

THE UNIVERSITY OF THE WEST INDIES

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REPORT FOR 1997

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EVALUATION

Genetic Basis of Resistance of cacao to *Phytophthora*

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This research is part of the CAOBISCO project on *Genome mapping and research linked to resistance to Phytophthora*, which commenced in June 1995. Its objectives are as follows: (1) to map the cacao genome and search for molecular markers linked to *Phytophthora* resistance, and (2) to accumulate alleles conferring resistance to *Phytophthora* diseases.

Development of an inoculation method to assess resistance to *Phytophthora*

Leaf resistance to *Phytophthora*, which was suggested as a predictor of pod resistance in cacao (Iwaro, 1995; Iwaro *et al.*, 1997), may be used to accelerate the screening of progeny in pre-breeding programmes. Nyassé *et al.* (1995) developed a leaf bioassay method utilising leaf discs for early screening for *Phytophthora* resistance. This method, developed in Cameroun, was modified at CRU, Trinidad.

The aims of this study were (1) to compare the method developed in Cameroun (Method A) with that in Trinidad (Method B), and (2) to determine the experimental conditions which need to be rigorously standardized.

Materials and methods

Plant material for this investigation was collected from seedlings and clones at CRU greenhouse at the St. Augustine Campus of the University of the West Indies or from clones at the International Cocoa Genebank, Trinidad (ICG,T) at Centeno. Eight experiments were conducted as outlined in Table 1, each with five replications per treatment. The factors investigated were leaf age, size of leaf material, the presence or absence of wounding, light regime, and zoospore concentration.

Leaf age: Leaves of different ages, described by Greathouse *et al.* (1971), were harvested: interflush 1 (I1) - soft fully expanded, light green or pink; interflush 2 (I2) - deep green leaves on green stems, and interflush 3 (I3) - green leaves on green-turning brown stems.

Leaf Material: The leaves were gently washed in distilled water and cut into longitudinal halves.

One half was left intact (representing one leaf treatment) and leaf discs (approx. 14 mm in diameter) were prepared from the other half representing a second treatment. Trays were lined with plastic onto which a wet sponge was placed. A single lining of wet paper towel was laid on top of the sponge. Leaf discs or half-leaves were placed with their adaxial surfaces on the paper towel. Discs and/or half-leaves from a particular clone were completely randomized within the trays. When both discs and half-leaves were used, those from a particular clone were placed adjacent to each other. When only leaf discs were used, three discs from each leaf were placed alongside each other.

Wounding: Wounding was performed by making a small hole in the half-leaf or disc with a fine needle.

Light regime: Two light regimes were investigated: (a) cycles of 24 hr dark and (b) normal light regime, 12 hr light, 12 hr dark. On the following day, *Phytophthora palmivora* cultures (10 days old) were induced to liberate zoospores by adding sterile distilled water at 4°C.

Zoospore concentration: Zoospore concentrations of 100,000, 250,000 and 500,000 zoospore ml^{-1} were used. These were checked with a haemocytometer. One drop (10 μL) of the appropriate zoospore suspension was deposited onto the abaxial interveinal mesophyll areas of the half-leaves and at the centre of each leaf disc. Trays were then enclosed in large plastic bags and placed in the incubation room at 25°C.

After incubation for three and six days, the reaction of the test material was assessed using two methods. Method A was modified from Nyassé *et al.* (1995) and used a scale of 0-5 where 0 = no symptoms; 1 = localised lesions; 2 = small, discrete lesions; 3 = coalescence of lesions; 4 = uniform lesion; 5 = large, dark brown lesion. Method B was the scale in use at CRU at the time of this study (1997) for the assessment of the resistance to penetration after an incubation period of three days. Method B uses a scale of 0-5 where 0 = no symptoms; 1 = 1-19 localized lesions; 2 = more than 20 localized lesions; 3 = 1-19 expanding lesions; 4 = more than 20 expanding lesions; 5 = coalesced lesions. This method was used for assessments at both three and six days (another method used at CRU at six days was not suitable for leaf discs).

Analyses of variance were conducted on the results obtained.

Results and Discussion

Results of some of the experiments are presented in Tables 2 and 3 and Figures 1 to 7.

Leaf age: Interflush 1 leaves were the most susceptible to *Phytophthora* at both three days and six days, and a trend of lower scores with leaf age was observed. However, the intermediate position of Interflush 2 was not consistently significantly different from that of Interflush 1 or 3. Young leaves are viewed as having imperfect barriers (Iwaro, 1995) which offer optimum conditions for infection.

