Chapter 9

Cocoa

☆ *Anitha Karun, V. Aparna, K.S. Muralikrishna and M.K. Rajesh*

1. Introduction

Cocoa (*Theobroma cacao* L., 2n=20) is one of the important commercial plantation crops mainly grown for its seeds or beans, which are an important raw materials for chocolate and beverage industries (Cooper *et al.*, 2008). It belongs to the family Malvaceae, categorized under genus *'Theobroma'*. The Latin name *'Theobroma'* literally means 'Food of gods'. This crop has played an important role in many ancient South American cultures (Hurst *et al.*, 2002).

The genus *'Theobroma'* contains 22 species among which *Theobroma cacao* L. is widely cultivated. *Theobroma grandiflorum* L. is the other closely related species of cocoa, which is also the source for a variety of chocolate known as *cupulate* or *cupuacu* (Venturieri, 2011). The tropical plant is a native of Amazon region of South America (Bartley, 2005; Cheesman, 1944); later its cultivation spread to the countries in Asia and Africa (Bartley, 2005; Zhang and Motilal, 2016). The main growing areas of the crop are situated approximately within 20° North and South latitude of the equator. Cocoa needs a temperature of 21-32°C and 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. Even though majority of the species are found in its native place of South America, half of the world's supply of cocoa comes from the two East African countries Cote D'Ivoire and Ghana, which contribute to 42.4 per cent and 17.4 per cent of world's supply, respectively (International Cocoa Organization, 2015).

Criollo, Forastero, Trinitario and Nacional are the four major types of cocoa cultivated around the world (Clement, 2010). The Criollo type produces very high quality cocoa beans, cultivated mainly in its native land South and Central

America but yields are fairly low. Well known cultivars of Criollo type are Chuao, Porcelana, Puerto Cabello and Carupano (Pohlan and Perez, 2008). Forastero is the other important cultivar contributing to nearly 80 per cent of the world's cocoa production. It is cultivated mostly in Africa and some parts of Central and South America. Its growth is vigorous and yields are higher than other types (Pohlan and Perez, 2008). A number of Forestero varieties are cultivated in different parts of the world and some of them are Carenero Superior, Caracas Natural, Rio Caribe and Forastero Amenolado (http://www.cacaoweb.net/cacao-tree.html). Trinitario is a crossbreed between the Forastero and Criollo. It has inherited qualities of aroma from Criollo type and disease resistance, productivity from Forastero type. This hybrid type is mainly cultivated in Central America, South America and Asia (Motilal *et al.*, 2010). Nacional is cultivated in South America and western parts of the Andes. This type is mostly prone to pest and diseases, but it has an excellent aroma (Solorzano *et al.*, 2012).

Cocoa is highly heterozygous crop because of its self-compatible nature (Pound, 1932; Knight and Rogers, 1953, 1955; Cope, 1958, 1959, 1962). In recent years, genetic advancements have been made in cocoa through different breeding approaches. In order to meet the growing demands of the cocoa farmers for true-to type planting material, there is a need for an efficient propagation system which should be cost-effective and produce a large number of true-to-type elite plants. *In vitro* multiplication tools are better choice over conventional propagation methods to meet the above demands. Plant regeneration *via* somatic embryogenesis provides an alternative approach for clonal propagation of cocoa (Li *et al.*, 1998), since the plants are derived from the genetically identical cells of donor parents. Centre de Coopération Internationale en Recherche Agronomique pour le Développement (CIRAD), Montpellier, France and Pennsylvania State University, USA, have developed viable protocols for *in vitro* multiplication through somatic embryogenesis (SE) in cocoa.

Early attempts for *in vitro* regeneration of cocoa through SE started during 1970s (Esan, 1977, 1992; Pence *et al.*, 1979, 1980) with immature embryo as an explant material. Later, a number of successful studies have been reported in cocoa with different explants, media and plant hormone combinations (Esan, 1977; Pence *et al.*, 1979; Adu-Ampomah *et al.*, 1988; Santos and Machado, 1989; Tan and Furtek, 2002). Axillary bud culture and SE are two basic approaches for clonal multiplication of cocoa.

2. *In vitro* Propagation of Cocoa

2.1. Collection and Sterilization of Cocoa Explant Material

Sterilization of explant material is the primary step in cocoa micropropagation since the explant material is highly prone to various types of contamination. Various sterilization methods have been used in cocoa to prevent the contamination caused by fungi, bacteria, epiphytes and other micro-organisms (Hall and Collin, 1975; Duhem *et al.*, 1988). More than 90 per cent contamination was observed in cocoa explant material collected from the field regardless of different procedures used for surface sterilization. Reduced contamination was reported with sodium

hypochlorite, calcium hypochlorite and its commercial formulations like Domestos (Passy and Jones, 1983; Esan, 1985a), Esan (1985b), Legrand and Mississo (1986) and Mallika *et al.* (1992) have suggested regular fungicidal sprays to the mother plants before collecting the explant material. Bavistin (0.2 per cent) and Dithane M-45 (0.3 per cent) sprays were recommended for the mother plants at an interval of three days before collecting explants for sterilization (Mallika *et al.*, 1992). Collection of explant material from the fungicide treated plants and surface sterilization with 70 per cent ethanol, 30 per cent calcium hypochlorite (15 minutes) followed by thorough washing with distilled water, help protect explants from contamination (Esan, 1985a). Surface sterilization of explants with 2 per cent orthodifolatan, followed by 15 minutes calcium hypochlorite rinsing and distilled water wash, was recommended by Legrand and Mississo (1986). Inoculation of surface sterilized material into a media containing the anti-microbial agents helped to minimise contamination chances in cocoa (Yidana *et al.*, 1987). However, Duhem *et al.* (1988) observed tissue necrosis with the addition of anti-microbial agents to the growing media.

Pods and buds can be sterilized by 0.2 per cent warm laundry detergent solution followed by 70 per cent alcohol (2-3 minutes), 10-20 per cent mild commercial bleach solution along with few drops of surfactant added to it and agitated thoroughly. In all sterilisation methods used, distilled water wash is essential at the end (Hall and Collin, 1975). In case of anthers, rinsing should be prolonged to last for 3-5 minutes (Esan, 1992). Pods can be sterilised by alcohol flaming or boiling saline water dip before excision of mature or immature embryos from the pods under aseptic condition (Esan, 1992). Excised embryos can be inoculated into a media without any further sterilization and 100 per cent contamination free cultures were obtained with this method (Esan, 1982). However, Novak *et al.* (1986) recommended further sterilization of excised embryos with NaOCl (5.25 per cent) along with surfactant for 40 minutes followed by sterile water rinsing. Embryos can also be treated with citric acid (50 mg/L) and ascorbic acid (40 mg/L) to check the explant browning as well as contamination (Novak *et al.*, 1986).

