Chapter 21

Cocoa

☆ *Anitha Karun, V. Aparna, M.K. Rajesh and P. Chowdappa*

1. Introduction

Cocoa (*Theobroma cocoa* L.) is an important tropical plantation crop worldwide. Cocoa seed is important raw material for the production of cocoa powder and butter; these are important ingredients for preparation of chocolate as well as various confectionery products (Li *et al.*, 1998). Cocoa is a native of Tropical America (Cheesman, 1944); later cultivation of this tropical crop spread to the countries in Asia and Africa (Zhang and Motilal, 2016). Cocoa requires a temperature of 21-32°C, well distributed rainfall of 100-250 cm for its optimal growth. It grows only below 1000 m of elevation, ideally below 300 m from the mean sea level. The main growing areas of the crop are situated approximately within 20° North and South latitude of the equator (http://www.cacaoweb.net/cacao-tree.html).

2. Cocoa Genetic Diversity

Genetic resources are a crucial element for the development of new and improved cultivars to achieve a more sustainable and cost-effective means of cocoa production. Cocoa genetic resources comprise the range of genetic variability that provides the raw material for breeding new and improved cultivars. The primary gene pool of cocoa is situated in the Amazon basin, ranging from French Guiana to Bolivia, where a large number of wild populations still exist. The centre of diversity of cocoa is in the upper Amazonian rainforest. Recent molecular studies suggest that the diversity of natural cocoa populations might be stratified by the major river basins (Thomas *et al.*, 2012). Within each river basin, wild cocoa is grouped in patches and separated by large spatial distances between patches. Gene flow is limited and mating is likely confined within patches due to short-distance seed

Table 21.1: Various Sections of *Theobroma* Species According to the Classification Proposed by Cuatrecasas (1964)

Sections and Theobroma Species	*Common Name*
Section *Andropetalum*	
T. mammosum Cuatr. and León	
Section *Glossopetalum*	
T. angustifolium Moçiño and Sessé	'cacao de mico'
T. canumanense Pires et Fróes	
T. chocoense Cuatr.	
T. cirmolinae Cuatr.	
T. grandiflorum (Willd. ex Spreng.) Schum.	'cupuassu'/'cupuaçu'/'Copoasu'/'Cupuasu'
T. hylaeum Cuatr.	
T. nemorale Cuatr.	
T. obovatum Klotzsch ex Bernoulli	' cabeça de urubu'/'Cacahuillo'/'Ushpa cacao'
T. simiarum Donn. Smith.	
T. sinuosum Pavón ex Hubber	
T. stipulatum Cuatr.	
T. subincanum Mart.	'cupuí'/'Macambillo'/'Macambo Sacha
Section *Oreanthes*	
T. bernouillii Pittier	
T. glaucum Karst.	
T. speciosum Willd.	'cacaui'
T. sylvestre Mart.	'cacau azul'
T. velutinum Benoist	
Section Rhytidocarpus	
Theobroma bicolor Humb. and Bonpl.	'mocambo'/'patashte'/'macambo'
Section *Telmatocarpus*	
T. gileri Cuatr.	
T. microcarpum Mart	'cacaurana'
Section *Theobroma*	
Theobroma cacao	'cacao'

and pollen dispersal. Only a very small fraction of the diversity was dispersed from the Amazon to Mesoamerica and thus the ancient cultivated materials have a narrow genetic background (CacaoNet, 2012). This genus '*Theobroma*' contains 22 species; out of which 15 species are edible (CacaoNet, 2012). All these related species possess potential commercial value, mainly because of the sweet seed-surrounding pulp of their fruits in addition to the beans and other crop related characteristics. *T. grandiflorum* (cupuassu), *T. bicolor* and *T. angustifolium* are generally cultivated in native areas of this crop. *T. grandiflorum* is considered an important fruit crop in various Amazonian countries and its cultivation has been increasing, especially

in Brazil (CacaoNet, 2012). Considerable work has been done on intergeneric and interspecific crosses involving these species and cocoa with variable levels of success. The 'tertiary genepool' germplasm consists mainly of various species of the genus *Herrania*.

3. Germplasm Conservation Strategies

For many crop plants, germplasm can be stored in the form of dried seeds at low temperature (*i.e.* so-called 'orthodox' seed storage) but this is not possible with cocoa because of its recalcitrant nature. The hygroscopic nature of the seeds further worsens possibility of seed storage in cocoa (Chandel *et al.*, 1995). Cocoa seeds are not even suitable for cold storage conditions; with increasing maturity of the seeds, freezing sensitivity also increases. Fully matured seeds survived desiccation up to 35 per cent of moisture level. But embryo axis was fully sensitive to freezing conditions at all physiological maturity stages (Chandel *et al.*, 1995). Cocoa germplasm can be conserved in two ways:

