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THE USE OF MOLECULAR BIOLOGY TECHNIQUES IN A SEARCH FOR VARIETIES RESISTANT TO WITCHES' BROOM DISEASE OF COCOA

PROJECT COMPLETION REPORT (PCR)

Note by the Secretariat:

The attached document was prepared by CEPLAC in Brazil, the Project Executing Agency (PEA). The ICCO Secretariat edited the document, resulting in some shortening, and added an introductory section.

CFC/ICCO/CEPLAC Project on:

The Use of Molecular Biology Techniques in a Search for Varieties Resistant to Witches' Broom Disease of Cocoa

Prepared by CEPLAC

CFC/ICCO/CEPLAC PROJECT ON
"THE USE OF MOLECULAR BIOLOGY TECHNIQUES IN A SEARCH FOR VARIETIES RESISTANT TO WITCHES' BROOM DISEASE OF COCOA"

Project Completion Report

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1. INTRODUCTION

1.1 PROJECT TITLE:	The Use of Molecular Biology Techniques in a Search for Varieties Resistant to Witches' Broom Disease of Cocoa
1.2 PROJECT NUMBER:	CFC/ICCO/04
1.3 PROJECT EXECUTING AGENCY:	*Comissão Executiva do Plano da Lavoura Cacaueira Centro de Pesquisas do Cacau* (CEPLAC/CEPEC) Ilhéus, Brazil
PARTICIPATING INSTITUTES:	UENF, Campos, Brazil. INIAP, Ecuador. ICT, Tarapoto; UNAS, Tingo Maria, Perú.
1.4 SUPERVISORY BODY :	International Cocoa Organization
1.5 PROJECT DURATION :	April 2000 – September 2005

1.6 PROJECT FINANCING PLAN :

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Counterpart Contribution	
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1.7 DATE OF REPORT : December 2006

1.8 AUTHORS OF REPORT :

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1.9 ABSTRACT

1. Witches' Broom Disease of cocoa (WB) has been present in Latin and South America for centuries, where it was known to devastate cocoa plantations and led to their decline and eventual abandonment. Its threat, although apparent for all to see, was not taken seriously and no adequate measures were put in place to control it. In the late eighties, a devastating outbreak of the disease occurred in the State of Bahia in Brazil with extremely serious economic, social and environmental consequences.

2. Cocoa production in Brazil was slashed from 380,000 tonnes per annum to 90,000 tonnes in the late nineties. Within a decade, the country went from the world's second largest cocoa producer to becoming a net importer. Mass abandonment of cocoa plantations led to a sharp decline in the area planted under cocoa from 600,000 ha to 300,000 ha within 15 years.

3. From a social perspective, an estimated 30,000 farm workers lost their employment in the State of Bahia alone. The indirect effects are estimated to have affected another 250,000 people who were dependent on cocoa production and related activities.

4. Regarding the environmental impact of the disease, mass deforestation followed. Indigenous trees that had been used to shade cocoa plantations were cut down for sale as timber to compensate for the loss of income from cocoa growing. This had negative effects on the unique Atlantic Rain Forest, which is recognized as having one of the world's largest biodiversities.

5. Faced with the scale of the damage, the Brazilian Government, through its Commission for Cocoa Planning and Development (CEPLAC), formulated a project proposal on the Use of Molecular Biology Techniques to Develop Cocoa Varieties Resistant to Witches' Broom Disease. The proposal was endorsed by ICCO and financed mainly by the Common Fund for Commodities.

6. The project was implemented in Brazil, Ecuador and Peru between 2000 and 2005 and was very successful. As a direct result, about 20 new cocoa clonal varieties were released on a large scale to farmers. The new techniques developed in the project ensured that the planting material released was more accurately evaluated both at phenotypic level (production, disease symptoms, etc.) and at molecular level, thus decreasing the risk of failure under field conditions.

7. As a direct result of the project, cocoa production in Brazil improved, and today there are clearly visible signs that abandoned farms have been reactivated, leading to re-employment of farm and factory workers. This in turn is reflected by re-migration into rural areas and increased commercial activities in the rural centres. With increased income, remnant forest is not being further destroyed for revenue.

