REPÈRES

Improvement of cocoa tree resistance to *Phytophthora* diseases

Christian Cilas and Denis Despréaux,
editors

CIRAD

THE EDITORS

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CIRAD

CIRAD, the "Centre de coopération internationale en recherche agronomique pour le développement", is the French Agricultural Research Centre for International Development. Its mission is to contribute to the economic development of the tropical and subtropical regions through research on agriculture, training, and dissemination of its results.

It employs 1 850 people, including 950 senior staff, working in the French overseas departments and some fifty other countries.

Cover photos
Bearing cocoa tree in Papua New Guinea (C. Cilas)
Cocoa pod with *Phytophthora* rot (C. Cilas)

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Foreword

CAOBISCO is the Brussels-based Association of the Chocolate, Biscuit and Confectionery Industries of the European Union. It is an association of national associations, based in 14 EU member states, plus 3 observer countries that are not yet members of the EU (Norway, Switzerland, Hungary). Within CAOBISCO, cocoa-related issues are dealt with by the Cocoa Committee, which is composed of cocoa specialists in CAOBISCO member companies. Its main objective is to ensure a sustainable supply of cocoa and to preserve the quality of beans at each stage of the cocoa chain, from production to industrial use.

With help from its Research and Quality Sub-Committees, and through international co-operation, the Cocoa Committee engages in numerous undertakings within the international cocoa sector. In 1995, the Cocoa Committee mandated the Research Sub-Committee to undertake a major research project aimed at improving the resistance of the cocoa crop to black pod, the most prevalent cocoa disease in the world. It is estimated that black pod can account for up to 30% losses in cocoa production worldwide, and is currently the cocoa disease that causes most loss to the farmer. Other diseases such as witches broom and monilia are potentially more dangerous to worldwide cocoa production, but these are currently restricted to the cocoa-producing countries of the Americas. Given the worldwide significance of black pod disease to cocoa production, and the expertise in both European research centres and in several cocoa-producing countries, CAOBISCO decided to fund a project aimed at producing cocoa varieties with increased resistance to black pod.

Black pod disease can be controlled by spraying with fungicide, by cultural practices, and, more fundamentally, by improving the genetic resistance of the tree through selective breeding. The high frequency of spraying needed to control the disease may be uneconomic and the rate of spread of the pathogen through a cocoa farm may make cultural practices insufficient to halt the disease. It is generally accepted that a combination of anti-fungal treatments, cultural practices and genetic resistance are needed to reduce the losses caused by the disease. Therefore, the CAOBISCO project concentrated on improving the genetic resistance of cocoa trees to both *Phytophthora palmivora* and *P. megakarya*. The concept was to identify the more resistant cocoa trees and cross them naturally together in the hope of producing more resistant progenies from these crosses.

A research team was set up in 1995, based at the Centre de cooperation internationale en recherché agronomique pour le développement (CIRAD) in France, with collaborating researchers at the Institut de recherche agricole pour le développement (IRAD) in Cameroon, the Centre national de recherche agronomique (CNRA) in Ivory Coast, and the Cocoa research Unit (CRU) in Trinidad. The project was completed in 2000 with a closing seminar at CIRAD on the major research findings.

This book presents the scientific results of five years of research and aims to make these results available to any researcher interested in using them.

To summarize, the research teams at CIRAD, CRU, CNRA, and at IRAD have made substantial advances in the search for ways to breed cocoa for increased resistance to

black pod disease. Phytopathological and genetic tests have been developed which will greatly speed up the rate at which resistant plants can be identified. Cocoa trees with greater resistance are already in the ground as a result of the project. However, for a worldwide benefit to accrue to cocoa as a result of such research, there is a need for an increased exchange of cocoa germplasm, and knowledge about germplasm, between producing country research centres. Therefore the importance of the International Cocoa Germplasm Database (ICGD), and the existing cocoa quarantine facilities at the University of Reading in England, and more recently the United States Department of Agriculture (USDA) in Miami, should be recognized by the whole cocoa sector. There are clearly lessons and techniques to be learned from this project for other cocoa diseases such as witches broom, monilia, and vascular streak dieback.

It is important to stress that the success of the project has been due to a number of factors. First of all, there has been strong collaboration among researchers in cocoa-producing and cocoa-consuming countries. This collaboration, North-South and East-West, has been unique, and was facilitated by each researcher's discipline in communicating results on a regular basis and the important coordination role played by CIRAD.

Then there has been the use of a number of valuable breeding populations in the producing countries, plus the effective combination of new molecular techniques with field observations, and an ability to coordinate the fast-moving parts of the project with those parts requiring more long-term experiments. Many of the techniques and expertise developed during the project are directly transferable to projects currently under way on other cocoa diseases, and it may even be the case that some of the genetic factors responsible for black pod resistance have a role in providing resistance to other cocoa diseases. It should not be forgotten that while resistance to disease is important, and probably an efficient way to improve cocoa yields, other traits such as fat content and composition, flavour, tree structure, photosynthetic efficiency, precocity, etc., are all important in breeding better cocoa trees.

In plant breeding, the long generating time for trees has restricted the speed at which new improved varieties become available. Nevertheless, tree crops such as apple, pear, poplar, and walnut have all been substantially improved through conventional plant breeding. In cocoa the situation is made more difficult by the fact that the crop is barely domesticated. Cocoa has also not had the same husbandry and horticultural advances common in other tree crops. Nevertheless, this project has shown that modern research techniques, applied in a coordinated way and having strong links to field experiments, can improve the cocoa crop.

The solution to poor yields on cocoa farms will be a mixture of improved genetic material, better tree husbandry, better management of soil fertility, better use of inputs (biocontrol agents, approved fungicides and pesticides), shade management, i.e. an integrated crop management approach. Through the CAOBISCO black pod project, we have made advances on the first of these and we now look towards building on this research and continuing to improve the development of the chocolate industry's most vital crop.

We wish all readers an enjoyable read and hope that this research will be usefully extended to all cocoa scientists and field implementation. In conclusion, we would like to thank the following people for the cooperation enjoyed throughout the five years of the project: Etienne Bidzanga, David Butler, Christian Cilas, Denis Despréaux, Michel Ducamp, Albertus Eskes, Marie-Henriette Flament, Ismael Kébé, Claire Lanaud, Nathalie Mercier, Jeanne N'Goran, Salomon Nyassé, Philippe Petithuguenin, Ange-Marie Risterucci, Olivier Sounigo, Mathias Tahi and Jean-Marc Thévenin.

Martin Gilmour, Cocoa Research Manager, Mars Incorporated
and Celine Anselme, former Raw Materials Manager at Caobisco

Preface

Phytophthora diseases are the main cause of harvest losses in existing cocoa plantings. They occur in all production zones and damage can amount each year to 20% of the world harvest. Locally, losses can exceed 50%, or even 90% in extreme cases. Several species of *Phytophthora* are implicated: *P. palmivora, P. megakarya, P. capsici* and *P. citrophthora*. The most widespread species is *P. palmivora,* whilst *P. megakarya* causes most damage and is only rife on the African continent.

The most common control methods are based on sanitation harvesting systems and fungicide treatments, to be applied during epidemics. Chemical treatments are relatively effective, but they are expensive, pollute the environment, and there is a risk of resistant strains appearing.

Numerous studies have shown the existence of substantial variability in cocoa tree resistance to *Phytophthora*. It has been revealed by artificially inoculating pods, leaves or young stems. Over 100 clones have been classed in Cameroon for their susceptibility, measured by artificial inoculation of pods, giving between 30 and 100% successful infections. Variations of between 15 and 90% of successful infections have been observed between families in Costa Rica. In addition, field observations over several consecutive years under plantation conditions revealed that resistance trait heredity was additive. Some genotypes transmit a higher than average resistance level to their progenies, thereby opening up the way for interesting genetic improvement possibilities. However, the difficulty in implementing breeding programmes still lies in the time needed to complete a selection cycle, around 12 years, with several cycles no doubt being essential for any notable varietal improvement. It also lies in the size of the areas required. Early, discriminant and repeatable screening tests are needed if these obstacles are to be overcome. Artificial inoculations under controlled conditions can be very useful tools in that respect. It merely means demonstrating the existence of a good relation between the degree of resistance in a genotype measured by the test, and its general performance in the field. With the cocoa tree-*Phytophthora* pair, it is difficult to demonstrate correlations between the results obtained with the different measuring methods, which suggests the existence of complex resistance mechanisms that may involve several resistance factors, or several more or less interdependent genes.

The scientific purpose of the project, whose studies are presented in this book, was to enhance knowledge of the genetic bases of cocoa tree resistance to *Phytophthora* diseases, in order to have reliable tools for developing varietal improvement programmes that are efficient within a limited time span. Its achievement was the fruit of an international partnership associating teams that already had extensive research experience on this subject: CNRA in Ivory Coast, IRAD in Cameroon, CRU in Trinidad and CIRAD in France. This partnership made it possible to pool human resources and the experimental bases already mobilized by these research establishments, along with additional financial resources generously provided by the European chocolate industry through its association, CAOBISCO. The project thus benefited from activities, programmes and experiments that formed a foundation on which the specific project operations hinged to create a synergy.

The aim was to use all the research tools available upstream, particularly in the molecular biology field, whilst remaining closely attached to linking laboratory results with whole plant field trials, in order to end up with products that could be used by farmers.

Three fields were covered at the same time:

• Host-parasite interactions, in order to identify the factors involved in resistance expression, along with effective indicators of the degree of resistance. This work also involved analysing the diversity of pathogen populations. It led to the development and validation of standardized evaluation methods that could be applied as early as possible, in order to speed up selection processes without systematically having to observe how trees performed in the field.

• Localization of the different regions of the genome involved in resistance traits. The initial work focused on finalizing the genome map, and particularly included the development of microsatellite markers in addition to the RAPD and RFLP markers already available. The map was then used to search for QTL by trying out inoculation tests and observing field resistance in trials planted in Cameroon and Ivory Coast. The QTL involved in the mechanisms of cocoa tree resistance to *Phytophthora* have been identified. A search for candidate genes, characterized in other species, was also undertaken at the same time.

• Creation and evaluation in the nursery of new progenies or new clones to check whether resistance genes could be cumulated and proceed with the first stages of a selection programme. An initial selection cycle was undertaken and the first set of preselected genotypes was made available to breeders in the partner countries.

Throughout its duration, the project was monitored by a technical committee, which met twice-yearly in Montpellier. The committee comprised representatives of CAOBISCO and of the four scientific partners involved. In general, all the scientists working on the project were invited to take part in the meetings, along with eminent people from outside whose views might prove valuable for subsequent research. Presentations and discussions were backed up by a report summarizing operations completed over the previous period, and proposing future action. A mid-term seminar and an end-of-project seminar were organized.

These regular contacts and the ready mobility of the researchers, who made frequent trips between the research sites, created a truly dynamic and united team based on true friendships that would easily outlive the duration of the project.

We should like to express our gratitude to the researchers and technicians who took part in this work, along with representatives of the chocolate industry, particularly Marc Fowler and Martin Gilmour who lent their expertise to the technical committee. We should also like to thank those who contributed towards this publication: the CIRAD publishing service, particularly Nicole Pons, the revisers and notably Brigitte Courtois, Didier Thareau, Jean Carlier, and Chantal Diaz, not forgetting Peter Biggins for his various translations and revisions.

Christian Cilas and Denis Despréaux

Résumé

La pourriture des cabosses de cacaoyer est responsable de près de 30 % des pertes de la production mondiale de cacao. Cette maladie est due à diverses espèces du genre *Phytophthora*. L'espèce la plus dommageable, *P. megakarya*, envahit actuellement la Côte d'Ivoire, premier pays producteur. Face à cette menace, un projet de recherche sur les bases génétiques de la résistance des cacaoyers aux maladies à *Phytophthora* a réuni des équipes de chercheurs du Cirad, en France, de l'Irad, au Cameroun, du Cnra, en Côte d'Ivoire et du Cru, à Trinidad . Ces recherches ont reçu l'appui financier des chocolatiers européens à travers l'association Caobisco.

Cet ouvrage de synthèse présente les résultats acquis lors des travaux conduits dans le cadre du projet. Il a pour principal objectif de mettre à la disposition de la communauté internationale les connaissances et les outils utilisables pour la sélection de cacaoyers plus résistants à *Phytophthora*.

Improvement of cocoa tree resistance to Phytophthora *diseases* fait le point sur la diversité du pathogène, les connaissances épidémiologiques, les paramètres génétiques de la résistance observée en champ, les aspects pratiques de la sélection. La pertinence de différents tests d'évaluation à partir d'inoculations artificielles et l'utilisation des marqueurs moléculaires dans la sélection de matériel résistant sont largement abordées.

Phytophthora diseases of *Theobroma* cacao

Denis Despréaux

Once the centre of economic activities in the Mayan and Aztec civilizations, cocoa has become one of the main modern-day agricultural exports from the humid tropics. The cocoa tree, from which it is produced, has adapted to numerous situations and, despite its high susceptibility to pests and diseases, it is grown throughout the equatorial and tropical belt of the planet. The best conditions for its expansion are to be found in Africa, especially in West Africa–the Ivory Coast and Ghana–which explains why more than two-thirds of world production now comes from that continent.

The large amount of research devoted to the cocoa tree has considerably enhanced our knowledge of its origin, its functioning, its requirements, and its potential, though it has not yet been possible to raise yields on a scale seen for many other cultivated crops. For instance, despite the existence of a few rare plantations based on an intensive system, the current average yield per hectare worldwide is no doubt not much more than it was in Central America prior to the Spanish conquest. Indeed, the enormous increase in volume has so far been achieved exclusively by increasing the areas planted, which still remains the most cost-effective solution. Most new plantations have been set up using traditional techniques on cleared forestland. This system was particularly advantageous when immense expanses of virtually virgin territory were available. Such zones still exist on a world scale even today, though they are becoming increasingly rare. However, this headlong pursuit will soon reach its limits. Major producing

countries, such as the Ivory Coast and Ghana, are already faced with a lack of new land for planting. Maintaining their production levels, which is as important for their economies as it is for world market stability, now entails the rehabilitation or renewal of plantations, many of which are already old (Petithuguenin and Despréaux, 1994). Moreover, environmental awareness is increasing among producers and consumers, and farming systems are now being considered for more than their productivity, with thought being given to the sustainable management of natural resources.

One of the main challenges for new crop management sequences will be their ability to control pests and diseases effectively, since the installation of a monoculture over long periods inevitably leads to an increase in the incidence of parasites associated with it. Cultivated ecosystems lead to a concentration of one species in a limited space, reducing natural biodiversity. Such conditions are propitious to pathogen multiplication. In some cases, parasite pressure can become such that a crop loses all its competitiveness. It is then abandoned, or becomes marginalized within the farming system.

Such devastating endemics have existed and continue to exist for cocoa. Witches' broom is very serious in Latin America, especially in Brazil, pod borers devastate plantations in Southeast Asia, but the most severe damage on a world scale remains that caused by *Phytophthora* diseases, which occur in all producing countries. The most serious situations are found in central Africa or West Africa, where a particularly destructive species develops, *Phytophthora megakarya*. Losses in some zones can amount to virtually the entire crop.

The ultimate aim of the international research project coordinated by the Centre de coopération internationale en recherche agronomique pour le développement (CIRAD) was to develop crop management sequences that sustainably limit the incidence of *Phytophthora* diseases in cocoa plantations. To that end, it was initially necessary to enhance scientific knowledge of cocoa genetic resistance to *Phytophthora*, in order to acquire the necessary tools for creating new cultivars less susceptible to epidemics.

This first chapter describes where research stood in terms of cocoa cultivation and *Phytophthora* diseases when the project was launched. The information provided enables the reader to see how the work conducted fits into a context of wider knowledge.

The cocoa tree and its cultivation

The cocoa tree

The cocoa tree belongs to the order of the Malvales, the family of the Sterculiaceae, the tribe of the Byttneriaceae and the genus *Theobroma*. This genus

includes around 20 species of trees, all from the Amazon forest and other humid tropical zones of Central and South America.

Cocoa trees have many morphological forms that may seem very different from each other. However, all these trees whether cultivated or wild, are cross-fertilizing, as are their progenies: they therefore all belong to the same species known today as *Theobroma cacao*. Cocoa trees are traditionally divided into three major groups: Criollo, Forastero and Trinitario; the last group contains crosses between the first two groups. For a clearer understanding of how this classification was defined, and what it still signifies today, it is worth looking back over the main features of the taxonomy work carried out on this subject over almost four centuries.

Morphological descriptors and their limitations for classification purposes

The first systematic review of cultivated cocoa varieties was drawn up by Morris (1882). Cocoa trees were listed in two classes, Criollo and Forastero, both terms taken from the current language. The words also had a geographical significance: Criollo corresponded to a local origin and Forastero to a foreign origin. Both names could therefore be opposites from one country to the next. For instance, in 1901, Preuss noted that the term Forastero in Trinidad tallied with Criollo in Venezuela and vice versa. In addition, his own research on cultivated cocoa trees in Central and South American countries led him to distinguish between three groups rather than two: a Criollo variety originating from Trinidad, and two cultivated varieties in Venezuela, Forastero and Trinitario[1].

The term Trinitario subsequently disappeared from classification proposals for more than 40 years. It was not used by Van Hall, who produced a detailed description of the variability of cultivated cocoa trees in 1914, and again in 1932, structuring the species again in two groups, Forastero and Criollo, each comprising several sub-varieties, or by Pittier, who published a key for the determination of known *Theobroma* species in 1935 and proposed the concept of a "cocoa complex" composed of several species The Trinitario name was only taken up again in 1944 by Cheeseman. After backing Pittier's theses for a time, Cheeseman finally concluded that there was genetic flow among wild, semi-wild and cultivated cocoa trees, and that all of them consequently belonged to a single species. According to him, the species can be split into two main morpho-geographical groups: Criollo and Forastero. The members of the first group are distributed North of the Andes, and those in the second group are distributed throughout the Amazon basin. Each group breaks down

1. In 1825, a Venezuelan grower introduced vigorous material from Trinidad. The seeds from those cocoa trees were then distributed in Venezuela under the Trinitario name. The precise genetic origin of the material is unknown, but it is likely that it involved crosses of ancient Criollos from Trinidad with Amelonados imported from the continent (Pittier, 1935).

into several sub-groups. The Criollos can be separated into two sub-groups: one originating from Central America, the other from South America. Likewise, the Forasteros can be divided into Amazon Forasteros, which are wild and cultivated almost everywhere, and Trinitarios, the result of a cross between Criollo and Amazon Forastero materials.

Cheeseman's proposal was taken up again and completed by Cuatrecasas who, in 1964, produced a detailed revision of the *Theobroma* genus. The genus was subdivided into 6 sections of 22 species, whose original geographical range extended on the American continent between 18° North and 15° South. The species *T. cacao* alone accounted for one of the sections, which contained the following sub-species and forms:

– subsp. *cacao* characterized by an elongated, claviform, fusiform or oblong ovoid-shaped fruit, with 5 to 10 more or less marked and warty ridges; a pericarp of moderate thickness and a thin woody endocarp; ovoid or ellipsoid seeds, usually with a rounded cross-section; white or yellowish-white cotyledons; the Criollos correspond to this sub-species. The following forms can be distinguished:

- forma *pentagonum* (5 ridges); common names: *cacao lagarto, alligator cacao*; known only in its cultivated state in Central America and southern Mexico; provides one of the best cocoas.
- forma *leïocarpum* (5 ridges); common names: *cunamaco* (Guatemala), *porcelana, java criollo* (trade name); provides a top quality cocoa.
- forma *lacandonense* (10 ridges); wild in the dense tropical forests of the north-eastern Chiapas, Mexico; could be an ancestor of cultivated cocoa trees.

– subsp *sphaerocarpum* characterized by an ellipsoid, almost globular, or more or less oblong fruit, rounded at both ends, smooth or very slightly warty, may have more or less slight furrows; very thick pericarp and a hard woody mesocarp; ovoid, more or less flattened seeds: purple or deep violet cotyledons. The Forasteros correspond to this sub-species: Calabacillo and Amelonado; this sub-species is found in its wild state from the Guyanas to mid Amazonia, to the north and east of the Andes.

The Trinitarios described by Cheeseman were classed here among the Forasteros, though they were identified as probably resulting from a cross between a Forastero originating from the Orinoco basin in Venezuela and Criollos from Trinidad.

Based on information from surveys by Pound (1938-1943) in Peru and Ecuador, in 1972 Toxopeus made a distinction between two types of Forasteros depending on their original location in the Amazon basin:

– Lower-Amazons, which are found in the lower section of the basin, and are relatively homogeneous around a major Amelonado morphological type. It is these that are most widely distributed and cultivated throughout the world.

– Upper-Amazons, from the upper section of the Basin, and which reveal substantial diversity, ranging from the Amelonado type to populations with a morphology very similar to that of the Criollos.

These different structuring proposals were gradually bolstered by increasing amounts of information on the diversity of the species. However, as time passed, the distinctions between the different groups based on their morphology became increasingly blurred. Indeed, no individual trait appeared to be typical of one or other of them. For example, the Criollos generally have elongated and rough pods. However, some populations, such as Porcelana in Venezuela, produce round, smooth pods of the Amelonado type. Likewise, the *Nacional* variety in Ecuador only differs from the Criollos through its pods, which are slightly less elongated and green, sometimes with slight traces of red pigmentation. This difficulty in setting more accurate limits to the different groups, based on morphological traits taken separately, resulted in attempts to combine the use of several descriptors. Engels (1986) thus carried out a principal components analysis using 39 independent foliar and floral descriptors, based on observations recorded in the collection of the Centro Agronómico Tropical de Investigación y Enseñanza (CATIE) in Costa Rica. While the analysis clearly indicated the existence of two groups, one primarily containing Criollos and the other Forasteros, the two groups still partially overlapped. Thus, in order to gain a clearer picture of how the species is structured, and thereby make more effective use of its genetic diversity, the use of additional tools proved essential.

Contributions of molecular tools

Studies on the genetic diversity of *T. cacao* populations, using molecular markers, began with the use of isozyme techniques. Only ten or so enzyme systems were found to be polymorphic and enzymatic polymorphism seemed to be very low: allelic diversity was from 1.9 to 2.2 alleles per locus, on average. However, allelic frequency varied substantially between the different groups. For instance, Amefia (1986), then Lanaud (1987), showed that the Upper-Amazon Forastero group was much more variable than the Lower-Amazon Forastero group. There was little polymorphism among the Criollos and it was completely overlaid by that of the Trinitarios. Later, Ronning and Schnell (1994) obtained similar results, making it possible to separate the Trinitarios and Criollos from the set of Forasteros.

Restriction fragment length polymorphism (RFLP) techniques also revealed substantial variability in nuclear DNA among the Upper-Amazon Forasteros (Laurent, 1993). In the Criollo and Trinitario materials, variability remained significant, albeit less, whereas that of the Lower-Amazon Forasteros was much less. An analysis of the chloroplastic genome only revealed slight diversity, unlike the mitochondrial genome, for which the greatest variability was found this time in the Criollos (Laurent, 1993). However, a clearer picture, with morpho-geographical structuring, was found with random amplification of polymorphic DNA (RAPD)

techniques, which distinguished between Criollos and Forasteros and, among the latter, between Lower-Amazons and Upper-Amazons (N'Goran *et al.*, 1994). The Trinitarios revealed wide variability, partially overlapping with the Criollos and Lower-Amazon Forasteros.

The highest rate of heterozygosity was found in the Trinitarios, which tended to confirm between-group hybridization of trees classed in this population. Criollos and Upper-Amazon Forasteros had a slightly lower level of heterozygosity. The Lower-Amazon Forasteros were the least heterozygous, and revealed a much larger percentage of fixed alleles than the other groups.

Thus, molecular tools can be used to corroborate the results obtained with morphological descriptors. There is, however, one exception: Criollo cocoa trees from Venezuela, which reveal substantial morphological variability, are very similar on a molecular level (Motamayor, 1996). One plausible explanation may be that the morphological variability of the Criollos from Venezuela depends on only a very limited number of genes. In that case, their strong morphological differentiation would not reflect substantial overall genetic diversity, but would be the result of the particular selection pressure exerted by man on this type of trait. Their genetic base could therefore be very narrow, or even reduced to virtually a single ancestral genotype.

On the whole, the results obtained with molecular markers tend to consolidate the hypothesis of the existence of two main groups, the Criollos and Forasteros, themselves subdivided into several morpho-geographical sub-groups. However, the limits of the groups and sub-groups still remain imprecise, as many individuals contain the alleles of several groups in different proportions. Human intervention may lie behind these hybridization phenomena. Indeed, intentional or accidental transportation of beans may have resulted in erratic introductions of exogenous cocoa trees, leading to mixes, and the differentiation that may have been marked at certain moments in the past has now been partially wiped out.

The existence of two main groups does not rule out the existence of other types of cocoa trees that are clearly distinct from the Criollos, and from Forasteros. This is particularly the case for Guianan trees that, in view of their geographical origin, ought to be among the Lower-Amazons. However, they have neither their morphological characteristics nor the specific alleles (Paulin *et al.*, 1996). Likewise, the traditional Nacional variety from Ecuador clearly stands out from the other populations (Lerceteau, 1996). This existence of other types of cocoa trees suggests that several diversification stages have occurred over time, more or less independently. Their chronological sequence remains to be determined.

Cocoa cultivation

Numerous depictions of the cocoa tree and its fruits in the sculptures found among the ruins of Mayan and Aztec cities bear witness to the importance of cocoa in the lives of those ancient peoples. Indeed, cocoa was probably the first

commercial agricultural produce in south-eastern Central America. It appeared in around 1000 BC and its central role in the regional economy was well established around 400 BC. Cocoa had become so important in Mayan society that the last three chiefs of the city of Tikal in Guatemala were called Mr Cocoa (Young, 1994).

The means of production consisted primarily of small plantations dispersed throughout the Mexican and Central American region. Only a few of them were intended to produce large quantities of beans for marketing. The first intensive farming systems seem to have been created by the Pipil-Nicarao Indians. Remnants of these crops survive in southern Guatemala and in the largest settlement of the Pipil at Izalco, on the Pacific coast of El Salvador. Cocoa was grown as a precious tree, on fertile soils, under regulated shade, in rows spaced 3 to 4 metres apart. They were exclusively Criollo type trees. Some of these cocoa trees could bear up to 100 pods per year. The existence of traces of irrigation systems, used to limit the harmful effects of long dry seasons in that part of Central America, reveal just how much care they took with these plantings.

When Hernando Cortès landed on the coast of Tabasco in 1519, he very quickly took an interest in cocoa; it was used to make a nourishing drink, which was widely consumed at the court of Montezuma. The beans were also the common currency in use throughout the provinces of Mexico, each of which paid a heavy tribute to the king. A rabbit could be bought on the market for 10 beans, a horse or a donkey for 50 beans, a slave for 100 beans.

The first introductions of cocoa to Spain began with small quantities in paste form. The first shipment of beans was not unloaded in that country until 1585. From that point onwards, demand in Europe continued to increase and, in order to satisfy this booming market, the Spaniards helped set up an export monoculture system.

The main types of cultivated cocoa trees

Most cultivated cocoa trees come from mass selections carried out by successive generations of growers who took seeds from trees with the most sought-after characteristics. In a given production zone, all the cultivated trees are usually derived from a limited number of individuals. Genotypes improved by these traditional methods are usually characterized by a relatively uniform morphology, a high level of self-compatibility, and much higher homozygosity rates than wild cocoa trees. However, they are still more or less heterogeneous populations, made up of individuals that are more or less heterozygous.

The oldest populations, from Central America and especially developed in that geographical zone, consist exclusively of Criollos. They were the only type of cocoa trees cultivated in the 17th century, but now account for less than 5% of world production. Trinitarios supply from 10-15% of world production. The largest percentage is from trees derived from the Forastero group. Two other groups of cultivated cocoa trees, which are distinct from the previous three, have

been inventoried: they are "Nacional" cocoa trees originating from Ecuador—for which the areas planted are in decline though they are still grown today—and Guianan cocoa trees, which appear to be vestiges of ancient plantations abandoned a long time ago.

Improved materials proposed by research centres are gradually taking over from traditional cocoa trees.

Criollo

Criollo trees produce a "pale break" cocoa, high in flavour, with only slight bitterness. However, the trees give low yields, are lacking in vigour, are highly susceptible to diseases and reveal low adaptability to conditions that differ from those of their respective sites of origin. For instance, despite the excellent quality of their beans for chocolate making, Criollos are virtually no longer cultivated. Moreover, the farms that still exist very rarely consist of pure Criollo plantings, but rather of mixtures that also include types of Forasteros or hybrids introduced more recently. Their beans are varying shades of violet, whereas they are white in the case of pure Criollos, and that is a good indication of the existence of Forastero alleles in cultivated populations.

At the time of the Spanish conquest, the main producing regions were Izalco, in El Salvador, and Sulla Valley, along the Caribbean coast of Honduras. Cocoa was also cultivated in Nicaragua, primarily in the Leon and Granada districts, where there are still a few small plantations. In those regions, the morphology of the fruits is either the cundeamor type, with long bright red pods with a bottleneck and curved tip, or the angoleta type, with small green pods, and much more rarely the pentagonum type.

There were also major production zones in Guatemala, particularly on the Pacific coast, near the Mexican border and at the foot of the mountains. Nowadays, the Suchitepéquez department is still a production zone, along with two other sites on the Pacific coast, but the trees grown are new hybrids.

In Mexico, around 20% of production comes from plantations where Criollos still dominate, primarily in the Tabasco region. However, pure Criollo plots are very rare apart from a few very old small plantations, where isolated groups of trees still exist that reveal considerable variability in the size and shape of their pods. The pods usually vary from light to dark green, but there are a few types with red pods. The pod shape is primarily angoleta, though cundeamor forms do exist.

In Colombia, there are still a few small plantations in the upper Cauca and Magdalena valleys. The mostly green pods are of the angoleta type. These Criollos were apparently imported in the past from Mexico.