1. ***In situ:*** The genetic resources are maintained in the natural habitat in which the diversity has evolved *viz.*, natural reserves and on-farm conservation. The advantage of in *situ* conservation is that it facilitates the ongoing processes of natural evolution.
2. ***Ex situ:*** Comprising all cocoa germplasm currently maintained in field genebanks as living trees and/or in *in vitro* collections as tissues, embryos pollen and DNA *etc.*

Ex situ collections play a crucial role in the conservation of many varieties, particularly those that have already disappeared from farmers fields. New germplasm can be introduced into a field genebank as seedling trees. *Ex situ* field genebank collections have the advantages that once the trees are established they can remain in the ground for many decades and can readily provide the bud wood, seed or pollen needed for evaluation and incorporation into breeding programmes. But they are under the risk of destruction due to natural calamities, pests, diseases and are costly for maintenance. Hence there is urgent need to develop safety duplicates of the living collections using alternate strategies of conservation such as *in vitro* conservation. There are 40 genebanks for cocoa around the world. Most of them are supported by public-private fundmg. There are currently only two international collections managed by the Cocoa Research Unit of the University of the West Indies (CRU/UWI), Trinidad and the Centro Agro-nómico Tropical de Investigacióny Enseñanza (CATIE), Costa Rica. These collections are called as the International Cocoa Genebank, Trinidad (ICG). These institutes concluded agreements with the International Treaty for Plant Genetic Resources for Food and Agriculture (ITPGRFA) to maintain their respective collections as global collections of cocoa genetic resources for the long term and to make the germplasm freely available to any professionally qualified institution or individual. The safe movement of germplasm at the global level, including virus indexing, is achieved through the International Cocoa Quarantine Centre (ICQC), an intermediate quarantine facility supported by the chocolate industry and USDA, at the University of Reading, UK. The USDA/ARS facility in Miami, USA, offers quarantine facilities for regional

transfers. The benefits of conserving and utilizing the cocoa genetic diversity will be realized if this diversity is of interest and is made available to researchers engaged in breeding programmes.

Despite the existence of over 24,370 cocoa germplasm accessions in *ex situ* collections worldwide, including 3500 accessions that are held in the two international collections, much of this germplasm is under-utilized or at risk, due to the lack of adequate long-term funding to conserve or utilize the existing germplasm effectively. Furthermore, genetic studies suggest that the material held in *ex situ* genebanks, particularly the international genebanks, does not fully represent the known range of diversity and still there are potential genetic variations yet be to be discovered in the rainforests and farmers fields of the Amazonian region. It has been estimated that even in Brazilian Amazon, where the greatest collecting activity has taken place, only some 20 per cent of the potential diversity has been explored (Bartley, 2005). Central American countries, especially Bolivia, Colombia, Ecuador, Peru and Venezuela, remain largely unexplored for cocoa diversity. With the rapid deforestation in this region, drastic changes in land use and replacement of traditional cocoa varieties with modern ones, both in the Amazon region and in other regions where cocoa is grown, there is the likelihood of irreversible genetic erosion unless further steps are taken to conserve materials *in situ*, or to collect and conserve them *ex situ*.

4. *In vitro* Conservation

Development of a cryopreservation technique for long term storage of cocoa germplasm is very important for preserving biological diversity and genetic fidelity. Cryopreservation is the preservation of viable cells, tissues and organ in liquid nitrogen (LN) at -196° C (Engelmann, 1991; Benson, 1999) and can be stored for indefinite periods without genetic erosion (Golmirzaie *et al.*, 1999). Cryopreservation method involves a sequence of treatments including encapsulation-dehydration, sucrose preculture, silica gel desiccation and liquid nitrogen storage *etc* (Fang *et al.*, 2004).

Cryopreservation of cocoa using zygotic embryos, embryonic axes, callus and somatic embryos as explants has been successfully demonstrated. Grout *et al.* (1983). tried desiccation storage for embryo axis and seeds in liquid nitrogen. Embryos are processed and frozen after aseptically removed from surface sterilized immature fruits. Extracted embryos are placed into a basal media containing cryoprotectant. Treated embryos are transferred to cryovials and then stored in liquid nitrogen container. Cryopreserved embryos were tested after certain storage by transferring the thawed embryos into a germination media (Duhem *et al.*, 1988). During desiccation and freezing, embryo axis was found to be severely damaged and fails to germinate on tissue culture medium. The immature zygotic embryos retains the ability to produce callus and undergo somatic embryogenesis after slow hydrated freezing and desiccated fast freezing in liquid nitrogen were reported by Pence (1991). Immature embryos of cocoa could be able to survive exposure to liquid nitrogen either in hydrated or desiccated state (Pence, 1991). Florin *et al.* (2000) standardized a cryopreservation technique for embryogenic callus of cocoa.

