In vitro collecting of coconut germplasm

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Introduction

In vitro collecting (i.e., the utilization of *in vitro* culture techniques for collecting plant germplasm) offers the plant collector an additional option for solving various problems which can be encountered during collecting expeditions. The application of *in vitro* collecting is particularly useful for the two main categories of problem crops (i.e., vegetatively propagated species and species with recalcitrant seeds) (Withers 2002). *In vitro* collecting protocols have now been developed for a number of different species (Pence *et al*. 2002).

In the case of coconut, seeds are bulky and heavy, making them costly to transport. They are also highly recalcitrant (Chin and Roberts 1980). These characteristics limit the amount of material that can be collected and restrict the geographic range of collecting missions. These limitations may have serious consequences for genetic resources conservation, since it is recognized that a large amount of the untapped genetic diversity in coconut is located in remote areas, such as atoll islands. The key to solving these problems, however, lies in recognizing that only the embryo is needed to propagate a coconut palm. Various efficient *in vitro* culture protocols are available which allow the production of whole plantlets from coconut zygotic embryos inoculated *in vitro* (Batugal and Engelmann 1998; Engelmann *et al*. 2002).

Status of work

Research on the adaptation of *in vitro* culture techniques to collecting coconut embryos was initiated 15 years ago under the aegis of the IBPGR (International Board for Plant Genetic Resources, the predecessor of International Plant Genetic Resources Institute -IPGRI), with the aim of facilitating not only the collecting but also the international exchange of coconut germplasm. In addition to the advantages offered by this technique for collecting genetic resources, *in vitro* collecting would also avoid the transmission of important coconut diseases, which do not pass through the embryo. This is particularly important with the expected increase in international exchange of coconut germplasm linked with the establishment of the multi-site International Coconut Genebank

(Ramanatha Rao and Batugal 1998). Various *in vitro* collecting techniques have been developed by different teams, thereby demonstrating not only the feasibility of collecting isolated embryos, but also the great flexibility that can be exercised within the basic concept (Engelmann 2002).

The *in vitro* culture of coconut embryos has been adapted by several researchers in collecting coconut germplasm from the field. The techniques basically include the following sequence of operations:

- Dehusking and cracking open the nut;
- Extracting a plug of endosperm containing the embryo by using a cork borer;
- Dissecting the embryo from the endosperm; and
- Inoculating the embryo into culture.

The methods developed differ in the degree to which attempts are made to reproduce laboratory conditions in the field, the amount of *in vitro* work actually performed in the field, and, therefore, the point at which sterilization is carried out. Their utilization requires varying levels of technical expertise, and the method selected will depend on the circumstances of the collecting mission and on the tissue culture expertise available among the collecting team.

The simplest methods, which do not require specific expertise at the collecting site, are one of the two methods developed in Côte d'Ivoire (Assy-Bah *et al*. 1987) and that established in the Philippines (Rillo and Paloma 1991; Rillo 1995). In the first protocol developed by Assy-Bah *et al*. (1987), after disinfection, the plugs of endosperm containing the embryos are placed in a solution of KCl (16.2 g l^{-1}), then brought back to the laboratory where they are redisinfected and inoculated *in vitro* under the laminar flow (see Protocol 1 below). In the protocol developed in the Philippines, plugs of endosperm containing the embryos are extracted in the field, brought to a simple isolation room close to the collecting site, disinfected with alcohol and commercial bleach, placed in sterile plastic bags with sterile, moist cotton and transported in cold storage. Upon arrival in the laboratory, subsequent manipulations are carried out aseptically, under the laminar flow hood. The cylinders of endosperm are resterilized with commercial bleach, and the embryos are extracted and inoculated *in vitro* for germination and growth. This protocol is used routinely in the Philippines in the framework of programmes for mass production of Makapuno embryos (Rillo 1999).

Another protocol, which has been established by Australian researchers, requires some tissue culture expertise because embryos have to be extracted from the albumen immediately after their collection, but allows transport time of up to six weeks (Ashburner *et al.* 1995, 1996;

Samosir *et al.* 1999). Plugs of endosperm are collected in the field and transported to an improvised laboratory close to the collecting site, where the embryos are extracted from the albumen, sterilized with commercial bleach, and inoculated into 2 ml sterile plastic cryotubes containing sterile water. Manipulations after arrival in the laboratory are performed aseptically under the laminar flow hood. The embryos are resterilized and inoculated *in vitro* for germination and growth