Venezuela is still reputed for its Criollo production:

– in the Cepe, Chuao and Choroni valleys, where the trees are very similar to the Nicaraguan Criollo, from which they could be derived. There are some-

times also pale green or pink *angoleta* pods, known locally as "criollo verde" or "criollo blanco".

– on the southwest shores of the Maracaibo lagoon, where Porcelana and Merida cocoas are found. The pods are very similar in shape to the Amelonado, with a slightly sharper tip, and a smooth surface with very shallow furrows. The pods are predominantly pale green-to-bright pink.

However, Criollos were also exported from the American continent, giving rise to other cultivation foci, though they remained very limited in number and size. For instance, there are Criollo plantings in the Comoro Islands (Ngazidja) and Madagascar (Sambirano region), characterized by small, orange, *angoleta*-shaped pods that produce rather small, round beans.

Trinitario

Trinitarios, which are hybrids of Criollos and usually Lower-Amazon Forasteros, have intermediate characteristics between the two groups. In the first generation, the trees can be very vigorous and high-yielding. Their bean colour, which is between the white of the Criollos and the dark colour of the Forasteros, make them much sought-after for powder manufacture.

The first known Trinitario population was developed in Trinidad. It was not introduced onto the American continent until the 19th century, first in Venezuela then in Ecuador. In the latter country, Trinitarios are more commonly known as Venezelanos, following massive introduction of Trinitarios from Venezuela between 1930 and 1940, in an attempt to control witches' broom. Nowadays, Trinitarios are cultivated in all regions where Criollos used to be grown (Mexico, Central America, Trinidad and the Caribbean islands, Colombia, Venezuela, etc.). There are few Trinitarios in Africa, except in Cameroon, which stands out from the other producing countries with its large population of that type of cocoa tree. Other Trinitarios come from Southeast Asia (Java, Sri Lanka) and Oceania (Papua New Guinea, Samoa, Fiji).

Through their variability, Trinitario populations are choice materials for breeders, who can pick combinations that bring together the maximum number of worthwhile traits from the multiple combinations encountered. Thus, numerous clones have been selected for breeding purposes, such as ICS clones (selection by Imperial College in Trinidad), UF clones (selection by United Fruit in Costa Rica), SNK clones (selection by the Nkoemvone station in Cameroon), or clone CCN51[2] in Ecuador. However, breeding these cocoa trees of hybrid origin from seed leads to substantial disjunction of traits in the progenies.

2. Clone CCN51 was selected in Ecuador at a private plantation from crosses between Trinitarios. Its notable production potential under intensive clonal cultivation conditions explains its major success with growers, and its spectacular development on a national scale. However, the cocoa it produces does not give the arriba flavour.

Forastero

The most frequently cultivated and common cocoa trees worldwide are the Amelonados, which come from the Lower-Amazon basin. Their pods are moderate in size, green in colour turning yellow as they ripen. They have a slight bottleneck and a smooth surface with very shallow furrows. The beans are medium-sized, and dark violet in colour. These varieties owe their success to their uniformity, their good agronomic traits and their excellent ability to adapt to new territories.

The largest cultivation areas are in Brazil, under the name cacao comum, which remains the most frequently grown planting material in the Bahia region, and in West Africa, where it is also the main means of production. The comum is generally considered to be the origin of the other Amelonados grown, often in mixes with Trinitarios, in most tropical American countries and the West Indies, Suriname, Costa Rica (under the Matina or Ceylon names), in Mexico, Guatemala and the Dominican Republic (under the Sanchez name), Colombia (under the Pajarito name) and Venezuela (under the Forastero de Barlovento name).

Two other types of Lower-Amazon Forasteros are grown in Brazil, with a more limited distribution; these are the *para* and maranhoa cocoas. Para pods are smaller, with a smooth surface barely marked by furrows, of the Calabacillo shape and pale green to whitish green in colour. Para is not very widely cultivated in plantations, though it is reputed to be very high yielding. The maranhao has elongated, large, Amelonado-shaped pods with a bottleneck, terminating in a blunt tip. The pods are green, marked with furrows and are slightly warty.

Two varieties, almeida and catongo, whose morphological characteristics are very similar to those of the *para* and comum have recently started being grown in the Bahia region. However, these two populations stand out from the previous two as they produce white beans. Nevertheless, the flowers and young shoots remain typically pigmented like Lower-Amazon Forasteros. These two populations appear to be derived from mutant individuals of the comum or para varieties.

Cocoa surveys in the upper reaches of the Amazon basin did not reveal the existence of any cocoa variety traditionally cultivated in those zones. However, of the numerous clones collected, and observed in Trinidad, a few have been selected and distributed to collections worldwide. These genotypes are very widely used as parents in most breeding schemes, since hybrids from crosses with Lower-Amazons or Trinitarios have often revealed excellent vigour, precocity and production traits. Most of the improved hybrid families proposed by research centres have been created with this type of cross.

Nacional

Nacional, a traditional variety from Ecuador, was long classed among the Forasteros. However, recent genetic diversity studies indicate that it is an independent population (Lerceteau, 1996). The green pods are large and oval with a slight bottleneck and a blunt tip. The pericarp is thick with deep furrows and a warty sur-

face. The beans are large and a paler violet than typical Amazon Forasteros. They produce a cocoa included in the "fine" cocoas with particular flavour characteristics, known on the market as *arriba,* and are responsible for the quality reputation of Ecuadorian cocoa.

Nacional is highly susceptible to witches' broom and is being used less and less in plantations. It is being replaced by Trinitarios disseminated either in seedling form, or as cuttings, as is the case for clone CCN51 that is accounting for an increasing share of Ecuadorian production.

Growing conditions

Climate

The ideal climate for the cocoa tree corresponds to the conditions prevailing in the rain forests from which it originated. The optimum average temperature is around 26°C, with a maximum not exceeding 30-32°C and a minimum no lower than 18-21°C. Temperatures below 10°C are fatal. The relative humidity must always be very high to prevent leaves from drying out and falling. Rainfall preferably exceeds 1,500 mm per year, but a regular rainfall pattern throughout the year is particularly important. The species is very sensitive to water deficits, and trees have difficulty withstanding dry seasons of more than three months. Cocoa trees can be found in temporarily flooded areas. Wind, particularly dry winds such as the Harmattan in West Africa, can cause considerable damage to the foliage.

Soil

In the pioneer front system, which is the main formation of the production zones, the criteria used when choosing to set up new plots are primarily the availability of forestland and the ability to assemble a sufficient work force. It is rare for planting sites to be determined according to the physicochemical characteristics of the soils. Cocoa trees are thus grown on extremely varied soils. An analysis of planting situations shows that this tree can perform very well in very different types of soils, and it is very difficult to deduce standard ideal growth conditions. Demands, particularly in terms of the physicochemical properties of the soil, largely depend on the rainfall pattern in which the tree grows, and adaptation of shading to the climate. Indeed, the aerial organs of the cocoa tree can grow well and yields can be good in shallow soils, through strong development of its secondary root system. However, under such conditions, it is highly sensitive to any dry season that lasts for a while. It is in soils with a depth of more than 1.5 m, in which it can develop a substantial taproot, that it will best resist the most unsuitable climatic conditions, and cultivation without shade.

Its soil texture requirements also depend on climatic conditions and cultivation methods. Indeed, good drainage is necessary to prevent root asphyxia during the rainy season. However, the water-holding capacity must be sufficient to prevent

the harmful effects of a water deficit during dry periods. For instance, in very wet climates, drainage quality will be an essential factor in the good performance of trees, while in drier conditions a good water-holding capacity becomes the most important factor.

As regards the chemical nature of the soils, it can be said that cocoa trees prefer soils that are somewhat rich in organic and neutral matter. By searching for a mineral nutrition balance, it is possible to recommend fertilizer programmes based on soil analyses (Jadin,1992).

Crop management sequences

The traditional and ancestral method of planting on cleared forest land consists of manual felling of a clearing to make way for the future cocoa trees. Partial felling retains a minimum of shading to protect seedlings from the harmful effects of full sunlight. Indeed, with total felling, young cocoa trees have to be protected with a system of temporary shade. Seeds are also often planted directly in the soil, which is still humus-rich. However, sowing can also be carried out in nursery bags, to take care of the seedlings as they grow and plant out seedlings that are already vigorous enough to resist the numerous physical or biotic aggressions they will have to face in the plots. Pesticide treatments often prove useful at these early stages, as the young organs are highly susceptible to insect attacks. In the early years prior to canopy formation, the main upkeep operation consists in controlling weeds, so as to encourage optimum development of the young trees. Cocoa tree pruning is not usually necessary, except for giving the tree a balanced shape and ensuring that future fruit-bearing branches are accessible. When planting is successfully carried out, the tree canopies join up after 5 to 7 years, providing dense shade at ground level, thereby preventing weeds and other undesirable plants from growing. Plantation upkeep becomes easy and only requires a few rounds a year, mostly to remove suckers that grow directly from the trunks.

It is also possible to set up plantations with highly perfected crop management sequences. In this case, the soil is tilled and improved, temporary shading systems, such as banana planted in the inter-row, are set up several months before cocoa seedlings are planted out in the plot. Seedlings are protected in the nursery, then in the field, by systematic insecticide treatments. The trees are lined up in such a way as to ensure that each of them has sufficient space for optimum expression of its production potential. Mature plantings are grown either without shade, when edapho-climatic conditions allow, or under shade trees planted for that purpose. In this context, the choice of the planting material used is very important. Nowadays, most producing countries have several widely distributed between-group hybrid families. In some countries, such as Ecuador, Malaysia and Papua New Guinea, clonal selections are also proposed. The best produce 2.5 to 3 t/ha under intensive cultivation conditions, as opposed to around 1 t/ha for the

best traditional varieties. They also have improved technological qualities, such as a larger bean size and higher butter content (Paulin and Eskes, 1995).

However, it is rare for intensive systems to be adopted in their entirety. It is exceptional in Africa, and mostly involves a few large estates in Malaysia, Papua New Guinea and Ecuador, along with some smallholdings in Sulawesi. Virtually everywhere else, the degree to which intensive cultivation has been adopted varies greatly and bears no relation to plot size. This limited success is undoubtedly partly due to the economic context—which has not been particularly favourable, with record low prices in the 1990s—but also to the strict relationship that exists between inputs (especially work loads and labour availability) and production, which does not show any true economies of scale. The most important change in recent years has primarily been the gradual replacement of old varieties by hybrid seedlings or their progenies. Nevertheless, the results obtained for commercial production with this type of planting material have generally been very slow in coming and yields of over 1.5 tonnes remain exceptional. The sensitivity of hybrids to climatic conditions or diseases and pests are often blamed for these average results.

Development of cocoa cultivation worldwide

Production in Central America declined towards the end of the 16th century, at a time when the Indian populations were decimated, due partly perhaps to a serious outbreak of plague. In order to try to extend cocoa cultivation to other areas, Capuchin monks introduced Criollo cocoa seeds to the state of Aragua in Venezuela in the 17th century, and set up the first plantations in South America. This new crop, which could easily be sold at a very lucrative price, soon caught on throughout the country, and Venezuela overtook Mexico to become the world's largest cocoa exporter. The original Criollo varieties hybridized with local wild cocoa trees, giving rise to a new Trinitario type population, which is still cultivated today in north-eastern South America (Young, 1994).

However, the Spanish did not stop at South America, and also successfully transferred cocoa seedlings at the same time to some of their other colonies, such as Trinidad and Jamaica, and the Philippines. That example was soon followed by other colonial nations, such as France, which attempted to develop cocoa cultivation in Martinique in 1690. For their part, the Dutch took seeds from the Philippines to set up an experimental botanical garden in Jakarta in 1778. From there, cocoa cultivation developed towards Sulawesi and Java, then to Sri Lanka, arriving in India towards the end of the 18th century. However, this initial wave of cocoa cultivation in Asia did not result in the creation of vast production zones, and its importance remained marginal on a world scale. On the other hand, production on the Caribbean islands was of prime importance up to the end of the 19th century.

Apart from Venezuela, cocoa cultivation in South America did not develop from Criollos, but through the domestication of local cocoa trees, firstly in

Ecuador, with the Nacional variety, then in the state of Pará in Brazil, with Bravo cocoa, a Lower-Amazon Forastero, in the state of Bahia. The seeds were apparently brought by a Frenchman, Frederick Warneau, in 1746. However, the rapid production increase in Bahia did not occur until the end of the 19th century. It continued into the 20th century, raising Brazil to second in the world production league table. Socio-economic changes, and the explosion in witches' broom disease, have contributed to the recent decline in Brazilian production. The entire American continent now accounts for less than 15% of world production.

The cocoa tree made its way to the African continent via the islands of the Gulf of Guinea. Some plants from Brazil were first planted on Príncipe in 1822 (Wood and Lass, 1985). The transfer to São Tomé occurred a few years later in 1830, followed by Fernando Po in 1854. However, the first seeds introduced on the African continent in 1857 seem to have been imported directly from Suriname to Ghana by Swiss missionaries, although this first attempt, which may have opened up the way for subsequent developers, was unsuccessful. In 1869, a Ghanaian labourer came back from Fernando Po with a few pods. This very limited introduction, backed by further contributions in 1886 from São Tomé, gave rise to the African Amelonados, whose cultivation zone extends throughout the forest zone of West Africa. In Cameroon, the history of cocoa cultivation began at Limbe, when a collection was set up by the German colonizers. This difference in origin explains why Cameroonian cocoa plantations have the particularity, for Africa, of not being primarily composed of Amelonados, but of Trinitarios. Cocoa cultivation prospered in Ghana, and to a lesser degree in Nigeria, right from the end of the 19th century. There was considerable development throughout the 20th century with the emergence of the Ivory Coast , which has now become the world's leading cocoa producer with an export volume of more than 40% of world production at the end of the 1990s. African countries as a whole account for more than 60% of global cocoa bean supplies.

The remaining production comes from the recent boom in cocoa cultivation in Southeast Asia and the Pacific. It first occurred in Malaysia and Papua New Guinea in the 1970s-80s, then spread to Indonesia, more particularly the island of Sulawesi.

Phytophthora diseases

Diseases on cultivated plants can be caused by pathogens that have evolved along with their hosts. *Crinipellis perniciosa,* a fungus responsible for witches' broom disease, belongs to this category on *T. cacao*. However, cocoa trees are also highly susceptible to attacks by numerous other organisms encountered during their dissemination throughout the world. Most of them remain largely limited to their zone of origin. For instance, each cocoa-growing zone has a par-

ticular parasite context. In the great majority of cases, parasite pressure is high and causes substantial harvest losses.

The damage caused to cocoa yields by diseases and pests has been estimated on numerous occasions. However, assessments are rarely based on large-scale observations, and usually remain fairly approximate. According to Cramer (1967), annual losses due to diseases and insect attacks amount to 20.8% and 25% of world production respectively. When damage caused by rodents, monkeys or birds is taken into account, apparently more than 50% of potential world production is lost each year. These data are old now and are only indicative, but they show the size of the problem faced by growers.

All cocoa tree organs, the stems, leaves, flower cushions, fruits and roots, can be affected by pathogens. Numerous fungus species are involved, such as *Crinipellis perniciosa, Moniliophthora roreri, Fusarium* spp., *Trachysphaera fructigena, Botryodiplodia theobromae, Macrophona* sp., *Geotrichum cancidum, Verticilium dahliae, Oncobasidium theobroma, Ceratocystis fimbriata, Corticium salmonicolor, Marasmius* spp., *Colletotrichum gloeosporioides, Phellinus noxius, Rigidoporus lignosus, Rosellinia pepo, Armillaria mellea* (Wood and Lass, 1985). Several viral infections have also been inventoried. The five diseases that cause the most serious damage are: diseases caused by *Phytophthora*, witches' broom, watery pod rot, swollen shoot (CSSV), and vascular streak dieback (VSD).

Diseases due to *Phytophthora* alone cause extremely serious damage, since they exist throughout the cocoa-growing zones. Such losses were estimated at more than 10% of world production by Padwick (1956) and 30% by Medeiros (1977). However, there are considerable disparities from one zone to another, with insignificant damage such as the 1.2% recorded by Hicks (1967) in Papua New Guinea, and up to 95% losses noted by Tollenaar (1958) in Cameroon.

Attacks on fruits are the most frequent signs of the disease, known as black pod. However, stems and trunks can also be affected, with cankers occurring on the bark, and the disappearance of flower cushions. Infections have also been found on leaves and roots, though less frequently and with a much lower incidence.

Phytophthora species attacking the cocoa tree

The causal agent of the disease was isolated for the first time in 1909 by Von Faber and described by Maublanc under the name *Phytophthora faberi* Maubl. (Waterhouse, 1970). The parasite was then classified by Butler (1924) under the name *Phytophthora palmivora*. Black pod throughout the world was long considered to be caused by this single species.

Initial work on structuring the pathogen population concentrated on the distribution of sexually compatible forms A1 and A2. For instance, Turner (1962) mentioned the existence of both types in Cameroon, but did not give any indication of the distribution and relative abundance of each type. In 1971, Huguenin

and Boccas indicated that type A1 was more frequent and that type A2 was widely distributed in clearly distinct locations in the south and east of the country. A few years later, Zentmyer *et al.* (1977) studied the sexual compatibility types of 38 isolates taken from the entire Cameroonian cocoa-growing zone: 29 proved to be of type A1 and 9 of type A2; the A1 and A2 types were uniformly distributed throughout the territory.

The multiplication of such research in the laboratory in different producing countries, using increasingly standardized methods, led to the discovery of considerable diversity among the morphological traits of isolates, linked to their geographical origins. In addition, the observation of metaphasal plates under a light microscope showed that certain karyotypes had 5 to 6 large chromosomes, while in others 10 to 12 small chromosomes were found (Sansome *et al.*, 1975). The combination of these elements led to doubts about the specific unity of *Phytophthora* likely to cause black pod, and an international workshop was held at the Rothamsted experimental station (UK) in 1976 to compare observations carried out in the main producing countries. In the final report, it was concluded that isolates taken from diseased pods could belong to distinct species of *Phytophthora*. Four species were then formally identified (Griffin, 1977; Zentmyer *et al.*, 1977; Babacauh, 1980; Kellam and Zentmyer, 1981):

– *P. palmivora* (proper) exists in virtually all cocoa-growing zones. This parasite is dominant in the Ivory Coast and Asia;

– *P. megakarya* was isolated in the countries of central and West Africa (Gabon, Cameroon, São Tomé, Nigeria, Togo, Ghana). These are countries where damage to fruits is very severe (up to 50% of potential production);

– *P. capsici* is the most common species on the American continent;

– *P. aff. citrophthora*, which is rarer, has been identified in the Ivory Coast and Brazil, but its economic importance is less.

Other species of *Phytophthora* have sometimes been determined, but they are relatively rare and their incidence on production remains negligible.

This relatively late discovery of the existence of several species of *Phytophthora* calls for careful reconsideration of results acquired earlier. Indeed, each species has its own characteristics, whether in biological, parasitic or epidemiological terms. For instance, *P. palmivora* is a highly polyphagous species that can attack all the organs of a cocoa tree, and also numerous other plants. *P. megakarya* seems to be primarily associated with cocoa and only seems to attack the fruits in the wild. There is in fact little to determine the changes that have occurred in the different populations in space and time, or the changes in their pathogenicity.

Phytophthora attacks on cocoa trees

Climatic conditions play an essential role in the start of epidemics, which can only develop in the presence of free water. However, the intensity of the diseases

and the speed with which they spread also depend on the susceptibility of the planting material, on cultural practices and on the one or more species of *Phytophthora* involved.

Development of epidemics

Only *Phytophthora* attacks on fruits have been covered by any real epidemiological studies. The most in-depth work was carried out between 1977 and 1981 by the "International Black Pod Project" team (Griffin *et al.*, 1981). Following that work, Gregory (1984) proposed the following scheme for explaining the initial phases of an epidemic: in the dry season, the parasite remains in latent form in cocoa tree roots. The first rainfall causes the parasite to resume its activity in the roots, and it emits sporocysts. Zoospores are released into the free water in cracks in the ground and they rise to the surface by negative geotaxis. Infectious propagules are transported to the fruits by the formation of a highly volatile aerosol suspension, during rainfall, which can reach all levels of the tree. The probability of fruit infection thus follows a linear gradient decreasing from bottom to top. Other less specific methods of infection can be added occasionally to this basic scheme. Thus, the existence of the fungus in certain flower cushions and a few cankers on the trunks can incidentally constitute a source of primary inoculum. Insects, particularly ants, or small mammals are no doubt also sometimes major vectors of the disease.

Several studies have since been conducted to confirm or refute the existence of a soil-borne phase in the infection cycle. The first fruits attacked can be at any level in the tree, but the probability of infection clearly follows a decreasing gradient in line with height. This phenomenon may be consecutive either to a soil-borne origin of the primary inoculum, or its transportation by water, which is subject to the laws of gravity, or by a combination of the two. However, irrespective of the precise origin of the primary inoculum, one thing is certain: the first infections occur as soon as there is a combination of fruits on the trees and a rainy season.

After 2 to 3 days, infection is seen through the appearance of a light brown patch, which then spreads to the entire surface of the fruit. A very wet environment encourages the spread of necroses. Thus, in the rainy season, a pod of mature size can be affected over its entire surface in under a fortnight. The first sporocysts occur between 4 and 5 days after infection and, if conditions are wet enough, they mature in a few hours. They release mobile zoospores after a few minutes' contact with free water. The rains thus generate highly volatile infectious mists that become the main vectors of inoculum propagation from infected pods to healthy pods (Maddisson and Idowu, 1981). However, daily removal of affected fruits in a plantation does not prevent a rapid increase in the number of rotten fruits during periods propitious to epidemics.

Ward *et al.* (1981) attempted to model disease development in the field. The model used was that for diseases of interest developed by Van der Planck

(1963). The author noted that the calculated infection rate (r) decreased rapidly during the epidemic. Ward felt that these variations ought to be corrected by taking into account the change in the number of pods over time, the latency period (p) and the duration of active sporulation of each fruit (i). Taking (p) = 6 days and (i) = 15 days, the author discovered that disease incidence on day (d) was correlated to the cumulated length of healthy pods on day (d), to rainfall on day (d-3) and to the cumulated length of sporulating pods on day (d-5). In addition, the increase in the rate of infection seemed to be favoured by high relative humidity and relatively low temperatures (optimum at 21°C).

Control methods involving phytosanitary intervention

Evaluation of efficiency made difficult by the heterogeneity of cocoa trees

The initial searches for control methods concentrated on developing means of phytosanitary intervention, such as fungicide treatments.

Hislop and Park (1962*a*, 1962*b*), in Nigeria, thus attempted to develop a fungicide selection method by combining two criteria measured on pods taken from trees. It involved the direct effect of the active ingredient on the infection process and, on the adhesive properties of the chemical formulas on the fruits. The direct effect on the infection process was assessed by comparing success rates for artificial infection using zoospore suspensions. The adhesion coefficient was determined in artificial rainfall and expressed by the equation:

$$\text{Log } W = \log Wo - B \log (R + I)$$

W : copper residue
Wo : initial deposit
B : constant specific to the fungicide
R : amount of rainfall
I : intensity of rainfall

This method was used to select fungicides based on cuprous oxide and copper sulphate, which seemed to have satisfactory properties. However, comparative trials set up in the field did not bring out any significant difference between the control plots and the plots in which the fungicides were applied on pods using a backpack sprayer every 21 days (Hislop and Park, 1962*c*).

In Cameroon, Marticou and Muller (1964) did not obtain any tangible results either after 5 years of observations in trials set up in Fisher blocks. Like Delassus *et al.* (1960) in the Ivory Coast , the authors blamed the extremely heterogeneous nature of cocoa trees—in terms of the trees, the soil and the shade—which cannot be controlled with experimental designs using blocks. Marticou and Muller then proposed observing the same plot for two years running, once without treatment, and once with treatment. A reference plot without treatment over the two years made it possible to assess the effect specific to each year. The production in the treated plot was to be defined by the equation:

P1 = Po.K1.I.E

Po : annual production
K1 : annual effect
I : effect of treatment
E : random variable

As this method did not provide the expected results, the block trials were abandoned and replaced by a technique developed by Muller *et al.* (1969): the pairs method. Its principle is to compare the relative efficacy of fungicides on several pairs of trees during production periods, each pair consisting of trees that are as similar as possible (general structure, morphological pod traits, number and distribution of fruits, etc.). One of the elements in the pair received the treatment to be studied, the other a known reference treatment. The results underwent statistical analysis by a non-parametric test, making it possible to compare the proportion of pods affected by the disease between the two treatments. The authors were thus able to show the activities of several copper-based products, captafol and two organostannic products in applications on pods a fortnight apart (Muller *et al.* , 1969 ; Muller and Njoumou, 1970). This technique also revealed the efficacy of 21-day intervals for a new active ingredient, metalaxyl, in Cameroon (Bakala, 1977; Bakala and Trocmé, 1979) and in Togo (Davous, 1982). However, the very promising control rate obtained with this pairs method (around 95%) proved to be much higher than the actual protection provided by fungicide treatments applied in the field under normal operating conditions.

In an attempt to bring the methods used to assess phytosanitary interventions more into line with their implementation in practice, Despréaux *et al.* (1988) invented a new method in Cameroon, known as "random targets", the principle of which is based on comparisons of disease development on batches of 100 trees chosen at random in plantations. Under these conditions, which enable comparisons with untreated control batches, it was shown that the degree of protection offered by fungicide treatments, combined with weekly removal of infected fruits, could vary between 33% and 77%, depending on how propitious conditions were for disease development.

Recommended interventions

Given the difficulty of assessing the true efficacy of control methods and the variability of the results obtained, recommendations are as much a matter of agricultural common sense as the application of proven research results.

Cultural practices must aim to make conditions less propitious to disease development and also reduce the quantity of inoculum in plantations. Pruning trees, reducing shading and controlling weeds, improve air circulation and reduce the relative humidity within a plantation. Regular removal of affected fruits before and during epidemics limits the possibilities of the disease spreading from diseased fruits. However, the application of such upkeep measures generally remains insufficient for controlling the disease.

Fungicides can be applied in several ways, depending on the active ingredients used. Spraying, or even better misting, copper-based products (cuprous oxide, copper sulphate) offers preventive protection. The persistence of these products which, under standard conditions (0.5 g of a.i./l), does not exceed 15 days, can be clearly improved by using much more concentrated solutions (Pereira and Lellis, 1984; Despréaux *et al.*, 1988). Metalaxyl or cymoxanil increase the efficacy or persistence of treatments through their ability to penetrate plant tissues. Lastly, aluminium ethylphosphite or phosphorous acid can be injected directly into the trunks, ensuring effective protection for periods ranging from 6 months to a year (Guest, 1994). However, this latter technique, which is also highly effective in controlling cankers, does not seem to act against all species of *Phytophthora*, particularly *P. megakarya*. It may also have a depressive effect on production potential. Hence, despite a substantial reduction in the number of rotten pods, final harvests would only seem to increase slightly.

Such phytosanitary intervention is restrictive and expensive. Moreover, the implementation of such measures does not guarantee acceptable control of losses in all cases in the most severely affected zones. In some plots, losses continue to exceed 50%. Thus, it seems essential to develop other approaches, particularly increasing the genetic resistance of cocoa trees, if a satisfactory solution is to be found for the *Phytophthora* disease problem.

Cocoa tree genetic resistance to *Phytophthora*

In order to strengthen the genetic resistance of cultivated cocoa trees, it is first necessary to characterize such resistance. Then, analysis of the parameters of transmission between generations makes it possible to draw up varietal improvement schemes.

Factors of genetic resistance can be many and varied in nature. Some are directly involved in host-parasite relations: during infection, on pathogen penetration of the tissues, the volume and duration of sporulation, etc. Others do not act directly on the infection cycle, but affect the conditions of disease expression. These include tree architecture, leaf area, the morphological characteristics of the fruits, distribution of the quantity of fruits, the time taken to ripen, and even resistance to other pathogens.

These factors may be linked or independent, and act in synergy or antagonistically. It is their combination that provides a given genotype with its overall potential resistance level. This level will only be expressed in interaction with the disease, which also depends on the pathogen populations, climatic conditions, farming system, etc. Thus, evaluating the resistance of a genotype is a complex business, which involves numerous interacting factors.

Individual assessment of genotypes for the resistance trait

A global approach can be taken to individual assessment of genotypes for the resistance trait, by monitoring harvests over several years. Observation of cocoa collections in this way has shown substantially different levels of disease incidence, which seem to be in relation to the genotypes. Soria (1974) and Rocha (1966) listed clones reported to be resistant in different environments nearly everywhere in producing countries. However, just as it is difficult to quantify the efficiency of phytosanitary intervention, resistance measurements in the field are highly variable, not particularly reproducible and remain highly dependent upon the observation sites and the methods used. In addition, the planting layout of collections is not generally designed for in-depth statistical analyses. Hence, this information can only be considered indicative.

Individual assessment can also be carried out taking an analytical approach that describes the different components of resistance and measures their respective importance in the ultimate resistance level of genotypes. This type of work provides greater knowledge of resistance mechanisms, makes it possible to focus the assessment on the most important components, and opens up the way to breeding programmes that ensure a better combination of these components. However, it is necessary to base the analytical approach on a global evaluation system that serves as a frame of reference.

Evaluation involving artificial infection

Artificial infection methods are simplified evaluation methods that offer the advantage of enabling more effective control of several parameters, in particular the type of inoculum and the inoculation method. They can reveal factors of resistance that are directly involved in the infection process, which will be called intrinsic resistance factors in the rest of this document.

Most of the work on artificial infection has been conducted on pods, *in situ* or in the laboratory. The susceptibility of the other organs (stems, leaves, roots, pericarp, pre-germinated beans) has also been covered by numerous experiments, using very different contamination methods: depositing calibrated zoospore suspensions, or mycelium disks grown on agar medium, or cultures blended in a liquid medium. During these studies, for all countries combined, no cocoa plant has yet revealed total resistance to *Phytophthora* attacks. However, the infection success rates and, where applicable, the speed with which symptoms developed, varied depending on the individuals, thus revealing the existence of genetic variability in cocoa for these traits.

