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# COCOA

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## 1. History, origin and distribution

Cocoa belongs to genus *Theobroma* that occurs wild in South and Central America along the Amazon basin, which is considered as its centre of origin and diversity. There are more than twenty species but *Theobroma cacao* L. is the only one, which is cultivated and has three basic populations or types. The Criollo type cocoa group evolved in Central America and the Forastero type in the Guyana region. The Trinitario type arose from hybridization between these two groups in Venezuela and the West Indies (Barrau, 1979). The Criollo type of cocoa had been cultivated since prehistoric times and is highly susceptible to diseases. Subsequently there was a shift to cultivation of Forastero types, which are hardy and vigorous and spread to commercial cultivation in West Africa and other regions. Original collections of Scavina populations were made along Ucayali river basin (Figuiera, 1997). In the Asia Pacific region cocoa populations are dominated by Trinitario types.

The germplasm collections have been done by several expeditions. A project was initiated in 1988 to produce a catalogue in the form of a computer database listing information on the origins and location of cocoa germplasm. The database contains information on over 2814 separate accessions of wild cocoa collected since 1938 and the current location of over 3000 clones from these collections. The International Cocoa Gene Bank, Trinidad and Tobago, is an international cocoa germplasm depository that conserves nearly 2500 accessions in its field collection. A portion of this germplasm was characterized for phenetic diversity with morphological descriptors from the Bioversity International (IPGRI) cocoa descriptor list. Data for 28 quantitative and 26 qualitative descriptors were obtained for 100 accessions representing 24 populations from Peru, Ecuador, Colombia, Venezuela, Brazil, Grenada, Costa Rica and Trinidad (4 of the populations were of unknown origin) (Bekele and Bekele, 1996). The computerized

International Cocoa Germplasm Database (ICGD) contains information on 8000 wild and selected clones of *Theobroma cacao*, their origins, characteristics and occurrence in germplasm collections (End *et al.*, 1992). The ICGD brings together information on cocoa clones from the literature and from data supplied by research stations. Information is provided on the names given to cocoa clones, their synonyms and homonyms, together with details of the origin of each clone, distinct characteristics, reaction to pests and diseases, photographs and SSR/ SNP profiles (Wadsworth *et al.*, 1997a; 1997b; 1997c). Some of the major germplasm collections are in Brazil, Colombia, Cost Rica, Ecuador, Trinidad, French Guyana, and Puerto Rico. To facilitate safe germplasm exchange, Intermediate Cocoa Quarantine Centre (ICQC) was established in 1987 in Europe, far away from native and productive zones of South America and Africa which are affected with debilitating diseases. University of Reading, UK with twin clad poly tunnels and virus indexing facilities taken up the responsibility of collecting clones from International Gene Banks, follows strict quarantine for two years with FAO/ Bioversity guidelines and supplying budsticks to the researchers around twenty countries (Hadley and Lee, 1992; Michelle End *et al.*, 2014) and helps in cocoa improvement programs.

Criollo cocoa (*Theobroma cacao* subsp. *cacao*) was cultivated by the Mayas over 1500 years ago and evolved independently from the cocoa populations in the Amazon basin (Motamayor *et al.*, 2002). The second morphogeographic group, Forastero cocoa was assigned to *T. cacao* subsp. *sphaerocarpum*. To gain further insight into the origin and genetic basis of Criollo cocoa from Central America, RFLP and microsatellite analyses were performed on a sample that avoided mixing pure Criollo individuals with individuals classified as Criollo, but which might have been introgressed with Forastero genes. These distinguished two types of individuals are classified as Ancient and Modern Criollo. In contrast to previous studies, Ancient Criollo individuals formerly classified as 'wild', were found to form a closely related group together with Ancient Criollo individuals from South America. The Ancient Criollo trees were also closer to Colombian- Ecuadorian Forastero individuals than these Colombian-Ecuadorian trees were to other South American Forastero individuals. RFLP and microsatellite analyses revealed a high level of homozygosity and significantly low genetic diversity within the Ancient Criollo group. The results suggest that the Ancient Criollo individuals represent the original Criollo group. The results also implies that this group does not represent a separate subspecies and that it probably originated from a few individuals in South America that may have been spread by man within Central America (Motamayor *et al.*, 2002 and 2003).

## 2. Botany

Cocao (*Theobroma cacao)* is a wide-branching evergreen tree, grows in the shade as an under storey crop in partially cleared forests and as a component crop in the agro forestry systems. It reaches to a height of 20-25 feet with a typical plant habit of branching in tiers (Wood and Lass, 1985). Recalcitrant seed of cocoa germinate as an epigeal plant with hypocotyl raising the cotyledon above ground and this first phase of development is termed as 'soldier' stage. Vertical growth continues until the plants reach a height of 1- 2 m and at this point, the vertical growth ceases and at the terminal end five buds will appear with short internodes and grow outwards uniformly as 'fan branches' and the process is called 'jorquetting'. Cocoa tree shows dimorphic growth habit where, vertical stem is 'orthotropic' and the fan branches are 'plagiotropic'. After some time of growth, from the jorquetting point upright shoots will arise which are called as 'chupons'. The chupons have capability of forming a jorquette in due course. This process will continue as the plant grows and it forms multiple tiers. Leaves present on the chupons have long petioles with symmetrical spiral arrangement whereas, fans have shorter petioles with asymmetrical alternate leaves. The petioles have marked pulvinus or swelling at each end which allows the leaf to be oriented in relation to the light. Leaves are pale green to red in colour depends on genotypes and flushing depends on the environmental factors. Medium to small trees with high yield is preferred in the selection programs.

Small white flowers come into bloom almost throughout the year and flowers, flushes and fruits can be seen at any time of the year, all together on the same tree. Two harvests are made per annum. The plant is "cauliflorous" with flowers (and later fruits) protruding directly from the woody branches and trunk (http://botit.botany. wisc.edu/ courses/ tour/ Roomeight-Th.html). The leaf axils become thicker after producing flowers and pods for several years are called as 'cushions'. Cocoa is a cross pollinated crop reproduces sexually. Flowers are about 15 mm in diameter, borne on long pedicels, have 5 free sepals, 5 free petals, 10 stamens and ovary of 5 united carpels. The petals have a distinct shape, they are very narrow at the base but expand into a cup shaped pouch and end in a broad tip or ligule. Androecium is in two whorls, the outer consisting of five long non fertile staminodes, the inner whorl with five fertile stamens. The stamens bear two anthers which lie in the pouch of the corresponding petal which is to be removed carefully while doing hand pollination. Style is single and the ovary contains 30 to 60 ovules and it is a highly heritable characteristic of cocoa. Though cocoa trees produce large number of

flowers, only 1-5 percent of the flowers are successfully pollinated to produce pods. Higher proportion of pod set reported in Amelonado type cocoa.

Self- incompatibility exist in cocoa which was first reported by Pound (1932), when he showed that certain trees in Trinidad could not set fruit with their own pollen nor with one another. In many plants incompatibility occurs in the style or stigma, preventing the development of pollen tubes, but in cocoa, the pollen tubes develop normally in all cases, but when the mating is incompatible the male gamete does not fuse with the female gamete and considered as 'gametophytic' (Cope, 1962 and Bartley and Cope, 1973). Cope (1962) in Trinidad proposed five different S-factors or S-alleles to explain the dominance relations and expressed in the formula: Sa=Sb=Sc>Sd>Sf. Previously Knight and Rogers (1955) studied the compatibility relations within few families of Amazon material in Ghana and explained the dominance relations as S1>S2=S3>S4>S5. In an incompatible pollination the proportion of non-fusion between the gametes will be 25, 50 or 100 per cent. In a failed set, the flower falls off three or four days after pollination.

The degree of incompatibility varies between different populations. Self-compatible genotypes are found in Lower Amazon Forastero, Criollo and Trinitario, whereas the Upper Amazon Forasteros are generally self-incompatible (Eskes and Lanaud, 2001). Amazon cultivars are all self-incompatible but are generally cross- compatible. Trinitario cultivars have a high proportion of self- incompatible trees, which will not cross with other self- incompatible trees, requiring pollen from self- compatible trees for successful pollination. The Amelonado population is entirely self-compatible. It is always suggested to plant mixture of populations. Self-incompatible but cross compatible clones are being used in production of hybrids and establishment of well-designed bi-clonal and poly-clonal orchards for production of hybrid seeds of known parentage and performance, which is essential in cocoa improvement and planting material production. Vegetative propagation through budding and grafting will speed up the evaluation trials.

Cocoa starts bearing after 4 or 5 years but yields most between 15 and 25 years of age. The immature fruit is called 'cherelle' and undergoing a natural thinning or physiological phenomena of cherelle wilt after 25, 50 and 75 days of pollination. It may be observed in the trees as pod mummies. The fruit, or "pod", reaches to one foot long and 2-4 inches in diameter. Criollo, Forastero and Trinitario types varied with pod characteristics.

Cocoa pod mature from green to yellow or red to orange while ripen and about 10 inches long, 6 inches wide and weighs about 500 g. Pod contains 30 to 50 beans surrounded by white mucilage, which is essential for fermentation and processing.

