

CRYOPRESERVATION OF COCOA SHOOT TIPS USING PRE GROWTH DESICCATION PROTOCOL

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Introduction

Conservation of germplasm as field gene banks is the most popular and practical method of conservation. This field bank has several disadvantages like it occupies a large land area, its labour and time intensive to maintain and controlled pollination for producing true-to-type, costs more. It is necessary to complement field gene bank with other conservation methods. The use of *in vitro* culture technique including slow growth and cryopreservation represents an important option for medium and long-term conservation of cocoa. Cryopreservation is a process where cells or whole tissues are preserved by cooling to sub-zero temperatures, (-196°C). At these low temperatures, any biological activity, including the biochemical reactions that would lead to cell death, is effectively stopped. Over the past 40 years, individual scientists developed and tested a range of cryopreservation techniques for preserving plant cells and tissues; but routine storage of plant germplasm other than seeds in liquid nitrogen is relatively new practice (Engelmann, 2004).

In this study, the main objective was to standardise the various cryopreservation techniques for shoot apex of cocoa, encapsulation dehydration technique, pregrowth-desiccation technique and droplet freezing method. The success of the procedure can be attributed to its easiness, high reproducibility and to the fact that it can be successfully applied to a wide range of tissues and plant species.

Materials and Methods

Cocoa shoot tips were collected from cocoa tree in the subspecies Forastero from CPCRI, Kasaragod. The shoot tips were collected from the field using a sterile blade and directly collected into a polyethylene bag without any hand contact.

Sterilization of explants

The outer leaflets of shoot tips removed completely for the proper sterilization of explant. The explants were kept in Bavistin (1g/L) for 1-3 hours to avoid fungal contamination. Then washed under running tap water for half an hour and treated with 2-3 drops of Tween-20 and subsequently washed under running tap water for one hour. Then the explants were transferred to laminar airflow chamber for further sterilization process. The explants were washed with 20% sodium hypochlorite solution, for 20 minutes with constant shaking and subsequently the explants were rinsed 2-3 times with distilled water. Then these explants were sterilized with 0.1% mercuric chloride for 5-7 minutes with constant shaking, subsequently rinsed with sterile water 4-5 times for the complete removal of the sterilant.

Culture media

McCown's Woody Plant Medium (WPM): The McCown's Woody Plant Medium (WPM) was used for preculture. The WPM contains macronutrients, micronutrients, organic supplements, 0.75M sucrose and 0.1g/L ascorbic acid.

Retrieval medium: The retrieval medium was prepared that contains the same components of WPM media but with 0.6M sucrose and growth hormones including BAP (1mg/L), GA₃ (0.5mg/L) and NAA (0.2mg/L).

Inoculation of explants: The external leaf whorls were removed using a sterile blade and the shoot tips were detached carefully. Using a sterile forceps each shoot tips were inoculated to WPM culture media.

Culture condition

After inoculation, the cultures were kept under dark. The temperature was maintained between 24-26°C and the relative humidity 80% for 7-10 days.

Cryopreservation

Three kinds of cryopreservation methods were used.

Encapsulation dehydration technique

Ten explants which grown in WPM media with 0.75M sucrose for one week were taken and dropped into 3% sodium alginate solution for one minute. Then each explant was transferred to 0.1M calcium chloride solution using a pipette to form the beads. The beads that contain explant were washed with sterile distilled water and wiped carefully using sterile tissue paper. All the beads were placed on the sterile aluminum foil and left it for 20 minutes in a bottle that contain autoclaved silica gel. After 20 minutes, transferred 5 beads to the retrieval media and stored under dark with temperature 24-26°C (-LN). This treatment contributes positive control. The rest five beads transferred to the autoclaved cryovials and kept it in liquid nitrogen for 24 hours. After cryopreservation, the cryovials were thawed at 40°C and inoculated into retrieval media using sterile forceps (+LN). Kept under desired conditions.