8. Over the five years of its implementation, the project achieved more than its initial objectives with several spin-offs including human capacity building. In total, twenty two (22) improved and resistant varieties were released by the project to farmers. More importantly, the procedures established to obtain resistant varieties to Witches' Broom Disease could easily be adapted for other diseases, thus opening a new way of investigation for cocoa pests and diseases. During the process, 1,300 plants were produced and transferred to the field, resulting from the cross of 10 plants carrying the QTL of resistance to Witches' Broom Disease. The nature of this multi-national project allowed for the joint evaluation of about 1,000 accessions for the benefit of the participating countries.

9. A highly saturated ***Genetic Linkage Map*** was developed for variety Sca-6 x ICS-1 and saturated maps were constructed for varieties CAB-208 x ICS-39 and CAB-214 x ICS-39. With the increase in knowledge on the new sources of resistance and considering the risk of fungal evolution and resistance "breakdown", it was considered important to map more genes for resistance. This risk factor was noted in the fungal pathogenic studies in the project and, therefore, three other populations were pre-selected to be mapped (ICS-39 x CAB-208, CAB-214 x ICS-39 and SIC-864 x CCN-51).

10. ***Molecular Markers*** related to genes resistant to Witches' Broom Disease were identified. A strong gene, controlling resistance to Witches' Broom Disease and markers associated with this gene were identified in Sca-6 x ICS-1. Three other genes associated with Witches' Broom Disease resistance were identified in the populations of ICS-39 x CAB-208 and ICS-39 x CAB-214. The markers associated to these genes and to that of Sca-6 x ICS-1 have a special use in producing varieties with durable resistance, through the accumulation of genes in a variety.

11. ***QTLs*** with important agronomic traits were identified. Three major QTLs associated with butter content and pod hardness were identified in Sca-6 x ICS-1. Markers associated with these QTLs were also

identified. QTLs were identified for all yield components (seed weight, number of seeds per pod) and pod traits (pod husk weight, pulp weight, pod size ad diameter) in Sca-6 x ICS-1. Three QTLs associated with resistance to black pod rot (*P. palmivora*) were identified in the crosses ICS-39 x CAB-208 and ICS-39 x CAB-214. One of these QTLs was found to be close to the QTL of resistance to Witches' Broom Disease.

12. Using ***Recurrent Selection***, about 1300 plants were produced and transferred to the field. The plants were the results of the cross of 10 plants carrying the QTL of resistance to Witches´ Broom Disease with three other sources of resistance to this disease or frosty pod rot. Thirty-one accessions of the germplasm collection were evaluated for the presence of the primer AV-14 (a marker associated with Witches' Broom Disease resistance). Analyses showed that AV-14 was quite frequent among other resistant accessions, including those apparently not related to *Scavina*. Sixty farmer selections were tested for the presence of markers associated to resistance (CIR-35, CIR-24). Some clones with high levels of resistance had those markers, suggesting that markers can be useful for selecting clones speeding up the process of evaluating clones to farmers. Twenty-seven crosses involving 23 parents, with different levels of resistance to Witches´ Broom Disease, Black Pod and yield were made and transferred to the field to be used in a marker assisted recurrent selection program.

13. ***Backcrossings in cocoa*** were conducted to advance backcross generations based on molecular and phenotypic data. A first generation of backcross was made between a source of resistance to Witches' Broom Disease (TSA-644) and a local, highly productive, but susceptible clone (SIC-19). Trees were selected and backcrossed in the populations TSA-644 x SIC-19 and TSH-1188 x TSA-644. Twelve individuals in the progeny TSH-1188 x TSA-644 and six on TSH-1188 x TSA-654 were selected for testing as clones. However, selection based on markers was not done.

14. On ***Germplasm evaluation,*** molecular data from 800 clones were collected using different types of markers, including SSRs and RAPD. Genetic diversity of the clones was assessed. It was found that genetic diversity existed among resistant germplasm, suggesting that they carry different genes of resistance. Some farmer selections were found to be genetically distant of the main source of resistance to Witches' Broom Disease, suggesting that some of them should be used more often to increase diversity in plantations. Diversity on more than 200 wild germplasm had shown that considerable diversity existed in the Amazon region. Some specific primers were developed for studies in cocoa and consequently, 123 new micro satellite (SSR) primers were developed for cocoa.

2. BACKGROUND AND CONTEXT OF THE PROJECT

2.1. SUMMARY OF PROJECT PROPOSAL

15. Conventional methods of cocoa breeding for obtaining varieties with desired traits have been standard practice since cultivation of cocoa became increasingly commercialized. With the global increase in cocoa consumption, cocoa trees from the Amazon Basin were introduced into other regions/countries suitable for its cultivation.