### 3. Species and Cultivars

Cocoa, *Theobroma cacao* L. belongs to family Sterculiaceae, reclassified to Malvaceae and its genome is sequenced (Lanaud *et al.*, 1992; Figueira *et al.*, 1992; Xavier *et al.*, 2011). *Theobroma* includes 22 species, some of the important ones are *T. bicolor, T.grandiflorum, T.angustifolium, T.microcarpum, T.mammosum, T.simiarum, T.speciosum* and *T.subincanum. T.grandiflorum* is consumed widely as 'cupuacu' in Brazil for its flavoured mucilage surrounding the beans. *T. cacao* is a diploid species with chromosome number 20 with a small genome of around 380 Mbp. The species has two sub-species viz., *T. cacao* spp *cacao* of Criollo populations and *T.cacao* spp *sphaerocarpum*, which includes all other types. There are three main types of *T.cacao viz.*, Criollo, Forastero and Trinitario (Wood and Lass, 1955). The Criollos (Fig.1) are characterised by soft red pod husk with 20-30 white, ivory or pale purple beans. The beans ferment faster with weak chocolate flavour. They are less vigorous and susceptible to diseases. Soria (1970) has described several types of Criollo like Mexican Criollo, Pentagona or Lagarto, Nicaraguan Criollo, and Columbian Criollo. The Forastero (Fig.2) belongs to large group of cultivated to semi-wild types. These are hardier than Criollos and most of present cultivated area comprises of Forastero. Amelonado, Comum, West African Amelonado, Cacao Nacional, Matina or Ceylan, Guiana wild Amelonado and Amazon populations are classified as Forasteros. Forastero has hard green pods and 30 or more, paler to deep purple beans. Majority of area in Brazil and West Africa is predominantly occupied by type known as Amelonado. Amelonados have uniform pod characteristics. The Trinitario type can be considered as Forastero and they have characters intermediate to Criollo and Forastero. They are believed to be cross populations of these two types of cocoa and are widely found in South East Asia and Oceania. Criollos are called as 'fine cocoa' whereas Forasteros and Trinitarios are called as 'bulk cocoa'. There are few old and new classifications like Angoleta, Cundeamor, Calabacillo and Refractarios. Cocoa populations and genotypes are grouped based on pod shapes, basal constriction, apex form, surface rugosity, prominence of ridges and furrows, husk thickness, pod size, presence of anthocyanin pigmentation etc. Diversity in the cocoa populations are widely utilized in the breeding programs.

Over the years several varieties of cocoa have been developed in different countries (Toxopeus, 1985). Some of the important characters that are considered for breeding are high yield, bean quality, vigour and tolerance to biotic and abiotic stresses. The countries developed their own varieties from germplasm collections maintained by them. Some of the important parent materials are described here. The ICS clones (Imperial College Selections) were developed in Trinidad from local Trinitario populations. Nearly hundred trees were selected and coded ICS 1 to ICS 100. Some of the best clones are ICS 39,40,48,60 and 95. Expeditions by Pound in Upper Amazon region resulted in some some witches broom disease resistant lines like SCA 6 and 12. Other materials are IMC (Iquitos Mixed Calabacillo), NA (Nanay), PA (Parinari) and SCA (Scavina) (Posnette, 1982).

In Brazil hybrid programme was started with local SIC clones and imported clones (Alvim, 1975). The EET series are from Costa Rica. In African countries *viz.*, Ghana, Nigeria and Ivory Coast the Amazon parents were used in crossing programmes. In Malaysia after initial set back of Amelonado material, Upper Amazon introductions were tried as planting materials.

**4. Genetics and Breeding**

The breeding of cocoa is aimed at improving yield, resistance to biotic and abiotic stresses and quality characteristics. Cocoa has long been a crop of near- mystical human importance among indigenous Mesoamerican cultures and its importance in modern culture is growing with new realizations of the potential health benefits of cocoa polyphenolic compounds. However, cultivated *T. cacao* is vulnerable to emerging disease pressures because it has a narrow genetic base, and systematic genetic improvement of the crop is imperative. A wide range of genomic tools and resources has been developed and are providing the basis for genome-based breeding and gene discovery (Bennett, 2003). Since the cocoa genome is sequenced, the most promising strategy in cocoa breeding is expected to be in increasing resistance or tolerance to *Phytophthora* pod rot, Witches' broom, *Moniliophthora* pod rot, and vascular streak dieback, the cocoa swollen shoot virus and the cocoa pod borer. Genetic improvement of cocoa began with the domestication of Criollo varieties in Central America. They were gradually superseded by Trinitario selections, and then by Forastero trees, which have better disease and insect resistance. In 1940, crossing Forastero lines, collected during survey in Upper Amazonia, with other genetic groups resulted in substantial increases in precocity and

productivity. Hybrids between different genetic groups were distributed widely (Paulin and Eskes, 1995). Four comparative multilocation hybrid trials of cocoa involving approximately sixty crosses between sixteen Upper Amazon clones and four locally selected Amelonado clones were evaluated in Cote d' Ivoire (Paulin *et al.*, 1993). The multilocation yield analysis revealed a significant hybrid-location interaction, although this was of minor importance in relation to the principal effects.

Breeding at the Indonesian Oil Palm Research Institute began in 1975 with introduced genetic materials. Hybrid seed for breeding was obtained after selection on the basis of trials of progenies and clones. Progress in breeding of new clones increased the yield by 20-40% over that of the hybrid seedling material (Napitupulu, 1993). The progenies of 17 double crosses of cocoa involving selected Nanay and Parinari hybrids were assessed, with 2 three-way crosses of variety Scavina and with F3 Amazon (CRIN Synthetic Series I) as controls, for survival, precocity, yields of pods and beans, pod-rot incidence and pod characters during 1983-85. The use of about 50 per cent of the progenies as a composite variety is suggested. The progenies (NA 173 x NA 387) x (PA 195 x PA 56) and (NA 387 x NA 20) x (PA 168 x PA 295) gave the highest yields of pods and beans (Ojo *et al.*, 1991). The performance of the cocoa variety Amelonado and its hybrids with Upper Amazon and Trinitario clones produced in Fiji in the early 1970s were evaluated in trials established in 1971-73 at several sites. Amelonado recorded the highest mean yield, with an annual yield of 2106 kg/ha during 1979-85 at Wainigata, although it was out yielded by the hybrids at some sites. Amelonado also showed acceptable pod value and bean weight, tolerance of black pod (*Phytophthora palmivora*) and adaptability to farmers' fields, justifying its current position as the only recommended variety for Fiji (Martin, 1987). Data on dry bean yield and vascular streak disease (*Oncobasidium theobromae)* infection in progenies of crosses planted during 1972-77 in 5 separate trials were studied in Malaysia (Ooi and Chew, 1985). Progenies are ranked on the basis of their yield relative to UIT 1 x NA 32 (62 entries with some duplicates between trials) and UIT 1 x NA 33 (48 entries). Individual hybrids showed considerable variation in performance between sites. Percentage vascular streak disease infection was generally higher in Amelonado clones and crosses with Amelonado parentage, which also tended to give yields inferior to those of the Trinitario and Upper Amazon hybrids. PA 7 was a parent of 7 of the 15 best crosses. The cumulative dry bean yield of seed garden mixed hybrids of unknown composition was inferior to that of hand-pollinated and seed garden identified hybrids by 12 and 8%, respectively.

Scavina (SCA) hybrids are Forastero selections of cocoa collections made in 1938 in the Upper Amazon region of Ecuador. In two trials, 18 Scavina hybrids were evaluated for yield, vegetative growth and resistance to vascular streak dieback caused by *Oncobasidium theobromae.* ICS 40 x SCA 9, ICS 40 x SCA 20, UIT 1 x SCA 9 and UIT 2 x SCA 20 were the best hybrids, giving a mean annual yield of 2.5-2.8 kg dry beans/ tree in the first 9 years (Yew *et al.*, 1991).

At the Cocoa Research Institute of Ghana, breeding work has been done on genetic resources, improving yield and establishment potential, and breeding for resistance to cocoa swollen shoot virus and black pod (Adu-Ampomah, 1996)). In terms of precocity, yield, combining ability and resistance to *Marasmius perniciosus* SCA 6 and SCA 12 are the outstanding parents tested to date in Trinidad. In Ecuador, ICS 6 x SCA 6 has proved outstanding for yield but both SCA clones and their hybrids have completely lost former resistance to *M. perniciosus*, probably owing to the development of more virulent strains. Much genetical material is now available in Trinidad, including F4 Scavinas, but the importance of collecting sources of resistance to the major diseases to broaden the basis of resistance breeding is urged (Chalmers *et al.*, 1972). Fifteen CRIN (Cocoa Research Institute of Nigeria) elite varieties have been produced as a result of a programme, the major objectives of which were to develop superior genotypes with good establishing ability, high levels of disease resistance, tolerance or escape, high yield and several other desirable commercial qualities. Concurrent projects included inbreeding of selected clones, diallel crossing of WACRI (West African Cocoa Research Institute) clones, establishment of a germplasm collection and the Nigeria-Trinidad introduction programme (Opeke *et al.*, 1972).

