IN VITRO MULTIPLICATION OF COCOA

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Introduction

In cocoa, clonal propagation is highly significant, because this alone can ensure the production of true-to-type progenies from any selected superior genotype. *In vitro* methods are expected to make cocoa improvement faster and more efficient. Because crop losses due to several diseases (like seedling die-back, black pod disease, cherelle rot, vascular streak dieback disease etc.) have been identified as major production constraints in the cocoa growing countries.

Research work on cocoa in India was initiated by the Research Cum Demonstration Unit of Cadbury India Pvt. Ltd. established at Chundale in Kerala in 1965. Most of the research activity was taken by the Central Plantation Crops Research Institute and Kerala Agricultural University. In the 50's and 60's plant tissue culture methods were developed for the propagation of a wide variety of species, but were not applied to cocoa until late 70's and then, with very limited success. Recently research conducted at Plant DNA technology CIRAD, Montpellier, France, Nestle, France and at the Pennsylvania State University, USA has led to the development of efficient methods for somatic embryogenesis of cocoa (Guiltinan and Maximova, 2001). A substantial number of technologies have been evolved by research institutes for improved productivity and higher income from cocoa. Clonal propagation of superior genotypes through *in vitro* methods is one of the identified thrust areas of cocoa biotechnology.

Thrust areas of cocoa biotechnology

- Clonal propagation of selected superior genotypes through *in vitro* multiplication such as somatic embryogenesis and micro propagation techniques.
- Characterization and cataloguing of genetic diversity and elimination of duplication in germplasm collections through *in vitro* exchange.
- *In vitro* conservation for long term storage, maintenance and transport of germplasm.
- Production of cocoa butter and cocoa flavours under *in vitro* condition.
- Homozygous diploids through anther culture and exploitation of hybrid vigour by crossing these.
- Overcoming incompatibility barriers through *in vitro* techniques of pollination, fertilization, embryo rescue, etc., for producing distant hybrids.
- Protoplast isolation, fusion and plant regeneration to assist in hybrid production, physiological investigations, genetic engineering etc.
- Developing techniques for genetic transformation.

Asexual propagation - Soft wood grafting

Cocoa being a self incompatible crop in general, the population will have lot of variations. Hence, softwood grafting is found successful and suitable in cocoa to breed true-to-type planting material. The method consists of cleft grafting of scions to seedlings of 75-90 days old raised in polybags. The scions are collected by prior defoliation of shoots of comparative thickness. The selected scions are inserted into the cleft portion of the root stocks so that they fit tightly and the grafted site is tied with polythene strips. These grafts are kept in shade and watered daily. Grafts union will take place within one month. All the shoots emerging from the rootstocks are to be removed periodically. The grafts are planted in the pits as in the case of seedlings after three months of hardening. About 80 to 90 per cent success is obtained when grafting is done during August to October, under Vittal, Karnataka conditions.

Research work in Kerala Agricultural University, India

The basal media, WPM (Mc Cown's Woody Plant Medium) (Mallika *et al.,* 1992) was found to be superior to half strength MS (Murashige and Skoog) in which cultures could be maintained for a long time without leaf necrosis and abscission. Addition of silver nitrate (5ppm) or $CoCl_2$ (0.5 mg/l) was suggested to overcome the problem of callus production at cut ends of explants and also to improve shoot regeneration. Experiments carried out by Mallika *et al.* (1992) reported the higher levels of endogenous auxins and gibberellins in the plant. The heavy callusing of cocoa plants also indicated higher levels of auxin. Growth supplement addition also found to have an influence on shoot growth and regeneration. Bud burst and sustained growth of shoots could be achieved when nodal explants were cultured in WPM containing additional supplements. One of the factor involved in positive growth response appeared to be the stage of leaf flush at which explants are harvested. Nodal position relative to the apex of the shoots appeared to have no significant effect on the results.

Mallika *et al.* (1992) reported that the roots formed by using IBA, NAA and Phloroglucinol hormones were thick, stubby and unbranched, resembling tubers and the plantlets failed to survive the hardening process. Anatomical studies revealed lack of vascular continuity between the root and shoot at the collar region and the presence of a layer of corky callus in between. Rooting of shoots derived from axillary buds, for shoots originating from seedling explants and for the field explants derived shoots could be accomplished by pulse treatment of IBA at various concentrations. The auxin treatment was followed by transfer of the materials to auxin free medium with charcoal, resulted in healthy roots. Shoots derived from juvenile plants rooted properly but in the field explants derived shoots the rooting percentage was poor. The rooted plantlets could be field planted successfully after a gradual process of hardening. Production of multiple shoots in the axillary bud culture will be ideal as it provides opportunity for obtaining a large number of plantlets from single explants. Mallika *et al.* (1996) could induce multiple shoots from nodal segments on WPM with supplements. They observed increased release of buds from the pre-existing axillary meristems. Continued presence of higher levels of 2-ip was detrimental to the sustained growth of shoots and was manifested as shoot-tip necrosis in such cultures.

