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GENERATION OF CACAO CLONES WITH DURABLE RESISTANT AGAINST FROSTY POD ROT (*Moniliophthora roreri* (Cif. & Par.) Evans *et al*.).

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SUMMARY

Cacao diseases are responsible for substantial losses at global scale, now fostered by the unprecedented effects of climate change. In a perennial crop such as cacao mostly grown by small farmers with limited resources, breeding for resistance to pathogens must have top priority worldwide and the durability of resistance must become central to sustainable disease management.

CATIE is developing a strategy for the generation and deployment of varieties with durable resistance against *Moniliophthora roreri*, the causal agent of moniliasis or frosty pod rot (FP), the most devastating disease in tropical America. The strategy is based on the sequential accumulation of FP resistant genes with different geographic and genetic origin into single varieties (pyramiding). This takes advantage of the polygenic and predominantly additive nature of FP resistance in cacao. CATIE is applying a holistic approach to enhance cacao performance and durability of resistance by simultaneously generating genotypes that combined FP resistance with agronomic relevant traits such as high yield, good quality and resistance to black pod rot (*Phytophthora palmivora*).

Screening for resistance to FP began at CATIE in the early 1980´s when a reliable inoculation method was consolidated. The method has been the cornerstone for the routine evaluation/selection of clones resistant/tolerant to FP. So far, 278 clones have been rated as resistant or moderately resistant. They belong or are related to the ten genetic groups described by Motamayor *et al*. in 2008. Since most of these clones originated in areas where FP was absent, it is probable that non-specific genes are responsible for FP resistance in cacao, and thus, they could protect the plant against other pathogens.

The Nacional, Criollo and Marañon genetic groups were the most important sources of FP resistant genes in the first stages of CATIE´s breeding program. Some of the resulting clones were released for farmer use in Central America and Mexico starting in 2007. They are mainly planted in polyclonal layouts that maximizes cross-pollination among the inter-compatible clones and reduce the natural pressure and impact of FP. It has been pointed out that resistant genes will last longer in cultivar mixtures than in pure stand simply owing to reduced exposure to the pathogen.

The current plant breeding approach at CATIE utilizes classical breeding techniques to incorporate into the Program novel resistant genes from other genetic groups. CATIE's efforts have global relevance in view of the growing threat of the spread of FP into other continents.

INTRODUCTION

The cacao crop and its associated multibillion-dollar value chain are at risk due to the growing spread and impact of diseases and the associated effects of climate change. The apparent predominance of susceptible varieties against frosty pod rot or moniliasis (FP) and other important diseases aggravates the situation. This contrasts with the status of crops grown in the United States where 75% of the varieties are resistant (Schumann & Darcy 2006).

FP caused by the basidiomycete *Moniliophthora roreri* is the most destructive cacao disease in tropical America. The aggressiveness of the fungus, its capacity to survive diverse environmental conditions, its rapid natural dispersal, its propensity for human-mediated dispersal, and the susceptibility of most commercial cacao genotypes, all indicate that FP presents a substantial threat to cacao cultivation worldwide (Phillips & Wilkinson 2007).

Disease resistance is a formidable, rate-limiting tool in disease management and has been responsible for some of the most important advances in production agriculture during the last century (Ploetz 2007). There is strong consensus, that growing genetically resistant cultivars is the most appropriated and cost effective means of managing pests and diseases as it has no cost to farmers and is environmentally safe (Niks & Rubiales 2002, Singh *et al*. 2004). The significance of resistance and its durability for plant production

especially in developing countries, justifies that breeding for resistance be given top priority worldwide (Hogenboom 1992). Durable resistance can be defined as resistance that remains effective during its prolonged and widespread use in an environment favorable to the disease (Johnson 1984).

Plants defend against pathogen invasion using a suite of highly conserved resistance (R) genes (Corrion & Day 2015), which can rightfully be considered one of the most important natural resources determining the survival of the human species (Mundt, 1994). There is broad agreement that combining resistant genes (gene pyramids) is a useful approach for increasing durability (Mundt 2014), and central to sustainable disease management (Vera Cruz *et al.* 2000).