In the Asia Pacific region, Malaysia, Indonesia, India and Papua New Guinea are involving in effective breeding programs for a long time with introduced collections followed by Philippines and Vietnam. Vascular Streak Dieback (VSD), *Phytophthora* pod rot (PPR) and Cocoa Pod Borer (CPB) are common among these countries and Indonesian and Malaysian breeding programs specifically concentrating on improvement of fine flavour cocoa. Indonesia is the major producer in Asia where cocoa was introduced way back in 1560 in Sulawesi and developed widely in Java in 1880. The varieties of 1948, DR-1, DR-2 and DR-38 are called as Java-Criollo with fine cocoa characters. Later resistance breeding combined fine flavour and PPR resistance and developed selections, ICCRI-01 and ICCRI-02 and hybrids ICCRI-03 and ICCRI-04 and ICCRI-05 for VSD resistance. Sulawesi 1, 2 and ICCRI-06 combined PPR and VSD resistance

(Susilo *et al.*, 2001). ICCRI-07 and Sulawesi 3 and farmer's varieties MCC 01 and MCC 02 are developed for cocoa pod borer (CPB) resistance. Malaysian Cocoa Board (MCB) initiated cocoa breeding with Amelonado hybrids in 1950's, with Trinitario and Forastero hybrids in 1970's and with local selections from 2000 onwards. The varieties MCB C-1 to MCB C-14 and the clones PBC 123, QH 1003 and KKM 22 contributed much to the cocoa economy in Malaysia. MCB also initiated some inter specific hybridization between *Theobroma cacao* with *T. grandiflorum* and *T. bicolor.* In Papua New Guinea, at the Cocoa and Coconut Institute (PNG-CCI), upper Amazon Forasteros were introduced in 1960's and Trinitarios in the 20th century. The varieties SG1 developed for VSD, SG2 for PPR and the current clonal selections and hybrids CCS1 to CCS5 aimed at CPB resistance. In Philippines, University of Southern Mindanao taken up farmers participatory cocoa breeding and released varieties BR 25, KI , K2, K9, ICS 40, UIT 1, DR 1, P7 and UF18 as selections from farmer's fields. Institute developed hybrids BR 25 X K2, K1 x K2, UF 18 x BR 25, UF 18 x PBC 123, UF 18 x K2 and BR 25 x S5 for pod borer resistance. Nong Lam University (University of Agriculture and Forestry), Vietnam initiated cocoa improvement specifically on clones suitable for single origin dark chocolates.

In India, Criollo cocoa was introduced way back in 1798 in Courtallam in Tirunelveli district of old Madras state (Ratnam, 1961), expanded to Western Ghats region covering Malabar and Mysore states (Wood, 1964) where there is more rainy days and short dry spells. Later with Forasteros and Trinitarios cocoa became an integral part of palm based cropping system, as a mixed crop in arecanut, coconut and oil palm gardens. Cocoa improvement work started during 1969 with few collections from Malaysia at ICAR- Central Plantation Crops Research Institute (CPCRI), Regional Station, Vittal, Karnataka and in Kerala Agriculture University (KAU), Vellanikara in the year 1980. Selection breeding resulted in development of 3 clones (VTLCC-1, VTLCS-1 and VTLCS-2) from CPCRI and 7 clones (CCRP selections) from KAU. Nine accessions collected from Lalbaugh gardens, Bangalore were evaluated for yield and related characters in 1986-87, among which ICS 1 and ICS 6 performed best for number of pods/ plant (71.3 and 69, respectively) and bean yield (3.5 and 2.2 kg/ plant), and had good plant heights and canopy spreads. Single- bean weight was the greatest in IMC 67 (2 g) and this accession had the best pod value (Nair *et al.*, 1990). Seven high yielding parental clones were selected from Malaysian and Nigerian collections based on compatibility reaction and utilized in five progeny trials during 1983-1994 which involved 76 cross

combinations (Bhat, 1999). These hybridization programs mostly aimed at more number of pods (less pod index), dry bean size (high bean index), dry bean yield and drought tolerance and resulted in development of 4 hybrids as varieties (Elain Apshara *et al.*, 2005; 2007). Though individual tree selections were made from seedling progenies they have to be further evaluated in clonal trials for confirmation. Heritability for yield is low or average when based on single tree harvests (Soria, 1975) but higher when based on yields from plots containing several trees (Lockwood and Pang, 1995) and so to assess the phenotypic value of genotype even in hybrid selection programmes, clonal trials are advised (Eskes and Lanaud, 2001). In the beginning of this century, clonal selection programmes initiated with pod index, bean size and disease resistance as selection criteria for cocoa. 20 best hybrids from CPCRI progeny trials were selected and further evaluated as clones, among which 17 progenies exhibited high vigour and cropping efficiency even at early years of development as clones (Elain Apshara *et al.*, 2008). Genetic analysis on 17 plant, pod and bean characters in 44 Nigerian cocoa clones resulted in selection of superior genotypes for higher performance with traits of high heritability with high genetic advance. Based on pod yield, NC-37, NC-23, NC-26, NC-50, NC-20, NC-51, NC-27 and NC-25 were identified as heavy bearers with optimal canopy. These clones recorded single bean weight of more than 1 gram, 10-15 percent shell, high nib recovery and more than 50 percent fat making them suitable for industries as well (Elain Apshara *et al.*, 2009 and Elain Apshara and Nair, 2011). Comparative study of parents and progenies as clones identified VTLCP-6, VTLCP-2 and VTLCP-20 and parents VTLC-1 and VTLC-56 as potential yielders (Elain Apshara, 2013a). Since Indian cocoa is under six months of rainless period breeding for drought tolerance has been done in CPCRI collections (Balasimha *et al.*, 1988; Balasimha *et al.*, 1999), which gained special attention among international cocoa researchers in the current climate change scenario. Morphological, physiological and molecular characteristics of cocoa genotypes from Central and South America conserved at CPCRI were studied in relation to drought tolerance (Elain Apshara *et al.*, 2013). Genotypic difference for morpho- physiological criteria and potential antioxidant enzymes depicting drought tolerance in young seedlings were determined with cocoa clones and hybrids under controlled low moisture stress conditions (M'bo Kacou *et al.*, 2015).

At KAU, Vellanikkara a total of 119 crosses were made and some of these have been assessed (Nair *et al.*, 1994) for resistance to vascular streak dieback. Hybridization program at KAU was initiated during 1983 and continued up to 1993. During this period, a total of 159 parents were

included to produce 239 crosses. Nursery evaluation of these hybrids were done with $HD^2$ (H- Height, D- Diameter) value. 3126 superior seedlings were field planted in series I,II,III,IV and progeny trials I,II,III,IV, 163 superior hybrids were selected, utilized in various breeding programme, release as hybrids and used as better combiners in clonal gardens. Three hybrids CCRP 8, 9 and 10 with high yield and good level of tolerance to Vascular Streak Dieback (VSD) were released as varieties (Minimol *et al.*, 2011).

Using path analysis, genotypic correlations and their direct and indirect effects were estimated for 10 traits of 12 hybrids of cocoa planted in 1978 and evaluated in 1986-87 at the ESPAM/ CEPLAC Experimental Station in Medicilandia, Para. A high coefficient of genotypic correlation was found between trunk height and bean dry weight/tree. However, path analysis revealed that trunk height had a negative direct effect on yield. Number of healthy fruits/tree and weight of dry bean/fruit showed direct effects of high value on dry bean weight/tree and thus should be considered as the main yield components. The number of seeds/fruit and bean dry weight constituted the main components of weight of dry bean/fruit. Since these traits showed direct and indirect effects of high value, they should be considered as secondary yield components (Almeida *et al.*, 1994). Several multivariate methods have been used in divergence analyses of populations. These studies have shown that statistics involving the error variance-covariance matrix should be preferred whenever its estimation is possible (Dias and Kageyama, 1998). The respective performances and temporal stability were studied depending on various yield components. There was substantial variability between cultivars for fruits per tree and fresh bean weight per hectare and per fruit. The only cultivar x year interaction was for fresh bean weight per hectare. The highest yields per hectare and per fruit were obtained with the improved cultivars (hybrids and ICS 1). In short, ICS 1 seemed to be the most appropriate cultivar (Santos Dias *et al.*, 1998). Cocoa bean size was studied in a 6 x 6 diallel cross established in southwestern Cameroon, planted in 1974. Data concerning black pod frequency and yield evaluated during 1987-89 were used to study correlations between these traits and bean size. General combining ability was the main factor of variation in bean size, and this trait was highly heritable. The genetic additive correlations between incidence of black pod and bean size showed that transmission of these traits was genetically linked (Fallo and Cilas, 1998).

Variability in pod characteristics in cocoa clones are also studied which are important in breeding progarammes. Significant differences in

bean size, pod weight, shell, bean weight, number of beans/pod, were found to be significantly different in cocoa clones (Enriquez and Soria, 1968). In India, nine characters were studied in 25 ten-year-old trees. Heritability estimates were high for weight of bean with pulp and cotyledon weight. No additive gene action was indicated for all characters. There was significant variation among the ten hybrids studied for the seven yield components measured and for percentage pulp per bean and total soluble solids (%) in the pulp (Subramonian and Balasimha, 1981). There was varietal and seasonal (Table 8) variations among cocoa hybrids and parents studied (Anil Kumar *et al.*, 1999). Correlation studies showed that there was a positive relationship of ovary weight to single bean weight. Carvalho *et al.* (2003) studied tree adaptability and yield temporal stability to screen agronomically superior cultivars for yield, bean quality and resistance to witches' broom under the ecological conditions of Ouro Preto do Oeste county, Rondonia, Brazil. At maturity, the SCA 6 x ICS 1, PA 150 x SIC 328 and IMC 67 x BE 8 hybrids were superior in the simultaneous analysis of total number of healthy fruits (TNHF), total weight of fresh beans (TWFB) and mean weight of fresh beans per fruit (MWFBF). The SCA 6 x ICS 1 hybrid also had general adaptability, in spite of indicated low predictability. The IMC 67 x BE 8 cross was adapted to unfavourable environments and had high predictability for TNHF. Carvalho *et al.* (2002) studied the repeatability estimates to define the evaluation period necessary to discriminate between yields of 20 cocoa hybrids on total number of fruits harvested, total number of healthy fruits, total weight of fresh seeds, mean weight of fresh seeds per fruit, percentage of wasted fruits and percentage of wasted fruits due to witches' broom disease (*Crinipellis perniciosa*). These estimates indicated that three evaluations are necessary from the 8th year after planting for efficient selection (accuracy >70%) for all the assessed traits. When the average of the assessments carried out in two pre-climax years was considered, it was possible to obtain a correlation greater than 0.70 between the real hybrid value and the traits total number of fruits collected and percentage of wasted fruits. Lachenaud and Montagnon (2002) developed a method developed in coffee tree breeding to assess family competition effects (partner effects) in comparative variety trials for application to the cocoa tree. The study was conducted in a hybrid comparative trial planted in French Guiana, involving twelve families of 50 trees in a totally randomized single-tree plot design, at a density of 1,667 trees per hectare. The trial was thinned at 10 years, at a rate of two out of four rows. Competition was studied with reference to juvenile and adult vegetative vigour and to periodic and cumulative yields (number of pods, potential weight, average weight of one pod, and the

production: vigour ratio). At the end of the trial, after thirteen years of monitoring, competition effects were revealed which explained 8 to 10% of the residual variance after removal of the hybrid and micro-environment effects. These effects, which were detected as early as 18 months, occurred earlier than generally acknowledged. Under the trial conditions, the families could be classed as aggressive, stimulating or passive for their neighbours. Vegetative vigour (trunk cross-section) explained 34% of the competition effects (partner effects), which, with hindsight, vindicated the use of the production: vigour ratio as the main selection criterion in cocoa breeding. The partner effects noted on the production variables were never explained by any production variable, hence non-aggressive high-yielding families can be selected.

