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A New Technique for Coconut (*Cocos nucifera*) Germplasm Collection from Remote Sites: Culturability of Embryos Following Low-temperature Incubation

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Abstract

A new technique for coconut (*Cocos nucifera* L.) germplasm collection was evaluated in the laboratory and tested in the field in Indonesia. The technique involved the non-sterile isolation of embryos, and incubation in sterile ascorbic acid solution (1 mg L^{-1}) at 5 ± 1°C in the dark. During this incubation period the embryos could be transported and/or stored for a period of up to 4 days without embryo viability loss. Following this period the embryos were surface sterilised with sodium hypochlorite (1.5% w/v) for 20 min, washed with sterile water and cultured in a liquid Y3 basal nutrient medium supplemented with Morel and Wetmore vitamins, sucrose (175 mM) and activated charcoal (2.5 g L^{-1}). After two weeks the embryos were subcultured onto a solid medium of similar constitution to encourage germination. Germinated embryos grew and produced healthy plants with normal morphology. Despite mild chilling injury as indicated by elevated ethylene production and solute leakage, the transported embryos retained viability with normal morphology. Using the low-temperature incubation treatment, the microorganism density in the ascorbic acid solution was kept low while that around other embryos kept at higher temperatures (25°C) increased. Even though embryos were exposed to a low-temperature treatment for up to 4 days they were able to germinate (95% viable) and grow in an identical fashion to freshly cultured embryos.

Introduction

Present coconut (*Cocos nucifera* L.) germplasm collection relies on the harvest and transport of the bulky fruits. For most collectors this is not cost effective and in some cases leads to the introduction of pests and diseases into new areas. The isolation, surface sterilisation and transportation of small pieces of endosperm containing the embryos (referred to as endosperm plugs) overcomes these problems but the technique does require sterile culture facilities to be available at or near the field collection site (De Guzman 1970; Assy Bah 1986; Samosir 1991). Often sterile culture facilities are not available as most collection sites are in remote locations. Some attempt has been made to use equipment that can be easily transported (Assy Bah *et al.* 1987; Sossou *et al.* 1987; Rillo and Paloma 1992; Karun *et al.* 1993). However, one of these techniques (Assy Bah *et al.* 1987) soaks the plugs in potassium chloride which later represses embryo growth while all techniques require a second, often damaging, laboratory sterilisation treatment. In addition, these techniques based on endosperm plug collection do not work for mutant kopyor or makapuno coconuts which do not have a firm endosperm at maturity. More recently, Ashburner *et al.* (1993) developed a method for coconut germplasm collection that only involves the isolation and transport of naked embryos. This method is suitable for mutant coconut forms but suffers from embryo loss through contamination. Thus, there is a need for a technique that isolates individual embryos but is able to protect them from contamination before they can be cultured.

The use of a low-temperature treatment is a well-known method to suppress micro-organism multiplication and to retain tissue quality (Ailan and Anon 1989; Berjak *et al.* 1992). It could be assumed that the use of an appropriate low-temperature treatment during

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embryo transport from the field to the laboratory may be a way of reducing embryo contamination. However, it is also possible that such a treatment would damage coconut embryos since a 3-month cold treatment is known to kill coconut embryos (Karunaratne 1988). It is unknown if shorter duration low-temperature treatments would damage coconut embryos and prevent them germinating.

This present study develops and tests a new, simple method for remote site coconut germplasm collection. Specifically, a low-temperature treatment was investigated as a way of suppressing microorganism activity while maintaining embryo viability prior to application of the standard tissue culture treatment.