The main purpose of gene pyramiding in breeding is to develop cultivars with durable resistance (Acquaah 2012). Resistance due to the additive action of many genes (also called polygenic, quantitative, horizontal resistance) is expected to be more durable than resistance due to the action of a single gene (also called monogenic, qualitative, vertical resistance) (Lo Iacono, van den Bosh & Gilligan 2013). Since multiple R genes are crossed into a single cultivar, it is very unlikely that a pathogen would overcome all of the R genes (Schumann & Darcy 2006).

Inheritance of the resistance for the main cacao diseases is variable. It is quantitative and mainly additive for black pod rot (Cilas *et al*. 1995). Research carried out in the 1970s revealed the presence of different levels of partial resistance to this disease based on polygenic systems (Nyasse *et al*. 2003). Conversely, resistance to witches´ broom is attributed to a single recessive major gene, which is homozygous in SCA-6 (Meinhart *et al.* 2008). The inheritance of resistance to *Moniliophthora roreri* is polygenic (Phillips-Mora & Galindo 1988), and predominantly additive (Cervantes-Martinez et al. 2006). Five QTLs for frosty pod resistance were reported by Brown *et al.* (2007), and 873 differentially expressed *M. roreri* genes with the primary difference being whether the clone was susceptible or tolerant were identified using RNA-Seq analysis (Bailey *et al*. 2014).

During the last two decades, CATIE's Cacao Genetic Improvement Program with the collaboration of the World Cocoa Foundation (WCF), USDA-ARS and MARS has developed a successful genetic improvement program with an increasing impact in Central America and México (Phillips-Mora *et al.* 2013). In 2007, the Program released superior clones that are currently available to more than 6.000 farmer families in the region. Emphasis has been placed on both, the selection of highly producing clones with good quality and resistance to frosty pod and black pod rots, and the generation of highly resistant clones by combining different sources of resistance.

The main goal of this paper is to describe the current strategy being carrying out at CATIE for the generation and deployment of superior varieties with durable resistance against *Moniliophthora roreri*.

DEVELOPMENT

The Cacao Breeding Program at CATIE is applying a holistic approach to enhance cacao performance and durability of resistance by generating genotypes that combined FP resistance with other relevant agronomic traits. Selection for resistance alone will not generate relevant varieties, unless it is simultaneously combined with other traits such as high yield and quality. The research was initiated 22 years ago but was intensified during the last few years as more resistant/tolerant materials were identified and more information on the genetic background of the materials became available.

The development of varieties with durable resistance involves the sequential accumulation of resistant genes with different geographic and genetic backgrounds into single varieties using conventional breeding procedures. Three basic steps are involved:

1. Mass screening and selection of FP resistant parents.
2. Production/testing of inter-clonal crosses among "resistant x resistant" parents with different genetic background.
3. Selection and released to farmers of superior clones combining high levels of disease resistant and other agronomic attributes.

A detailed description of the mentioned aspects is giving as follows.

1. MASS SCREENING AND SELECTION OF FP RESISTANT PARENTS

In order to identify genetic resistance in germplasm, it is necessary to expose a large collection of more or less exotic accessions of the crop species to inoculum of the target pathogen species (Niks & Rubiales 2002). The accessibility of a broad genetic diversity, the selection of virulent isolates for testing the genotypes, and the availability of reliable artificial inoculation techniques are essential elements for the effective identification of resistant/tolerant genotypes. CATIE meets these requirements.

The International Cacao Collection at CATIE (IC3) preserves a broad representation of the genetic diversity of *Theobroma cacao,* which comprises 1,235 clones with different geographic/genetic origin. IC3 is one of the two universal depositories of cacao, and a central part of the "Global Strategy for the Conservation and Use of Cacao Genetic Resources" (CacaoNet 2012). The collection is genetically enriched by the annual introduction of new germplasm from different sources, particularly from the Intermediate Quarantine Station at the University of Reading, UK (Phillips-Mora *et al.* 2013).