Wound: The effect of wounding was to generally increase the scores obtained, even if the difference of resistance to *Phytophthora* was not always significant between presence or absence of wounding.

Abrasions of the abaxial surface by mechanical factors or pests would facilitate the entry of the pathogen in wounded discs; this could account for the non-significant differences observed between unwounded and wounded discs.

Table 1 Synopsis of experiments conducted.

Expt. #	Plant material ^a	Leaf age	Wound	Light regime	Zoospore MI ⁻¹ (x 10 ³)	Leaf treatment	Assessment method
60	15 clones greenhouse	I3	Absent	Normal	250	Half-leaf, Disc	A
64	20 clones ICG, T	I3	Absent	Normal/ Dark	114	Disc	A
69	10 clones ICG, T	I2	Present/ Absent	Normal	100, 250	Half-leaf, Disc	A, B
77	20 progeny greenhouse	I2, I3	Present/ Absent	Normal	100, 250, 500	Half-leaf, Disc	A, B
79	7 clones ICG, T	I1, I2, I3	Absent	Normal	250, 500	Disc	A, B
81	12 clones greenhouse	I1, I2, I3	Absent	Normal	100, 250, 500	Disc	A, B
83	30 progeny greenhouse	I1, I2, I3	Present/ Absent	Normal	100, 250, 500	Half-leaf, Disc	A
85	10 clones ICG, T	I2, I3	Absent	Normal/ Dark	100, 250, 500	Disc	A

^a- Progeny = seedling material

Zoospore concentration: Increasing inoculum concentration resulted in higher score indices. An inoculum concentration of 100,000 zoospores/ml gave the lowest score, which was significantly different ($P < 0.05$) from those obtained from 250,000 or 500,000 zoospores/ml.

Table 2a Mean value of scores of *Phytophthora palmivora* resistance from leaf bioassay test after incubation for three days under normal light conditions (Experiment #69).

Clones	Wound		Zsp MI ⁻¹ x10 ³		Leaf treatment		Assessment method		Means
	Yes	No	100	250	Half-leaf	Disc	A	B	
IMC 57	2.78	1.01	1.59	2.20	1.15	2.64	1.60	2.19	1.89
B 721	2.36	0.93	1.09	2.20	1.23	2.06	1.36	1.93	1.64
IMC 103	1.78	0.81	0.89	1.70	0.94	1.65	1.11	1.48	1.29
ICS 1	3.38	1.76	2.06	3.08	2.20	2.94	2.11	3.03	2:57
ICS 95	3.11	1.54	2.00	2.65	1.69	2.96	1.85	2.80	2.33
PA 121	2.53	1.20	1.51	2.21	1.56	2.16	1.54	2.19	1.86
ICS 60	3.73	2.73	3.01	3.44	2.83	3.63	2.61	3.84	3.22
ICS 84	3.01	1.35	1.95	2.41	1.90	2.46	1.78	2.59	2.18
P 25A	3.59	1.75	2.54	2.80	2.15	3.19	2.14	3.20	2.67
UF 11	3.51	2.37	2.65	3.24	2.11	3.78	2.43	3.46	2.94
Means	2.98	1.54	1.93	2.59	1.78	2.75	1.85	2.67	

LSD_{0.05} = 0.25; LSD_{0.05} (means) = 0.36

Table 2b Mean value of scores of *Phytophthora palmivora* resistance from leaf bioassay tests after incubation for six days under normal light conditions (Experiment #69).

Clones	Wound		Zsp MI ⁻¹ x10 ³		Leaf treatment		Assessment method		Means
	Yes	No	100	250	Half-leaf	Disc	A	B	
IMC 57	3.19	1.09	1.88	2.40	1.54	2.74	1.86	2.41	2.14
B 721	2.89	1.20	1.54	2.55	1.78	2.31	1.73	2.36	2.04
IMC 103	2.14	0.89	1.04	1.99	1.24	1.79	1.30	1.73	1.51
ICS 1	4.07	2.56	2.75	3.89	3.20	3.44	2.81	3.82	3.32
ICS 95	3.89	2.10	2.65	3.34	2.32	3.66	2.53	3.46	2.99
PA 121	2.85	1.62	1.98	2.50	1.89	2.59	1.89	2.59	2.24
ICS 60	4.16	3.10	3.43	3.84	3.23	4.04	3.10	4.16	3.63
ICS 84	3.44	1.64	2.40	2.68	2.36	2.71	2.14	2.94	2.54
P 25A	4.43	2.31	3.21	3.53	2.89	3.85	3.03	3.71	3.37
UF 11	4.10	2.94	3.15	3.89	2.97	4.06	3.04	4.00	3.52
Means	3.52	1.94	2.40	3.06	2.34	3.12	2.34	3.11	

LSD_{0.05} = 0.27; LSD_{0.05} (means) = 0.38

Table 3a Mean value of scores of *Phytophthora palmivora* resistance from leaf bioassay tests after an incubation period of three days (Experiment #85).

