

Storage and transport of zygotic embryos of *Cocos nucifera* L. for *in vitro* culture

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Summary

Embryo culture of coconuts can circumvent problems of storage and transport caused by the weight and bulk of the nuts and the absence of dormancy. This paper describes a technique that can be routinely used for collecting large numbers of embryos. The embryos were extracted in the field, brought to a simple isolation room, washed in ethyl alcohol, disinfected in 100% commercial bleach for 20 minutes and then rinsed again. Sterilized embryos were sealed in sterile plastic bags and then transported in cold storage. Redisinfection in 100% commercial bleach for 5 minutes prior to culture under aseptic conditions was necessary to reduce contamination rates. This procedure did not adversely affect embryo germination.

Embryo culture of coconuts is being developed as a means of moving coconut germplasm around the world for coconut improvement work or disease studies. The heavy and bulky nature of coconuts is the main reason to develop this technology, but another is the absence of the phenomenon of dormancy, which results in quick germination *in situ*. *In vitro* culture requires a fastidiously clean working environment and the use of laboratory equipment. Collecting embryos during prospection, which unfortunately is not always in accessible places, will require the embryos to be transported to the tissue culture facility for culturing. It is during these prospecting and collecting trips that the embryos must be kept alive and cultivable after a number of days.

Some researchers have tried to develop techniques for easier and effective methods of storage and transport of embryos for long periods and over long distances. Notable among these efforts were those of Assy Bah (1988). She tried different methods such as direct *in vitro* culture in the collecting area, where the contamination rate was observed at 10% after one month, compared with 2.5% for laboratory conditions. The germination rate was good, at 78% after four months.

Cold storage (1° to 5°C) of solid endosperm cylinders still containing the embryos and placed in hermetically sealed jars was also found satisfactory with regard to contamination and germination rates as long as the storage period did not exceed 14 days and the temperature remained consistently low.

Storage of core samples in sterile distilled water was found to result in embryos with more or less marked stains which did not affect the survival rate of the embryos, but may affect their later development. The addition of potassium chloride at a rate of 16.2 g/l was reported to prevent the burns and to improve germination. However, embryo germination slows down in proportion to storage time.

These methods, tried by Assy Bah on small samples, could greatly improve the ease of storing and transporting embryos. But the need to use jars and culture vessels during field collection might still be cumbersome and its efficiency has still to be confirmed, especially when large numbers of embryos from different cultivars are needed.

Sossou *et al.* (1987) also developed a procedure for initiating *in vitro* cultures in the field for zygotic embryos of some palm species like coconut. This procedure requires the use of a portable, collapsible glove-box instead of a laminar air-flow hood, a sterile metal box and special culture vessels with the medium.

At the Albay Research Center of the Philippine Coconut Authority (PCA) a storage and transportation method has been satisfactorily developed and used routinely. This came about through studies on the screening of cultivars for resistance to the cadang-cadang disease of coconut using an *in vitro* system involving the collecting of thousands of embryos from different cultivars at one time. Embryos from different coconut cultivars were collected at the Zamboanga Research Center (ZRC) on Mindanao Island and brought to Albay Research Center on Luzon Island for *in vitro* culture. The travel time is usually three days by plane between the two places. This paper describes the collecting, storage and transportation methods developed and now routinely used for embryo collecting.

Materials used

- No. 14 cork borer
- Autoclavable plastic bags, each of which contains a piece of wet cotton wool
- 3 litres ethyl alcohol
- 2 gallons commercial bleach
- Sterile water
- Ordinary plastic bags
- Ice chest
- Packing tapes
- Pressure cooker

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Methodology

Nine- to 11-month-old coconut seednuts were harvested from the palms and then split into halves with the use of a 'bolo'. The embryos were extracted in the field with the use of a No. 14 cork borer (Fig. 1A). The embryos, still enclosed in the solid endosperm, were immersed in liquid coconut endosperm contained in ordinary plastic bags. When the desired number of embryos was collected, these were brought to a simple isolation room with still air to minimize contamination.

Meanwhile, the cotton in autoclavable plastic bags was soaked with water and the bags were sealed by twisting the ends of each bag and tying them into a knot. The plastic bags were sterilized using an ordinary pressure cooker for 20 minutes. Water for rinsing the embryos later was sterilized in the same manner.

The extracted embryos within the solid endosperm were initially washed in tap water to remove dirt and any soil particles. Using ordinary 1- to 2-litre glass jars, the cylinders were quickly washed in 95% ethyl alcohol for one to two minutes, stirring the contents every now and then to remove fatty substances. The alcohol was used twice. The cylinders were then disinfected in 100% commercial bleach for 20 minutes to disinfect the cylinders thoroughly. The contents were occasionally stirred. After 20 minutes, the cylinders were washed several times with sterile water and then placed inside the sterile autoclavable plastic bags with cotton (Fig. 1B). About 100 embryos in solid endosperm can be put into one 19.5 × 12.0 in. autoclavable plastic bag. The bags were then tied and placed in an ice chest packed with several packs of ice or stored in a refrigerator before transport. The ice packs are removed when air transport is used, as the airline does not allow packed ice to be transported. The ice chest is sealed properly with packing tapes to keep it cold.

