SEVENTH INGENIC WORKSHOP

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ABSTRACTS

Genotypic plasticity of the chocolate tree *Theobroma cacao:* Aroma diversity

Daniel Kadow[a]*, Joerg Bohlmann[b], Wilberth Phillips[c] and Reinhard Lieberei[a] [Presented by Christina Rohsius]

[a]University of Hamburg, Biocenter Klein Flottbek, Ohnhorststr. 18, 22609 Hamburg, Germany, [b]University of British Columbia, Michael Smith Laboratories, 321 - 2185 East Mall, Vancouver B.C., Canada V6T 1Z4, [c]Centro Agronómico Tropical de Investigación y Enseñanza (CATIE), 7170 Cartago, Turrialba 30501, Costa Rica

*Corresponding Author: daniel.kadow@uni-hamburg.de

Abstract

The seeds of *Theobroma cacao* L. are the key raw material in chocolate manufacturing. Depending on the variety, traders separate them into bulk and fine or flavour cocoa. The latter is characterized by the presence of special aroma notes such as fruity and floral. In contrast to chocolate aroma that derives from seed endogenous components (e.g. storage proteins and carbohydrates) fine aroma has been linked to the fruit pulp. However, detailed information on its molecular background is lacking.

In the present study we analyzed fruit pulp of two fine or flavour cocoas (SCA 6 and EET 62) and a bulk cocoa (CCN 51) using GCMS.

β-myrcene, β-trans-ocimene, β-cis-ocimene and β-linalool were characteristic for the SCA 6 pulp volatile composition. Regarding EET 62 2-heptanol, 2-heptanol acetate, 2-heptanone and 2-nonanone were typic. We conclude that these molecules are the main components of SCA 6 and EET 62 fine aroma. Accordingly, fine aroma components apparently derive from different metabolic pathways depending on the genotype.

Genome Enabled Tools for Cacao Functional Genomics

Mark Guiltinan
Penn State University , Department of Horticulture, 422 Life Sciences Building, University Park, PA 16802-5807 email: mjg9@psu.edu, Web Site: http://guiltinanlab.cas.psu.edu

The cacao genome sequence has enabled a new era of cacao science, providing tools and resources that can be used in many different strategies to advance our knowledge of the genetics and molecular biology of cacao. Functional genomics is the study of gene function at a mechanistic level, which enables the discovery of the roles genes play in plant development, growth, defense response and reproduction, and to discover candidate genes potentially useful for breeding of enhanced genotypes for production. I will discuss briefly some of these resources, including: genome browsers and other databases, whole genome microarray, proteome database and proteomics capability, transient gene expression method and the use of Arabidopsis to perform transgenic complementation analysis.

Integrating genomics in future approaches to cocoa selection and propagation in Côte d'Ivoire

Pokou, N. Desire.[1], Tahi, Mathias.[2], Gutierrez, Osman. [3], Motamayor Juan Carlos.[4], Schnell, Raymond[4], and Broun,Pierre[5].
1CNRA, Laboratoire Central de Biotechnologie 01 BP 1740 Abidjan 01 Côte d'Ivoire
2CNRA, Station de Divo B.P 808, Divo, Côte d'Ivoire ; 3. USDA-ARS, 13601 Old Cutler Road, Miami, Florida; 4. MARS-Inc ,. C/o USDA-ARS, 13601 Old Cutler Road, Miami, Florida
5 Nestlé, Centre R&D 101, Avenue Gustave-EIFFEL B.P. 49716 37097 TOURS CEDEX 2.

In Côte-d'Ivoire, the cocoa breeding is based on a reciprocal recurrent scheme that has been set up in the aim of improving simultaneously the characteristic of the two main populations: Upper Amazon and Lower Amazon+Trinitario. Resistance to Phytophthora and Cacao Swollen Shoot Virus has becoming the main criterion of the breeding program. The development of SSR markers linked to resistance gene has been carried out during this decade to identify QTLs for resistances to diseases such as Black Pod, Frosty Pod and Witches' Broom. Black Pod affects cocoa production worldwide, and a particularly severe species is spreading in West Africa from where 70 % of world cocoa is produced, while the two last diseases are only found in the Americas but there is the constant threat that they could spread to new regions and threaten world cacao production. The objective of the genomic assisted breeding in Côte-d'Ivoire is to develop varieties with resistance to current and potential disease threats. New mapping populations are being creating to identify additional QTLs. The utility of Association Mapping for quantitative trait has been demonstrated providing an alternative method to the traditional mapping. More recently, the development of SNP has facilitated genetic mapping and fingerprinting. SNPs are being used to certify the quality of crosses made in seed gardens to produce the planting material for farmers.

