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Completion report on “The Use of Molecular Biology Techniques in Search for Varieties Resistant to Witches’ Broom Disease of Cocoa” project

CFC/ICCO/CEPLAC Project on:

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

Prepared by CEPLAC, August 2006

CFC/ICCO/CEPLAC Project on The Use of Molecular Biology Techniques in Search for Varieties Resistant to Witches' Broom Disease of Cocoa

Project Completion Report

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1.2	**PROJECT NUMBER:**	CFC/ICCO/04
1.3	**PEA:**	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	**SUPERVISOR BODY :**	International Cocoa Organization
1.5	**PROJECT DURATION :**	03/04/2000 (Starting Date) till 30/09/2005 (Completion Date)
1.6	**PROJECT FINANCING PLAN :**	

Total Cost of which:	US$ 3,191,824
CFC Grant	US$ 816,197
Co-Financing Contribution	US$ 800,100
Counterpart Contribution	
CEPLAC:	US$ 1,175,527
UENF:	US$ 400,000

1.7	**PERIOD COVERED :**	May 2005 – July 2006 (incl. authorised extension period)
1.8	**DATE OF REPORT :**	21st August 2006
1.9	**AUTHORS OF REPORT :**	Ph.D. João Louis Pereira — Project co-ordinator Ph.D. Uilson Vanderlei Lopes — Technical co-ordinator Ph.D. Messias Gonzaga Pereira — Author

1.10 EXTENDED ABSTRACT :

Introduction to the Completion Report

1. The first part of this Completion Report (Section 2) provides background information on the project objectives, structure, financing and beneficiaries. Sections 3 to 5 are based on the results of the final reports provided by CEPLAC and the Participating Institutes. An abstract of the Project Impact was produced after the Final Project Workshop held in Brazil during July 2005.

2. The original Completion Date of this five year project (30/03/2005) received an extension of six months by the CFC. This was justified to complete certain project activities and to organise and hold the Final Project Evaluation Workshop on July 2005.

Main Project Achievements

3. To deal with serious disease pressure, shown to cause the ruin of cocoa industries in the South American continent, the project had to be ambitious in seeking to apply molecular biology

techniques in search for varieties resistant to witches' broom in three countries. Although the independent evaluators at the early proposal stage of the Project were not fully convinced that applying cutting-edge technology would attain results, an initial bias approach to the use of *basic research* in combination with academic studies was necessary to venture into a new field of disease management in a tropical tree crop. However, not for a moment, over the five years duration did the project loose track that basic research was a tool to applied research to obtain a supply of improved techniques in support of conventional breeding to formulate recommendations – goals of the project.

4. The aim of the project was a search for varieties resistant to witches' broom disease through the use of molecular marker techniques. Project execution in activities developed exceeded requirements as determined in the project proposal. There was an extremely urgent need to secure what was left from abandoned cocoa in Brazil, which at the start of the project was about 50% of the total cultivated area of 600,000 ha. Witches' broom does not kill the cocoa tree but after several years of crop loss and weakening of trees, non-productive farms were abandoned and trees uprooted to be replaced with pasture or other profitable crops. With a loss of existing cocoa trees it would be impossible to graft improved material on existing rootstock, gaining time in reaching production.

5. Before the project commenced some varieties that appeared promising were released. However, during the project life, considerable more material was released that was not only evaluated for phenotypic qualities but also at molecular level (projects purpose) resulting in more reliable recommendations to farmers. The numbers of varieties released doubled and were precisely evaluated for phenotypic data (production, disease symptoms, etc.) and also at molecular level (sources of resistance). Also, an important project impact was the increase varietal diversity in farms thus, providing stability to the farmer.

6. Brazil experienced a contemporary witches' broom disease outbreak; therefore, compared to Ecuador and Peru, more data was available to assess the project impact over periods until the culmination of the project. From a total production value of 380,000 tons before the outbreak of witches' broom, productions drop to 90,000 tons. However, as a project impact the release of resistant material resulted in an increase in cocoa production to 144,000 tons. Considering that renovation of farms with resistant material was undertaken by cloning on the existing rootstock of old trees and by seedlings (pre-cloned in the nursery and then transferred to the field) there was an expected time lag to attain full production. Yet, the results obtained from 2000 onwards demonstrate a yield increase in trees only 3-5 years old and therefore, will reach full potential production at 7 years of age.