Cryopreservation using encapsulation-dehydration technique for somatic embryos was developed by Fang *et al.* (2004). for long-term conservation of cocoa (*Theobroma cacao* L.) germplasm. Survival of individually encapsulated somatic embryos after desiccation and cryopreservation was achieved through optimization of concentration of cryoprotectant (abscisic acid and sugar), duration of osmotic and evaporative dehydration and development stage of the embryo. Up to 63 per cent somatic embryos of the genotype SPA4, in early-cotyledonary stage, survived cryopreservation following seven days preculture with 1 M sucrose and silica exposure for 4 hours (16 per cent moisture content in bead). Recovered SPA4 somatic embryos were converted to plantlets at a rate of 33 per cent and the regenerated plants were phenotypically comparable to non-cryopreserved somatic embryo-derived plants. This optimized protocol was successfully applied to three other genotypes *e.g.* EET272, IMC14 and AMAZ12 with recovery frequencies of 25, 40 and 72 per cent, respectively (but the latter two genotypes using 0.75M sucrose).

Fang *et al.* (2008) also evaluated the role of ethylene and oxidative stress for the recovery of cryopreserved embryos. Dimethyl sulfoxide (DMSO), a free radical scavenger, and an anti-oxidant compound, quercetin, were supplemented to media for enhancing the recovery of cryopreserved embryos. Wetten *et al.* (2008) tested somaclonal variations in cryopreserved somatic embryos with the help of microsatellite markers and none of the embryos exhibited the aberrations in their DNA profiles. Electron microscopic studies revealed that, primary somatic embryos arise from intermediary callus unlike secondary somatic embryos which were originated from the cells of epidermal calli (Wetten *et al.*, 2008). Secondary somatic embryos, thus pose low risk of undergoing mutations than primary somatic embryos, since they are derived from epidermal cell rather than callus cells. No polymorphism was observed in cryopreserved secondary somatic embryos when they are tested for genetic fidelity with microsatellite markers (Fang *et al.*, 2009).

Quainoo (2009) investigated the effect of liquid nitrogen storage time on survival and regeneration of somatic embryos of cocoa (*Theobroma cacao* L.). Somatic embryos from different cocoa genotypes (AMAZ 3-2, AMAZ 10-1, AMAZ 12, SIAL 93, and IMC 14), at 15.45 per cent moisture content, were cryopreserved in LN for one hour, four and eight weeks. Somatic embryos of the genotypes emerged from the alginate beads at different periods 4 to 12 week's post-cryopreservation. Individual genotypes subjected to low temperature storage time did not show significant differences in post-cryopreservation survival, although different genotypes responded differently with AMAZ 12 and IMC 14 recorded the highest and lowest mean survival rates of 58 per cent and 35 per cent, respectively. Plantlets originating from five genotypes were weaned and these plantlets developed normally and were comparable to non-cryopreserved somatic embryo-derived plantlets in the glasshouse.

Fang and Wetten (2011) studied the structural integrity of cryopreserved somatic embryos of cocoa following encapsulation dehydration method. Results showed that the parenchyma cells of the hypocotyls and radicle were the major sites of injury possibly due to their large size and non-cytoplasmic nature whereas the shoot meristem and provascular strand were well preserved throughout the

treatments. The extent of damage increased with each step of the encapsulation dehydration procedure. Even though post thaw regrowth of injured embryos was possible, it often failed to convert into plantlets. The authors suggested the maintenance of structural integrity of the somatic embryos at each treatment step for the successful cryopreservation of cocoa germplasm.

Adu-Gyamfi and Wetten (2012) used vitrification technique for cryopreservation of secondary somatic embryos derived from floral organs. About 74.5 per cent post-cryostorage survival rate of secondary somatic embryos (SSE) could be obtained by pre-culturing SSE on 0.5 M sucrose medium followed by 60 minutes dehydration in cold PVS2. Cation sources were removed from the embryo development solution or the recovery medium in order to reduce the free radical related cryo-injury to the material. This treatment gave a significant benefit during recovery stage. After optimization of this protocol with cocoa genotype AMAZ 15, the same protocol was tested in five other additional lines and successful results were obtained with this vitrification procedure

Adu-Gyamfi *et al.* (2016) studied the epigenetic variations among *in vitro* multiplied somatic embryos, cryopreserved and post- cryopreservation generated somatic embryos along with the ortet trees, using methylation sensitive amplified polymorphism technique. He observed higher level of epigenetic changes in post-cryopreservation generated somatic embryos compared to *in vitro* multiplied and cryopreserved somatic embryos. Furthermore, the passage of cryopreserved embryos through another embryogenic stage led to further increase in variations. Interestingly, these epigenetic variations were reversible to a certain extent.

5. Conclusion

Early attempts for *in vitro* conservation of cocoa started during early 1980s and most of the cryopreservation work was carried out using somatic embryos as well as immature embryos. Though cryopreservation of cocoa tissues appears feasible, a protocol with good recovery of plantlets, along with lowers levels of phenotypic, genetic and epigenetic variation, needs to be standardized for proper maintenance as well as sustainable use of cocoa germplasm. Development of new protocols is also required to conserve different explant material like shoot meristem, embryos, somatic embryos, pollen and DNA samples of wild as well as cultivated species of cocoa.

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