Clones	Light regime		Zoospore conc. $MI^{-1} \times 10^3$			Leaf age		Means
	Dark	Normal	100	250	500	I2	I3	
ICS 1	3.75	3.00	3.25	3.37	3.50	3.50	3.25	3.37
ICS 95	2.83	2.77	2.83	2.75	2.83	2.69	2.91	2.81
ICS 6	2.88	2.58	2.54	2.75	2.91	2.52	2.94	2.74
ICS 60	3.13	2.44	2.83	2.75	2.79	2.97	2.61	2.79
IMC 67	2.22	1.50	1.66	1.95	1.95	2.00	1.72	1.86
IMC 6	2.97	2.33	2.62	2.75	2.58	3.33	1.97	2.65
IMC 57	1.33	0.86	1.20	1.04	1.04	1.22	0.97	1.10
IMC 103	2.25	2.05	1.66	2.45	2.33	3.30	1.00	2.15
UF 11	2.38	1.36	2.00	1.79	1.83	1.97	1.77	1.87
Na 286	2.36	1.61	1.33	2.41	2.20	2.19	1.77	1.99
Means	2.61	2.05	2.19	2.40	2.40	2.57	2.09	
LSD _{0.05}	0.27		0.11			0.27		0.39

Table 3b Mean value of scores of *Phytophthora palmivora* resistance from leaf bioassay tests after an incubation period of six days (Experiment #85).