2. BACKGROUND AND CONTEXT OF THE PROJECT

2.1. SUMMARY OF PROJEC PROPOSAL

7. Conventional methods of cocoa breeding aimed to obtain varieties with desired traits had early origins, probably since cultivation of cocoa became increasing commercialised. With global increase in cocoa consumption, trees from the Amazon Basin were introduced into other regions/countries suitable for its cultivation. Selected material from the existing wealth of cocoa germplasm in the PPC, although diverse, required to be subject to a process of breeding, over an extended period of time to ensure the presence of traits necessary for economic cultivation, adaptation to specific climatic conditions, inherent presence of protection mechanisms to pests and diseases and potentially high yielding while meeting the demand in quality factors required by the cocoa industry (bean size, butter fat etc.).

8. Conventional breeding, for a tree-crop like cocoa, calls for long-term enhancement programs to achieve desired needs. To obtain results candidate trees in experiments had to reach a required stage of maturity to record their full potential; this puts 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. Both methods not only require many years of investigations but also produce, as final result, a questionable level of precision, when the depth in study observes the tree, as a whole. Genes within the cell, which carry transferable attributes in genetic composition, remain unknown. Thus, material believed to be 'improved', may be short-term and puts at risk large investments in new or renewed plantations.

9. Pests and diseases, presently a limiting factor to world cocoa production, is exemplified in one case were conventional breeding on its own is not able to determine the existences of resistance with clarity in candidate material, nor identify the source(s) of resistance. Plantations may be deemed to lose their short-term disease resistance mechanism or believe that a source of resistance is present, when not. In the case of the latter, not knowing the nature in source of resistance may cause the trees to be vulnerable to attack, known to occur, resulting from changes in the pathogen and overcoming the trees resistance mechanism.

10. The proposed project was designed to apply cutting-edge technology at gene level in support to conventional breeding thus, considerably hastening 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 largest problem in Latin America. This disease caused the ruin of some of the world's largest cocoa industries in producing countries of the region, over its 100 years of existence. The project aimed efforts in The Use of Molecular Biology Techniques in Search for Varieties Resistant to Witches' Broom Disease of Cocoa, with the objective to recover cocoa production in endemic countries and widen knowledge on cocoa disease management.

11. However, since the greatest part of world production comes from countries other than the Americas, information from this project would allow for a preparatory/preventive strategy to a possible onslaught of witches' broom in countries free of the disease, applying techniques, protocols and a field operational strategy to transform farms with introduced clonal material resistant to witches' broom.

2.2. COMMODITY ISSUES ADDRESSED BY THE PROJECT

12. Cocoa is a commodity cultivated in the humid tropic, mainly by farmers of smallholdings that depend on the revenue derived to meet their every-day needs, including food. Therefore, the greater part of the world's cocoa farming community is commodity dependent. Farmers of smallholdings require to apply inputs routinely, more so, in management of pests and diseases. These repeated inputs involve a high cost, often incompatible to their scale of operation. Also, this group of farmers, due to their shear numbers, call for an efficient technology transfer mechanism to reach all, which has to combine with an easily adaptable technology.

13. Therefore, one purpose that the project addressed was the reduction of the inputs the farmers need to apply in managing pests and diseases by making available clonal material developed on by research institutes and then passed on for cloning on existing cocoa root-stock. As a project, the case study on witches' broom of cocoa, concentrated on supplying growers with improved genetic material, employing molecular biology techniques, with an intrinsic high selective quality, and made available in a considerably reduced period of time. Since the disease was one that posed a challenge for many decades with limited success, including recently in 1989, reducing Brazil's status from the world's 2nd largest producer to importer, there is reason to believe that the same techniques can be applied in other countries that may experience high disease pressure.

