Planting material screening by controlled inoculation

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It is not possible to distinguish rapidly and reliably between planting materials for their field performance in relation to different species of *Phytophthora* by observing natural infection in the field. Indeed, to obtain reliable data from natural infections, it is necessary to set up field trials with appropriate statistical designs, wait for the first sample yields (around four years after planting), then collect at least four years' data on natural infections occurring in the field. It thus takes eight years to obtain a more or less reliable classification of genotypes and parents for mating designs. Regionalized effects may also disrupt the reliability of results during assessments under natural infection conditions, and attack rates are not always sufficient, notably with *P. palmivora,* to obtain contrasting results on the planting material being tested. For these reasons, researchers have attempted to develop tests to assess differences in resistance between genotypes—clones or crosses as effectively as possible. Such tests involve artificially inoculating different organs of the plants being tested. Among the organs tested, particular attention has been paid to pods, the organs targeted by the disease, and leaves, which are available from a very young age.

One of the aims of this project was to develop an early, reliable test of resistance to the disease. After describing the pod test, we shall go on to see how the resistance test using leaf discs was developed. The protocol is described, and the adjustments that are made, depending on selection objectives and on the populations undergoing selection, are then discussed.

Pod test

This chapter gives a brief history of the different methods used to test pod resistance. It then details the improvements made during the CAOBISCO project, along with the conditions under which this test is applied.

Background

The disease mainly develops on pods, causing substantial yield losses, so the first work on tests to assess resistance began on pods. Pod tests can be carried out in different ways: pods still on the trees or removed, with or without wounding; several inoculums and symptom assessment methods can also be used. The main methods used to test fruit reactions to the pathogen are briefly described.

Attached pods (*in situ*) or detached pods

Most of the tests were developed on detached pods, in the laboratory, so as to be able to control the main environmental conditions mainly temperature, humidity and lighting, and to ensure good repeatability of successive tests. All the work indicated that detached pods were more susceptible than pods left *in situ* on the tree. In Brazil, Rocha and Mariano (1969) showed that resistance in the Catongo cultivar was considerably less when pods were inoculated after being removed from the tree. On Amelonado, Blaha (1967) showed on several occasions that pods were more susceptible after harvesting. Tarjot (1969) also obtained indications of greater susceptibility when detached pods were inoculated in the laboratory. In Trinidad, Iwaro *et al.* (1997*b*) also reported results indicating the greater susceptibility of detached fruits.

In Ghana, Wharton (1960) preferred working on pods *in situ*. According to him, inoculating pods still on the tree was the best way of reducing variations in performance induced by pod harvesting. The main advantage of testing attached pods rather than laboratory inoculations was to work on fruits that expressed physiological reactions during the test that were closest to reality under natural infection conditions.

The degree of *in vitro* fruit resistance under laboratory conditions can be used to indicate a higher level of resistance in the field (Lellis and Peixoto, 1960). However, given the greater susceptibility of detached pods, the different degrees of resistance become less apparent, since the scale of reaction is compressed.

In order to try to combine the advantages of both methods, some authors (Blaha, 1967 and 1971; Dakwa, 1968) developed ways of assessing attached pods that minimized the impact of environmental conditions on test repeatability. After inoculation, the pods were wrapped in plastic bags, with or without perforations, to protect the inoculum from adverse climatic conditions over the first two days. Symptoms were expressed more rapidly when the bags were not perforated.

In 1967, Medeiros recommended carrying out simultaneous inoculations for the same tree on pods left on the tree and on pods that had been removed. In such cases, the field had to be near the laboratory, so that all the pods could be inoculated with the same inoculum possessing equivalent viability. In such a context, it can also be difficult to find enough pods to carry out both types of inoculation on one tree.

Testing pods still on the tree also means that any fungicide treatment is impossible in the plot for the duration of the assessment.

Prendergast (1965) used an assessment method involving square pieces of pod (5 cm x 5 cm) from the equatorial zone of mature pods. The blocks were inoculated with a zoospore suspension. The criterion studied was the successful infection rate. Spence and Bartley (1966), along with Rocha and Vello (1971), also used this method, which gave results similar to those obtained with inoculations on detached pods without wounding; using pieces of pod made it possible to test a larger number of replicates.

Pods inoculated with or without wounding

Pods were wounded, by removing the epidermal barrier, so that the pathogen could penetrate the tissues. This was generally carried out by making a circular hole of standard diameter and depth in the pod cortex. The inoculum, consisting of a fragment of infected pod (Turner, 1963), or a pure *Phytophthora* culture disc (Spence, 1961*a*, 1961*b*; Prendergast, 1965; Tarjot, 1965) was then inserted into the hole. The criterion observed for cortex tissue reaction to this type of inoculation was the size of the patches developing several days after inoculation (Turner, 1963; Prendergast, 1965; Tarjot, 1965), or an estimation of the degree of sporocyst production (Turner, 1963).

Some authors (Orellana, 1953*a,b*; Wharton, 1957; Tarjot, 1967*a,b*) showed that it was preferable to assess pod resistance to *Phytophthora* on unwounded pods. Indeed, the disease developed immediately after wounding, but when the epidermis remained intact, an incubation period was necessary before infection symptoms could be seen (Tarjot, 1967*a*). In addition, the pods of certain clones became susceptible after wounding, whether attached or detached: SIC 28 (Thorold, 1955), P 7 (Prendergast and Spence, 1967); SIC 802 and 848 (Rocha and Mariano, 1969). Wounding therefore appeared to favour the pathogen by eliminating one of the essential elements of fruit resistance: resistance to penetration. Some clones resistant to penetration might therefore be overlooked during selection if the tests were carried out with wounding. The deeper the wound, the lower was the resistance of certain clones. For example, with a depth of 1 mm, Sca 6 was highly resistant, but became more susceptible with a depth of 4 mm and was classed as highly susceptible with a depth of more than 8 mm (Rocha and Vello, 1971).

However, when the test without wounding was used alone, it did not reveal the degree of pathogen propagation in cocoa tree tissues (resistance to post-

penetration). This situation led Blaha (1974) to recommend using both methods simultaneously, so as to be able to class clones in different categories: clones resistant to penetration and post-penetration, clones resistant to penetration only, clones resistant to post-penetration only, and susceptible clones.

Susceptibility criteria used

In Ivory Coast, Tarjot (1967*a*, 1969) invented a susceptibility index that took three parameters into account simultaneously: the percentage of successful infections, the incubation period (time lapse between depositing the drop of zoospore suspension and patch development) and the speed with which the patch spread (the departure point being the date on which the patch began to develop, not the inoculation date). Inoculations were carried out on unwounded pods, using a zoospore suspension. This work primarily concerned resistance to penetration. Various authors in Trinidad (Prendergast, 1965; Iwaro, 1995) and Brazil (Rocha and Mariano, 1969) estimated the number of lesions formed after spraying zoospores onto unwounded pods. Disease severity was scored 4 days after inoculation on the following scale (Iwaro *et al.*, 2000).

1: no symptoms (resistant to penetration)
2: 1 to 5 localized lesions (resistant)
3: 6 to 15 localized lesions (moderately resistant)
4: 15 localized lesions (partially resistant to post-penetration)
5: 1 to 5 expanding lesions (resistant to penetration only)
6: 6 to 15 expanding lesions (moderately susceptible)
7: 15 expanding lesions (susceptible)
8: coalescing lesions (highly susceptible)

However, with this type of inoculation, it could be difficult to separate clones that were highly resistant to penetration and post-penetration from those with only strong resistance to penetration, especially if a low inoculum concentration were used.

Other authors (Blaha, 1971; Turner, 1963; Tarjot, 1965) proceeded with droplet inoculations with or without wounding, and used the percentage of successful infections, the daily rate of patch expansion, or the necrotic area of the patch on different days (from 2 to 11 days after inoculation) as the criteria for assessing resistance. In 1996, Luz *et al.* only measured lesion size once the average patch diameter on the pods of the susceptible control reached 5 cm, which was a fixed point for the different replicates. They optionally observed the frequency with which lesions were obtained 2 days after inoculation (the number of infection points per inoculation site was assessed on a scale of 0 to 5).

In 1963, Turner estimated the sporocyst production rate by washing necrotic areas each day and counting the sporocysts with a hematocytometer.

Numerous microscopic examinations of the pod epidermis (Tarjot, 1972*a*) did not reveal any correlations between the susceptibility index and the number of

stomata or epidermal hairs present on the pod. However, Iwaro *et al.* (1997*a*) did find a positive correlation between both lesion and stomatal frequency.

Host x parasite interactions

The resistance levels found in inoculation tests could seem different from one country to the next for the same clone. Whilst the methods used were the same, one factor that might have been involved was the degree of aggressiveness of the strains used. Consequently, before using such a screening test on a large scale it was necessary to assess any host x parasite interactions.

Chowdappa and Chandra Mohanan (1994) compared the aggressiveness of 5 strains of *P. capsici* on pods removed from 20 clones, by inoculating them with a mycelium disc, with and without wounding the pod tissues. A highly significant host x parasite interaction was found for lesion size and for the abundance of aerial mycelium when pods were wounded prior to inoculation.

Luz *et al.* (1996) evaluated the resistance of 82 cocoa tree genotypes to 3 species of *Phytophthora* encountered in Brazil (*P. capsici, P. palmivora* and *P. citrophthora*) using an inoculation test on detached pods, without wounding. All in all, *P. citrophthora* and *P. capsici* proved to be the most and the least aggressive respectively. Only 4 resistant clones (PA 30, PA 150, SIC 864 and SIAL 105) and 6 susceptible clones (AM 2, BE 3, BE 5, MA 11, TSA 644 and SIC 23) displayed the same reaction to the 3 species. Moreover, those authors specified that a major variation was found in the degrees of resistance to the 3 species of *Phytophthora,* thereby suggesting that *Phytophthora* clone x species interactions do exist: such an example was SCA 6 and EET 59, which were resistant to *P. palmivora* and *P. capsici*, but susceptible to *P. citrophthora*. Yet, in Trinidad and Tobago, a study conducted by Iwaro *et al.* (1998), on 10 clones using a strain of *P. palmivora* and a strain of *P. capsici* to inoculate unripe but adult-sized pods, showed that there was no species x clone interaction, and the clone classification based on lesion size was only slightly modified depending on the species used. Nevertheless, *P. palmivora* proved to be more aggressive.

Pod test carried out in Cameroon with *P. megakarya*

This work was undertaken by Nyassé (1997) and Flament (1998).

Preliminary trials

Preliminary trials were carried out to choose the *P. megakarya* strain and the inoculation method.

Choice of strain

Wounded fruits of the same cocoa genotype were inoculated with five strains of *P. megakarya*, at a rate of 5 fruits per strain, and 1 inoculation point per fruit.

The growth rate of the strains was obtained by measuring the diameter of the patches each day from day 3 to day 7 after inoculation. The strain with the average growth rate was chosen for further testing on the NS 231 planting material. In fact, that strain provided better discrimination between the individuals of a given progeny for their resistance, notably during QTL searches.

Choice of inoculation technique

Four inoculation methods (2 with wounding and 2 without wounding) were compared on the fruits of 4 cocoa clones (SNK 413, SNK 10, UPA 134 and ICS 84). These clones were the parents of the progenies studied in the search for resistance quantitative trait loci (QTL). The 4 methods were combined with 3 inoculum concentrations, i.e. a total of 12 inoculation techniques were tested.