2.2. Inoculation and *in vitro* Multiplication of Explants

Starting from the pioneering work of Evans (1951)., there are many reports (Archibald, 1954; Esan, 1977; Townsley, 1974; Hall and Collin, 1975; Prior, 1977; Pence *et al.*, 1979; Novak *et al.*, 1986; Bhavyashree, 2009) of callus induction from almost all types of explant material, *viz.*, stem, bark, nodal cuttings, petiole, leaf, flower, shoot apex, embryos, anthers, floral parts, in various types of media and conditions (Figure 9.1). Some of the workers (Orchard *et al.*, 1979; Passey and Jones, 1983; Flynn *et al.*, 1990) have used shoot tip material of seedlings as an explant. Stem and nodal regions of *in vitro* raised seedlings have also been used as explant material by Esan (1985a), Legrand and Mississo (1986) and Figueira *et al.* (1990). Mallika *et al.* (1992) used explant material collected from budded plants of elite cocoa cultivars maintained under the controlled glass house conditions. Legrand and Mississo (1986) and Flynn *et al.* (1990) observed a positive correlation between the explant size and *in vitro* growth response. A single node stem cutting, with length ranging from 2-3 cm having maximum length towards the lower internode with subtending leaf above, is an ideal explant for cocoa tissue culture (Mallika *et*

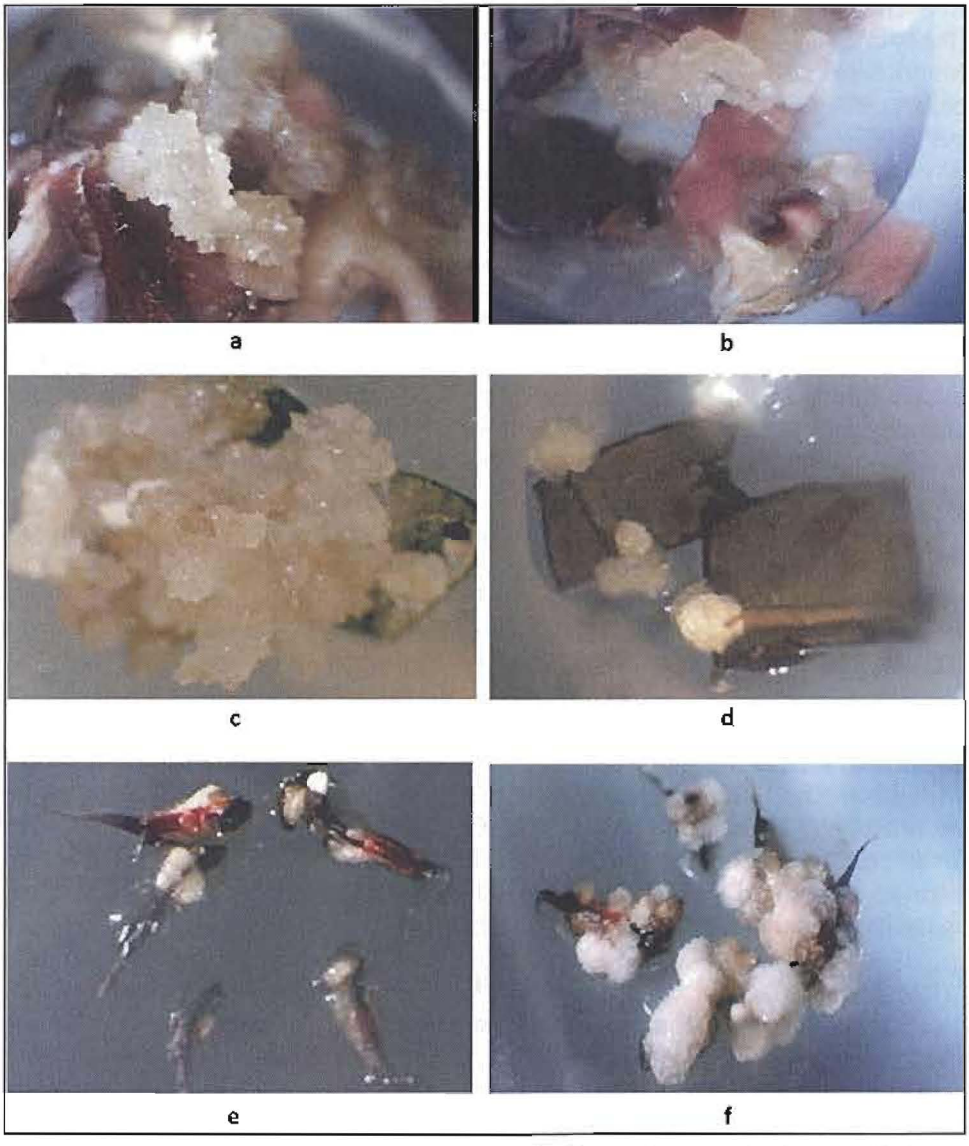

Figure 9.1: Callus Initiation from Different Explants of Cocoa. Callogenesis in cotyledonary explants (a, b), in tender leaves (c, d) and staminodal (e, f) explants.

al., 1992). Explant collection during leaf flushing stage may show some positive effects on *in vitro* grown cultures (Mallika *et al.*, 1992). Many researchers have observed improved bud initiation from pre-existing meristems. Maximova *et al.* (2003) also tried micropropagation of cocoa from somatic embryo derived plants Salzar *et al.* (2006) studied plant regeneration *via* SE technique. Different protocols were standardized in cocoa in order to use staminodes, petal, anther and immature cotyledon as explant materials. Tan *et al.* (1998) reported root and shoot formation

from staminodes of young flower buds. Bhavyashree (2009) studied the callus initiation and SE from the leaf, nodal cuttings, cotyledon and staminodal explants of cocoa using MS and DKW basal media. Among different explants used, better embryogenic callus initiation was observed from the leaf explant and more browning was reported with DKW media compared to MS basal media. Reports suggest that callus induction from the cocoa is possible in almost all types of basal media with or without any plant growth regulator. But organogenesis from the callus has been found to be difficult in cocoa. Esan (1985a) observed callus initiation from plumular, radical and hypocotyl regions of cultured embryo axis. Among these three types of callus induced, plumular callus gave rise to shoots and roots.