A successful resistance/tolerance screening program also requires an appropriate inoculation technique. The technique has to be consistent, provide a stable inoculum concentration, be easily and rapidly applied, and should mimic natural (field) infections as closely as possible (Rudgard, Maddison & Andebrhan 1993). A reliable inoculation method was consolidated at CATIE early in the 1980´s based on previous experiences from Colombia and Ecuador (Sánchez *et al*. 1987). The methodology is summarized as follows (Phillips-Mora *et al.* 2005): Two- to 3-month-old pods are spray-inoculated in the field with 0.5 mL of a freshly prepared spore suspension (1.2×10^5 spores mL^{-1}). Pods are then covered with a transparent plastic bag containing a paper towel and 50 mL water for 2 days to maintain humidity. Nine weeks after inoculation, the percentage of internal necrosis in each fruit when sectioned longitudinally is measured using the following scale: 0 = 0%; 1 = 1–20%; 2 = 21–40%; 3 = 41–60%; 4 = 61–80%; and 5 > 80%. Cacao clones are classified according to mean internal severity values, as follows: highly resistant (IS = 0.0 – 1.0), resistant (IS = 1.1 – 2.0), moderately resistant (2.1 – 3.0), moderately susceptible (3.1 – 4.0) and susceptible (4.1 – 5.0).

This methodology has been the cornerstone for the routine evaluation and selection of resistant/tolerant (R/MR) clones against FP at CATIE for more than three decades. The frequency of these clones in CATIE´s genebank is higher than initially estimated (Phillips-Mora *et al*. 2009). As many as 278 clones have been rated as R/MR, but no immune or highly resistant genotype except CATIE-R6 has been identified so far (Phillips-Mora, unpublished results). However, since the experimental conditions and particularly the fungal isolate varied during such long period, a re-evaluation of the entire group of R/MR clones is currently in progress at CATIE under uniform conditions.

The first results indicate that within the group of 278 clones some have a moderate susceptible or susceptible reaction; however, most of them apparently maintain their R/MR status. The reaction of 50 clones was already corroborated. Information on the genetic background of these clones is summarized in Table 1. A very relevant finding is the fact that within this group there are representatives of eight of the ten genetic groups described by Motamayor *et al*. (2008): Amelonado, Criollo, Curaray, Guiana, Iquitos, Marañon, Nacional, and Purús. Within the two missing groups (Contamaná and Nanay) there are clones with an apparent R/MR reaction, however their final rating will be determined as more information is collected. This means that resistance to FPD is widely spread in the complete known genetic diversity of *Theobroma cacao*, and involves different countries and contrasting regions and environments.

The predominance of R/MR clones from French Guiana suggests that an important selection pressure have led the emerging of resistant genotypes in this country, apparently associated with organisms different to *M. roreri*, which is not present there. Interestingly, the only resistant clone already identified in the Amelonado Group, also originated in French Guiana. In Peru, at the western side of South America, a similar process occurred in different genetic groups. The consistent emergence of R/MR clones in regions where FP is not present, suggest that non-specific R-genes are mostly responsible for FP resistance in cacao. It can be conjectured that the accumulation of these genes in single individuals would protect the plants against both, FP and a series of endemic and foreign pathogens.

None of the R/MR clones originated from the proposed center of origin and genetic diversity of *M. roreri* in Eastern Colombia (Phillips-Mora, Aime & Wilkinson, 2007; Ali *et al.* 2015), a region of high priority for collection expeditions seeking wild cacao trees with potentially novel sources of resistance (Phillips-Mora 2003).