Micrografting technique standardized at KAU helps to save time and resources in the micropropagation of cocoa. Rooting could be surmounted by this technique. The *in vitro* raised shoots were cleft-side-grafted on to *in vitro* or *ex vitro* root stock raised from open pollinated seeds. But the *ex vitro* grafting appeared to be more effective. Success was obtained only with shoots having one or more leaves. The *ex vitro* grafts exhibited rapid and extensive elongation of the shoots and therefore the plants could be transplanted to the field within five months. The field performance of these plants was also found to be satisfactory. A successful hardening and field transplanting procedure was developed. After culturing for about one month in the rooting medium, the plantlets were transferred to a potting mixture. The polythene bags covering the plants provided 100% humidity and thereby protecting the plants from desiccation. Small holes provided in these bags helped in facilitating air circulation. Following acclimatization for 3-4 months, the plants were successfully transferred to the bigger pots containing standard potting mixture. Initial growth of these plantlets was very slow but gradually the growth rate improved. The growth behaviour and morphological appearance of these plantlets were comparable after 6 months. Mallika *et al.* (1992) reported that Bavistin (0.2%) and Dithane M-45 (0.3%) were sprayed regularly on mother trees at three days interval. The explants were collected, washed thoroughly in tap water containing few drops of teepol and allowed it to dry. The explants were then swabbed with cotton dipped in 70% ethanol. Single node cuttings were prepared under laminar air flow and then surface sterilized in freshly prepared chlorine water for four minutes followed by washing twice in sterile water. Morales (1995) could completely check contamination of zygotic embryos and apical buds by 100% sodium hypochlorite treatment for 15 minutes.

Research work abroad

An efficient somatic embryogenesis method was reported which was capable of propagating a wide variety of cocoa genotypes (Li *et al.,* 1998). Somatic embryo from cocoa staminodes was produced from all genotypes. Cotyledon explants for secondary embryogenesis were selected from primary embryo (from staminodes), and cultured for 14 days on modified secondary callus growth medium containing 2.4 μM, 2,4-D and 1.4 μ M 6-Benzylaminopurine. Somatic embryogenesis of cocoa was studied by Tan *et al.* (1998) in order to develop a shoot regeneration protocol which could be exploited for genetic transformation. Staminodes from young flower buds were excised on an MS-based medium with the addition of 1.0 mg NAA/l. Nodular calli were initiated after 3-4 weeks of culture; 2 types of calli were observed, yellow friable and compact white. Callus was transferred to a differentiation medium supplemented with 1.0 mg IAA, 1.0 mg IBA and 0.5 mg gibberellic acid (GA3)/l for 4-8 weeks in the dark. Somatic embryos formed 4-8 weeks after transfer and developed from the globular to the torpedo and finally to the cotyledonary stage before they germinated. According to them, the conversion to whole plant was still problematical.

Traore and Abdoulaye (2000), Traore and Guiltinan (2006) studied the effect of five carbon sources (glucose, fructose, maltose, sorbitol, sucrose) on cocoa somatic embryogenesis. Glucose, fructose and sucrose were found to support cocoa somatic embryo production, while no embryos were produced with sorbitol or maltose in the media. Four different media (MS, WP, DKW and DKW supplemented with potassium nitrate and amino acids) were tested for their ability to support and promote shoot development during embryo conversion. DKW medium supplemented with potassium nitrate and amino acids yielded the highest percentage of normal and total shoot production (59% and 92% respectively). DKW and WPM media supported a higher percentage of conversion (77% each) than did MS medium (59%). The addition of TDZ to the medium did not affect shoot production from internodal and apical explants. Higher rooting efficiencies of shoot explants were achieved using IBA. The basal media, WPM (Flynn *et al.,* 1990) was found to be superior to half strength MS in which cultures could be maintained for a long time without leaf necrosis and abscission. Liquid MS medium was beneficial for subculturing shoots for elongation and growth (Adu-Ampomah *et al.,* 1992). Flynn *et al.* (1990) observed formation of new leaves in cultures similar to the *in vitro* flushing characterized by pinkish leaves. Legrant and Mississo (1986) cited the problem of bud dormancy as a significant disadvantage in cocoa micro propagation. Somatic embryogenesis has been reported from nucellar tissues also. A protocol for conversion of nucellar somatic embryos into plantlets was developed that involves preculture of somatic embryos in liquid medium and transfer to semi- solid medium in chambers receiving 20,000 ppm CO_2.