2.3. Somatic Embryogenesis

Somatic embryogenesis offers scope for *in vitro* seedling production by direct embryogenesis (Esan, 1977; Pence *et al.*, 1979; Elhag *et al.*, 1987; Ad-Ampomah *et al.*, 1988; Santos and Machado, 1989) as well as indirect embryogenesis from embryogenic competent callus (Kononowicz and Kononowicz, 1984). There are many reports on direct embryogenesis from zygotic embryos (Kononowicz and Kononowicz., 1984; Kononowicz and Janick, 1984a, b; Wen *et al.*, 1984). But plantlets obtained from zygotic embryos were not true to type. When somatic embryos are induced from the vegetative tissues or other than zygotic tissue of non-sexual origin, this process allows the mass multiplication of elite and uniform plantlets.

A protocol was developed for indirect SE of cocoa by Tahardi and Mardiana (1995). Embryogenic calli was initiated from the immature flower buds, which was followed by development of somatic embryoids from nodular calli. After two subcultures in a hormone free medium, embryo maturation and germination were achieved. A detailed study was conducted for cocoa flower bud tissues with respect to its physiological age, floral explant types, genotypes, phytohormones and media compositions in order to improve SE (Tan and Furtek, 2002). Salzar *et al.* (2006) reported SE from the Venezuelan cocoa cultivars. Li *et al.* (1998) observed SE and plant regeneration from floral tissues of a number of cocoa cultivars. Effect of different carbon sources (glucose, sucrose, fructose, maltose and sorbitol) on cocoa SE was studied by Guiltinan *et al.* (2002). Among the five carbon sources tested, glucose, fructose and sucrose were found to show positive effect on somatic embryo production, while no somatic embryo production was observed in medium supplemented with maltose or sorbitol. Maximova *et al.* (2002) developed the secondary SE technique in cocoa using primary somatic embryos developed from the cotyledon explants. Primary somatic embryos collected from two different locations were primarily cultured on callus multiplication medium containing 2.4 µM 2,4-D along with 1.4 µM BA. Cultures were further transferred to hormone free embryo development medium for induction of secondary SE.

Scanning electron microscopic studies have been conducted to monitor different developmental stages of SE from immature embryo explant in cocoa (Santos and Machado, 1989). A typical cocoa embryo is characterized by an elongated axis with two well-developed cotyledons (Santos and Machado, 1989). MS basal medium along with auxin component is sufficient for induction of embryoids in cocoa.

Kononowicz and Kononowicz (1984) observed highest number of embryoids with MS medium, containing 2,4-D alone. Improved mitotic index of callus was observed with increased concentrations of 2,4-D (Kononowicz and Kononowicz, 1984). Callus transition towards embryogenesis was accompanied by increased DNA replication and RNA synthesis (Knonowicz and Janick, 1984b). Promotive effect of embryogenesis was observed with the addition of casein hydrolysate and coconut water to the media containing auxin component. Induced somatic embryos were cultured in a basal media containing cytokinin and auxins; this improved maturation and germination rate of somatic embryos. Absicic acid also aided maturation of embryoids (Alemano *et al.*, 1996). Proper germination was reported in embryoids of more than 4 mm size. Sub culturing process could be continued until one or more leaflets appeared from germinated embryos (Alemanno *et al.*, 1996). An efficient *in vitro* clonal propagation method for cocoa was developed by Guiltinan *et al.* (2002), who have also reported the occurrence of primary and secondary SE processes in cocoa.

2.4. Organogenesis

Woody Plant Medium (WPM) was superior over half-MS medium for long term maintenance of cultures without tissue necrosis and abscission (Flynn *et al.*, 1990; Mallika *et al.*, 1992). MS liquid medium was found to be beneficial for shoot growth and elongation (Adu-Ampomah *et al.*, 1987). Addition of ethylene inhibitors (5 ppm $AgNO_3$ or 0.5 mg/L $CoCl_2$) was suggested to overcome the problems of excess callus production at the cut ends of explants, as well as for subsequent improvement in shoot regeneration (Mallika *et al.*, 1992). Addition of additional amino acids and anti-oxidants to basal media helped sustained growth of *in vitro* regenerated shoots in cocoa (Flynn *et al.*, 1990). Addition of vitamins and casein hydrolysate to the basal media improved the proliferation of axillary shoots from cotyledonary node explant. Adu-Ampomah *et al.* (1987) observed plantlet regeneration from shoot tips of *in vitro* grown seedlings. Addition of cytokinin to the media helped axillary shoot induction, shoot elongation and leaf formation from the cotyledon as well as root explants (Figu *et al.*, 1990, 1991). But prolonged treatment with high levels of cytokinins (2-iP) led to shoot tip necrosis instead of its sustained growth (Figueira *et al.*, 1990, 1991).

Increased levels of illumination and CO_2 promoted *in vitro* shoot development in cocoa (Figueira *et al.*, 1991; Figueira and Janick, 1990). Shoot elongation and production of normal leaves from the detached axillary shoots were observed when the explants were exposed to higher levels of CO_2 (20,000 ppm) and 150-200 µM $s^{-1}m^{-2}$ of PPFD (Photosynthetic Photon Flux Density) (Figueira *et al.*, 1991). Shoot elongation as well as leaf development was enhanced when concentration of CO_2 was increased from the ambient to 24,000 ppm (Figueira and Janick, 1990). The same developmental changes were not true under dark, implying that photosynthesis stimulation is an important deciding factor for the performance of *in vitro* cultures. Maintenance of higher CO_2 may also act as an ethylene inhibitor, in addition to its role in stomatal regulation and cellular pH maintenance. Removal of cotyledons from embryos also improved plantlet recovery under dark conditions (Mallika *et al.*, 1992).

Shoots are normally kept for one month in a rooting medium containing auxin. *In vitro* rooting was obtained in cocoa using plant hormones *viz.*, IBA, IAA and phloroglucinol (Passey and Jones, 1983). Sometimes, the roots formed from *in vitro* multiplied shoos were thick, stubby and unbranched resembling tubers and the plantlets failed to survive in hardening stage (Mallika *et al.*, 1992). Anatomical studies revealed lack of vascular continuity between shoot and roots at the collar region. Rooting was normally induced in shoots developed from different explant material by pulsing treatment with various concentrations of IBA. Thin and healthy roots were observed on shoots when auxin treated material was further transferred to hormone free charcoal medium. The rooted plants could be field planted after gradual hardening process (Mallika *et al.*, 1992).

2.5. Induction of Multiple Shoots

Adu-Ampomah *et al.* (1988) developed a protocol for the production of cocoa plantlets using shoot tip culture. Multiple shoot production from the axillary bud culture will be an ideal technique for obtaining large number of plantlets from a single explant. When nodal explants were grown on the medium containing thidiazuron, axillary buds were proliferated and shoot development progressed (Figueira *et al.*, 1990, 1991). Mallika *et al.* (1996) induced the multiple shoots from nodal segments on WPM media with additional supplements.