Lawrence (1978) considered that the most reliable and most discriminant test for revealing differences between mature clones was that developed in Cameroon by Blaha and Lotodé (1976). This technique consists in maintaining a few drops of calibrated zoospore suspension in contact with immature but adult-sized pods in

plasticine pots, and monitoring disease development over several days. The authors thus showed that pods of different clones did not all respond uniformly to artificial contamination on pods. Two scales of susceptibility were defined on a little over 100 genotypes tested, based on two criteria:

– epidermal resistance, assessed by the infection success rate;

– internal resistance, assessed (after successful infection) by the speed of necrosis development.

The two scales proved to be very similar to each other, with a few exceptions. The authors proposed that preference should be given to epidermal resistance in selection schemes.

Search for biochemical markers of resistance

Other researchers have attempted to relate the variation in resistance levels to the existence of biochemical compounds. The aim of this work was to characterize one or more cellular metabolism compounds that were likely to serve as biochemical markers of resistance in a selection programme. Initially, the presence of polyphenols or other anti-fungal products was sought and found in the cortex of healthy cocoa pods (Prendergast and Spence, 1965; Rocha and Saenz, 1966; Meiffren and Tanguy, 1967; Reyes *et al.*, 1977). However, no strict relation could be established between polyphenol contents and the resistance of a cultivar.

After infection, Daguenet and Parvais (1981) sought the existence of phytoalexinic type molecules in the epicotyls of *T. cacao*, *T. bicolor* and *T. grandiflora* seedlings. These authors found substances with an anti-fungal action in *T. grandiflora*, though they were unable to determine their precise nature. The search drew a blank for *T. cacao*. Attempts at interspecific hybridization between *T. cacao* and *T. grandiflora* have remained unsuccessful.

Debost *et al.* (1988) compared carbohydrate, lipid and phenol compositions and contents in the cortex of healthy pods, of wounded pods and of pods first wounded then infected. On the healthy pods no substantial differences in carbohydrate and lipid contents were found between SNK 413 and SNK 10, classed resistant and susceptible respectively on Blaha and Lotodé's scale. However, the two clones showed different reactions to parasite aggression. The reactions to infection were stronger in SNK 413 than in SNK 10. The main phenomena observed were: a decrease in fructose, glucose and sucrose (by 40 to 60%) and a reduction in the least polar lipid band. This went hand in hand with an accumulation of the other more polar lipid compounds. However, none of them showed any anti-fungal activity against *P. megakarya*. Infection in SNK 413 also caused a substantial drop in the proportion of linoleic and linolenic fatty acids, to the benefit of oleic acid. An analysis of the purified methanol phase revealed the appearance of 11 bands of phenolic compounds in SNK 413 pod extracts when the pods were infected. Phenolic compounds were also accumulated in infected SNK 10 fruits, but in smaller quantities. Some

of these bands revealed marked anti-fungal activity against *P. megakarya*: 5 in SNK 413 and 2 in SNK 10.

Genetic parameters of resistance transmission

The first cocoa breeding programmes were launched at the beginning of the 20th century to control witches' broom disease, which was devastating plantations in the Andean countries and Trinidad. International surveys were carried out in the Amazon basin, the species' zone of origin, to seek genotypes with resistance to this disease. In terms of genetic control, research did not achieve the creation of varieties with true resistance to *C. perniciosa*. However, it did make it possible to set up collections, and acquire a basic knowledge of the vigour and precocity of hybrids between clones derived from distinct morpho-geographical groups, which are the basis for all the selection schemes developed since then.

Most major producing countries have embarked upon their own varietal improvement programmes, based primarily on production criteria. These programmes have usually been implemented in zones where *Phytophthora* disease incidence was rather moderated, so as to be in a position to assess production potential without phytosanitary constraints. This type of selection is not appropriate for the characterization of resistance factors. Thus, none of the clone hybrids currently recommended has been specifically selected for its resistance to *Phytophthora*, even if that criterion is actually included in the assessment of production.

However, geneticists have tried various ways of studying the genetic parameters of resistance transmission, with a view to creating new breeding programmes.

Use of early artificial inoculation tests on pre-germinated beans

In order to speed up the selection stages, methods have been developed for early analysis of the genetic parameters of resistance transmission to a progeny. For instance, hulled, pre-germinated beans were tested by subjecting them to contamination with a drop of zoospore suspension (Amponsah and Asare Nyako, 1973) or soaked in liquidized mycelium (Partiot, 1975; Tarjot, 1977). A comparison was made of germination failure between the families. It was not possible to develop a simple way of interpreting the phenomena observed from the results obtained.

Use of an artificial inoculation test on pods

Blaha and Lotodé (1977) applied their artificial inoculation method on pods to study the transmission of resistance traits in 56 clone hybrid families selected according to agronomic criteria. It was not possible from the statistical analysis to class the families according to the mean percentages of successful infections observed in each progeny, due to the excessive variability within each family. The authors concluded that there was strong parental heterozygosity for resistance

traits, which led to high heterogeneity in the progenies. They therefore deduced that selection based on crosses involving clones led to hybrid families that were highly splintered for the desired trait: the individuals had variable resistance levels, from strongest to weakest, even though some progenies seemed to be more resistant than others. They therefore felt that improvement through hybridization was less worthwhile, at least initially, than cloning individuals revealing the best characteristics.

Observation of disease development in the field

The genetic parameters of resistance transmission in the field were studied in a 6 x 6 diallel trial set up in Cameroon in 1974 (Despréaux *et al.*, 1989; Berry and Cilas, 1994). Weekly monitoring of natural disease development tree by tree, over several years in succession, revealed the existence of significant differences in performance between progenies, related to the general combining ability (GCA) of the parents. Resistance transmission was mainly additive.

Relations between individual evaluation and the genetic parameters of resistance

An analysis of the diallel trial in Cameroon, based on weekly production records, made it possible to differentiate between and class parents according to their GCA. There thus exists an overall resistance trait that can be transmitted from one generation to the next.

However, the parents in this diallel also underwent individual evaluation by Blaha and Lotodé's artificial inoculation method, which also led to a classification of the clones in relation to each other.

The classification of genotypes according to their GCA with respect to overall resistance, and the classification obtained following artificial inoculations were substantially different. In particular, clone UPA 134, which was highly susceptible in the artificial inoculation trial, was the genotype that transmitted the best overall resistance level to its progenies (Despréaux *et al.*, 1989). There therefore undoubtedly exist other factors not taken into account by the artificial inoculation method, which are expressed in a determinant manner under natural conditions in the field. These factors may be directly linked to the way the infection process takes place, with artificial inoculation adding uncontrolled bias. But they may also be of a totally different nature. It is possible to put forward a large number of hypotheses: foliage density may play a role in local micro-climatic conditions, the distribution of yields throughout the year, the number and size of fruits may encourage or discourage the development of an epidemic, the time taken to ripen may shorten or lengthen the period during which the host is "receptive" to the parasite, etc.

Lastly, it cannot be certain that the same observations are reproduced under different conditions, in another climate and another pathosystem. The relative

importance of the different components of resistance may vary depending on the environment in which they are expressed.

Conclusion

Cocoa cultivation has been extended to virtually everywhere in the humid tropics. The tree has proved to be highly susceptible to diseases and pests, especially *Phytophthora* diseases. Several species of *Phytophthora* can attack *T. cacao*, but the most serious damage is caused by *P. palmivora*, which exists in all production zones, and by *P. megakarya*, which is very aggressive on the African continent.

Most of the damage follows attacks on fruits, but the parasite can also infect the tree's other organs, such as the trunk and stems. Disease incidence under natural conditions depends on many factors and it has so far proved impossible to establish a satisfactory epidemiological model. The heterogeneity of the system is such that even the effectiveness of the simplest phytosanitary interventions is difficult to assess. In any event, this type of intervention is not enough to control epidemics once conditions are propitious to disease development. It is therefore important to develop resistant cocoa trees that are able to substantially reduce the incidence of the parasite.

No known genotypes have proved to be totally resistant to *Phytophthora*. The breeding programmes implemented so far have not concentrated on this trait, and clone hybrid selections distributed by research centres are as susceptible as traditional varieties.

However, there is variability among genotypes for resistance levels. This variability is expressed in the results of artificial inoculation tests, and during family evaluations based on yield records over several years running. The overall resistance trait is primarily transmitted in an additive way and it is no doubt possible to implement effective breeding schemes. As this resistance is partial, it can be hoped that this trait is polygenic and sustainable.

Even so, the evaluation of overall resistance remains very laborious, requires appropriate experimental designs and takes at least 10 or so years per selection cycle. In addition, this type of evaluation does not provide information about resistance mechanisms and it is not possible to characterize the different genetic components. Lastly, the differences observed between artificial inoculation tests and evaluations based on production indicate that the relationships are not simple, and that it is no doubt essential to further our knowledge in this field in order to develop early selection schemes based on relevant criteria.

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Genetic diversity of cocoa tree *Phytophthora* pathogens

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Before searching for individuals resistant to pod rot disease caused by various species of the genus *Phytophthora*, it is essential to study pathogen diversity. Good knowledge of that diversity provides a clearer understanding of the diversity of attacks, notably of "host x pathogen" interactions that might sometimes complicate the search for resistance. This chapter therefore proposes a review of the genetic diversity of cocoa tree *Phytophthora* pathogens. This genetic diversity is investigated using various recently developed molecular tools. Following an introduction to the different cocoa tree pathogen species, the diversity of the most widespread species will be examined.

The different pathogenic species of the genus *Phytophthora* found on cocoa

Black pod rot is a cocoa disease found worldwide, and was initially thought to be caused by a single species, *P. palmivora* (Butler, 1919). The taxonomic history of *Phytophthora*, which was first described by Butler (1910), has been very eventful.

The first descriptions and identifications of *Phytophthora* isolated from cocoa, coconut or rubber were based on morphological characteristics. These charac-

teristics involved vegetative or reproductive organs (Rosenbaum, 1917; Butler 1925; Ashby, 1922; Turner 1960, 1961), followed by characters linked to the physiology or the pathogenicity of the strains (Tucker, 1931; Gadd, 1924, 1927; Ashby, 1929). Substantial diversity was already found among strains; depending on the criteria used, the strains studied, and the degree of within-species diversity accorded by the author, the limits of the species were often questioned. Some species, such as *P. arecae* and *P. meadii* were included, then withdrawn from the *P. palmivora* group (Tucker, 1931, Waterhouse, 1963). For its part, *P. botryosa* was created from that group (Chee, 1969).

A key stage in the taxonomic history of *P. palmivora* was the cocoa *Phytophthora* workshop held in May 1976 at Rothamsted Experimental Station (UK). The workshop was organized following a presentation by Brasier and Sansome, at the International Cocoa Research Conference (Nigeria, 1975), reporting that at least two chromosomal types of *P. palmivora* existed in West Africa. During those meetings, the *P. palmivora* group was subdivided into four morphological types (MF1, MF2, MF3 et MF4) based on precise morphological criteria and on the size and number of chromosomes; as the other strains of *P. palmivora* did not correspond to any of these morphological types, they were considered to be atypical (Griffin, 1977). A new key for the identification of *Phytophthora* species was then published (Newhook *et al.*, 1978). However, the work by Brasier and Griffin (1979), involving 1,104 strains, 892 of which had been isolated in Nigeria, led those authors to consider morphological type MF3 (5-6 large chromosomes, corresponding to Turner's type 'N', 1960) as a new species: *P. megakarya*. Type MF4 too was a subject of controversy: as similarities were found between MF4 strains and *P. capsici*, Zentmyer *et al.* (1981), Idosu and Zentmyer (1978), Kaosiri (1978) and Kaosiri *et al.* (1978) proposed attaching these strains to *P. capsici* Leonian; Tsao and Alizadeh (1988) then redescribed *P. capsici* in order to include all *P. palmivora* MF4 isolates that are pathogenic on cocoa.

At this stage, it should be noted that in most of the work mentioned, zygote formation was considered to be proof that strains exposed to each other were interfertile. However, Brasier (1972) described the concept of selfing in heteroallelic *Phytophthora* when the oogonia of one strain were in fact fertilized by the antheridia of the same strain, being influenced by the presence of a strain with a complementary mating type, or other factors. It is therefore essential to check that the progenies obtained from a cross between two strains carry the genetic traits of both parents, before concluding on interfertility between those strains, hence that they belong to the same species. Boccas (1981) thus tested crosses between mature species of heteroallelic *Phytophthora* (*P. palmivora*, *P. megakarya*, *P. capsici*, *P. nicotianae* var. *parasitica*, *P. cinnamomi* and *P. cambivora*). The progenies from those crosses displayed heterogeneous and recombined morphological, phenotypical and physiological traits, thereby suggesting the hypothesis of hybridization between species. However, an analysis of the composition between soluble proteins revealed that those progenies came in fact

from selfing of the parental strains. Hence, with the strains and methods used, there was no hybridization; it therefore seems that there are interspecificity barriers between these different species of *Phytophthora*. However, the formation of selfed oospores increases the chances of a species surviving under adverse environmental conditions.

These analysis techniques have developed and new biochemical and molecular tools are now available for describing intra- and interspecific variability. Techniques such as immunology (Burrel *et al.*, 1966; Savage *et al.*, 1968; Merz *et al.*, 1969), electrophoresis of total proteins (Zentmyer *et al.*, 1977; Erselius and Shaw, 1982; Hansen and Maxwell, 1991), isozyme analysis (Clare and Zentmyer, 1966; Hall *et al.*, 1969; Blaha, 1990; Oudemans and Coffey, 1991*a*, 1991*b*), in situ DNA-DNA hybridization (Goodwin *et al.*, 1989), DNA restriction fragment length polymorphism (RFLP) (Klimczack and Prell, 1984; Carter *et al.*, 1990; Goodwin, 1991; Förster *et al.*, 1987), and random amplification of polymorphic DNA (RAPD) (Nyassé *et al.*, 1999; Sackey *et al.*, 1994), contribute towards more effective discrimination of genotypes and more effective characterization of population structures. In fact, these different methods often give complementary results, enabling more effective characterization of the limits between species. It was in this way that an isozyme determination key was proposed by Ortiz-Garcia (1996), for the *Phytophthora* species involved in cocoa and coconut diseases. This key was drawn up from an isozyme analysis of 220 strains of *Phytophthora,* 150 of which were reference strains from various international collections identified by morphological criteria. Of the 26 isozyme systems studied, 21 proved to be polymorphic, and *Phytophthora* strains pathogenic on cocoa and coconut can be identified by exploiting only 3 loci (Idh-2, Pgi, Mdh-1) (figure 1). This key is based on the extraction of proteins which, unfortunately, cannot be used to take the genome study any further, though new techniques based on DNA analysis do make it possible to fine-tune genome analysis.

The evolution of cocoa pathogenic *Phytophthora* populations over time depending on their geographical origin, type of reproduction, and control methods in the field, is currently being investigated by ITS sequence polymorphism studies (nucleotide sequences of untranscribed intergene regions of ribosomal DNA), (Ducamp *et al.*, 2002; Lee *et al.*, 1993). This ITS-RFLP technique was utilized in comparison to the other techniques used earlier to confirm that the strains described as belonging to species of *P. palmivora* from different host-plants are indeed part of the same species (figure 2). The closest species is *P. megakarya,* which, itself, is also homogeneous, whether it is isolated from cocoa or cola. The species *P. capsici* and *P. citrophthora* are close to the species *P. citricola* and *P. colocasiae*. On cocoa, it is possible to isolate *P. capsici sensu stricto* and a population we shall call *P. capsici* "cocoa", which seems to be particularly adapted to cocoa in terms of aggressiveness. Their ITS sequences differ very little (10 pairs on the 835 studied), but they can cross, giving hybrids (Ortiz-Garcia, 1996). In Brazil, it is possible to isolate *P. citrophthora "cocoa"*, which has a very

similar ITS to that of *P. citrophthora sensu stricto,* which can be isolated from citrus. This technique therefore makes it possible to characterize the different species of *Phytophthora* that are pathogenic on cocoa.

These different methods also provide increasingly precise data that can be used not merely to assess the diversity of *Phytophthora*, but also genetic diversity within the species of *Phytophthora* that are pathogenic on cocoa. These studies may provide a clearer understanding of the differences found between epidemics (Oliveira, 1990). Indeed, the variation in damage from one country to another depends on environmental conditions and the type of material planted, but also on the species of *Phytophthora* involved, or even the strains within species.

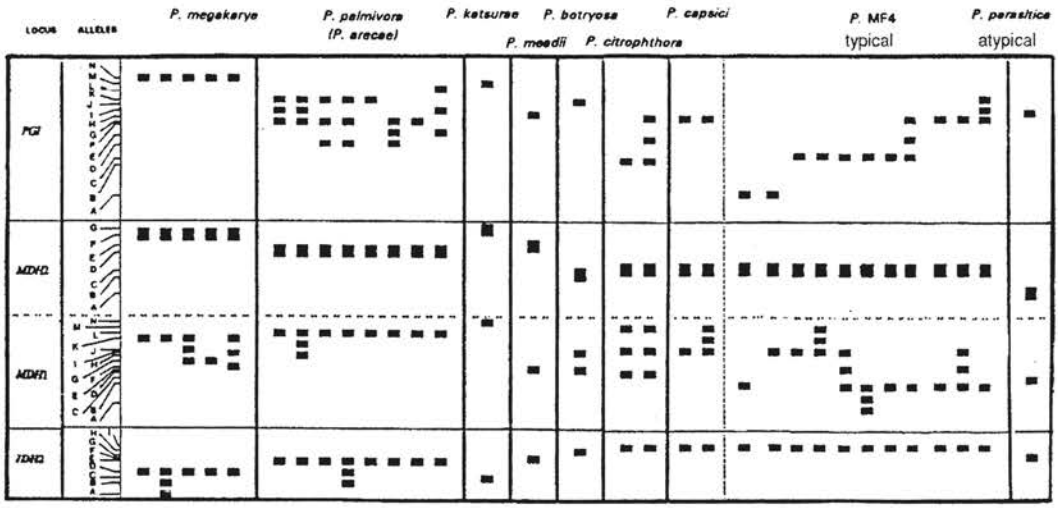

Figure 1. Biochemical key for the determination of Phytophthora *species parasitizing cocoa and coconut.*

Idh-2	if allele C	*P. katsurae*	Pgi	if allele A	*P. MF4 typical*
	if allele D	*P. megakarya*		if allele B	*P. citrophthora* Smith & Smith Leonian
	if allele E	*P. palmivora/P. arecae*		if allele C	*P. MF4 typical*
	if allele F	*P. meadii*		if allele G	Mdh-1
	if allele G	*P. parasitica*		if allele B	*P. MF4 atypical*
	if allele H	*P. botryosa*		if others	*P. capsici*
	if allele I				

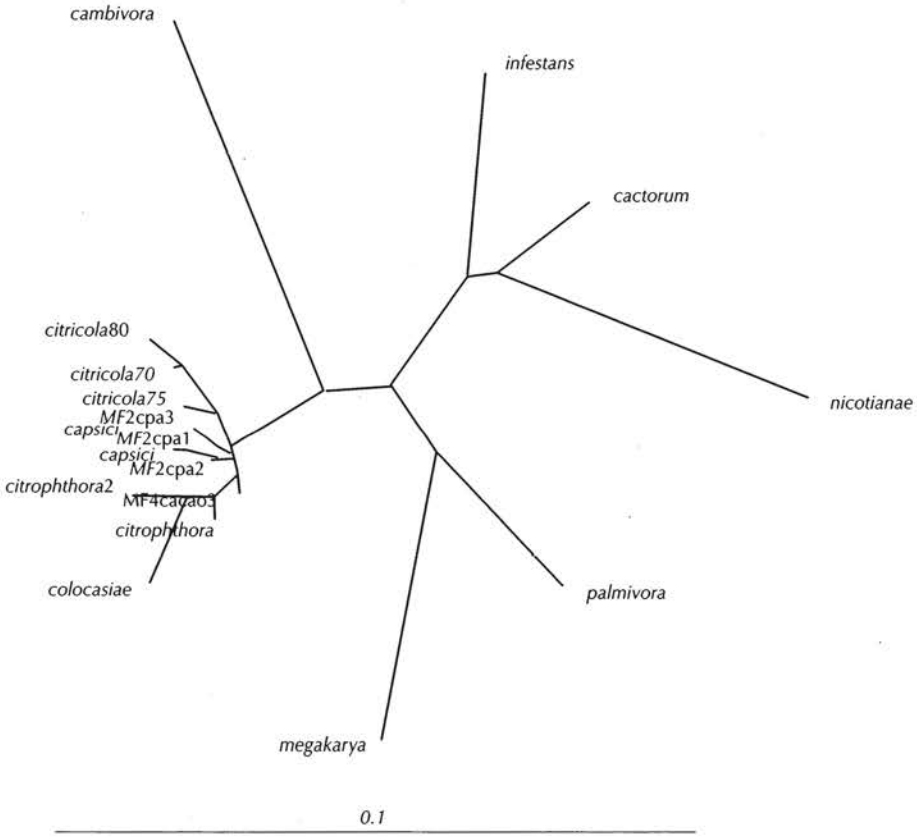

Figure 2. Classification of the different Phytophthora *species from ITS sequences.*

Genetic diversity of *P. palmivora*

The species *P. palmivora* exists virtually throughout the world's cocoa-growing zone, even though it is not always the majority species (*P. capsici* being the most common in Latin America and *P. megakarya* in Central Africa). Neither is it rare to see different attack levels within the same species, depending on the strains used (Surujdeo-Maharaj *et al.*, 2001; Appiah *et al.*, 2002).

Genetic studies were recently carried out on *P. palmivora* along with taxonomic studies; they date from the 1990s and focused on isozyme criteria (Oudemans and Coffey, 1991; Ortiz-Garcia, 1996).

Oudemans and Coffey (1991*b,c*), working on 393 strains of 12 species of *Phytophthora,* studied interspecific and intraspecific diversity by isozyme analysis. An initial conclusion indicated that no distinction could be made between the

species *P. palmivora* and *P. arecae*. The 100 *P. palmivora* strains studied, which came from such varied hosts as *Theobroma cacao, Cocos nucifera, Carica papaya, Durio zibethinus* etc., and from different regions of the world (Asia, Africa, Latin America), proved to be relatively uniform: only 2 loci (PGI and IDH-1) out of 18 were polymorphic. UPGMA analysis (Sokal and Sneath, 1963) using Rogers' genetic distance (1972) modified by Wright (1978), gave a maximum distance of 0.3 between strains of the *P. palmivora* group and the genetic diversity measured with Nei's index (1978) was evaluated at 0.08 within this group.

The work by Mchau and Coffey (1994*a,b*) on 93 strains of *P. palmivora* and 6 strains of *P. arecae*, also originating from highly varied hosts and countries, gave 18 ETs, established from 6 polymorphic loci (PGI, HEX-2, IDH-1, MDH-1, PEP and SOD), the most polymorphic loci being once again PGI and IDH-1. As previously, the *P. arecae* strains shared the ETs most represented in *P. palmivora*. Maximum genetic diversity was found in strains isolated from coconut and durian in Indonesia, Malaysia and Thailand. As these plants came from Southeast Asia, the authors propose this region as the centre of origin of *P. palmivora*.

Ortiz-Garcia (1996) worked on 631 strains of *P. palmivora* and *P. arecae* from different parts of the world (West Africa, Latin America, Caribbean, Southeast Asia and the Pacific), isolated from *Theobroma cacao, Cocos nucifera*, and from soil in coconut plantings. Forty-six different ETs were obtained using 7 loci. Once again, the strains morphologically identified with *P. arecae* shared common ETs with *P. palmivora*. A cross carried out between a *P. palmivora* strain and a *P. arecae* strain revealed genetic recombination, thereby indicating interfertility between the two "species". Nei's genetic diversity index was then evaluated at 0.229.

An examination of the UPGMA dendrogram established from the matrix of Rogers distances showed that there was no preferential distribution of strains according to their geographical origin or those from which they came (figure 3). However, examination of a sub-set comprising 179 coconut strains and 69 cocoa strains, all from Southeast Asia, showed that these two populations are genetically differentiated (they are separated by a distance of 0.202) and suggested the existence of parasitic specialization within this species. This parasitic specialization was confirmed by crossed artificial inoculations.

Indeed, tests carried out at Balai Penelitian Kelapa, Manado, Indonesia, showed that strains isolated from pods more easily attacked pods (between 55 and 94% success rate) than coconuts (between 0 and 22%), whilst strains isolated from coconut attacked pods and nuts indifferently (between 11 and 94% for pods and between 28 and 55% for coconuts). However, in the latter case, lesions developed much more quickly on nuts (lesion diameters of between 22 and 62 mm, 8 days after inoculation) than on pods (between 1 and 15 mm) (unpublished results). These results tally with those obtained by Steer and Coates-Beckford (1990) and by Warokka and Maskar (1991).

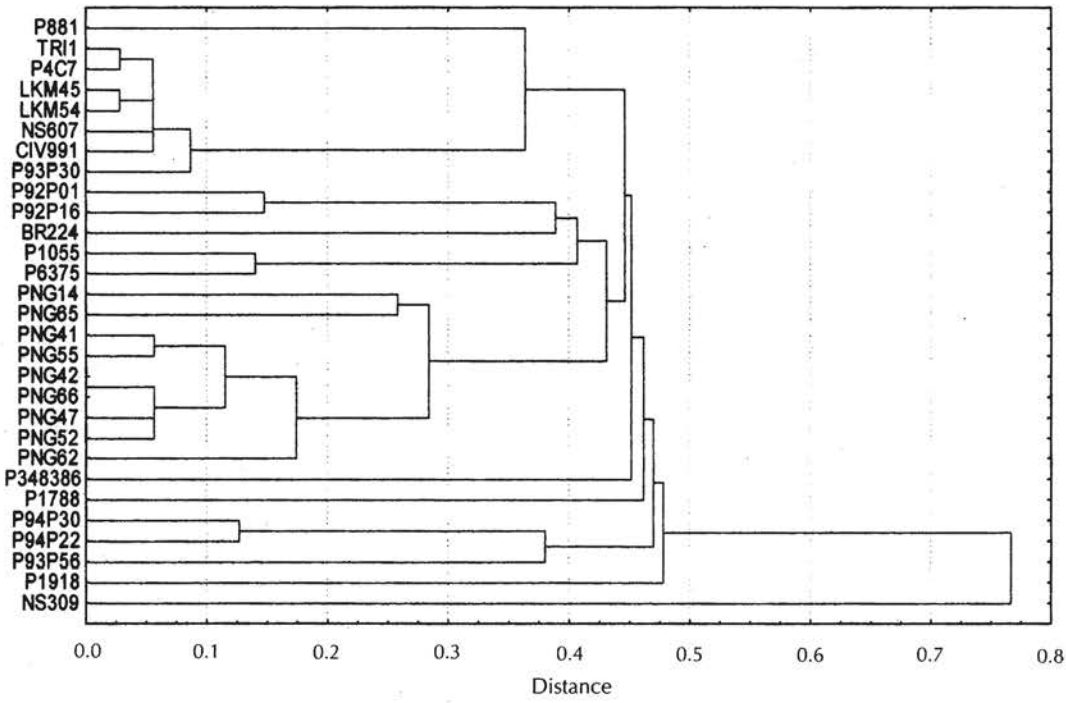

Figure 3. Genetic diversity of P. palmivora.

P. palmivora: Cocoa tree strains: P881: Jamaica (A1), TRI 1: Trinidad (A2), P4C7: Cuba (A2), LKM 45: Malaysia (A2), LKM 54: Malaysia (A2), CIV 991: Ivory Coast (A2), NS 607: Cameroon (A2), P93P30: Indonesia-South Sumatra (A2), P92P01: Indonesia-North Sulawesi (A2), P92P16: Indonesia-North Sulawesi (A2), BR 224: Brazil (A2), PNG 14, PNG 65, PNG 41, PNG 55, PNG 62: Papua New Guinea.

Coconut strains: P94P30, P94P22, P93P56: Indonesia.

Others: P1055: rubber, Thailand (A1), P6325: durian, Malaysia (A1), P34386: bamboo, USA (A1), P1788: papaw, Hawaii (A1), P1819: vanilla, Polynesia.

P. megakarya : NS309: cocoa, Cameroon (A1).

The cocoa population study (198 strains) revealed genetic proximity between the strains from West Africa and those from Latin America (distance of 0.016). Maximum genetic diversity was found in the Southeast Asian population (0.229, minimum: 0.104 in the Pacific), and more particularly in the North Sulawesi region of Indonesia (0.258, minimum: 0.163 in North Sumatra). Similar results were obtained with the coconut population, for which maximum genetic diversity was found in the regions of North and Central Sulawesi, in Indonesia.

To conclude, all this different research argues in favour of merging *P. palmivora* and *P. arecae* in a single species, *P. palmivora,* and of a centre of origin for the species in Southeast Asia. However, it disagrees with the work by Zentmyer (1988), who based his conclusions on diversity linked to the morphological traits of *P. palmivora* and who, on observing that most of the host plants were of American origin, proposed Central America as the centre of origin of *P. palmivora*.

Latin America and West Africa could therefore be two zones into which *P. palmivora* was introduced from Southeast Asia, right from the first exchanges of planting material between those regions (Harries, 1978; Wood, 1991). The Pacific islands would seem to be another, older, centre of introduction, from Southeast Asia, linked to maritime trading by Polynesians (Harries, 1978). Wood (1991) states that "Criollo" cocoa trees from Venezuela were first of all introduced into Sulawesi and the "Criollos" from Mexico were introduced into the Philippines, and that it is from one of those regions that the cocoa tree was then introduced into Java. Ortiz-Garcia (1996) believes that the structuring found in Southeast Asia comes from an adaptation of naturally present *P. palmivora* strains. Adaptation, then differentiation, would seem to depend on the material planted; the genetic proximity of Javan strains with those from Sulawesi, and of those from the Philippines with those from the other regions of Indonesia would seem to be related to the two types of Criollo initially introduced.