2.3. ICCO STRATEGY

14. The International Cocoa Organization, in its role of international commodity body, is responsible for the well being of cocoa farmers, reflected in a sustainable production of cocoa. The International Cocoa Agreement 2001, which came into force on October 2003, explicitly requires the Organization to encourage and promote scientific research and development in the areas of cocoa production, transportation, processing and consumption as well as the dissemination and practical application of the results obtained in the field. The ICCO identified among one of its priority areas the implementation of sustainable cocoa practices, supporting any initiative from one or more Members to develop project to reduce the losses from one or more pests or pathogens, possibly at a regional level. This would involve, among other things, the development of scientific techniques to produce cocoa varieties resistant to pests and diseases to assure a stable production among regions.

15. The ICCO global strategy, envisaged under the concept of sustainable cocoa economy, is focused in giving due consideration to the sustainable management of cocoa resources in order to provide fair economic returns to all stakeholders in the cocoa economy, bearing in mind the principles and objectives of a sustainable development.

2.4. OBJECTIVES AND EXPECTED OUTPUTS

2.4.1. Objectives Identified

16. The overall objective of the project was to apply molecular biology techniques in cocoa germplasm evaluation, more precisely as 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.

17. The expected output would guide and strengthen the conventional breeding programmes, by ensuring a shortening of the standard long-term breeding process while also ensuring a higher quality of enhanced genetic material generated in the process, for release to farmers.

The ***specific project objectives*** are:

a. Construct saturated linkage maps in cocoa, using molecular markers (RAPD, AFLP and microsatellites).
b. Quantify variation in the fungal population.
c. Identify molecular markers closely linked to genes of resistance to witches' broom.
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. To advance backcross generations based on molecular and phenotypic data.
g. Evaluate germplasm collections in Brazil, Peru and Ecuador.

2.4.2. Project Components and Outputs

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

Component 1: Construction of a Genetic Linkage Map based on RFLPs and PCR based techniques (RAPD, AFLP and microsatellites)

19. Linkage maps are the arrangement of markers, with known positions, alongside the chromosomes of a given species. These maps are very useful when associated to phenotypic data, allowing the selection of varieties combining different characteristics of interest as well as to understand the genetic profile of these characteristics. Linkage maps have been established for many species. The objective of this component is to construct a saturated linkage maps in cocoa, using molecular markers (RAPD, AFLP and microsatellites)

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

20. Witches' broom ranks as the most devastating disease of cocoa in Latin America, and has a potential of spreading globally, threatening the economies of major cocoa producing countries. Selection of varieties resistant to witches' broom are not easily obtained by conventional methods, because the effect of environment on the expression of this resistance. Equally important, is the very nature of long breeding cycle of cocoa – a tree crop. When markers associated to genes of resistance are found, the process of breeding for resistance is accelerated, assisted by selections done in the early stages of plant development, at the DNA level, minimizing the environmental effects. Further, it is essential that good knowledge on the diversity the fungal population is generated and made available. This would allow the development of varieties resistant to witches' broom and also ensure implementation of strategies to increase durable resistance. The objective of this component is to quantify variation in the fungal population and to identify molecular markers closely linked to genes of resistance to witches' broom

Component C: Identification of QTL, related to Agronomic Traits

21. The continued cultivation of cocoa is dependent on economic returns to the farmer, aggravated by severe disease pressure. Therefore, it is essential that, besides the planted material being resistant to witches' broom, farmers receive varieties that also have incorporated improved characteristics of agronomic interest, such as high yielding and high bean quality, among other traits. The objective of this component is to identify molecular markers closely linked to genes that control other trait of agronomic importance.

Component D: Use of Recurrent Selection to obtain Improved Populations

22. Rarely in nature does a single individual or variety has all the necessary genes controlling traits of commercial interest (i.e. high resistance to diseases, high yield, and high quality). Recurrent selection is one of the tools the breeder use to gradually add genes of interest in a population, maximizing the chances of finding individuals with all useful traits. The objective of this component is to obtain improved populations through recurrent selection assisted by molecular markers.

Component E: Backcrossing in Cocoa

23. Backcrossing is a tool used by breeders to transfer one or more genes from a donor parent to a variety agronomically beneficial (the recurrent parent), which lacks those specific genes. In using this strategy, selected individuals are backcrossed with the recurrent parent until most of the genes of that parent are recovered. Without the use of markers as a tool in the use of backcrosses, it would be almost impossible to advance in this area, because of the time factor involved in conventional cocoa breeding programmes. The objective of this component is to advance backcross generations based on molecular and phenotypic data.