16. Conventional breeding, for a tree-crop like cocoa, calls for long-term enhancement programmes to achieve desired results. In conventional breeding, candidate trees in experiments have to reach a required stage of maturity to establish their full potential and this places a high demand on time, space and resources. Further, the very nature of this form of obtaining genetically improved material is based on phenotypic observations and intensive data collection. This method requires many years of investigations and in most cases, produces an end product with a questionable level of precision. Genes within the cell, which carry transferable attributes in genetic composition, remain unknown. Thus, material believed to be 'improved', may be short-lived and places at risk large investments in new or renewed plantations.

17. Conventional breeding on its own is not able to determine the existences of resistance with clarity in candidate materials, nor is it perfectly able to identify the source(s) of resistance to pests and diseases. It is such that plantations may be deemed to lose their short-term disease resistance mechanism or believe that a source of resistance is present, when it is truly not. In the case of the latter, not knowing the nature of the source of resistance may cause the trees to be vulnerable to attacks as a result of changes in the pathogen overcoming the trees' resistance mechanism.

18. The project was designed to apply cutting-edge technology at gene level to complement the conventional breeding to speed-up the process of cocoa breeding while also providing a high level of precision. In the Project Proposal, Witches' Broom Disease of cocoa was chosen as a case study, being the most pressing in Latin America. This disease had ruined some of the world's largest cocoa industries in producing countries of the region over its 100 years of existence. The project directed its studies on The Use of Molecular Biology Techniques in a search for Varieties Resistant to Witches' Broom Disease of Cocoa, with the objective to recover cocoa production in endemic countries and widen the knowledge on cocoa disease management.

19. However, since most of world cocoa production comes from countries other than the Americas, information resulting from this project would allow for a preventive strategy to a possible onslaught of Witches' Broom Disease in countries free of the disease, by applying techniques, protocols and a field operational strategy to transform farms with introduced clonal material resistant to Witches' Broom Disease.

2.2. OBJECTIVES AND EXPECTED OUTPUTS

2.2.1. Objectives Identified

20. The overall objective of the project was to apply molecular biology techniques in cocoa germplasm evaluation, more precisely using molecular markers at DNA level, to increase knowledge on the relationships among genotypes including studies on heterozygosity, pedigree, characterization of genes controlling the inheritance of economic traits and identification of genes controlling disease resistance.

21. The expected output would guide and strengthen the conventional breeding programmes, by ensuring a reduction of the time-frame in the breeding process and also ensuring a higher quality of enhanced genetic material generated for release to the farmers.

The ***specific project objectives*** were:

a. Construct saturated linkage maps in cocoa, using molecular markers (RAPD, AFLP and micro satellites).
b. Quantify variation in the fungal population.
c. Identify molecular markers closely linked to genes of resistance to Witches' Broom Disease.
d. Identify molecular markers closely linked to genes that control other traits of agronomic importance.
e. Obtain improved populations through recurrent selection assisted by molecular markers.
f. Advance backcross generations based on molecular and phenotypic data.
g. Evaluate germplasm collections in Brazil, Peru and Ecuador.

2.2.2. Project Components and Outputs

22. The project Components and Outputs related to the immediate project objectives were identified as follows:

Component A: Construction of a Genetic Linkage Map based on RFLPs and PCR based techniques (RAPD, AFLP and micro satellites)

23. Linkage maps are the arrangement of markers, with known positions, alongside the chromosomes of a given species. This component would establish the ideal population of selected genetic material to be mapped. The output of this component was to construct a saturated linkage map in cocoa, using molecular markers (RAPD, AFLP and micro satellites) that will help to identify gene location.

Component B: Identification and Characterization of Molecular Markers associated with Resistance to Witches' Broom Disease

24. Screening was to be conducted looking for probes or primer markers which could probably be linked to resistant genes to Witches' Broom Disease. The output of this component was to quantify variation in the fungal population and to identify molecular markers closely linked to genes of resistance to Witches' Broom Disease.

Component C: Identification of QTL, related to Agronomic Traits

25. A group of QTLs was expected to be identified for agronomic important traits. With the determination of the number of QTLs, the inheritance and the genome location, it would be possible to improve the breeding procedures. The objective of this component was to identify molecular markers closely linked to genes that control other traits of agronomic importance.

Component D: Use of Recurrent Selection to obtain Improved Populations

26. Identification of individuals with genes of interest using markers was to be undertaken. The project would make estimates of the genetic distance among parents used in the breeding process. The output of this component was to obtain improved populations through recurrent selection assisted by molecular markers.