Recent DNA study techniques should make it possible to confirm all these results. In fact, enzyme electrophoresis can be used to distinguish charge differences between proteins, but cannot detect amino acid substitutions if the protein charge is not modified. Moreover, these enzymes do not always form a representative sample of the genome (Hartl, 1987). However, studying DNA, which is a source of genetic variability, makes it possible to establish a veritable identity card for each individual, thereby going right to the heart of the genome.

Of the work currently under way on cocoa tree *Phytophthora,* we would mention the RAPD studies undertaken by Sackey *et al.* (1999), who suggest the existence of genetic variations between and within *P. megakarya* and *P. palmivora*. Recent genetic diversity studies in the same laboratory, using RAPD according to the protocol used by Nyassé (1997) on 28 strains of *P. palmivora* isolated from different plants (figure 3), showed that the strains from bamboo, papaw, coconut and vanilla, were clearly separate from strains isolated from cocoa. The strains isolated from rubber and durian were closer to the strains from cocoa; being of opposite mating types, their crossing when these crops are grown together might lie at the origin of further diversification of the species *P. palmivora* (Ducamp, 2002).

Genetic diversity of *Phytophthora megakarya*

The species *P. megakarya* is clearly distinct from the other species of *Phytophthora* through the size of its chromosomes. It seems endemic to Africa, since it has never been detected on other continents (Ortiz-Garcia *et al.*, 1994). It is found in Cameroon, Gabon, São Tomé, Nigeria, Togo and Ghana, and appeared in Ivory Coast in 2000. This species often exists alongside *P. palmivora* (Brasier and Griffin, 1979; Zentmyer, 1988). In Cameroon, the characterization of more than 2,000 *Phytophthora* isolates seems to indicate that *P. megakarya* is virtually alone responsible for pod rot (Nyassé, 1992), though *P. palmivora* was mentioned in that country at the

end of the 1970s (Bakala, 1981) and mixes with *P. palmivora* exist in some plots. In West Africa, *P. megakarya* is spreading westwards and it has been reported in Togo (Djiekpor *et al.*, 1982), followed by Ghana (Dakwa, 1988; Luterbacher and Akrofi, 1994), and consequently more recently in Ivory Coast (Kébé, pers. comm.).

Phytophthora megakarya is the pod rot pathogen that causes most damage on the species *Theobroma cacao* L. Losses have reached 80% in Cameroon (Despréaux *et al.*, 1988; Berry and Cilas, 1994) and Gabon (Anon., 1990), and 100% losses were reported in Ghana (Dakwa, 1988). In Togo, 80% of cocoa plantations have been infected by this species (Djiekpor *et al.*, 1982). In comparison, the incidence of pod rot due to *P. palmivora* is more limited, with losses of around 20-30%. For optimum control of *P. megakarya*, it is necessary to acquire a clearer understanding of the pathogen's epidemiology, reproduction and diversity. The last aspect takes on particular importance for characterizing host-pathogen interactions, and therefore for developing efficient breeding strategies for resistance to this disease.

It proved important to study isolates of the species *P. megakarya* from the African countries in which it has been reported, using several types of markers (biochemical and molecular). From the results of earlier work, it is in fact possible to choose the tools required to study *P. megakarya* diversity. Isozymes appear to be a powerful tool for describing the intraspecific and interspecific diversity of cocoa *Phytophthora* (Blaha, 1990; Nyassé, 1992; Blaha, 1994). An isozyme analysis of 15 *P. megakarya* isolates from Nigeria and Cameroon with 16 enzyme systems enabled a clear separation of the isolates from the two countries (Oudemans and Coffey, 1991*a*). The intraspecific diversity of *P. megakarya* was characterized from mitochondrial DNA on a sample of 12 isolates (Förster *et al.*, 1990); this study enabled effective differentiation between the isolates from Cameroon and those from Nigeria. The genetic diversity of 161 isolates from various African countries (Cameroon, Gabon, São Tomé, Nigeria, Togo and Ghana) was studied using 13 isozyme systems and 9 RAPD primers, after determining the compatibility of their mating types. The degree of correlation between the two types of markers was determined by the Mantel test. Although RAPD are not the most appropriate markers for genetic studies of populations on diploid individuals, these molecular markers do provide a rapid overall picture of population structure. We therefore used them as a quick way of screening a large number of genotypes on numerous loci.

The *P. megakarya* isolates studied

One hundred and sixty-one isolates were collected from naturally infected pods in Cameroon (72), Gabon (11), Ghana (10), Nigeria (50), São Tomé (14) and Togo (4) between 1982 and 1995 (table 1). The distribution of the sampling zones is indicated in figure 4. In Cameroon, samples were taken from all the production zones. In Nigeria, samples were taken on six different agricultural stations, with the help of O.A. Olunoyo, K. Badaru and E.B. Esan from the Cocoa Research Institute of Nigeria. The isolates from Ghana and Togo were collected from

Table 1. Regions sampled for *Phytophthora megakarya* in West and Central Africa, number of isolates collected, year of collect, mating types detected and isozymes and RAPD genotypes identified.

Location	Nb[1]	Year [2]	Mating type[3]	Isozyme genotype[3]	RAPD genotype[3]
Cameroon (72, 19, 28)[4]					
Fako (FK)	7	1995	A1	I33(3), I35(2), I136(2)	R7, R8(3), R28(2), R29
Haut Nyong (HN)	5	1995	A1	I31(5)	R17(4), R18
Haute Sanaga (HS)	6	1995	A1	I31(6)	R17(6)
Manyu (MA)	5	1995	A1	I31(4), I34	R1, R31, R33, R34(2)
Mbam (MB)	15	1994 & 1995	A1	I1, I4, I9, I10, I14	R11, R12, R13, R14(3), R15, R17(4)
				I31(5), I32(4), I33	R18, R20, R27, R39
Mefou (MEF)	5	1990 & 1994	A1	I1(5)	R17(5)
Meme (MM)	9	1994 & 1995	A1	13, 17, I31(3), I37(4)	R28(4), R30, R31, R32, R33(2)
Mfoundi (MF)	4	1989 & 1994	A1(3), A2	I1(3), I2	R17(2), R21, R26
Ndé (NDE)	1	1994	A1	I7	R22
Ndian (ND)	3	1995	A1	I37(3)	R28(3)
Ntem (NT)	1	1994	A1	I8	R24
Nyong et Kellé (NK)	1	1994	A1	I1	R19
Nyong et Mfoumou (NM)	6	1995	A1	I31(6)	R17(5), R18
Nyong et So'o (NS)	1	1994	A1	I1	R17
Océan (OC)	2	1990 & 1994	A1	15, I11	R25, R44
Unknown (isolate 184)	1	-	A2	I15	R16
Gabon (11, 5, 6)[4]					
Koulamoutou (KO)	2	1982	A1	I12, I20	R40, R41
Makokou-Est (MA)	3	1982	A1	I12(3)	R41, R42, R43
Oyem-CM (OYC)	1	1982	A1	I17	R43
Oyem-Est (OYE)	5	1982	A1	I12(2), I18, I19(2)	R12, R24, R41(3)

(Contd.)

Location	Nb[1]	Year[2]	Mating type[3]	Isozyme genotype[3]	RAPD genotype[3]
São Tomé (14, 4, 4)[4]					
Clara Dias (CL)	1	1994	A1	I21	R35
Pedroma (PE)	3	1994 & 1995	A1	I21(2), I30	R36(3)
Poto (PO)	7	1994 & 1995	A1	I7, I9, I21(2), I30(3)	R35(2), R36(4), R38
Queluz (QU)	3	1995	A1	I30(3)	R35, R37(2)
Nigeria (50, 9, 8)[4]					
Ibeku (IBE)	9	1995	A1	I22(9)	R1(9)
Ibule (IBU)	6	1995	A1, A2(5)	I24, I25, I26, I27(3)	R4(5), R5
Idi-Ayunre (ID)	15	1995	A1	I22(13), I28(2)	R1(10), R2, R3(2), R10(2)
Ikom (IK)	6	1995	A1	I22(6)	R1(6)
Owena (OW)	3	1995	A1	I22, I28, I29	R1, R6, R9
Uhonmora (UH)	10	1995	A1	I22(9), I23	R1(10)
Unknown (isolate P1663)	1	-	A1	I13	R1
Ghana (10, 2, 2)[4]					
Ashanti (AS)	1	1994	A1	I13	R4
Brong Ahafo (BR)	5	1993	A1	I13(5)	R1(5)
Volta (VO)	2	1994	A1	I16(2)	R1(2)
Western (WE)	2	1994	A1	I13(2)	R1(2)
Togo (4, 1, 2)[4]					
Kloto (KL)	2	1988 & 1991	A1	I13(2)	R1(2)
Litimé (LI)	2	1991	A1	I13(2)	R1, R2

1. Number of isolates collected.
2. Isolate collection year(s).
3. A number in brackets corresponds to the number of isolates for a given genotype when there is more than one. For mating type, this is valid only when type A2 is present.
4. (x, y, z); x = Total number of isolates from the country; y = Number of isozyme genotypes; z = Number of RAPD genotypes.

several production zones in those countries; they were supplied by S.T. Sackey (Cocoa Research Institute of Ghana) and E.K. Djiekpor (Institut Togolais de la Recherche Agronomique) respectively. From Cameroon, two reference strains with known mating types were studied: strain 309, of mating type A1, collected from the Mbam zone and strain 184, of mating type A2 (Blaha, 1995), along with two strains, NS130 and NS131, taken from naturally infected cola fruits (*Cola nitida*). The mating types were determined by exposing each strain to strains 309 and 184, characterized as being type A1 and A2 respectively, on carrot-based culture medium (Ribeiro, 1978). The existence of oospores was noted between the 15th and 30th days after inoculation.

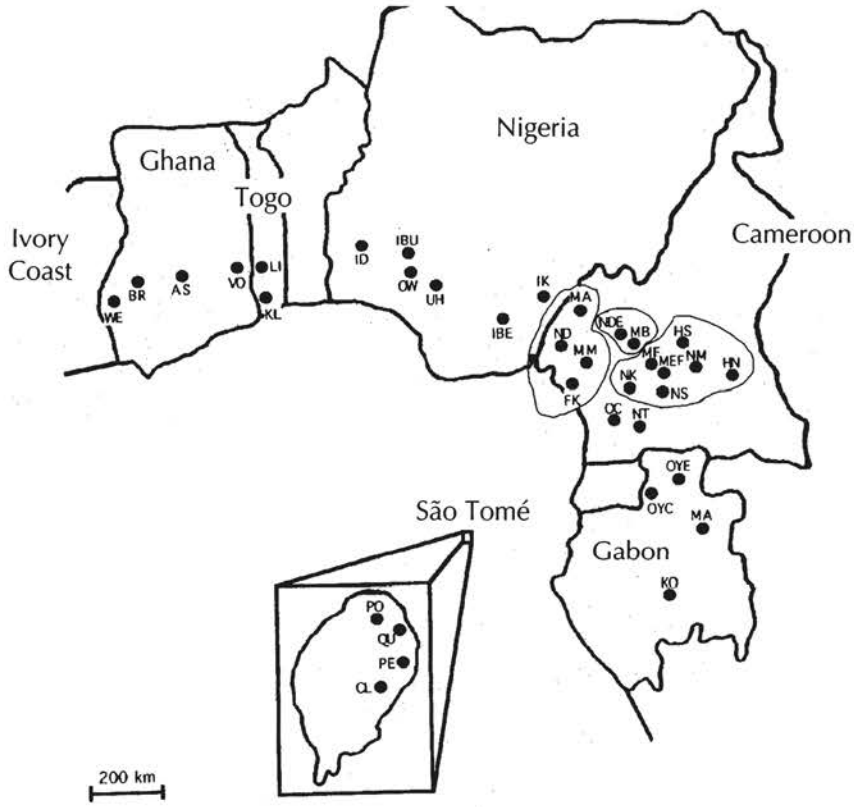

Figure 4. Distribution of sampled P. megakarya *strains.*

The analyses: isozymes and RAPD, material and methods

The different methods used to produce mycelium, and the isozyme and RAPD analysis techniques, have been described (Nyassé *et al.*, 1999). For each allozyme, the configurations observed were considered as different modalities of the same genetic descriptor, since it was impossible to identify the loci and alleles

for several of them. For RAPD, we considered that each band represented a locus. Each polymorphic band was given a score of 1 for presence and 0 for absence. Similarity indexes were calculated between all the possible pairs of isolates by simple matching. The calculations were carried out with Numerical Taxonomy System (NTSYS) software (Rohlf, 1993). The correspondence between the similarity matrix of the isozymes and that for RAPD was examined by the Mantel test (1967). Significance levels were determined from 500 permutations. The distances between genotypes were summarized using two statistical techniques. Firstly, factorial correspondence analyses (FCA) were carried out with Addad software (Escofier and Pagès, 1988). Secondly, classifications were performed with NTSYS, using the simple matching index and the unweighted pair-group method using arithmetic averages (UPGMA). The reliability limits of the nodes produced by the dendrograms were evaluated using the robust bootstrap technique with Winboot software (Yap and Nelson, 1996). A measurement of genetic diversity in each region was given by:

$G=1 / \sum p_i^2$

where p_i is the frequency of genotype i in the region considered (Stoddart and Taylor, 1988). For a given region, G is equal to 1 when all the isolates have the same genotype and is equal to 1/N when all the isolates are represented by a single genotype. The maximum possible diversity percentage (G/N) is used to compare regions with different sample sizes (McDonald *et al.*, 1994; Drenth *et al.*, 1996).

Genetic diversity from isozyme analysis

Thirteen enzyme systems were studied on 161 strains. Ten of them gave between two and four different configurations (ICD: 2, MDH1: 3, MDH2: 4, G6PDH: 3, MPI: 2, HK: 3, FUM: 3, ADA: 2, PEP(L-T): 3, PEP(G-L):3) and three were monomorphic (figure 5). These analyses therefore gave a total of 28 polymorphic descriptors, enabling the isolates to be classed into 36 different phenotypes. The number of isolates with the same phenotype varied from 1 to 38. Three phenotypes, I22, I31 and I13, were very frequent, since they alone accounted for around 50% of the isolates. Only 18 isolates had unique phenotypes. Nineteen different phenotypes were detected in Cameroon, five in Gabon, four in São Tomé, nine in Nigeria, two in Ghana and one in Togo. There were two phenotypes common to São Tomé and Cameroon and one to Nigeria, Ghana and Togo. The factorial correspondence analysis identified groups (figure 6).

– Two main groups stood out along the first axis, explaining 29% of total variability. This first group comprised phenotypes corresponding to the West African isolates (Togo, Ghana, Nigeria) and the second group comprised the isolates from Central Africa (São Tomé, Gabon, Cameroon). One isolate, I34, had an intermediate phenotype between the two previous groups. This isolate had an enzyme configuration typical of Central Africa for three isozymes, PEP(L-T)-1, PEP(G-L)-1 and MDH2-2, but it was the only Central African isolate displaying

the two enzyme configurations ADA-2 and HK-3, which are very frequent in West Africa. This isolate came from the Manyu region of Cameroon near the Nigerian border.

– Axis 2 explained 12% of the variability and separated the West African isolates into two groups, with two intermediate isolates. One group comprised isolates collected at Ibule in Nigeria, the other comprised genotypes scattered throughout West Africa.

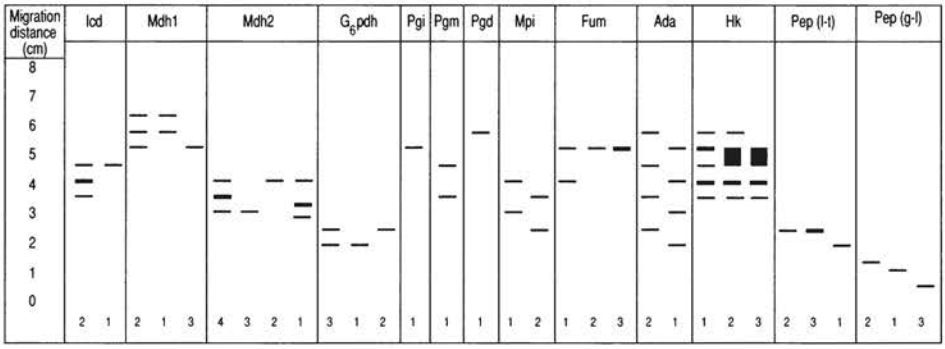

Figure 5. Description of the observed patterns for the 13 isozymes in P. megakarya.

The analysis of the UPGMA classification provided a complementary point of view. It also distinguished between the same two geographical groups indicated by the first FCA axis (figure 7). Intermediate isolate I34 is attached to the West African group. The bootstrap values associated with the nodes corresponding to the two groups were 43% and 46% for the West African and Central African groups respectively. These values became 83% and 76% when intermediate genotype I34 was removed from the analysis. No structure was detected within each group.

Genetic diversity from RAPD analysis

Nine RAPD primers revealed 33 reliable polymorphic bands among the 161 isolates. The size of the amplified products ranged from 0.5 to 3.0 KB. The isolates were divided into 44 RAPD genotypes. The number of isolates displaying the same RAPD genotype varied from 1 to 50. Two genotypes were very frequent: R1 corresponding to 49 isolates from West Africa and one isolate from western Cameroon, and R17 corresponding to 27 isolates from Cameroon. For the remaining genotypes, the number of isolates per genotype was less than nine. Twenty-eight different RAPD genotypes were identified in Cameroon, six in São Tomé, four in Gabon, eight in Nigeria, two in Ghana and two in Togo. One genotype was common to Nigeria, Togo and Ghana, another was common to Nigeria and Togo, and one was common to Nigeria and Ghana.

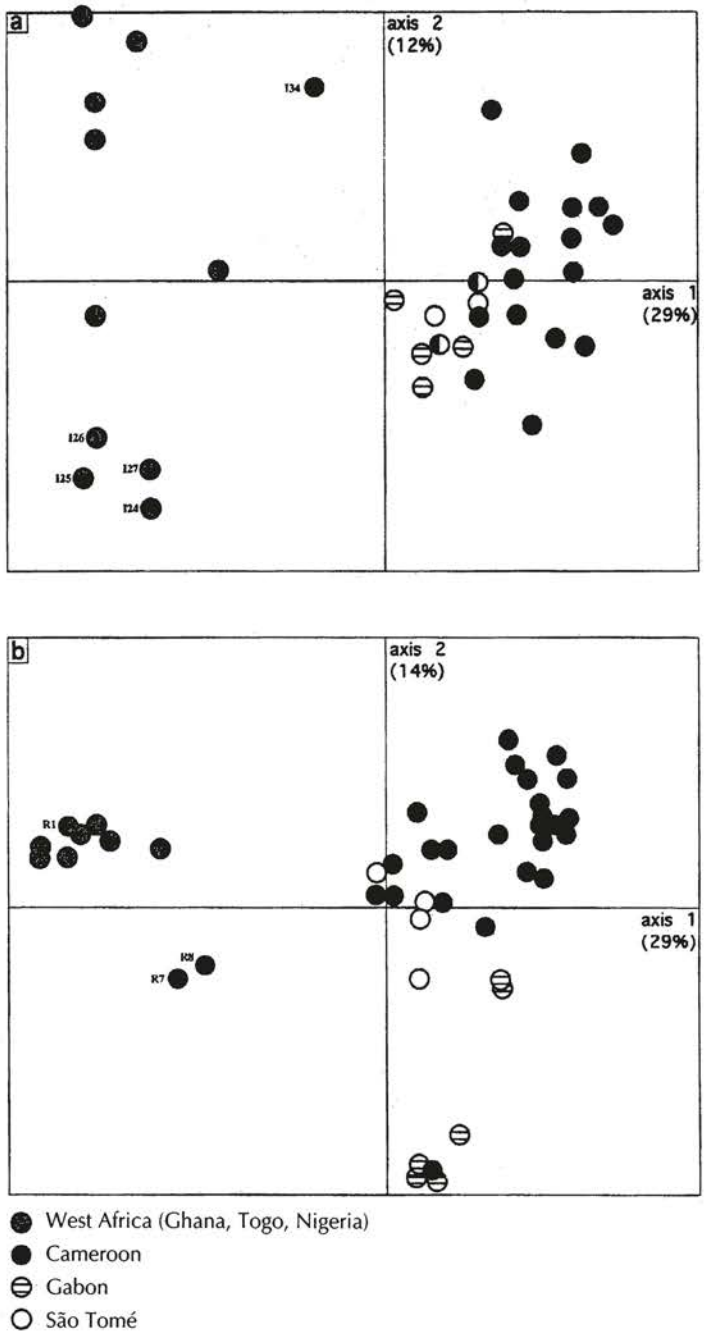

Figure 6. Plots of the first two axes generated by the Factorial Correspondence Analysis conducted on the isozymes (a) and RAPD (b) genotype data.

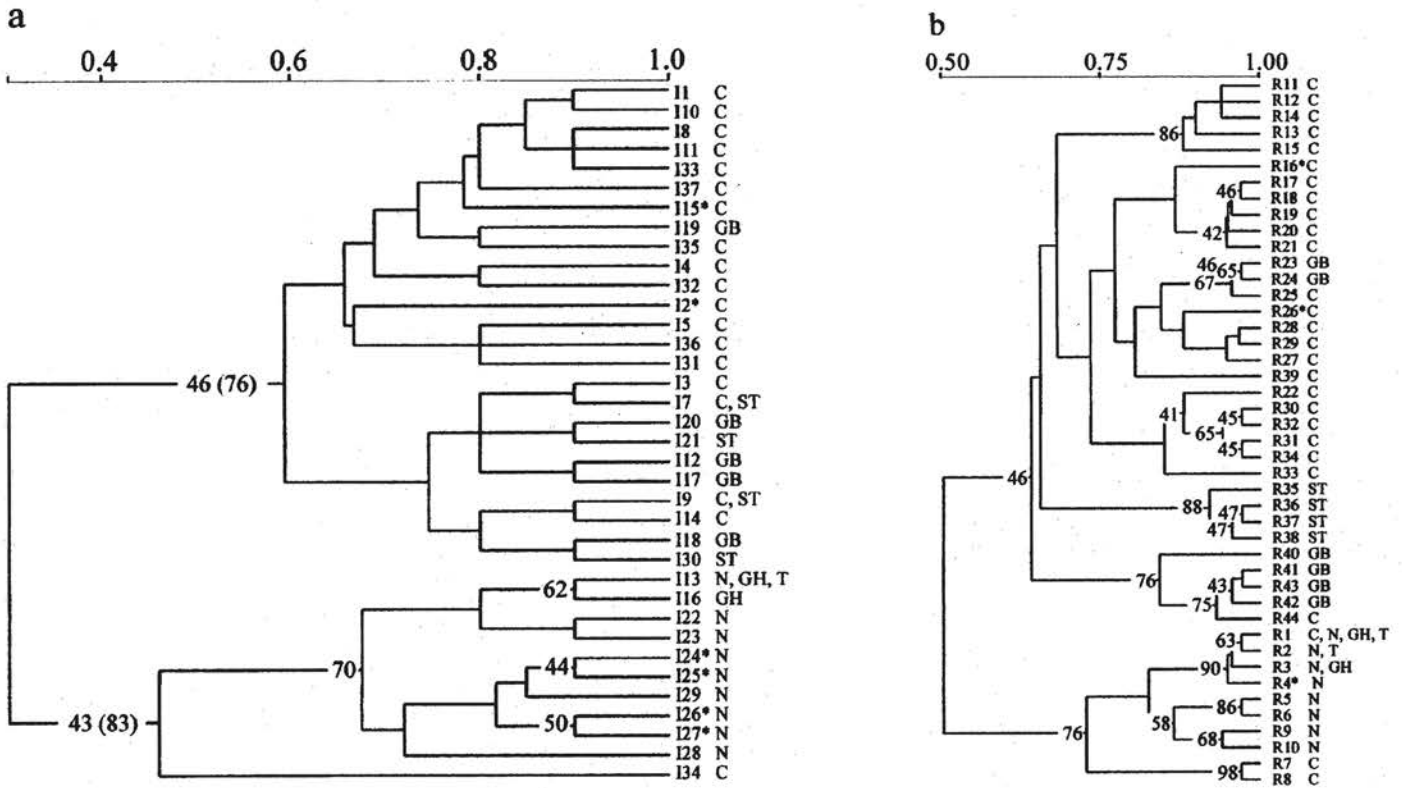

Figure 7. UPGMA dendrograms performed with the isozymes (a) and RAPD (b) genotype data. Bootstrap node values are given when they exceed 40%. The countries where genotypes have been detected are indicated as follows: C: Cameroon, GB: Gabon, GH: Ghana, N: Nigeria, ST: São Tomé, T: Togo. Genotypes including isolates of the A2 mating type are identified with an asterisk. On the isozyme dendrogram, values in brackets are obtained when genotype I34 is removed from the analysis.

Axis 1 of the factorial correspondence analysis, which explained 29% of total variability, separated the set of isolates into two groups. The first group was heterogeneous and contained 34 genotypes corresponding to the isolates collected in Central Africa (São Tomé, Gabon, Cameroon). The second group comprised eight genotypes corresponding to all the isolates collected in West Africa (Togo, Ghana, Nigeria), containing an isolate of genotype R1, sampled in the Manyu region of western Cameroon. This group also contained two genotypes, R7 and R8, corresponding to the isolates collected at Fako in Cameroon, near the Nigerian border. These two genotypes were closely linked to the West African genotypes, but had some specificities. In particular, they displayed four specific bands of the Central African genotypes, which were absent from the eight West African genotypes. A few genotypes of the first group were divided into sub-groups along axes 2, 3 and 4.

Axis 2 explained 14% of the variability. It made it possible to distinguish an initial sub-group containing four genotypes from Gabon (R40, R41, R42, R43) and one from Ocean in southern Cameroon (R44).

Axis 3 explained 12% of the variability and made it possible to distinguish a second sub-group containing four genotypes from São Tomé (R35, R36, R37, R38).

Axis 4 explained 11% of the variability and brought out a third sub-group containing four genotypes from Mbam (R11, R12, R13, R14), a sampling zone located in Central Cameroon.

The UPGMA analysis indicated two main groups corresponding to those distinguished by axis 1 of the FCA (figure 7). The bootstrap values associated with the nodes of the West African and Central African isolates were 76% and 46% respectively. The Central African group was therefore more heterogeneous, and sub-groups revealed by axes 2, 3 and 4 corresponded to the genotypes from Gabon and southern Cameroon, and also to those from São Tomé and Mbam.

Correspondence between the isozyme and RAPD analyses

The correspondence between the similarity matrices obtained for isozymes and RAPD on all the isolates was estimated by the Mantel test. The normal Mantel statistic Z was $r = 0.82$ with $p = 0.004$ (calculated from more than 500 permutations), indicating good general agreement between the two datasets. The correspondence between the isozyme and RAPD analyses is shown in diagram 5. The total number of genotypes determined simultaneously on the isozymes and RAPD amounted to 63, of which 47 corresponded to the central African isolates and 16 to the West African isolates. Nevertheless, four genotypes did not respect this geographical classification. It involved five isolates collected at Manyu and Fako in western Cameroon, near the

Nigerian border. In fact, they had intermediate configurations between the two previously mentioned groups. These isolates (NS259, NS269, NS270, NS267 and NS268) had intermediate characteristics for the isozymes and the RAPD.

For the isolates from São Tomé, the structuring found with RAPD analysis was different from that found with isozyme analysis; this situation revealed a problem with isozyme analysis power, as they were less numerous. In fact, with isozymes, the isolates from São Tomé were identical or closely linked to those from Cameroon, while with RAPD these isolates were clearly differentiated from all the others. In addition, the isolates from Gabon were separated into two groups based on both types of markers (figure 7). The first group contained two isolates from Oyem in eastern Gabon, while the second contained all the other isolates collected in Gabon. However, these two groups remained closely linked to the isolates from southern Cameroon: Ocean and Ntem.

Geographical distribution of genotypic diversity

Calculation of the genetic diversity indexes G and G/N, based simultaneously on isozymes and RAPD, showed that genetic variability was lower in West Africa than in Central Africa (table 2). In West Africa, variability distribution was heterogeneous; it was very low in Ghana, Togo, at Idi-Ayunre (western Nigeria) and at Ikom, Ibeku and Uhonmora (eastern Nigeria). Two isozymes associated with genotype RAPD R1 were strongly dominant in these two regions (I13, R1) and (I22, R1). At Ibule and Owena, in central eastern Nigeria, genetic variability appeared to be much greater, though few isolates were sampled. In Central Africa, diversity was high in the three countries studied: Cameroon, São Tomé and Gabon (table 2). In Cameroon, where the largest number of isolates was collected, variability depended on the region considered. Three geographical groups were formed: region 1 corresponding to the western mountainous region, region 2 to the east of the Bamiléké plateau and region 3 to the east of the River Sanaga. Genetic diversity was much greater in regions 1 and 2 than that estimated in region 3. In region 3, two genotypes were very frequent: (I31, R17) and (I1, R17). Greater variability could be detected by studying the 23 places in which several isolates were collected from the same plot. In four of those places, two different genotypes were detected with isozymes (two cases in West Africa and two cases in Cameroon) and in 11 places, from two to three different genotypes were detected with RAPD (two cases in West Africa and nine cases in Cameroon).

Distribution of mating types

Of the 159 isolates for which the mating type was determined, 153 were type A1 and 6 were type A2: one in Mfoundi in Cameroon and five at Ibule in Nigeria (table 1). This confirms the results previously obtained by Maddison and Griffin (1981), indicating the predominance of mating type A1 in

Table 2. Genotypic diversity G and percentage of maximum possible diversity G/N of geographical samples of *P. megakarya* observed at different scales. Computation of G is based on the multilocus isozyme and RAPD genotypes.

Location	No. of isolates	*G*	(*G*/N) %
Central Africa	97	15.0	16
Cameroon	72	10.0	14
Region 1*	24	7.4	31
Region 2*	16	9.2	57
Region 3*	28	2.6	9
Gabon	11	5.3	48
São Tomé	14	6.1	44
West Africa	64	3.0	5
Nigeria	50	2.0	4
Ibeku	9	1.0	11
Ibule	6	3.0	50
Idi-Ayunre	15	1.3	9
Ikom	6	1.0	17
Owena	3	3.0	100
Uhonmora	10	1.2	12
Togo	4	1.6	40
Ghana	10	1.9	18

* see text.