Curatest type (drop of inoculum applied to the unwounded pod and protected with a Curatest type adhesive strip)

1	5×10^5 zoospores/ml
2	8×10^5 zoospores/ml
3	1×10^6 zoospores/ml

Plasticene cup type (the drop of inoculum was deposited in a small plasticene cup against the pod surface, without wounding)

4	5×10^5
5	8×10^5
6	1×10^6

Wounding with a nail (the inoculum was deposited in a hole made with a 5-mm long nail)

7	5×10^5
8	8×10^5
9	1×10^6

Wounding with a cork borer (the inoculum was deposited in a hole made with cork borers of different diameters, but at a standardized depth)

10 (4 mm diameter)	5×10^5
11 (6 mm diameter)	5×10^5
12 (7 mm diameter)	5×10^5

Technique 7 was the most discriminating. The classification of clones obtained by artificially inoculating clones with this technique was identical to that obtained by observing their field performance under natural infection conditions. With both assessment systems, UPA 134 was the least susceptible and SNK 10 was the most susceptible.

A positive correlation was found between the methods with wounding and the methods without wounding (e.g. technique 4 was correlated to technique 7) for

the 4 clones studied. The clone-concentration interactions for the 12 techniques studied were not significant.

Technique 7 was the easiest and fastest technique to use when compared to the Curatest, where the adhesive strips sometimes had trouble sticking to the surface of the pod, and the plasticene cup test, which required lengthy preparation. It was very difficult to achieve a constant wound depth with the cork borer, while it was easier using a nail with a fixed length. In this protocol, the nail passed through a plank and only protruded 5 mm on the other side, so the nail could only penetrate the pod to that depth.

Application

The test on attached fruits could be applied using inoculation techniques with or without wounding indifferently, provided the inoculum strain and concentration chosen gave rise to symptoms.

As this test appeared to be highly sensitive to environmental conditions (the results considerably differed from one plot to another and from one set of inoculations to the next), it was necessary to perfectly control environmental conditions, or to develop a test on detached pods under perfectly standardized conditions from one replicate to another.

Development of the pod test

Methodology used for the pod test (PT) on 12 clones and a progeny

Implementation of these pod tests confirmed their main drawbacks: they could only be applied in a production period and the number of replicates depended on the number of fruits available for inoculation.

The test was carried out on mature and immature fruits, around 4 months old. The technique used was inoculation with a drop of zoospore suspension deposited in holes (2 mm in diameter and 5 mm deep) made in the cortex, on the median section of the fruit, using a nail (technique 7). Each fruit had two infection points located opposite each other on the largest diameter, so as to facilitate the measurement of lesion diameters. Inoculation involved depositing 30 µl of a calibrated suspension of 3×10^5 zoospores/ml in each hole forming an infection point. The hole was then plugged with plasticene for at least 24 hours.

This inoculation technique with wounding was used to assess susceptibility using fruits of 12 clones in the same locality but spread over several plots; 5 clones (SNK 10, SNK 413, ICS 84, ICS 95 and UPA 134) were in the same trial planted in a totally randomized single-tree plot design (plot called IRAD) and 7 clones (SNK 13, SNK 30, SNK 64, ICS 1, T 79/467, Sca 12 and UPA 143) spread over different plots, planted in row designs (plots called PSCC). This test was also used to assess the susceptibility of the individuals of the progeny (UPA 134 x ICS 84) derived from a 6 x 6 diallel trial planted in 1974 at Barombi-Kang.

The 12 clones were tested at a rate of 10 trees per clone, 5 fruits per tree and 2 inoculation points per fruit. The hybrids were tested in 3 series of inoculations at a rate of 18 fruits per tree (5 fruits for the first and third series, 8 for the second) and 2 inoculation points per fruit. The parent clones, UPA 134 and ICS 84, were inoculated for each series of inoculations to serve as controls.

Strain NS 231 was kept from one year to the next on a V8 culture medium in a Petri dish and regularly subcultured on pods so as to preserve a constant level of aggressiveness for that strain.

Inoculations were carried out either on fruits left on the tree, in which case incubation took place under natural conditions, or on detached fruits, in which case incubation took place in the laboratory at 26-28°C.

Resistance traits were studied by carrying out daily observations from the third to seventh day after inoculation, and more precisely by measuring patch width (in millimeters) to determine its mean diameter; at the same time, the intensity of fungus sporulation on the pods was quantified.

Statistical analyses were carried out with SAS software using the generalized linear model (GLM) procedure. Heritability values were calculated with optimally partitioned electric properties (OPEP) software (Baradat and Labbé, 1995).

Results of the study on 12 clones

A principal components analysis (PCA) showed, for the same clone, that the observations (patch diameter, sporulation intensity) carried out on attached fruits, 3, 4, 5, 6 and 7 days after inoculation, were positively and significantly correlated (correlation > 0.89 for patch diameter and correlation > 0.83 for sporulation intensity). Sporulation intensity on the fruit was also positively and significantly correlated to patch diameter (correlation of between 0.65 and 0.96). This suggested that these variables could be considered linked to each other. The strong correlation found between the average diameters of patches on fruits, 3, 4, 5, 6 and 7 days after inoculation ($r = 0.89$), suggested that expansion between 3 and 7 days was correlated to the measurement obtained on one of those different dates, e.g. 7 days (table 1).

Repeatability of the pod test was studied in the IRAD plot on the 5 clones. Ten trees were tested per clone. Repeatability was estimated from broad sense heritability (h^2 = genetic variance/phenotypic variance) calculated from the genetic analysis of the 5 clones. For the pod test, the heritability values were 0.62, 0.63 and 0.56 for the mean diameter at 3, 5 and 7 days respectively. These high heritability values suggested that selection could be effective for these criteria, so long as trial conditions were sufficiently uniform. A strong genetic effect was therefore detected by this test.

Results of the study on progeny UPA 134 x ICS 84

The 60 individuals of the progeny used came from two reciprocal crosses (UPA 134 x ICS 84 and ICS 84 x UPA 134). An analysis of variance on fruits was

carried out on the fifth day of observations after inoculation. These two reciprocal crosses were not significantly different for their reaction to the test on attached fruits (table 2); this made it possible to consider these two families as a single progeny, so as to increase the overall number of individuals. A significant difference was found between trees, and between fruits on the same tree.

Table 1. Average diameter of the rot patch developed on the pods of 13 cocoa tree clones, 7 days after inoculation.

Clones	Average patch diameter (mm) at 7 days	
IRAD Plot		
UPA 134	47.62	a
SNK 413	52.80	a b
ICS 95	65.46	b c
ICS 84	75.95	c d
SNK 10	83.76	d e
PSCC Plot		
ICS 1	91.67	d e f
Sca 12	100.33	e f g
T 79/467	102.97	e f g
SNK 13	110.66	f g h
SNK 64	119.39	g h
UPA 143	119.95	g h
SNK 30	123.64	h

Values followed by the same letter are not significantly different at 5% according to the Newman-Keuls test.

Table 2. Results of the analyses of variance on rot patch measurements on the pods of 60 hybrid plants (UPA 134 x ICS 84), 5 days after inoculation.

Sources of variation	DF	ESS	MS	F	S
Estimation of the family, tree and pod effects					
Family	1	18.98	18.98	22.23	NS
Tree (family)	77	5758.5	74.78	14.1	**
Pod in tree	663	3516.7	5.3	6.21	**
Error	735	627.7	0.85		
Estimation of the series and tree effects of the pod test					
Tree	78	5232.8	67.08	35.39	**
Series	1	1016.5	1016.5	536.3	**
Tree x Series	62	423.3	6.82	3.6	**
Error	1335	2530.4	1.89		
Estimation of the plot and tree effects of the pod test					
Plot	1	1853.5	1853.5	625.23	**
Tree (plot)	77	3971	51.57	17.4	**
Error	1398	4144.4	2.96		

DF: degrees of freedom
ESS: estimated sum of squares
MS: mean square
F: F-ratio
S: Level of significance
NS: not significant
**: significant at 5% level

Table 2 shows that there was an effect linked to the test series. The tree effect in each series was highly significant, and significant interactions were found between the trees and the inoculation series. The progeny was in two plots (A and B). Plot and tree effects in each plot were highly significant. The mean patch diameters on the fruits were significantly higher for plot A (88.2 mm) than for plot B (55.6 mm), hence a strong environmental effect was involved in symptom expression on pods. Plot A had uniform shade, whereas plot B had heterogeneous shade and a large number of missing trees. Regarding parents of the progeny, clone UPA 134 appeared to be more resistant than parent clone ICS 84, on the fifth day after inoculation (patch diameter of 1.21 mm and 3.04 mm respectively).

Screening for resistance on the attached pods of a progeny by artificial inoculation was dominated by the extent of plot effects. The inoculation series effect explained a large share of variation, as did the tree effect. The significant, or even highly significant, estimation of environmental effects influencing the expression of resistance traits justified adjusting the resistance data in relation to those effects, so as to estimate the genetic share of tree resistance more effectively. Thus assessment of a progeny using the pod test needed to be done in strict and, if possible, balanced experimental designs.

Consequently, a study on the effect of some parameters that might affect the degree of tree response needed to be taken into account, e.g. pod age. It is difficult to obtain pods of the same age, and carrying out controlled pollinations only partly solves the problem since pods ripen at different times for each clone. Luz *et al.* (1996) recommended recording the formation of new cherelles each week on the trees to be studied. The importance of other resistance traits—such as fruiting periods and their length, which might be likened to disease avoidance factors—could also be more effectively quantified. Morphological components, such as pod shape, the existence or absence of epidermal hairs might be involved, for example, in water retention on the surface, hence in greater pathogen development.

Pathogenicity on fruits of isolates from clones with different susceptibility

In this study, we examined the pathogenicity of fifteen isolates of *P. megakarya* (NS 310 to NS 324) in relation to 5 clones. These clones had been planted in the biclonal seed gardens at Barombi-Kang for more than 20 years. Each plot had two clones planted in alternate rows. The fruits of 5 clones (Sca 6, SNK 64, ICS 84, SNK 413 and SNK 10) were cross-inoculated with 3 isolates taken from the same clones, in plots where those clones were in the majority. This design was to enable detection of any pathogen adaptation to clone resistance. The reaction of the fruits of these clones to artificial inoculations and to natural infections was known: SNK 10 was the only one of the five considered to be susceptible.

Detached fruits from these 5 clones were inoculated with a 30 µl drop of a suspension calibrated at 5×10^5 zoospores/ml. Inoculations were carried out on fruits

wounded by technique 7, and observations were made daily from the third to seventh day after inoculation, measuring patch length and width.

The results of the analysis of variance on the mean patch diameter (table 3) showed a highly significant clone-and-isolate effect in the 2 replications. It turned out that the replicate effect found on the third day after inoculation was no longer significant on the fifth and seventh days. No interaction was found between isolate and clone. However, in trial 2 there was an isolate x clone interaction on the fifth day, but it disappeared on the seventh day, and the clone classification on the fifth day was not significantly modified by the isolate.