Pregrowth desiccation method

Total six explants from the Woody Plant Media were transferred into 1M sucrose solution and kept it for 16 hours. After the osmotic dehydration the explants transferred to the autoclaved silica gel for 2 hours. Two of the dried explant directly inoculated into the retrieval media (-LN), kept under dark at 26°C and observed daily. The rest four explants were transferred to the autoclaved cryovials and quenched in liquid nitrogen for 24 hours. After cryopreservation, the explants were transferred to the retrieval media (+LN). Stored under dark and the temperature maintained between 24°C and 26°C and the observation was taken every day for morphological changes. Using the 1.5M sucrose solution and the time ranges to 10 hours in sucrose solution carried out in the same procedure. Stored under similar conditions and observations were recorded every day.

Droplet freezing method

Six explants were taken from the woody plant media. Three drops of 50% DMSO solution was placed on a sterile aluminum foil and three explants were transferred into it, kept for 2 minutes. Then dipped it in liquid nitrogen for 2-3 minutes. Transferred to the autoclaved cryovials and cryopreserved in liquid nitrogen for 24 hours. After 24 hours, the cryovials was thawed at 40°C and inoculated into retrieval media with a sterile forceps. These tubes were labeled as +LN. The rest three explants were dipped in 50% DMSO solution for 2-3 minutes. Then inoculated into the retrieval media under sterile conditions. These tubes were labeled as -LN. All the six tubes were stored under dark with the temperature varies from 24-26°C, and the observation were noted down daily for physiological changes.

Histological study

The samples were fixed in standard fixative Carnoy's 'B' fluid (Chloroform-30ml; absolute alcohol-60 ml; glacial acetic acid-10 ml) to localize RNA, DNA, proteins and polysaccharide. Fixed materials were dehydrated serially using alcohol and butanol, which replaces the water in the material. A mixture of paraffin wax and bee's wax of melting point 58-60°C was used for infiltration and embedding. Melted paraffin along with the explants was poured into the mould to prepare paraffin block for section cutting.

Microtome sectioning

After cooling, the edge of the paraffin block was trimmed to form a cube, and fixed on the wooden block. Uniformly thin sections of 10µm thickness were cut using Leica RM 2145 rotary microtome.

Affixing the sections on slide

3% gelatin was prepared were put on the surface of a pre cleaned micro slide. Appropriate portions of the paraffin ribbons were cut into convenient length and floated over 3% gelatin over the slide. Then the slides were placed over a warming plate, maintained at nearly 45°C to facilitate stretching of the ribbon.

Deparaffinisation and dehydration of the section

Slides were deparaffinised using xylene. Subsequent dehydration was done with butanol and alcohol. Then they were transferred to 100% alcohol and subjected to histochemical staining either directly or after hydration depending on the stain used. Toluidine blue and Periodic acid Schiff's reagents were used to localize the nucleic acids and polysaccharides respectively. The sites of nucleic acid appear dark blue in colour against faintly stained background. The intensity of which is the measure of the amount of RNA or DNA present in tissues. The sites of polysaccharides and starch granules take red colour.

Assessment of cell viability

The cell viability was assessed by using TTC (2, 3, 5 Triphenyl Tetrazolium Chloride) solution. For checking the viability, the shoot tips after cryopreservation by different methods, were incubated in TTC solution in a glass vial and covered with aluminum foil. After 24 hours, the sections of shoot tips were taken and observed. The appearance pink colour indicates that the tissues are viable.

Photodocumentation

The photographs of the cultures and histochemical sections were taken with the help of automatic Leica Qwin Standard Image Analyzer connected to Leica DMLS Microscope.

Results

The extracted shoot tips after proper sterilization stored in Woody Plant Media (WPM) for 2-3 weeks. During dark incubation 76.73% fungal contamination was noticed.

Encapsulation dehydration method

A total of nine cultures which encapsulated with 3% sodium alginate solution to form beads were used for the experiment. Out of which 4 beads were not cryopreserved (-LN) and 5 beads were cryopreserved in liquid nitrogen for 24 hours (+LN). Cryopreserved materials inoculated in retrieval medium, were not increased in size when observed after 2-4 weeks (Fig. 7).

Pregrowth desiccation method

The shoot tips which were incubated in 1M and 1.5M sucrose solution for 24 hours, showed slight enlargement (5%) (Fig. 6).