An Example of Association Mapping in Cacao

[1]Gutierrez, O.A., [2]Schnell , R., [2]Motamayor, J.C., [1]Kuhn, D.N., [1]Tondo, C., [1]Boza, Edward, [2]Livingstone, Donald, [1]Royaert, Stefan, [3]Nagai, Chifumi, [4]Phillips, Wilbert , [5]Amores, Freddy, [6]Lopes, Uilson, [7]Takrama, Jemmy, [7]Padi, Francis, [8]Aikpokpodion, Peter, [9]Pokou, Desire, [10]Efombagan, Ives, [11] Sounigo Olivier and [12]Epaina, Peter

[1]USDA-ARS, Subtropical Horticulture Research Station, 13601 Old Cutler Road, Miami, FL 33158, USA. [2]MARS Inc., Elizabethtown PA, USA. [3]Hawaii Agriculture Research Center, P.O. Box 100, Kunia, HI 96759. [4]Centro Agronómico Tropical de Investigación y Enseñanza (CATIE), Turrialba, Costa Rica. [5]INIAP, Estación Experimental Tropical Pichilingue Quevedo, Los Ríos, Ecuador. [6]Centro de Pesquisas do Cacau (CEPEC/CEPLAC), Itabuna, Bahia, Brasil. [7]Cocoa Research Institute of Ghana (CRIG) New Akim Tafo, Ghana. [8]Department of Genetics & Biotechnology, University of Calabar, Calabar, Nigeria. [9]Centre National de Recherché Agronomique (CNRA), Laboratoire Central de Biotechnologie (CNRA-LCB), Abidjan, Cote d'Ivoire. [10]Institut de la Recherche Agricole pour le Développement (IRAD) Centre de Nkolbisson, Yaoundé, Cameroon. [11]IRAD CIRAD, Yaoundé Cameroon. [12]Cocoa and Coconut Institute of Papua New Guinea, Rabaul, East New Britain Province, Papua New Guinea.

Association mapping and genomic selection have become important methodologies in perennial crop breeding improvement programs for accelerating breeding efforts and increasing the efficiency of selection. They are good alternatives to the classical Quantitative Trait Loci (QTL) mapping approach.The USDA-ARS and MARS Inc. joint cacao research improvement program has developed cacao breeding populations in several countries that are segregating for resistance to Witches' Broom, Frosty Pod and Black Pod disease, as well as for productivity traits. QTLs associated with these traits have been identified; however, some of these associations are specific to certain populations and they are influenced by their interaction with the environment. Additionally, the majority of these traits are controlled by many genes with small effects. Due to these constraints, Marker Assisted Selection (MAS) and traditional plant breeding methodologies have not been very effective in accelerating the development of cacao varieties with improved yields, excellent flavor traits and superb disease and insect resistance. Advances in high throughput genomic technologies at reduced cost and the sequencing of the cacao genome have allowed the development of thousands of Single Nucleotide Polymorphism (SNP) markers that are currently being used in parental identification, germplasm characterization and the fine mapping of QTLs. Furthermore, the genotype-by-sequencing approach is also currently being used in cacao. Finally, the recent development of robust statistical methodologies for association mapping and genomic selection (GS) make them very attractive for use in cacao breeding. Currently in our research program we are using previously identified QTLs and applying association mapping techniques to evaluate the marker-trait associations across different sets of cacao germplasm and breeding populations from South and Central America, Africa, and Asia. Another goal is to identify loci that could be significantly associated with disease resistance, agronomic and quality traits, in order to later use them as predictors of performance. Selection based on these predictors could lead to faster genetic gains and reduction in the time and cost of releasing superior varieties with high yield, disease resistance and the quality attributes required by the confectionary industry.

[NOT PRESENTED]

Genome Based Comparative Analysis of Carotenoid Biosynthetic Pathway in Cocoa.