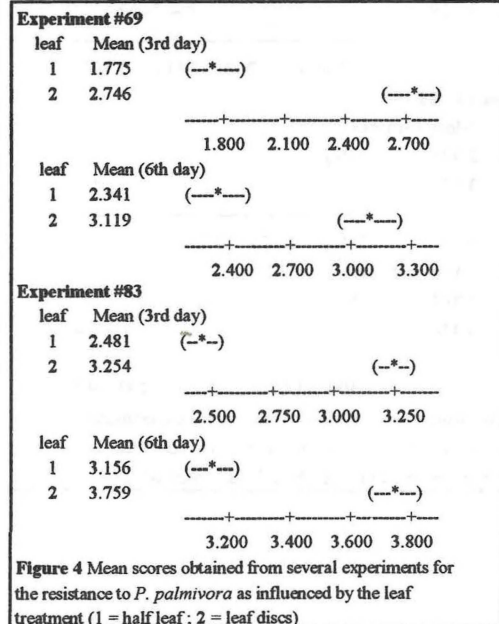
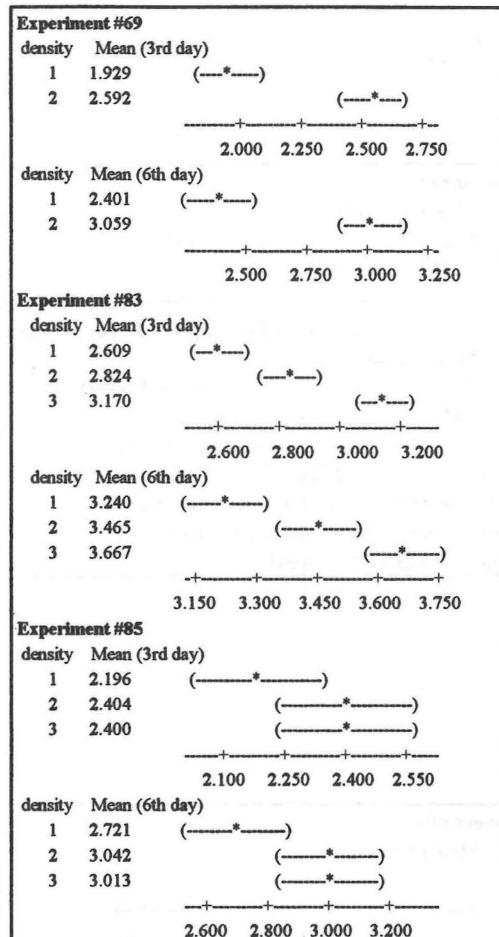
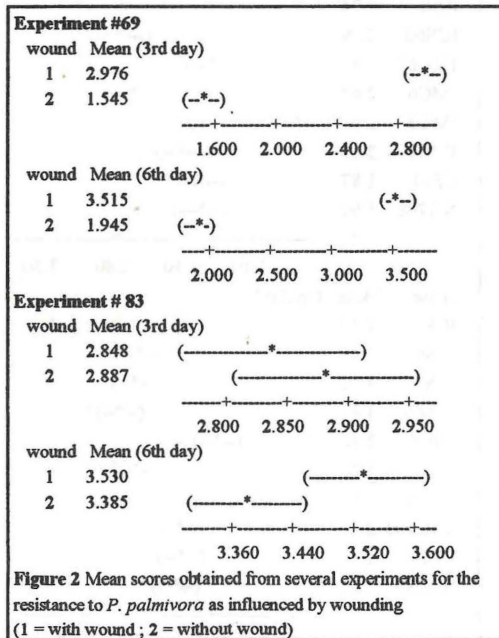
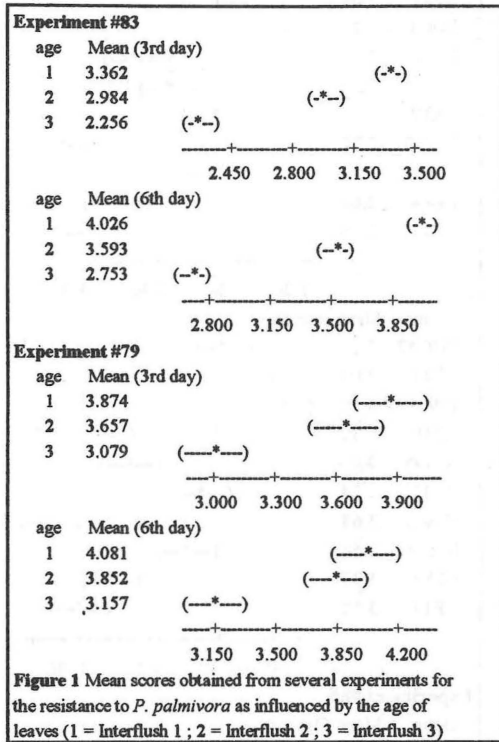
Clones	Light regime		Zoospore conc. $MI^{-1} \times 10^3$			Leaf age		Means
	Dark	Normal	100	250	500	I2	I3	
ICS 1	4.33	4.00	3.79	4.29	4.41	4.38	3.94	4.17
ICS 95	3.25	3.13	3.08	3.12	3.37	3.05	3.33	3.19
ICS 6	3.19	3.25	3.00	3.29	3.37	3.05	3.38	3.22
ICS 60	3.61	3.27	3.41	3.50	3.41	3.61	3.27	3.44
IMC 67	2.61	2.13	2.16	2.54	2.41	2.63	2.11	2.38
IMC 6	3.63	2.94	3.33	3.41	3.12	4.00	2.58	3.29
IMC 57	1.91	1.25	1.41	1.75	1.58	1.66	1.50	1.58
IMC 103	2.66	2.58	2.29	2.87	2.70	3.83	1.41	2.62
UF 11	2.91	2.33	2.66	2.54	2.66	2.83	2.41	2.62
Na 286	2.83	2.61	2.04	3.08	3.04	3.02	2.41	2.72
Means	3.09	2.75	2.72	3.04	3.01	3.21	2.63	
LSD _{0.05}	0.25		0.20			0.25		0.36

The reactions caused by the latter two concentrations were often but not always different from each other ($p < 0.05$). Resistance to *P. palmivora* can be assessed at 250,000 zoospores/ml or lower since the ranking of clones was not significantly affected and concentrations greater than this may be difficult to attain under sub-optimal conditions.