Droplet freezing method

In this method, the shoot tips were treated with 50% DMSO solution for 2 minutes and cryopreserved. These shoot tips did not show any tissue swelling and high browning (Fig. 5).

Histological studies

Encapsulation-dehydration method

The cocoa shoot tips that were cryopreserved after the encapsulation- dehydration method were observed under low and high magnification power and there is no fine staining with Toluidine blue on cell walls and nuclei. The cell wall breakage and cell shrinkage were observed in almost all cells (Fig. 10).

Pregrowth-desiccation method

The tissues that were treated in 1M and 1.5M sucrose solutions observed under light microscope after Toluidine blue staining. There was no cell wall breakage or cell shrinkage observed. Also the nuclei were appeared as darkly stained (Fig. 9).

Droplet-freezing method

The Toluidine blue stained tissues which were cryopreserved after treated in 50% DMSO solution for 2 minutes. No cell wall breakage and stained nuclei were observed. The cell shrinking observed in high percentage (Fig. 8).

Assessment of cell viability

Encapsulation dehydration method

The sections of the cryopreserved shoot tips of encapsulation-dehydration method which were treated in TTC solution for 24 hours do not possess pink colour and concluded that were dead tissues (1% survival). The tissue had freezing injury and the tissue was flaccid (Fig. 13).

Pregrowth desiccation method

The section of the shoot tips which were cryopreserved after treated in 1.5M sucrose solution had pink colour when treated in TTC solution for 24 hours and they were found to be viable (5%) (Fig. 12).

Droplet freezing method

The section of the shoot tips that were cryopreserved after treated in 50% DMSO solution had no pink colour and they were found to be flaccid and dead (2% survival) (Fig. 11)

Discussion

Most of the tissue culture work on cocoa reported the high percentage of bacterial and fungal contamination. During the period of preculture of cocoa shoot apices in WPM media, 76.73% of culture was fungal contaminated. The fungal contamination may be due to the high humidity and rainfall during the time of material collection and relative humidity varied 83-98%. Also, 80% humidity was observed during preculture. However, Dulbin (1984) pointed out that with explants detached from the field, the percentage of infection was 90% regardless of procedure used for surface sterilization. Mallika *et al.* (1992) reported a procedure for reducing the rate of contamination in explants collected from the field-grown plants. The treatment in 1% Bavistin, an anti-fungal agent, for 1-2 hours reduced the high chance of contamination. The results reported by Duhem *et al.* (1988) indicated that the above antimicrobial agents induced explant necrosis. Though various surface sterilants were tried at different concentrations alone or in combination with antibiotics and fungicide, they could not completely control contamination. They obtain partial control of contamination by pre-treatment of clonal material grown in glass house.

The shoot apices are widely used for cryopreservation, because they are totipotent and contain organized tissues which minimize the risk of genetic stability. The cryopreservation of cocoa shoot apices were tried through three different techniques *viz.*, Encapsulation- dehydration, Pregrowth- desiccation method and Droplet- freezing techniques.

The TTC test and histological studies gave positive results to the shoot apices that incubated in sucrose solution (1.5M) comparing with the shoot apices that treated with DMSO solution and encapsulation-dehydration technique. *In vitro* grown young lateral buds when treated in 1M sucrose solution induced a large number of uniform young lateral buds in cryopreserved. The shoot apices that encapsulated with

3% sodium alginate and dehydrated with silica gel had no enlargement in size and high browning was observed. But encapsulation dehydration was successfully implemented with large number of species both from temperate and tropical origin. Later it was experimented in cocoa somatic embryo.