Component F: Germplasm Evaluation

24. Germplasm collections are the reservoir of genes used by breeders in developing superior varieties. A full knowledge of the germplasms available is essential for the success of a breeding programme. For some time now, emphasis was put in increasing the size of germplasm collections, however, with little emphasis on evaluation of the same. In this project, a large number of germplasms were evaluated covering several phenotypic traits and also molecular markers. The objective of this component is to evaluate germplasm collections in Brazil, Peru and Ecuador.

2.4.3. Milestones Identified

25. Identified milestones in the 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 change through out space and time.
- Identification of markers associated to genes with resistance to witches' broom.
- QTLs were identified for trait of agronomic importance such as butter content, hardness, yield component, high vigour and resistance to black pod rot.
- The nature of this multiple-national project allowed for joint evaluation of germplasm and exchange of elite material from approximately 1,000 accessions for mutual benefit.
- In total, 123 new microsatellites primers were developed for cocoa.
- Transfer of technology and training activities implemented.

2.5. BENEFICIARIES AND ESTIMATED BENEFITS

26. For many years conventional breeding at scientific research institutions in cocoa producing countries and other institutions had being the main process, aimed at generating recommendations to farmers of improved planting material. This was directed to all aspects of crop protection and crop production; all with the singular interest of ensuring that cocoa farmers are able to cultivate cocoa economically. However, with increased plantings over time, diseases and pests have been escalating in severity and continue to progressively disseminate over geographically areas at a more rapid pace. Therefore, to overcome this situation, greater technological advances had to be achieved at fundamental level (basic research) to then be transformed to outputs in the form of technological recommendations (results of applied research). The project visualised the possibility of reverting the situation by borrowing from cutting-edge technology.

27. The main beneficiaries in project output are the cocoa farmers that gain from studies designed to assure greater sustainability. However, 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 few crops that is grown in a forest environment. Thus, it's very continued existence in this surrounding promotes ecological preservation in harmony with economic and agronomic ventures.

28. While cocoa is not a 'food-crop' in its true sense; it is a cash-crop for millions of farmers of small holdings in the cocoa growing regions of Africa, Asia, Central and South America. Revenues derived from cocoa assuming that fair trade prevails result in benefits that could influence the living standard of people in these regions. In a recent estimate of financial losses due to witches' broom in Bahia, Brazil since the disease was first registered in 1989, reached a value of about US$10 billion.

2.6. PROJECT STRUCTURE AND FINANCING PLAN

2.6.1. Project Structure

29. Project structure, in this section under the main heading 'Background and Context of the Project' was initiated even before the project commenced. A series of preparatory requirements were in place, such as adequately trained personnel in molecular biology, genetics, laboratories in molecular biology and plant pathology, germplasm collections, field experimental area and green/screen houses.

2.6.2. Project Financing

30. Application of resources followed the guidelines of the Detailed Cost Tables of the Prodoc. 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.

31. The main financing of the project was received from CFC. CEPLAC and UENF contributed funds and also in-kind. However, even before the start of the project (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. Some field populations in experimental areas for use in DNA studies and field screening of the disease were also established.

2.6.3. Co-ordination and Project Management

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

33. Financial operations with support personnel of Foundation Pau Brazil, ensured the funds were efficiently utilised according to the financial manual as well as establishing compatibility between the work plan and financial plan. Parallel to this, careful management of finances was undertaken to accomplish even more than that required in the Prodoc, within the funds available in all PPC. Exceeding required output was recorded among many activities, attributed to team effort and project management.

34. The Technical Coordinator was responsible for planning all aspect of the scientific programme, guided by the objectives and goals pre-determined. At certain stages, weekly meetings were held by the PEA together with research teams to establish time frames for conclusion of activities and determined intervals, re-evaluate progress achieved, implement adjustments required to attain programmed goals and keep abreast with advances in this relatively new field, increasing the quality of the Project output. These activities were implemented by the PEA, as well as in every PPC.