Component E: Backcrossing in Cocoa

27. With a complete genetic linkage map and an identified genomic region determining qualitative traits, genomic regions would be introgressed from the donor parent to the recurrent parent by using two or three generations of backcrossing. The output of this component was to advance backcross generations based on molecular and phenotypic data.

Component F: Germplasm Evaluation

28. A finger print study in the germplasm collection would provide valuable information such as heterozygosity level, genetic distance among genotypes, heterotic group and pedigree. DNA from each genotype of the germplasm would be isolated. After obtaining the genotypic data, the analysis would indicate genetic distance, pedigree, homozygosity level and possibly a source of important genes. The output of this component was to evaluate germplasm collections in Brazil, Peru and Ecuador.

2.2.3. Milestones Identified

29. Identified milestones in project implementation were:

- Saturated maps were produced for varieties Sca-6 x ICS-1 and CAB-208 x ICS-39.
- Identification of different races of the fungus and their changes through space and time.
- Identification of markers associated to genes with resistance to Witches' Broom Disease.
- QTLs were identified for traits of agronomic importance such as butter content, hardness, yield component, high vigour and resistance to black pod rot.
- The nature of this multinational project allowed for joint evaluation of germplasm and exchange of elite material from approximately 1,000 accessions for mutual benefit.
- In total, 123 new micro satellites primers were developed for cocoa.
- Transfer of technology and training activities implemented.

2.3. BENEFICIARIES AND ESTIMATED BENEFITS

30. For many years, conventional breeding at research institutions in cocoa producing countries has been the main process to produce improved planting material. Cocoa breeding has been directed at all aspects of crop protection and crop production, to ensure that cocoa farmers are able to cultivate cocoa in an economic and sustainable way. However, with increased plantings over time, pests and diseases have escalated in severity and continue to progressively disseminate across borders at a more rapid pace. To overcome these problems, greater technological advances have to be achieved at fundamental level (basic research).

31. The main beneficiaries of the project are the cocoa farmers who would receive improved planting material as a result of the investigations. This material would provide greater sustainability in the production systems applied, thus increasing farmers' income. Other beneficiaries include the cocoa industry and through them, a stable supply of cocoa and its by-products to consumers. Globally, cocoa is one of the few crops grown in a forest environment. Thus, its continued existence in this environment would promote ecological preservation in harmony with economic and agronomic ventures.

32. While cocoa is not considered an essential 'food-crop', it is the source of income for millions of smallholders in the cocoa growing regions of Africa, Asia, Central and South America. Revenues derived from cocoa would result in benefits that could influence the living standards of people in these regions. In a recent estimate of financial losses due to Witches' Broom Disease in Bahia, the overall losses have been estimated at about US$10 billion since the disease was first registered in 1989.

2.4. PROJECT STRUCTURE AND FINANCING PLAN

2.4.1. Project Structure

33. Before the start of the project, a series of preparatory requirements had been put in place such as: adequately trained personnel in molecular biology and genetics, laboratories for molecular biology and plant pathology, germplasm collections, field experimental areas and green/screen houses.

2.4.2. Project Financing

34. The use of resources followed the provisions outlined in the Detailed Cost Tables. There were no operative/administrative obstacles in acquiring the necessary supplies that influenced the time for execution of project activities in Brazil, Ecuador or Peru.

35. The main financing of the project was received from the CFC. CEPLAC and UENF made contributions in cash as well as in kind. However, even before the start of the project (see 2.6.1. Project Structure) an investment of approximately US$1 million was made to establish a Molecular Biology Laboratory and a semi-automated ‘assembly-line’ inoculation system was installed for mass disease screening. Field populations in experimental areas for use in DNA studies and field screening of the disease were also established.

2.4.3. Co-ordination and Project Management

36. Management of the Project was through a General Co-ordinator, responsible for the overall efficient execution of operations and management at PEA level and in the participating institutes/countries. At the same time, the co-ordinator was responsible for constant communication between the PEA, ICCO and CFC.

37. Financial operations were supported by personnel from Foundation Pau Brazil, ensuring the funds were efficiently used according to the financial programme as well as establishing compatibility between the work plan and the financial plan. Parallel to this, careful management of finances was undertaken to accomplish even more than initially envisaged. This could be attributed to a real team effort and good project management.