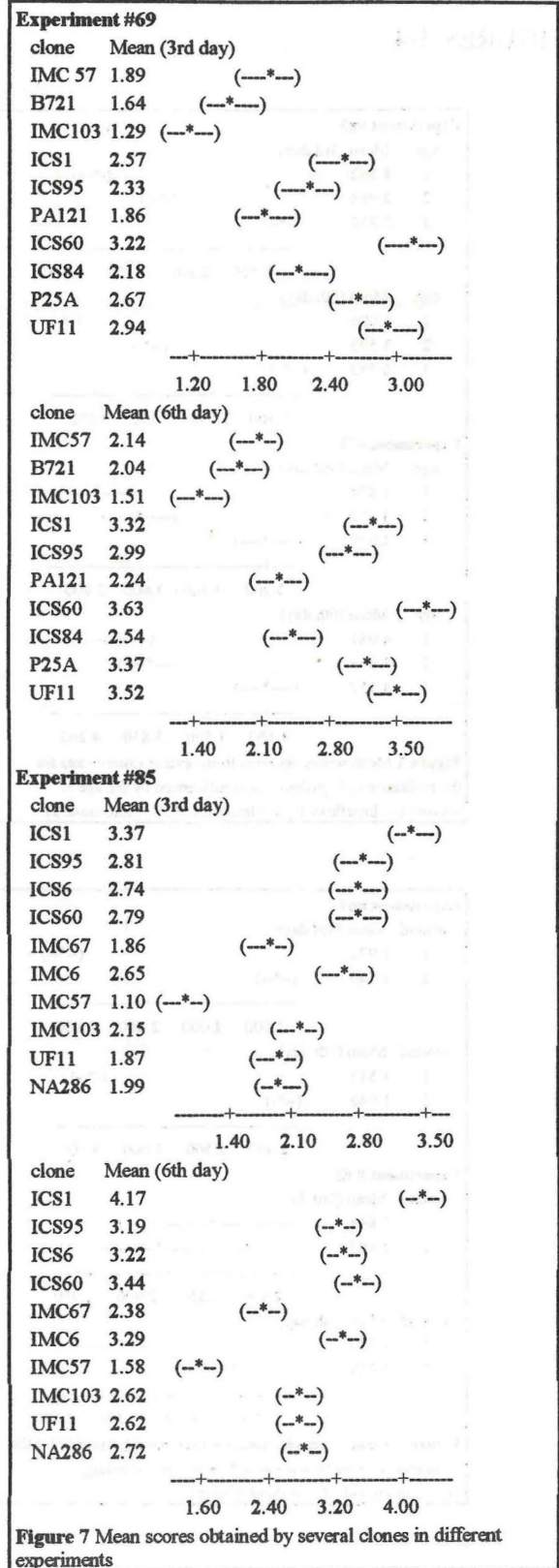
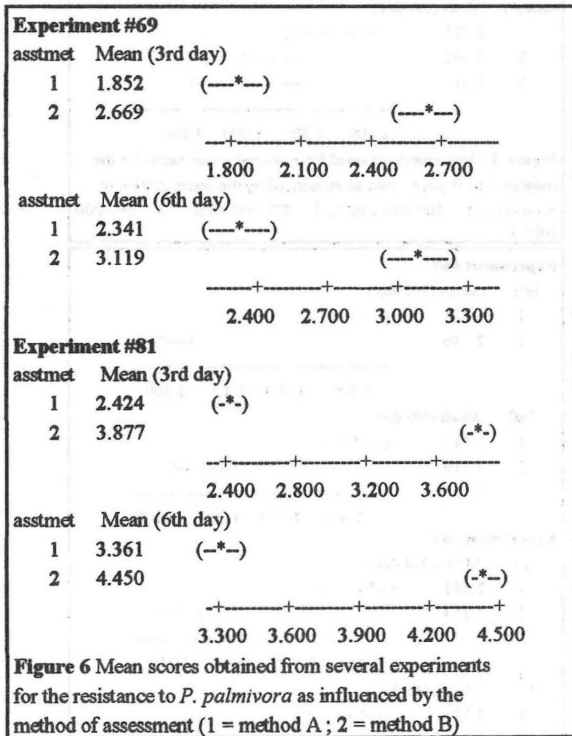
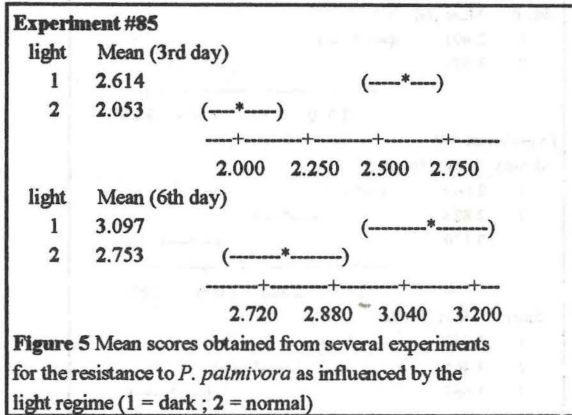
Leaf treatment: Higher disease indices were consistently obtained on discs compared to half-leaves ($P < 0.001$). In leaf bioassays, the resistance of whole, detached leaves was well correlated with attached leaves (Iwano, 1997). However, it appeared that as the integrity of the leaf is reduced (from half-leaves to 14 mm discs), there was a significant increase in the disease index. This may be explained by the involvement of anti-microbial compounds from surrounding as well as distant areas of the leaf in the defense reaction.