35. The Author of the Project, at UENF, Campos, Rio de Janeiro, served in the capacity of a Project Consultant. The same was the case of the International Consultant. Ecuador and Peru had their own Country Co-ordinators that maintained constant communication with the General Coordinator and the Technical Co-ordinator.

3. PROJECT IMPLEMENTATION AND RESULTS ACHIEVED

3.1. PROJECT IMPLEMENTATION

3.1.1. Structure and Rationale of Project Implementation

<u>Implementation structures</u>

36. Geneticists identified suitable populations of cocoa genetic material either in existence at research institutes' experimental areas or established in selected populations in the field. As the project advanced, progressively crosses were made with promising candidate plants, widening the reaches of genetic material. Also, a few years after the outbreak of witches' broom in Bahia, some cocoa trees on farms were observed to be resistance under high disease pressure; these were also selected for analysis. DNA was extracted from field material and molecular marker assisted processes, identifying promising genes for sources of resistance and other economic useful traits.

37. At the same time techniques were improved, including outcome from MS and Ph.D. thesis of Project Scholars, orientated to contribute in developing and perfecting techniques to achieve an increasing higher level of project output quality, again greater than determined in the Prodoc.

38. Pathologists interacted with geneticists, evaluating mature trees in the field to identify signs of resistance to the disease and also maintaining a continuous process of artificially inoculating seedlings, screened for disease resistance. Improvement on existing screening techniques increased precision aimed at higher quality in methodology.

<u>Rationale in Project Implementation</u>

39. This multi-national project put together institutions/countries aimed at dealing with a common problem of witches' broom that continued to reduce cocoa production in the three countries, resulting in abandoned farms. The causal agent of witches' broom, *Crinipellis perniciosa,* ranks high in the list of serious plant pathogens worldwide, and poses a continued challenge. This is more likely based on the fact that the pathogen has its origin in the Amazon region; having co-evolved with cocoa (the host) from the same origin thus, the pathogen is adaptable to changes in the host and maintains domination.

40. The project had to ensure that activities were accomplished within the time-frame determined and therefore, sufficiently well programmed to ensure that project goals were achieved. But, the projects' research personnel also had the driving force of meeting an urgent demand from growers and associated communities over the project execution duration. Thus, a need to ensure that worked on identified resistant material was immediately transferred to commercial farms for cloning, through facilities in mass production of propagules, to maintain farmers' interest through increasing income gained from cocoa cultivation, before all tree-stock was lost in the field.

41. Efficient project implementation was through an operational structure that had to be in place to allow for project advances in activities, on all fronts and in all countries, from the start.

3.1.2. *Modus Operandi* of Project Activities

<u>Training</u>

42. Since the Project contemplated a new approach in disease management of a tropical tree-crop - cocoa, project operations required to start correcting existing deficiencies in comparable excellence on the subject of molecular biology. With this common goal, training of personnel was conducted in two complementary areas:

a. Basic academic training (MS 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; 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 that obtained higher degrees through the project was more than required in the Prodoc.

b. Basic training courses, including for Ecuador and Peru on the use of molecular biology techniques, was undertaken initially at UESC. Operational training continued, in greater part at the Molecular Biology Laboratory at the Cocoa Research Centre, 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, immediately at 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 graduates students that worked in the laboratory proceeded to obtain higher degrees from the nearby UESC in molecular biology/pathology related subjects.

3.1.3. Project Monitoring and Supervision

43. The General Coordinator, Technical Co-ordinator the Author of the Project visited the PPC almost every year, ensuring that compatible advances were made. The International Consultant visited the PEA facilities during the early part of the Project and during the Mid-term Evaluation. Visits by personnel of CFC and ICCO were carried out in three occasions.

3.1.4. Resource Utilisation

44. The following table shows Funds received from CFC during the course of the project, ensuring the efficient execution with the work plan.