38. The Technical Co-ordinator was responsible for planning all aspects of the scientific programme, guided by the objectives and pre-determined goals of the project. At a certain stage, weekly meetings were held by the PEA with the research teams involved to establish time frames for conclusion of activities and to determine intervals, re-evaluate progress achieved, implement adjustments required to attain programmed goals and to keep abreast with advances in this relatively new field. These activities were implemented by the PEA, as well as by every institution in the PPC.

39. The Author of the Project, at UENF, Campos, Rio de Janeiro, served in the capacity of a Project Consultant. This was also the case for the International Consultant. Ecuador and Peru had their own Country Co-ordinators who maintained constant communications with the General Co-ordinator and the Technical Co-ordinator.

3. PROJECT IMPLEMENTATION AND RESULTS ACHIEVED

3.1. PROJECT IMPLEMENTATION

3.1.1. *Modus Operandi* of Project Activities

Training

40. Since the Project applied a new approach in disease management of a tropical tree-crop, project operations were required to correct existing deficiencies in molecular biology techniques. With this common goal, training of personnel was conducted in two complementary areas:

a. Basic academic training (MSc and Ph.D. level) was undertaken at the *Universidade Estadual Norte Fluminense* (UENF) Campos, Rio de Janeiro, under the guidance of the author of the project and his team. Other universities included were: *Universidade Estadual de Santa Cruz* (UESC) and *Universidade Estadual de São Paulo* (USP) – ESALQ, Piracicaba, University of Guayaquil (Ecuador) and *Universidade Federal de Viçosa* (UFV). The number of research personnel who obtained higher degrees through the project was larger than initially envisaged in the project proposal.

b. Basic training courses on the use of molecular biology techniques were undertaken initially at UESC. Operational training continued in greater part at the Molecular Biology Laboratory at the Cocoa Research Centre in Bahia, Brazil. This laboratory was fully equipped and appropriately staffed even before the start of the project, thus able to serve the other PPC and local researchers right from the start of the project. In time, with technical support from the project co-ordinators, the participating countries established their own laboratories, and the project progressed effectively in Brazil, Peru, and Ecuador. In addition, a number of under-graduates and graduate students who worked in the laboratory proceeded to obtain higher degrees from the nearby UESC in molecular biology/pathology related subjects.

3.1.2. Project Monitoring and Supervision

41. The General Co-ordinator, Technical Co-ordinator and other project experts visited the PPC almost every year, ensuring that compatible advances were made. The International Consultant visited the PEA facilities during the early stage of the project and during the Mid-term Evaluation. Visits by personnel of CFC and ICCO were carried out on three occasions.

3.1.3. Resource Utilization

42. A grand total of US$725,334.33 was released by the CFC to the project in fourteen instalments throughout the five-year period of project implementation.

3.2. PROJECT RESULTS ACHIEVED

3.2.1. Qualitative Analysis of Results

43. The project achieved the following results:

- Precisely evaluated material for phenotypic data (production, disease symptoms, etc.) and also at molecular level (sources of resistance).

- Wider diversity in cocoa varieties in farms, thus providing greater stability to farmers.

- Studies in Brazil allowed for a better understanding of the population dynamics of *M. roreri* in Ecuador and Peru.

- A strong gene controlling resistance to Witches´ Broom Disease and markers associated to this gene was identified in Sca-6 x ICS-1.

- Results on other components of the project (high diversity and selection in the fungal population) convincingly showed that less effort should be applied in trying to introduce single genes through each backcrossing. A better approach would be recurrent selection involving different sources to accumulate many genes. Hence efforts were focused on this strategy and rather less on backcrossing.

Other qualitative results were divided into four areas described as follows:

Socio-economic:

- Abandoned farms reactivated.

- Farm labour was re-employed, registering a return towards original rural population levels.

- An increase in commercial activities in the urban areas.

- With increased income, remnant forest was not further felled to obtain emergency revenue, and forests were again used as shade for cloned cocoa.

- Investment in mixed cropping diversification programmes was re-initiated.

Perfection and development of molecular biology techniques in cocoa:

- Mapping genes and QTL identification.

- New markers were developed, including: SSRs for cocoa; SSRs for *Crinipellis* (and *Moniliophthora*).

- Elimination of the risk associated to plants of a single resistance source, before release to farmers.

- Farmer selections non-related to *Scavina* identified.

- Selections in *Scavina* descendant populations, assisted by markers.