Materials and Methods

Development of a Germplasm Collection Protocol

Coconut (*Cocos nucifera* L.) fruits were purchased from a local supermarket following importation from Western Samoa. The fruits were halved and endosperm plugs (2.5 cm diameter surrounding the embryo) removed by a clean but non-sterile method. This technique, suitable for field collection, used a cork borer that had been cleaned with 70% ethanol and washed with tap water. The embryos were then removed from the plugs with clean dissecting instruments, weighed and individually placed, without surface sterilisation, into sterile polycarbonate, screw cap culture tubes (8.0 × 2.5 cm diameter) containing 10 mL of a filter-sterilised ascorbic acid solution (1 mg L^{-1}). Any visibly damaged or abnormal embryos were discarded prior to incubation in ascorbic acid. The embryos were incubated in the dark at one of two temperatures (5 or 26 ± 1°C) for 0, 1, 2 or 4 days. After the appropriate temperature treatment and incubation time, 10 replicate embryos were weighed and their health assessed. Health assessment was achieved by placing them individually into sterile glass tubes (4.8 × 2.5 cm diameter), then closing them with Subaseal stoppers (No. 49; Selby Anex, Brisbane, Australia) and incubating them in the dark at 26 ± 1°C for 12 h. Embryo-free tubes were set up as controls. After the first 4 h of incubation a 0.5-mL headspace gas sample was removed from each of the treatment and control tubes with a sterile hypodermic needle attached to a sterile plastic syringe (1 mL) and analysed for carbon dioxide. The removed gas samples were replaced with sterile air and the vessels incubated for a further 8 h before a second 1-mL headspace gas sample was removed for ethylene analysis. The two gases were analysed with a gas chromatograph (GC; Model 5830A, Hewlett Packard Ltd, Wilmington, USA) containing a Porapak N (Waters Association Inc., MA, City, USA) column (1.8 m × 3 mm) with the oven and injectors set at 90°C and 95°C, respectively (Adkins *et al.* 1990). A flame ionisation detector (95°C) was used for ethylene analysis, while a thermal conductivity detector (65°C) was used for carbon dioxide analysis. Standards of ethylene (0.5–10 ppm) and carbon dioxide (1000 ppm) were used for the calibration of the GC.

The ascorbic acid solutions remaining in the polycarbonate tubes after embryo health assessment were shaken and sampled (1 mL) for microorganism density using a counting chamber (model WSI; Weber Scientific International, London, England) viewed under a microscope (method of Jenkins 1992). Dilution of the incubation solution was undertaken in some cases when the microorganism density was too great in the initial solution to provide an accurate analysis (*viz.* most 26 ± 1°C incubated samples). The remaining 9 mL of ascorbic acid solution in each of the tubes was subjected to electrical conductivity (TPS conductivity meter, TPS Instruments model 2100, TPS Pty Ltd, Brisbane, Australia) and pH (Model 6071 Jenco Electronics Ltd, San Diego, USA) analyses at 26 ± 1°C to assess further aspects of the embryos' health.

After health assessment, embryos were surface sterilised using sodium hypochlorite (1.5% w/v, 20 min) and washed in sterile deionised water. They were then individually placed into sterile polycarbonate screw cap tubes (8.0 × 2.5 cm diameter) containing 10 mL of an autoclaved (120°C, 15 min) liquid culture medium consisting of Y3 basal nutrient medium (Eeuwens 1976) supplemented with Morel and Wetmore vitamins (Morel and Wetmore 1951), sucrose (175 mM) and activated charcoal powder (2.5 g L^{-1}, Sigma Chemical Co, St Louis, USA). The medium pH was adjusted to 5.3 prior to the addition of the activated charcoal powder. The embryos were then incubated, without shaking, in the dark at 28 ± 1°C for 2 weeks. After this time the embryos were individually subcultured onto a solid medium (7 g L^{-1} agar, Sigma Chemical Co, St Louis, USA) of similar constitution, in similar culture vessels, placed under the same environmental conditions for a further 4 weeks. During this period embryos were scored for germination (visible emergence of plumule or radicle from cotyledonous sheath) and, after 6 weeks

growth, were evaluated by measuring fresh weight. Subculture onto media of a similar type was undertaken every 4 weeks and after four such subcultures the seedlings could be planted into soil (not undertaken in this present study).