Mutation breeding is also being attempted in cocoa. The possible interaction between nuclear and cytoplasmic genes of a cacao growth mutant, MJ 12-226 was reported from Papua Newguinea (Efron *et al.*, 2003a). Controlled hand pollinations were done between cocoa mutant MJ12-26 as the female parent and 4 normal clones as males (OTC-1, Matina-1-9, KA2-101 and EET 308). Reciprocal crosses were made using KA2-101 and EET 308 as females and the mutant MJ12-226 as male. All crosses with the mutant as the female parent showed a significant fit to a 1:1 ratio between the normal and the mutant phenotype, similar to the ratio obtained in open pollinated pods. When the mutant was used as a male parent, all seedlings except 10 (out of 585) showed a mutant phenotype. The average height of these 10 normal seedlings from the cross EET 308 x MJ 12-226 was significantly higher by 24.1% than the average height of the 10 normal seedlings from the reciprocal cross, MJ 12-226 x EET 308. Efron *et al.* (2003b) tested the effect of the cocoa growth mutant (MJ 12-226) rootstock on bud sprouting as tested in 2 different experiments in Papua New Guinea. In the first experiment, the mutant rootstock was compared with 2-week-old normal rootstocks of open pollinated pods of genotype RST and 2-month-old normal segregants from open pollinated pods of MJ 12-226; and the scions used were plagiotropic buds from clone 21-4-8, MJ 12-226 and 33-15/1, and orthotropic buds from the clone 33-15/1. In the second experiment, the rootstocks tested were normal and mutant seggregants obtained from controlled hand pollination of the mutant MJ 12-226 (female) and several other clones as males; and the scions were plagiotropic and orthotropic buds of the clones 17-3/1, 33-15/1 and 37-13/1. Results confirmed the hypothesis that buds grafted into the growth mutant MJ 12-226 sprouted faster than those on normal rootstocks.

Inbreeding forms a part of the breeding activities, not only to breed parents with some degree of homozygosity for the production of hybrids, but also to breed materials homozygous for desirable traits like disease resistance. Existence of self- incompatibility in cocoa makes inbreeding efforts difficult but with self-compatible trees, selfing is possible which should be continued upto six to seven generations to attain homozygosity and thereafter to be utilized for crossing to exploit the hybrid vigour. KAU taken up this task of selfing self compatible plants over 20 years of continuous effort and maintains two genotypes of S4 generation, 5 of S3, 9 of S2 and 51 genotypes of S1 (Mallika *et al.*, 2002; Vikraman Nair *et al.*, 2002; Minimol *et al.*, 2011).

### 4.1. Resistance Breeding for diseases

A large amount of research is going on in cocoa to breed resistant cultivars since cocoa bean production is experiencing heavy loss due to pests and diseases. Five major diseases *viz.*, Witches' broom (WB), black pod rot/ black pod disease (BPR/ BPD), *Moniliasis* pod rot (MO), cocoa swollen shoot virus (CSSV) and vascular streak die back (VSD) affect the crop causing about 40% yield loss per year. To discover and understand the stability of putative natural resistance mechanisms in this commodity crop, Jones *et al.* (2002) undertook a gene-discovery programme and demonstrated its use in gene-expression arrays. Sequencing and assembling bean and leaf cDNA library inserts produced a unique contig set of 1380 members. High-quality annotation of this gene set using Blast and MetaFam produced annotation for 75% of the contigs and allowed us to identify the types of gene expressed in cocoa beans and leaves. Microarrays were constructed using amplified inserts of the uni-gene set and challenged with bean and leaf RNA from five cocoa varieties. The microarray performed well across the five randomly chosen cocoa genotypes and did not show a bias towards either leaf or bean tissues. This demonstrates that the gene sequences are useful for microarray analysis across cocoa genotypes and tissue types. The array results, when compared with real-time PCR results for selected genes, showed a correlation with differential gene-expression patterns. We intend that the resultant DNA sequences and molecular microarray platform will help the cocoa community to understand the basis, likely stability and pathotype resistance range of candidate cocoa plants. Resistance gene homologue (RGH) sequences have been developed into useful genetic markers for marker-assisted selection (MAS) of disease resistant cocoa (Kuhn *et al.*, 2003). A plasmid library of amplified fragments was created from seven different cultivars of cocoa. Over 600 cloned recombinant amplicons were evaluated. From these, 74 unique RGHs were

identified that could be placed into 11 categories based on sequence analysis. Primers specific to each category were designed. The primers specific for a single RGH category amplified fragments of equal length from the seven different cultivars used to create the library. However, these fragments exhibited single-strand conformational polymorphism (SSCP), which allowed us to map six of the RGH categories in an F2 population of cocoa. RGHs 1, 4 and 5 were in the same linkage group, with RGH 4 and 5 separated by less than 4 cM. SSCP can be efficiently performed on our automated sequencer, a convenient and rapid high throughput assay for RGH alleles (Kuhn *et al.*, 2003).

### 4.1.1. Resistance to *Phytophthora*

Resistance to *P. megakarya* in cocoa tree genotypes was assessed at an early stage by inoculation of leaf discs taken from nursery plants (Nyasse *et al.*, 2003). Four hybrid progenies introduced from Cote d' Ivoire and known to be resistant to *P. palmivora* in that country were compared to local control clones known for their lower susceptibility to *P. megakarya*, and to progenies produced in Cameroon. There was highly significant variability for resistance between progenies, and between individual plants within the progenies. The progenies from Cote d' Ivoire were more resistant to *P. megakarya* than the control clones from Cameroon, and also when compared with the progenies produced in Cameroon. This shows the potential of the progenies from Cote d' Ivoire for controlling black pod in Cameroon and the reliability of the leaf disc inoculation method (Nyasse *et al.*, 2003).

Field infection of 25 selected Trinitario x Upper Amazonian hybrid progenies was monitored for five consecutive years (from 1990 to 1994) at the Cocoa and Coconut Research Institute in Papua New Guinea by Saul Maora *et al.* (2003). The resistance durability of the various hybrids was analysed using a factorial split- plot model with years as the main factor. The reaction of the hybrid progenies remained stable and similar throughout the five years. The analyses clearly showed maternal inheritance under field conditions, and the progenies from what are believed to be resistant females showed good resistance.

In order to evaluate twelve doubled haploids (DHs) of cocoa used as parents, Sounigo *et al.* (2003) conducted a trial with several traits such as yield, vigour, yield: vigour ratios, resistance to the black pod disease caused by *Phytophthora* sp., percentage of flat beans and mean weight of 100 cocoa beans. Out of the three progenies derived from crosses between two DHs, two showed severe drawbacks. A reduction of the heterogeneity

within these progenies was occasionally observed for some of the traits, but failed to be consistent. When tested as female parents in combination with diploid testers, some of the DHs showed a significantly higher combining value than their parents for traits such as the mean weight of 100 beans and the yield: canopy surface ratio. The results showed the potential of DHs to improve selected parents in only one cycle of selection but more crosses between two DHs need to be tested to evaluate the potential of the resulting F1 progenies.

Amino acids and soluble carbohydrates were analyzed in cocoa pods to demonstrate whether or not there is a relationship between these compounds and the susceptibility of the different cocoa clones to *P. megakarya* (Omokolo *et al.*, 2002). Nine cocoa clones were used: SNK 10, UPA 134, SNK 13, SNK 213, SNK 64, ICS 95, SNK 416, ICS 84 and SNK 413. Analyses were carried out 5 days after pod infection. The clones SNK 213 and SNK 416, documented as mildly susceptible and less susceptible respectively, were found to be highly susceptible. Seven amino acids (asparagine, cysteine, glycine, isoleucine, proline, serine and tyrosine) were identified but the occurrence of each in the pods varied with the genotype and with the treatment. Total amino acid content was 745% higher in the less susceptible clone SNK 413 than in the highly susceptible clone SNK 10. A significant negative correlation ($rp=-0.646$, $P < 0.05$) was found between the level of amino acids and the lesion size. Glucose, fructose and sucrose were also identified in pod extracts. Their abundance and number were genotype dependent and varied with pod treatment; they decreased with wounding and with infection. A negative relationship ($rp=-0.60$, $P < 0.1$) was found between the level of carbohydrates and the lesion size. Pod infection was characterized by the disappearance of sucrose in the pod extracts. Our findings suggest that the variations in amino acids and soluble carbohydrates may, at least in part, account for the differences in the susceptibility of the different cocoa clones to *P. megakarya* black pod disease and reflect the polygenic character of cocoa resistance to this disease (Omokolo *et al.*, 2002).