Plant pathology:

- Better understanding of host/pathogen relationships

- Improved techniques and quicker screening processes for resistance.

Renewed interest in cocoa:

- Renewed confidence in cocoa cultivation was registered in all the cocoa producing countries – Ecuador, Peru and Brazil.

- In Brazil, an unprecedented support system was mounted by the CEPLAC Extension Services to establish clonal gardens on selected farms to supply farmers with millions of pieces of enhanced propagative material for grafting.

- A mass production facility was installed - Bio-factory (*Biofabrica)* with funds from the State Government for production of bud-wood for cloning and pre-grafted seedlings.

- Similar structures commenced, presently on a smaller scale, in Ecuador and Peru, however, with programmed expansion on the same lines as in Brazil.

- With a smaller structured tree, planting density on farms was also increased (about 600 to 1,100 trees/ha).

3.2.2. Quantitative Analysis of Results Compared to Planned Outputs

44. Besides the main population (Sca-6 x ICS-1) selected at the start of the project, three additional populations were pre-selected to be mapped. These were ICS-39 x CAB-208, CAB-214 x ICS-39 and SIC-864 x CCN-51. By increasing the population, the project safeguarded the risk of fungal evolution caused by a breakdown in the main (*Scavina*) source of resistance. New marker tools generated during the project were RAPD, SSR and RGH primers amplified (hybridized) in the population, generating about 700 markers in the main population and 300 in the other populations.

45. A highly saturated map was produced for Sca-6 x ICS-1 and in addition, saturated maps were produced for CAB-208 x ICS-39 and CAB-214 x ICS-39. The primer (RFLP), originally proposed, was substituted by four new primer types (RAPD, AFLP, SSR and RGH), in accordance with the updated technology available. A differential series of six clones was established during the project. These series allowed the identification of pathotypes of *C. perniciosa*, assisting breeders in selection of resistance to different strains of the fungus. Three populations of the fungus, with different pathogenicity were identified. Populations derived from drier climatic areas were found to be more aggressive.

46. The nature of this multinational project allowed for joint evaluation of germplasm and exchange of elite material from approximately 1,000 accessions for the mutual benefit of the PPC. In Peru, 600 selections were obtained from farmers' plantations. DNA analysis was undertaken in 500 isolates of *C. perniciosa* to obtain a better understanding of fungal population changes. The results from this analysis showed that a fungal population may change through space, (countries, regions, among trees within a farm and within a tree) and over time. Key results from these studies were:

- Most diversity of the fungus occurs in a single region and often in a single farm. Thus, varieties recommended to farmers have to be resistant to different strains of the fungus. The same applies to fungicides and biological control agents.

- Fungal populations change from country to country (at least in PPC), requiring quarantine measures to avoid the introduction of new strains of the fungus in countries were they do not exist.

- Sixty specific micro satellite (SSR) primers were developed for studies on populations of the fungus *C. perniciosa*. Some of these primers also proved useful for population studies of *Moniliophthora roreri*, (cause of frosty pod rot disease in cocoa, in Latin America).

- A collection of 1,200 isolates was registered and preserved as a permanent reference source on diversity in *Crinipellis perniciosa.*

- Three other genes associated to Witches' Broom Disease resistance were identified in the populations of ICS-39 x CAB-208 and ICS-39 x CAB-214. These may lead to varieties with durable resistance, through the accumulation of genes in a single variety.

47. As a standard procedure, phenotypic data were collected for yield components, resistance to other diseases and bean quality in populations aimed to identify genes associated to these traits. Apart from the development of planting material resistant to Witches' Broom Disease, characteristics of agronomic interest were incorporated into the research of new varieties. This was achieved by identifying Quality Trait Locis (QTL) for different characteristics. Three major QTLs associated with butter content and hardness were identified in Sca-6 x ICS-1. With these results, selection for butter content and hardness could be conducted at an early stage, when plants have their first leaves (2-3 months), not requiring years until plants produce pods. QTLs were identified for all yield components (seed weight, number of seeds per pod) and pod traits measured (pod husk weight, pulp weight, pod size and diameter) in Sca-6 x ICS-1, thus speeding up the process of developing more productive varieties for farmers. One QTL identified allowed early selection for lesser or higher vigour. Three QTLs associated with resistance to Black Pod Rot (*P. palmivora*) were found in the crosses ICS-39 x CAB-208 and ICS-39 x CAB-214. One of these QTLs is close to the QTL of resistance to Witches´ Broom Disease. This opened the way for possible resistance to two diseases.