Table 3. Analysis of variance for the pod test combining the 2 trials, F test and significance limits.

Source		F test (group of 3 isolates from the same clone)		
	DF	3 days	5 days	7 days
Trial	1	42.91*	0	0.01
Clone	4	91.52*	90.56*	27.48*
Isolate	4	6.10*	7.32*	33*
Clone x Isolate	16	1.06	0.70	0.91
Trial x Clone	4	3.02*	5.46**	3.81**
Trial x Isolate	4	5.28*	71**	2.98*

DF: degrees of freedom; * : significant at 5% level; **: significant at 5% level.

The scores and classifications of the 5 clones and of the groups of 3 isolates from the same clone are shown in table 4.

Table 4. Mean patch diameter (mm) at 5 days, developed on pods of cocoa tree clones during 2 trials.

Groups	Mean diameter
Clone	
SNK 64	167.07 a
Sca 6	149.71 b
SNK 10	146.65 b
ICS 84	145.77 b
SNK 73	128.60 c
Origin of the groups of 3 isolates	
1 (SNK 10)	155.54 a
2 (SNK 64)	150.61 a b
3 (SNK 413)	145.35 b
4 (ICS 84)	143.98 b
5 (Sca 6)	142.35 b

Values followed by the same letter are not significantly different at 5% according to the Newman-Keuls test.

Clone SNK 64 was the most susceptible clone, whereas SNK 413 was the most resistant. Clone Sca 6 had the same level of susceptibility as another susceptible clone, SNK 10. This was undoubtedly due to the type of test used with wounding,

which eliminated the high degree of resistance to penetration. The leaf test on clone Sca 6 with the same strains revealed, without wounding, that the level of resistance in this clone was high. It would therefore be very important to repeat this type of study, with wounding and without wounding, to truly determine the degree of clone resistance, and also the degree of isolate aggressiveness on penetration and post-penetration. During this study, the strains from clone SNK 10 were the most aggressive, although no significant difference was found between the different groups of isolates during trial 2.

Pod test carried out in Ivory Coast (*P. palmivora*)

A study on the correlation between pod susceptibility in the field and pod susceptibility in the greenhouse was carried out at the CNRA (ex IDEFOR-DCC) station at Bingerville (Tahi, 2003). It involved 45 hybrid trees and 2 control clones: IFC 5 (susceptible) and P 7 (resistant).

Five immature adult pods were taken from each of the 45 trees assessed and placed in trays together with control pods. All the pods were inoculated, with wounding, using 30 µl of a zoospore solution calibrated at 5×10^5 zoospores/ml.

In the field, around ten wounded pods were inoculated on each hybrid with 30 µl of a zoospore suspension (5×10^5 zoospores/ml).

Observations began on the second day after inoculation, in the greenhouse and in the field. They consisted in measuring the large and small diameter of every rot patch each day, until the pod had been totally invaded.

There was a significant correlation between the patch propagation rate on pods in the greenhouse and that on pods in the field. The correlation was also significant between the total area invaded on pods in the greenhouse and in the field.

However, the areas on the fifth day and the patch expansion rates were significantly greater on the pods in the greenhouse than on the pods in the field: 151 cm^2 for 7.67 cm^2 and 92 cm for 3.82 cm respectively. This result confirmed the greater susceptibility of detached pods compared to that of pods left on the tree.

Pod test carried out in Trinidad and Tobago (*P. palmivora*)

Methods for assessing pod susceptibility to *P. palmivora* were studied at the Cocoa Research Unit plant pathology laboratory. Five pods were taken from trees of the 45 clones studied and inoculated under standard laboratory conditions. Three series of inoculations were carried out.

One inoculation was carried out on each pod, without wounding, by depositing a calibrated drop of zoospore suspension (3×10^5 zoospores/ml), and another inoculation was carried out opposite, wounding to a depth of 3 mm and

depositing a drop of calibrated suspension. The inoculated pods were placed on damp sponges in trays with a lid, to maintain constant humidity, and kept in the dark at 25°C. The areas of the patches forming on the pods were measured 6 days after inoculation.

The areas of the rot patches occurring during pod inoculations with wounding (PODWW) and with no wounding (PODNW) were significantly correlated. However, the Catongo, P 25A, ICS 40 and B 184 clones had a comparatively lower susceptibility level with wounding than without wounding (figure 1). In these clones, it may have been that wounding induced greater defence mechanisms than for all the other clones tested, which might have hindered subsequent pathogen development.

Clones GU 265, GU 305, Sca 11 and JA 59, however, had a comparatively higher susceptibility level with wounding than that obtained without wounding. In these clones, wounding apparently enabled the resistance mechanisms expressed on the surface (i.e. in terms of penetration) to be bypassed.

The merits of carrying out a pod test with and without wounding are clearly shown in figure 1. Many clones were resistant to penetration (PODNW < 2 mm^2), but in the test with wounding, those clones could be separated according to their level of resistance after penetration, thereby enabling a more effective assessment of clones resistant to penetration. GU 305 and Sca 11 also had a high level of resistance to penetration, but the test with wounding revealed an average level of resistance at the post-penetration stage.

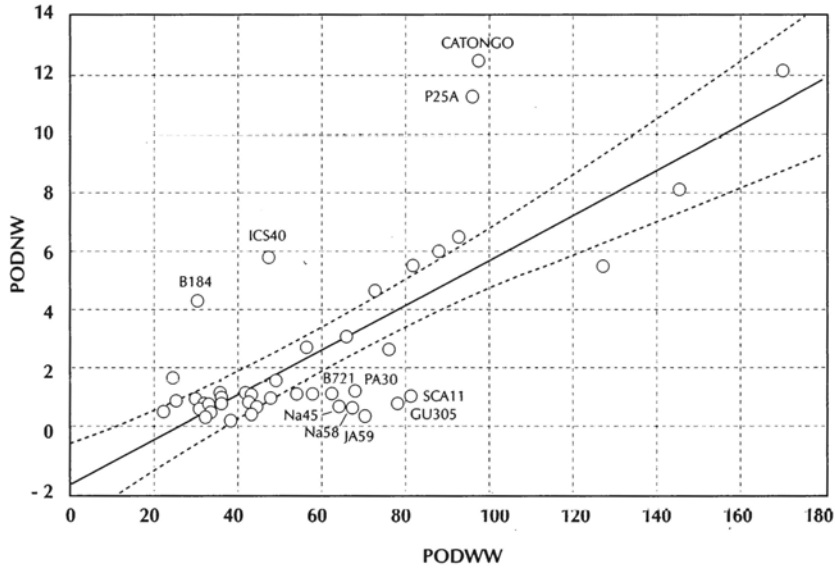


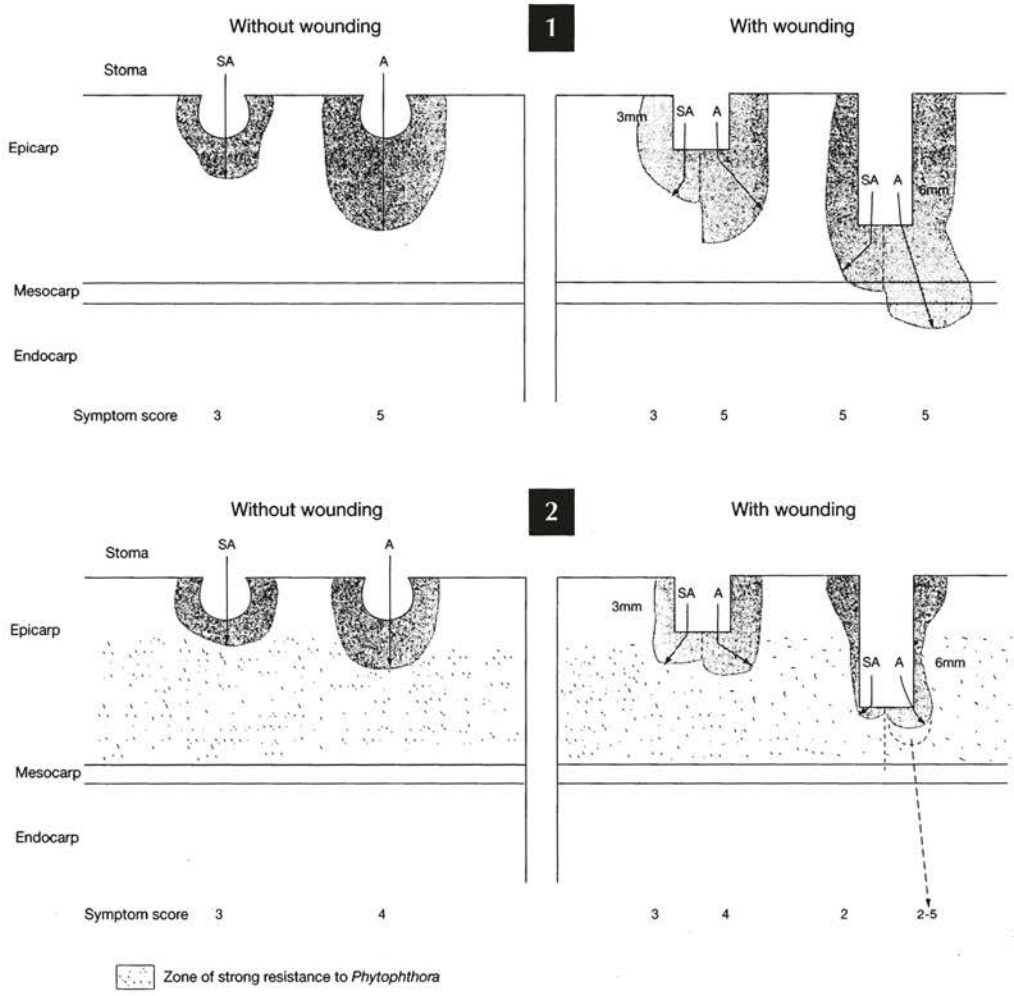
Figure 1. Comparison of areas (cm^2) necrotized by P. palmivora *on pods 6 days after inoculation with wounding (PODWW) and without wounding (PODNW).*

Conclusions on the pod test

For a given clone, it was very difficult to compare the resistance levels obtained with the pod test, as different methods were used. Figure 2 provides a clearer understanding of why the level of resistance for some clones was totally different depending on the method used.

For a clone susceptible to penetration and post-penetration (figure 2.1), the same score was obtained 5 days after inoculation, with or without wounding, irrespective of strain aggressiveness.