Light regime: Incubation in the dark resulted in significantly higher score indices ($P < 0.001$); the effect may be indirect since the relative humidity in the incubation chamber would be greater in the dark than the light.

FIGURES 1-4



FIGURES 5-7



Method of assessment: Higher disease indices were consistently obtained with method B compared to method A due to the fact that the former had maximum scores when coalescence was present. Method A relied primarily on the spatial development and pattern of the lesion(s). This method (A) is recommended since it produced valid results, took into account the pattern of symptom development, and was easier and more convenient to use since it did not require the estimation of lesion area or counts of lesions.

Clone: The clone effect was highly significant ($P < 0.001$) in all experiments. Ranking was hardly affected by the presence/absence of the wound, zoospore concentration, light regime, assessment method, leaf treatment, leaf age or time of assessment. The results invariably agreed with the known resistance levels of resistant clones observed in the field (IMC 57, B 721, IMC 103, PA 121) and susceptible clones (ICS 60, ICS 1, IMC 6).

However, some significant interactions were observed between clone and other factors such as leaf age, leaf treatment or the presence/absence of wounds. These emphasize two things; the importance of proper sampling and the number of replications. Leaves must be free from insect attack to avoid penetration of the pathogen through wounds and they must be sampled at a similar stage to avoid irregular responses due to physical and physiological changes during the maturation of the leaves. The number of discs should be enough to reduce the effect of the heterogeneity of the leaf as well as to allow a sufficient number of replications (thereby reducing the “tray” effect due to the variation in moisture content of the sponges).

Conclusion

The results obtained have enabled the standardisation of a test procedure for further trials:

Age of leaves: Interflush 2

Zoospore concentration: 250,000 zoospores/ml

Light regime: 12h daylight/12h dark

Plant material: Leaf discs

Wound: without

Scale: 0 : no symptom

1 : localised lesions

2 : small discrete lesions

3 : coalesced lesions

4 : uniform lesion

5 : large dark brown lesion

The score index is calculated as following:

$$I = (a/b) \times c, \text{ where}$$

a: 5th or 6th day score according to the experiment

- b: mean of the population during each trial
c: grand mean of the population over experiment

Resistance classes are assigned as:

0	≤	Index	<	1	:	very resistant (VR)
1	≤	Index	<	2	:	resistant (R)
2	≤	Index	<	3	:	moderately resistant (MR)
3	≤	Index	<	4	:	susceptible (S)
4	≤	Index	≤	5	:	very susceptible (VS)

Evaluation of the resistance to *P. palmivora* of the IMC 57 x Catongo progeny for the search of markers linked to resistance to *Phytophthora*

This study was performed jointly with the Biochemistry section.

Materials and methods

Two leaves (I2) were sampled from each seedling, and *Phytophthora* resistance was assessed with the leaf disc bioassay method as described above. One tray represented one replication and contained three discs from each plant, as well as three discs from each parent. There were five replications. Assessments were made three and six days after inoculation.

Results and discussion

From a total of 222 seedlings in the progeny, 170 (76.6%) were evaluated (1st trial: 49 plants; 2nd trial: 50 plants; 3rd trial: 46 plants; 4th trial: 25 plants). The other seedlings did not have leaves of a satisfactory condition at the time of sampling. The distribution of the ranked classes in the progeny is shown in Table 4 and is presented in Figure 8. The sixth day score was used in the Score index calculation to obtain resistant classes.

Means six days after inoculation :

IMC 57	:	2.59
Catongo	:	3.81
Progeny	:	2.76

Table 4 Number of seedlings according to their resistance level to *P. palmivora*.