Figueira *et al.* (1991) studied the effect of CO_2 level in *in vitro* development of shoot. Axillary shoots of cocoa, induced *in vitro* with cytokinins (thidiazuron), elongated and produced leaves only in the presence of cotyledons and/or roots. Detached axillary shoots, which do not grow *in vitro* under conventional tissue culture protocols, rooted with auxin (IBA or NAA) and developed normally *in vivo.* Detached axillary shoots from cotyledonary nodes and single-node cuttings from mature plants were induced to elongate and produce normal leaves in the presence of 20,000 ppm. CO_2 and a photosynthetic photon flux density (PPFD) of 150-200 $\mu mols^{-1}m^{-2}$. Subcultured nodal cuttings continued to elongate and produce leaves under enriched CO_2 levels and higher irradiances, and some formed roots. Subculture of microcuttings under CO_2 enrichment could be the basis for a rapid system of micropropagation for cocoa. Using cotyledon or roots as explants, axillary shoot induction, elongation and leaves formation with the action of cytokinin were observed by Figueira *et al.* (1991). Shoot elongation and leaf development increased with carbon dioxide from the ambient to 32,000 ppm. The carbon dioxide effect did not occur in the dark, implying that photosynthesis stimulation is an important factor of enhanced *in vitro* performance. Some synergistic effect of high carbon dioxide might be acting either on ethylene inhibition, reduction in photorespiration, and reduction in cellular pH or control of stomata. Maximova *et al.* (2002), developed a procedure for the regeneration of cocoa. Rapidly growing callus were induced on a DKW- based primary callus growth (PCG) medium supplemented with 20 g glucose/litre, 9 μM 2,4-D and Thidiazuron (TDZ) at various concentration by culturing a staminode as an explant. Calli were subcultured onto a WPM-based secondary callus growth medium supplemented with 20 g glucose/litre, 9 μM 2,4-D and 1.4 nm kinetin. Somatic embryogenic callus following transfer to a hormone-free DKW-based embryo development medium containing sucrose. The concentration of TDZ used in PCG medium significantly affected the rate of callus growth, the frequency of embryogenesis and the number of somatic embryos produced from each responsive explants. A TDZ concentration of 22.7 nm was found to be the optimal concentration for effective induction of somatic embryos from various cocoa genotypes.

Secondary embryogenesis system was developed by Maximova *et al.* (2002) 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. Repetitive somatic embryogenesis in cocoa was studied. In order to achieve repetitive somatic embryogenesis in cocoa callus derived from floral tissues were continuously cultured in a medium containing 2,4-D. In 5% of the explants, repetitive somatic embryogenesis was observed after 8 weeks and maintained in a globular stage for several weeks. Maximova *et al.* (2008), studied the micropropagation of cocoa using somatic embryo derived plants. When nodal explants were cultured on thidiazuron medium, axillary buds proliferated and developed into shoots, which were excised and rooted.

During root induction, short treatment with indole-3-butyric acid (IBA) increased the total percentage of rooted microcuttings upto 89%. Longer exposure to IBA increased the average number of roots per microcutting. Plant regeneration *via* somatic embryogenesis was studied which provides an alternative method for clonally propagating cocoa. Therefore, a protocol was developed in order to use staminode, petal, anther and immature cotyledon explants of genotypes Choroni-42, Ocumare-77, Ocumare-61 and Porcelana. Callus formation was induced with Thidiazuron (TDZ), high sucrose concentration and 2,4-D. Somatic embryos were induced with kinetin and coconut water. Embryos were transferred to a medium containing amino acids and placed under dark conditions and 26-29°C. Cotyledonary embryos were subcultivated under photoperiodic conditions of 16/8 h light-dark for posterior formation of plants.

Orchard *et al.,* 1979; Passey and Jones, 1983; Flynn *et al.,* 1990 used shoot tips from seedlings as explants. A critical review of literature suggests that callus induction is possible practically in all types of basal media with or without any growth regulator. However, organogenesis from callus is reported to be rare. In studies by Esan (1982a) the cultured embryo axis of mature zygotic embryos could be induced to form callus from plumule, radicle and roots. Plantlets have been raised from the field grown vegetative parts, whole and parts of flower and mature seeds, whole immature and zygotic embryos (Esan, 1982b). The problems hindering the development of a viable protocol are variations in genotypes, physiological maturity of explants and seasonal variations. Though somatic embryogenesis appears feasible, protocol for recovery of plantlets is still not perfectly standardized. Cryopreservation techniques once perfected will be a boon to ease maintenance and exchange of the germplasm. The prospects of making use of the various biotechnological approaches in cocoa hybridization programmes also seem to be very bright.

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