For a clone susceptible to penetration and resistant to pathogen spread within the plant tissues (figure 2.2), the same score was obtained, with and without wounding, irrespective of pathogen aggressiveness. However, if the wound was



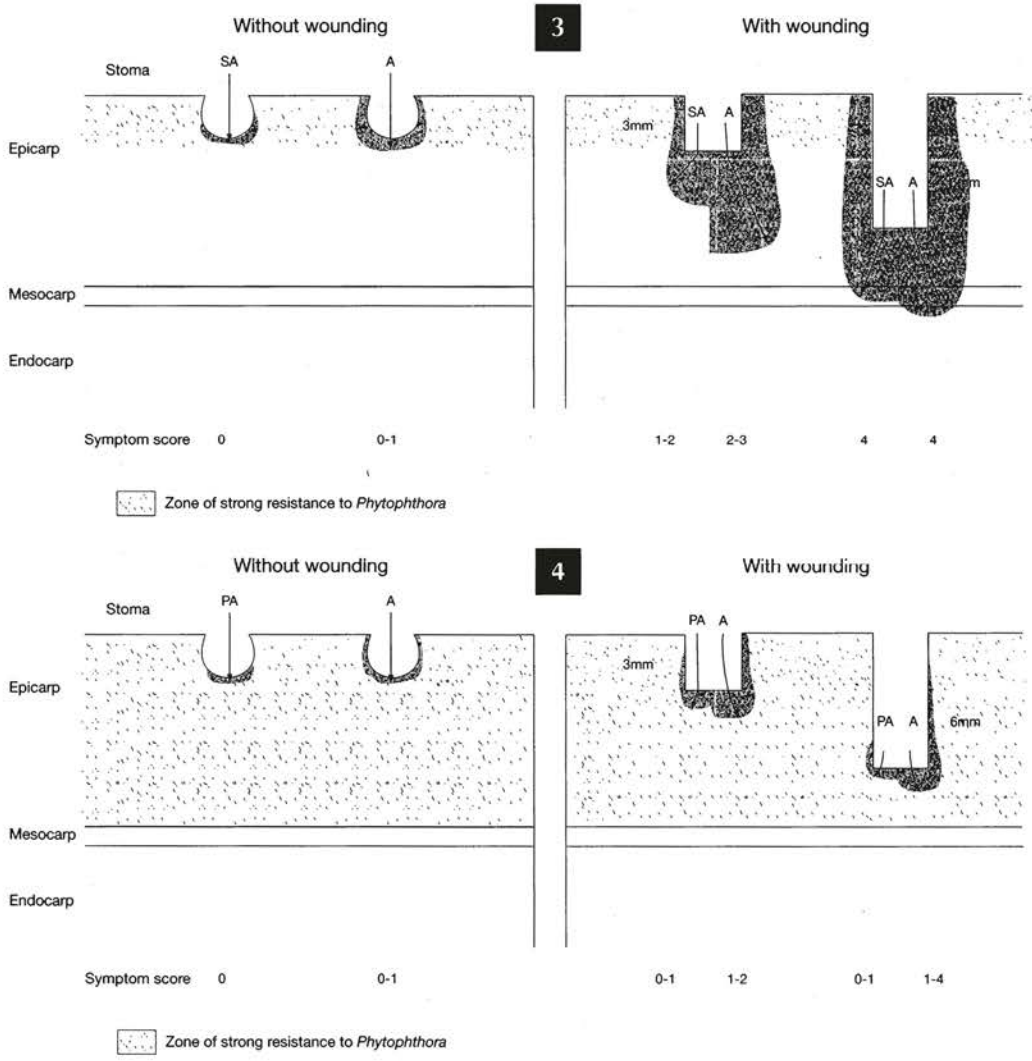


Figure 2. Symptom intensity (scale of 0 to 5) depending on the type of pod inoculation (with or without wounding) and on the aggressiveness of the Phytophthora *strains (SA: slightly aggressive; A: aggressive).*

1. Clone susceptible to the penetration and post-penetration stages.
2. Clone susceptible to the penetration stage and resistant to the post-penetration stage.
3. Clone resistant to the penetration stage and susceptible to the post-penetration stage.
4. Clone resistant to the penetration and post-penetration stages.

SA: slightly aggressive strain
A: aggressive strain

too deep and the isolate very aggressive, the latter could pass through the mesocarp barrier in some cases and develop, without encountering any resistance in the endocarp. The thickness of the pod cortex for all the clones inoculated was an important parameter to know before carrying out a pod test with wounding. In that way, the ideal depth and location of the inoculation (ridge, inter-ridge) for each clone could be determined, so as to reduce scoring variability for that category of clones.

However, for a clone that was resistant to penetration and susceptible to post-penetration, the scores differed depending on the method used and the aggressiveness of the strain inoculated (figure 2.3). Without wounding, the score varied from 0 to 1 depending on the aggressiveness of the pathogen. With wounding, it varied from 1 to 4 depending on the depth of the wound and the aggressiveness of the strain. The deeper the wound was, the higher the score was.

For a clone that was resistant for both penetration and post-penetration phases (figure 2.4), the same score was obtained irrespective of the method or the aggressiveness of the *Phytophthora* inoculated. However, for some pods, if the wound was too deep, the very aggressive strain was able to develop enough to invade the endocarp, hence the entire pod.

To obtain a reliable test to screen for pod resistance to *Phytophthora,* it therefore seemed essential to carry out inoculations, with and without wounding, simultaneously on the same pod. This double inoculation made it possible to define the levels and sites of resistance in the pods of a clone (penetration and post-penetration). Knowing the average cortex thickness of the pods of a clone in the study was also a very important factor. This method was all the more reliable if it was applied on detached pods inoculated under standard conditions. It should always be borne in mind that the level of resistance obtained on detached pods is underestimated when compared to that found on pods left on the tree. As there was a good level of correlation between the patch expansion rate and the level of sporulation, it was possible to do away with laborious quantification of the quantity of zoospores produced on a pod. However, observation of the quantity of chlamydospores formed on a pod of a clone was also very important information to know, in order to determine the possibilities of pathogen survival in a cocoa planting. In zones where sexual reproduction of the pathogen is possible, observation of oospore formation on pods should be considered.

This double inoculation method is ideal for studying clones, but when studying hybrid pods an appropriate experimental design is essential for obtaining a reliable assessment of the degree of resistance in all the progenies. Nevertheless, a certain number of parameters still has to be studied to optimize the reliability of this pod test:

- Effect of the *Phytophthora* species inoculated. Some are more adapted to pod attacks than others.
- Effect of the inoculum type (zoospores, mycelium, chlamydospores, oospores) and of inoculum concentration.

- Effect of the physiological stage (flower emission, flush emission, etc.) of the tree from which pods are taken.
- Age of pods and impact of insect wounds (e.g. capsids).

Leaf test

The use of leaves to develop an early, non-destructive test that can be repeated on the same plants virtually at will is a particularly attractive idea; this is justified by the fact that young leaves can be attacked naturally by *Phytophthora*, especially by *P. palmivora,* and that the histological structure of the underside of leaves is similar to that of the superficial layers of pods (Van der Vossen, 1997).

Several teams had already studied the possibility of using cocoa tree leaves to predict the level of resistance in the plant to *Phytophthora* (Tarjot, 1972*b*, Tondje *et al.*, 1988), but leaf tests only became operational when Nyassé *et al.* (1995) and Iwaro *et al.* (1997*b*) developed and demonstrated the merits of their methods. The tests proposed by those authors are ideal early tests, can be carried out in the nursery, and are easy and cheap to implement. They would also make it possible to considerably shorten cocoa breeding cycles by selecting resistant plants at an early stage from the progenies created in pre-breeding programmes. They were based on the use of parasites belonging to two distinct species, and differed in many aspects, in terms of both inoculation conditions and incubation, and in the actual description of symptoms.

The work by Nyassé *et al.* (1995) led to the recommended use of leaves borne by a very slightly lignified twig. Leaf discs with a diameter of 15 mm were inoculated with a 10 µl drop of *P. megakarya* suspension calibrated at 300,000 zoospores/ml, and covered with a disc of filter paper. Scoring was based on the development of penetration points into a necrotic patch, rated on a scale of 0 to 5.

Iwaro *et al.* (1997*b*) used whole adult leaves that were dark green in colour and borne by a green twig; resistance to penetration and to post-penetration were assessed in two separate tests.

For the "resistance to penetration" test, inoculation was carried out with a 30 µl drop of a *P. palmivora* suspension calibrated at 150,000 zoospores/ml and 400,000 zoospores/ml for inoculation of the underside and upper side of leaves respectively; the drop was then covered with a 1 cm^2 piece of filter paper (0.23 mm thick). Symptoms were scored 3 days after inoculation by counting the number of lesions.

For the "resistance to post-penetration" test, whole leaves were perforated with holes of 4 mm in diameter; a plaster was stuck over the upper side of the leaf and a filter paper disc of a 4 mm diameter imbibed with a *P. palmivora* suspension calibrated at 200,000 zoospores/ml, was placed over the hole on the underside

of the leaf. Symptoms were scored 6 days after inoculation, measuring the area of the lesions.

Although a test developed in one country cannot usually be directly transposed to another country where conditions are not identical (different *Phytophthora* species or isolates with different degrees of aggressiveness, environmental conditions in the nursery or fields, and different laboratory conditions), it is often requested in international projects that standardized tests be used as much as possible, so as to be able to compare the results from one country to another. On this basis, it was essential to study all the factors that might be involved in disease expression in the laboratory.

Plant material preparation

Leaf collection and sampling

Effect of the sampling zone in the canopy

In Ivory Coast, Tahi (2003) studied the effect of leaf exposure to light on susceptibility to *P. palmivora* in the laboratory, as exposure to sunlight could have an effect on leaf tissue turgor and on receptivity to infection. Leaves were taken from trees in the field (clones PA 150 resistant, T 60/887 moderately resistant and NA 79 susceptible) from three zones in the canopy: upper section (direct sunlight), lower section (dominant shade) and middle section (half-shade). The results showed that leaves taken from the upper section of the canopy had higher susceptibility scores than those in the shaded zones. However, there was a significant clone x sampling zone interaction, and the best correlation with the field rot rates observed over a 10-year period was obtained with leaves taken from the middle section of the canopy.

Effect of sampling time

For the same reasons as mentioned above, the leaf sampling time was suspected of affecting plant material susceptibility observed in the laboratory. In the same work, Tahi (2003) took leaf samples at 7:30 am and 1:30 pm, times of the day when temperature and relative humidity conditions were very different. Half of the leaf discs were prepared and inoculated late in the afternoon on the same day, and the other half the following morning. The analysis results showed that if leaves were kept overnight before cutting and inoculation, there was no difference between the sampling times. However, if leaves were prepared and inoculated the same day as collection, significant differences occurred between the collection times: the leaves that were collected in the morning displayed lower susceptibility than those collected in the afternoon. The best correlation with the field rot rate was obtained with leaves collected in the morning.

Effect of leaf age

The leaf stages used were generally defined using the nomenclature described by Greathouse *et al.* (1971). The leaves of Interflush 1 correspond to adult size leaves and are supple and pale green or red depending on the planting material. The leaves of Interflush 2 correspond to adult size leaves that are dark green and borne by a green twig. The leaves of Interflush 3 correspond to adult size leaves that are dark green but borne by a twig undergoing lignification. In the field, under particularly humid conditions, it is not unusual to see *Phytophthora* attacks on young supple leaves, but not on adult leaves.

In Trinidad and Tobago, studies by Thévenin and Motilal (1999, 2000) showed that the youngest leaves, corresponding to Interflush 1, were always the most susceptible and that decreasing scores were obtained as leaf age increased (table 5); this phenomenon tallied with the observations of Nyassé *et al.* (1995) and Iwaro (1995). Two hypotheses were put forward to explain this: young leaves possessed imperfect barriers, thereby providing better conditions for infection; the defence mechanisms of the leaves were not yet fully expressed.

Table 5. Effect of leaf age on the susceptibility of leaf discs.