S. Naganeeswaran and S. Elain Apshara
Central Plantation Crops Research Institute, Regional Station Vittal, D.K.Dt., Karnataka- 574 243, India. E-mail: naganeeswaran@gmail.com, elain_apshara@yahoo.co.in

Agri-bioinformatics is a newly initiated program of Department of Information Technology, Govt. of India, under which CPCRI is undertaking a project on bioinformatics and molecular approaches to understand the biosynthetic pathways in cocoa. Cocoa EST sequences available in the public domain are used initially to identify metabolic pathways. Since the cocoa genome has been sequenced and published, we are now utilizing this information for comparative studies. Carotenoids are biotechnologically important colour pigments which play a key role in photosynthesis, as antioxidants and in photo-oxidative stress resistance. In plants they are the precursors for the synthesis of abscisic acid and alpha and beta carotenoids, which are the precursor for Vitamin A. The objective of the present study is to annotate the important genes involved in the carotenoid biosynthetic pathway from the cocoa genome and reconstruction of the pathway and to study the comparative and evolutionary relation between the important genes in carotenoid biosynthesis. The carotenoid biosynthetic pathway enzymes with known functions are identified from the literature and corresponding protein sequences belonging to model organisms are retrieved from Uniprot, Genbank and KEGG databases. The mRNA sequences of corresponding enzymes, ~25,000 cocoa whole genome short gun sequences are retrieved from Genbank and the standalone cocoa specific blast database is created using farmatdb program for comparative genomics. Carotenoid biosynthetic pathway enzyme sequences are aligned with cocoa EST sequences using the TBLASTN program. We have annotated the putative enzymes and predicted 16 important enzymes and their isoforms involved in carotenoid biosynthesis pathway. The functional annotation of the predicted genes is done and pathway is reconstructed based on related enzymes. Six major plant model organisms, *Arabidopsis thaliana, Populus trichocarpa, Vitis vinifera, Oryza sativa japonica, Sorghum bicolor and Zea mays* are used for comparative genomics study. We used gene model of these plants and EST sequence of cocoa for genome annotation. Comparative genomics results showed that 6 major plants share the similar pathway for the synthesis of lutein, beta-carotene and violoxanthin. A downstream pathway enzyme, neoxanthin synthase (NSY), not annotated in other model plants, is found in cocoa genome. Phylogenetic analysis is carried out for rate limiting lycopene cyclase family of enzymes such as Lycopene beta cyclase (LYC) and Lycopene epsilon cyclase (LUT1). Phylogram is constructed using neighbor-joining method implemented in MEGA software. The phylogenetic tree showed that LYC and LUT1 clustered separately in two groups and they have a common ancestor revealing that they might acquire the different functions during the evolutionary process. This annotated information can be used for cocoa improvement and genetic engineering works in future.

Cocoa Genome Browser Workshop

Xavier Argout

CIRAD, Département Systèmes biologiques, UMR 1334 AGAP, Avenue Agropolis - TA 108/03 (Bat. 3, Bur. 14), 34398 Montpellier CEDEX 5, France
xavier.argout@cirad.fr

Hands on workshop to introduce the main functionality of the cocoa genome database (http://cocoagendb.cirad.fr). A brief overview of the development and use of the browser interface will be presented. Participants will be instructed how to use the main features of the database and will work through specific analyses with workshop instructors in hands on sessions using the online Genome Database. Please bring your own laptop. Several additional computers will be available for attendees who cannot bring a computer.

We will cover:

- Basic genome viewing
- Understanding gene annotation displays
- Searching for specific genes
- BLAST analysis
- Exploring protein predictions
- Finding QTL regions and analysis of candidate genes
- Identification of SSR, SNP
- Downloading sequencing data

Potentials for Application and Utilization of Cacao Somatic Embryogenesis for Commercial Plant Propagation

Siela Maximova and Mark Guiltinan

Penn State University , Department of Horticulture, 422 Life Sciences Building, University Park, PA 16802-5807 email: mjg9@psu.edu, Web Site: http://guiltinanlab.cas.psu.edu

