ix. Ex vitro establishment

Keep the plantlets (18-25 cm) with balanced root (well developed secondary and tertiary roots, 5-6 ml root volume) and shoot (3-4 leaves) system in carbendazim 1g/L and IBA (1000 ppm solution) for 1 hour each

Transfer the plantlets to pots filled with sterilized sand, soil, and coir dust in equal proportions

Cover the pots with polythene bags to maintain high humidity for initial establishment

Keep the plants in ambient condition under illumination

After two weeks make small perforations on polythene cover to gradually reduce humidity

After two weeks remove the cover completely. Keep the plants with proper watering inside a lab for 1-2 weeks

Shift the potted plants to the green house under 50% shade

Keep the plantlets in the nursery for two months

Transplant it to large polythene bag filled with soil and organic manure

Proper care should be taken for the healthy growth of plantlets by regular irrigation and manuring. Remedial measures are to be taken in case the plant is showing any disease symptoms

Fig.2. *In vitro* and *ex vitro* establishment of cryo-preserved coconut zygotic embryos

Conservation of coconut germplasm using vitrification protocol

The vitrification based protocol was used for the following genotypes viz. ADOT, AGT, BENT, CALT, CGD, COD, CRD, FJT, GBGD, Kalpa haritha, KPDT, KTOD, LCT, LMT, MOD, Kalpa jyothi, NDRT,NLGD, PHOT, RKBT, SKGT, SLT, STVT, TPT, WCT, GZB, CART, SUT, SYCT, WAT, AGT, MOD, NLGD, SYCT, KPDT for long term conservation of embryos at National Gene Bank, New Delhi

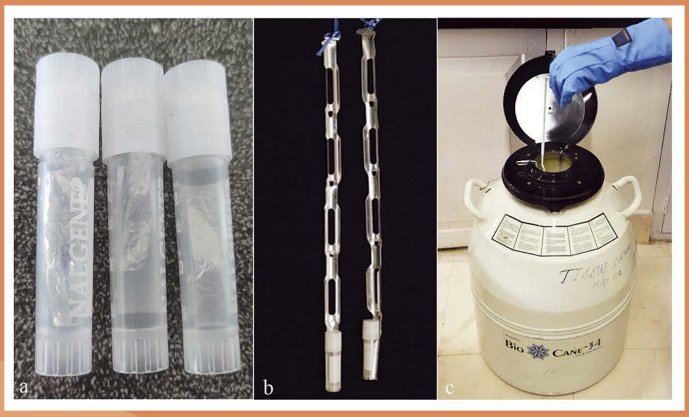

Fig. 3.Cryopreservation of coconut zygotic embryos in cryotank

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Cryopreservation of coconut zygotic embryos

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Introduction

Coconut zygotic embryos provide an ideal material of choice for collection and conservation of coconut genetic diversity. ICAR-CPCRI has developed different methods such as air desiccation, pregrowth desiccation, encapsulation dehydration, and vitrification techniques for cryopreservation of coconut zygotic embryos (Karun *et al.*, 2005; Sajini *et al.*, 2006, 2010). Out of four standardized techniques, vitrification based protocol (PVS3) is found to be more effective (Sajini *et al.*, 2010) and easiest method cryopreservation of coconut embryos.

In this method, the embryos is to be precultured first in the high sucrose that result in mild osmotic shock and protect the cellular membrane that later will help in mitigation of the effect of highly concentrated vitrification solution. The choice of vitrification solution, duration of treatment with vitrification solution is very critical for survival of the explant after cryopreservation. PVS3 vitrification solution is found to be best for the coconut embryos (Sajini et al., 2010) and treating the embryos with PVS3 solution for duration of 16 hours reduces the moisture content to the safer level (16.8%). Various steps involved in the method are embryo extraction, sterilization, preculture, dehydration with vitrification solution, unloading, rapid cooling and rapid rewarming. The vitrification protocol developed at ICAR-CPCRI is detailed in the following

Checklist for vitrification method

Items needed for extraction and surface sterilization of embryos

i. Knife
ii. Cork borer
iii. Small knife
iv. Beaker (100 ml, 1000 ml)
v. Tray
vi. Measuring cylinder

Prepare in advance

- Sterile cryovials
- Sterile conical flasks
- Y3 medium containing 0.6M sucrose-solid (Preculture medium); 1.2 M sucrose-liquid (unloading solution); 60 g/L sucrose-liquid (Retrieval medium); Y3 medium containing 40 g/L sucrose-solid (Retrival medium-2)

Procedure

i. Excision of embryos

- Split open the nuts
- Scoope out the embryo along with endosperm from beneath the large soft eye of the nuts using cork borer
- Collect the endosperm plugs
- Making a small incision in the endosperm with the help of knife, excise the embryos from the endosperms and collect it in a beaker filled with distilled water

ii.Surface sterilization of embryos

- Wash the embryos in distilled water two to three times to remove coconut milk and small pieces of endosperm
- Put the embryos in sterile conical flask (100 ml) inside the laminar air flow
- Add 20% of sodium hypochlorite solution in sterile water and kept it for twenty minutes with occasional stirring
- Drain off the sterilant and wash the embryos in sterile water 4 to 5 times

Fig.1. Zygotic embryo extraction and sterilization from coconut

iii. Pregrowth incubation

Inoculate surface sterilized coconut embryos into full strength Y3 medium supplimented with 0.6 M sucrose and incubate for three days

iv. Dehydration

Place the embryos in plant vitrification solution (PVS3) containing Y3 medium supplemented with 50% sucrose and 50% glycerol in a conical flask (25 embryos in 100 ml conical flask containing 25 ml PVS3)

Place the conical flask in a rotary shaker (90 rpm) for 16 hours

v. Cryopreservation

Put the embryos in sterile cryovials filled with fresh PVS3 solution

Plunge the cryovials directly in liquid nitrogen for minimum of 24 hours

vi. Thawing

Takeout the vial from the cryotank and immerse the cryovials in warm water (40°C) for two minutes

vii. Unloading

Incubate the embryos in full strength liquid Y3 medium supplemented with 1.2M sucrose for 90 minutes with one or two changes with the same medium in between

viii. *In vitro* retrieval

- Inoculate the embryos in full strength Y3 liquid medium supplemented with 60g/L sucrose.
- After one month, subculture in to medium (solid) containing 40g/L sucrose, 1g/L activated charcoal. The cultures were kept under dark until the emergence of first leaf. In case of tall varieties of coconut germination starts within 30-35 days. Transfer the cultures to illuminated room with 16 hours photoperiod (temperature 27±2°C, relative humidity 65-70%). Additional amount of BAP or NAA were added (BAP 2mg/L, NAA 5 mg/L)