	Leaf stage		
	Interflush 1	Interflush 2	Interflush 3
Trial on 30 seedlings	4.03a	3.59 b	2.75 c
Trial on 7 clones	4.08a	3.85a	3.16 b
Trial on 10 clones	—	3.21a	2.63 b

Scores 6 days after inoculation on a scale of 0 to 5, according to Thévenin and Motilal (2000). The values in the same row, followed by the same letter, are not significantly different at the 5% level (Newman-Keuls test).

The two stages, Interflush 2 and Interflush 3, were suitable for distinguishing between clones throughout the experiment, the former being recommended by Thévenin and Motilal (2000) and Iwaro (1995), the latter by Nyassé *et al.* (1995).

Sampling

As leaf disc tests are mostly intended for use on young seedlings, the number of leaves that can be used is limited by the number of leaves available. When seedlings are involved, a minimum of two leaves is recommended. For older trees it is difficult to make recommendations for the number of leaves to be tested per tree, because environmental conditions can affect symptom expression on leaves. A larger number of leaves is therefore required from a tree in a heterogeneous environment. Generally speaking, a minimum of ten leaves per tree is recommended under standardized light exposure conditions and at an identical age. Three replications over time appeared to be necessary to smooth out the variations found.

Disc preparation

Discs have to be prepared as quickly as possible after leaf sampling, to avoid any deterioration of the leaves. Use of a semi-automatic instrument is also recommended for disc cutting, to guarantee their uniformity and quality. Using such an instrument saves valuable time when numerous plants have to be assessed in the same test, which is not a negligible factor in avoiding leaf desiccation due to overlong preparation times.

The bottom of the trays used for the experiment was covered with a damp sponge on which the leaf discs were placed underside upwards, as the underside with its stomata is more receptive to infection (Iwaro *et al.*, 1997*b*).

Disc sampling zone

In order to standardize the leaf disc assessment method, a study was undertaken to compare the different zones of leaves from which the disc samples were taken. As stomata open first of all in the apical zone of the leaf, differences in reactivity to the pathogen were suspected of existing between the different leaf zones.

Tahi (2003) therefore investigated the influence of the sampling zone from which leaf discs were cut (section near the petiole, median section of the lamina, section near the tip) on symptom expression, using the clones PA 150, T 60/887 and NA 79. No difference was detected between these different sampling zones, which simplified the disc sampling method.

Effect of disc size

There were two major drawbacks when using whole leaves for such a test: depending on the size of the leaves and the trays, only 4 to 5 leaves could be placed in the same tray, hence not all the plants would be represented; when assessing young nursery plants, the number of leaves per plant at the same stage is limited, which in turn limits the number of replicates. It therefore appeared necessary to compare the responses of leaves and discs to inoculation, along with the effects that disc size had on symptom expression. This work was carried out in Trinidad and Tobago by Thévenin and Motilal (2000).

Inoculation of half-leaves compared to discs cut from the other half-leaves consistently and significantly gave lower scores (table 6). Cutting discs probably led to greater physiological modifications in the leaf tissues than simple longitudinal cutting of the leaf into two parts. However, clone classification did not change.

Table 6. Comparison of the inoculation response between half-leaves and discs.

	Half-leaves	Discs
Trial on 30 seedlings	3.16b	3.76a
Trial on 10 clones	2.34b	3.12a

Scores 6 days after inoculation on a scale of 0 to 5, according to Thévenin and Motilal (2000). The values in the same row followed by the same letter are not significantly different at the 5% level (Newman-Keuls test).

Several disc sizes varying from 10 to 26 mm in diameter were compared. No regular trend was observed between one trial and the next (table 7): while in one trial the scores obtained decreased as the diameter increased, the trend was reversed in another trial. Although "diameter x clone" interactions were often significant, they were negligible when compared to the "clone" or "diameter" effects themselves, and clone classification according to diameters did not alter much. The best correlation between the inoculation data and the field classification was obtained with the 14 mm diameter.

Table 7. Effect of disc size on response to inoculation.

	Disc diameter				
	10 mm	14 mm	18 mm	22 mm	26 mm
Trial on 6 clones	3.62 a	3.26 b	2.80 c	2.79 c	2.65 c
Trial on 6 clones	2.63 a b	2.42 b	2.26 b	2.68 a	2.83 a
Trial on 7 clones	3.64 b	3.74 a b	3.85 a b	3.91 a b	4.05 a

Scores 6 days after inoculation on a scale of 0 to 5, according to Thévenin and Motilal (2000). The values in the same row followed by the same letter are not significantly different at the 5% level (Newman-Keuls test).

Effect of wounding

Experience has shown that leaves attacked by insect often develop more symptoms than undamaged leaves. Thévenin and Motilal (2000) conducted experiments to check how wounds influenced symptom expression. Leaf discs were wounded by simple perforation of the leaf tissues with a thin needle, in the middle of the disc. Wounding generally led to higher scores, even though the differences between "with" and "without" wounding were not always significant; the clone classification underwent little or no change.

In order to have intact leaves at all times, it may be necessary to protect trees by spraying with insecticide. The residual effect of routinely used insecticides (Perfekthion® [dimethoate], Karate® [lambda-cyhalothrin] and Vydate® [oxamyl], either alone or combined) was proved not to have any effect on the expression of *Phytophthora* symptoms when leaf disc inoculations were carried out in Trinidad and Tobago (unpublished results).

Number of discs

A minimum of 4 discs per leaf is recommended. If the leaf disc test is to be performed on seedlings, the number of discs per leaf can be increased if the number of leaves per seedling is limited.

Inoculation and incubation

The choice of strain to be used for inoculation was of the greatest importance. It needed to have a degree of aggressiveness enabling it to clearly distinguish between resistant and susceptible control clones, at a given zoospore con-

centration. It also had to be representative of the *Phytophthora* existing in the geographical zones studied. The inoculum was prepared over a period of 10 days, including a phase of mycelium growth in the dark on V8 medium, and a phase of sporocyst formation in alternating light. The culture incubation temperatures required for infectious propagule growth and production could vary from one *Phytophthora* species to another. To release spores, cultures had to undergo a thermal shock: the cultures were covered with distilled water and left in a cold place (4°C) for 15 min, then at ambient temperature in the dark for 30 to 60 min.

Choice of strain and host x parasite interactions

It has often been suggested that cocoa tree resistance to *Phytophthora* was of the horizontal or quantitative type (Tan and Tan, 1990; Simmonds, 1994) and that consequently, it was governed by a pool of genes. This type of resistance is considered to be more sustainable than so-called vertical or complete resistance, due to the absence of significant host x parasite interactions, hence the absence of physiological races (Agrios, 1988). A study of host x parasite interactions is therefore an important stage for developing a test to assess plant resistance to a parasite. Indeed, it is essential for checking that the planting material created in breeding programmes is resistant to all strains or species existing in a given territory.

Using the leaf test in Cameroon, Nyassé *et al.* (1995) inoculated 13 clones with a strain of *P. megakarya* and a strain of *P. palmivora* and obtained a significant strain x clone interaction. However, it should be noted that the four most susceptible clones and the two most resistant clones retained their classification from one strain to the next and that the interaction came from clones with an intermediate performance. The same author (Nyassé, 1997) did not find any significant interaction (3 strains, 12 clones) within the species *P. megakarya*.

In Trinidad and Tobago, Surujdeo-Maharaj *et al.* (2001) inoculated leaves at the Interflush 2 stage (Greathouse *et al.*, 1971) from 18 clones with 10 *P. palmivora* isolates from various regions of the country and showed that there were substantial differences in strain aggressiveness. However, once again the strain x clone interaction was not significant for the two variables used, namely the number of lesions (resistance to penetration) and necrotic area (post-penetration resistance).

Appiah *et al.* (2002) inoculated leaf discs from 24 clones using strains from several countries and several species (3 *P. palmivora*, 1 *P. capsici*, 7 *P. megakarya*) and obtained a strain x clone interaction that was once again significant.

Work at CIRAD (Ducamp, 1999 and 2000*a,b*), carried out in France, involving the inoculation of 34 different clones with 61 strains of *P. megakarya*, showed no significant interaction within the populations of West Africa (24 strains isolated in Ghana and Nigeria) and central Africa (37 strains from Cameroon,

São Tomé and Gabon) taken separately. However, a very slight interaction, significant at the 1% level, which was due to 3 particular strains (from Ghana and from Nigeria), was found when all the strains were analysed at the same time.

The strains from São Tomé and Gabon displayed a low level of aggressiveness and all were of mating type A1; among this group, the most aggressive ones were isolated in Gabon on the border with Cameroon (table 8). The isolates from Cameroon varied in their degree of aggressiveness (between 1.11 and 3.97); the most aggressive isolates were those with a hybrid RAPD profile between the West and central Africa populations, and those from western Cameroon, where genetic diversity is greatest. Isolates of the A2 mating type had low aggressiveness, which may explain their gradual disappearance, to the benefit of A1 strains, as no A2 strain has been isolated in Cameroon since 1994.

Table 8. Level of aggressiveness of 61 isolates of *P. megakarya*.

Isolate		Score	Isolate		Score
Code	Origin		Code	Origin	
G 10.302	Gabon	0.71	ASH 36	Ghana	2.26
G 4.94	Gabon	0.78	BA 5	Ghana	2.28
G 8.222	Gabon	0.79	NS 260	Cameroon	2.28
G 9.195	Gabon	0.86	BA 14	Ghana	2.41
G 1.14	Gabon	0.87	NS 308	Cameroon	2.41
NS 69	Cameroon	1.11	NGR 47	Nigeria, East	2.43
NGR 33	Nigeria, Ibadan	1.12	G 107	Gabon	2.45
NGR 44	Nigeria, East	1.34	NS 264	Cameroon	2.59
4 ST 23	São Tomé	1.48	ASH 12	Ghana	2.60
4 ST 34	São Tomé	1.49	G 112	Gabon	2.66
M 184	Cameroon (A2)	1.49	NS 229	Cameroon	2.66
WR 7	Ghana	1.51	NS 261	Cameroon	2.68
4 ST 15	São Tomé	1.54	NGR 53	Nigeria, East	2.68
VR 37	Ghana	1.57	NS 309	Cameroon	2.74
NS 128	Cameroon	1.57	NS 275	Cameroon	2.78
4 ST 3A	São Tomé	1.58	NS 270	Cameroon (H)	2.83
4 ST 4	São Tomé	1.60	NS 266	Cameroon	2.94
POT 1	São Tomé	1.64	NS 285	Cameroon	3.02
4 ST 39	São Tomé	1.72	NGR 11	Nigeria, Ibule (A2)	3.08
NGR 19	Nigeria, Owena	1.79	NS 287	Cameroon	3.29
VR 64	Ghana	1.85	M 309	Cameroon	3.56
NGR 36	Nigeria, East	1.92	NGR 29	Nigeria, Ibadan	3.66
NGR 22	Nigeria, Ibadan	1.94	NGR 16	Nigeria, Ibule (A2)	3.76
4 ST 22	São Tomé	1.97	NGR 15	Nigeria, Ibule (A2)	3.78
3 ST 23	São Tomé	2.00	NGR 12	Nigeria, Ibule (A2)	3.81
4 ST 8	São Tomé	2.02	NGR 14	Nigeria, Ibule (A2)	3.82
4 ST 2C	São Tomé	2.07	NS 269	Cameroon (H)	3.87
NGR 17	Nigeria, Ibule	2.18	NS 268	Cameroon (H)	3.88
NGR 10	Nigeria, West	2.19	NS 259	Cameroon (H)	3.97
BA 1	Ghana	2.21	NGR 20	Nigeria, Owena	4.32
NS 203	Cameroon (A2)	2.22			

Scores 7 days after inoculation on a scale of 0 to 5.
A2: strains of mating type A2; H: strains with a hybrid RAPD profile between the two populations, West Africa and Central Africa.