The shoot apices that treated in 50% DMSO solution for 2 minutes observed high browning and no cell expansion. This may be due to the use of high concentration of DMSO solution even though it is cryoprotectants. However, Rajasekaran (1996) achieved successful regeneration of cotton (*Gossypium hirsutum* L.) plants from cryopreserved embryogenic callus and cell suspension cultures by using the cryoprotectant mixture consisting of a modified Murashige and Skoog (1962) medium with sucrose (5% w/v), DMSO (5% v/v) and glycerol (5% v/v). Bernard and Jane (1979) studied the cryoprotection of suspension cultures of sugarcane cells by using various cryoprotectants in various combinations and highest viability value was achieved by employing glucose and DMSO. The embryonic axes, which treated in 10% DMSO solution for 2 minutes does not have any cell expansion when observed after 2-3 weeks of inoculation in retrieval media. But in case of 10% DMSO treated embryonic axes which then cryopreserved in liquid nitrogen for 24 hours (+LN) had high rate of browning which is not present in non-cryopreserved one(-LN). This indicates that the cryopreservation may damage or kill the embryonic axes even if treated in DMSO. Even though DMSO acts as cryoprotectant its treatment is critical for the post cryopreservation survival. The optimum treatment in droplet-freezing method might have led to the shrinkage of cells and breakage of cell walls after thaw recovery.

The histological study of the cryopreserved shoot apices revealed that the tissues of fresh and pregrowth technique possess the proper staining of Toluidine blue and cell walls and nucleus stained darkly. But in the case of liquid nitrogen treated cells some of the cells of apical dome retained its structure but some of the cell walls were broken. In case of encapsulation-dehydration and droplet-freezing method, the cells of the apical dome were disrupted by breakage of cell wall. The loss of cellular integrity may be the cause of death of cells in cryopreserved materials in all the methods tested above. The localized presence of intact cells in the pregrowth-desiccation method showed their positive responses to sucrose incubation. The tissues that undergone encapsulation-dehydration and droplet-freezing method results in cell wall breakage and cell shrinkage and most of the tissues were unstained. Almanno *et al.* (1996) carried out the histology of the somatic embryogenesis from floral tissue of cocoa, which showed that only staminodes and stamen filaments were able to produce somatic embryos in expression medium.

The viability checking of both cryopreserved and fresh shoot apices carried by TTC test, which gave pink colour on viable cells. The fresh shoot apices and +LN shoot apices of pregrowth method gave the pink colour and shoot apices of other two techniques gave the negative result as it was dead. However, much attention is also given to cryopreservation of shoot-tips and buds from *in vitro* seedlings/explants, especially for those recalcitrant species whose embryonic axes have failed to survive. Positive TTC tests are most promising; however, longer-term studies must be performed concerning the development and regeneration of cryopreserved material. The ultimate result of fresh shoot tip is comparable with pregrowth apices, but not much intensity as fresh axes.

The TTC test is showing slight appearance of pink color, its actual survival depends on the ability of shoot to regenerate. The intensity and extent of TTC staining are successfully employed to predict the germinability percentage of a sampled lot of seeds (Bennett and Loomis, 1949; Parker, 1953). Only the fresh shoot apices and shoot apices that treated with sucrose (pregrowth-desiccation technique) had red color which indicates the cell respiration. In addition, TTC reduction has been widely used in the viability assay of plant tissues exposed to stressful conditions. By comparing the respiration rate, Parker (1953) suggested that one of the best methods in determining the viability of conifer leaves after various drying and freezing treatments is the *in vivo* reduction of TTC.

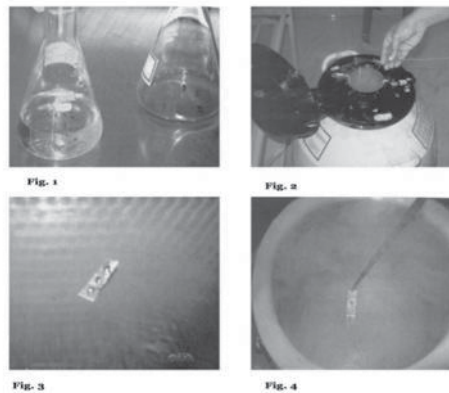


Fig. 1. Encapsulation dehydration, Fig. 2. Pregrowth desiccation, Fig. 3. Droplet freezing, Fig. 4. Dipping into the liquid nitrogen