The small holders in the central region of Cameroon, rarely cultivate varieties developed by research. There were two reasons for this: a level of resistance to pod rot that was judged insufficient, and the inefficiency of seed distribution systems. The generalized use of endogenous seeds slowed down the progress in pod rot control that could have been provided by genetic improvement (Paulin *et al.*, 2003).

A significant relationship was observed between resistance to

*Phytophthora* pod rot (measured as the frequency of localised lesions) and bean number (r=-0.45, p_0.001) showing that the two traits may complement to each other (Iwaro *et al.,* 2003). The combination of low to intermediate pod index with moderate to high resistance to *Phytophthora* pod rot was found in 87 genotypes, 12 of which were also reported to have resistance to witches' broom disease (Iwaro *et al.,* 2003).

Countries in Asia Pacific region are free of viral diseases. In India since the main harvest season coincides with monsoon, incidence of black pod rot caused by *Phytophthora palmivora* is higher. Through field screening clones are categorised into having <10%, 10-25% and >25% damage levels due to pod rot for resistance studies. *In-vitro* screening using isolates of *P. palmivora*, *P. capsici*, *P. citrophthora* indicated that collections of Nigerian origin exhibit certain degree of tolerance (Chandramohanan, 1982). 21 exotic clones collected exclusively for *Phytophthora* pod rot resistance and are being utilized for screening and hybridization programs.

### 4.1.2. Witches broom

Surujdeo Maharaj *et al.* (2003) studied the effects of host age, leaf number, host type (clone or seedling), pathogen spore concentration and incubation time on inoculation with *Crinipellis perniciosa* (witches' broom disease of cocoa) in greenhouse experiments using susceptible cocoa genotypes. Three methods of inoculation (agar-drop, water-drop and spray) were also tested. An optimized inoculation method was selected and tested for its repeatability as well as its ability to discriminate between various levels of resistance to *C. perniciosa* in cocoa. The optimized method (350 000 viable basidiospores per ml, 60 h incubation, agar-drop technique) produced 100% infection repeatedly, on both clonal and seedling plants of a susceptible genotype. Seedling age (2-12 months) and leaf number did not significantly affect the percentage of plants with symptoms or broom characteristics. This method discriminated effectively between the various levels of resistance in 14 cocoa genotypes and is recommended as an inoculation method to identify levels of resistance in germplasm collections. Symptom severity was shown to be a better measure of resistance than infection success. Queiroz *et al.* (2003) mapped genomic regions associated with resistance to *C. perniciosa* using an F2 population derived from a cross between 'SCA-6' (resistant) and 'ICS-1' (susceptible). The phenotypic index was determined as the average number of vegetative witches' brooms per canopy area of each plant, the witches' brooms were counted and eliminated during six field evaluations between May 1998 and August 1999. A total of 124 random amplified polymorphic DNA (RAPD) and 69

amplified fragment length polymorphism (AFLP) markers were mapped along 25 linkage groups covering 1713 cM of cocoa genome. After employing single factor and composite interval mapping analyses, a major quantitative trait loci (QTL) flanked by the marker AV14.940 was identified in the linkage group 11, explaining almost 35% of the resistance to witches' broom. The present result suggests that this QTL acts as a major dominant component of resistance to this pathogen, with great potential for use in marker-assisted selection procedures in cocoa breeding programmes.

### 4.1.3. Vascular-streak

Segregating progenies of five hybrid crosses of cocoa were screened in the field for resistance to *Oncobasidium theobromae,* the causal agent of cocoa vascular-streak dieback (VSD) disease, under natural infection conditions (Efron *et al.,* 2002). Four-month old seedlings were planted in the field in Madang, Papua New Guinea in December 1999. The seedlings were individually assessed for VSD 11 months after planting and thereafter at monthly intervals for 10 months. Infection was evenly distributed within the plot, reaching 100% of the trees with infection symptoms at the end of the testing period. Crosses differed in the incidence of symptoms, the percentage of susceptible plants and their Susceptibility Index. Progenies of the Trinitario clone K82 were more susceptible than those of the clone KA2-106. High rainfall increased the rate of infection. Plants of the same cross ranged from highly resistant to highly susceptible. It was concluded that the conditions at the testing site were adequate for large-scale practical screening of cocoa genotypes for resistance to VSD. Guidelines for future testing are outlined. This study showed that there is great potential for further selection of cocoa clones from hybrid progenies with enhanced resistance to VSD.

VSD is the most important disease in the South East Asia in India (Kerala), Indonesia, Malaysia, Philippines and Papua New Guinea. The threat of the disease is very much reduced with the detection of partial levels of resistance in several Upper Amazon and Trinitario genotypes. In Sabah, an *in vitro* dual culture system of cocoa callus and the pathogen, using mycelial colonization and callus growth as parameters for resistance/ susceptibility of the genotypes was developed. This method is very effective in early screening in selection/ hybridization programmes and for accurate studies on host- pathogen interactions (Ang and Shepherd, 1980; Lamin *et al.*, 1999). In Malaysia PBC 123 showed resistance over 200 clones tested (Nuraziawati *et al.*, 2013) and in Indonesia resistant clones tested were rich in terpene pinene, decane, myrcene, octadeconoic acid, trichome

density of flushes, polyphenol content and chitinase activity (Adi Prawoto and Teguh Iman Santoso, 2013). In Kerala, several crosses were evaluated for VSD resistance with SCA 6 as one of the parents (Mallika *et al.*, 2000). A high level of resistance exists in SCA-6, SCA-12, NA-33 and KA 2-106. The resistance is inherited in an additive manner and heritability is high. Around 1500 hybrids were evaluated at Kerala Agricultural University and 10 resistant varieties, CCRP-1 to CCRP-10 were released.

### 4.2. Resistance to Insect pests

#### 4.2.1. Homopterous insect pests

Mechanism of plant resistance to insects is complex. Plant and pod attractiveness to some extent affects the level of infestation, antixenosis prevents feeding, while antibiosis disturbs the pest development and finally cocoa tolerance is linked to the ability of a tree to contain damage and recover from it. The breeding value of ten Upper Amazon cocoa selections was evaluated with respect to their attractiveness to four homopterous insect pests (*Toxoptera aurantii*, *Tyro tessmannii*, *Planococcus citri* and *Planococcoides njalensis*) in Ghana (Adomako and Ackonor, 2003). Ten female parents (Alph. B36, IMC 60, PA 7, PA 150, Pound 7, Pound 26, T17/524, T65/238, T60/887 and T85/799), each crossed with three male parents (ICS 6, P 30 and SCA 9). The females came from one broad Upper Amazon population including Nanay, Parinari, Iquitos Mixed Calabacillo and T (Trinidad) types and males are from Imperial College Selection of Trinitario population, West African Amelonado selection from the Lower Amazon Forastero and Scavina of Upper Amazon population. There were significant differences in the attractiveness of the 10 Upper Amazon cocoa selections to the homopterous insect pests but none of the Nanay, Parinari, IMC and T types was consistently preferred, when the female parents were considered. For the male parents, SCA 9 (Upper Amazon) was markedly more attractive to the insect pests than either ICS 6 (Trinitario) or P 30 (Amelonado).

#### 4.2.2. Trunk longicorn (*Glenea aluensis*)

The response of cocoa clones derived from diallele crosses of ten Trinitario clones to infestation by the trunk longicorn, *Glenea aluensis*, was investigated in Papua New Guinea (Efron and Epaina, 2003). The average longicorn damage score on the test clones was in the range 6-40 and 6.3-38 in two trials. Genotypes obtained from the cross between clones K20 and KA2-101 had the lowest average scores. The highest average damage scores were observed in genotypes from the crosses KT140 x KA5-201, KT140 x 58/24 and 58/24 x KA2-101.

### 4.2.3. Cocoa mirids

Cocoa mirids alone can cause an average of 20-50% crop loss while black pod disease contributes to about 10-25% crop loss in Cote d' Ivoire (Kebe *et al.*, 2002). The development and use of mirid resistant cocoa varieties is one of the alternatives to chemical control and resistance studies mostly concentrated on assessment of ûeld damage (N' Guessan *et al.*, 2004). Sounigo *et al.* (2003) assessed the susceptibility to mirids (*Distantiella theobromae* Distant and *Sahlbergella singularis* Haglund) in four seedling progeny trials planted in Cote d' Ivoire by visual estimation of the cumulative damage on a 5 point scale. Although a significant effect of the progenies was identified in all trials, a clear discrimination between entries was only observed in one trial. Broad and narrow sense heritability's were identical, varying between 0.09 and 0.38, according to the trial. The highest value was found for the trial comparing genetically diverse parents, represented by a high number of offspring. Tolerance to *Sahlbergella singularis* and *Distantiella theobromae* is measured by observing cumulative and recent damage in field trials in Cameroon and Ivory Coast. This method permits ranking of genotypes according to their global reaction to mirid attacks (Anikwe *et al.*, 2009).

Tea mosquito bug (TMB) (*Helopeltis* sp.) incidence became severe in the recent years and became a major pest in Asia Pacific region. *Helopeltis antonii, H. theivora* and *H. bradyi* are reported in cocoa in South India and the population build up of this pest is highly influenced by temperature, water stress, humidity, shade level, density of cocoa trees and a shift in host plants. Damage on flushes, cherelles and pods are being measured at different grade levels of infection as screening method for TMB tolerance among genotypes. Red coloured, smooth surfaced pods exhibited certain levels of tolerance.