The strains isolated in Ghana (Ducamp, 1998) formed a uniform population with low (East Volta region) to moderate (Ashanti and Brong Ahafo region) aggressiveness, whereas the isolates from Nigeria varied in aggressiveness. The most aggressive came from the Ibule and Owena zones in central-western Nigeria, a zone where *P. megakarya* genetic diversity is substantial and where the 2 mating types exist side by side, enabling genetic recombination through sexual reproduction. A comparison of aggressiveness levels in strains isolated in 1994 and 1998 in Ghana revealed a worrying increase, especially in the Brong Ahafo region, for which the average score increased from 2.42 to 3.90 during that period.

In the same work, 16 clones were inoculated with 22 strains of *P. palmivora* representative of genetic diversity in that species. No significant interaction was found, thereby suggesting that any strain of *P. palmivora* could be used to assess the level of cocoa tree resistance to black pod rot, provided its level of aggressiveness has matched the objectives of the experiment. Isolates from Trinidad, Indonesia and Ivory Coast were among the most aggressive whereas isolates from Malaysia and Venezuela, among others, were less aggressive.

Lastly, a slight significant interaction at 1% was found in an experiment comparing the level of aggressiveness of 6 strains of *P. capsici* used to inoculate 16 clones; this interaction was due to a Brazilian strain, which was also the most aggressive one.

Other trials using one or more strains of *P. palmivora* and *P. megakarya* to inoculate the same range of clones did not reveal any significant strain x clone interactions.

Inoculation conditions

Several studies, including those by Thévenin and Motilal (2000), were undertaken to investigate the effect of zoospore concentration on symptom expression. Inoculations with *P. palmivora* using 10 µl drops per disc showed that the resulting symptom intensity generally increased as the zoospore concentration increased from 100,000 to 500,000 zoospores/ml. However, differences between the highest two concentrations were not significant (table 9).

Table 9. Effect of zoospore concentration on symptom expression.

	Zoospore concentration 10^3/ml		
	100	250	500
Test on 10 clones, 1	2.40a	3.06 b	—
Test on 30 seedlings	3.24a	3.46 b	3.67 b
Test on 10 clones, 2	2.72a	3.04 b	3.01 b

Scores 6 days after inoculation on a scale of 0 to 5, according to Thévenin and Motilal (2000). Values in the same row followed by the same letter are not significantly different at 5% (Newman-Keuls test).

These results were confirmed by work undertaken at CIRAD in Montpellier, where the maximum symptom intensity levels were obtained with concentrations of 200,000 and 300,000 zoospores/ml of *P. megakarya*. With the same parasite, concentrations ranging up to 2 million zoospores per millitre did not lead to any increase in the susceptibility scores compared to those obtained with a concentration of 200,000 zoospores/ml, on 10 clones judged to be resistant by the leaf test and in the field. However, with concentrations under 200,000 zoospores/ml, maximum symptom intensity was obtained with 50,000 zoospores/ml for some clones and with 150,000 zoospores/ml for others.

Incubation conditions

Effect of tray humidity

Fungi of the genus *Phytophthora* are known to form chlamydospores, a kind of resistance to climatic adversities, which are necessary for surviving a marked dry season. When the rains return in the tropics, the fungus is reactivated and resumes intense asexual reproduction cycles, producing zoospores, which cause sometimes dramatic damage. Moisture conditions play a substantial role in symptom development in plantations.

This aspect was also studied in the laboratory by Tahi (1998) under standardized conditions. Two levels of incubation tray humidity (800 ml and 1,500 ml of water per 50 x 70 cm sponge) and two incubation temperatures (25-26°C in an air-conditioned room and 30-31°C in a room without air-conditioning) were compared, inoculating 3 clones (PA 150, T 60/887 and NA 79).

Lighting effect

In Trinidad and Tobago, Thévenin and Motilal (2000) showed that incubating trays in the dark throughout the experiment led to higher scores than with incubation under alternating light conditions (12 h of light, 12 h of dark): 2.75 (alternating light) and 3.09 (darkness), 6 days after inoculation on 10 clones. Darkness has a favourable effect on mycelium growth, while alternating light/dark is known to favour the formation of fungus reproductive structures, which might explain this phenomenon. As it was easier to keep the trays in darkness than to maintain standardized alternating light conditions, it was possible to adopt this option insofar as symptoms were not completely levelled off at the upper end of the evaluation scale.

Incubation temperature effect

As not all species of *Phytophthora* have the same optimum growth temperature, it was assumed that symptom expression after inoculation in the laboratory might also depend on the incubation temperature.

Ducamp (2000*b*) inoculated 6 clones (ICS48, ICS84, LAF1, ICS95, VENC4 and SNK10) with three *Phytophthora* species to determine the optimum temperature for symptom expression.

Temperatures leading to maximum symptom expression were 22°C for *P. megakarya*, 25°C for *P. palmivora* and 28°C for *P. capsici* "cacao" (table 10). These results therefore differed from those obtained in Ivory Coast, which showed more severe symptoms at 30-31°C. However, when comparing the aggressiveness levels of several species, a temperature of 25°C was preferred since it enabled each species to express a level of aggressiveness close to its maximum level and also enabled a clear distinction to be made between the plant materials being compared.

Table 10. Effect of incubation temperature on symptoms development.

	Incubation temperature				
	20°C	22°C	25°C	28°C	30°C
P. megakarya (strain M309)	3.46	3.55	3.05	1.56	1.05
P. palmivora (strain TRI1)	3.55	3.97	4.25	4.00	3.56
P. capsici "cacao" (strain TRI3)	3.10	3.14	3.58	3.95	3.67

Scores 7 days after inoculation on a scale of 0 to 5.

Symptom assessment

The two methods developed almost simultaneously by Nyassé *et al.* (1995) and Iwaro (1995) used scales to assess symptoms based on different criteria: development of symptoms from localized penetration points to the formation of a necrotic patch in the first case, and the number of penetration points or lesions and the area of the necrotic patch in the second case. It seemed worthwhile comparing the two scales; however, as it was not possible to measure the lesion area on the leaf discs because of the small size of the discs, two scales were compared by Thévenin and Motilal (2000).

Scale "A"
0: no symptoms
1: localized penetration points
2: network of points (small developing lesions sometimes in contact with each other)
3: weblike patch (merging lesions)
4: mottled patch (more or less uniform lesion, pale brown colour, sometimes still with isolated lesions)
5: true patch (large uniform lesion, dark brown colour typical of necrosis)

Scale "B"
0: no lesions
1: 1-19 localized lesions
2: 20 or more localized lesions

3: 1-19 expanding lesions
4: 20 or more expanding lesions
5: merged lesions

The authors found that method "B" uniformly and significantly gave higher scores than method "A". In fact, this came as no surprise because method "A" was based on the shape of lesions and their gradual development, whereas method "B" gave a maximum score of "5" as soon as the lesions could not be distinguished from each other, hence could not be counted. However, clone classification remained the same irrespective of the method used, especially 3 days after inoculation, when symptoms had yet to develop much.

It is worth noting that CIRAD Montpellier is developing a computer software (OPTIMAS) combined with an image analyser, in order to precisely quantify necrotized areas and the number of penetration points per inoculated disc 3 days after inoculation, so as to have information on resistance to penetration, and 7 days after inoculation to have information on resistance to symptom development.

Repeatability of results

Statistical analyses of the trials using the leaf test revealed the importance of the experimental design and of replicates (Tahi, 2003). A minimum of 4 trays per experiment is recommended, with all the clones or plants represented in each of them. For optimum estimation of within-clone or within-family variance, which are important selection parameters, it was important that the design be analysed taking the tree as the elementary unit. At the same time as observing variations between trays, it was necessary to study the stability of responses to inoculation over time, and over several series of inoculations.

The results obtained by Tahi (2003) on three crosses (P7 x T60/8887 resistant, IFC1 x IFC1 susceptible and PA150 x IFC1 moderately resistant) and with 3 series of inoculations one month apart, showed that the Pearson and Spearman coefficients of correlation between series were positive but not always significant; however, the correlations between a given series and the mean of the other two series were always significant (table 11). These results revealed that susceptibility observed in the laboratory depended on the environmental conditions in which the plants were growing.

The same author also studied the family performances derived from the same factorial mating design between 4 female parents (PA13, PA121, P19C and ICS89) and 2 male parents (PA150 and IMC67) during three series of inoculations over a period of three months. He showed that even though a strong series effect was found, the correlations between the series were always positive and significant, whether for the progenies, parents or all the trees combined.

Test stability was also studied in Cameroon by Nyassé (1997), who carried out 6 series of inoculations on 20 trees of cross UPA134 x ICS34 (along with the parents), from a diallel planted at Barombi Kang. Pearson's correlation coefficients between these 6 series scaled over a period of 12 months were all positive but not always significant. However, all the series were significantly correlated with the mean values of the 6 series of inoculations (0.70-0.83); based on these results, the minimum number of series required was 3.

Table 11. Relationship between inoculation series.

	Series (S)					
	S1/S2	S1/S3	S2/S3	S1/S2S3	S2/S1S3	S3/S1S2
Pearson's coefficient	0.28NS	0.46*	0.44*	0.46*	0.45*	0.56**
Spearman's coefficient	0.47**	0.35NS	0.58***	0.47**	0.62***	0.56**

Pearson's and Spearman's coefficients of correlation on the scores observed 7 days after inoculation, according to Tahi (2003).

NS: not significant at 5%; *, ** , ***: significant at 5%, 1% and 0.1% respectively.

Conclusion and recommendations

It appeared that host x parasite interactions could become significant depending on the planting material or inoculation techniques used. They could come from clones with intermediate performances, as clones in the extreme classes kept their classification. Zadoks (1997) and Agrios (1988) mentioned that significant interactions might come from an environmental effect, if the experimental conditions were not clearly enough defined. The first thing that comes to mind is sampling conditions: plot heterogeneity, position of pods or leaves in the tree, sampling time, or incubation conditions: e.g. tray effects if not all the treatments can be represented in the same tray (for example, the pods). However, if observed interactions cannot be attributed to the environment, and they persist in successive tests, a gradual evolution of the parasite towards physiological specialization cannot be ruled out.

Even in the absence of a significant clone x parasite interaction, the isolates displayed levels of aggressiveness that could differe substantially. Selecting clones using a very aggressive strain might lead to the elimination of clones that are of interest for other traits, such as resistance to another disease, yield, or flavour quality; on the other hand, using an isolate with low aggressiveness will not enable sufficiently strict selection, during extensive pre-breeding programmes for example. The choice of *Phytophthora* strain(s) (species/level of aggressiveness) for carrying out tests to assess planting material resistance will have to be made in accordance with the objectives fixed, or with local selection or network breeding programmes.