2.6. Problems Associated with Cocoa Micropropagation

Plantlet production is possible through *in vitro* multiplication in various genotypes of cocoa. Still cocoa is considered as a recalcitrant species because of certain factors, which hinder multiplication rate *in vitro* and limit the elite planting material production through tissue culture methods. Presence of number of phenolic compounds and their oxidation hinders the callus multiplication and SE in cocoa. Naturally, cocoa contains large amounts of polyphenolics and their oxidation could be one of the limiting factors preventing proper tissue multiplication and maintenance (Griffiths, 1958; Kim and Keeney, 1983). In non-embryogenic calli, production of phenolic compounds has been found to be higher than embryogenic calli (Alemonno *et al.*, 1996). Profuse callusing at the cut ends of explant material after bud break is found to arrest shoot growth. Heavy callusing from the cocoa explant material may be due to the presence of higher concentrations of endogenous plant hormones *i.e.* auxin and cytokinins (Mallika, 1992). Callus appearance has been cited as another interfering factor for the success of cocoa *in vitro* cultures (Passey and Jones, 1983; Dublin, 1984; Legrand and Mississo, 1986; Mallika *et al.*, 1992) Khalid *et al.* (1991) observed the inhibitory action of ethylene on tissue organogenesis from callus. Browning, excessive production of slimy exudates, callus over growth, poor organogenesis from the callus, slow multiplication rate and bud dormancy are some of the reported problems during the *in vitro* multiplication of cocoa (Hall and Collin, 1975; Legrand and Mississo, 1986).

2.7. Hardening, Acclimatization and Field Transplanting of Rooted Plantlets

Hardening and transplanting procedures for cocoa for tissue cultured plantlets have been standardized by many workers. After root initiation, plantlets were transferred to a potting mixture and protected from desiccation by covering with polythene bags. Air circulation to the plants could be maintained by making small holes on the polythene bag. After 3-4 months of acclimatization, the plants could be successfully transferred to the bigger pots with standard potting mixture (Mallika *et al.*, 1996). Growth rate was initially slower, which improved gradually. Growth behaviour and morphological appearance of plantlets were comparable after six months of initial establishment. Tissue cultured plantlets in the field was found to be more vigorous than *ex vitro* derived plants (Mallika *et al.*, 1996). Flowering and fruiting of the tissue culture derived plants were on par with the budded or grafted plants. Pollen fertility and viability was reported to be more in budded and seedling derived plants compared to micro propagated ones. However, this did not influence the cocoa pod yield. Variations were observed in case of pod morphology, bean number and bean weight (Mallika *et al.*, 1996).

2.8. Anther Culture

Esan (1977) and Prior (1977) made the earliest attempts for standardizing cocoa anther culture technique. Esan (1982) reported the production of haploid and diploid types of callus from cocoa anther cultures. Prior (1977) observed induction of callus from anther somatic tissues and also demonstrated its use as a nurse tissue for the growth and reproduction of a cocoa fungal pathogen *Oncobasidium theobromae*. To date, no androgenic cocoa plants production has been reported under *in vitro* conditions, although many of the haploid plants have been found in nature (Esan, 1992). Callus induction and root development was observed after 60 and 90-120 days of anthers inoculation, on MS and double strength white medium supplemented with 20 gl^{-1} sucrose,100 mgl^{-1} inositol, 0.2 mgl^{-1} kinetin and 5 $mg1^{-1}$ NAA. With the addition of casein hydrolysate or coconut milk extract, callus development was improved, but rooting was either delayed or even prevented (Esan, 1992). Mallika *et al.* (1992) also could induce callus from anthers; however they could not obtain plant regeneration from anther callus. MS medium supplemented with 2 mg 1^{-1} 2-iP, 0.1 mg 1^{-1} NAA and 126 mg 1^{-1} phloroglucinol was found to be most favourable combination for root development in anther callus induced proembryoids (Sunil, 1992).

A detailed study on cocoa anther culture and influence of different factors for successful plantlet regeneration was conducted by Sunil (1992), who reported that the development stage of anther, minimum temperature in the field of donor plants, type and strength of basal medium, type and concentration of auxins and cytokinins, carbohydrate source, sucrose level, physical environment and gamma and UV rays were main factors that influence anther callus induction (Sunil, 1992). Anther callus multiplication was influenced by type of basal medium, type and concentration of growth regulators, carbohydrate source, sucrose level, presence of amino acids, organic supplements, ethylene releasing and inhibiting chemicals,

adenine and its derivatives, unusual regulants, gibberellins and growth inhibitors, light and gamma rays. The factors influencing indirect embryogenesis were found to be stage of anther development and light. Hybrid genotypes responded more favourably to callus induction, callus multiplication, callus rhizogenesis than traditional cultivars and they were also found to be stable with respect to seasonal changes (Sunil, 1992).

Sunil (1992) first reported plantlet recovery from cocoa anthers via indirect embryogenesis. Anthers at tetrad stage were subjected to a two-stage culture procedure involving incubation and subculture (after 4 weeks) on modified H3 basal medium supplemented with 1 mgl^{-1} NAA and 0.1 mgl^{-1} 2-iP for 50 days (stage 1) and subsequent weekly transfer to ½ MS basal media supplemented with 1mgl^{-1} 2-iP and 3 mgl^{-1} GA_3 (stage II). Proembryoids were obtained in stage 1 medium *via* callus within 45 days of culture, only when anthers derived from Criollo, Trinitario and hybrid (H2) were used. Serial subculturing of embryoids in stage II media led to formation of shootlets and rootlets. With four sub-cultures in stage II in a span of one month, the embryoids could be germinated into plantlets of size 2.5 cm with two leaflets and one rootlet (Sunil, 1992).

2.9. Protoplast Isolation and Culture

Thompson *et al.* (1987) used protoplast isolation and fusion techniques for cocoa regeneration. When excised protoplasts from the tissues were grown in dark, cell wall regeneration and embryoids formation was observed further. Studies were also conducted on factors influencing the protoplast isolation from young cocoa leaves at the early F2 stage. Chantrapradist (1999) isolated protoplasts from rapidly growing cocoa cell suspensions using 2 per cent (w/v) driselase, 0.5 M sorbitol, 1 mM MES (2-N-morpholino ethanesulfonic acid) and 10 mM $CaCl_2.2H_2O$ at pH 5. After 3 hours of cell incubation with the enzyme solution, protoplasts were separated with an average yield of $4.5x10^5$ per gram fresh weight. Best results were obtained when protoplasts were grown in MS basal medium supplemented with 2.3 µM 2, 4-D under dark conditions. Cell wall formation and cell division were observed after 8-10 days of culture. Continuous cell division leads to a formation of cell colonies and small microcalli within four weeks.