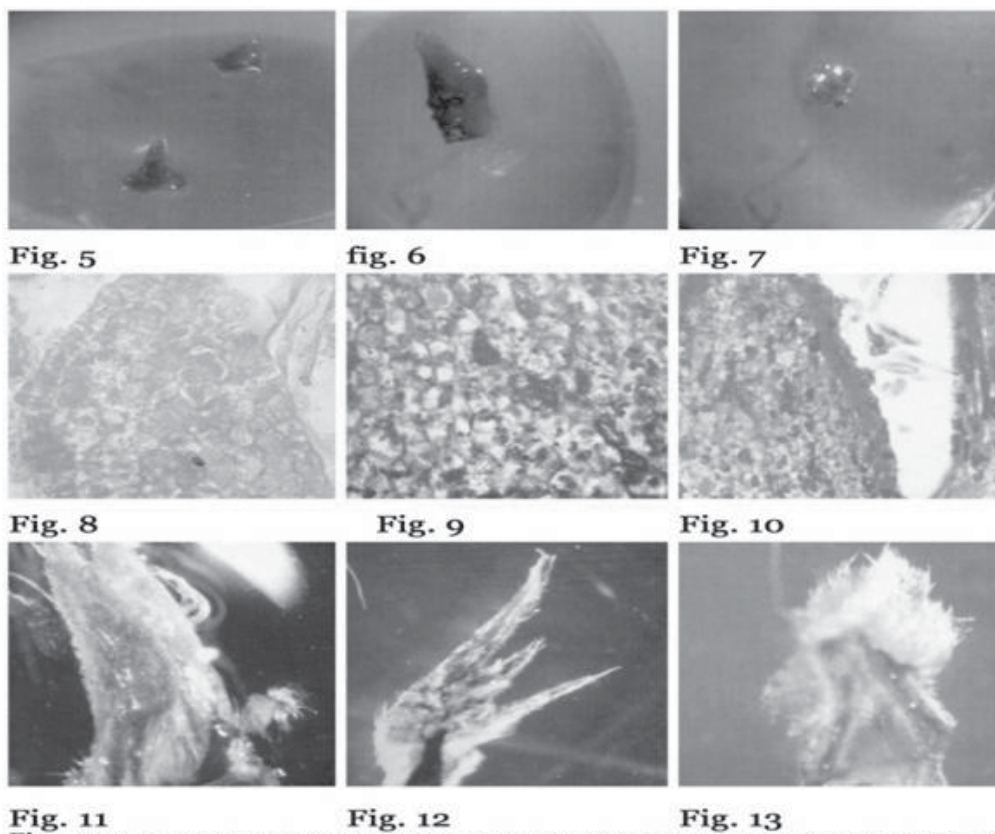


Fig. 5. Shoot tip treated with droplet freezing method, Fig. 6. Shoot tip treated with pregrowth desiccation method, Fig. 7. Shoot tip treated with encapsulation dehydration method, Fig. 8. Histological section of shoot tip treated with droplet freezing method, Fig. 9. Histological section of shoot tip treated with pregrowth desiccation method, Fig. 10. Histological section of shoot tip treated with encapsulation dehydration method, Fig. 11. Negative result obtained in droplet freezing method, Fig. 12. Positive result obtained in pregrowth desiccation method Fig. 13. Negative result obtained in encapsulation dehydration method.

When compared to encapsulation-dehydration and droplet-freezing method, the pregrowth-desiccation method followed for cryopreservation of cocoa shoot tips shows response with respect to its viability using TTC and cell structure. The preliminary studies revealed that in pregrowth desiccation, survival was 55 whereas in droplet freezing and encapsulation dehydration survival was very low (1% and 2% respectively).

Conclusion

Histological studies revealed that the shoot tips of cocoa after cryopreservation, the meristematic region of the shoot apices precultured in sucrose solution (pregrowth desiccation) showed similar type of actively dividing cells as that of fresh apices. Most of the tissues of encapsulation-dehydration and droplet-freezing technique showed cell breakage and shrinkage of tissues. The TTC test conducted for the viability checking of cryopreserved shoot apices and only the sucrose treated shoot apices gave positive result and the other two treatments gave negative result. From these results, pregrowth-desiccation method found to be one of the potential methods for cryopreservation studies using cocoa shoot tips.

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