### 4.2.4. Cocoa Pod borer

Cocoa pod borer (CPB) (*Conopomorpha cramerella* Snellan) is the major debilitating pest which is affecting the cocoa economy in the Asia- Pacific region covering Malaysia, Indonesia, Philippines, Vietnam and Papua New Guinea. In Malaysia differences have been shown in the survival rates of cocoa pod borer larvae inside pods depending on the clones (Lamin and Saed, 1995). Larval mortality associated with sclerotic layer hardness and husk thickness is one of the criteria for selection of *C.cramerella* resistant cocoa clones (Azhar and Long, 1996). Penetrometer readings for determining the hardness of sclerotic layer, thickness at primary (ridge) and secondary furrows of pod husk are being used to correlate with

pod borer resistance. At CPCRI, 100 genotypes were assessed for favourable husk traits with penetrometer for possible interpretation with insect resistance (Elain Apshara, 2013). Malaysian Cocoa Board and Penn State University, USA initiated a project to introduce the insecticidal genes into cocoa trees to combat the infestation of CPB (Tan *et al.*, 2013). Several insecticidal genes wing bean trypsin inhibitor gene (WBTI), mustard trypsin inhibitor gene (MTI) and *Bacillus thuringiensis* gene (*Cry1Ab*) were constructed into binary vectors using recombinational cloning. The binary vector contained selective marker (hygromycin/ kanamycin) and reporter gene (GUS/GFP) were transformed into *Agrobacterium tumefaciens* supervirulent strain AGLI. Immature embryo explants were inoculated with *Agrobacterium* strains harbouring these genes. The main target of this project is to express the insecticidal protein on the pod tissue and not into the cocoa seed.

### 4.3. Genetic variation on chemical composition

Cocoa cotyledons contain vicilin (7S)-class globulin (VCG), a major storage protein. It is the native source of oligopeptides and free amino acids which have been identified as precursors of cocoa-specific aroma and are formed through proteolysis during fermentation. High-resolution electrophoresis of native proteins isolated from ripe, unfermented cocoa cotyledons harvested from different cultivars was used to determine the genetic differences among the genotypes. Flavour differences have been reported to exist after standard fermentation in cocoa beans harvested from various genotypes. Amin *et al.* (2002a) found cotyledon storage proteins from various genetically different cocoa trees are, within methodological limits, the same aroma differences in raw cocoa harvested from various genotypes are the result of other genetic, physiological or curing-related factors, but are not due to genetic differences of aroma precursors derived from storage proteins. Aspartic proteinase (EC 3.4.23) activity plays a pivotal role in the degradation of *T. cocoa* seed proteins during the fermentation step of cocoa bean processing. Therefore, this enzyme is believed to be critical for the formation of the peptide and amino acid cocoa flavour precursors that occurs during fermentation. Laloi *et al.* (2002) found using coda cloning and northern blot analysis, that there are at least two distinct aspartic proteins genes (TcAP1 and TcAP2) expressed during cocoa seed development. Both genes are expressed early during seed development and their mRNA levels decrease towards the end of seed maturation. TcAP2 is expressed at a much higher level than TcAP1, although the expression of TcAP1 increases slightly during germination. The proteins encoded by TcAP1 and TcAP2 are relatively different from each other

(73% identity). This, and the fact that the two corresponding genes have different expression patterns, suggests that the TcAP1 and TcAP2 proteins may have different functions in the maturing seeds and during germination. Because the TcAP2 gene is expressed at a much higher level during seed development than TcAP1, it is likely that the TcAP2 protein is primarily responsible for the majority of the industrially important protein hydrolysis that occurs during cocoa bean fermentation. Finally, TcAP2 has been functionally expressed in the yeast *Yarrow lipolytica*. The secreted recombinant protein is able to hydrolyse bovine haemoglobin at acidic pH and is sensitive to pepstatin A, confirming that TcAP2 encodes an aspartic proteinase, and strongly suggests that this gene encodes the well-characterized aspartic proteinase of mature cocoa seeds.

Amin *et al.* (2002b) prepared acetone dry powder (AcDP) from six cocoa genotypes, namely, Forastero (Amelonado type), Criollo, Trinitario, SCA 12, UIT 1 and PBC 140. Hydrophobic oligopeptides were produced when autolysis of AcDP was conducted at pH 3.5. Comparative HPLC analysis showed that autolysis of AcDP from various genotypes revealed a similar pattern of oligopeptides. Most of the hydrophobic oligopeptides were not generated during autolysis of AcDP in the presence of protease inhibitor (Pepstatin A), indicating that the generation of these oligopeptides was due to the action of cocoa cotyledon aspartic endoprotease. This finding implies that the splitting action of aspartic endoprotease on vicilin (7S)-class globulin (VCG) from various genotypes was the same. The information from the study provides additional evidence that there are no obvious differences in VCG composition between various genotypes.

Adomako and Adu Ampomah (2003) evaluated flavour using fermented and dried cocoa beans. The utilization of Upper Amazon pollen parents resulted in increased percentage of shell content of beans and fat content of the nibs. Few of the inter-Upper Amazon hybrids lacked the chocolate flavour, based on the evaluation of the chocolate manufacturers. In general, the bean characteristics of the new hybrids did not differ significantly from the control crosses. Although the bean characters studied appeared to be largely genetically determined, these parameters could also be influenced markedly by environmental factors. The chances of Ghanaian cocoa beans falling short of the acceptable flavour range are not high provided that breeding is within the Amazonian Forastero group.

Cocoa is rich in polyphenols and oligomers of polyphenol molecules named procyanidins, fat and theobromine, contributed to the

health beneficial effects and exhibits high antioxidant activity both *in vivo* (Kondo *et al.,* 1996) and *in vitro* (Baba *et al.,* 2000) conditions. Cocoa beans of different clones conserved in India also exhibited distinct differences. Polyphenol content ranged from 82-136 mg/g, procyanidin from 49-64 mg/g, fat content from 24-54% and antioxidant activity was found to be in a wide range from 77-98% among cocoa clones of Malaysia, Kew and Trinidad collections. Principal component analysis divided the high polyphenol, procyanidin and antioxidant in PC1 and high fat content and moderate polyphenol contents of cocoa clones in PC2. In general, cocoa beans with high polyphenol and procyanidin contents exhibited high antioxidant activities which is observed to be high in EET-272, ICS-1, I-21, II-67 and I-56, which will be used for selective breeding (Senthil Amudhan and Elain Apshara, 2015).

## 5. Biotechnology

Research on tissue culture methods has been done in several countries. The tissue culture in cocoa serves two important purposes (i) safe means of distribution among countries using disease free meristematic tissues; (ii) develop selected parental material for rapid multiplication to establish seed gardens. The use of micro propagation methods for commercial planting material has limited impact as at present. A detailed review is presented by Mallika *et al.* (2001).

The developments in cocoa biotechnology within the next 10 to 25 years are expected to be in the areas of modifying cocoa bean yield and quality (Gotsch, 1999). Another important aspect in biotechnology is for producing cocoa components *in vitro* or cocoa butter substitutes with the help of crops other than cocoa. There is good scope for breeding cocoa cultivars with improved resistance to pests and diseases. Furthermore, varieties with modified quality characteristics may be available, in particular with increased cocoa butter content and modified fatty acid patterns. Significant reduction of yield losses caused by *Phytophthora* pod rot, vascular streak dieback, Witches' broom, and the cocoa pod borer may be achieved.

### 5.1. Tissue Culture

A protocol for the production of cocoa plants by shoot tip culture has been developed (Adu-Ampomah *et al.*, 1988). The liquid half strength MS medium containing 5% sucrose gave the highest germination. Transfer of germinated embryoids to solid media with $GA_3$ resulted in an excessive development of embryoid cotyledon while the embryo axis ceased to

develop. However, removal of cotyledons of germinated embryoids, followed by sub culturing in liquid medium with high $GA_3$ enhanced differentiation of embryoids into shoots and leaves. There were no differences between cultivars in the ability of their embryoids to produce plantlets. With orthotropic explants, use of benzyl adenine and zeatin helped in breaking bud dormancy and growth of explants (Legrand and Mississo, 1986). Gibberellic acid also promoted embryos formation from callus while CCC inhibited (Legrand *et al.*, 1984; Kononowicz and Janick, 1984). Shoot proliferation occurred during the first 6 weeks on media containing BA, zeatin or zeatin riboside. The shoots were rooted on a medium with IBA, NAA and phloroglucinol (Passey and Jones, 1983).

Recent progress has been made on the development of methods for the production of somatic embryos (SE) from non-sexual explants (petals and nucellus tissues) of cocoa. Large numbers of SE were produced and resulting plants have been raised to maturity. The regeneration rate was 4.3% for petals (9000 explants) and 2% for nucellus (29 000 explants). Since the SE is of non-sexual origin, the progress reported allows the micro propagation of elite cocoa trees. Mature donor plants could be selected from segregating field collections and submitted to this cloning process. These improved methods will be of use in cocoa improvement programmes that rely on cellular and molecular genetic techniques (Sondahl *et al.*, 1993).