Remote Site Germplasm Collection

The protocol developed and tested in the laboratory was field evaluated in West Lombok, Indonesia. The coconuts used in this study were cultivar Batu Layar and came from smallholder plantations located along the island's west coast road (approximately 20 km north-west of Mataram). Mature fruits were harvested from randomly sampled trees, dehusked, halved and endosperm plugs removed using a non-sterile but clean cork borer (as described above). The embryos were then isolated from the plugs (as described above) and placed (in lots of 150) in six sterile polycarbonate screw cap tubes (11.0 × 4.5 cm diameter) each containing 50 mL of a sterile ascorbic acid solution (1 mg L^{-1}). Three of these tubes were placed immediately onto a cotton wool layer sitting over bags of ice housed in an insulated plastic cooler tank (Lion Star Co, Jakarta, Indonesia). In a preliminary study, this apparatus, when closed and with ice replaced every 24 h, had been shown to provide a constant temperature of 5 ± 2°C inside the culture tube for at least 4 days. The other three tubes were placed into a similar tank but without ice, providing an average temperature when closed of 25 ± 5°C. Both tanks were then transported (*c.* 20 km; *the collection and transportation took about 6 h*) to the University of Mataram. Once in the laboratory the embryos from the tank with ice were removed and placed individually, without surface sterilisation, into sterile polycarbonate, screw cap culture tubes (8.0 × 2.5 cm diameter) containing 10 mL of a filter sterilised ascorbic acid solution (1 mg L^{-1}). The embryos were incubated in the dark in a refrigerator (5 ± 2°C). The embryos from the second tank were similarly treated but placed on a laboratory shelf (*c.* 28/25 ± 2°C, day/night) in the dark. From both storage treatments and after 0, 1, 2 and 4 days of incubation tubes were removed from the treatments, embryos removed, surface sterilised, weighed (as previously described) and individually cultured in liquid, then onto solid medium (as described above). The percentage of embryos that had become contaminated was assessed and the germination rate of the remainder determined 6 weeks after the start of culture (as described above).

Experimental Design and Statistical Analysis

Both studies were undertaken using a completely randomised design. In the first study, on protocol development, each incubation period consisted of 10 replicate tubes each containing one embryo. In the second study, on remote site germplasm collection, each incubation period consisted of three replications each consisting of 50 tubes containing one embryo. The data from both experiments were statistically analysed for variance and mean compared with the Student–Newman–Keuls test using SigmaStat (Jandel Corporation, San Rafael, USA). The data of contamination, germination rates and microbial density underwent a square-root transformation prior to statistical analysis.

Results

Development of a Germplasm Collection Protocol

The initial fresh weight of the embryos at the time of isolation was 170 ± 10 mg and this did not significantly change during the 4-day incubation period at 5°C or 26°C (data not shown). The embryos incubated at 5°C showed no visible morphological changes, they remained white with a firm texture. In contrast, the embryos incubated at 26°C turned yellow with a soft texture, the intensity of both features increasing with time. The embryo incubation solution kept at 5°C remained clear while that from around the embryos kept at 26°C became turbid. The pH and electrical conductivity of both incubation solutions significantly increased ($F = 31.8$, $P < 0.01$ for pH and $F = 63.0$, $P < 0.01$ for electrical conductivity) during the course of incubation (Fig. 1*A*, *B*); however, over the 4-day period a significantly greater increase in both parameters was observed at 5°C than at 26°C. The pH and electrical conductivity values of the solutions from the embryo-free culture vessels did not significantly change regardless of the time of incubation or temperature of incubation (data not shown). Microorganism density in the 5°C incubation solution did not significantly increase during the 4-day period; however, at 26°C microorganism density increased 10-fold (10.8 to 109.4 × 10^6 cells mL^{-1}; Fig. 1*C*). An increase in the control microorganism density could not be detected.