Payment made	**Value * (US$)**	**Deposit date**	**Project Year**
1st	79,990.00	04/04/2000	**1st year**
2nd	32,490.00	07/02/2001	**1st year**
Sub-total	**112,480.00**		**1st year**
3rd	36,756.16	17/04/2001	**2nd year**
4th	45,909.66	10/07/2001	**2nd year**
5th	13,818.34	26/07/2001	**2nd year**
Sub-total	**96,484.16**		**2ndyear**
6th	78,953.69	29/04/2002	**3rd year**
7th	74,757.65	22/01/2003	**3rd year**
Sub-total	**153,711.34**		**3rd year**
8th Mid-term	11,620.00	28/05/2003	**4th year**
9th	76,614.00	06/10/2003	**4th year**
10th	76,413.29	22/04/2004	**4th year**
11th	61,013.30	05/10/2004	**4th year**
Sub-total	**225,660.59**		**4th year**
12th	63,762.39	27/04/2005	**5th year**
13th Final workshop	31,314.00	03/08/2005	**5th year**
14th	41,921.85	24/10/2005	**5th year**
Sub-total	**136,998.24**		**5th year**
Grant Total	**725,334.33**		

*The values in the Grant Total column refer to that shown on the bank deposit-slip at source and does not reflect the actual lower value received after local bank deductions (commissions, etc). Also, this Grant Total needs to be adjusted for funds applied at source in backstopping and international consultancy.

3.2. PROJECT RESULTS ACHIEVED

45. The 'Objectives and Expected Outputs' (Item 2.4) would seem unconnected, to attain desired field applicable results. However, this was a calculated approach dedicating time and effort in molecular biology techniques - part of a new strategy in search of resistance to witches' broom; restructured on current scientific inputs adjusted to cocoa.

3.2.1. Qualitative Analysis of Results

A summary of the qualitative analysis results achieved are described as follows:

46. Planting material was precisely evaluated for phenotypic data (production, disease symptoms, etc.) and also at molecular level (sources of resistance). As a result of a more precise evaluation, the project was able to produce a wider diversity in cocoa varieties in farms thus, providing greater stability to farmers. As a secondary result, the studies in Brazil allowed for a better understanding of the population dynamics of *M. roreri* in Ecuador and Peru. The identification of a strong genes controlling resistance to witches´ broom and markers associated to this genes were found in Sca-6 x ICS-1.

47. Results on other components of the project (high diversity and selection in the fungal population) convincingly showed that less effort should be put into trying to introduce single genes through each backcrossing. A better approach would be recurrent selection involving different sources to accumulate many genes. Therefore, efforts were focused on this strategy and less in backcrossing. Other qualitative results were divided into four areas, described as follows:

Socio-economic:

- Abandoned farms reactivated as well as cocoa grinding industry in Brazil.
- Farm labour was reemployed, 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 forest were given due importance as required shade for introduced cloned cocoa to be productive.
- Investment in mixed cropping diversification programs 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 *Monliophthora*).
- 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 relationship
- 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 millions of enhanced propagative material for grafting.
- A mass production facility was installed - Bio-factory (*Biofabrica)* with a 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

48. Besides the main population (Sca-6 x ICS-1) selected at the start of the project, further three 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, generating about 700 markers in the main population and 300 in the other populations.

49. 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 4 new primer types (RAPD, AFLP, SSR and RGH), in accordance with the updated technology now available. A series of 6 differential clones were 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 more aggressive.

50. The nature of this multiple-national 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 originated from farmers plantations were obtained. DNA analysis was undertaken in 500 isolates of *C. perniciosa* to obtain a better understanding in fungal population changes. The results from theses analysis showed that the fungal population may change through out space, (countries, regions, among trees within a farm and within a tree) and in time, when a new resistant variety is recommended to farmers. From these studies some key results are:

- 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, which require to have a level in efficacy to all these strains.

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

- Sixty specific microsatellite (SSR) primers were developed for studies on populations of the fungus *C. perniciosa*.

- Some of these primers proved to also useful for population studies of *Moniliophthora roreri*, (cause of frosty pod rot disease in cocoa, in Latin America).

- A collection of 1200 isolates were registered and preserved as a permanent reference source on diversity in *Crinipellis perniciosa.*

- Three other genes associated to WB 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.

51. As standard procedure, phenotypic data was collected for yield components. Information regarding resistance to other diseases and bean quality in populations aimed to identify genes associated to these traits was recollected. Besides the development of planting material resistant to witches' broom disease, characteristics of agronomic interest were incorporated to the research of new varieties. This was done by identifying Quality Trait Locis (QTL) for different characteristics. Three major QTLs associated to butter content and hardness were identified in Sca-6 x ICS-1. With these results, selection for butter content and hardness can be done early, 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 to farmers. One QTL identified allowed early selection for less or high vigour. Three QTLs associated to 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. This opened the way for possible resistance to two diseases.