Somatic embryogenesis in cultures derived from immature zygotic embryos has limited value for propagation because the zygote is an untested genotype. Furthermore, conversion of somatic embryos into seedlings has so far proved marginal. Recently, somatic embryogenesis has been reported from nucellar tissue, which is maternal in origin. The induction of nucellar embryony is here confirmed and a protocol for conversion of nucellar somatic embryos into seedlings was developed that involves preculture of somatic embryos in liquid medium and transfer to semi-solid medium in chambers receiving 20,000 ppm carbon dioxide (Figuira and Janik, 1993). Somatic embryogenesis from floral buds was tested on 25 cocoa genotypes under defined culture conditions, revealing genotypic variations. An efficient *in vitro* clonal propagation method for cocoa using somatic embryogenesis has been developed (Guiltinan and Maximova, 2001). Induction of somatic embryogenesis from tissues in unopened flower buds of cocoa with respect to physiological age, type of floral explant, genotype, and medium composition and phytohormones indicated that two-to-three-week-old staminodes were found to be the best explants for embryogenesis. Embryogenesis was affected by genotype and sugars (Tan and Furtek, 2003). Two main types of abnormalities of the embryos were observed: fusion of

the hypocotyls and multiple cotyledons. These embryos have a lower rate of conversion into plantlets. Cytological analyses of somatic embryo-regenerated plants revealed a somatic chromosome number of 2n=2x=20, similar to seed-derived plants. Maximova *et al.* (2002) developed a secondary embryogenesis system using primary somatic embryo cotyledon explants. Primary somatic embryo cotyledon explants from 14 cocoa genotypes growing in 2 different locations in France were cultured for 14 days on modified secondary callus growth medium with 2.4 µM 2,4-D and 1.4 µM benzyladenine and then transferred to a growth regulator-free embryo development medium. The genotypes include: UVE 1, 5, 9 and 22, EEG 29, TSH 565, GU 143, GF 23, IFC 5, KER 1, IFC 705, NA 32, NA 79 and Scavina-6. The secondary embryogenesis system resulted in up to a 30-fold increase in somatic embryo production compared to primary somatic embryogenesis. All genotypes produced embryos but at different intensities. The start of embryo formation was observed between 4 and 6 weeks after culture. The peak in embryo production was between weeks 8 and 18. Studies have shown that while primary somatic embryos arise from a multi-cellular pathway, secondary somatic embryos arise predominantly from single cells. Secondary somatic embryos also exhibit a higher quality and conversion rate compared to primary somatic embryos as well as a higher production rate for most genotypes. Histological monitoring of somatic embryo ontogenesis revealed that the somatic embryos were of multicellular origin. The right conditions for the selection and maintenance of meristematic cells (from which somatic embryos are derived) and embryogenic cells were sought by culturing in a liquid medium using a temporary immersion system. A friable embryogenic callus was obtained. A study of zygotic embryogenesis was carried out with a view to improving the later stages of somatic embryogenesis. Zygotic embryogenesis was characterized by a period of embryo growth, followed by accumulation of starch and protein reserves, during which slow and moderate desiccation occurred. By adding a growth phase to the somatic embryogenesis protocol, and defining a maturation medium containing abscisic acid, embryo maturation was improved (Alemanno *et al.*, 1996, 1997).

Anthers, ovules, nucelli, embryos and embryo axes of cocoa were cultured *in vitro*. Explants produced callus when cultured on MS medium. Weak seedlings and plantlets were obtained from nucelli, immature embryos and mature embryo axes. Adventive embryos, which budded from all parts of immature embryos, were mostly epidermal in origin. Callus production sometimes preceded adventive embryogenesis. Growth was generally better

on agar gelled nutrient media than on Heller's filter paper support in liquid media. The axes of mature embryos on Heller's supports remained dormant for over 12 months, but when they were transferred to agar medium containing gibberellic acid, active growth was resumed and seedlings were produced within 4 weeks of reculture (Esan, 1977). Somaclonal variation was reported in *in vitro* multiplication of cocoa. Detection and quantification of *in vitro* culture induced chimerism using simple sequence repeat(SSR) analysis in cocoa (Lopez *et al.*, 2004). Induction of somatic embryogenesis from tissues in unopened flower buds of cocoa was studied with respect to physiological age, type of floral explants, genotype, and medium composition and phytohormones (Tan and Furtek, 2003). Somatic embryogenesis in Venezuelan cocoa cultivars and culture conditions were reported by Velasquez *et al.* (2006).

*In vitro* methods are expected to make cocoa improvement faster and more efficient. Firstly, these methods form the basis for more effective propagation of improved clones, and for the conservation and more efficient exchange of valuable breeding materials. The micro propagation and *in vitro* regeneration provide the foundation for genetic engineering. The considerable chances attributed to *in vitro* regeneration/ multiplication techniques are consistent with the fact that not only has somatic embryogenesis been achieved with cocoa, but also the ability to induce somatic embryos to develop into rooted plantlets (Lopez Baez *et al.*, 1993).

### 5.2. Molecular markers

Molecular markers can be used for fingerprinting and mapping. The value of these markers is that selection for traits expressed in mature plants (for instance resistance of pods to rot) can be made at the seedling stage. Molecular markers are preferred over botanical and biochemical markers because the environment does not influence their expression. Since so little of the conventional genetics of even the simplest characteristics is known, adequate linkage maps are going to be very difficult to construct. Genetic characterization of four resistance/tolerance characteristics to pathogens and insect pests: *Phytophthora* pod rot, vascular streak dieback, Witches' broom, and cocoa pod borer are going to be valuable. In fact, the development of a molecular linkage map has been reported in the literature (Crouzillat *et al.*, 1996) and the organization of genetic diversity in *T. cacao* is being investigated with the help of molecular marker technology (Lerceteau *et al.*, 1997).

Examination of the phosphoglucoisomerase (PGI) and malate dehydrogenase (MDH) enzyme systems allowed clear distinction between

the cocoa fruit pathogens *P. palmivora*, *P. megakarya* and 2 species close to *P. capsici* and *P. citrophthora*, respectively. The number of constant and variable MDH bands in *P. megakarya* suggests that there are at least 4 populations of the species. *Phytophthora palmivora*, with heterozygous PGIs and segregation in its progeny, seems to be a naturally selected hybrid of certain specially related intraspecific entities. Restriction fragment length polymorphism (RFLP) analysis of nuclear DNA gave the most distinctive profiles for the enzyme BamH1 (Blaha, 1990).

Restriction fragment length of the rRNA genes was studied in cocoa using heterologous rDNA probes. One hundred and ninety-two individuals including both cultivated and wild clones were analysed. DraI and EcoRI restriction sites were mapped. Both length heterogeneity and restriction site polymorphism have been found. Fifteen different types of rDNA units have been characterized. As opposed to previous enzymatic studies, the rRNA gene analysis indicates a clear distinction of the three genetic groups Criollo, Forastero and Trinitario within cocoa and points out the hybrid origin of Trinitario (Laurent *et al.*, 1993). A total of 203 cocoa clones were surveyed for restriction fragment length polymorphisms (RFLPs) using four restriction endonuclease and 31 seed cDNA probes. A high level of polymorphism has been found. This study points to a structuring of the species that fits with the distinction between the Criollo and Forastero populations. These results combined with previously obtained nuclear rDNA and mtDNA data allow us to propose new hypotheses on the origin and evolution of the different cocoa populations (Laurent *et al.*, 1994). Analysis of genetic distances indicated that, in the case of RAPD (random amplified polymorphic DNA), no individual primer successfully differentiated all varieties, with only 7 of the 20 primers producing polymorphism. In contrast, all Amplified Fragment Length Polymorphism (AFLP) primer pair combinations generated considerable polymorphisms, enabling all varieties to be differentiated with only one primer pair (Perry *et al.*, 1998).

Characterization of cocoa germplasm using 24 enzyme markers for descriptor purpose has been done (Atkinson *et al.*, 1986). Allozyme data for eight polymorphic loci encoding six enzymes were used to describe 86 clones from the USDA/American Cocoa Research Institute cocoa germplasm collection (Ronning and Schnell, 1994). To further characterize gene diversity, the total population was subdivided two ways: by geographical origin and by morphological type. Contingency $Chi^2$ analysis showed the Caribbean and Central American groups to be distinct from South American clones. Among types, the Forasteros differed from the

Trinitarios at four of six loci. Some differences were due to the presence or absence of certain alleles. Phenetic trees were constructed using gene frequency data averaged over groups. Most gene diversity was found to exist within, rather than between, groups; this differentiation was somewhat higher among morphological types than among geographical origins. A core collection of clones selected across all morphological types, using both morphological and allozyme descriptors, might provide an optimal method of maintaining a germplasm collection of this species. An experiment was conducted to analyse the relationship between Central and South American cacao cultivars ancient Criollo, modern Criollo, Trinitario, Amelonado, Guyana Forastero, Upper Amazon Forastero and hybrids with at least one Upper Amazon Forastero parent (Lanaud *et al.*, 2001; Motamayor and Lanaud, 2002; Risterucci *et al.*, 2001). The average number of alleles was highest for the Forastero group, as well as the percentage of polymorphic loci and the observed heterozygosity. For RFLP markers, all ancient Criollo individuals (92) mapped in the left half of the FAC with a cluster of homogeneous clones with several unresolved, near identical individuals in the fourth quadrant. Only 8 ancient Criollo genotypes were observed in 92 individuals whereas each Forastero individual had a unique RFLP genotype. Despite the increased number of alleles, genetic diversity of the ancient Criollo group observed from microsatellite data was still very low compared with that observed for the Forastero group. Within geographic regions, the gene diversity values for 13 and 5 individuals from the Peru and Colombia-Ecuador regions were similarly high (0.70). The observed heterozygosity was 0.00 for the ancient Criollo and 0.34 for Forastero. Recent studies using restriction fragment length polymorphism probes indicated that Nacional cocoa genotypes are genetically different from the Forastero, Criollo or Trinitario groups (Crouzillat *et al.*, 2001). Phenotypic data analysis from germplasm collection in Brazil indicated a strong association between bean weight, number of pods and witches' broom resistance (Pires *et al.*, 2001).

Molecular markers are important when phenotypic evaluations are not efficient in identifying resistance genes. Most recently, microsatellite markers (SSR's) have gained acceptance as the most accurate and reliable method. Quantitative traits refer to phenotypes or characteristics that vary in degree and can be attributed to polygenic effects, *i.e.,* product of two or more genes, and their environment. Quantitative trait loci (QTLs) are stretches of DNA containing or linked to the genes that underlie a quantitative trait. Mapping of regions of the genome that contain genes involved in specifying a trait can be done using molecular tags or markers.