P. megakarya. This observation argues in favour of a primarily asexual type of reproduction in this species. In Central Africa, mating type A2 of the Mfoundi isolate corresponded to genotype (I2, R26), which was not linked to any genotype of mating type 1, be it with isozymes or RAPD (figures 6 and 7). This was also true for the reference strain (184), which was used to identify the mating types and which was of genotype (I15, R16). In West Africa, Ibule was the only place where mating type A2 was detected. At that sampling site, five out of six isolates had that type, which was therefore dominant. The five A2 isolates had the same RAPD genotype (R4), but different isozymes (I24, I25, I26 and I27). Of them, only two isozymes were polymorphic, ICD and MPI, with two band configurations for each of them. The four genotypes therefore corresponded to the two possible pairs of combinations. The isolate of mating type A1 was of genotype (I27, R5), it differed from genotype R4 through the existence/absence of two bands. These indexes indicated that sexual reproduction seems possible in the Ibule region.

Discussion

The intraspecific genetic variation of *P. megakarya* was studied with isozymes and RAPD, using 161 isolates collected from six African countries. This study led to the identification of two strongly differentiated genetic groups. These two groups were divided either side of a line roughly corresponding to the

border between Cameroon and Nigeria, with one group corresponding to isolates from West Africa and the other to isolates from Central Africa. This geographical structuring tallied with the results obtained by Oudemans and by Coffey (1991a) with isozymes, and those obtained by Förster *et al.* (1990) with mitochondrial DNA collected from isolate samples from Cameroon and Nigeria.

Five isolates collected near the contact zone displayed bands specific to each of the two groups for different isozyme of RAPD markers. This indicated possible exchanges between these two groups, through sexual reproduction or through heterokaryosis. This study also confirmed the overall dominance of mating type A1, already observed by Maddison and Griffin (1981), in the two genetic groups. The substantial disparity in frequency between the two mating types argued in favour of predominantly asexual reproduction. This was corroborated by the high frequency of a few genotypes in extensive geographical zones in both West Africa and Cameroon. Nevertheless, the other modes of reproduction cannot be ruled out locally:

– the existence of the two mating types at Ibule in Nigeria, and the enzymatic polymorphism revealed by isozymes indicate that sexual reproduction is possible in that zone,

– the intermediate genotypes, originating from the border between Cameroon and Nigeria, might be the result of hybridizations or of heterokaryosis,

– in the Volta region of eastern Ghana, selfing could have occurred. The two isolates collected in that region had the genotype I16. It only differs from I13, the only other genotype found in Togo and Ghana, through a single allozyme, ICD, which has simple genetic determinism with one locus and two alleles. I16 is homozygous (configuration ICD-1) and might result from selfing of I13, which is heterozygous (configuration ICD-2).

The overall genetic diversity found in West Africa (Nigeria, Togo, Ghana) is low. Few genotypes have been detected in Ghana and Togo, either with isozymes or RAPD. Moreover, all the genotypes from those two countries also existed in Nigeria, apart from one (I16). *P. megakarya* seems to be spreading westwards on the continent. The species was reported for the first time in 1982 in Togo (Djiekpor *et al.*, 1982) then in 1985 in Ghana (Dakwa, 1988; Luterbacher and Akrofi, 1994). It is likely that this extension is primarily due to vegetative propagation of a small number of individuals. *P. megakarya* was recently detected in Ivory Coast, a serious threat for that country, the world's leading cocoa producer.

In Central Africa, overall genetic diversity is greater than in West Africa. No clear structuring was revealed with isozymes, and the genotypes from São Tomé and Gabon appeared to be very similar to those from Cameroon. With RAPD, geographical structuring of the isolates clearly appeared. The difference in structuring found between isozymes and RAPD, might be due to a difference in the number

of loci explored (around 10 with isozymes and 33 with RAPD) or to a difference in the evolution rates of the sequences targeted by these two methods.

The division of *P. megakarya* into two highly differentiated groups, corresponding to two geographical zones, can be explained by the so-called refuge zone theory. Although its most important host from an economic viewpoint, *Theobroma cacao*, comes from Latin America, *P. megakarya* is probably endemic in Africa. In fact, *T. cacao* is a Sterculiaceae and, notably in this study, it was shown that *P. megakarya* can also be isolated from fruits of other Sterculiaceae, such as *Cola nitida,* which comes from West Africa. Consequently, as there are numerous wild Sterculiaceae in African tropical forests, the transfer of *P. megakarya* from an indigenous species to cocoa seems to be a reasonable hypothesis.

In addition, during the Quaternary Period, glacial cycles induced an arid climate in the tropical zones, reducing the dense African forest to a series of refuges, in places where rainfall had remained high (Maley, 1996). This led to fragmentation in the distribution of indigenous species and their pathogens, involving isolation and differentiation of populations. Once milder conditions returned, the forest species spread out from their refuge zones. In the zone covered by this study, two main biogeographical domains resulting from these climate events can be distinguished based on the distribution of plant species (White, 1979; Berthaud, 1984) and animal species (Moreau, 1966). The boundary between these two domains is in eastern Nigeria, near the Cameroon border, and corresponds approximately to the geographical separation between the two *P. megakarya* groups detected in this study. The convergence seen in the structuring of diversity between different plant, animal and fungus taxons in Africa is a fresh argument in favour of ancient development of *P. megakarya* in this region.

Genetic diversity of the pathogen and host-pathogen interactions

Work on the genetic diversity of *Phytophthora* indicates that the species *P. megakarya* is currently the predominant species in Cameroon and Nigeria, since very few *P. palmivora* isolates have been detected in those countries. This suggests that the species *P. megakarya* could also become the predominant species in the other African countries, where it currently exists alongside *P. palmivora* (Togo, Ghana, Gabon, São Tomé and Ivory Coast). With the dissemination of *P. megakarya*, it can be feared that there will be an increase in rot damage in those countries, as currently seen in Ghana. It is also to be feared that this species will invade Ivory Coast, the world's leading cocoa producer, from the east of the country where the first *P. megakarya* have been reported.

Two distinct geographical groups have been identified, between which there has apparently been very little mixing, indicating limited isolate mobility between the two geographical regions. This is undoubtedly due to the geographical barrier of eastern Nigeria, to which can be added the natural division at the river Sanaga in

Cameroon. The latter barrier might explain the difference between the isolates from Bafia and those from sites located south of the river Sanaga.

Although *P. megakarya* multiplication seems to be primarily asexual, there are indications that other mechanisms involving recombination (sexual reproduction or mitotic recombination) are possible locally at Ibule in Nigeria and in western Cameroon. Genetic recombinations are potentially a threat, since they are a source of variability, notably for strain pathogenicity. This has been shown in *P. infestans* (Drenth *et al.*, 1994). This could also be the case for *P. megakarya*, as strain NS269, the most aggressive in the study, is genetically an intermediate strain between the two populations that might be derived from a recombination.

Use of the leaf test to measure the intraspecific aggressiveness of the pathogen should be considered, as results on leaves can be positively correlated with the level of attacks seen in the field. Pathogenicity tests carried out on leaves with a sample of 11 *P. megakarya* strains revealed a large variation in the degree of aggressiveness, but there did not seem to be any link between genetic diversity and the aggressiveness of the strains. This tallied with attack levels, which can also be high in all the countries affected by the disease.

Host-parasite interaction studies suggest that the relations between *P. megakarya* strains and cocoa clones are only slightly specific or not at all. This might be explained by the fact that contact between the parasite and its host is recent. The cocoa tree was introduced into Africa in 1857 and it was not until 1979 that *P. megakarya* was identified with certainty on cocoa (Brasier and Griffin, 1979). Another reason might be the fact that *P. megakarya* is a parasite that attacks several host plants. Often with polyphagous parasites, pathosystem specificity is less marked. Further work is required to acquire a clearer understanding of possible interactions between cocoa trees and strains of *Phytophthora* spp.

Conclusion and prospects

Studies on the genetic diversity of *P. megakarya* are continuing. Some of this work is being conducted in the CIRAD plant pathology laboratory in Montpellier, notably with a view to monitoring the evolution of pathogenic populations. The most recent results confirm those presented in this book. New strains have been studied and the most recent classification is proposed in figure 5.

The different results obtained by Nyassé (1997) have been confirmed and, in particular, the methodology has been fine-tuned:

– As regards the West African population, genotypes R1, R2 and R3 obtained by Nyassé can be grouped into a single genotype R1. Strain NS259 from the Cameroon-Nigeria border is therefore identical to those existing in Nigeria. Strain NS328 representing the five strains collected in 1999 in that border zone, is included in the West African strains. We shall call it R2. In that zone, it seems that the only genotypes that can be characterized are of the West African

type for RAPD. It would seem paramount to monitor this possible extension throughout western Cameroon.

– The other new strains characterized, which came from the other zones of Cameroon, are all of the Central African type for RAPD. Genotypes R13, R14 and R15 can be grouped into a single genotype R13. The same applies for genotypes R17, R18 and R20, which should be grouped in genotype R17. Genotypes R22, R31, R32 and R34 will be grouped in genotype R22. Genotype R31 will be attributed to genotype 2.

– The strains from Gabon are well grouped in a single group, with one strain from Cameroon, but isolated on the Gabon border.

– The strains from São Tomé are also placed in a single group.

Phytophthora capsici

Other *Phytophthora* species affect cocoa trees, causing fruit rot. In Brazil and India, there have been attacks by *P. citrophthora*, and *P. capsici* is seen as the dominant pathogen in Latin America and the Caribbean.

Although UPGMA classification of the isozyme patterns on five loci showed a genetic relation between *P. capsici* and *P. citrophthora* (common allele is C for Mdh-2, I for Idh-2 and F for Sod-2) (Oudemans and Coffey, 1991*a*; Oudemans *et al.*, 1994; Mchau and Coffey, 1995; Ortiz-Garcia, 1996), the analysis involving eight loci (Pgi, Mdh-1, Mdh-2, Idh-2, Sod-2, Me, Pgm and G_6pdh) separated the two species *P. capsici* and *P. citrophthora* into four groups, three of which were more closely linked (Ortiz-Garcia, 1996):

– *P. citrophthora* at a distance of 0.46 (strains from *Citrus* and rubber),

– *P. capsici* at a distance of 0.36, but which subdivided into three subgroups:
  - strains of *P. capsici* from market garden plants and strains of "typical group A" *P. capsici* strains from pepper and cocoa,
  - "typical group B" *P. capsici* from cocoa (allele Mdh-1 different from "typical group A" strains),
  - "atypical" *P. capsici,* with strains from pepper and cocoa.

Following this study, two initial certainties prove founded: firstly the parallel between typical gr. B *P. capsici* and atypical *P. capsici* in agreement with the work by Goodwin *et al.* (1990) and by Mchau and Coffey (1994*b*) showing that these morpho-taxonomic groups are linked more to each other than to *P. citrophthora* Smith & Smith (strains from Citrus*)*; secondly the division of *P. capsici* strains (from cocoa and pepper) into three closely linked genetic units belonging to the same species, *P. capsici.*

Other information will be acquired by using genetic parameters of the populations (Ortiz-Garcia, 1996).

The structuring of the isozyme group *P. capsici* typical group A was studied on seven geographical populations: *P. capsici* from market garden plants, 26 strains (Mediterranean countries and North America, including the southern USA and northern Mexico), and from pepper, 5 strains (Southeast Asia), *P. capsici* typical group A from cocoa, 42 strains (northern Amazonia, eastern Brazil, southern Mexico).

The allele frequencies showed that the typical group A *P. capsici* population from eastern Brazil could be distinguished from all the other populations through the predominance of alleles A for Pgi and H for Me. However, the *P. capsici* populations from the Mediterranean countries, North America and Southeast Asia had in common a homozygous GG pattern, and allele G was not found with the typical group A *P. capsici* populations. The allele differentiation test and the Rogers distances consequently led to a division into three genetic groups defined by:

– a link between *P. capsici* strains from the Mediterranean countries and North America, though stronger than with *P. capsici* strains from Southeast Asia,

– individualization of typical group A *P. capsici* strains from cocoa in eastern Brazil,

– grouping of typical group A *P. capsici* strains from cocoa in northern Amazonia and southern Mexico.

The within-population genetic parameters applied to three *P. capsici* populations from eastern Brazil (Bahia), typical group A (10 strains), typical group B (14 strains) and atypical (16 strains), showed that the typical group B *P. capsici* population had the highest percentage of polymorphic loci and greater genetic and genotypic diversity. These last two parameters differed little from those of the atypical *P. capsici* population. The allele differentiation test between populations, which was highly significant for five out of six loci in each pair of populations (allele A for Pgi only being present with the typical group A *P. capsici* population), the Rogers distances and the coefficient of genetic divergence (both high), confirmed that the three populations are distinct.

The structuring studied on 173 typical group A *P. capsici* strains from southern Mexico, originating from four geographical sites, coastal Tabasco (17 strains), central Tabasco (93 strains), western Tabasco (23 strains) and northern Chiapas/southern Tabasco (40 strains), revealed very low genetic diversities (under 10% on 8 loci within each of the populations).

The third certainty, given by the study of between and within-population structuring, on both a world and Latin American level, is the diversification of *P. capsici* strains into three large groups:

– *P. capsici* from temperate regions (strains from market garden crops) or tropical zones, Southeast Asia (strains from pepper), Trinidad, Costa Rica (strains from cocoa) and typical group A *P. capsici* from northern Amazonia and southern Mexico (strains from cocoa),

– typical group A *P. capsici* from eastern Brazil (strains from cocoa),

– typical group B *P. capsici* and atypical *P. capsici* (strains from cocoa).

To conclude, all these genetically linked groups and subgroups would seem to have a common ancestor from Amazonia and, for the majority of them, the greater between-population diversity and existence of unusual alleles such as A for Pgi are found in eastern Brazil. However, outside Latin America, these taxonomic groups are dispersed: *P. capsici* in Southeast Asia on pepper, in Central America and the Caribbean on cocoa, and in temperate regions on plants such as tomato and capsicum, though the origin is Latin America; typical *P. capsici* and atypical PMF4 on pepper and on cocoa in West Africa and Southeast Asia.

The first numerous trading exchanges between continents, which led to major mixing of plants, and also of their parasites, might explain this worldwide dispersion. New constraints, be they edapho-climatic or plant-related, might well have favoured the adaptation and evolution of individuals capable of finding a "return trip" to their zones of origin (Ortiz-Garcia, 1996). A detailed analysis of allozymes can rapidly provide an evaluation of the genetic diversity of a species (Forster and Coffey, 1991), since over and above the species level, isolates displaying common electrophoretypes segregate into sub-groups assimilable in distinct populations, which often cannot be differentiated through their morphological traits alone. Like *P. palmivora* / *P. arecae* (Oudemans and Coffey, 1991*b*, 1991*c*; Blaha *et al.*, 1994; Mchau and Coffey, 1994*a*) and *P. megakarya* (Nyassé, 1999), *P. capsici* would seem to comprise populations derived from the same phylum but having acquired a specific evolution, through isolation due to particular events. The risks of introduction, which are sources of notorious cocoa crop losses in some countries, such as Brazil, call for constant surveillance and strict quarantine for other producing countries yet to be affected by *Phytophthora capsici sensu* Tsao.

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Disease incidence and field resistance

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The main purpose of the overall research project covered by this book was to reduce disease incidence in new cocoa plantings by selecting resistant material. In this context, it was necessary to show that sources of resistance existed in cocoa tree populations. It was also necessary to develop ways of assessing resistance in the field that made it possible to distinguish between genetic resistance and other factors that might be involved in disease expression. To that end, it was essential to understand clearly the epidemiology of the disease and assess genetic parameters involved in transmission of the resistance trait.

Indeed, before considering genetic improvement of the cocoa tree for resistance to *Phytophthora,* it was necessary to have reliable and reproducible ways of assessing planting material. The first step in developing such methods was to itemize the different factors that might be involved in disease expression in cocoa plantations. Once these factors were known, it might be possible to check their effects using appropriate experimental designs intended to evaluate cultivated planting material under natural infestation conditions. It might also be possible to correct some of these factors using appropriate statistical analyses, such as an analysis of covariance, if those factors corresponded to quantitative variables measured during the evaluation; this amounted to checking after the event the factors involved in disease incidence.

We then give a rundown of the genetic parameters of resistance measured in different mating designs, and we take a look at the merits of these estimations in choosing cocoa tree breeding strategies for disease resistance. The different genetic parameters, estimated from rot rates measured in trial plots under natural infestation conditions, were also used to determine the relation between productivity and disease resistance. Examples of index-based selection, combining productivity and resistance to *Phytophthora,* can be found at the end of the chapter.

Factors involved in disease expression

Pod rot, caused by different species of the genus *Phytophthora,* is rife in all producing countries. The pathogen therefore finds the conditions for its development in all the ecological zones in which cocoa is grown. Several species of this pathogen have been identified in the different production zones. The most widespread species, *P. palmivora,* exists in virtually all producing countries. Certain studies in Cameroon revealed the existence of a single species, *P. megakarya* (Nyassé, 1992), but although that species is preponderant, *P. palmivora* has been detected alongside *P. megakarya* in cocoa plantings (Ducamp, pers. comm.). *P. megakarya* is considered to be the most aggressive species (Brasier and Griffin, 1979) and can lead to almost total destruction of the harvest. This species is also found in Nigeria, Togo and Ghana, and was recently reported in Ivory Coast. On the American continent, the species *P. capsici* has been detected in numerous production zones. These different species all cause the same symptoms on pods, namely rotting black patches that sometimes spread to the entire fruit.

Numerous factors, both environmental and genetic, are involved in disease expression in the field, i.e. they affect the rotten pod rate. These different factors may also interact with each other. For example, some genotypes may prove to be resistant in a given environment, yet react like susceptible genotypes in other environments; we then speak of "genotype x environment" interactions. Interactions between different factors that might affect disease intensity are sometimes difficult to estimate, as many different conditions exist that result in situations propitious to disease development. Attack severity therefore varies depending on such different factors as:

- environmental conditions in the plantations;
- pathogen conservation and its transmission;
- the pathogen species and strain involved;
- the genetic nature of the host.

Many conditions propitious to disease development therefore result from combinations of these different factors, which might interact with each other, to promote or, conversely, slow down disease development.

Although this disease can affect various organs, such as the roots or the trunk, it mainly attacks fruits, so it is worth taking another look at the different stages involved in the cocoa tree fruiting cycle, and in the pathogen cycle.

Cocoa tree fruiting cycle

The flowers grow on the trunk, branches and defoliated parts of secondary branches. A cocoa tree starts flowering at around two years old, and the flowering rhythm depends on climatic conditions. However, there is substantial variability from one tree to another as regards the number of flowers produced and the flowering periods. An unpollinated flower lives for no more than three days. The cocoa tree is considered to be a cross-fertilizing plant, though selfing is possible. All Upper Amazon Forasteros are self-incompatible, whereas Lower Amazon Forasteros are usually self-compatible. There are many cases of self-incompatibility in Trinitarios and Criollos. However, self-incompatibility is not strict; fruit-setting from selfing sometimes occurs on self-incompatible trees (Lanaud *et al.*, 1987; Lanaud, 1987). Pollination is exclusively by insects: the main pollinating insects for selfing are midges of the family Ceratopogonidae (*Forcipomyia* sp.), thrips (*Frankliniella parvula* Hood), aphids (*Toxoptera aurantii* B.), and for cross-fertilization *Forcipomyia* sp. When pollination is effective, a young fruit known as a cherelle develops.

Pathogen cycle

Phytophthora were long classed as fungi. The name *Phytophthora* is derived from Greek, and literally means plant *(phyto)* destroyer *(phthora)*. *Phytophthora* belongs to the kingdom *Chromista* and class *Oomycete*. *Phytophthora* is fungus-like, is commonly referred to as a fungus and is studied by mycologists, but it is in fact a protist. The biological cycle of *Phytophthora* (figure 1) comprises two phases, a vegetative or asexual phase, and a sexual phase (Blaha, 1995).

Vegetative phase

Sporocysts, which are vegetative multiplication organs par excellence, form from a mycelial thallus. The sporocysts germinate directly on a rich substrate, but on a poor substrate (rainwater), they germinate indirectly, releasing mobile zoospores that encyst for a few hours then germinate, which marks the start of the parasitic attack phase. Under unfavourable conditions, so-called "conservation" spores are produced; these are known as chlamydospores, which germinate once conditions are suitable for giving a mycelial thallus.

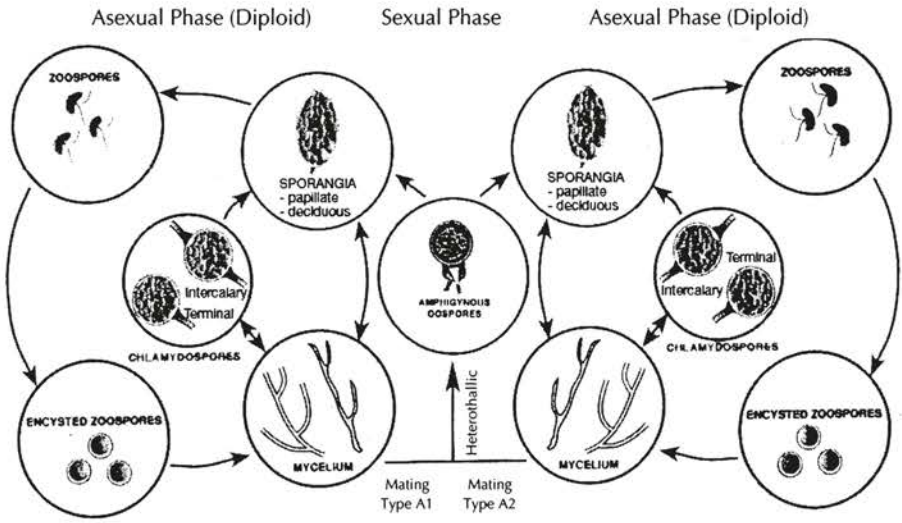

Figure 1. Phytophthora *biological cycle.*

SEXUAL PHASE

When two sexually complementary mycelial thalli, A1 and A2, come into contact they give rise to male sexual organs (antheridia) and female sexual organs (oogonia). Frequent selfing occurs. Gametocyst formation (haploid part of the cycle) is followed by their fusion during amphigenesis, leading to the formation of a zygote, whose germination will give another mycelial thallus (diploid): almost all the cycle is therefore diploid (2n), the haploid part (n) being limited to the gametocysts.

We shall now determine the different factors involved in disease expression in the field. The impact of these factors is sometimes poorly estimated and their effects are sometimes highly variable depending on the different growing conditions prevailing in plantations worldwide.

Environmental conditions in plantations

There are many environmental factors involved in disease expression in the field, and they may not all have been identified yet. These environmental factors can be divided into two types: edapho-climatic, and agricultural linked to cultural practices.

The climatic factors propitious to disease development in the field are relatively well known. On the other hand, the role of pedological factors has yet to be studied; in theory, these factors do not appear to play a dominant role in disease development. As soil has been suspected of being the pathogen conservation site, it would probably be worth mentioning that the physico-chemical constraints required for such conservation, and studies on this

subject might provide useful information. Soil moisture has turned out to be an important factor in *P. capsici* development on field-planted pepper (Gumpertz *et al.,* 1997). This factor has not been examined for the *Phytophthora* spp. - cocoa combination. Soil water-holding properties, the existence of leaf litter, and weed cover are all factors that need to be taken into account to acquire a clearer understanding of disease development in the field.

Of the climatic factors, rainfall, and especially relative humidity, are factors that favour disease development (NDoumbé, 2002). Indeed, pathogen sporulation is encouraged by high humidity, and rainfall also acts as a disease vector. Reproduction by zoospores, dependent upon an aquatic life, requires heavy rainfall. Temperature also has an effect on different parts of the pathogen cycle. For example, during artificial inoculations, optimum temperatures propitious to symptom development have been determined from several experiments. Light has been identified as a growth-inhibiting factor in different species of *Phytophthora* (Blaha, 1983). All these observations were carried out in the laboratory, and it is difficult to assess the impact of such factors (temperature and light) on epidemic development in the field. The effect of solar radiation on the survival of *Phytophthora* living off other species has also been studied (Mizubuti *et al.,* 2000), but this work has not generally made it possible to model how these factors affect natural epidemics.

Cultural techniques can have an effect on disease intensity in the field. High planting densities or dense shading are propitious to disease development, due to a lack of aeration in the plots caused by such conditions. Conditions favouring high humidity rates contribute towards high rot rates. Slight shading is therefore recommended when environments are favourable to disease development. Excessive planting densities could also facilitate the spread of the inoculum between neighbouring trees. However, such transmission between trees has never been demonstrated, and that type of propagation does not seem to be particularly important, as there are usually no correlations between neighbouring trees. In addition, certain agricultural practices can lead to pathogen transmission by the tools used, or through the transport of infected pods.

Pathogen conservation and transmission

The pathogen usually develops during the rainy season, which is propitious to sporulation. No pathogen activity has been detected during dry seasons, even though fruits are present on trees. However, the pathogen is conserved during such dry periods, since its activity resumes shortly after the first rain (Gregory, 1974; Gregory and Maddison, 1981). The quantity of pathogen conserved during dry seasons constitutes the primary inoculum available at the start of the infection period. The conservation sites are not known with any certainty. In

Nigeria, it has been shown that the parasite can be obtained from soil even outside epidemic periods (Thorold, 1955) and that the amount of underground inoculum experiences substantial seasonal variations (Okaisabor, 1969). Work on sources of primary infection (Maddison and Griffin, 1981; Ward and Griffin, 1981) showed that some of the pods that triggered the disease were near the soil and were contaminated by splashes from the ground, but most were more than 70 cm from the ground. An analysis of how pods affected by primary infection were distributed in trees indicated that the probability of contamination, depending on the height of the pods, followed a linear gradient. It was more likely the nearer the fruits were to the ground, but it was never zero irrespective of the height of the pods in the tree.

Some authors state that soil is the preponderant pathogen conservation site, while others mention different tree organs: bark, flower cushions (Babacauh, 1982). Today, all these sites seem to be possible conservation sites, but no indications backed up by figures and based on observations or experiments have yet been proposed for primary inoculum distribution between these different sites. Cleaning trees after major harvesting periods (pruning of suckers and dead branches, removal of rotten, dry or mummified fruits) apparently helps to reduce attack rates by reducing the amount of primary inoculum conserved in different tree organs. A proportion of the inoculum may also be conserved in other plants existing in cocoa plantations. Such pathogen conservation in other species is particularly important in intercropping systems. Coconut-cocoa intercropping systems in Southeast Asia (Malaysia and Indonesia) or in Papua New Guinea are particularly problematic for *Phytophthora* disease management. In fact, some species such as *P. palmivora* are pathogenic for both species, and coconut palms serving as shade trees for cocoa trees are an additional source of inoculum for cocoa trees. This cropping system therefore raises health problems characterized by cankers on cocoa tree trunks and by severe pod losses due to *P. palmivora*. A study of pathogen conservation sites in both plant species would surely make it possible to envisage appropriate control methods more effectively, notably for postponing the epidemic period or reducing the amount of primary inoculum.

Pods affected by *Phytophthora* rot constitute the secondary inoculum. Infected pods become necrotic and sporulation occurs on the surface of the epidermis. Released spores can inoculate new pods. The overall amount of inoculum can be reduced by removing infected pods: this is known as sanitary harvesting. However, the efficacy of such preventive measures is limited. A trial on the effect of sanitary harvesting was conducted in Litimé, a region of Togo in which *P. megakarya* is rife. The cumulated percentage of rotten pods was 78% on control trees, and 66% on trees on which weekly sanitary harvesting had been carried out. These unpublished results would be worth confirming in other ecosystems.

Different types of pathogen transmission have been mentioned, but most authors agree that rainwater is the main vector. During rainfall, runoff along branches would seem to carry spores from the upper storeys of the trees down to the

lower zones, at ground level. This downward transmission by rainwater would clearly be accompanied by the humidity favourable to pathogen germination and penetration. Other authors have suggested upward transmission by rainwater from the ground to the lower parts of the trees. In this case, rainwater loaded with spores would be splashed upwards, reaching pods on the lower sections of the trunk. This second hypothesis does not explain how the pathogen then reaches the upper parts of the tree; and other ways of transmission are undoubtedly involved in the contamination of pods on branches. Among other suspected vectors of disease propagation, certain insects are thought to play a predominant role, particularly ants of the genus *Crematogaster,* primarily *C. striatula* (Evans, 1971). Trials on the chemical control of these ants led to a reduction in attack rates in the experimental plots treated (Dufour, pers. comm.). These insects move from the ground into the foliage of the trees, and from tree to tree when the canopies touch, i.e. once the cocoa trees are 4 or 5 years old. Other crawling or flying insects may also be involved as vectors; quantifying their role would undoubtedly be very useful for developing integrated control systems. Other animals, notably rodents such as rats, are often mentioned as potential vectors of the disease (Muller, 1974).

Human intervention may also be responsible for transporting the pathogen and thereby promoting disease development. During pruning or harvesting, the tools used are rarely cleaned during the working day; these tools may therefore be contaminants during different upkeep or harvesting operations.

Wind is also suspected of transporting spores, but no work on the subject has yet made it possible to establish its role in spreading the disease. Whilst numerous potential vectors are incriminated in disease propagation, their relative importance is not yet known, and that is a missing link for establishing an epidemic model.

Precise identification of pathogen conservation sites and pathogen transmission methods remains of paramount importance for defining effective control strategies. This research, combined with genetic improvement of the cocoa tree, should make it possible to set up new cocoa plantations that are much less susceptible to pod rot.

