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Embryo culture of coconut: the CPCRI protocol

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INTRODUCTION

It is widely accepted that the movement of coconut germplasm should be through embryo culture because of phytosanitary requirements and for easy transport. Following a series of experiments, a protocol for coconut embryo culture was developed at CPCRI. Its different components include direct field collection of the coconut embroys of 8-11 months, short-term storage (Karun and Sajini, 5), in *vitro* retrieval (Karun *et al.*, 9) and *ex vitro* establishment (Karun and Sajini, 6). The protocol was further tested during 1994 by bringing 86 embryos of six coconut accessions maintained at World Coconut Germplasm Centre (WCGC), Andamans to CPCRI, Kasaragod and their subsequent *in vitro* retrieval (Karun *et al.*,8). It has also been successfully utilized during a germplasm expedition in the Indian Ocean Islands during 1997. All the collections were made in that expedition in the form of embryos and this is the first attempt of its kind (Kumaran *et al.*, 11). Short-term storage of embryos, rescue of immature embryos and the higher rate of acclimatization of seedlings retrieved *in vitro* are the distinct characteristics of the protocol developed at CPCRI. Though the different components of the CPCRI protocol were discussed separately in earlier publications, its complete account is not described elsewhere.

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MATERIAL AND METHODS

For standardizing the retrieval medium, two experiments were conducted with embryos of West Coast Tall (WCT). Conventionally matured nuts (11-12 months after fertilization) are being used for seed-nut collection. This restricts the size of collection in a germplasm expedition. However, it is possible to extract embryos of 8 months old onwards and their germination *in vitro* was studied. Table 1 provides the experimental details. The collected embryos in these two experiments were kept in the laboratory under ambient conditions (temperature 30-35°C) before transferring to fresh Eeuwen's (4) Y3 solid medium supplemented with sucrose and charcoal. To standardize the retrieval medium for the dwarf type, in experiment III, 11 months old embryos of Chowghat Orange Dwarf (COD) were used. The treatments consisted of the strengths of Y3 basal medium supplemented with 4 levels of sucrose and charcoal, 10 embryos per treatment combination replicated twice (Table 2).

Initially the embryos were kept in the dark room (temperature 27±1 °C, relative humidity 85%) with a photoperiod of 16 hr. Subculturing was done once in every 21-25 days. Well-developed root system was induced by supplementing the medium with IBA 5 ppm and NAA 1 ppm in all the experiments after about 4 months of inoculation. Two attempts were made to bring coconut germplasms in the form of embryo culture from World Coconut Germplasm Centre (WCGC), Andamans to CPCRI, Kasarogod. In the

Table 1. Plants obtained after 4 months of inoculation in nutrient medium.

Experiment	Description	Age of embryos	Plants obtained (%)
I	Y3 medium supplemented with 30 g/l sucrose and 1 g/l charcoal. Ten embryos per treatment replicated thrice.	8 months 9 months 11 months	84.6 70.0 70.0
II	Y3 medium supplemented with 60 g/l sucrose and 2.5 g/l charcoal. Twenty embryos per treatment replicated thrice.	8 months 9 months 11 months	58.2 76.3 89.8

Table 2. Plants retrieved (%) in experiment conducted for standardizing the protocol for dwarf embryos.

Sucrose	30 g/l	40 g/l	60 g/l	60 g/l*
Y3 medium	75	75	80	70
Half Y3 medium	85	85	95	70
Half MS medium	70	55	65	80

* Sucrose and glucose in the ratio 1:1 and level of charcoal was 2.5 g/l; the level of charcoal for other combination was 2 g/l.

Table 3. Details of coconut germplasm brought from WCGC, Andamans to CPCRI, Kasaragod in the form of embryo cultures.

Accessions	Embryos collected	germination (%)	survival after 4 months (%)
Bora Bora Tall	17	58	52.94
Niu Ui	15	100	60.00
Rangiroa Tiputa Tall	9	78	66.66
Nikkore	10	100	80.00
Rangiroa Avatoru Tall	19	100	84.21
Niu Hako	13	100	92.32

first attempt, 15 embryos were brought in liquid Y3 medium during 1992. During 1994, 86 embryos of six Pacific Ocean Island accessions were brought from WCGC in sterile water (Table 3).

RESULTS AND DISCUSSION

The complete protocol is illustrated in Fig. 1-13. The contamination of embryos in the course of *in vitro* culturing was very low in all the three experiments (less than 4%). However, when embryos were transported from WCGC, Andamans in nutrient medium, 12 out of 15 embryos were contaminated (Anon, 1). When sterile water was used instead of nutrient medium, only three embryos out of 86 embryos were found contaminated when they were brought from WCGC to CPCRI. It is, thus desirable to inoculate the embryos first into sterile water at the collection site and later into the retrieval medium after bringing them to the laboratory. The mature coconut embryos can be stored in sterile water for 2 to 4 months without affecting their viability (Karun and Sajini, 5; Karun *et al.*, 7).