48. The genetic distance among 90 clones used as parents of breeding populations was estimated. High genetic diversity was noted, even among clones with very high resistance to Witches' Broom Disease, suggesting the occurrence of different resistant genes available for pyramiding. Crosses among these clones were made and established in the field for advanced selection of clones to be released to farmers. About 1,300 plants were produced and transferred to the field, resulting from the cross of 10 plants carrying the QTL of resistance to Witches´ Broom Disease with three other sources of resistance to this disease or Frosty Pod Rot.

49. Thirty-one accessions of the germplasm collection were evaluated for the presence of the primer AV-14 (a marker associated to Witches' Broom Disease resistance). Analyses showed that AV-14 was quite frequent among other resistant accessions, including those not related to *Scavina*. Either they carry the same *Scavina* gene or the markers were not associated to resistance in those clones as found in *Scavina*. Sixty farmer selections were tested for the presence of markers associated to resistance (CIR-35, CIR-24). Some clones with a high level of resistance had these markers, suggesting that markers can be useful for selecting clones, speeding up the process of evaluating clones for farmers.

50. Twenty-seven crosses involving 23 parents totalling around 3,200 plants, with different levels of resistance to Witches´ Broom Disease, Black Pod and yield were produced and transferred to the field to be used in a marker assisted recurrent selection program,. A first generation of backcross was made between a source of resistance to Witches´ Broom Disease (TSA-644) and a local, highly productive, but susceptible clone (SIC-19). Around 120 trees of this backcross were produced and planted in the field for

field evaluation and marker assisted selection. Trees selected and backcrossed were made in populations TSA-644 x SIC-19 and TSH-1188 x TSA-644. Twelve individuals in the progeny TSH-1188 x TSA-644 and six on TSH-1188 x TSA-654 were tested as clones.

51. DNA of around 800 clones was isolated for genetic analysis. The clones were selected considering breeders' demands: choosing parents to make crosses, checking for mislabelling, assessing diversity in clones to be planted by farmers and assessing the diversity in some specific populations. Molecular data from the 800 clones were collected using different types of markers, including SSRs and RAPD.

52. The genetic diversity of around 800 clones was assessed, thus guiding breeders in decision-making with greater confidence. Diversity analysis became a routine strategy for breeding. Some key results of these analyses were:

- Genetic diversity existed among resistant germplasms, suggesting that those germplasms carried different genes of resistance. This was very important, not only in increasing the genetic base of the resistance, but also in pyramiding genes in varieties aiming at increased durability.

- Some farmer selections were genetically distant of the main source of resistance to Witches´ Broom Disease, suggesting some of them could be used more often to increase diversity in plantations.

- Experimental areas and breeding efforts could be saved by avoiding the use of genetically related germplasms in trials and crosses, when assisted by diversity analysis.

- Diversity on more than 200 wild germplasm showed that considerable diversity existed in the Amazon region and that this diversity is fairly regionalized.

- Among 200 plants selected on farms in Peru for high yield and resistance to Witches´ Broom Disease and Frosty Pod were genetically very diverse.

- Early results of the project determined the need for developing specific primers for studies in cocoa diversity. Therefore, 123 new micro satellite (SSR) primers were developed for cocoa. This was a very important contribution to the international community, since before this project, only 200 SSR primers were developed and made available by CIRAD. Hence the project was responsible for about a 30% increase in the SSR primers available for cocoa, worldwide.

53. Genetically enhanced material against Witches' Broom Disease, with identified source(s) of resistance released for large-scale use, and which continued to hold their resistance to the disease are:

- 1st. lot: earlier screened but worked-on in the project: nine (9) clones (TSH-516, TSH-565, TSH-1188, EET-397, CEPEC-42, TSH-774, TSH-654, TSH-656 and TSH-792);
- 2nd. lot: eleven (11) clones (CEPEC-2001, CEPEC-2002, CEPEC-2003, CEPEC-2004, CEPEC-2005, CEPEC-2006, CEPEC-2007, CEPEC-2008, CEPEC-2009, CEPEC-2010 and CEPEC-2011);
- 3rd. lot: eleven (11) clones CP-38, CP49, CP-53, PH-16, VB-276, VB-679, CNN-10, EET-392, CP-40, CP-39, and PS-13. A total of 31 clones; more then expected.