Access to this knowledge would also provide a clearer understanding of disease epidemiology and make it possible to model it. No precise epidemic model has yet been proposed, but disease progress has been studied. With a view to determining the consequences of phytosanitary intervention, Medeiros (1976) sought to define the parameters that govern rot disease progress in the field. The author observed pod infection trends over two consecutive years in two regions: one with a "severe" epidemic and the other with a "slight" epidemic. In both cases, infection followed the same system of compound interest diseases (Van Der Plank, 1963). Thus, at any given moment, the proportion of diseased pods is defined by the equation:

$$x = x_0 e^{rt}$$

where: x_0 = quantity of primary inoculum; r = infection progress rate; t = time

Computation of r gave the following results:

– "severe" epidemic region: r = 0.033

– "slight" epidemic region: r = 0.066

The "severe" epidemic regions had a lower progress rate, but were characterized by a much longer season propitious to the disease. In addition, disease development seemed to be linked to climatic conditions, particularly relative humidity and rainfall.

A general epidemic system was proposed in Nigeria by Maddison and Griffin (1981). In the dry season, the parasite remains in latent form in cocoa tree roots. As soon as the first rain falls, zoospores emitted from root sporoscysts rise to the surface by negative geotaxis. Transport to the fruits is then ensured by highly volatile aerosol suspension, which can reach all the fruits in the trees, but particularly those nearest the ground. This phase of the infection cycle alone can cause substantial damage. If diseased fruits are not removed, disease propagation continues from pod to pod, in sequences of varying size, at an estimated average of 3.5 pods. It is splashing caused by rainfall that carries infectious propagules. The fungus has also been found in certain flower cushions, and in some cankers, which can also be an accessory source of primary inoculum. Neither is rainfall alone in transporting propagules; insects, particularly ants, can be included among the major causes of contamination.

Pathogen species and strains

The species *P. palmivora* exists in all growing zones and causes losses ranging from 5 to 25%. The most aggressive species is *P. megakarya,* which is found in central Africa and is spreading westwards on the continent; the advancing front is currently in eastern Ivory Coast. The other species, such as *P. capsici* or *P. affine capsici* are less widespread. Although *P. megakarya* is the most aggressive species overall, there is genetic variability within each of these species (see Chapter 2), which is reflected, among other things, in variable aggressiveness. In the species *P. megakarya,* the most aggressive strains seem to come from natural hybridization between forms A1 and A2, which are in contact with each other in western Cameroon (Nyassé, 1997). Differences in aggressiveness between strains of the same species have often been reported (Blaha, 1967). Such pathogen variations are seen during artificial inoculations, on leaves or pods, but variations in field losses depend on many factors, and it is therefore difficult to estimate how much variations in rot rate are due to the strain. Although differences have been observed between species or strains, no interaction has been detected between species (strains) and cocoa tree clones (or hybrid crosses). The level of resistance in the host thus seems to be independent from the strain or species of *Phytophthora*. Whilst such an interaction may exist, it is of less importance than the genetic effects of the host or pathogen.

Genetic nature of the host

Before going into the influence of cocoa tree genetics in the following chapters, it is important to mention at this stage that the genetic nature of the host can undoubtedly play a role in the susceptibility of tissues, and also in the different connected traits that will or will not be propitious to disease development. The variability in tissue susceptibility, which we call "inherent resistance", is the main source of variation used in genetic improvement. Assessing this inherent resistance involves various tests that are described in the following chapters. Not all of the other traits that might be involved in disease expression in the field have yet been identified, but a certain number of them have been studied, or at least suspected.

Phytophthora rot disease is primarily expressed on fruits. While some symptoms occur on other organs, such as cankers on the trunk in Papua New Guinea, pods are the main organs affected. Pod rot generally leads to their destruction, causing fruit losses, hence lower incomes for farmers. Disease development is therefore linked to tree fruiting. Fruiting intensity, the length of the fruiting cycle, and its possible offset from the pathogen cycle are all factors that influence disease intensity in plantations. Although Trinitarios are generally more susceptible to the disease than Forasteros, perhaps this difference might be due to different fruiting cycle lengths. On average there is an interval of five and one-half to six months between pollination and ripe fruit harvesting of Trinitarios, whereas for Forasteros the interval is around four and one-half to five months (Berry and Cilas, 1994*b*). Trinitario pods thus remain about one month longer on the trees and, as the disease targets pods, longer exposure could lead to greater losses.

The differences between fruiting cycle lengths can only be blamed if the cycles are synchronous, i.e. if the fruiting peaks coincide between the two genotypes compared. Although fruiting is generally synchronous among most genotypes—as synchronization is governed by the climate cycle, notably alternating dry and rainy seasons the fruiting cycles of some genotypes are staggered in relation to the cycles of their neighbours in a plantation (Jagoret *et al.*, 1994). In fact, most genotypes have a fruiting cycle that coincides with that of the pathogen, corresponding to the rainy season, though a few genotypes have a fruiting cycle that is offset from that of the pathogen. Consequently, for the same inherent susceptibility, some genotypes may have low rot rates in the field merely because they have a different fruiting period. Some genotypes may therefore bear fruits while the pathogen is inactive; this is known as an escape phenomenon. This resistance through escape is often difficult to transpose from one growing area to another. Indeed, this type of behaviour is usually linked to an interaction between the genotype and the climate, i.e. genotype x environment interaction, which is therefore more difficult to manage than a genetic effect involving little interaction with the environment. In addition, the crop load may affect disease intensity, as pods are the secondary source of inoculum. Genetic and environmental correlations between production and rot rate in the field are investigated in the next section, which will shed further light on the relation between fruit load and susceptibility to the disease.

Conclusion

The factors involved in disease expression in the field have been identified. However, the relative share of these different factors in disease severity has yet to be quantified. This is a missing link that future research efforts will have to uncover. A clearer understanding of disease propagation and development is necessary for any integrated control system to be considered. The project that gave rise to this book primarily focused on the genetic resistance of cocoa trees to various species of *Phytophthora*. While genetic resistance appears to be the key component for integrated control, it is nonetheless essential to integrate other control methods—such as chemical control of the pathogen or its vectors, and the use of certain cultural practices, such as shade regulation—for more effective control of the disease. Development of these other control methods will require increased knowledge of the epidemiology of this disease. Constructing an epidemic model is one research objective that should make it possible to quantify the effect of the different factors involved in disease expression, thereby helping to establish true integrated control.

Genetic parameters of resistance

Selecting cocoa trees displaying less susceptibility to black pod rot remains a priority objective for reducing disease impact. Despite a great deal of work (Thorold, 1953; Tarjot, 1969; Blaha and Lotodé, 1976), the search for cocoa trees displaying total resistance to this disease has so far drawn a blank. Numerous authors suggest that differences in reaction to *Phytophthora* spp. arise from partial, probably polygenic, resistance (Partiot, 1975; Blaha and Lotodé, 1977). In addition, it has been demonstrated for *P. megakarya* (Despréaux *et al.*, 1989; Berry and Cilas, 1994*b*) and for *P. palmivora* (Cilas *et al.*, 1999) that transmission of the field resistance trait, under natural infection conditions, is primarily additive. Different ways of assessing planting material have been tested (Blaha, 1974). The observation of the field performance of trees under natural infection conditions, and artificial inoculation tests on pods or leaves, remain the main methods adopted. In this section, we shall particularly be concentrating on the genetic parameters of resistance assessed in the field.

Field resistance heritability

The results came from mating designs set up in three countries:

– a 6 x 6 complete diallel, planted at the Barombi-Kang station (IRAD) in Cameroon in 1974;

– a 12 x 12 triangular diallel, planted at the Tové station (CRA/F, ex-IRCC) in Togo in 1987;

– a 16 ♀x 4♂ factorial mating design (North Carolina 2 Design), set up at the Bingerville station (CNRA) in Ivory Coast in 1978.

Description of the trials and analyses

The cocoa trees observed in Cameroon were therefore derived from a 6 x 6 complete diallel design (without the selfs). This trial, which was planted at the Barombi-Kang station in 1974, comprised 6 blocks. Each block contained 12 trees per family set out totally at random, at a density of 1,330 plants/ha. The 6 parents used were: SNK 10, UPA 134, IMC 67, ICS 95, SNK 413 and ICS 84. SNK 10 and SNK 413 were local Trinitarios, ICS 95 and ICS 84 were Trinitarios from Trinidad selected by ICTA, UPA 134 was a clone derived from an Upper Amazon Forastero progeny from Ghana, and IMC 67 was an Upper Amazon collected at Iquitos (Peru).

The trees observed in Togo came from a 12 x 12 triangular diallel design (without the selfs). The plot, which was planted in 1987 in the Litimé region, comprised 2 blocks. Each block contained 6 trees per family set out totally at random, at a density of 1,330 plants/ha.

The 12 parents used were:

Sca 6	Amazon of wild origin
IMC 67	Upper Amazon of wild origin
Na 32	Upper Amazon of wild origin
T60/887	Upper Amazon derived from cross Pa 7 x Na 32
T85/799	Upper Amazon derived from cross IMC 60 x Na 34
T86/45	Upper Amazon derived from cross Pa 35 x Pa 7
UPA 134	progeny of Upper Amazon selected in Ghana
IFC 5	Lower Amazon selected in Ivory Coast
SNK 64	Lower Amazon selected in Cameroon
UF 676	Trinitario selected in Costa Rica
ICS 40	Trinitario from Trinidad selected by ICTA
ICS 100	Trinitario from Trinidad selected by ICTA

In Togo, the two species (*P. palmivora* and *P. megakarya*) exist (Djiekpor *et al.*, 1982), but the species found in Litimé was *P. megakarya*.

The cocoa trees observed in Ivory Coast came from a factorial mating design between 16 Upper Amazon female parents and 4 Lower Amazon male parents. The trees of the different crosses were planted in a total random design of plots each comprising a single individual.

The 16 female parents used were:

P 7	Upper Amazon of wild origin
Na 79	Upper Amazon of wild origin
Sca 6	Upper Amazon of wild origin
IMC 67	Upper Amazon of wild origin
Pa 150	Upper Amazon of wild origin
Na 32	Upper Amazon of wild origin
Pa 35*	poorly identified Trinitario
Pa 7	Upper Amazon of wild origin
IMC 78	Upper Amazon of wild origin
T60/887	Upper Amazon derived from cross Pa 7 x Na 32
T79/501	Upper Amazon derived from cross Na 32 x Pa 7
T79/416	Upper Amazon derived from cross Na 32 x Pa 7
T79/467	Upper Amazon derived from cross Na 32 x Pa 7
T63/971	Upper Amazon derived from cross Pa 35 x Na 32
T63/967	Upper Amazon derived from cross Pa 35 x Na 32
T85/799	Upper Amazon derived from cross IMC 60 x Na 34

The 4 male parents used were:

IFC 1	Lower Amazon selected locally
IFC 2	Lower Amazon selected locally
IFC 5	Lower Amazon selected locally
IFC 15	Lower Amazon selected locally

Each trial tree was observed during the fruiting period (May-November), for six years (1986, 1987, 1988, 1989, 1990, 1995 and 1996) in Cameroon, and in 1991 in Togo. Rotten pods (affected by black pod), wilted pods (physiological desiccation), rodent-damaged pods and healthy ripe pods were counted each week. A sanitary harvest was carried out during the counting operations. The topographical situation of the different trees was also recorded in Togo, so that pod rot distribution in the plot could be visualized.

Losses due to *Phytophthora* spp. were estimated in relation to potential production by the formula:

$$prr = \frac{\text{number of rotten pods}}{\text{number of rotten pods} + \text{number of ripe pods} + \text{number of healthy pods on last count}}$$

In Ivory Coast, the number of rotten pods and the number of healthy ripe pods were recorded on each harvesting round during the fruiting period. For each of the trial trees, the total number of pods, production in kilos of fresh beans, and the pod rot rate, were estimated from 9 years of observations.

After adjusting any block effects, diallel analyses were carried out using Keuls and Garretsen's general method (1977) adapted to unbalanced mating designs, possibly with missing crosses:

$$P_{ijk} = \mu + g_i + g_j + m_i - m_j + s_{ij} + r_{ij} + E_{ijk}$$

where: g_i: general combining ability of parent i; m_i: general reciprocal effect (or maternal effect) of parent i; s_{ij}: specific combining ability of pair ij; r_{ij}: specific reciprocal effect of pair ij.

The different characters studied were:

– potential production: total number of pods formed, Log transformed to satisfy conditions for application of analysis of variance models, *Log(yp);*

– actual production: number of ripe pods harvested, Log transformed, *Log(y);*

– pod rot rate, *prr*.

Analysis of the factorial mating design in Ivory Coast was based on the "North Carolina 2" model:

$$P_{ijk} = \mu + f_i + m_j + s_{ij} + E_{ijk}$$

where: f_i: effect of female parent i; m_j: effect of male parent i; s_{ij}: interaction or specific combining ability of pair ij.

The different characters studied were:

– potential production (total number of pods formed Log transformed, *Log(yp);*

– actual production (fresh bean weight), *yb;*

– pod rot rate, *prr*.

Narrow and broad sense heritabilities were estimated for each of the mating designs studied (Cilas, 1995). Phenotypic, genetic and environmental correlations were calculated between the pod rot rates and the production variables. The trial analyses were mostly carried out with OPEP genetics software (Baradat *et al.*, 1995).

Trial analyses and results

Cameroon

There were substantial pod losses due to rodents, with average rates of 20% to 50%. The block effects were highly significant for all the characters and years, indicating that the blocks controlled some major environmental effects (Berry and Cilas, 1994*a*).

The complete diallel analyses are shown in table 1. The genetic effects were limited for potential production: *Log(yp);* this character therefore appeared to have quite low heritability. However, the general combining ability (GCA) effect was substantial for the actual production characters, *Log(y),* and especially for the pod rot rate, *prr*.

Table 1. Analysis of the complete diallel for the different variables considered in Cameroon.

Variables effects		*Log(y)*	*Log(yp)*	*prr*
GCA	F	4.126	1. 465	11.976
	α (%)	(0.114)	(19.779)	(< 0.001)
SCA	F	2.644	2.847	1.604
	α (%)	(0.516)	(0.271)	(10.894)
GRE	F	5.231	4.136	3.818
	α (%)	(0.012)	(0.112)	(0.213)
SRE	F	0.448	0.434	1.245
	α (%)	(92.250)	(93.014)	(25.741)

GCA: general combining ability; SCA: specific combining ability; GRE: general reciprocal effect (maternal); SRE: specific reciprocal effect.

The GCAs of the different parents for the pod rot rate in the field could be estimated and compared by a multiple comparison of means test (table 5).

Togo

The diallel analyses were carried out after fitting to the block effects based on the trial design (table 2). These analyses involved actual and potential production (Log transformed) and the pod rot rates per tree.

Table 2. Analysis of the triangular diallel in Togo.

Variables effects		*Log(yp)*	*Log(y)*	*prr*
GCA	F	4.27	5.23	2.73
	α (%)	(< 0.001)	(0.001)	(0.20)
SCA	Γ	1.71	1.73	1.01
	α (%)	(0.176)	(0.145)	(45.17)

GCA: general combining ability; SCA: specific combining ability.

As previously, the GCAs were preponderant, meaning that these characters were primarily transmitted additively. However, the effects relative to the reaction to black pod were less marked than in the Cameroon trial, probably because the observations were only carried out over one year.

The GCAs of the different parents could be estimated and compared by a multiple comparison of means test (table 4).

Ivory Coast

The analysis of the factorial mating design is shown in table 3. Production (in kilos of fresh beans) and the pod rot rate per tree were analysed in line with the factorial model.

Table 3. Analysis of the 16 ♀ x 4 ♂ factorial mating design (Ivory Coast).

Variables effects		*Log(yp)*	*yb*	*prr*
Female	F	5.86	8.15	13.57
	α (%)	(< 0.001)	(< 0.001)	(< 0.001)
Male	F	1.37	1.24	3.37
	α (%)	(28.40)	(29.35)	(1.82)
SCA	F	1.27	1.63	0.912
	α (%)	(13.59)	(1.18)	(62.13)

The female parent effects were the main variation factors, confirming primarily additive transmission of the characters considered.

Classification of the parents in the different countries

Based on the above analyses, a parent classification was established, based on the pod rot rate observed in their progeny. For the diallels, it involved a multiple comparison of the estimated GCA per parent. For the factorial design in Ivory Coast, independent classifications for the female and male parents are presented (table 4).

Table 4. Classification of the different parents for their susceptibility to *Phytophthora* spp. Newman and Keuls test (5 %).

	Cameroon (3 years) *P. megakarya*		Togo (1 year) *P. megakarya*		Ivory Coast (9 years) *P. palmivora*	
– susceptible						
			IFC 5	a	Pa 150	a
			SNK 64	ab	Sca 6	a
			T86/45	ab	P 7	a
			T85/799	ab	T85/799	b
			T60/887	ab	T60/887	bc
			Sca 6	ab	T79/416	bc
			ICS 100	ab	T79/501	bc
	UPA 134	a	UPA 134	abc	Pa 7	bcd
			ICS 40	abc	T63/967	bcde
			Na 32	abc	Na32	bcde
			UF 676	bc		
	IMC67	b	IMC 67	c	IMC 67	bcde
	SNK 413	b			T63/971	bcde
	ICS 84	b			T79/467	bcde
	ICS 95	c			Na 79	cde
	SNK 10	c			IMC 78	de
					Pa 35	e
+ susceptible						

The lower susceptibility to black pod rot due to parent UPA 134 was confirmed in Cameroon (Despréaux *et al.*, 1988). It should be noted that this classification, obtained from the GCA, only involved a single inversion compared

to that for the specific values of clones estimated in the clonal trial at the same experimental station (Berry and Cilas, 1994*a*). On the other hand, these classifications differed from those observed in pod inoculation tests (Blaha and Lotodé, 1976). The relative classifications of parents UPA 134 and IMC 67 were confirmed in the diallel trial in Togo. However, according to the Togo results, more worthwhile parents than UPA 134 should be used in Cameroon. The better performance of the Lower Amazons in Togo needs to be confirmed after several years of observations. The classifications obtained in Ivory Coast tallied with those obtained in Togo, though the pathogen species was different. The superiority of parents Sca 6, T85/799 and T60/887 over parents Na 32 and IMC 67 was confirmed. Narrow sense and broad sense heritabilities were estimated for the pod rot rate (table 5).

Table 5. Individual heritability values for pod rot rate (*prr*).

	Pathogen species	Number of years	h^2_n	h^2_b
Cameroon	*P. megakarya*	7	0.155	0.195
Togo	*P. megakarya*	1	0.061	0.061
Ivory Coast	*P. palmivora*	9	0.681	0.681

h^2_n: narrow sense heritability; h^2_b : broad sense heritability.

The broad sense heritabilities were identical to the narrow sense heritabilities in Togo and Ivory Coast, and of the same magnitude in Cameroon. Primarily additive type heritability therefore seemed to govern transmission of these characters, which confirmed earlier studies (Tan and Tan, 1990; Berry and Cilas, 1994*b*). The heritability values increased in line with the number of years taken into account. The precision of the individual values, hence the family values, were indeed better when observations covered a larger number of years. In that respect, the data for the design in Ivory Coast should be considered the most reliable, while further observations need to be carried out in Togo.

Phenotypic, genetic and environmental correlations were calculated for the potential production and rot rate variables. It was mostly a matter of assessing how the quantity of fruits produced in the trees affected disease expression in the field. These different correlations are shown in table 6. It should be remembered that the phenotypic correlations corresponded to correlations tree by tree, not taking into account the genetic structure of the study population. In the 3 designs, this population consisted of full-sib families connected to each other by half-sib relations. The genetic correlations corresponded to links between the trees that were explained by the family structure of the study populations. If the parents were homozygous, these genetic correlations would correspond to the correlations between the family means, and the environmental correlations would correspond to the correlations between the residuals, once the family effects were removed. The environmental correlations therefore corresponded to the correlations between trees, which could not be explained by kinship relations.

Table 6. Phenotypic, genetic and environmental correlations between pod load and pod rot rate (*Log(yp)* and *prr*).

	Phenotypic	Genetic	Environmental
Cameroon	0.397	0.339	0.407
Togo	0.041	- 0.278	0.296
Ivory Coast	0.090	- 0.236	0.449

The phenotypic correlations between the production and pod rot rate variables were not stable according to site. This correlation was maximum for Cameroon, where the pod rot rates were highest: in that country, high-yielding trees were in fact more severely attacked, irrespective of the family to which they belonged.

The genetic correlations were negative in Ivory Coast and Togo, meaning that the good parents for production were also good for the disease resistance trait. It may be that the severely attacked families bore fewer pods due to attacks that might have occurred on young fruits or flowers. This correlation was positive in Cameroon, indicating that under severe attack conditions it will be more difficult to select planting material that is both high yielding and resistant.

The environmental correlations between pod rot rate and potential production were systematically positive. This means that the highest-yielding trees in a given family also tended to be the most severely attacked. This correlation was doubtless due to secondary infections, from pod to pod, which increased in line with fruit density on the trees.

Index-based selection of individuals

The main aim of cocoa genetic improvement is to increase production per unit area planted. In addition, sustainable development of cocoa cultivation means providing planting material that is less susceptible to diseases, so as to reduce phytosanitary inputs. With this dual objective in mind, it is necessary for *Theobroma cacao* L. breeding programmes to take into account adversities that might affect production. Among these adversities, pod rot caused by various species of the genus *Phytophthora* is the disease that causes the greatest losses. With a view to disseminating high-yielding clonal material that is less susceptible to the disease, selection based on an index combining productivity and resistance to *Phytophthora* spp. has been undertaken in the experimental designs previously studied in Ivory Coast and Cameroon. The manner of constructing the index was to try different weights on the target traits and successively to introduce predictor traits in order to obtain the desired genetic progress on each target trait (Cotterill and Jackson, 1985; Cunningham *et al.*, 1970).

Ivory Coast

The three selection criteria adopted for this study were:

- production, measured as the weight of pods produced per tree over 9 years *(yb);*
- resistance to rot, assessed by the pod rot rate *(prr);*
- bulk, estimated by the canopy diameter at 10 years *(cd).*

Under plantation conditions, cocoa trees are generally subjected to competition from the age of 5 to 6 years. It is therefore necessary to limit the bulkiness of the trees so as to lessen competition at the current planting densities of 1,330 trees/ha. This is why we included a bulkiness aspect in the selection index.

This study therefore involves combined individual-family selection, for subsequent dissemination of clonal material. It is multi-trait selection based on an index combining the three target traits already mentioned. Predictor traits were added to these criteria to improve index precision; the predictor traits adopted were girth diameter increase between 1 and 2 years *(dg)* and trunk circumference at 11 years *(tc)*. This study used the (16 females x 4 males) factorial mating design used to evaluate the genetic parameters of resistance.

In the immature period, girth diameter increase between 1 and 2 years was calculated for each tree in the trial *(dg)*. In 1989, i.e. 11 years after planting, tree vigour and bulk were measured: trunk circumference *(tc)* and canopy diameter in a horizontal plane *(cd)*.

Genetic prediction coefficients were used to quantify the efficacy of indirect selection (Baradat *et al.,* 1995); for example, selection efficacy for girth diameter increase *(dg)* against production *(yb)* (table 7).

Table 7. Genetic prediction coefficients, and broad sense heritability on the diagonal.

	dg	*tc*	*cd*	*yb*	*prr*
dg	0.186				
tc	0.196	0.292			
cd	0.159	0.255	0.416		
yb	0.312	0.261	0.230	0.524	
prr	- 0.138	- 0.067	- 0.035	- 0.335	0.681

The genetic parameters previously estimated were used to construct a selection index with a view to selecting trees that perform well with regard to their dissemination in budding or cutting form. Total genetic values were used to identify high-yielding trees with low susceptibility to black pod rot, and with limited vegetative development comparable to the plot mean.

It was possible to test several ways of weighting the target traits. The choice of weighting system depended on the relative genetic gains desired for each of the traits and the quality of the index, estimated by r(h,i) (correlation between merit and the index). This correlation was a measurement of the stability of the calculated indexes.

First of all, we estimated the genetic gains obtained by performing the maximum genetic gain for each of the main two traits (*yb* and *prr*) (table 8).

Table 8. Relative genetic gains for selection based on rot rate and production, with different selection rates.

Trait	Weighting	10% rate	5% rate	1% rate
dg	0	15.69	18.45	23.83
tc	0	4.00	4.70	6.08
cd	0	6.62	8.01	10.35
yb	0	44.51	52.33	67.60
prr	- 1	- 69.73	- 81.97	- 105.89
				r(h,i) = 0.861
dg	0	25.60	32.44	41.91
tc	0	10.94	12.86	16.62
cd	0	23.61	27.76	35.86
yb	1	63.66	74.83	96.66
prr	0	- 48.76	- 57.32	- 74.05
				r(h,i) = 0.824

r(h,i): correlation between the calculated index and the merit (theoretical index, if the genetic values were known.

Selection with maximum genetic gain for *prr* involved substantial genetic gain, not only for the trait (- 105.89% for a 1% selection rate), but also for production, due to genetic correlation. This genetic progress corresponded to a reduction of more than half of the pod rot rates per tree.

Selection with maximum genetic gain for *yb* acted in the same way, obviously with a higher genetic gain for that trait. However, selection for production alone induced a substantial genetic gain for the vigour traits, notably the *surfa* variable, which represented the ground area projection of the canopy of each tree. To select trees to be planted at the same planting density, it was therefore necessary to limit genetic gain for that trait. In addition, it also appeared advisable to balance the genetic gains for the main two traits: *yb* and *prr*. After several simulations, the choice of weighting coefficients was fixed in accordance with those objectives (table 9).

Table 9. Relative genetic gains depending on the chosen weighting.

Traits	Weighting	10% rate	5% rate	1% rate
dg	0	22.67	26.65	34.42
tc	0	6.59	7.75	10.00
cd	- 1	5.99	7.04	9.09
yb	1	57.41	67.48	87.18
prr	- 20	- 57.88	- 68.03	- 87.89
				r(h,i) = 0.835

r(h,i): correlation between the calculated index and the merit (theoretical index, if the genetic values were known.

This selection index was therefore chosen to best meet the selection objectives previously mentioned. The normality of index distribution was checked. The list of trees selected with a 1% selection rate is given in table 10.

Table 10. List of index-selected trees at a 1% selection rate.

Female parent	Male parent	Row No.	Tree No.	Index
Sca 6	IFC 1	18	33	2.387
Sca 6	IFC 15	7	3	2.398
P 7	IFC 5	6	24	2.491
Sca 6	IFC 15	7	25	2.505
Sca 6	IFC 2	16	21	2.520
T79/501	IFC 5	18	6	2.588
P 7	IFC 5	3	32	2.593
Sca 6	IFC 2	13	4	2.598
Sca 6	IFC 2	15	39	2.598
Sca 6	IFC 5	8	31	2.767
Sca 6	IFC 2	13	2	2.803
Na 32	IFC 5	4	21	2.947
Sca 6	IFC 2	10	37	3.081
Sca 6	IFC 5	10	41	3.392
Pa 150	IFC 1	7	35	3.728

A selection index was therefore established from a factorial mating design in a process of combined individual-family selection based on total genetic values. It amounted to the selection of individuals for vegetative multiplication, which need, first of all, to be confirmed in a clonal trial. Substantial genetic progress can be expected for both the production trait and for less susceptibility to pod rot.

Cameroon

The purpose of this selection operation was to choose high-yielding planting material with resistance to pod rot, for propagation in cutting or budding form. It thus consisted in combined individual-family selection based on an index integrating total genetic effects (additive + dominance).

The two traits to be improved were therefore *y* (↗) and *ppr* (↘), *yp* being a trait that could be used as an associated predictor.

It was possible to test several ways of weighting the two target traits. The choice of weighting system depended on the relative genetic gains desired for each of the target traits and the quality of the index (estimated by r(h,i)). In fact, h, called merit, corresponded to the theoretical value of the index (i), if the genetic values were known and not estimated. The correlation r(h,i) was therefore a measurement of the stability of the calculated indexes. First of all, the genetic gains

obtained by performing maximum genetic gain for each of the main two traits (*y* and *prr*) were estimated without using any predictor (table 11).

Table 11. Relative genetic gains for selection based on production and on pod rot rate respectively.

Trait	Weighting	10% rate	5% rate	1% rate
y	1	27.42	32.11	41.48
prr	0	- 3.67	- 4.31	- 5.57
				r(h,i) = 0.541
y	0	3.77	4.43	5.72
prr	- 1	- 24.92	- 29.29	- 37.84
				r(h,i) = 0.692

Introducing the potential production trait as a predictor resulted in increased genetic gains and gave better correlations between merit and index (table 12).

Table 12. Relative genetic gains for selection based on production and on pod rot rate respectively, with the introduction of *yp* as a predictor.

Traits	Weighting	10% rate	5% rate	1% rate
y	1	27.43	32.24	41.65
yp	0	25.46	29.93	38.66
prr	0	- 4.46	- 5.25	- 6.78
				r(h,i) = 0.543
y	0	4.21	4.95	6.39
yp	0	- 9.46	- 11.12	- 14.36
prr	- 1	- 27.32	- 32.11	- 41.48
				r(h,i) = 0.758

The coefficients were then weighted to best meet the selection objectives fixed by the breeders (table 13).

Table 13. Relative genetic gains depending on the chosen weighting.

Traits	Weighting	10% rate	5% rate	1% rate
y	1	17.63	20.72	26.77
yp	0	5.39	6.33	8.18
prr	- 3	- 23.21	- 27.28	- 35.24
				r(h,i) = 0.697

This selection index gave worthwhile genetic gains for each of the target traits. The normality of index distribution was checked. The list of trees selected for a 1% selection rate is given in table 14.

Table 14. List of index-selected trees with a 1% selection rate.

Female parent	Male parent	Block No.	Tree No.	Index
SNK 413	UPA 134	1	140	2.338
UPA 134	SNK 413	6	1,606	2.371
UPA 134	IMC 67	4	943	2.380
UPA 134	SNK 413	3	377	2.397
IMC 67	UPA 134	2	726	2.467
UPA 134	SNK 413	3	693	2.541
SNK 413	UPA 134	4	887	2.594
UPA 134	IMC 67	3	347	2.667

Index quality, which was measured by the correlation between merit and the index, increased with the use and the number of predictors. This situation was not a generality, as introducing many traits often led to problems with the inversion of variance-covariance matrices between phenotypic predictors. Associated predictors need to be added gradually (Baradat *et al.,* 1995), until the best prediction of merit (H) by the index (I) is obtained.