Progeny	Number of plants evaluated	Resistance level				
		VR	R	M	S	VS
IMC 57 x Catongo	170	0	29	82	47	12

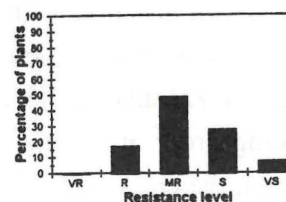


Figure 8 Distribution of *P. palmivora* resistance in the seedling progeny of IMC 57 x Catongo

The distribution of resistance to *P. palmivora* in the progeny of IMC 57 x Catongo appeared to be approximately normal. The majority of seedlings (48%) were moderately resistant.

All plants were cut back in November 1997. Two other assessments are planned in 1998 to confirm the individual plant resistance levels.

Phytophthora pre-breeding activities

Materials and methods

The distribution of resistance classes was assessed in the progeny of 10 selected crosses: ICS 84 x TSH 1077, UF 11 x SCA 9, IMC 65 x SPEC 194-103, EET 162 x NA 26, IMC 57 x TSH 1077, NA 45 x B 721, NA 45 x GU 175, B 721 x IMC 103, NA 45 x IMC 57 and EET 162 x SPEC 194-103. Two leaves (I2) were sampled from each seedling and *Phytophthora* resistance was assessed with the leaf disc bioassay method as described above. One tray represented one replication and contained two discs of each plant as well as two discs of the available parents and/or controls. There were 10 replications and assessments were done at three, five and seven days after inoculation. The fifth day score was used in the Score index calculation to obtain resistant classes.

Results and discussion

The numbers of seedlings per resistance class are presented in Table 5 and Figure 9. All five classes were observed and the distribution of resistance to *Phytophthora* was approximately normal. However, very resistant and very susceptible plants were few in number, and the progeny of the crosses ICS 84 x TSH 1077 and NA 45 x B 721 did not have any very resistant seedlings (Figure 9). Moderately resistant and susceptible seedlings were well represented among all progeny.

Resistant seedlings have been identified in the progeny of several crosses, of which IMC 57 x TSH 1077, NA 45 x GU 175, B 721 x IMC 103, NA 45 x IMC 57 and EET 162 x SPEC 194-103

appeared the most promising. The large numbers of resistant seedlings obtained in the cross IMC 57 x TSH 1077 were compatible with the known resistance of these two clones.

The contribution of parental effect to resistance was demonstrated by the crosses NA 45 x IMC 57, NA 45 x B 721 and NA 45 x GU 175. The second parent can be ranked (from IMC 57 to GU 175) in descending order of resistance, and this order was coincident with the mean score ranking of the progeny from the crosses (Table 6).

Breeding programmes usually generate more “unwanted” (susceptible plants) than “desired” (resistant) plants. Thus, the leaf bioassay test, which can be performed on seedlings, is useful for screening large numbers of seedlings in order to identify resistant and very resistant individuals. This allows the breeding programme to be accelerated, this being particularly important for cacao, a perennial tree crop. However, the plant material was only tested with a locally available *Phytophthora* isolate, and the susceptibility of the seedlings to other isolates of the same species or to other *Phytophthora* species could vary. Thus, all highly promising material should be tested with local isolates in other countries before being included in further selection programmes.

All the progeny of these 10 crosses were cut back in January 1998 to allow a second assessment, and a third assessment is planned for the most resistant plants.

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Table 5 Number of plants classified by their resistance level to *P. palmivora*.

Progeny	Number of plants evaluated	Resistance level				
		VR	R	MR	S	VS
ICS 84 x TSH 1077	64	0	3	20	38	3
UF 11 x SCA 9	73	2	8	45	13	5
IMC 65 x SPEC 194-103	81	1	2	13	54	11
EET 162 x NA 26	73	1	8	51	12	1
IMC 57 x TSH 1077	78	6	27	22	22	1
NA 45 x B 721	72	0	6	45	18	3
NA 45 x GU 175	60	3	13	31	11	2
B 721 x IMC 103	72	2	13	44	9	4
NA 45 x IMC 57	71	1	14	42	12	2
EET 162 x SPEC 194-103	84	2	10	59	8	5

Table 6 Mean score of *P. palmivora* resistance five days after inoculation of leaf discs.