This is an early step in identifying and sequencing the actual genes underlying trait variation. QTL analysis of agronomic traits, quality parameters and disease resistance are under taken and results have been obtained on trunk diameter, canopy height, earliness in flowering, number of ovules per ovary, pod length, bean number, bean weight and resistance to *Phytophthora*. This will enable introgression of particular traits in varieties using marker assisted selection (N'goran *et al.*, 1995; Crouzillat *et al.*, 1996). TropgeneDB is maintained by CIRAD, France which comprised of genetic and physical maps, marker information, QTL's, sequence data and molecular data on genetic resources (www.tropgenedb.cirad.fr). Penn State University conducted an extensive comparative study on flower development in *Arabidopsis thaliana* and *Theobroma cacao* and examined the expression of several key floral regulatory genes (Swanson, 2005) in continuation of the reclassification of cocoa from Sterculiaceae to Malvaceae (Alverson *et al.*, 1999; Bayer *et al.*, 1999; Whitlock *et al.*, 2001). The INGENIC Study Group for Molecular Biology is chartered in 2003 to coordinate the activities of the members interested in molecular approaches (Guiltinan, 2007). At CPCRI, DNA fingerprinting with RAPD markers was done earlier on 76 collections and recently 16 SSR primers specific to cocoa were used to assess 44 Nigerian collections. An attempt was made to identify the markers for drought with susceptible and tolerant parents and progenies of cocoa (M'bo Kacou *et al.*, 2014). Cocoa genome is successfully sequenced in the year 2010 by CIRAD, France and Penn State University, USA along with a group of institutes and 75% of it is available in the public domain which paved way for analyzing genes related to specific needs (Xavier *et al.*, 2011). CPCRI is one of the Agri Bioinformatics centre under Department of Information Technology and through bioinformatics tools, Expressed Sequence Tag (EST's) and genome analysis were done. Proteins involved in drought tolerance, *Phytophthora* resistance and carotenoid biosynthetic pathways were identified and databases, CocoaESTdb, CocoaSTRESSdb were developed (Naganeeswaran and Elain Apshara, 2011; Naganeeswaran *et al.*, 2012, 2014, 2015).

Faleiro *et al.* (2002) studied the genetic similarity of 18 cocoa accessions maintained in 37 rows in the germplasm collection using RAPD markers. Genetic similarities of 0.70, 0.94 and 0.96 were observed between plants of the accessions RB 29, Ca 1 and C. Sul 4, respectively. Dias *et al.* (2003) studied the genetic distance among 5 cocoa clones (CC 41, SIAL 169, CEPEC 1, ICS 1 and SIC 19) with random amplified polymorphic DNA (RAPD) data (genetic distance, GD) and yield components data *i.e.*

number of healthy fruits per plant, number of collected fruits per plant, wet seed weight per plant, wet seed weight per fruit and percentage of infected fruits per plant. Both distances were related to heterotic performance of hybrids for wet seed weight per plant and wet seed weight per fruit. The average hybrid performance for the same 2 yield components was correlated with only MD. Hence, genetic distances measured by RAPD and yield components can be used as a guide to the choice of the superior crosses.

Microsatellite markers are becoming increasingly important. Motilal and Butler (2003) reported the presence of mislabeled trees from data compiled in the International Cocoa Germplasm Database (ICGD), which contains the published records of cocoa accessions in global holdings. The identification of mislabeled trees should depend ultimately on molecular analysis. A method for cocoa DNA fingerprinting ring test was developed by Swanson *et al.* (2003) using genotypes PA 30 T1, LX 31, PA 30T10, GU 114P, PA 30 T5, GS 4/4A, LCTEEN 68-1 and IMC 47. Eleven fluorescent microsatellite primers were used for distinguishing among the genotypes. PCR and electrophoresis studies were conducted. The 11 primers used were more than sufficient for clearly differentiating the genotypes. Results showed that a total of 61 polymorphic markers were scored, among which 33 contained markers that were seen in only 2 of 3 amplifications, indicating some variation in the reproducible production of these fragments.

Yamada *et al.* (2003) determined the genetic variability and the heterozygosity in 34 of accessions of cocoa using microsatellite markers. Twelve microsatellite loci were analysed, generating a total of 49 alleles. Results indicated genetic variability among the Parinari accessions with genetic distances varying from 0.00 to 0.64 and heterozygosity levels between 11 and 72%. About 40% of the accessions presented heterozygosity equal or greater than 50%.

Quantitative trait loci (QTL) mapping for bean traits and the number of ovules per ovary was carried out in cocoa using three test cross progenies derived from crosses between a lower Amazon Forastero male parent (Catongo) and three female parents: one upper Amazon Forastero (IMC 78) and two Trinitario (DR 1 and S 52). Restriction fragment length polymorphism (RFLP), microsatellite (SSR) and amplified fragment length polymorphism (AFLP) markers were used for mapping (Clement *et al.*, 2003). Between one and six QTL for bean traits (length, weight, and shape index) and one and four QTL for the number of ovules per ovary were detected using composite interval mapping. Individual QTL explained

between 5 and 24% of the phenotypic variation. QTL clusters were identified on several chromosomes, but particularly on chromosome 4. QTL related to bean traits were detected in the same region in both Trinitario parents and in a close region in the upper Amazon Forastero parent. In reference to a previous diversity study where alleles specific to Criollo and Forastero genotypes were identified, it was possible to speculate on the putative origin (Criollo or Forastero) of favorable QTL alleles segregating in both Trinitario studied (Clement *et al.*, 2003).

### 5 .3. Transgenics

The genetic engineering has to contribute considerably in the improvement of resistance of cocoa to some pathogens. Best chances are predicted for a contribution of genetic engineering to resistance/tolerance to cocoa swollen shoot virus, the cocoa pod borer, and Witches' broom. Also for the other three diseases investigated (*Phytophthora* pod rot, *Moniliophthora* pod rot, and vascular streak dieback) good prospects are anticipated. These findings are consistent with the arguments brought up in the survey, where a broad consensus exists that first attempts will concern insect resistance, virus resistance (swollen shoot), and simple characteristics. One of the aims is to genetically engineer cocoa for resistance to fungal pathogens and insect pests (Gotsch, 1999). However such studies on creating transgenic cocoa trees using are going to take at least 4-5 years. During genetic transformation studies cocoa tissues are frequently destroyed due to *Agrobacterium* overgrowth following co-cultivation. Furthermore, the addition of the antibiotic cefotaxime, commonly applied to tissue culture media to eliminate *Agrobacterium* post infection, decreased cocoa somatic embryo production by 86% (Mayolo *et al.*, 2003). Two cephalosporins, cefotaxime and moxalactam and two penicillins, amoxicillin and carbenicillin, moxalactam, a beta-lactam antibiotic, was proven to effectively suppress *Agrobacterium* growth. Furthermore, at certain concentrations, it also significantly enhanced the efficiency of cocoa SE (Mayolo *et al.*, 2003).

Another aspect to the transgenic cocoa is for the *in vitro* production of economically important cocoa components. This is likely to be through (i) *in vitro* production with the help of cocoa cells or parts of them or (ii) *in vitro* production with the help of cell cultures other than cocoa or using microorganisms. The *in vitro* production of cocoa components with the help of cocoa cells or parts of them is likely to be less promising. The chances of producing flavour components are considered less favourable than those of producing cocoa butter components. Flavour formation is a

complex process about which little is known. Highly purified oil bodies have been isolated from mature seeds of cocoa. Characterization of the proteins by SDS-PAGE analysis indicated that the purified oil bodies contain a minimum of seven polypeptides, with a polypeptide of approximately 16.1 kDa being the most abundant. At least five of the oil body proteins were in the size range for oleosin proteins (15-30 kDa). Peptide sequencing showed that the approximately 16.1 kDa polypeptide and an approximately 15.0 kDa polypeptide were in fact oleosins, and indicated that an approximately 26.5 kDa polypeptide was probably a caleosin. cDNA encoding the 16.1 kDa polypeptide (TcOleo 16.9) and the 15.0 kDa polypeptide (TcOleo 15.8) were isolated and characterized (Guilloteau *et al.,* 2003). Analysis of the protein sequences encoded by these two cDNA indicates that they belong to two different classes of oleosin proteins. Northern blots showed that TcOleo 16.9 and TcOleo 15.8 have relatively similar expression patterns during seed development, although the overall expression of TcOleo 16.9 was significantly higher than that of TcOleo 15.8, in agreement with the observation that the 16.1 kDa polypeptide is more abundant than the 15.0 polypeptide in purified seed oil bodies. The expression of both genes was also induced briefly in the cotyledons during germination. Southern blot analysis showed that TcOleo 16.9 and TcOleo 15.8 were probably single copy genes in the cacao genome. Because the data presented here shows that the oil bodies of cocoa seeds contain oleosin proteins, it is unlikely that the 'recalcitrant' nature of these seeds is due to the absence of oleosin proteins in cacao seed oil bodies as previously proposed.

The prospects of *in vitro* production of cocoa components with the help of cell cultures other than cocoa or with the help of microorganisms are rated slightly more favourably than those of *in vitro* production with the help of cocoa cells or parts of them. Through the co-operation of biochemistry and molecular biology genes involved in characteristics such as flavour and fatty acid profiles could be identified, isolated, and introduced into bacteria, and yeasts and components may be produced in bioreactors. The cloning of a gene for the cocoa seed storage protein and its expression in yeast has been reported (Yavuz *et al.*, 1996).

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