Once they had reached the Albay Research Center, the embryos were stored in the refrigerator at 5°C for

up to five to seven days if *in vitro* culture of the embryos could not be done immediately.

Prior to culture under aseptic conditions in the laminar flow hood, the embryos were re-disinfected with commercial bleach (Fig. 1C). Two methods were tried. One was re-disinfection in 20% commercial bleach for 20 minutes and the other was in 100% commercial bleach for five minutes. After the embryos within the solid endosperms were re-disinfected, they were washed several times in sterile distilled water. The embryos were then ready for excision.

The embryos were aseptically excised from the solid endosperm. Then they were sterilized with 10% commercial bleach for one minute and washed three times with sterile distilled water. They were blotted dry on sterile petri dishes with filter paper and then inoculated singly (Fig. 1D) onto liquid Y3 media with 2.5 g/l activated charcoal (Rillo and Paloma, 1990).

Contamination rates were recorded one month later and analyzed using ANOVA in Completely Randomized Design (CRD). Germination rates were noted after two months.

Results and Discussion

It can be observed from Table 1 that re-disinfection of transported embryos in 20% commercial bleach for 20 minutes resulted in higher contamination rates than re-disinfection in 100% commercial bleach for five minutes. This was significant at 5% using ANOVA in CRD. Consequently, the former re-disinfection procedure yielded fewer germinated embryos for all cultivars tested because many were lost through contamination. Re-disinfection using 100% commercial bleach for five minutes resulted in much lower contamination rates. The high concentration of disinfectant did not have an adverse effect on the germination of embryos because these were still covered with solid endosperm (Fig. 1E). Differences in germination rates between cultivars may be varietal in nature. Moreover,

Table 1. Contamination and germination rates of embryos from different coconut cultivars collected from PCA-ZRC using two different secondary disinfection methods

Cultivar	20% commercial bleach for 20 minutes			100% commercial bleach for 5 minutes		
	No. embryos collected	Contamination (%)	Germination (%)	No. embryos collected	Contamination (%)	Germination (%)
Tagnanan	120	45.0	40.8	122	6.5	77.8
Zamboanga Tall	103	41.7	46.6	104	4.8	83.6
West African Tall	107	47.6	36.4	114	4.3	67.5
Karkar	236	39.8	40.6	288	3.8	64.9
Pilipog	167	49.1	32.9	182	3.3	65.9
Sn. Ramon	118	40.6	48.3	119	1.6	77.3
Spikata	145	48.9	40.0	149	4.0	69.8
Rennel	105	46.6	45.7	115	3.4	73.0
Equatorial Green Dwarf	246	37.8	43.5	255	2.7	70.9
Bago-Oshiro	106	44.3	42.4	121	0.8	71.9
Tahiti	107	33.6	53.2	117	1.7	76.0
Total	1560			1686		
Overall		43.2	42.7		3.4	72.6