54. From a production of 380,000 tonnes before the outbreak of Witches' Broom Disease, production declined to 90,000 tonnes. The release of resistant material resulted in an increase in production to 144,000 tonnes. Full production is yet to be expected from resistant material due to the time lag to maturity. Farm productivity which was at its lowest at the start of the project, rose rapidly to between US$

600 and US$ 800/ha for farms with paid employees and share-croppers, respectively. At the end of the project, land value in farms with cloned cocoa rose to about US$1,600/ha as opposed to a decline in non-cloned cocoa to about US$200/ha. Employment demand in the cocoa producing region increased from about 20,000 tonnes to about 40,000 tonnes with projections of 80,000 tonnes and 115,000 tonnes in 2011 and 2014, respectively.

55. Over the project period, 1,000 selected farmers were trained to establish clonal gardens. As a result 5,500 farmers had established clonal gardens at the end of the project.

Training:

- Four PhD students (Ioná Santos, Jay Wallace, Ricardo Moreira, Paulo Albuquerque and Luis Cordeiro- parcial).
- Five MSc students (Ioná Santos, Karina Solis, Alfredo Dantas, Ronaldo Santos, Cássia Bahia).
- Three specialists.

3.3. DISSEMINATION OF PROJECT RESULTS

56. Due to the devastating effects of Witches' Broom Disease in the project participating countries (PPC), the urgent need to provide resistant planting material was the driving force from the very beginning of the project. This made it necessary to transfer improved material to growers, as soon as available, during the whole project period. One important element of project execution was the immediate feedback received, allowing for adjustments. The releases of material were accompanied with a number of agronomic practices initially passed on to the extension personnel and then, in turn, to the growers. The demand was such that the thirst for more information on grafting methods, the nature of resistance, planting systems and densities, pruning procedures for a compact structured tree and others still continues. The farmers recognized that this new approach to modernize cocoa cultivation within a renovation process was necessary for the survival of cocoa growers in these regions.

57. Dissemination of results on an international scale was achieved through presentations at important international scientific meetings – many of which were requested to be attended by project members as guest speakers.

4. CONCLUSIONS AND RECOMMENDATIONS

58. The impact of Witches' Broom Disease in Bahia was no different to the experiences encountered in other countries in South America, which occurred over 100 years. As before, knowledge of disease management was inadequate to reverse the situation. Despite a scaled eradication operation, the disease spread unchecked. Intensive research was needed to strengthen components in integrated disease management.

59. It was therefore seen as very ambitious to initiate a project with a research programme consisting of the use of molecular biology techniques to complement conventional breeding methods. This approach added a new dimension to breeding for disease resistance in cocoa, which not only accelerated the pace in which resistant material was made available to growers, but also ensured that sources of resistance could be precisely identified at DNA level. As an outcome of this strategy, cocoa genotypes with an inherent degree of resistance could be made available, leading to increased production levels. The introduced material was supplied to growers as cuttings (clones) or pre-grafted seedlings, increasing tree density from about 600 to 1,100 per ha.

60. The project opened up a new field in preventive cocoa breeding, thanks to the identification of molecular markers associated to genes of resistance. This approach helped countries to carry out the selection of resistant varieties even in the absence of the pathogen. The project also generated new technical and scientific expertise that is now readily available and transferable to other countries. The knowledge gained in this project can be applied in an early recovery programme for other equally devastating cocoa diseases. While more needs to be done to ensure durable resistance in some of the new cocoa varieties produced, the spin-offs of this project are available and can readily applied to other cocoa producing countries worldwide.

61. Finally, the institutional, organizational and logistic arrangements in the project which made it possible to renovate and modernize about 140,000 ha of cocoa farms, in a relatively short period for a tree crop, is another major achievement that deserves to be mentioned. Indeed, this project has enabled the successful implementation of an otherwise lengthy and difficult process, whereby positive outputs in molecular biology, genetics, pathology and agronomy were obtained, leading to results that were subsequently supported by mass production of planting material, training in methodology of cloning root-stock and establishment of clonal gardens over the whole region, all aimed at accelerating the process, with no significant restrictions or delays.

62. In an age of globalization where pests and diseases are easily transported from one region/country/continent to another, either knowingly or unknowingly, the experience of Witches' Broom Disease of cocoa serves as a perfect case study on how to tackle a disease outbreak.

63. In the words of the Project International Consultant, Dr Mark Guiltnan, the CFC/ICCO/CEPLAC Project on Witches' Broom Disease; "*turned a dream into reality*".

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