. This differential rate of development could partly be responsible for the different shapes of cacao pods from different clones.

At maturity, SPh in the cortex was higher in the less susceptible than in the highly susceptible clones. This result is in agreement with the general view of the significance of phenolic compounds in disease resistance (Meiffren and Tanguy 1967, Daguenet and Parvais 1981, Matern and Kneusel 1988, Luthra *et al.* 1989, Jeun and Hwang 1991). Phenols of the hydroxycinnamic group were not detected in the SNK10 clone and this character was transmitted to the progeny when SNK10 was male (F1). Phenols of the flavanol group were detected in all the five genotypes studied. In each case, their quantity was at least 10 times higher than those of the hydroxycinnamic group. Among the phenol compounds, constitutively produced flavanols have been shown to have a pronounced antimicrobial properties (Mansfield 1982, Wink 1988). Our results suggest that higher SPh in the cortex may act as a retardant of fungus development during infection: this may explain why pods of SNK413 mature before they are damaged.

Based on these results, it seems that there is a variation in POD, SPOD, SPh, flavanol and hydroxycinnamic compounds which may be related to the stage of maturation and to the susceptibility of a given cacao genotype to black pod disease. Moreover, crossing two clones far from each other on the susceptibility scale gives

hybrids with lower SPh in the cortex as well as soluble saccharides; this may enhance their susceptibility to black pod disease compared to the parents.

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