The leaf test was useful in several countries. However, it cannot be completely standardized, though certain parameters can be fixed.

Sampling and condition of leaves

Leaves sampled in the field must be located in a half-shade section of the tree. In the nursery, particular care must be taken to achieve conditions that are as uniform as possible, particularly as regards shading. Leaves in good condition and without any visible insect attacks should be sampled over a short space of time, preferably early in the morning (07:00-09:00). They should be adult sized, dark green, and be borne by a green or slightly lignified twig.

Leaf preparation

Discs 14 mm in diameter should be cut from the median section of the leaves and placed with the upper side against a damp sponge in the bottom of the inoculation tray. Special care should be taken when cutting the leaf discs to ensure that they are not allowed to dry out while the experiment is being set up. The inoculation trays should be wrapped in plastic bags to maintain high relative humidity and placed in a temperature-controlled room. As far as possible, all the plants/clones being studied should be represented in the same tray alongside control clones; common control clones should be present in each tray. At least 3 series of inoculations should be carried out over time, each comprising 4 replicates (4 trays).

Inoculation and symptom assessment

Inoculation should be carried out the following day, depositing a 10µl drop of calibrated zoospore suspension on the underside of the leaves, without wounding. Depending on the species of *Phytophthora* and the level of aggressiveness of the selected strain, the zoospore concentration may vary from one country to another.

Symptoms should be assessed according to the following scale:
0: no symptoms
1: localized penetration points
2: small developing lesions, sometimes in contact with each other
3: merging lesions
4: more or less uniform lesion, sometimes still with isolated lesions
5: large uniform lesion

Expected genetic gains through integration of rapid resistance tests in conventional cocoa breeding

The progress obtained up to 1995 in breeding for black pod resistance was relatively limited (Eskes and Lanaud, 2001; INGENIC, 1999). Though variation

in disease resistance has been observed in collections and in breeding populations, major difficulties identified in obtaining resistant varieties were (Eskes and Lanaud, 2001):

– lack of reliable early screening tests,

– lack of knowledge on the environmental versus genetic factors determining field resistance,

– lack of knowledge on the stability of resistance (interaction between *Phytophthora* isolates and/or species with cocoa genotypes),

– complexity of selection for several quantitative traits simultaneously in a perennial crop such as cocoa.

Recent advances in research (INGENIC, 1999), including the CAOBISCO project, have helped to overcome several of these obstacles. The objective of this subchapter is to analyse progress that can be expected from selection for black pod resistance and how rapid screening tests, such as the leaf disc and detached pod inoculation tests (Nyassé *et al.*, 1995; Iwaro *et al.*, 2000) can be integrated into cocoa breeding.

Cocoa breeding methods

Only a summary of conventional breeding methods applied to disease resistance will be given here. The possible use of markers related to selection traits (QTL) will be covered in another chapter of this book.

Clone selection

Cocoa breeding started in the 1920s by selecting clones in commercial plantations. Since then, clone selection has been carried out in most cocoa producing countries. Most of these clones were used to establish collections of local material, aiming at their further use in "speculative" crosses with introduced genotypes to obtain new hybrid cultivars. In some cases, selected clones with high yield and quality were used commercially, as is the case with Trinitario clones (ICS, DR) that are still being used as cultivars in Trinidad and Indonesia, respectively.

From the 1970s onwards, there was fresh interest in the selection of new clone cultivars, mainly to obtain rapid progress for resistance to devastating diseases such as vascular streak dieback (VSD) in Southeast Asia and to witches' broom (Trinidad Selected Hybrid clones) in Trinidad and, more recently, in Brazil. Such selection has been successful, because VSD and witches' broom resistance can be identified in the field relatively easily under severe attack conditions, such as prevail in Malaysia and Brazil. Large-scale selections of commercial clones with high resistance to black pod have only been launched more recently (Eskes *et al.*, 1998; Blaha *et al.*, 2001; Efron, 2000).

Hybrid selection

Between the 1950s and the 1990s, the selection of new hybrid cultivars was the main activity in most cocoa breeding programmes worldwide. Hybrid selection is based on heterosis observed in crosses between genetically distinct genotypes. As parental materials, local and introduced clones available in the germplasm collections were generally used. There was effective progress in most countries for early production (precocity), yield capacity and vigour and such hybrids have been used on a large scale. However, the hybrid selection method has generally not provided satisfaction in obtaining good disease resistance. Hybrid varieties are also sometimes too vigorous, at least under favourable growing conditions found in Papua New Guinea and Ecuador; this leads to pronounced "yield decline" after 5 to 10 years. The mixed hybrid varieties have also shown large phenotypic variation for all traits, often not desired by farmers, and making rational management of cocoa plantations more difficult.

Recurrent selection and pre-breeding

Traditional selection of hybrids does not lead to continuous genetic progress. Breeders have therefore proposed using successive breeding cycles to increase the frequency of favourable alleles in parental populations (Toxopeus, 1972; Kennedy *et al.*, 1987). Recurrent selection exploits general combining ability, predominating for most selection traits in cocoa, when populations are based on genetically related individuals, such as the Lower Amazon types. Both general and specific combining ability is exploited in reciprocal recurrent selection (Baudouin *et al.*, 1997). In this case, the base populations are genetically divergent, making it possible to obtain heterosis for yield and vigour in the between-population crosses.

A recurrent selection programme with two base populations has been launched in Ivory Coast (Clément *et al.*, 1994). In this programme, two cycles of recurrent selection have been proposed in order to increase the frequency of favourable alleles for traits with relatively high heritability in the base population (e.g. disease resistance, self-compatibility and pod index). After two selection cycles, a comparison of the value of within-group and between-group crosses will help in deciding whether it is worth continuing recurrent selection separately in the base populations or starting reciprocal recurrent selection. Other countries (e.g. Brazil, Ghana and Malaysia) have also initiated recurrent selection programmes adapted to the locally available germplasm.

Pre-breeding can be considered as a specific form of recurrent selection. Its objective is to genetically improve distinct base populations for specific traits in large germplasm collections before distributing these populations to user countries. The Cocoa Research Unit of the University of the West Indies, which is managing the International Cocoa Genebank in Trinidad, has recently embarked upon a pre-breeding programme with emphasis on disease resistance (Iwaro and Butler, 2002).

Breeding progress for black pod resistance up to 1995

A comprehensive review was made of the progress obtained and problems encountered in selection for resistance to *Phytophthora* during the INGENIC workshop on the contribution of disease resistance in cocoa variety improvement (INGENIC, 1999). The results reported at that workshop can be summarized as follows:

- Despite significant efforts, only a few cocoa cultivars have been selected with effective resistance to black pod disease.

- Significant variation in levels of black pod attacks in the field has been identified in germplasm collections and breeding trials in all countries. Typically, the average percentage of field attacks on cocoa genotypes varied between 10 and 40% for *P. palmivora* (e.g. in Ivory Coast) and between 20 and 80% for *P. megakarya* (e.g. Nigeria and Cameroon).

- The level of field resistance in different environments and in relation to different *Phytophthora* species was quite stable for several clones, such as for the resistant clones P7, Sca6, PA150 and PA30 and for the susceptible clone PA81. This suggests that progress in one country can be useful in other countries too. It would justify regional or international programmes on breeding for black pod resistance.

- Significant correlations between the average degree of field attack, period of pod production and number of pods have been observed, indicating that in some cases the level of field resistance can partly be explained by escape mechanisms (pod production outside the epidemic season or low pod production).

- Although average results obtained by inoculation of either pods or germinating seeds of hybrid progenies could be correlated with average levels of resistance in the field, selection of individual trees or seedlings for a better level of resistance by these methods appeared to be inconsistent. This could be due to environmental effects on pod and seed susceptibility, or to a lack of replications (a seed can only be inoculated once).

Significant correlations between artificial inoculation tests and field resistance have been established more recently, for example in Ivory Coast (Tahi *et al.*, 2000) and in Cameroon (Nyassé *et al.*, 2002). The CAOBISCO project has led to clearer identification of the conditions required to obtain consistent and repeatable results, mainly with the leaf disc test. A detached pod test, which has recently been developed (Iwaro *et al.*, 2000; Blaha *et al.*, 2001), is also giving consistent results that are apparently correlated to the level of field infection (Blaha *et al.*, 2001). This suggests that rapid screening tests, using standardized inoculations of leaves or pods, can be effectively integrated into cocoa breeding to enhance progress in selection for resistance to black pod.

Progress possible through selection for black pod resistance

Before analysing the integration of rapid resistance screening tests in cocoa breeding, an empirical analysis is made on progress that can be expected in selecting for resistance to black pod. An example of progress for black pod resistance in Africa is given in table 12 based on natural disease incidence and known variation for resistance. The expected level of disease incidence (% of rotten pods) is compared for varieties with resistance levels varying from highly resistant (HR) to highly susceptible (HS).

Table 12. Estimated variation in the resistance of cocoa varieties to black pod disease expressed by the percentage of rotten pods and yield in the presence of Phytophthora palmivora and P. megakarya in Africa.

	Resistance level of cocoa varieties[1]				
	HS	S	MS	R	HR
Variation in attacks observed in breeding trials, due to the pathogen species					
P. palmivora	40	30	20	10	5
P. megakarya	80	60	40	20	10
Variation encountered in germplasm collections (examples)[2]					
Trinidad, Ghana, Ivory Coast	+	++	++	+	+
Selected clones (T60/877, UPA134, PA150, P7, Sca6, IMC47...)			+	+	+
Expected variation in resistance for traditional varieties in Africa[2]					
Amelonado (A)		++			
Trinitario (T)	++	++	+		
Mixed Upper-Amazon (UA)		++	+	+	
Variation in distributed hybrids in Africa (T x A, UA x A, Ua x UA)[2]	+	++	+	+	
Expected variation among selected hybrid varieties (MS x R, MR x MR, MR x R, R x R)[2]			+	++	+
Variation in net yield (kg dry cocoa per ha) for farmers by using varieties with different resistance levels and with a potential yield, in the absence of black pod disease, of 800 kg/ha for:					
P. palmivora	480	560	640	720	760
P. megakarya	160	320	480	640	720

1. HS = highly susceptible; S = susceptible; MS = moderately susceptible; R = resistant; HR = highly resistant)
2. ++ = frequent; + = less frequent

This example implies that average disease incidence is roughly twice as high with *P. megakarya* as with *P. palmivora*. This corresponds to reported situations in Cameroon (Ndoumbé *et al.*, 2001), with variation from 20 to 60% rotten pods between the most susceptible and most resistant hybrid varieties, and in Ivory

Coast (Tahi *et al.*, 2000) with 10 to 30% rotten pods for the same type of varieties. In table 12, more extreme levels of resistance (HR) and susceptibility (HS) have been postulated than found in the above hybrid trials. This is justified by known variation existing in germplasm collections, such as in the International Cocoa Genebank in Trinidad (ICG,T) or in the larger collections in African countries. Some cocoa populations (e.g. Trinitario) are known to be highly susceptible whereas selected clones in germplasm collections are known to be highly resistant (e.g. Sca6, P7 and IMC47).