2.10. Embryo Rescue

Embryo culture helps to overcome the problems of seed abortion and improves the germination ability of difficult to germinate types. Poor germination of cocoa seeds may be due to toxic or lethal factors of cotyledonary tissue (Ibanez, 1964). Despite high heterozygosity in cocoa, disease resistant genotypes among the cultivated types are limited in cocoa. Distant hybridization techniques are normally used for improving the disease resistance, agronomical traits or other qualitative traits of beans in cocoa (Kononowicz and Janick, 1984c). An *in vitro* embryo culture technique helps to maintain the embryos resulting from distant crosses. Kononowicz and Janik (1984b) successfully excised 100 days old embryo, which could be maintained *in vitro* up to its maturity using embryo culture technique. Palma and Villalobos (1989) successfully obtained 16-87 per cent of plantlets along with 2 per cent of haploid types from two different crosses by embryo rescue technique.

2.11. Suspension Cultures for Somatic Embryogenesis and Secondary Metabolite Production in Cocoa

Hall and Collin (1975) first initiated cocoa suspension cultures from seedling tissues. Jalal and Collin (1978).and Tsai and Kinsella (1981) formulated a synthetic media for maintaining cocoa callus and suspension cultures. MS basal media supplemented with 0.5 mgl^{-1} 2, 4-D and 0.1 mgl^{-1} kinetin was found to be the most successful medium for growing cocoa suspension (Tsai and Kinsella, 1981). Wen *et al.* (1984) established cell suspension using cocoa bean callus tissues and investigated lipid composition in liquid cell suspension in comparison with cocoa beans at different maturities. Proliferations of asexual embryos were observed from immature zygotic embryos when they are cultured on semi-solid or liquid medium (Wen *et al.*, 1984). Niemenak *et al.* (2008) used temporary immersion bioreactor system (TIS) for multiplication of cocoa somatic embryos; better results were obtained with TIS system compared to solid media. TIS also improved the formation of somatic embryos as well as their development regarding their conversion to torpedo shaped forms. High speed multiplication was obtained in cocoa cell suspension upon enzymatic treatment of explant material in liquid suspensions. (Rojas *et al.*, 2012).

Cocoa suspension cultures have been mainly initiated in order to synthesize flavour (purine alkaloids, theobromine, caffeine and theophylline) and butter components (triglycerides, cis-palmito-oleostearin) of cocoa. Townsley (1974) produced a chocolate aromatic product from mature suspension cell of cocoa. But, suspension cultures were found to have low polyphenolic content in comparison with callus and explant tissue (Jalal and Collin, 1997). No purine alkaloids were identified in suspension cultures of cocoa (Jalal and Collin, 1979). When purine precursors (7-methylxanthosine and methionine) were artificially added to callus, theobromine was synthesized; it shows the activity of a part of the purine biosynthetic pathway (Jalal and Collin, 1979). Later, Gurney *et al.* (1992) tested and observed low amount of purine alkaloids production and accumulation in suspensions in comparison with the callus cultures. Leathers and Scragg (1989) observed the effect of different temperatures on suspension growth, lipid content and fatty acid composition of cocoa cell suspension cultures. The optimal temperature for growth of cell suspension was found to be 30°C. Lipid and fatty acid biosynthesis were maximal at temperatures ranging from 15-20°C. The lipid composition of cocoa butter is different from the lipids which were identified in cocoa suspensions (Tsai and Kinsella, 1982). During development of the embryo, fatty acid composition becomes more saturated as cocoa butter is synthesized. An increased sucrose concentration to a medium containing somatic embryos induces synthesis of triglycerides, which constitute cocoa butter (Pence *et al.*, 1981).

2.12. Gene Expression Studies Related to Somatic Embryogenesis

Somatic embryogenesis is an efficient propagation system for rapid and mass multiplication of cocoa. One of the major bottleneck in cocoa SE is that the efficiency of somatic embryo production is highly genotypic dependent. Further, only a lower percentage of plantlet regeneration is achieved from somatic embryos due to improper cotyledon development in cocoa. Studies were conducted to

understand the SE process in cocoa by examining the genes related to the process of. Maximova *et al.* (2014) studied the gene expression patterns during differential developmental stages of cocoa somatic and zygotic embryogenesis. The expression of 28,752 genes was determined at four developmental time points during zygotic embryos and two time points during cocoa somatic embryogenesis. During zygotic embryogenesis, 10,288 differentially expressed genes were enriched for functions related to responses to abiotic and biotic stimulus, metabolic and cellular processes. In total, 10,175 genes were differentially expressed in zygotic and SE. Many TF genes, related to ethylene metabolism and response, were more strongly expressed in somatic embryogenesis as compared to zygotic embryogenesis. Genes related to fatty acid metabolism, flavonoid biosynthesis and seed storage functions were also found to be differentially expressed between two stages. The insights gained from the differential gene expression patterns might enable designing of more efficient protocols for cocoa SE.

An orthologue of the *Arabidopsis* Leafy Cotyledon-2 gene (*AtLEC2*) was characterized in *Theobroma cocoa* (*TcLEC2*) and its expression studies were conducted in cocoa cultures (Zhang *et al.*, 2014). The expression pattern of *TcLEC2* was reported to be higher in embryogenic than non-embryogenic calli. Transient overexpression of *TcLEC2* in immature zygotic embryos of cocoa causes a change in the gene expression profiles and fatty acid composition. The overexpression of *TcLEC2* in cocoa explants was found to improve the frequency of regeneration of stably transformed somatic embryos. Another important gene BABY BOOM (*BBM*) was characterized (*TcBBM*) and tested in cocoa (Florez *et al.*, 2015). *TcBBM* gene expression was observed in entire embryo development stages. Expression level of *TcBBM* was reported to be high in SE compared to zygotic embryogenesis. *TcBBM* over-expression alone in cocoa led to the formation of embryogenic structures without addition of any exogenous plant growth regulators. Only moderate enhancements in embryogenic potential was observed with transient ectopic expression of *TcBBM*. Constitutive overexpression of this gene greatly increased SE proliferation but also appeared to inhibit subsequent development of embryo development and regeneration. *TcBBM* and *TcLEC2* could potentially be used as a biomarker for the improvement of the SE process and screen for elite varieties in cacao germplasm (Zhang *et al.*, 2014; Florez *et al.*, 2015).