Crosses		Parents/Controls	
Progeny	Score	Clone	Score
ICS 84 x TSH 1077	2.28	ICS 84	3.7
UF 11 x SCA 9	2.55	NA 45	2.7
IMC 65 x SPEC 194-103	2.51	GU 175	2.5
EET 162 x NA 26	2.76	B 721	2.0
IMC 57 x TSH 1077	2.74	IMC 57	1.7
NA 45 x B 721	3.09	Catongo	2.2
NA 45 x GU 175	3.37	IMC 103	1.9
B 721 x IMC 103	2.57	UF 11	3.4
NA 45 x IMC 57	2.64	ICS 1	3.2
EET 162 x SPEC 194-103	2.46	IMC 67	3.1

FIGURE 9

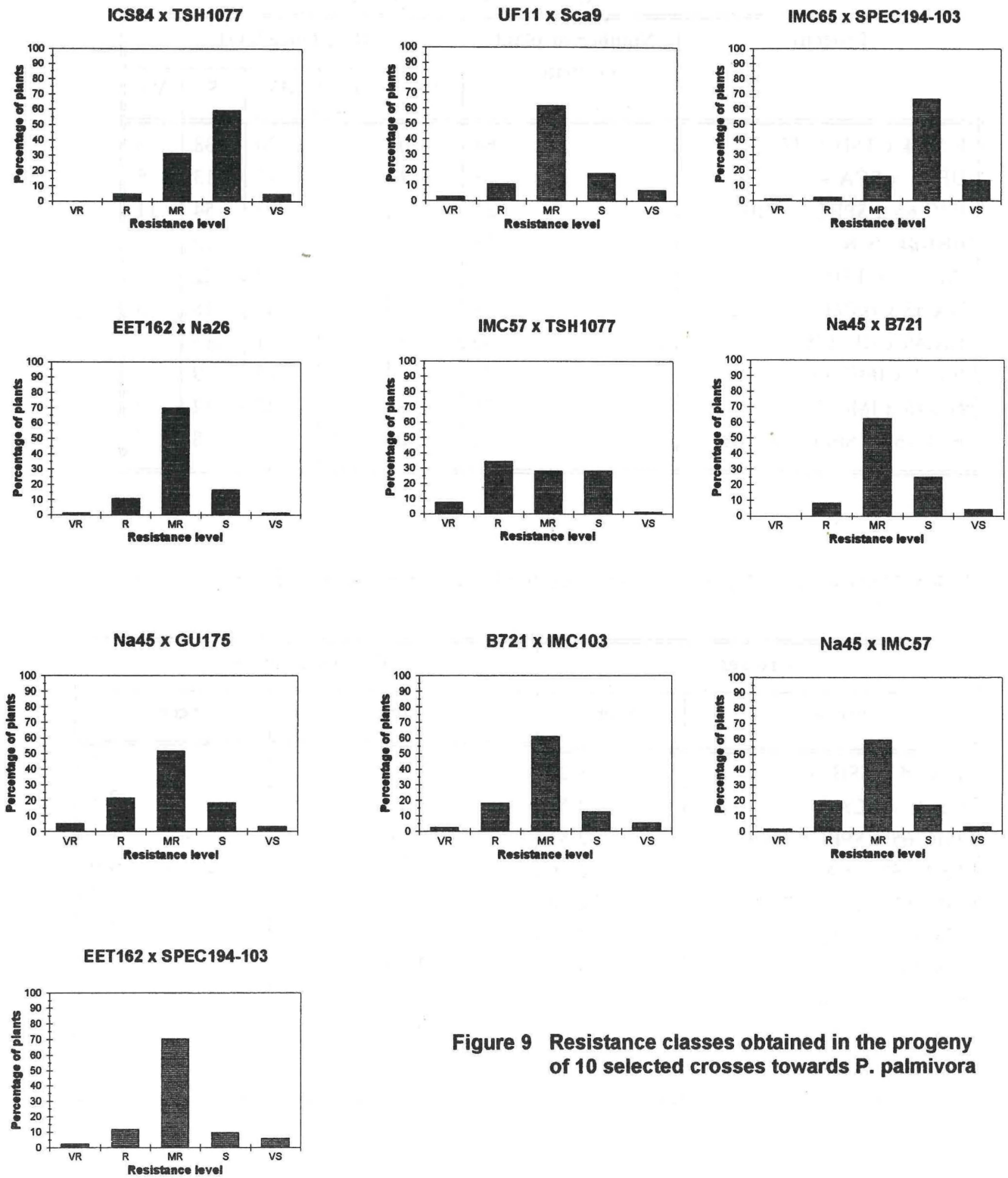


Figure 9 Resistance classes obtained in the progeny of 10 selected crosses towards *P. palmivora*