Present knowledge indicates that the prevailing cocoa planting material in Africa (Amelonado, Trinitario and mixed Upper Amazon populations, as well as hybrid varieties distributed to farmers) mainly contains highly susceptible (HS), susceptible (S) and moderately susceptible (MS) varieties. Based on the variation that is found in germplasm collections, it can be expected that new hybrid varieties can be created, with higher levels of resistance than found in the existing breeding trials, in crosses between resistant and highly resistant clones (such as IMC47 x Sca6 or P7 x PA150).

The difference in susceptibility between the varieties currently cultivated in Africa and selected resistant varieties could represent a three-fold reduction in losses due to black pod, from 30 to 10% for *P. palmivora* and from 60 to 20 % for *P. megakarya*. This represents a gain in net yield for the farmer of approximately 30% in the case of *P. palmivora* (720 kg instead of 560 kg per hectare in the example given in table 12) and of 100% for *P. megakarya* (640 kg instead of 320 kg per hectare).

Integration of rapid resistance tests in cocoa breeding

A prerequisite for effective and rapid selection for black pod resistance is close collaboration between breeders and pathologists. Furthermore, the correct conditions for carrying out resistance tests need to be respected. The best proof of working under correct conditions is obtained by calculating the rank correlation of genotypes between individual inoculation series. Existing experience indicates that coefficients of rank correlation can be as high as 0.7 to 0.9 for average levels of resistance in clones or hybrid progenies, if test conditions are adequate. The same correlations are expectedly lower if tests are applied on individual seedlings in the nursery or on adult trees in the field: variations in the correlation coefficient of between 0.25 0.40 are found in this case under satisfactory test conditions.

Within the context of this section, two types of resistance testing are identified: "screening" and "evaluation". Screening for resistance is considered to involve a large number of plants and few inoculation series. The objective is to select the most promising genotypes from a large population. Evaluation of resistance is normally required to confirm the resistance of parents to be used in breeding or of candidate varieties for multi-site testing.

Screening of accessions in germplasm collections

Screening with the leaf disc test would involve two inoculation series, with at least 10 leaves per accession and per series, carried out with enough time between series to allow for variation in the growing conditions of the accessions (right stage of leaves should be available). Unfavourable conditions, such as the dry season, should be avoided for leaf disc inoculations. Screening with the detached pod test would involve the inoculation of at least four pods (Iwaro *et al.*, 2000). Confirmation of the resistance of the most promising accessions would involve duplication of this screening effort (two more inoculation series for the leaf disc method, and four more pods inoculated for the detached pod test).

A prerequisite for effective and rapid selection for black pod resistance is close collaboration between breeders and pathologists. Such collaboration is currently being promoted in international collaborative projects, such as the joint project on Cocoa Germplasm Utilization and Conservation of the International Cocoa Organization (ICCO), the Common Fund for Commodities (CFC) and the International Plant Genetic Resources Institute (IPGRI).

Within the context of this section, two types of resistance testing are identified. Screening for resistance is considered to involve a large number of plants and few inoculation series. Evaluation of resistance is normally required to confirm the resistance of new parents to be used in breeding or of candidate varieties for multi-site testing.

Screening of accessions in germplasm collections

Screening with the leaf disc test would involve two inoculation series, with at least 10 leaves per accession and per series, carried out with enough time between series to allow for variation in the growing conditions of the accessions (right stage of leaves should be available). Unfavourable conditions, such as the dry season, should be avoided for leaf disc inoculations. Screening with the detached pod test would involve the inoculation of at least four pods (Iwaro *et al.*, 2000). Confirmation of the resistance of the most promising accessions would involve duplication of this screening effort (two more inoculation series for the leaf disc method, and four more pods inoculated for the detached pod test).

Selection of hybrid progenies

Screening for the most resistant progenies can probably best be done by the leaf disc test. Discs will be obtained from at least one leaf of each of 15 seedlings per cross (hybrid progeny). If the test conditions are correct, two inoculation series (replicates) might be enough to identify the most worthwhile progenies. Confirmation of the resistance of the best progenies will normally be effectively obtained by one or two more inoculation series.

Clone selection in hybrid progenies

The best use of rapid screening tests for selection of superior individuals in segregating seedling progenies is probably to first evaluate the average level of resistance in the progenies, and then select the best individual seedlings or trees within the best progenies. For screening of the best seedlings or trees within the best progenies, the leaf disc test can be used on seedlings, and both leaf or pod tests can be used on adult trees.

For effective screening of individual plants within progenies, with the leaf disc test, at least three replicate inoculations will be required (with the use of at least two leaves per seedling or five leaves per adult tree for each replicate). One or two more replicates may be required to confirm the resistance of the most worthwhile plants. If results between series are consistent, one more inoculation series may be sufficient.

To evaluate the resistance of individual adult trees, the detached pod test appears to be adequate (Blaha *et al.*, 2001; Iwaro *et al.*, 2003). Apparently, the consistency in results with this test would mean good results can be obtained with the inoculation of only four pods per tree. For screening purposes, even two pods per tree may be considered, with confirmation of the most interesting trees through inoculation of two or four more pods.

Selection in pre-breeding and recurrent selection programmes

As described above, the pre-breeding and recurrent selection programmes involve selection cycles to improve populations. Use of rapid resistance tests is fundamental in order to obtain effective selection progress (Iwaro and Butler, 2002).

Selection cycles would probably be most efficient with alternate use of the detached pod test (to select the best parents for a new selection cycle) and of the leaf disc test (to select the best seedlings within the best progenies already in the nursery). Each of these methods appears to be correlated with field results, although resistance mechanisms revealed by these tests can be different and therefore complementary.

Multi-trait selection

Cocoa breeding involves selection for multiple traits (yield, resistance and quality). By using efficient resistance screening methods, the probability of recombining genes for resistance with genes for yield capacity and quality increases.

By applying early screening tests on nursery materials, breeders can save time and the number of cocoa progenies or clones to be sent for field evaluations can be reduced. In the field, selection pressure can therefore concentrate on other selection traits, although resistance evaluated by the detached pod test will become a trait to be introduced into a selection index. The use of selection indexes will help to maximize genetic gain for all traits considered.

Genetic progress by applying rapid screening tests

Use of genetic resources

Cocoa genebanks are typically made up of hundreds of accessions that are represented by 5 to 10 trees each. In general, the age of the accessions and the growing conditions (shade, fertility) are variable. This means that the environment probably plays a greater role in the variation for natural infection with black pod. It is therefore difficult to obtain reliable data on the genetic level of resistance of such a collection using only field data. Furthermore, only a small percentage of accessions in germplasm collections displays worthwhile levels of resistance to *Phytophthora* (Phillips-Mora, 1999; Iwaro *et al.*, 2003; Blaha *et al.*, 2001). Hence, effective use of germplasm collections in breeding for resistance to black pod will be largely dependent on the application of reliable rapid screening tests (leaf disc and detached pod tests).

Selection of individual trees within hybrid progenies in breeding trials

Heritability for the level of field resistance depends on the uniformity of growing conditions, on the quality of observations, and the number of years they are carried out. Heritability after 10 years of observation can vary between 0.19 and 0.7, as noted in Cameroon and in Ivory Coast, respectively (Cilas *et al.*, 1999; Nyassé *et al.*, 2002). This means that many years of field observations are required to obtain selection progress.

Broad sense heritabilities of the leaf disc and pod inoculation tests appear to be around 0.6 (Nyassé, 1997; Iwaro *et al.*, 1997*b*). This means that efficient selection for intrinsic resistance is possible by using such rapid screening tests, resulting in substantial genetic gains.

One example is provided here with regard to the genetic gain in field resistance that can be expected from one selection round using the leaf disc test to aim for the selection of individual seedlings in segregating populations. The calculations are based on the significant regression observed between field resistance and the leaf disc test in Cameroon and Ivory Coast (Tahi *et al.*, 2000; Nyassé *et al.*, 2002). In Ivory Coast, the average infection level of progenies from nine different parents in a factorial mating design in the field varied from 10 to 30%, whereas the average scores in the leaf disc test for the same parents increased from about 1.4 to 3.2 (Tahi *et al.*, 2000). In Cameroon, with an average infection level of about 40% in a diallel mating design (Ndoumbé *et al.*, 2001), the variation in general combining ability at a 10% infection level approximately corresponded to a one point variation on the 0-5-point scale used for scoring the leaf discs. Both results tallied as a one point variation on the 0-5-point evaluation scale approximately corresponded to a 10% variation for the level of field infection.

Iwaro and Butler (2002) estimated the genetic gain obtained in selection with leaf disc testing of 1 000 seedlings inoculated together with parental clones. With an average susceptibility level of 3.4 on the 0-5 point scale, an observed broad sense heritability of 0.51, and a standard deviation of 1.1, the estimated genetic gain was 0.98 on the 0-5 point scale for a selection intensity of 10%. In the study in Trinidad, this would mean that the selected population was expected to have a slightly higher level of resistance than Sca6, one of the most resistant control clones. With an average infection level of 20% rotten pods on the original population in the field, as often observed with *P. palmivora*, a 10% reduction can be expected in the field infection of the selected population. With an average field infection level of 40%, as can be observed with *P. megakarya* in Cameroon (Ndoumbé *et al.*, 2001), the selected population would have around 20% of infection. For the farmer, this would mean an increase in net yield of 10% and 25% respectively (table 12).

Implementing successive selection rounds with the most resistant individuals in segregating populations, such as will be done in the pre-breeding programme in Trinidad (Iwaro and Butler, 2002), would enable a cumulative reduction in the susceptibility of the selected population. Taking the cumulative selection gain, a relative reduction in the average level of susceptibility of the base population 0.98/3.4 = 29%, as observed in the pre-breeding programme in Trinidad, is assumed for each selection round. The selected population would then have 71% of the susceptibility level of the base population, on average. Applying three selection cycles, and assuming the same relative genetic gain of 29% for each selection cycle by using rapid screening tests, the average level of susceptibility would become (0.71 x 0.71 x 0.71) x 3.4 = 1.22 on the 0-5 point scale. Applying the same logic, one could translate this into a reduction from 20% pod infection in the field for the original population to (0.71 x 0.71 x 0.71) x 20 = 7% in the selected population. For the more destructive *P. megakarya*, one could expect a reduction from an original average infection of 40% in the unselected population to 14% in the selected population. For farmers, this would correspond to increases of 16% and 43% in net yield respectively.

Conclusions

Although significant variation has been demonstrated in several countries between cocoa genotypes for field infection levels, no significant progress in breeding for black pod resistance has been obtained until now (INGENIC, 1999). The integration of rapid screening tests is fundamental to obtaining rapid selection progress for intrinsic resistance. This is true for all stages in the cocoa breeding process, i.e. for screening of germplasm collections, for population breeding and pre-breeding programmes as well as for rapid selection of new cocoa varieties (clone or hybrid varieties). Furthermore, rapid screening tests enable an evaluation of resistance in the absence of the disease, as in quarantine centres.

Expected genetic gains can be expressed as a significant reduction in the level of field incidence, e.g. of 30 to 10% or 60 to 20% rotten pods for *P. palmivora* and *P. megakarya* respectively. This represents an increase in net cocoa yield for farmers (30 to 100% respectively), as can be deduced from data on resistance variation in Africa.

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