Plant tissue culture offers a suite of new approaches to speed up the development and deployment of genetically improved genotypes. Research conducted at Plant DNA Technology; CIRAD, Montpellier, France; Nestles Inc., Tours, France; and at The Pennsylvania State University has led to the development of efficient methods for multiplying cacao by somatic embryogenesis (SE). The main advantages of this tissue culture method include the possibility of rapidly generating asexually propagated, uniform plants with highly valued genetic traits. Additionally, for cacao, somatic embryogenesis offers a system for clonal production of orthotropic plants with normal dimorphic architecture and taproot formation. The production and testing of disease free materials and germplasm conservation via cryo-preservation are other important potential applications of plant tissue culture to the improvement, preservation and distribution of cacao germplasm, which is currently preserved only in living collections in the tropics.

A review has been commissioned by Bioversity International with the goal to synthesize publically available information and literature describing the research and development activities on cacao tissue culture and of field-test evaluations of SE-derived plants. Current activities in cacao producing countries involving adoption and scale-up for large-scale propagation of important genotypes were also reviewed.

The review of the existing research literature and results of 12 years of field testing by different groups in Ecuador (Nestle, INIAP, Penn State), Saint Lucia (Penn State) and Puerto Rico (USDA, Penn State), ascertained that in general SE plants have normal growth morphology and demonstrate high levels of field performance equal to or better then other forms of vegetative propagation. It was concluded that cacao tissue culture holds a very strong potential to help address the future demand for cacao planting material and to supporting a truly sustainable and profitable cocoa supply chain. Furthermore, with the rapid pace of scientific advancement in the fields of cocoa genetics and breeding, it will become even more important to develop rapid clonal propagation systems for cacao to aid the more efficient and rapid testing and deployment new varieties. Perhaps SE technology would be most important in the initial scale up of new varieties by saving two or three years in a varietal scale up, when only one or a few mother trees exist. Cacao SE technology can be to be utilized as a stand-alone technology or in conjunction with other methods of propagation such as rooted cuttings and grafting. The potential to use SE for germplasm conservation and movement is also of great importance and should be exploited.

The Current Status of Selected Cacao Material and Future Approaches to the Development of Cocoa Varieties in Côte d'Ivoire

G. M. Tahi[1], Ph. Lachenaud[3], J. A. K. N'Goran[1†], P. N. Désiré[1], I. B. Kébé[1], D. Paulin[2†], K. F., C. Cilas[2]., N'Guessan[1] and A. B. Eskes[2]

[1]CNRA, B. P 808, Divo, Côte d'Ivoire, [2]CIRAD (UPR 106), Av. Agropolis, 34398, Montpellier, France CIRAD (UPR 106), BP 701, 97398 Kourou Cedex, France

In Côte d'Ivoire, the genetic improvement of cacao began in the late 1960s with the introduction of Upper Amazon clones. The first crosses between Upper Amazon and Trinitario clones were multiplied in seed gardens and distributed to farmers from 1975 onwards. These hybrids were very popular among farmers due their early vigour, precocity, productivity and bean quality. From 1990, a Reciprocal Recurrent Selection program was adopted and new criteria such as resistance to black pod and mirids, and the relationship between yield and vigour (cropping efficiency) have been taken into account to achieve the greatest genetic gains. This new breeding scheme is currently in its second cycle of selection. At the Divo Research Station three breeding trials involving intra- and inter-population crosses were planted in 2001, together with a complementary clone trial.

This article includes the following: the list of the twenty hybrids selected and currently distributed in Côte d'Ivoire, and the quantities of improved seeds distributed over the past ten (10) crop years as well as the constraints relating to this process. Following the analysis of data from the breeding trials, the most promising hybrids and candidates for elite clones have been identified for confirmation testing. In addition, a preliminary trial based on intra- and inter-population crosses, including the best trees of each group, is planned to allow the selection of new high performing hybrids.