2.13. Micrografting

In this method, *in vitro* raised shoots are grafted on to root stock of *in vitro* or *ex vitro* origin. This technique helps to save time as well as resources in the micropropagation of cocoa. Aguilar *et al.* (1992) attempted a micrografting technique in cocoa using somatic embryos and young cocoa seedlings as a scion and rootstock material. Best results were observed on simple culture medium with three months old rootstock and somatic embryos without cotyledons. Nearly 10 month time period was required for complete plant regeration after a successful graft attempt was done. Bindu (1997) tried micrografting using *in vitro* raised shoots from nodal segments as a scion material. Axenic seedlings cultured on half MS liquid medium devoid of sucrose was found to be best as rootstock when *in vitro* raised shoots used as a scion material. Side grafting was the most ideal for micrografting procedure among the different grafting techniques available. Success was highest when scions with

two or more hardened leaves were grafted 4 cm below the cotyledons in 4-5 weeks old axenic seedlings with a few hardened leaves. Anatomical studies revealed that the graft union was complete in about a month. Successful grafts could be obtained when scion material had one or two hardened leaves. Grafting on *ex vitro* root stock material was more successful and exhibited the rapid and extensive elongation of shoots. Field performance of micro grafted plants was also found to be satisfactory (Bindu and Mallika, 2008).

3. Future Prospects

Plantlets have been raised from the cocoa vegetative or floral plant parts collected from the field, seeds, mature and immature zygotic embryos *etc.* Variations in plant genotype, physiological maturity of explants and seasonal variations are some of the problems hindering the development of a viable protocol. Though SE appears feasible, protocol for recovery of plantlet has still not been perfectly standardized. A 'perfect' protocol for the clonal multiplication and cryopreservation techniques need to be standardized for the multiplication as well as maintenance of elite cocoa gemrplasm. Since cocoa whole genome sequencing has already been completed, the data can be used for selection and modification of different genes which are involved in SE process through different transformation approaches. The prospects for making use of the various biotechnological approaches in cocoa hybridization programme also seem to be very bright.

References

Adu-Ampomah, Y., Novak, F.J., Afza, R. and Van Durren, M. (1987). Embryoid and plant production from cultured cocoa explant. *Proceedings of Tenth International Cocoa Research Conference*, Santo Domingo. pp.129-136.

Adu-Ampomah, Y., Novak, F.J., Afza, R., Van Durren, M. and Perea- Dollos, M. (1988). Initiation and growth of somatic embryos of cocoa (*Theobroma cocoa* L.). *Café Cacao The'*, **32:** 187-200.

Aguilar, M.E., Villalobos, V.M. and Vasquez, N. (1992). Production of cocoa plants (*Theobroma cacao* L.) via micrografting of somatic embryos. In *Vitro Cellular and Developmental Biology- Plant*, **28**: 15-19.

Alemanno, L., Berthouly, M. and Ferrière, M.N. (1996). Histology of somatic embryogenesis from floral tissues in *Theobroma cacao* L. *Plant Cell Tissue and Organ Culture*, **46**: 187-194.

Archibald, J.F. (1954). Culture *in vitro* cambial tissues of cocoa. *Nature*, **173**: 351-352.

Bartley, B.G.D. (2005). The genetic diversity of cacao and its utilization. In: Lockwood, R. (Ed.), *Experimental Agriculture, Volume.42, Issue.1*, CABI Publishing, Wallingford, U.K. p. 341.

Bhavyashree, U. (2009). *In vitro* multiplication of cocoa (*Theobroma cacao* L.). M.Sc. Thesis, Department of Biosciences, Mangalore University, Karnataka, India.

Bindu, M.R. and Mallika, V.K. (2008). Micrografting in cocoa (*Theobroma cacao* L.). *International Journal of Agriculture Sciences*, **4**: 601-603.

Bindu, M.R. (1997). Standardization of *in vitro* techniques for rooting, hardening and micrografting in cocoa (*Theobroma cacao* L.). Ph.D thesis, Faculty of Agriculture, Kerala Agricultural University, Kerala, India.

Chantrapradist, C. and Kanchanapoom, K. (1999). Cell suspension, isolation and culture of cacao (*Theobroma cocoa* L.) protoplasts. *Songklanakarin Journal of Science and Technology*, **21**: 1-8.

Cheesman, E. (1944). Notes on the nomenclature, classiûcation and possible relationships of cocoa populations. *Tropical Agriculture*, **21**: 145-146.

Clement, C.R., Cristo-Arau ´jo, M., d'Eeckenbrugge, G.C., Pereira, A.A., and Rodrigues, D.P. (2010). *Origin and domestication of native Amazonian crops. Diversity*, **2**: 72-106.

Cooper, K.A., Donovan, J.L., Waterhouse, A.L and Williamson, G. (2008). Cocoa and health: A decade of research. *British Journal of Nutrition*, **99**: 1-11.

Cope, F.W. (1962). Mechanism of self incompatibility in *Theobroma cocoa* L. *Heredity*, **17**: 157-182.

Cope, F.W. (1958). Incompatibility in *Theobroma cocoa* L. *Nature*, **181**: 279.

Cope, F.W. (1959). Nuclear fusion and non-fusion in *Theobroma cocoa* L. *Nature*, **183**: 1540.

Dublin, P. (1984). Techniques de reproduction végétative in vitro etamélioration génétique chezles caféiers cultivés. *Café Cacao Thé*, **28**: 231-244.

Duhem, K., Le Mercier, N. and Boxus, P. (1988). Difficulties in the establishment of axenic *in vitro* cultures of field collected coffee and cacao germplasm. *Acta Horticulture*, **225**: 67-76.

Elhag, H.M., Whipkey, A. and Janik, J. (1987). Induction of somatic embryogenesis from callus in cocoa (*Theobroma cacao* L.) in response to carbon source and concentration. *Revista Theobroma*, **17**: 153-162.

Esan, E.B. (1977). Tissue culture studies on cacao (*Theobroma cacao* L.): A supplementation of current research. *Proceedings of the 5th International Cacao Research Conference*, Ibadan, Nigeria. pp. 153-162.

Esan, E.B. (1982). Shoot regeneration from a callus derived from embryo axes cultures of *Theobroma cacao* L. *in vitro*. *Turrialba*, **32**: 359-364.

Esan, E.B. (1985a). Axillary bud transformation into asexual embryos from seedling node cultures of cocoa. *Nigeria Agricultural Journal*, **20**: 13-22.

Esan, E.B. (1985b). Clonal multiplication of *Theobroma cocoa* L. zygotic embryos *in vitro* asexual embryogenesis. *Nigeria Agricultural Journal*, **20**: 23-38.

Esan, E.B. (1992). Micropropagation of Cocoa (*Theobroma cacao* L.). In: Y.P.S. Bajaj (Ed.), *Biotechnology in Agriculture and Forestry, Vol. 18, High-Tech and Micropropagation II*. Springer-Verlag, Berlin Heidelberg. pp. 96-122.

Evans, H. (1951). Investigations on propagation of cocoa. *Tropical Agriculture*, **28**: 147-203.