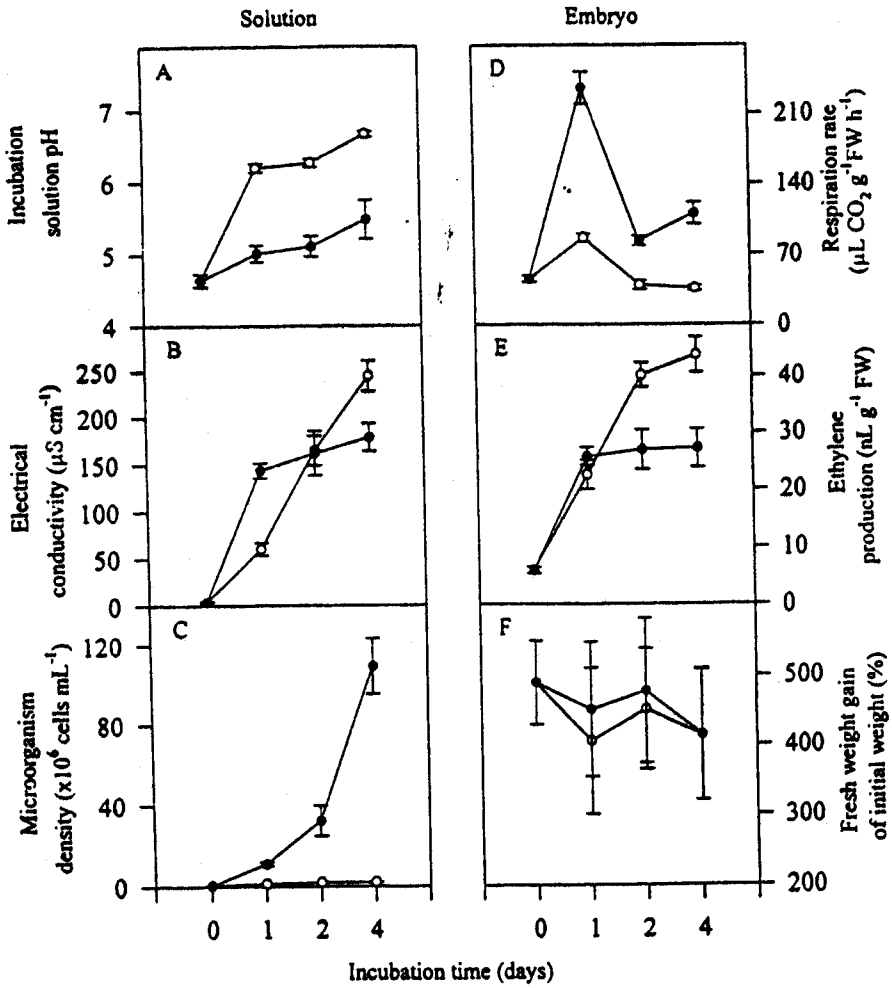

Fig. 1. Condition of the incubation solution (*A*, *B* and *C*) and coconut embryos (*D*, *E* and *F*) following incubation at 5 ± 1°C (○) or 26 ± 1°C (●). After the indicated time the solutions were analysed for pH (*A*), electrical conductivity (*B*) and microorganism density (*C*). Embryo respiration rate (*D*) and ethylene production (*E*) were measured after transferring the embryos from the solution into sealed tubes and incubating at 26 ± 1°C in the darkness. The fresh weight (*F*) of embryos was recorded after culture on a tissue culture medium for 6 weeks. All data points are means ± s.e. (vertical bars).

The respiration rate of the 5°C incubated embryos doubled after only 1 day, then declined over the next 3 days to be significantly less than the initial rate (Fig. 1*D*). In contrast, the 26°C incubated embryos underwent an approximate five-fold increase in their respiration rates in the first day before declining over the remaining 3 days to a value that was double the original rate. Ethylene emission, used here as an indicator of tissue stress, significantly ($F = 23.2$, $P < 0.01$) increased in response to both incubation temperatures (Fig. 1*E*). For the 5°C incubated embryos ethylene emission rose rapidly during the first 2 days and then more gradually over the next 2 days. The 26°C treated embryos showed a similar trend over the first day then did not increase over the remaining 3 days. There was no change in the measured environment gases in control vessels over the 4 days of incubation.

The remaining viable embryos from both temperature treatments germinated and grew in a manner similar to those cultured fresh. In addition, there was no significant difference in individual embryo growth (as measured by fresh weight gain) 6 weeks after the start of cold incubation as compared to 26°C treated embryos (Fig. 1*F*). All the plants produced had normal morphology, indicating that the cold treatment had not damaged the embryos or upset their subsequent growth potential.

Remote Site Germplasm Collection

All embryos incubated at 5°C remained white and firm while those under the conditions without temperature control (first 25 ± 5°C then 28/25 ± 2°C; day/night) turned yellow, with colour intensity increasing with time. Although detailed analysis of the microorganism density in incubation solutions was not undertaken, visual observations indicated that the incubation solution kept at 5°C remained clear over the incubation period, while the incubation solution from the treatment without temperature control became turbid.