CRINTc-1 to 8: New Cocoa Genetic Resources for productive boost of Nigerian Cocoa economy

Adewale, B. D. and Aikpokpodion, P. O.
Crop Improvement Division, Cocoa Research Institute of Nigeria, PMB 5244, Ibadan, Nigeria
d.adewale@gmail.com

The CFC/ICCO/Bioversity participatory breeding project ultimately resulted in the generation and release of eight outstanding cocoa genetic materials for Nigeria in 2011. These varieties are hoped to transform the Nigerian cocoa economy. All the eight cocoa hybrids (CRINTc-1, CRINTc-2, CRINTc-3, CRINTc-4, CRINTc-5, CRINTc-6, CRINTc-7 and CRINTc-8) had Amazonian maternal genes. Generally, their pollen donors were either Amazon, Amelonado or Trinitario. The varieties combined high yield (1 – 2.0t/ha) with precosity (attaining first pod production within 24 months of field establishment), tolerance to *Phytophthora* pod rot and Mirids, higher butter content (≥ 55%) and higher individual bean weight (> 1.0g). The National Cocoa Seed Garden (NCSG) and a World Cocoa Foundation (WCF) community based Cocoa Seed Garden projects were programme outlets for reaching the Nigerian farmers with these materials. The Nigerian Cocoa Transformation Agenda (CocTA) stemmed up the breeding success with targets to improve cocoa productivity. Seedlings from thousands of generated hybrid pods will become established during the planting season of 2013. The low quantity of the parental stock remains a challenge, however, a programme for widening the parental stock in forty locations within the cocoa ecologies of Nigeria is being proposed. The national scheme on old cocoa plantation rehabilitation would be implemented using CRIN Tc-1 to 8 as the clonal materials.

Cacao breeding and planting material situation in Trinidad and Tobago and some of the other Caribbean islands

Kamaldeo Maharaj
Ministry of Agriculture, Land, and Marine Resources, Central Experiment Station, Centeno, Via Arima P.O., Republic of Trinidad and Tobago, West Indies. kama1@tstt.net.tt

Cacao breeding activities in Trinidad and Tobago revolves around the successful breeding of the Trinidad Selected Hybrids (TSH). These varieties have low pod index, large beans, and good disease resistance under adequate management. Over 21 TSH clones are commercially grown by local farmers. Further breeding is underway to improve the BPD resistance of the TSHs by incorporating pooled gene resistance from specific populations derived from the Germplasm Enhancement Programme of CRU/UWI. No formal breeding activities take place in Jamaica, Grenada, Dominica, St Lucia and St Vincent. Cultivars of ICS types were mainly acquired from Trinidad. The propagation of cacao planting material is described for Trinidad and the other islands.

CACAO BREEDING ACTIVITIES AT CATIE, COSTA RICA

Wilbert Phillips- Mora, Allan Mata, Aldo Sánchez, Adriana Arciniegas, José Castillo
[1/]Centro Agronómico Tropical de Investigación y Enseñanza (CATIE), 7170 Turrialba, Costa Rica.

CATIE, with support from the World Cocoa Foundation (WCF), initiated a breeding program in 1996 aimed at identifying sources of resistance to black pod (*Phytophthora palmivora*) and frosty pod rot (*Moniliophthora roreri*) from its international genebank, and creating new varieties that can overcome the principal biotic problems limiting cacao production in tropical America. The program was strengthened with side projects in partnership with USDA / MARS (2002 -) and Bioversity-CFC (2005-2009).

In 2007, CATIE selected the following group of high yielding, disease tolerant and good quality Trinitario clones: CATIE-R1, CATIE-R4, CATIE-R6, CC-137, ICS-95 and PMCT-58 for farmer observation and use. These materials currently constitute the base of the genetic strategy of the Central American Cocoa Project (PCC) and other regional initiatives aimed at the complete modernisation of plantations, and improving the incomes and living conditions of farming families. The clones were also recently introduced in Mexico with the support of the chocolate industry (Hershey) and local actors (ECOM, INIFAP and Cacao México Foundation) to palliate the severe effect of *M. roreri* in that country.

In collaboration with local organizations, the clones are being established in an informal net of clonal gardens, which have the following goals: evaluate the behavior and adaptation of the clones in different agro-environments, determine the genotype x environment interaction, serve as observation parcels for farmers, and provide budwood for new plantings. Starting in 2008, 35 hectares of clonal gardens have been planted in the region. So far, they have provided vegetative material for the planting of approximately 300 ha of commercial plantations, principally in Costa Rica, Panama and Honduras.

CATIE recently published a catalogue containing comprehensive information on the six clones. The photographic, morpho-physiological and molecular information included in the catalogue will allow the clones to be distinguished, compared to other materials of interest and confirm their identity. The document also contains information on the potential productivity and quality of the materials and also on their reaction to black pod and frosty pod under natural and artificial test conditions.