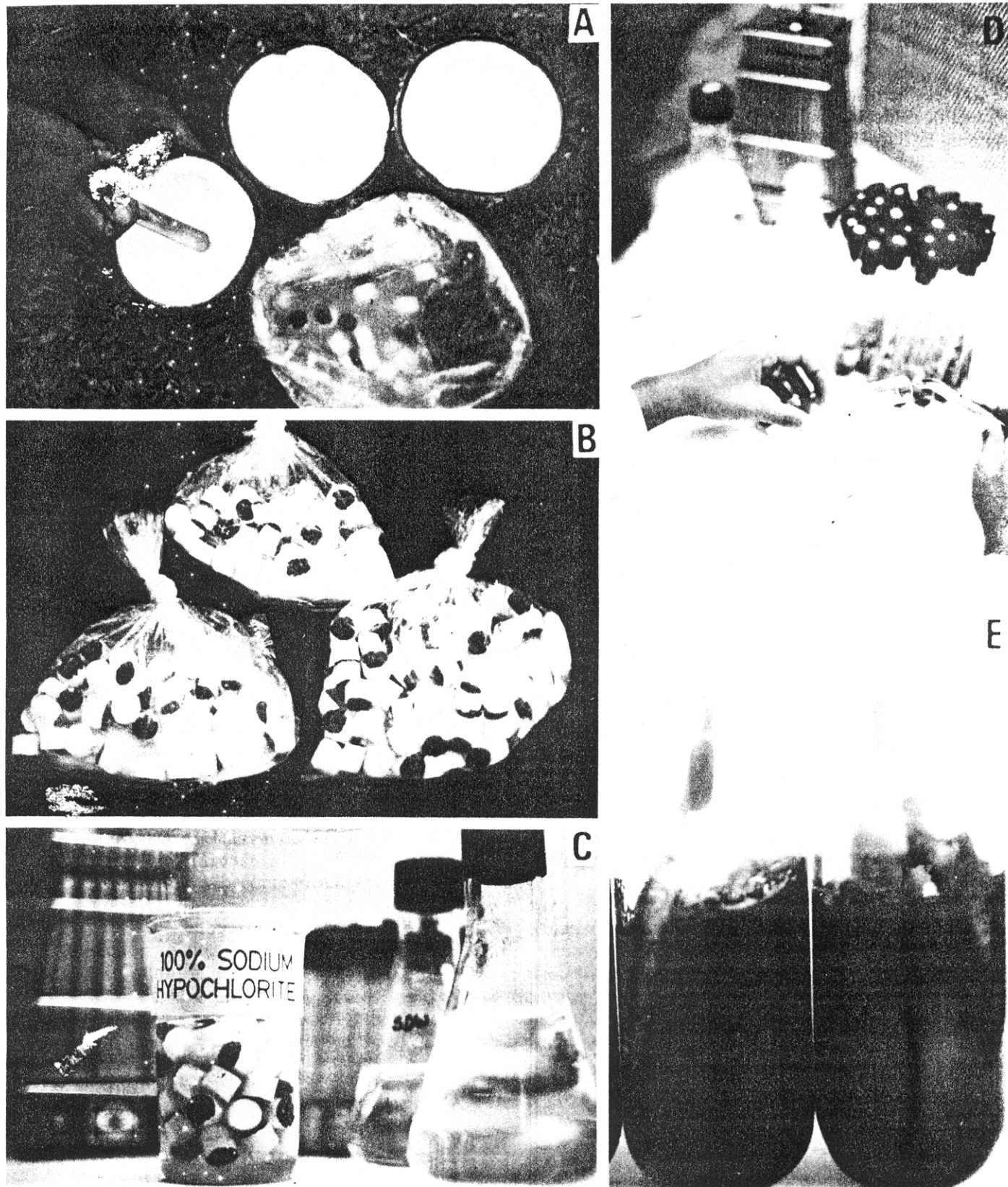


Fig. 1. A - The embryo is extracted from the nut using a cork borer and placed in an ordinary plastic bag; B - The embryos, enclosed in solid endosperm, are sealed in sterile plastic bags lined with wet cotton wool; C - Upon reaching the research centre, the embryos are redisinfecting with 100% commercial bleach for five minutes; D - The embryos are aseptically excised and inoculated singly on to the medium; E - Good germination of embryos was observed five weeks after inoculation on to solid medium

embryo age significantly affects germination rate, the older embryos germinating better.

This storage and transportation method was found to be rapid, easy and requires simple and inexpensive equipment that must be brought to the field. This makes possible the bulk collection of embryos, even running into thousands. It is also a versatile technique. For example, if it is desired to identify the specific palm source of the embryos, smaller autoclavable plastic bags can be used. The only critical factor in this technique is that the embryos must be transported in cold storage. The disinfection of the coconut cylinders with 100% commercial bleach for five minutes was adequate to eliminate whatever microbial contamination had set in during transport.

Conclusion and Recommendation

The collecting method described above works well when collecting large numbers of embryos from remote places. Care should be taken though that the

embryo, when extracted from the nut with the cork borer, should remain enclosed within the solid endosperm and not be exposed through any cracks. It would also be convenient for the collector if linkages could be established beforehand so that a clean room could be made available near the collecting site. This would save the collector from having to carry cumbersome materials and equipment to the collecting site.

References

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Résumé

Stockage et transport d'embryons zygotiques de Cocos nucifera L. pour la culture in vitro

L'embryoculture de noix de coco est un moyen de circonvenir les problèmes de stockage et de transport imputables au poids et au volume des noix et à l'absence de dormance. Cette communication décrit une technique utilisable de façon courante pour la collecte d'embryons en grands nombres. Des embryons extraits sur le terrain et isolés dans un local de type simple, ont été lavés dans l'alcool éthylique, désinfectés pendant 20 minutes dans un produit de nettoyage commercial à 100 pour cent, puis rincés à nouveau. Les embryons stérilisés ont été déposés dans des sacs en plastique stériles, hermétiquement fermés puis transportés dans un entrepôt frigorifique. Une nouvelle désinfection de 5 minutes dans un produit de nettoyage commercial à 100 pour cent a été nécessaire avant la culture en conditions aseptiques pour réduire les taux de contamination. Cette procédure n'a pas eu d'effet négatif sur la germination des embryons.

Resumen

Almacenamiento y transporte de embriones zigóticos de Cocos nucifera L. para su cultivo in vitro

El cultivo embrionario de cocos puede evitar problemas de almacenamiento y transporte causados por el peso y volumen de las nueces y la ausencia de latencia. En este trabajo se describe una técnica que puede utilizarse de forma rutinaria para recolectar grandes cantidades de embriones. Los embriones se extrajeron en el campo, se llevaron a una habitación para su aislamiento, se lavaron en alcohol etílico, se desinfectaron en lejía comercial al 100% durante veinte minutos y luego se volvieron a enjuagar. Los embriones esterilizados se sellaron en bolsas estériles de plástico y luego se transportaron para almacenarlos en frío. Para reducir los índices de contaminación se hizo necesaria su desinfección de nuevo en lejía comercial al 100% durante cinco minutos antes de su cultivo en condiciones asepticas. Este procedimiento no afectó a la germinación de los embriones.