2. PRODUCTION/TESTING OF INTER-CLONAL CROSSES.

CATIE, with support from the World Cocoa Foundation (WCF), began in 1996 a genetic improvement program focused on the generation of high-yielding and FP resistant varieties (Phillips-Mora *et al*. 2013). The Program was strengthened with parallel projects in partnership with MARS, USDA and other associates. In the last 22 years, CATIE has established at least one inter-clonal field trial per year in La Lola farm located on the Atlantic Coast of Costa Rica (40 m.a.s.l., 24.5 °C average annual temperature, and 3,560 mm average annual precipitation). The trials are evaluated monthly and by tree using parameters related to production, such as the number of healthy fruits and the fresh weight of the seeds, or the reaction to moniliasis and black pod for which the number of diseased fruits is counted. When the clonal or progeny trials accumulate five years of data, a first selection is made of the best trees based primarily on their productive performance and reaction to FP. The trees selected are vegetative propagated and established in a clonal trial along with other pre-selected clones and local or international controls such as CCN-51. For their part, the selected clones go on to more advanced stages of improvement, such as the establishment of clonal gardens, observation plots, multi-location trials or tests on producers' farms (Phillips-Mora *et al*. 2013).

The most promising long-term disease control strategy is to breed and deploy clones carrying durable resistance based on minor genes with additive effects (Singh *et al.* 2004). Breeding for resistance is mostly a cumulative process called gene pyramiding, cultivars with resistance being the basis for later cultivars with multiple resistances (Hogenboom 1992). Thus, developing elite varieties requires breeders to combine traits from multiple parents (Francis *et al.* 2012).

A program of crossing and selection for the accumulation of FP genes in single individuals is an integral part of the CATIE´s breeding efforts since the beginning. However, the recent information available on the genetic background and parentage of many cacao clones (Motamayor *et al.* 2008; http://www.cacaogenomedb.org/shrs/exportgenotypes.php) has opened novel avenues for focusing the crosses in a more effective way. The generation of resistant varieties exploits the predominantly additive character that resistance to FP has in cacao (Cervantes-Martinez *et al*. 2006), and the fact that hybridisation between distant individuals or distinct populations lead to increased chances of achieving heterosis and genetic gain (Baudouin et al. 1997; Lachenaud *et al*. 2007). In this sense, the corroboration of the R/MR reaction of clones belonging to different genetic groups indicated in the previous section became very useful to re-direct the breeding efforts at CATIE.

During the first phases of CATIE´s Breeding Program, the most extensively genotype used in the crosses was UF-273, a clone early identified as resistant at CATIE (Phillips-Mora 1986), which was included in approximately 42% of the crosses. UF-273 has a genetic composition of 66% Nacional and 33% Amelonado (Zhang *et al*., unpublished results). The putative Amelonado parent is Matina, a highly susceptible variety prevalent in Costa Rica when FP caused a devastating impact after its arriving in 1978 (Phillips-Mora, Ortiz & Aime 2007). Concordantly, it is very feasible that FP resistant of UF-273 originated from its Ecuadorian National background. In fact, resistance to FP was reported in pure stands of cacao Nacional in Ecuador as early as 1926 (Rorer 1926). The reaction of UF-712 supports this hypothesis since it is a resistant clone with a pure Nacional ancestry. Approximately 10% of the crosses evaluated at CATIE have UF-712 as parent. UF-712 has a positive combining ability for FR resistance and UF-273 an important additive gene effects for productivity (Cervantes-Martinez *et al*. 2006).

Conversely, the presence of Amelonado lineage in UF-273 probably has a detrimental effect on the resistance to FP. The putative parent of UF-273 is Matina, a highly susceptible variety (Phillips-Mora, Ortiz & Aime 2007) closely related to the African Amelonado and the Común variety from Bahia. Matina was widely used in Costa Rica as a parental variety in diferent crosses, consequently several clones from the Series UF, CC, ARF, and PMCT have a Matina parentage.

Aside from UF-273, each of the following clones has a 11-13% contribution in the crosses evaluated at CATIE: CC-137 (a moderately susceptible clone with 73% Amelonado and 24% Criollo ancestry), and PA-169 (a moderate resistant clone with a 100% Marañon ancestry). The following hybrid clones participated in less than 5% of the crosses: ARF-22, ARF-37, CC-124, CC-252, EET-75, and ICS-95.