Figueira, A., Whipkey, A. and Janick, J. (1991). Increased CO_2 and light promote *in vitro* shoot growth and development of *Theobroma cacao* L. *Journal of the American Society of Horticultural Science*, 116: 585-589.

Figueira, A., Whipkey, A., and Janick, J. (1990). Micropropagation of *Theobroma cocoa L. a recalcitrant species in high* CO_2. *Horticultural Science 87th Annual Meet, ASHS*, Tueson, Arizona. pp. 608.

Florez, S.L., Erwin R.L., Maximova S.N., Guiltinan M.J. and Curtis W. (2015). Enhanced somatic embryogenesis in *Theobroma cacao* L. using the homologous BABY BOOM transcription factor. *BMC Plant Biology*, 15: 2-12.

Flynn, W.P., Glicenstein, L.J. and Fritz, P.J. (1990). *Theobroma cacao* L.: An axillary bud *in vitro* propagation procedure. *Plant Cell, Tissue and Organ Culture*, 20: 111-117.

Griffiths, L.A. (1958). Phenolic acids and flavonoids of *Theobroma cacao* L.: Separation and identification by paper chromatography. *Biochemical Journal*, 70: 120-125.

Guiltinan, M., Maximova, S.N., Traore, A., Miller, C., Robinson, M., Antunez de Mayolo, G., Barreto, E., Young, A. and Pishak, S. (2002). Integrated systems for the propagation of cacao: Tissue culture, bentwood gardens and rooted cuttings. In: *Proceedings of the 13th International Cocoa Research Conference, Workshop on the New Technologies and Cocoa Breeding*, Kota Kinabalu, Sabah, Malaysia. Cocoa Producers Alliance, Lagos, Nigeria. pp. 323-330.

Gurney, K.A., Evans, L.V. and Robinson, D.S. (1992). Purine alkaloid production and accumulation in cocoa callus and suspension cultures. *Journal of Experimental Botany*, 43: 769-775.

Hall, I.R.H. and Collin, H.A. (1975). Initiation and growth of tissue cultures of *Theobroma cacao* L. *Annals of Botany*, 39: 555-570.

Hurst, W.J., Tarka, S.M., Powis, T.G., Valdez, F. and Hester, T.R. (2002). Cacao usage by the earliest Maya civilization. *Nature*, 418: 289-90.

Ibanez, M.L. (1964). Role of the cotyledon in sensitivity to cold of cacao seed. *Nature*, 201: 414-415.

International Cocoa Organization (2015/16). *Quarterly Bulletin of Cocoa Statistics*, Vol. XLII, No. 1, Cocoa.

Jalal, M.A.F and Collin, H.A. (1978). Secondary metabolism in tissue culture cultures of *Theobroma cacao* L. *New Phytologist*, 83: 343-349.

Khalid, M., Charaibi, B., Latche, A., Roustan, J.P. and Fallot, J. (1991). Stimulation of shoot regeneration from cotyledons of *Helianthus annus* by ethylene inhibitors, silver nitrate and cobalt. *Plant Cell Reports*, 10: 204-207.

Kim, H. and Keeney, P. (1983). *Polyphenols-tannins in Cocoa Beans*. 37th PMCA Production Conference, Lancaster, PA. pp. 60-63.

Knight, R. and Rogers, H.H. (1953). Sterility in *Theobroma* cocoa L. Nature, 172:164.

Knight, R. and Rogers, H.H. (1955). Incompatibility in *Theobroma* cocoa *L. Heredity*, 9: 69-77

Kononowicz, A.K. and Janick, J. (1984b). The influence of carbon source on the growth and development of somatic embryos of *Theobroma cocoa* L. *Physiologia Plantarum*, 61: 155-162.

Kononowicz, A.K and Janick, J. (1984). *In vitro* development of zygotic embryos of *Theobroma cocoa* L. *Journal of American Society of Horticultural Sciences*, 109: 266-269.

Kononowiczh, J. and Janick, J. (1984). Response of embryogenic callus of *Theobroma cacao* L. to gibberellic acid and inhibitors of gibberellic acid synthesis. *Journal of Plant Physiology*, 113: 359- 366.

Kononowicz, H. and Kononowicz, A.K. (1984). Asexual embryogenesis via callus of *Theobroma cacao* L. *Journal of Plant Physiology*, 113: 347-358.

Leathers, R.R. and Scragg, A.H. (1989). The effect of different temperatures on the growth, lipid content and fatty acid composition of *Theobroma cacao* L. cell suspension cultures. *Plant Science*, 2: 217-222.

Legrand, B. and Mississo, E. (1986). Influence de la taille des explantats et des régulateurs de croissance sur le développement des tissus de *Theobroma cacao* L., var. Amelonado cultivés *in vitro*. Café Cacao The', 30: 239-246.

Li, Z., Traore, A., Maximova, S. and Guiltinan, M.J. (1998). Somatic embryogenesis and plant regeneration from floral explants of cacao (*Theobroma cacao* L.) using thidiazuron. In *Vitro Cellular and Developmental Biology-Plant*, 34: 293-299.

Mallika, V.K., Mathew, J.P., Chacko, D.,Vijaykumar, N.K. and Nair, R.V. (1996). Induction of multiple shoots in nodal explants of fully grown trees of cocoa (*Theobroma cocoa* L.). *Journal of Plantation Crops*, 24: 503-510.

Mallika, V.K., Shankar, A., Sindhu, L., Rekha, M.C., Vijaykumar, N.K. and Nair R.V. (1992). Plantlet regeneration *in vitro* from nodal segments of cocoa (*Theobroma cocoa* L.) and field planting. *Journal of Plantation Crops*, 20: 114-122.

Maximova, S.N., Alemanno, L., Young, A., Feffiere, N., Traore, A. and Guiltinan, M.J. (2002). Efficiency, genotypic variability, and cellular origin of primary and secondary somatic embryogenesis of *Theobroma cacao* L. In *Vitro Cellular and Developmental Biology-Plant*, 38: 252-259.

Maximova, S.N., Florez, S., Shen, X., Niemenak, N., Zhang, Y., Curtis, W. and Guiltinan, M.J. (2014). Genome wide analysis reveals divergent patterns of gene expression during zygotic and somatic embryo maturation of *Theobroma cacao* L., the chocolate tree. *BMC Plant Biology*, 14: 1-17.

Maximova, S.N., Miller, C.R., Antúnez de Mayolo, G., Pishak, S., Young, A. and Guiltinan, M.J. (2003). Stable transformation of *Theobroma cacao* L. and influence of matrix attachment regions on GFP expression. *Plant Cell Reports*, 21: 872-883.