52. 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 WB, 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 with other three sources of resistance to this disease or frosty pod rot.

53. Thirty-one accessions of the germplasm collection were evaluated for the presence of the primer AV-14 (a marker associated to WB resistance). Analyses showed that AV-14 was quite frequent among other resistant accessions, including those apparently not related to scavina. Either they carry the same scavina's gene or the markers were not associated to resistance in those clones as is the case in scavina. Sixty farmer selections currently in on-farm clonal trials were tested for the presence of markers associated to resistance (CIR-35, CIR-24). Some clones with high level of resistance had these markers, suggesting that markers can be useful for selecting clones, speeding up the process of evaluating clones to farmers.

54. Twenty-seven crosses involving 23 parents, with different levels of resistance to witches´ broom, black pod and yield were made and transferred to the field to be used in a marker assisted recurrent selection program, totaling around 3,200 plants. A first generation of backcross was made between a source of resistance to witches´ broom (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.

55. 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 was collected using different types of markers, including SSRs and RAPD.

56. The genetic diversity of around 800 clones was assessed, thus guiding breeders on 'decision making' with greater confidence. Diversity analysis became a routine strategy of breeding, even after the project ended. Some key results of these analyses were:

- Genetic diversity exists among resistant germplasms, suggesting that those germplasms carry different genes of resistance. This is very important not only in broaden the genetic base of the resistance, but also in pyramiding genes in varieties aiming to increased durability.

- Some farmer selections are genetically distant of the main source of resistance to witches´ broom, suggesting some of them could be used more often to increase diversity in plantations.

- Experimental areas and breeding effort can 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 exists in the Amazon region and this diversity is fairly regionalized.

- Among 200 plants selected on farms in Peru for high yield and resistance to witches´ broom 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 microsatellite (SSR) primers were developed for cocoa. This is a very important contribution to the international community, since before this project only 200 SSR primers were developed and made available by CIRAD. So, the project was responsible for about a 30% increase in the SSR primers available for cocoa, worldwide.

57. Genetically enhanced material against witches' broom, with identified source(s) of resistance, was released for large-scale use, and which continue 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; ore then expected.

58. From a production value of 380,000 tons before the outbreak of witches' broom, productions drop to 90,000 tons. Releases of resistant material resulted in an increase in production to 144,000 tons. Full production is yet to be expected from resistant material due following a time lag to maturity. Farm productivity that 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 of about US$200/ha.Employment demand in the cocoa producing region from about 20,000 rose to about 40,000 with projected values of 80,000 and 115,000 in years 2011 and 2014, respectively.

59. Over the project period, 1,000 selected farmers were trained to establish clonal gardens, and as a result saw an increase to 5,500 farmers at the end of the project.

Training:

- 4 Ph D (Ioná Santos, Jay Wallace, Ricardo Moreira, Paulo Albuquerque and Luis Cordeiro- parcial).
- 5 MS (Ioná Santos, Karina Solis, Alfredo Dantas, Ronaldo Santos, Cássia Bahia).
- 3 specialists.

60. Therefore, in this quantitative analysis of results compared to planned outputs, in virtually all cases, the project produced more than originally planed.

3.2.3. Extended Impact on Beneficiaries

61. The beneficiaries of the project, in terms of extend of impact were the world cocoa farming community. Gains from advances made in the use of molecular marker technology directed spin-offs to be used in other crop protection and production areas, provided by:

- Readily transferable expertise.
- Markers made available worldwide.
- Reduction in time in renewing farms with genetically improved material.
- An early start in preventive breeding and pathology, before the presence of the pathogen(s) in a country.
- New approaches readily transferable.
- Availability of an increased numbers of microsatellites developed during the project.