Some successful examples exists at CATIE demonstrating the positive impact of combining different sources of resistance. For instance, the clone CATIE-R6 derives from the combination of two genetic backgrounds: UF-273 a clone with a high parentage of Nacional from Ecuador, and PA-169, an upper amazon clone belonging to the Marañón group. Along the same lines, the cross between UF-273 and Pound-7 produced an outstanding group of clones with double resistance to FP and black pod rot. A heterozygous F1 mapping population between these clones revealed that there are five QTLs associated

with FP resistance, with UF 273 appearing to be the source of resistance. Three QTLs for black pod resistance were also identified with the most favorable alleles coming from Pound 7 (Brown *et al*. 2007).

3. SELECTION AND RELEASED OF SUPERIOR CLONES

CATIE selected a group of six high yielding, moniliasis-tolerant clones for commercial use in 2007 (Phillips-Mora *et al*. 2013). The clones CATIE-R1, CATIE-R4, CATIE-R6, CC-137, ICS-95 T1 and PMCT-58 show an increasing expansion and impact in Central America and Mexico, where they are part of different regional initiatives to modernize plantations and improve incomes and living conditions of farm families. The clones were also introduced in Brasil for preventive breeding in 2013 and 2015. Several other clones are candidates for future releasing to complement or substitute some of the mentioned materials.

It has been pointed out that resistant genes will last longer in cultivar mixtures than in pure stand simply owing to reduced exposure to the pathogen (Mundt 2014). In accordance with this concept, the deployment strategy recommended by CATIE includes the establishing of the clones in mixtures or polyclonal layouts that minimize disease spread and impact, and maximize pollen exchange among inter-compatible clones (Figure 1). If genetic uniformity makes a crop more vulnerable to diseases, then one potential, low cost method of suppressing disease is to increase the genetic diversity of the crop. A simple way to enhance genetic diversity and increase resistance durability is to mix in the field different genotypes that vary in their susceptibility to the pathogens. This method ensures genetic diversification with the advantage that it can be used in addition to any other form of disease control (Wolfe 1988). In fact, when resistance genes are used, the probability of selecting a pathogen strain that can overcome the resistance is reduced by a good cultural program (Schumann & Darcy 2006).

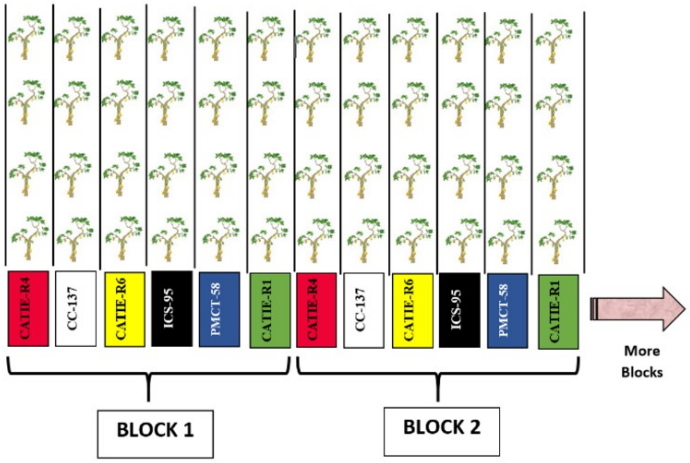

Figure 1: Polyclonal layout recommended by CATIE for commercial plantations to minimize the spread and impact of frosty pod and black pod rots and maximize the exchange of pollen among inter-compatible clones.

Another situation in which mixtures may be of economic interest is for the protection of susceptible host genotypes with superior agronomic characteristics. In that case, the deployment of the susceptible host, for instance, a high-quality but susceptible cacao variety, in combination with an agronomic inferior, but disease resistant genotype, may be a solution (Garrett & Mundt 1999).

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Table 1. Genetic background of 50 resistant/moderately resistant clones against *Moniliophthora roreri* (moniliasis = frosty pod disease). CATIE, Turrialba, Costa Rica. 2017.