The progressive increasing of new plantations based on the CATIE clones leads to the reasonable expectation that these varieties will become an important source of Mesoamerican Trinitarian cacao in the near future. Other clones that are in different breeding phases will soon complement the current range of CATIE´s improved germplasm.

Evaluation of Cocoa lines for Desirable Traits and Recommendation of Varieties from Central Plantation Crops Research Institute, India

S. Elain Apshara, K.S. Ananda and D. Balasimha

Central Plantation Crops Research Institute, Regional Station, Vittal – 574243, Karnataka, India.
E-mail: balasimhad@rediffmail.com

Cocoa as a commercial crop gained importance in India from 1970 onwards and since then there has been a continuous effort by Central Plantation Crops Research Institute in introducing cocoa germplasm, their conservation, characterization and evaluation for desirable traits. Cocoa in the present growing situation in South India experiences long rainless period upto a maximum of six months, which necessitated the screening for drought tolerance and further utilization in hybridization programs.

After evaluating 65 lines of initial introductions through physiological parameters and yield characters, four high yielding trees and three drought tolerant types were selected and subjected to crossing. High yielding lines, VTLC-1, VTLC-10, VTLC-10A and VTLC-5 of Malaysian origin were used as female parents, while VTLC-15, VTLC-19 and VTLC-30 of Pound collections were used as pollen parents. Resulted nine hybrids along with seven parents were assessed for their performance and tolerance to water stress, in a replicated trial at CPCRI Research Centre, Kidu, Karnataka under arecanut. Rapid screening technique was followed with seedlings as well as field planted trees. Variability in photosynthetic parameters and yields were observed and the hybrids VTLCH-3 and VTLCH-4 were selected as drought tolerant hybrids along with favorable pod and dry bean yields. The average dry bean yields of the hybrids VTLCH-3 and VTLCH-4 were 2.048 kg and 1.515 kg/plant/year resulting in 1331 kg and 985 kg/ha/year respectively. They were multiplied as grafts, planted under coconut in two locations and assessment of initial six years of bearing showed high yield efficiency with dry weights of 1.07 and 1.01 g/bean, 13.7 and 12.1% shell and 50 and 49% fat contents respectively. These two hybrids were recommended as varieties in 2006 for regions with periods of water stress.

The average productivity of Indian cocoa is only 350 kg/ ha and it should be sufficiently increased for sustainable production and so in another trial, mixture of 25 lines imported as seeds were evaluated for their adaptability and yield potential and individual tree selections were made. They were further multiplied as clones planted during 1995 in two locations both under arecanut and coconut and evaluated for their growth and yield parameters. Among them VTLC-1 and VTLC-57, yields average of 55 and 54.5 pods/tree/year at the age of 12 years as clones with medium canopy, both under arecanut and coconut as well as in farmer's gardens. Pods are of 360 g and 400 g size with 1.13 and 1.21 g dry weights, 11 and 15% shell, 52 and 53% fat contents and showed a yield potential of 2.52 and 2.70 kg dry beans/tree/year contributing 1700 and 1840 kg/ha/year respectively. Since these lines are there in the system for a long period their reactions to black pod rot, tea mosquito bug and water stress were also documented, which showed considerable tolerance to both biotic and abiotic stresses. These two selections were recommended as varieties during 2011. These were utilized in establishment of seed gardens, in development of hybrids as well as for supply and demonstration in farmer's gardens.

NEW COCOA HYBRID SELECTIONS WITH SUPERIOR QUALITY OBTAINED AT TULUMAYO STATION, HUANUCO-PERU

Luis F. García Carrión[1]; David Guarda Sotelo[1]; Patricia V. García Rodríguez[2]

[PRESENTED BY DARIN SUKHA]

1 Researchers in cacao plant breeding of the Universidad Nacional Agraria de la Selva, Tingo Maria-Perú, 2 Postgraduated in plant breeding of the Universidad Nacional Agraria-La Molina, Lima-Perú

Between 2005 and 2010, the area cultivated with cacao grew significantly due to high demand and good prices in the international market, but average yields have remained low. Plant breeding efforts to obtain new materials have continued but have not yet been sufficient to increase the production of cacao in Perú. Sixteen years ago, the program of cacao improvement of the Universidad Nacional Agraria de la Selva - Tingo María, began by selecting parents to create hybrids and to select superior clones which will soon be released on account of their productivity, tolerance to main diseases and superior quality.