3.3. DISSEMINATION OF PROJECT RESULTS

62. Due to the devastating effects of the presence of witches' broom in PPC, the very nature of the situation was urgent from the start of the project. This made it necessary to transfer improved material to growers, as soon as available, during the whole project period. The important advantage to project execution was the immediate feedback received, allowing for adjustments. These releases of material were accompanied with a 'technological packet' initially passed on to the extension personnel and them, 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 other practices still continues. The farmers recognise that this approach to modernise cocoa cultivation within a renovation process is necessary for the survival of cocoa growers in these regions.

63. Dissemination of results on an international scale has been through presentations at important international meetings – many of which were requested to project members as guest speakers, to relate experiences gained.

3.4. PROJECT PUBLICATIONS

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3.5. PROPOSAL FOR THE CONTENTS OF THE FINAL PROJECT PUBLICATION

64. The Final Project Publication will use as guidelines previous CFC publications, copies of which were received from CFC. The process of gathering information started following the Final Workshop in which presentations from all PPC were made and electronically filed.

4. LESSONS LEARNT

4.1. DEVELOPMENT LESSONS

65. It took five years to convince independent project reviewers that the project proposal was feasible. Admittedly, the analysis was critical, as they had reason to believe that without substantial documented evidence in the use of molecular markers leading to a search for material resistant to witches' broom, specifically in cocoa, would result in a poor project success criterion.

66. However, if over 100 years of methods in management of this disease, including through conventional breeding for resistance, had a very poor record, then cutting-edge technology had to be explored. This, in the project proposal was substantiated with qualified personnel in this

area with experience in using similar techniques, although not in a tropical tree crop. Probably there also was concern that the project might yield good results in basic research and not applied research. However, the three PPC were applied research establishments with urgent demands from the cocoa farming community in these countries. The strong driving force was to generated recommendations, and thus save the industry and what its significance to the regions.

67. The Project Co-ordinators realised that while dealing with a relatively new technology, with few researcher specialised in this area, it was necessary to ensure a high level of academic and operational training. Further, for this regional project to succeed there was a need for compatible excellence on all fronts of project activities, in all PPC countries.

68. Further, good results would serve little purpose if certain aspects were not contemplated and in place. This included a farmers' training program to apply for the first time grafting technology and, at the same time, mass production facilities for producing clonal material. Work in this direction was undertaken with support from other institutes' researchers and extension personnel.

4.2. OPERATIONAL LESSONS

69. There were no specific operational lessons learnt, except aim for results in all activities, well above originally planned.

5. CONCLUSIONS AND RECOMMENDATIONS

70. The impact on socio–economic upheavals in a cocoa-growing region, resulting from an outbreak of witches' broom in 1989, Bahia, Brazil, was no different to the experiences in other countries in South America, which occurred over 100 years. Even in a contemporary disease outbreak, knowledge in disease management was inadequate to revert the situation. This was in spite of implementing a scaled eradication operation, disease spread unchecked. Intensive research was needed to strengthen components in integrated disease management (IDM).

71. Therefore, the Project aimed at initiating an ambitious research program to complement conventional breeding methods, in the use of molecular biology techniques borrowed from cutting-edge technology. This added a new dimension to breeding for disease resistance in cocoa, which not only quicken the pace in which resistant material was made available to growers, but also ensured that sources of resistance can be precisely identified at DNA level. As an outcome of this strategy, cocoa genotypes incorporated with an inherent degree of resistance were made available thus, production levels rose. The introduced material was supplied to growers as cuttings (clones) or pre-grafted seedling for renovating farms, increasing tree density from about 600 to 1,100 per ha. Also, with this intrinsic advantage in improved material, other IDM components operated more effectively with lower input cost.

72. The tendency on the way to recovery validates the hypothesis that a new era in cocoa disease management is being witnessed. The methods, techniques and rationale introduced in this project case study serve as spin-offs to other situations in cocoa producing countries, as virtually all of these are readily transferable expertise.

73. The organisational aspects that made the whole operation viable in renovating and modernising about 140,000 ha of cocoa farms, in a relatively short period for a tree crop, deserves comment. It was the project that initiated the whole process, which involved outputs in molecular biology, genetics, pathology and agronomy, leading to results that were then supported by mass production of planting material, training in methodology of cloning on existing root-stock and establishment of clonal gardens over the whole region, all aimed at accelerating the process, with no significant restrictions/hold-ups.

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