The index adopted, using an associated predictor trait, suggests that a genetic gain of 26.77% can be expected for the total number of pods, and a genetic gain of -35.24% for the pod rot rate, with a 1% selection rate.

A selection index was therefore established from a diallel mating design in a process of combined individual-family selection based on total genetic values. It involved selecting individuals for vegetative propagation, which need to be confirmed in a clonal trial. Substantial genetic progress can be expected for both the production trait and for less susceptibility to *P. megakarya*.

The selected trees were multiplied by budding and a confirmation trial was set up at the IRAD Barombi-Kang station in Cameroon. These trees will also have to be multiplied by the somatic embryogenesis technique (Alemanno *et al.,* 1996), with a view to setting up multi-site trials in several countries.

Conclusion

The genetic study of cocoa tree resistance to rot diseases caused by *Phytophthora* in plots, in Cameroon, Togo and Ivory Coast, confirmed that trait transmission is primarily additive under natural infection conditions (Despréaux *et al.,* 1989; Tan and Tan, 1990). Indeed, analyses of the variance of these three experimental designs indicated that GCAs were preponderant, meaning that resistance trait transmission is mostly additive.

The parent classifications tallied between these three countries, despite different pathogen species. Consequently, the selection work carried out in Ivory

Coast for resistance to *P. palmivora* will be useful if ever that country is invaded by the species *P. megakarya*.

Trinitario parents are generally more susceptible to the disease. The long fruiting cycles of that species may have something to do with the poor field performance (Berry and Cilas, 1994*b*). Amelonado type Lower Amazon parents, and some Upper Amazons such as Sca 6, P 7, Pa 150 or T85/799 should help in the creation of less susceptible varieties, notably in Cameroon, where those parents have yet to be used.

The heritability of pod rot rates per tree increased in line with the number of years taken into account. Heritability of around 0.7 was obtained in the experimental design in Ivory Coast, which was observed over nine consecutive years. Substantial genetic progress can therefore be expected through selection targeting the *Phytophthora* resistance criterion. Genetic correlations between potential production and the pod rot rate were rather weak. It is therefore possible to proceed with combined selection for these two traits. Combined individual/family selection based on an index combining production and resistance to pod rot caused by *Phytophthora* spp. was therefore proposed in Ivory Coast and Cameroon, with a view to selecting from interesting families those individuals suitable for use as clones, or as parents for new crosses (Cilas *et al.*, 1995; Cilas *et al.*, 1999; NDoumbé *et al.*, 2001). This selection led to the identification of trees in each country, and a confirmation trial has been set up in Cameroon based on this selection. Grouping the different clones selected in Ivory Coast and Cameroon in the same clonal trials will enable a useful comparison to be made of the results obtained in these studies.

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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.

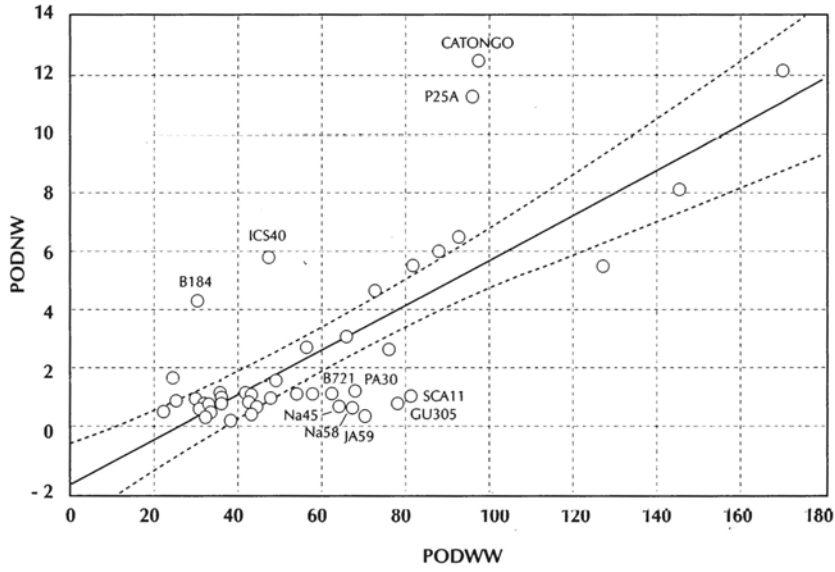


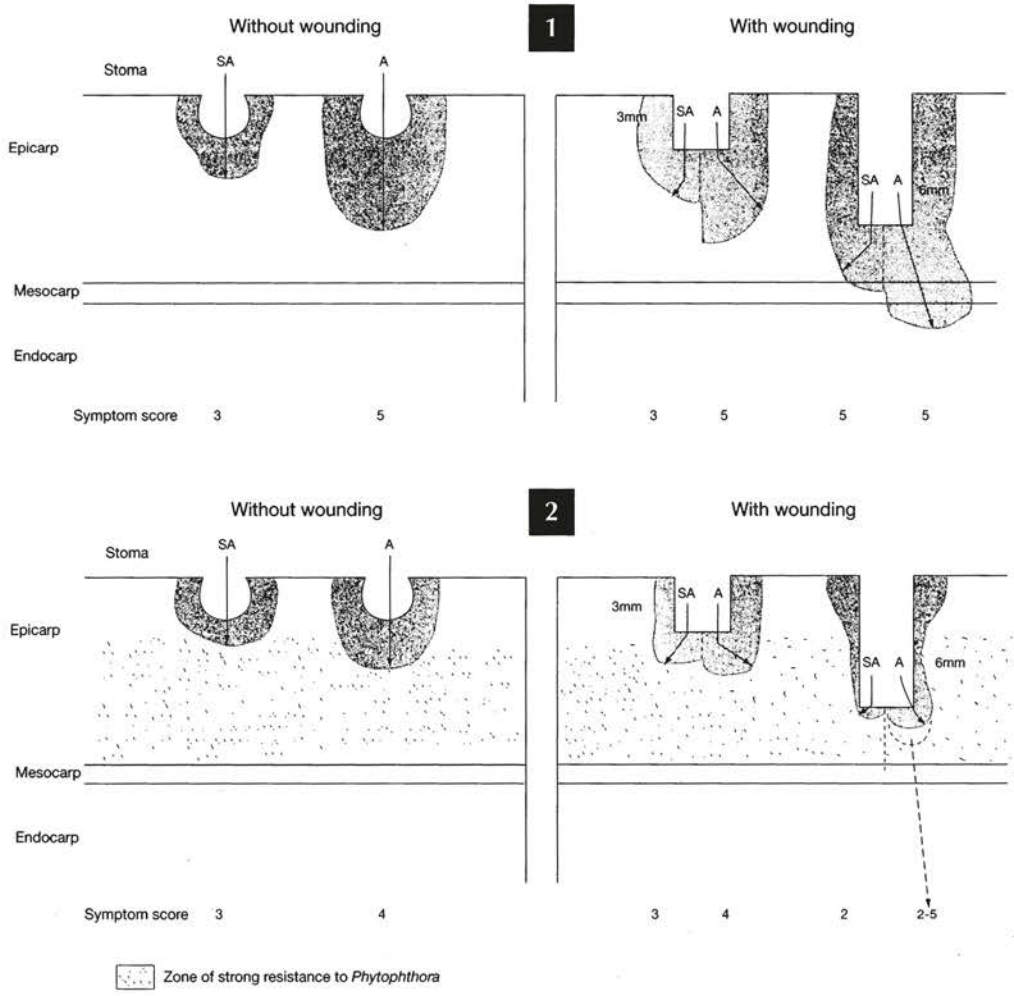
Figure 1. Comparison of areas (cm^2) necrotized by P. palmivora *on pods 6 days after inoculation with wounding (PODWW) and without wounding (PODNW).*

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



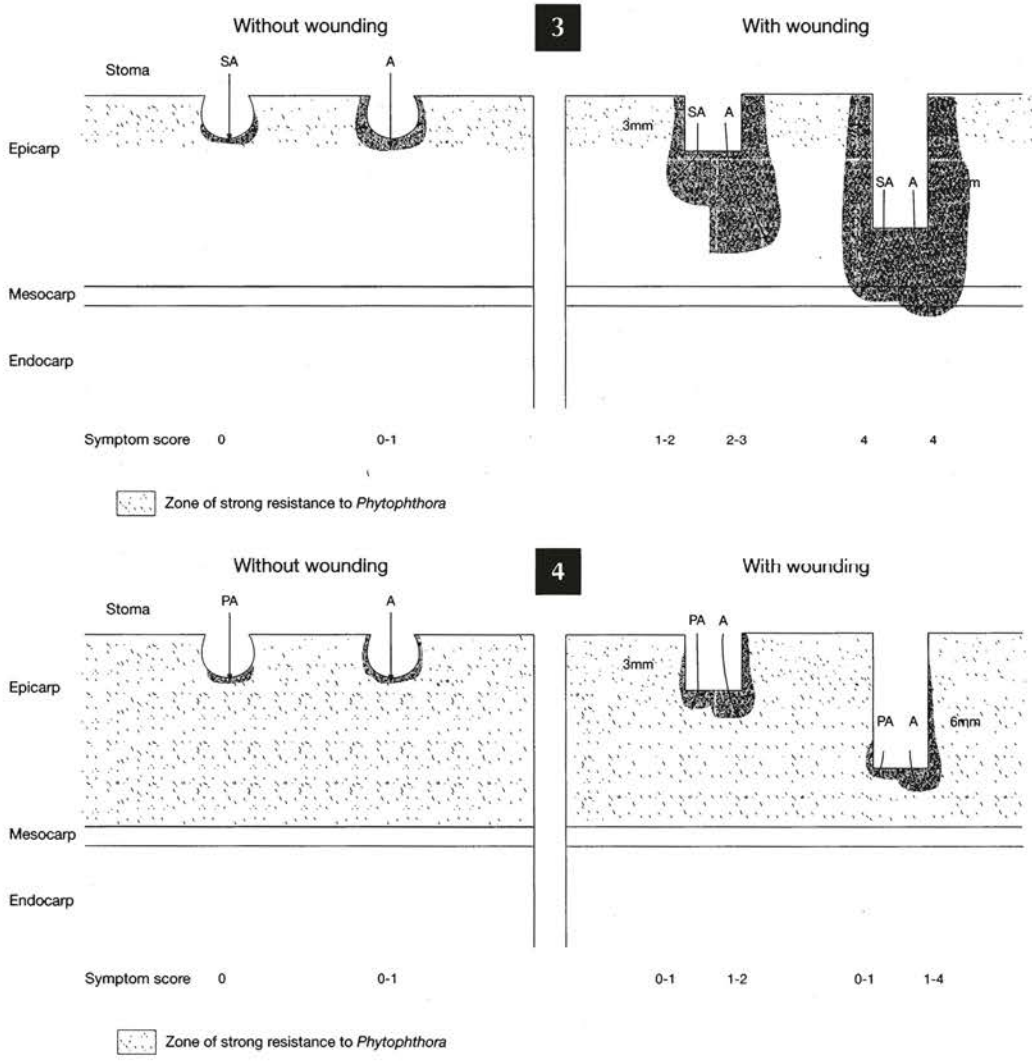


Figure 2. Symptom intensity (scale of 0 to 5) depending on the type of pod inoculation (with or without wounding) and on the aggressiveness of the Phytophthora *strains (SA: slightly aggressive; A: aggressive).*

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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Genetic mapping of quantitative trait loci for black pod resistance in cocoa

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Black pod on cocoa is caused by different *Phytophthora* species. *P. palmivora* (Butler, 1919) has a worldwide distribution and losses can be as high as 30%. Several other species have been described, namely *P. megakarya* (Brasier and Griffin, 1979), responsible for large losses in central Africa, *P. capsici* (Tsao and Alizadeh, 1988) and *P. citrophthora* (Babacauh, 1980). Like many other agronomic traits, resistance to *Phytophthora* exhibits a continuum of phenotypic variations in the species *T. cacao,* suggesting the implication of several genes. A polygenic control of resistance has already been suggested by several authors (Blaha and Lotodé, 1976; Enriquez and Salazar, 1980; Rodriguez *et al.*, 1985; Tan and Tan, 1990; Warren, 1994; Enriquez and Soria, 1996; Cilas *et al.*, 1996). Cilas *et al.* (1999) also showed that genetic factors involved in resistance in the field were additive.

Genome mapping studies were developed under the CAOBISCO project in order to specify the genetic bases of cocoa resistance to *Phytophthora*. Using this approach, it is possible to identify the several genetic components, or QTL, that

affect a complex quantitative trait. The QTL approach allows the calculation of the number of genetic factors involved in the resistance trait and the localization of them in the cocoa genome. It is also possible to estimate, for each one, the particular phenotypic variation it demonstrates, and to determine the parental origin of favourable alleles. The plant-pathogen interaction could be analysed in detail, along with the possible identification of genes involved successively in various stages of interaction, with respect to different strains or species of *Phytophthora*. Such an approach was widely used to study the resistance of plant species to various types of pathogens—virus, fungus, bacteria (Young, 1996).

QTL analyses allow the variability of resistance genes among several cocoa clones known for their good level of resistance to be determined, and the possibility of increasing the resistance level in cocoa trees by cumulating different resistance genes to be evaluated. Several types of resistance evaluation were carried out (artificial tests on leaves or pods, rot observations in the field), and the approach based on genome mapping made it possible to observe whether the same genes were involved in the different observations of resistance. In addition, other traits linked to production or to the biological cycle of the trees, were analysed in order to study their possible interactions with resistance traits.

To try to clarify the functional role of QTL, markers corresponding to genes with a known biological function were mapped and a co-segregation between these genes and the QTL was looked for. Such a "candidate genes" approach, based on known resistance or defence genes already isolated from other species, was developed in this project to try to understand resistance mechanisms and find selection markers located in the genes directly involved in resistance. This information is likely to facilitate the combining of the most useful QTL for cocoa resistance improvement using tightly linked markers for efficient marker-assisted selection. It could also be a starting point for map-based cloning of alleles at QTL of interest.

Progenies analysed and *Phytophthora* species involved

Several progenies located in Ivory Coast, Cameroon, Trinidad and France (Montpellier) were analysed to identify, in several clones, the regions of the genome (QTL) involved in quantitative resistance to black pod due to various species of *Phytophthora* (table 1). The progeny studied in Montpellier was used for a simultaneous study of resistance to three different *Phytophthora* species: *P. palmivora, P. megakarya, P. capsici.*

Resistance was evaluated either by the rot rate in the field or by artificial inoculation tests on pods or leaves taken from adult or nursery trees. The progenies in Ivory Coast and Cameroon were mainly used to study resistance in the field, plus

resistance evaluated by artificial inoculation tests (on pods or leaves from adult trees), whereas the progenies located in Trinidad and Montpellier were only used to study resistance evaluated by leaf tests on young nursery plants (table 1).

Table 1. Progenies analysed during the project, nature of resistance traits observed and species of *Phytophthora* involved in black pod disease.

Progenies	Country	Resistance traits observed			Species of *Phytophthora* involved in black pod
		Field rot rate	Leaf test	Pod test	
UPA 402 x UF 676	Ivory Coast	x	x		*P. palmivora*
T60/887 x Amelonado		x	x	x	
IMC78 x Catongo		x			
DR1 x Catongo		x			
S52 x Catongo		x			
IMC 57 x Catongo	Trinidad		x		*P. palmivora*
TSH1077 x Catongo					
ICS 84 x UPA 134	Cameroon	x	x	x	*P. megakarya*
SNK 10 x UPA 134		x	x		
IMC 67 x SNK 413		x	x		
(Sca 6 x H) x IFC 1	France				*P. palmivora* (2 strains)
			x		*P. megakarya* (2 strains)
					P. capsici (2 strains)

Markers used for mapping

A high-density reference map had already been established for cocoa by Risterucci *et al.* (2000), using the progeny UPA402 x UF676 located in Ivory Coast. UPA402 is an Upper Amazon Forastero and UF676 is a Trinitario. The map is mostly composed of RFLP and AFLP, along with a few RAPD and microsatellite markers.

Efficient molecular markers are needed to establish the maps and compare QTL locations easily among them. AFLP were used for rapid saturation of the maps. They require only a small amount of DNA, but they do not allow a comparison to be made between different maps when used alone. Microsatellite markers were developed to make these comparisons possible. Microsatellites are particularly useful for these purposes: they are PCR markers that require only a small amount of DNA, they are co-dominant, locus-specific, highly polymorphic, and offer repeatable results or analyses. A first set of microsatellites was produced by Lanaud *et al.* (1999*b*). The production of new microsatellites markers was carried out at CIRAD in collaboration with the French Centre National de Séquençage (CNS) and CAOBISCO.

Thirty-five new polymorphic microsatellites were isolated using two methods of enrichment: a protocol adapted from that by Edwards *et al.* (1996), and a modified protocol of the Streptavidin MagneSphere Paramagnetic Particles kit, Promega (Billotte *et al.*, 1999). Twenty-five newly produced microsatellites were mapped on the UPA402 x UF676 reference map. A total of 46 microsatellites produced by CIRAD and called mTcCIRx have been mapped. Nineteen microsatellites, named Cx or Cax, identified by D. Crouzillat of Nestlé, were also mapped. In all, 64 microsatellites have now been mapped on the reference map (Lanaud *et al.*, in press).

These markers were distributed on all the chromosomes. The map length remained stable at 887.3 cM. The new reference map now includes 468 markers: 64 microsatellites, 191 AFLP, 178 RFLP, 30 RAPD and 5 isozymes. Microsatellite markers will be easier to use for a marker-assisted selection made in tropical countries.

Mapping QTL for resistance to *Phytophthora palmivora* on progenies from Ivory Coast

In Ivory Coast, only *P. palmivora* was detected during our experiments, even though *P. megakarya* existed at the border between Ghana and Ivory Coast. *P. palmivora* causes between 10 and 25% pod losses (Kébé, 1994; Cilas *et al.*, 1999). Five progenies located in Ivory Coast were studied for their resistance to *P. palmivora* (Lanaud *et al.*, 1999*a*; Flament *et al.*, 2002; Clément *et al.*, 2003*a,b*). These were located in four different places: Bingerville, Zagné, Divo and Abengourou. Disease pressure is higher in Bingerville and Zagné than in Divo and Abengourou. One progeny was studied in both Zagné and Divo. QTL analyses for resistance traits were carried out on clones belonging to different genetic groups: Trinitario (UF676, DR1, S52) and Upper Amazon Forastero (UPA402, T60/887, IMC78). Traits for resistance, yield and morphology were studied in most of the progenies to test possible relationships among traits.

Several significant QTL of resistance evaluated by the percentage of rotten pods in the field were identified in the different parents: one QTL was located on chromosome 1 of UF676, another was located on chromosome 10 of T60/887 and another was located on chromosome 4 of IMC78. Other putative QTL with lower LOD score values were also identified and need to be confirmed—like those identified on chromosome 8 of T60/887, on chromosomes 1 and 9 of UPA402, on chromosome 4 of DR1, and on chromosome 10 of S52. However the putative QTL identified on chromosome 4 of DR1 was located in the same region as significant QTL related to that trait identified in IMC78, and the putative QTL identified on chromosome 1 of UPA402 was located in the same region as the significant QTL related to resistance identified in UF676. An improved trial design, adapted to QTL

analyses (larger number of individuals, reduced environmental effects) is required to increase the power of QTL detection.

In both progenies UPA402 x UF676 and T60/887 x Amelonado from Zagné, QTL of resistance evaluated by the leaf test on adult trees were identified on several chromosomes, but low repeatability was found between the different sets of experiments. Some QTL were nevertheless identified on the mean of the sets of experiments. These tests are very sensitive to environmental factors, which may prevent detection of significant effects, and which may also explain the lack of correlation observed between the different resistance evaluation methods used on these old trees: only one co-localization with a putative QTL related to resistance evaluated in field was observed in UPA402.

The identification of different regions involved in resistance to *P. palmivora* suggests that a pyramiding strategy of different resistance genes from various parents is possible to improve their level of resistance to *P. palmivora*. Marker-Assisted Selection would facilitate this accumulation of resistance genes.

The possible interaction with other morphological or agronomic traits of the trees was also studied in the five progenies from Ivory Coast. In IMC78 and DR1, a co-localization was found between the QTL related to field resistance and the QTL related to the mean pod weight; this QTL was also co-localized with a QTL related to vigour (trunk circumference) and yield in IMC78. In T60/887, studied in a progeny located in Zagné, for which vigour and yield traits were also observed, the bigger QTL for field resistance located on chromosome 10 of T60/887 was not co-localized with other QTL related to yield or vigour. However a putative QTL explaining 10% of the variation of the field resistance trait was located in the same region of 30 cM of chromosome 8 as a QTL involved in pod number and trunk circumference. In this same region, a QTL for mean pod weight was identified in T60/887, studying a progeny located in Divo. These co-localizations could correspond to direct interactions between the vigour and mean pod weight of the tree and its resistance, or could also correspond to a close linkage between different genes involved in these traits. A larger population is needed to confirm these results.

Progenies from Cameroon

Phytophthora megakarya, which is found only in Africa, is the most destructive *Phytophthora* species on cocoa. When no treatment is used, losses can be as high as 80% in Cameroon (Despréaux *et al.*, 1989; Berry and Cilas, 1994). Using an artificial inoculation test on fruits still attached to the tree, 100 clones were evaluated for resistance and classified (Blaha and Lotodé, 1976). Among these clones, six were chosen based on their different levels of susceptibility. Then a diallel trial was composed with the six clones used as parents. Despréaux *et al.* (1989) studied this trial and showed that genetic factors involved in the resistance

to *P. megakarya* were additive and could imply polygenic resistance. Three progenies of the diallel trial, involving five different cocoa clones, were studied to specify the genetic control of cocoa resistance to *P. megakarya* (Flament, 1998).

The diallel trial was put in place in 1979. After nearly 20 years in the plantation, these old trees were not always in good, general state, which could explain the large heterogeneity observed for all observations made on these progenies and the difficulty in having clear, significative, statistical results. For this reason, an adjustment with the Papadakis method (Papadakis, 1937) was chosen to analyse field data.

The results show that methods of resistance evaluation in the field, and fruit and leaf tests, are strongly affected by environmental conditions. Resistance expression in the field, as indicated by a variance analysis, showed the existence of a plot-and-year effect. Moreover the heritability of this trait was low (0.2). Also, no correlation was detected between artificial inoculation tests and the pod rot rate estimated in the field. However Nyassé (1995, 1997) showed that when the test was carried out on five clones, there was a correlation between the leaf resistance trait and the pod rot rate in the field. It is possible that a strong environmental effect might mask a real correlation that could not be detected as it involved a small number of individuals. It can also be imagined that different genes were involved in the resistance mechanisms evaluated by the three different methods and that each method could measure a different type of resistance trait.

In spite of these strong environmental effects, five QTL were detected for all traits and for different parental clones. As resistance trait distributions were not normal, QTL detected by interval mapping were also analysed by a non-parametric approach of the Kruskal and Wallis type. Three of the five QTL detected were detected by the two methods, but two were detected at a higher probability than the other. Indeed a QTL was detected for leaf resistance traits in UPA134 on chromosome 9 by interval mapping at a LOD of 4 and confirmed by Kruskal and Wallis at $P = 0.0005$. Moreover this QTL was also detected by variance analysis when the number of individuals was increased to 104. Another QTL was detected for fruit resistance traits in UPA134 on chromosome 2. This QTL was detected for two different sets of experiments, confirming its existence. The threshold was intentionally chosen low, since our number of individuals was small, to increase QTL detection, but increasing the risk of detecting a false QTL.

SNK413 is a very resistant clone in Cameroon. The lack of QTL identification in this clone could be due to strong environmental heterogeneity, but also to the possible homozygous status of resistance alleles in this clone, which could explain the absence of segregation of resistance alleles in the progeny and the high resistance of SNK413. No QTL was common to the three methods of resistance evaluation. Different genes could therefore be involved in the genetic control of resistance to *Phytophthora*, as suggested by the absence of correlation. However, other common QTL could exist without being detected because of the low trial power (few individuals) and the strong environmental impact.

Due to many off-types detected in the progenies, the low number of individuals per progeny (58 to 78) limited the power of QTL detection. Only a common parent (UPA134) present in two progenies allowed, after adjustment, the cumulation of data for both progenies and the confirmation of QTL detected by leaf test on chromosome 9. This fact revealed the difficulty in working on trees already planted. They were planted in 1974 with a different objective than QTL detection. Moreover, the trial was not really adapted, since the absence of tree replication is a disadvantage in QTL detection. These results are preliminary and need to be confirmed. Other factors could interact with resistance evaluation, such as the ripening period, which was not taken into account during this study. Indeed this factor is assumed to act as resistance by escape (Berry and Cilas, 1994), whereby trees produce their fruit before the zoospore production period.

Progeny from Trinidad

Among diseases affecting cocoa in Trinidad, black pod rot caused by *Phytophthora* is currently the main cause of production losses. The two species of *Phytophthora* existing in Trinidad are *P. palmivora* and *P. capsici*, the former being the more widespread and more aggressive in Trinidad (Iwaro *et al.*, 1998).

A progeny composed of 155 plants derived from the cross between IMC 57 (female parent) and Catongo (male parent) was studied (Motilal *et al.*, 2002). IMC57 is an Upper Amazon Forastero and was chosen for its resistance to *P. palmivora* (assessed by an artificial inoculation test on leaves) and its high heterozygosity rate. Catongo is a Lower Amazon Forastero and was chosen for its low level of resistance to *P. palmivora* (assessed by the leaf test). This study enabled us to detect QTL for resistance to *P. palmivora* in IMC57.

Resistance to *P. palmivora* was assessed in the nursery, using the leaf inoculation test developed by Thévenin and Motilal (1998). Each plant was subjected to four series of inoculations. This led to the identification of numerous significative QTL located on chromosomes 1, 2, 4, 6, 9, and 3 or 8. Each of the QTL accounts for between 6.5 and 10% of the total variation. In most cases, the QTL were identified on the means obtained from five series of inoculations, and they account for a very large part (almost 80%) of the variation for this trait.

The studied progeny was planted out in the field in July 2000. Its level of resistance to *P. palmivora* will be also tested by artificial inoculations on pods, in accordance with the protocol described in Iwaro *et al.* (2000), and then by estimating the percentage of rotten pods under natural infection conditions. The QTL obtained from these analyses will be compared to those obtained by the leaf inoculation test and presented in this work. This QTL analysis confirms that many genes are involved in cocoa resistance to *P. palmivora*.

Thus, a cumulation of several favorable alleles using a marker-assisted selection could permit the level of resistance of cocoa clones to be greatly improved.

Progeny studied in France

This study aimed to compare the genetic control of cocoa resistance to three different species of *Phytophthora*: *P. palmivora*, *P. megakarya* and *P. capsici* (Risterucci *et al.*, in press). It is important to know whether selection for resistance to one of the *Phytophthora* species could increase the level of resistance to the other species, and whether common genes of resistance usable as a priority for marker-assisted selection can be located on the genome. The study was conducted on 151 hybrid progenies from the cross (Sca6 x H) x IFC1, created in Ivory Coast and grown in a greenhouse in Montpellier. Using microsatellite markers, the clone H, initially unknown, was identified as a Trinitario clone close to the clone GS36. As not all *Phytophthora* species exist in a single growing zone, leaf tests were carried out in the greenhouse in Montpellier, where a large collection of different *Phytophthora* strains and species exists. *Phytophthora* resistance was screened by leaf test inoculation with two different strains per species.

Selection of the best individuals for resistance to *P. palmivora* at a 10% selection rate would lead to a genetic gain of 47% for *P. palmivora,* 21% for *P. megakarya* and 42% for *P. capsici*. A comparison of the 30 most resistant individuals selected for each species revealed that six trees on average were common, i.e. 20% of the resistant trees. This result confirms that there is little interaction between species and genotype and that selection for resistance to a single species (e.g. *P. palmivora*) would provide genetic gains for improving resistance to the other species. These results are in agreement with the QTL analysis. Indeed, some QTL for resistance to three and two species were detected in common regions. QTL were identified using composite interval mapping and located in six genomic regions. One of these QTL was detected on chromosome 5 with five strains from the three *Phytophthora* species. Another was detected on chromosome 6 with three strains of two species and one other QTL was detected on chromosome 1 for two strains of two different species. Three additional QTL were detected for only one strain of *Phytophthora* species. Each QTL accounted for between 8 and 12% of the phenotypic variation. For each strain, between 11.5 % and 29.6 % of the total phenotypic variation could be accounted for by the QTL identified.

For some QTL, the Sca6 or H (Trinitario) origin of favourable resistance alleles was determined by very close markers. Not all the favourable QTL were provided by Sca6 alone, but the Trinitario H involved also provided some favourable alleles. This was particularly the case for those of chromosomes 1 and 5 provided by the Trinitario; the favourable resistance alleles located on chromosomes 3 and 6 were provided by Sca6. Observation of the molecular banding patterns for Sca6 also showed that these favourable alleles were in the homozygous state in Sca6.

These results show that both specific and non-specific QTL were identified in both the Trinitario and the Forastero clones. This is particularly interesting in the present situation of progression of *P. megakarya* in Africa. Indeed, *P. megakarya* is responsible for the largest yield losses, and even though only *P. palmivora* is currently present in Ivory Coast, when selecting for resistance to *P. palmivora*, it should be possible to increase the resistance of clones to *P. megakarya*. It could be particularly worthwhile transferring these common QTL into an elite clone through a marker-assisted selection scheme.