Motilal, L., Zhang, D., Umaharan, P., Mischke, S., Mooleedhar, V. and Meinhardt, L.W. (2010). The relic Criollo cacao in Belize-genetic diversity and relationship with Trinitario and other cacao clones held in the International Cocoa Genebank, Trinidad. *Plant Genetic Resources: Characterization and Utilization*, 8: 106-115.

Niemenak, N., Saare-Surminski, K., Rohsius, C., Ndoumou, D.O. and Lieberei, R. (2008). Regeneration of somatic embryos in *Theobroma cacao* L. in temporary immersion bioreactor and analyses of free amino acids in different tissues. *Plant Cell Reports*, 27: 667–676.

Novak, F.J., Donini, B. and Owusu, G. (1986). Somatic embryogenesis and *in vitro* plant development of cocoa (*Theobroma cacao* L.). *Proceedings on International symposium on nuclear techniques and in vitro culture for plant improvement. IAEA*, Vienna, Austria. pp. 443-449.

Orchard, J.E., Collin, H.A. and Hardwick, K. (1978).Culture of shoot apices of *Theobroma cacao* L. *Physiologia Plantarum*, 47: 207-210.

Palma,T. and Villalobos, V.M. (1989). Rescate *in vitro* de embriones provenientes de semillas aplanadas de cacao (*Theobroma cacao* L.). *Turrialba*, 39: 525-529.

Passey, A.J. and Jones, O.P. (1983). Shoot proliferation and rooting *in vitro* of *Theobroma cacao* L. type Amelonado. *The Journal of Horticultural Science and Biotechnology*, 58: 589-592.

Pence, V.C., Hasegawa, P.M. and Janick, J. (1978).Asexual embryogenesis in *Theobroma cacao* L. *Journal of American Society of Horticultural Sciences*,104: 145-148.

Pence, V.C., Hasegawa, P.M. and Janick, J. (1980). Initiation and development of asexual embryos of *Theobroma cacao* L. *in vitro*. *Journal of Plant Physiology*, 98: 1-14.

Pence, V.C., Hasegawa, P.M. and Janick, J. (1981). Sucrose-mediated regulation of fatty acid composition in asexual embryos of *Theobroma cacao* L. *Physiologia Plantarum*, 3: 378-384.

Pohlan, H.A.J. and Perez, V.D. (2008). Soil Plant Growth and Crop Production. Vol-III, Growth and Production of Cacao. *Encyclopaedia of Life Supporting Systems*. pp. 1-32.

Pound, F.J. (1932). Studies on Fruitfulness in Cocoa -II, *First Annual Report on Cocoa Research Trinidad*, pp. 26-28.

Prior, C. (1977). Growth of *Oncobasdium theobroma* Talbot and Keane in dual culture with the callus tissues of *Theobroma cocoa* L. *Journal of General Microbiology*, 99: 219-222.

Rojas, L.F., Florez, C., Atehortua, L., Gil, A. and Londono, J. (2012). Application of a new method to obtain cell biomass of *Theobroma cacao* L. seeds for metabolites production in flasks and Bioreactor. *Acta Horticulture*, 961: 435-440.

Salzar, V.R., Betancourt, C., Mata, J and Garcia, F. (2006). Somatic embryogenesis in Venezulan cocoa cultivars. *Agronomia-Tropical-Maracay*, 56: 61-74.

Santos, A.V.P and Machado, R.D. (1989). A scanning electron microscopy study of *Theobroma cacao* L. somatic embryogenesis. *Annals of Botany*, 64: 293-296.

Solorzano, R.G.L., Fouet, O., Lemainque, A., Pavek, S., Boccara, M., Argout, X., Amore, F., Courtois, B., Risterucci, A.M. and Lanaud, C. (2012). Insight into the

wild origin, migration and domestication history of the fine flavour Nacional *Theobroma cocoa* L. variety from Ecuador. *PLoS ONE*, 7: 1-11.

Sunil, K.P. (1992). Anther culture in cocoa (*Theobroma cacao* L.). M.Sc. Thesis. Vellanikkara, *Department of Agricultural Botany, College of Horticulture*, Kerala, India.

Tahardi, J.S. and Mardiana, N. (1995). Cocoa regeneration via somatic embryogenesis. Menara Perkebunan, 52: 174- 178.

Tan, C.L. and Furtek, D.B. (2002). Development of *in vitro* regeneration system for *Theobroma cacao* L. from mature tissues. *Plant Science*, 164: 407-412.

Tan, C.L., Davey, M.R., Mulligan, B.J., Power, J.B., Lowe, K.C., Jaafar, H., Yeang, Y.H. and Lee, M.T. (1998). Somatic embryogenesis in cocoa as a basis for genetic transformation. In: Davey, M.R., Alden, P.G., Lowe, R.C. and Power, J.B. (Eds.), *Tree Biotechnology- Towards the Millennium*. Nottingham University Press, Nottingham, UK. pp.149-154.

Thompson, W., Collins, H.A., Issac, S. and Hardwick, K. (1987). Isolation of protoplasts from cocoa (*Theobroma cacao* L.) leaves. *Café Cacao, The'*, 31: 115-120.

Townsley, P.M. (1974). Chocolate aroma from plant cells. *Canadian Institute of Food Science and Technology Journal, 7: 76-78.*

Tsai, C.H and Kinsella, J.E. (1982). Tissue culture of cocoa bean (*Theobroma cacao* L.): Incorporation of fatty acids into lipids of cultured cells. *Lipids*, 17: 848-852.

Tsai, C.H. and Kinsella, J.E. (1981). Initiation and growth of callus and cell suspensions of *Theobroma cacao* L. *Annals of Botany*, 48: 549-558.

Venturieri, G.A. (2011). Flowering levels, harvest season and yields of cupuassu (*Theobroma grandiflorum*). *Acta Amezonica*, 41: 143-152.

Wen, M.C., Germen, B., Kinsella, J.E. (1984). Cocoa bean cell and embryo culture. *Journal of the American Oil Chemists Society*, 61: 1720-1724.

Yidana, J.A., Withers, L.A. and Ivins, J.D. (1987). Development of single method for collection and propagating cocoa germplasm *in vitro*. *Acta Horticulture*, 212: 95-98.

Zhang, D. and Motilal, L. (2016). Origin, dispersal and current global distribution of cacao genetic diversity In: Baily, L.W. and Meinhardt, L.W. (Eds.), *Cocoa Diseases*. Springer International Publishing, Switzerland. pp 1-31.

Zhang, Y., Clemens, A., Maximova S.N. and Guiltinan, M.J. (2014). The *Theobroma cacao* B3 domain transcription factor TcLEC2 plays a dual role in control of embryo development and maturation. *BMC Plant Biology*, 14: 2-16.