The *in vitro* germination of coconut embryos was similar to that of seed nuts except for the enlargement of haustorium. In the cultured embryos, the surface of the haustorium darkened and became brittle. This portion was removed at the time of subculturing. The number of days taken for germination was not the same in different experiments. The embryos of 9 and 11 months of WCT were germinated within 45 days of inoculation, but those of 8 months took 60 to 80 days. In the embryos of dwarf type, the emergence of plumule was noticed on the 15th day after inoculation. The embryos of the six accessions at WCGC, Andamans germinated within 35 days of inoculation in the retrieval medium except for Bora Bora Tall (45-60 days).

Not all the germinated embryos usually grew into complete plants, a few of them got contaminated. However, after 2 months from germination the loss was observed to be very low. Hence, the number of plants survived 4 months after inoculation was taken as an indication to compare the treatments regarding retrieval of embryos. The number of plants derived *in vitro* in different experiments are shown in Tables 1 to 3.

The germination of 8 months old embryos was found to be less in the second experiment (Table 1). One factor affecting the germination of embryos could be their size. The dimension of 8 months old embryos was less (1-4 mm) compared with the size of 9 and 11 months old (4-5 mm and 7-8 mm respectively). Similarly, the dimension of the embryos of Bora Bora Tall was also less (2.5 mm) compared with other accessions (the average being 7.5 mm) and the percentage plants derived was also the lowest (Table 3). It seems that embryos of smaller size required a special treatment. In another experiment, it was found that germination of immature embryos was more in Y3 medium supplemented with 60 g/l glucose instead of sucrose.

Higher levels of sucrose in the nutrient medium were found beneficial for improved rhizogenesis in some coconut cultivars (Del Rosaio and de Guzman, 3; Karunaratne *et al.*, 10; Assy Bah *et al.*, 2). But in Experiment II, in which high levels of sucrose was used, the growth of plants was not satisfactory. Even after 8 months of inoculation, only 9.4% cultures showed satisfactory growth compared with 70.6% cultures in Experiment I. Hence, the levels of sucrose and charcoal in the basal medium were brought down to that of Experiment I. The growth of plants was then improved. Hence, it is concluded that for embryos of tall cultivars, 30 g/l sucrose and 1 g/l charcoal need only be supplemented in the Y3 medium. There was no deleterious effect due to higher concentration of sucrose with regard to embryos of dwarf type (Table 2), which confirms the studies conducted in other laboratories. In all the experiments, the rhizogenesis was enhanced on transferring the plants, having a height of 7-10 cm to the medium supplemented with IBA 5 ppm and NAA 1 ppm.