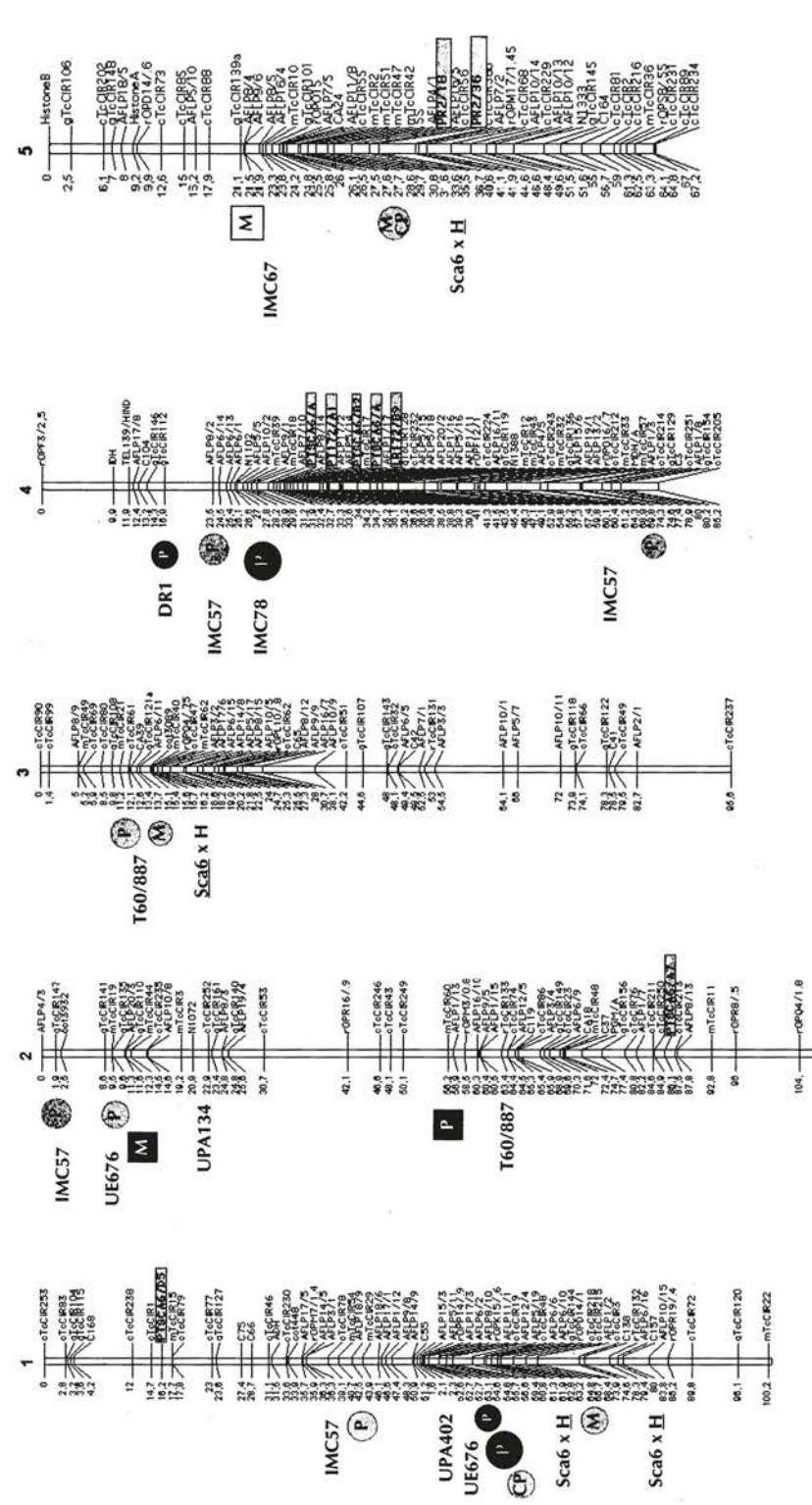
Characterization and genetic mapping of resistance and defence gene analogues in cocoa

Disease resistance and defence gene analogue (RGA/DGA) sequences were isolated in cocoa (Lanaud *et al.*, in press) using a polymerase chain reaction (PCR) approach with degenerate primers designed from conserved domains of plant resistance and defence genes: the NBS (nucleotide binding site) motif present in a number of resistance genes such as the tobacco *N*, sub-domains of plant serine/threonine kinases such as the *Pto* tomato gene and conserved domains of two defence gene families (pathogenesis-related proteins (PR) of classes 2 and 5). Nucleotide identity between 36 sequences isolated from cocoa and known resistance or defence genes varied from 58 to 80%. Amino acid sequences translated from corresponding coding sequences produced sequences without stop codons, except for one NBS-like sequence.

Most of the RGA could be mapped on the cocoa genome and three clusters of genes could be observed: NBS-like sequences clustered in two regions located on chromosomes 7 and 10; *Pto*-like sequences mapped in five genome regions of which one, located on chromosome 4, corresponded to a cluster of five different sequences; PR2-like sequences mapped in two regions located on chromosome 5 and 9 respectively.

Co-localizations observed between QTL related to *Phytophthora* resistance and candidate resistance and defence genes

Results of QTL and RGA/DGA mapping are reported in figure 1.


1
2
3
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IMC57
UE676
UPA134
T60/887
Sca6 x H
DR1
IMC78
UPA402
IMC67

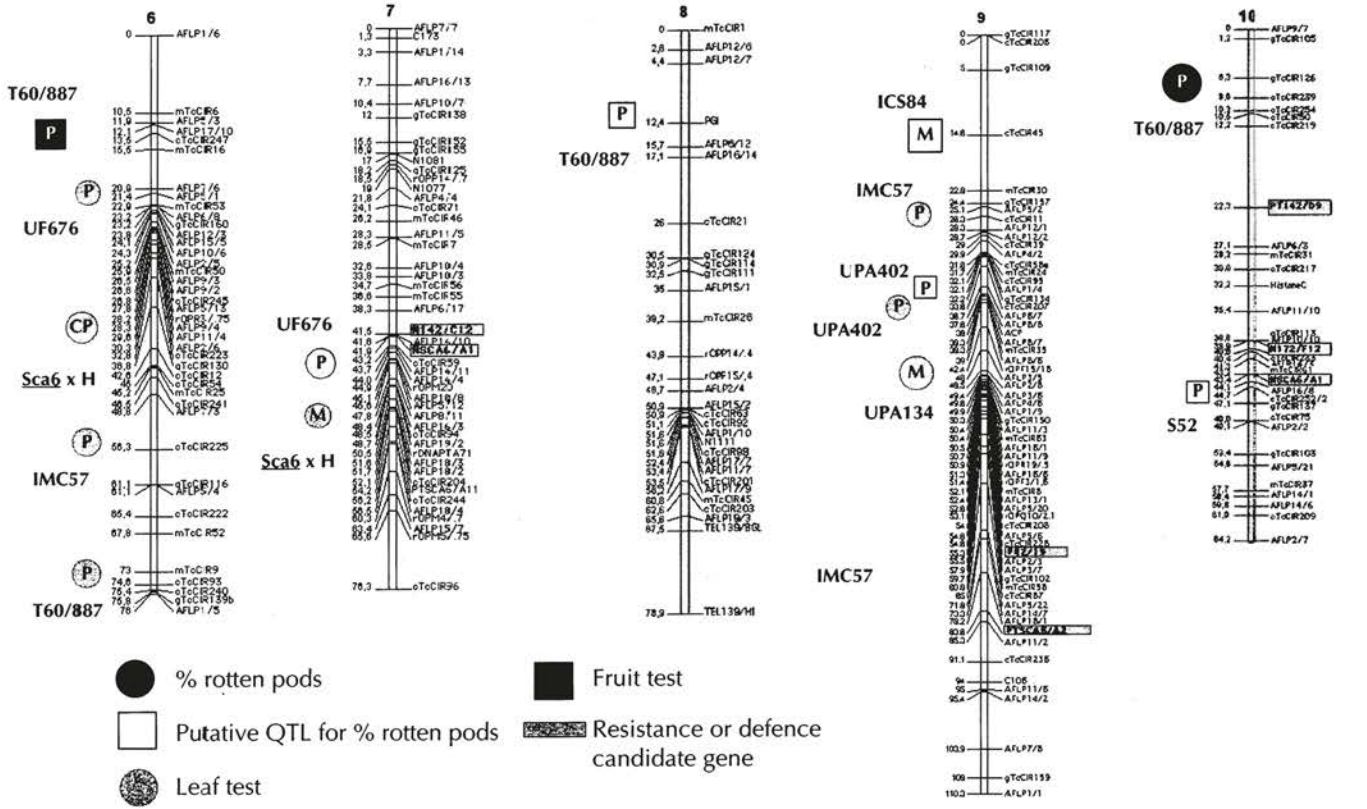

Figure 1. Mapping of QTL for resistance to P. palmivora *(P),* P. megakarya *(M) or* P. capsici *(C). The name of the clones where the QTL has been identified is mentioned near the QTL. In the case of the hybrid Sca6 x H, the parental origin of the favourable allele (Sca6 or H) is underlined.*

Co-localizations between QTL related to *Phytophthora* resistance

Several QTL related to resistance evaluated by the percentage of rotten pods were identified on chromosomes 1, 4, 5, 8, 9, and 10. Co-localizations between these QTL were found on chromosome 1 for UF676 and UPA402 (putative QTL), chromosome 4 for IMC78 and DR1 (putative QTL), and chromosome 9 for ICS84 and UPA402 (both putative QTL). These co-localizations were observed each time on clones belonging to different genetic groups and were not specific to one particular genetic group.

Co-localizations among QTL identified by the leaf test were also noted on chromosome 2 for IMC57 and UF676 (putative QTL), chromosome 3 for Sca6 and T60/887, chromosome 4 for IMC57 and UPA402 (putative QTL), chromosome 6 for Sca6 and IMC57 and chromosome 9 for IMC57, UPA402 and UPA134.

Some co-localizations were also observed between QTL related to resistance evaluated on different clones by the leaf test, pod test or by the percentage of rotten pods in the field, on chromosome 1, chromosome 2, chromosome 4 and chromosome 9.

Co-localizations between RGA, DGA and QTL regions for resistance to *Phytophthora*

Using common markers (particularly microsatellites) located in the several maps established, several co-localizations between RGA and DGA identified in this project and QTL for resistance could be observed.

This is the case for the cluster of RGA located in chromosome 4. In the same region of about 10 cM, four QTL for resistance to *P. palmivora* have been identified: one QTL explaining 13.2% of the variability of resistance to *P. palmivora* evaluated by a fruit test was identified in a Forastero clone (Pound 12) at LOD 3.4 by Crouzillat *et al.* (2000) while studying a progeny located in Costa Rica. Two other QTL of resistance to *P. palmivora* were identified in a Forastero clone (IMC78) and in a Trinitario clone (DR1) at LOD 7.4 and 2.5 respectively by Clément *et al.* (2003*b*) while studying progenies located in Ivory Coast. These QTL accounted for 22.6% and 10.1% respectively of the variability of the percentage of rotten pods observed in the field on cumulated data over a six-year harvesting period. Another QTL, located in this same chromosome region, and accounting for 8.1% of the variability of resistance evaluated by leaf test, was identified at LOD 2.2 by Motilal *et al.* (2002) in a Forastero clone (IMC57) by studying the progeny located at Trinidad.

Another region of 15 cM located in chromosome 5 gathers two DGA (PR2 analogues) and QTL for resistance evaluated by leaf test by Risterucci *et al.* (in press) toward three different species of *Phytophthora* (*P. palmivora*,

P. megakarya, P. capsici). These QTL were identified at LOD 2.9 to 3.9 and accounted for between 7.5 and 12.4% of the variability of the trait according to strains and *Phytophthora* species studied.

Another case of co-localization could be observed in chromosome 7 where 2 RGA containing NBS and two QTL for resistance evaluated by leaf test are co-localized, identified by Risterucci *et al.* (in press) in a Trinitario clone (H), and by Lanaud *et al.*, (1999a) in another Trinitario clone (UF676). These QTL were identified at LOD 3.1 and LOD 2.5 respectively, and accounted for 8% and 10% respectively of the variability of this resistance trait.

Discussion and general conclusion

Resistance to *Phytophthora,* which is already known to be partial, is a complex trait depending on several genes. The mapping approach was developed to acquire more precise knowledge of its genetic bases and to localize in the genome the regions (QTL) involved in resistance expression. This approach could also make it possible to compare the various sources of resistance, depending on the clones.

Several progenies involving clones belonging to different genetic groups were studied. Most of the cocoa clones recognized for their resistance have various levels of heterozygosity and QTL mapping studies have mainly been carried out using direct crosses already existing between those clones. Hence, only the resistance genes present with a heterozygous status could segregate in the progenies and be revealed from these direct crosses. The more resistant clones could have their resistant alleles in a homozygous status, and in that case direct crosses involving those clones are not appropriate for revealing their resistant alleles. This might explain why no QTL was identified in a very resistant clone, such as SNK413 in Cameroon, which was studied on a direct cross. The study of such resistant clones would require an examination of backcrosses or test cross models involving these clones. Scavina 6 is a very resistant clone with a high level of homozygosity. A test cross was produced with Scavina 6 during the project, and the study of the progeny (Sca6 x H) x IFC1 led to the identification in the genome of several QTL of resistance whose favourable alleles, originated from Sca6, are in a homozygous state in the Scavina 6 parent.

A large number of QTL related to resistance to *Phytophthora*, evaluated in the field or by artificial inoculations, were identified in the various progenies. Some chromosome regions appeared to be particularly involved, as on chromosomes 1, 4 and 9, where several co-localizations were observed. Other strong genetic effects were identified on chromosomes 5 and 10 for some clones studied. QTL for resistance to *P. palmivora* were also identified in the same regions of chro-

mosomes 1 and 4 by Crouzillat *et al.* (2000), studying by pod tests a progeny located in Costa Rica and resulting from a cross involving Pound12 and Catongo.

As a first step, existing trials, for which observations had been carried out for several years, were used for these analyses. However, the designs of these trials were not optimized for QTL analyses that require a large number of individuals analysed per progeny and plants observed under uniform conditions. While several significant QTL related to field resistance were identified, many other QTL appeared as putative QTL. New trials, with appropriate designs involving larger progenies, need to be planted for further QTL analyses.

Early artificial tests on leaves have been developed as an alternative method to evaluate the resistance of clones without needing to observe several years of harvests. The repeatability of leaf tests on leaves from adult trees in the field or on young trees in the nursery appeared to be very different. In the first case, low repeatability was observed between several sets of experiments carried out on the same plants at different periods, and the QTL were identified with low LOD score values. It was not the case for leaf tests on young nursery plants, for which high repeatability of genetic effects was observed between experiments associated with higher LOD score values. These differences could be due to several reasons: the larger number of plants studied in young progenies and also the stronger environmental effects on adult trees in the field, which might induce variations in leaf reactions after artificial inoculations.

This fact probably explains the lack of co-localisation between QTL related to field resistance and artificial inoculation tests on adult trees. For these reasons, the QTL studies based on data from leaf tests carried out on adult trees and reported here did not really permit the establishment of links between resistance evaluated by the percentage of rotten pods and the leaf tests; and they could not be used to test the predictive value of the leaf test to select resistant plants. In view of their greater stability, the leaf tests applied to young nursery plants seemed to be more promising and experiments were set up during the project in order to establish, on a genome level, the relationship between leaf tests applied to young plants and the percentage of rotten pods on adult trees.

Both specific and non-specific QTL for resistance to two or three different species of *Phytophthora* (*P. palmivora*, *P. megakarya* and *P. capsici*), were identified by leaf tests in the two genotypes belonging to two different genetic groups: Scavina 6, a Forastero clone and H, a Trinitario clone. These results are particularly interesting and, when selecting for resistance to *P. palmivora*, they should make it possible to increase the resistance of clones to *P. megakarya*, which is responsible for the largest yield losses in Africa, even though only *P. palmivora* is currently present in most of producing countries.

The search for candidate genes has been another approach developed under this project, to try to identify genes involved in cocoa resistance and provide markers for early screening of resistant plants among all the germplasm. Several cocoa DNA fragments homologous to resistance or defence genes have been isolated

and mapped, and some co-localizations between these loci and QTL for resistance have been observed. Due to the large number of QTL and candidate genes identified, some co-localizations could also be artefacts. However, three chromosome regions gathering RGA or DGA, with several QTL of resistance from different clones, could be of particular interest: one is located on chromosome 4, and gathers cocoa sequences homologous to the Pto R gene from tomato, to the rust resistance gene LRK10 from wheat, and to four QTL related to resistance evaluated in field or by leaf test. Another region of chromosome 7 gathers wo RGA and two QTL for resistance evaluated by leaf tests, and another region gathers defence PR2-related genes and QTL related to resistance to several species of *Phytophthora*.

However, other factors depending on the biological traits of the trees could also be important in resistance expression. Biological traits related to vigour and yield were studied on five progenies from Ivory Coast. Co-localizations were observed on chromosome 4 of IMC78 between QTL related to vigour and mean pod weight and a QTL of field resistance. Another co-localization was found on chromosome 8 of T60/887 between a QTL for pod number and a QTL for field resistance. Larger progenies would be necessary to determine whether it is a unique gene involved in several traits or different, closely linked genes.

The 10 progenies studied revealed the existence of various resistance genes. This situation is favourable for improving resistance in parents by possible pyramiding of different resistance genes using a marker-assisted selection strategy. Several QTL of resistance evaluated in the field or by the leaf test in some parents may be good candidates for marker-assisted selection. That is the case for the QTL of resistance to several species of *Phytophthora* identified in Sca6 and in the Trinitario clone H. Other QTL identified with strong genetic effects observed after leaf tests, or observations over several years of harvests, could also be good candidates for initial testing of marker-assisted selection. Such is the case, for example, for the QTL identified by leaf tests in IMC57 or for the QTL for field resistance identified in UF676, T60/887 or IMC78.

Improving clones for resistance cannot be carried out independently from the improvement of other important traits, such as yield or quality. The usefulness of a mapping approach involving the main traits of interest also includes the ability to identify the linkage between favourable and unfavourable alleles of each trait of interest, and to more effectively estimate and manage the number of plants needed to select worthwhile combinations. On chromosome 4 of IMC78, an association was found between favourable alleles for field resistance, vigour and yield, and selection of all that area of the genome will be interesting to carry out with Marker-Assisted Selection. In other areas, recombinations would be needed to recombine favourable alleles of several traits.

Marker-assisted selection could make it possible to optimize selection for resistance traits and to construct favourable associations in a controlled way using markers close to the QTL. Locus-specific markers, like microsatellites, are highly

polymorphic and easy to reveal by PCR, and will be particularly useful in this strategy.

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Conclusion

Christian Cilas

The project entitled "Genetic bases of cocoa tree resistance to *Phytophthora* diseases", which received financial backing from European chocolate makers through CAOBISCO, came to an end in September 2000. Working conditions and cooperation between the different partners were very good during the project, which was launched in June 1995. Before concluding this book, certain scientific and organizational aspects that helped to make the project a success should be highlighted.

Numerous scientific results were obtained in the different fields involved in the search for cocoa trees that are resistant to *Phytophthora*; these results have been published in various scientific journals and now in this book. This project led to major advances in the methodological problems of screening planting material for its reaction to *Phytophthora* diseases. An early screening test on leaves was developed and validated; this will enable faster, more reliable assessments for future selection of clones or hybrid combinations. Some work is still needed to validate this test for selecting individuals within full-sib families.

Based on this test, and the results from field trials, QTL (Quantitative Trait Loci) were identified on different parts of the cocoa tree genome. Detection of the zones of the genome involved in expression of the disease resistance trait will also facilitate the selection of more resistant planting material, notably by making it possible to associate different favourable QTL. In addition to the methodological

advances made during this project, clones and hybrid families were preselected for their good level of resistance to different strains of *Phytophthora*.

This planting material is now planted in the nursery or in the field and amounts to major capital for future research and projects, and forms the basis for establishing integrated control of this disease. In particular, new trials have been set up in Cameroon with a view to confirming the selections carried out, and carrying out crosses between the most worthwhile individuals. It should be noted that the selections came partly from the leaf test carried out on around 160 clones and 3,500 seedlings from 75 new crosses, and partly from a field assessment of more than 4,000 trees. Exchanging and utilizing this planting material are therefore priorities for giving substance to the results obtained in the different partner countries.

This project brought together several research organizations and strengthened existing cooperation links between the partners. The applied objective of this research project enabled a scientific community to share, exchange and pool methodologies, ideas and planting material over five years, in order to tackle the problem it faced in the best possible way. Throughout its duration, the project was monitored by a technical committee that met half-yearly in Montpellier. The committee was made up of representatives of CAOBISCO and of the scientific partners involved. Generally speaking, all the scientists working on the project, and present when the committee meeting was held, were invited to take part, as were eminent people from outside, whose views might prove valuable for furthering the research. The presentations and discussions were backed up each time by a report summing up activities for the previous period and proposing how to proceed thereafter. A mid-term seminar and a final debriefing seminar were organized. The major scientific advances achieved were largely due to the enterprise of the team of researchers involved, which now needs to be prolonged and maintained.

It is important to make optimum use of the achievements to ensure long-lasting cooperation between the partners and promote ongoing progress in controlling coconut pod rot caused by *Phytophthora*. The planting material bred in the different countries has been propagated to safeguard it. This material needs to be exchanged and tested in different environments to assess the stability of its resistance. New varieties derived from crosses between preselected clones should make it possible to cumulate the different favourable genes. QTL can be used to guide the choice of crosses to be carried out. It is a matter of identifying the different resistance mechanisms involved, possibly linked to the different QTL identified, so as to combine different resistance mechanisms in future crosses. The answers obtained during the project are bound to give rise to new research questions to which answers will have to be found, in order to continue making progress in searching for and understanding resistance, and in order to move towards more effective control of cocoa pod rot caused by various types of *Phytophthora*.

To conclude, a certain number of questions to which future research will need to provide answers are listed below:

❒ How does the behaviour of resistant plants change as the different *Phytophthora* evolve?

❒ What are the different resistance mechanisms involved in resistance to *Phytophthora*?

❒ Are some resistance mechanisms more stable over time than others?

❒ How can different resistance mechanisms be combined to guarantee durable protection of plantations?

❒ Are avoidance phenomena usable and sustainable?

❒ How do plantings with more resistant planting material need to be managed in the context of integrated disease control?

Answering these questions will be one of the objectives of future research and should lead to more effective disease control, hence to a more lucrative farming system for cocoa producers.

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Colour plates

I – Aspect of *Phytophthora* pod rot diseases

❑ Rot symptoms in the field

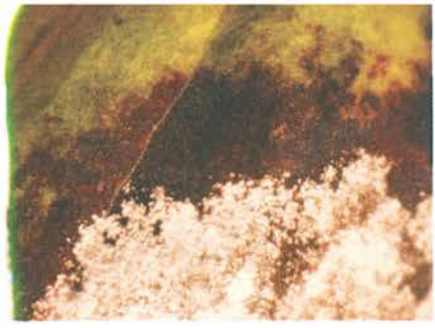
Phytophthora palmivora

Phytophthora megakarya

❑ Necrosis symptoms on pods

Phytophthora palmivora

Phytophthora megakarya



❑ Cross-sections of infected pods

Phytophthora palmivora

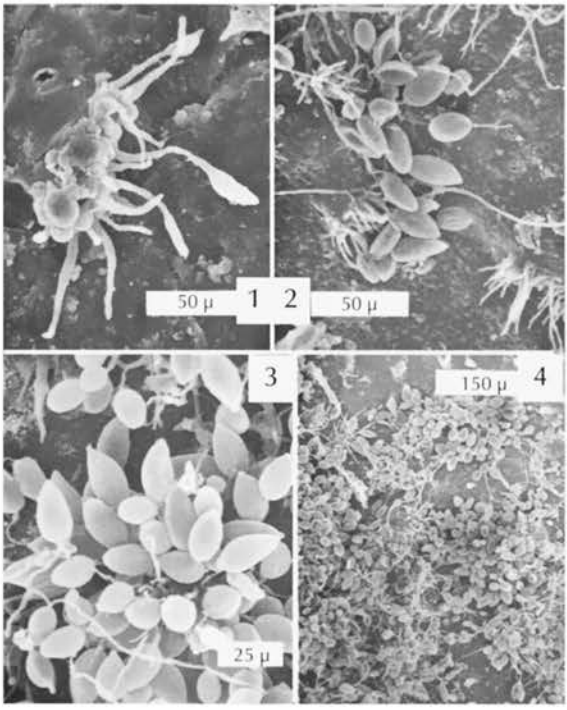
Phytophthora megakarya

II – Infected pod tissues under the scanning electron microscope

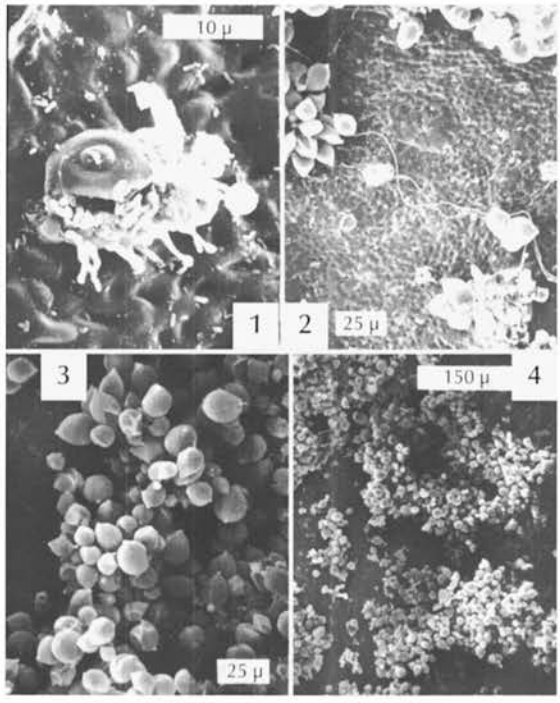
❑ *Phytophthora* sporulation dynamics on a pod under the scanning electron microscope

1. Appearance of spore-bearing filaments.
2. Sporangia masses.
3. Mature sporangia.
4. Proliferation of sporangia.

Phytophthora palmivora

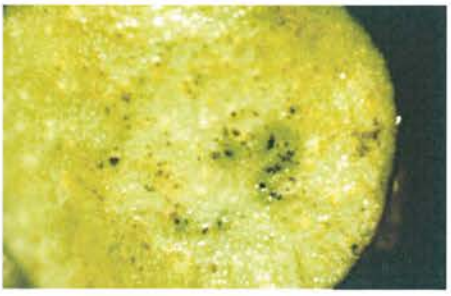


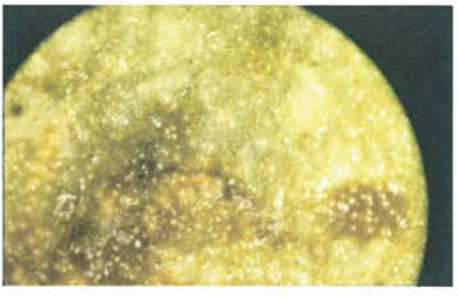
Phytophthora megakarya



III – *Phytophthora* susceptibility tests on pods

- Susceptibility test on pods *(Phytophthora palmivora)*.
- Symptom expression levels 1, 3 and 5 on susceptibility scale.

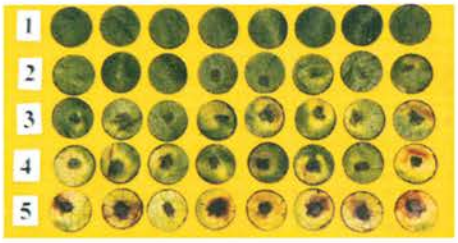




IV – *Phytophthora* susceptibility tests on leaves

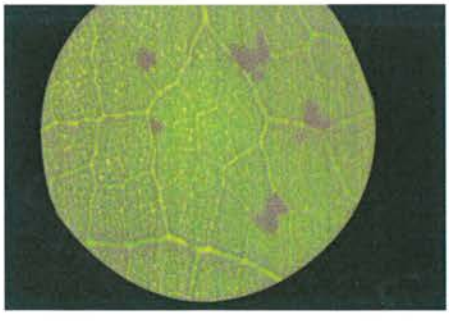
Preparing leaf discs with a mecanic cork borer.

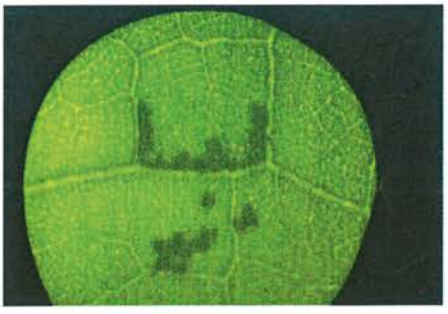
Leaf disc inoculations.

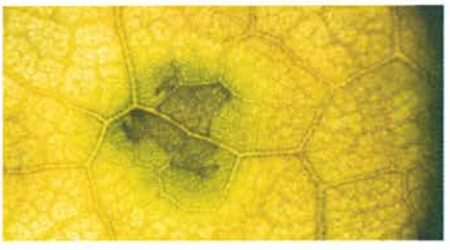


Symptom susceptibility scoring.

❑ Symptom expression levels 1 (left), 3 and 5 (right) on susceptibility scale.







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Cocoa black pod rot causes almost 30% losses in world cocoa production. This disease is caused by various species of the genus *Phytophthora*. The most harmful species, *P. megakarya*, is currently invading Ivory Coast, the leading producing country. Faced with this threat, teams of researchers from CIRAD in France, IRAD in Cameroon, CNRA in Ivory Coast and CRU in Trinidad came together in a research project on the genetic bases of cocoa tree resistance to *Phytophthora* diseases. This research received financial backing from European chocolate makers through the Caobisco association.

This overview presents the results obtained in work undertaken during the project. Its main purpose is to provide the international community with knowledge and tools that can be used to breed cocoa trees with greater resistance to *Phytophthora*.

Improvement of cocoa tree resistance to Phytophthora *diseases* covers pathogen diversity, epidemiological knowledge, the genetic parameters of resistance observed in the field, and practical breeding aspects. The relevance of different screening tests involving artificial inoculations and the use of molecular markers for the selection of resistant material are widely covered.

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