Eight new selections (hybrid trees) were selected based on their superior quality. The trees labelled as B, C, D, E, F y G, showed intensities from middle to high for floral and fruity (specific flavors), highlighting the "E" (U-68 x IMC-67) x CCN-51 and "G" (U-68 x IMC-67 x ICS-95) trees, for their strong intensity of floral aroma. In addition, these are characterized by low to very low scores for acidity, astringency and bitter (basic/undesirable flavors) intensities. The "F" (EET-62 x U-1) and "H" (U-68 x IMC-67) x CCN-51 trees, was selected for their strong fruity flavor. The "H" tree, highlighted also for its low to very low intensities of the basic flavors. Finally, the "J" (U-68 x IMC-67 x ICS-95) tree, is noted for mid-range fruity flavour intensity,but with low to very low scores for undesirable flavor characteristics.

The implementation of the national cacao improvement program with a focus on productivity, quality and climate change adaptation, is opportune and strategic. We must continue to encourage the regional and local authorities to support the scientific research in alliance with the international technical cooperation, to contribute to the cocoa sustainable production.

Cacao breeding strategies for developing improved varieties in Ceplac, Brazil - Current status and future approaches.

Wilson Reis Monteiro, Uilson Vanderlei Lopes, José Luis Pires and Milton Macoto Yamada [Not Presented]

Ceplac/Cepec, Caixa Postal 7, Ilheus, Bahia, Brazil
wrmonteiro@cepec.gov.br

A synthesis of the cacao breeding strategies for developing improved varieties that are pursued by Ceplac, in Bahia, Brazil is here presented. The development of high yielding varieties, with good seed quality, resistant to important local diseases such as *Phytophthora* pod rot, witches' broom, *Ceratocystis* wilt are the main objectives of breeders. To achieve these objectives, a recurrent selection programme was put in practice, to produce breeding populations that allow not only the association of very important agronomic traits, but also pyramiding genes related to disease resistance, in particular. To facilitate the understanding, the breeding program was split in two subprograms: SP-1 and SP-2.

In the SP-1 some synthetic populations were generated by taking advantage of the botanical infrastructure built a long time ago by the breeders, e.g., old hybrid progenies. Plants were selected within these hybrid progenies to produce the families of the first cycle populations. Only some few accessions of the germplasm collection were accessed. At each cycle of breeding the pedigree of the progenies that compound the populations increased in complexity, involving, thus, many parents, representing also distinct source of resistance to witches' broom, without necessarily recourse to accessions of germplasm collection. Early selection was practiced for selecting new genotypes to be used as parents for advancing generations more rapidly. Genetic and/or statistical designs were used for producing the progenies. These populations were established in the field under old cacao plantings, heavily infected by these local diseases, at the spacing distance of 1.5 to 3.0 m. At each cycle, all cacao trees were evaluated individually, collecting data on vigor, pod and seed production, pod losses for disease, disease incidence on canopy and flower cushions, etc. As the main focus was given to clone selection, almost 500 plants were selected in these populations, and they were coded as CP series. Also, emphasis was given to selection to self-compatibility.

In the SP-2, populations are being improved through recurrent reciprocal selection. Progenies were produced according to North Carolina II mating design, involving 16 distinct clones from the germplasm collection, chosen on the basis on phenotypic and molecular evaluation. Plants selected within these progenies were used for advancing generation to a second cycle of recombination. Also, marker assisted selections is being considered for genotype selection.

Clones selected in the two subprograms and some farm selections are being tested either in multilocational trials or in five networks of clone evaluation. It involves many farms, distributed in the cocoa region of Bahia. Statistical designs were adopted.

New breeding populations are being produced focusing also moniliasis, an exotic disease caused by *Moniliophthora roreri*. CP selections with high resistance in pods to witches' broom are being used as parents together with others known sources regarded as resistant to moniliasis. Some of selected clones are being recommended as planting material to the cacao growers.