After 12 months of *in vitro* culture, plants were

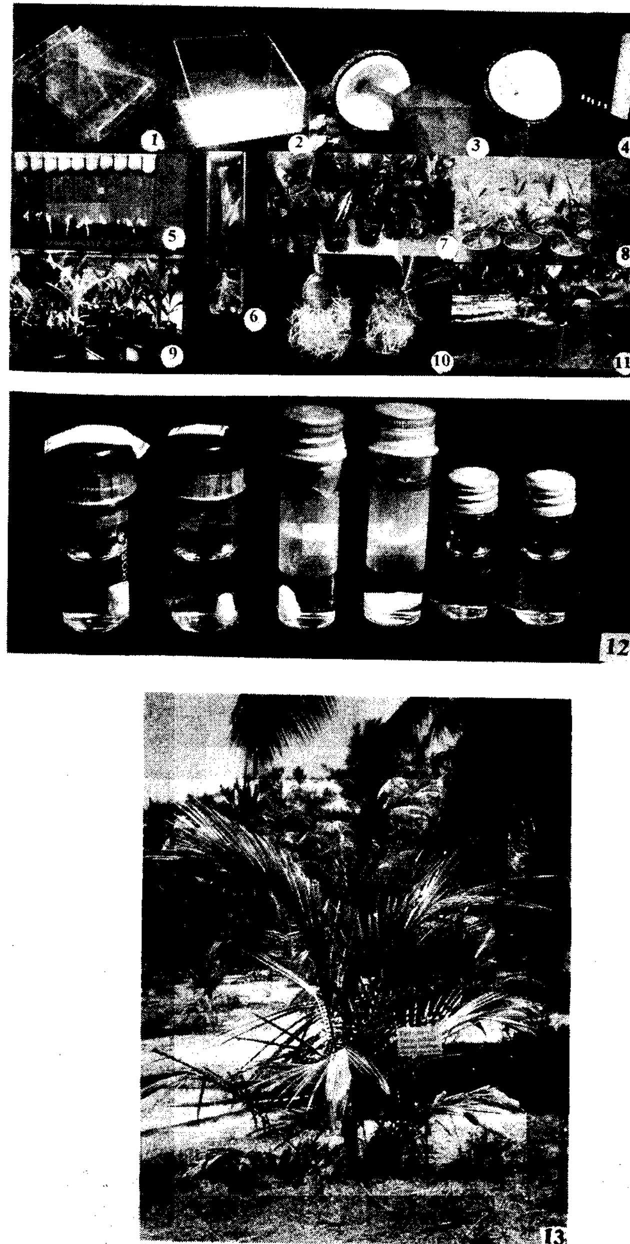

Fig. Field collection, *in vitro* retrieval and field establishment of coconut zygotic embryos: 1, Folding type of portable inoculation hood; 2; Front view of inoculation hood; 3, Scooping of embryos from splitted nut with the help of cork borer; 4, Extracted embryos; 5, Embryos in retrieval medium after 35-40 days of inoculation; 6, A complete plant; 7, *Ex vitro* establishment; 8-9, Pot and poly bag establishment; 10, Comparison of root intensity of seed nut sown and embryo cultured seedling; 11, Well-established plants in the field (after 6 months of planting); 12, Field-collected embryos in a vial containing sterile water; 13, Established plant in the field after 2 years of planting.

Table 4. Correlations between characters of *in vitro* retrieved plantlets.

Characters	Experiment I			Different accessions		
	No. of leaves	Root volume	Plant height	No. of leaves	Root volume	Plant height
At the time of transfer						
Plant height	0.16	0.51**	0.51**	0.70**	0.61	0.52
No. of leaves		0.43	0.16		0.40	0.47
Root volume			0.34*			0.68**

ready for transferring to pots. The plants were treated with Bavistin (carbendazim) 1 g/l and thereafter with IBA 1,000 ppm solution for 1 hr each before transferring to flower pots. The potting mixture consisted of sterile soil, sand and coir dust in equal parts. Higher humidity in the vicinity of the plants was maintained by covering them with polythene bag for 2-3 weeks. Humidity was gradually reduced by providing perforations to the polythene bag and later removing the bag during night time. This process was continued for 1 month. Before removal of the polybag, Hoagland's solution was applied once in 15 days but on observing less growth, it was replaced with Euwen's Y3 macro nutrient viz., NH_4Cl, NaH_2PO_4 and KCl. The plants in pots were then shifted to the net-house (50% shade) for further hardening.

The procedure followed for the *ex vitro* establishment of plant was found satisfactory. A well-developed root system is advantageous for the establishment of plants. The correlation between the plant characters at the time of transfer to pots with the height of plants after 8 months in pots is shown in Table 4. The volume of root was found to have positive correlation with the growth of plants in pots. After 6 months inside the net-house, the plants were ready for field planting. A total of 31 plants were planted in the field during 1995 and all of them were well established (Fig. 13). The remaining plants were being utilized as experimental material for various projects of the institute.

SUMMARY

The field collection of embryos from mature nuts (9 months onwards), which can be easily identified for its hard eye and thick kernel, is done by means of a surface-sterilized cork borer. From the endosperm plug thus scooped out, the embryo is separated by using knife. The collected embryos are placed in distilled water. Once all the embryos are extracted, they are subjected to surface sterilization with 50% chlorine water for 20 minutes. The embryos are washed thoroughly in sterile water 4-5 times and inoculated individually into screw-cap bottles containing 2-5 ml sterile water (pH 5.7). The entire operation is to be done in surface-sterilized (absolute alcohol) inoculation hood (portable). The field-collected embryos are then transferred to Y3 solid medium within 2 months. The medium is supplemented with 30 g/l sucrose and 1 g/l charcoal for embryos of tall types and 60 g/l sucrose and 2.5 g/l charcoal for dwarf types.

For the 8 months old nuts, embryos being small in size (1.5 to 4 mm), surface sterilization was done for 10 minutes. These embryos can be stored in half-strength Y3 medium for 1 month. Initially these embryos are inoculated in Y3 medium supplemented with 60 g/l glucose and 2.5 g/l charcoal. After 1 month, on attaining the full size, the embryo were transferred to regular retrieval medium.

Cultures in the retrieval medium are initially kept in dark room till germination and later transferred to the illuminated room. Periodical sub-

culturing was followed at monthly intervals. Once the plants attain the length of 5-7 cm, they were transferred to liquid medium. For rhizogenesis, the medium was supplemented with IBA (5 ppm) and NAA (1 ppm). For pot establishment, sterilized sand, soil and coir dust in equal proportion were used as the potting mixture. The humidity was controlled in the initial stage of *ex vitro* establishment and macro nutrients were provided.

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