Clone	Genetic and/or Geographic origin	Genetic group [1/]
ELP-37A	River Elepoussing, French Guiana	AMELONADO
CHUAO-120	Chuao, Venezuela	CRIOLLO?
LCTEEN-37	Rio Anangu, Napo River, Ecuador	CURARAY
B7-A2	French Guiana	GUIANA
B-13/1 (RUQ348)	French Guiana	GUIANA
ELP-16A	River Elepoussing, French Guiana	GUIANA
ELP-20A	River Elepoussing, French Guiana	GUIANA
GU 139-A	French Guiana	GUIANA
GU 147-N	French Guiana	GUIANA
GU-185G	French Guiana	GUIANA
GU 249-H (RUQ228)	French Guiana	GUIANA
GU 285-B	French Guiana	GUIANA
GU 296-H	French Guiana	GUIANA
GU 310-P (RUQ815)	French Guiana	GUIANA
GU 355-N	French Guiana	GUIANA
AMAZ-3/2 (RUQ91)	AMAZ-3 Open, Amazonas River near Iquitos (Chalmers collection at INIAP, Ecuador)	IQUITOS
IMC-60	Iquitos, Peru	IQUITOS
PA-169	Parinari River, Perú	MARAÑON
UF-712	United Fruit Co., Atlantic Coast of Costa Rica	NACIONAL
Nacional-1 (A3)	Offspring of an open pollinated Nacional Clone	NACIONAL?
Nacional-2 (A26)	Offspring of an open pollinated Nacional Clone	NACIONAL?
Nacional-2 (A27)	Offspring of an open pollinated Nacional Clone	NACIONAL?
Nacional-3 (A38)	Offspring of an open pollinated Nacional Clone	NACIONAL?
RB-33/3 (RUQ40)	Rio Branco, Brazil	PURUS
ARF-37	Catongo x POUND-12. Experimento Central, La Montaña, CATIE	"Amelonado x Iquitos" hybrid
HY-2714202	IMC-67 x TSAN-792. Trinidad	"Iquitos x Uknown" hybrid
ICS-95 T1	Hacienda La Reconnaissance, North Range, Trinidad	"Criollo x Amelonado" hybrid
UF-273 T1	United Fruit Co., Atlantic Coast of Costa Rica	"Nacional x Amelonado" hybrid
ARF-33	POUND-7 x SCA-6. Experimento 14, Rep IV Árb 2. CATIE, Turrialba, CR	"Nanay x Contamaná" hybrid
FHIA-330	UF-273 X P-23 (seeds provided by CATIE)	"(Nacional x Amelonado) x Unknown" hybrid
FHIA-707	UF-273 X PA-169 (seeds provided by CATIE)	"(Nacional x Amelonado) x Marañon" hybrid
CATIE-R4	UF-273 X PA-169	"(Nacional x Amelonado) x Marañon" hybrid
CATIE-R6	UF-273 X PA-169	"(Nacional x Amelonado) x Marañon" hybrid

ARF-2	F. Th. P. Árb Viejo, Finca Theobroma, Changuinola, Panamá	??
ARF-6	Hershey, Belmopán, Belice, 111195	??
EET-75	La Pretoria, Guayas, Ecuador	??
EET-233	Pichlingue, Los Ríos, Ecuador	??
EET-401	Selección Patología 7321. Pichilingue	??
EET-407	Selección Patología 7581. Pichilingue	??
EET-610	Pichlingue, Los Ríos, Ecuador	??
EQX-27 RUQ-857	Ecuador	??
GN-20	??	??
FHIA-577	FHIA, Honduras (seeds provided from CATIE)	??
ICS-10	Imperial College, Trinidad	??
IMC-45	Iquitos, Peru	??
Laranja	Bahía, Brasil	??
Pound-16/A (RUQ26)	Nanay River, Loreto, Perú	??
Pound-18 (RUQ874)	Amazonas River, Perú	??
VM-Z	??	??
Santa Clara-3	Upala, Costa Rica	??

[1/] Motamayor *et al*. 2008; http://www.cacaogenomedb.org/shrs/exportgenotypes.php.