The highest rate of embryo contamination (35%) was observed when the embryos were incubated without temperature control while the cold-treated embryos exhibited a very low rate of contamination (< 5%; Fig. 2). The germination and subsequent growth of the uncontaminated embryos from both temperature treatments was similar (Fig. 2).

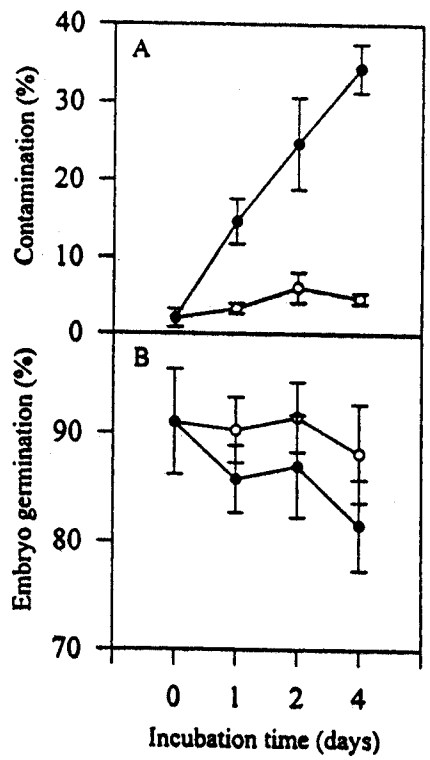

Fig. 2. Condition of field-collected coconut embryos following incubation at 5 ± 1°C (○) or without temperature control 18/25 ± 2°C (●). After the indicated time and culture on a tissue culture medium for 6 weeks, embryo contamination (*A*) and germination (*B*) were determined. All data points are means ± s.e. (vertical bars).

Discussion

Since no change occurred in the incubation solutions taken from tubes without embryos, the increases in pH and electrical conductivity (Fig. 1*A*, *B*) may indicate that under both temperature conditions solutes were released from the incubated embryos. Since this leakage was greatest at 5°C, this may indicate that chilling injury had occurred. This is not surprising since similar low-temperature treatments have been shown to affect coconut (Karunaratne 1988) and other tropical crops in a similar way (e.g. *Glycine max*, Leopold 1980; *Hevea brasiliensis*, Hu *et al.* 1985; *Cucumis sativus*, Sobczyk *et al.* 1985). The occurrence of chilling injury is further indicated by the higher ethylene production (Fig. 1*E*) and the lower respiration (Fig. 1*D*) in this condition as compared to the warmer incubation condition (Fig. 1*E*). Such increases in ethylene emission from somatic embryos of *Elaeis guineensis* Jacq. (Corbineau *et al.* 1990) and production of 1-aminocyclopropane-1-carboxylic acid, an immediate ethylene precursor, by embryogenic axes of chickpea (*Cicer arietinum* L.) seeds (Delgado and Matilla 1994) have previously been observed following a low-temperature treatment.

Even though some chilling injury may have occurred this was not sufficient to prevent the low-temperature-treated embryos from germinating and growing in a fashion identical to embryos that were not low-temperature treated (Figs 1 and 2). Short-term low-temperature treatments did not adversely affect other species depending on the moisture of seeds and embryos (see Cal and Obendorf 1972; Bramlage *et al.* 1978; Bedi and Basra 1993). The preservation of the health of the embryos under the low-temperature treatment may be because this condition prevented microorganism multiplication (Fig. 1*C*) similar to that observed on many other occasions (see Ailan and Anon 1989; Berjak *et al.* 1992). As the indicators of chilling injury used here are still increasing after 4 days it may be unwise to store coconut embryos for much longer at 5°C. Under most germplasm collection conditions, 4 days would be ample time to return the embryos to a tissue culture laboratory for surface sterilisation and culture.

The advantage of the present remote-site germplasm collection technique is that no sterilisation or aseptic culture is required in the field. This is different from most other previously described techniques for coconut (Assy Bah *et al.* 1987; Sossou *et al.* 1987). In addition, the present technique can be undertaken by unskilled people in the field and requires little equipment and chemicals (*c.* 3 kg in total weight for the isolation and transport of at least 500 embryos).

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