In the other protocols (i.e. the second protocol developed in Côte d'Ivoire, see below) (Assy-Bah *et al.* 1987) and those established by Sossou *et al.* (1987) and Karun *et al.* (1993), *in vitro* inoculation of the embryos is performed directly at the collecting site, thus requiring the relevant expertise to be available within the collecting team. The field equipment requirements are greater than in the protocols described above, but even these methods range in complexity. The technique of Sossou *et al.* (1987) attempts to simulate laboratory facilities and methods in the field using an inflatable glove box. The protocols established by Assy-Bah *et al.* (1987) and Karun *et al.* (1993), however, accept the limitations of working in the field and present a lower-technology approach. Endosperm plugs are extracted from the nuts and disinfected with commercial bleach. The embryos are then dissected and inoculated inside a wooden or plexiglass box (to protect from airborne contaminants) and transferred into sterile culture tubes. With the protocol developed by the research team from India, embryos are either directly inoculated on growth medium or kept for 2-4 months in sterile water (Karun *et al.* 1996). This protocol has been used successfully by Indian researchers to collect several thousand embryos from remote Indian Ocean islands (Karun *et al.* 1998; 2002). All these protocols give good results, with contamination percentages below 10% of the inoculated embryos.

Detailed description of the in vitro *collecting protocols developed by Assy-Bah* et al. *(1987)*

Assy-Bah *et al.* (1987) developed two coconut embryo *in vitro* collecting protocols - one consisting of storing the disinfected embryos in a KCl solution until they are brought back to the laboratory, where they are redisinfected and inoculated *in vitro* under sterile conditions, and the other including *in vitro* inoculation of the embryos in the field. Details of the protocols are as follows:

Protocol 1 (inoculation of embryos in the laboratory)

Preliminary operations are performed in the open air, on a folding table that has been washed and disinfected with a bleaching solution.

1. Select and dehusk mature nuts.
2. Break nuts open with a clean hammer.

3. Use a cork borer to remove a cylinder of solid endosperm containing the embryo, and use forceps to transfer the cylinder to a jar containing 500 ml of commercial bleach. Disinfect all instruments with commercial bleach and sterilize in the flame of the gas burner.
4. Immerse batches of 25 cylinders in commercial bleach for 20 minutes.
5. Immediately after disinfecting, transfer endosperm cylinders without rinsing in individual 30 ml containers containing 15 ml KCl solution (16.2 g/l).

The following steps are performed in the laboratory, under the laminar airflow cabinet.

1. Remove endosperm cylinders from the KCl solution and immerse in batches of 25 cylinders in commercial bleach for 20 minutes.
2. Place one cylinder in a sterile Petri dish and dissect out the embryo using forceps and a scalpel. Flame dissecting tools before manipulating a new embryo to reduce the risk of cross-contamination.
3. Rinse the embryo once in sterile water (using one flask per embryo to reduce the risk of cross-contamination) and transfer it to solid medium in a culture tube.
4. Seal the tube with cling film and place it on a rack for culture in the growth room.

Protocol 2 (inoculation of embryos in the field)

Steps 1-5 are the same as in Protocol 1 above.

The following steps are performed inside a wooden box, which provides some protection from external contaminants. The inside walls of the box are disinfected with bleach.

1. Place one cylinder in a sterile Petri dish and dissect out the embryo using forceps and a scalpel. Flame dissecting tools before manipulating a new embryo to reduce the risk of cross-contamination.
2. Rinse the embryo once in sterile water (using one flask per embryo to reduce the risk of cross-contamination) and transfer it to solid medium in a culture tube.
3. Seal the tube with cling film and place it on a rack for transport to the laboratory.

Using Protocol 2, contamination was around 10%, while it was only around 5% with Protocol 1. No differences were noted in germination

and development between embryos treated following Protocols 1 and 2. Embryos could be stored for up to 14 days in the KCl solution without any effect on their further development. After direct inoculation in the field (following Protocol 2), embryos could be kept in semi-solid medium under non-controlled environmental conditions for two months before being grown in the culture room of a laboratory (Engelmann and Assy-Bah 1992). These results were confirmed recently by N'Nan (personal communication) following a series of *in vitro* collecting experiments performed in Côte d'Ivoire in 2001. *In vitro* collecting has been used routinely to collect and send over 20000 embryos from Côte d'Ivoire to France over the last two years.

In vitro *culture of embryos*

After inoculation *in vitro*, embryos have to be germinated and grown into weanable (acclimatized and hardened) plantlets. Research towards the development of *in vitro* culture protocols has been performed over the last 30 years by various research teams worldwide. An assessment of the available protocols, carried out during the IPGRI/COGENT-funded International Coconut Embryo Culture and Acclimatization Workshop held in the Philippines in 1997, revealed a large discrepancy in the performance of these *in vitro* culture protocols, with 14 to 55% of the inoculated embryos giving rise to plantlets growing *in vivo* (Engelmann 1998). The main bottleneck was the low efficiency of *in vitro* embryo germination and plantlet development. The protocols developed also differed in the culture conditions, composition and sequence of media employed and the stage of plantlets selected for weaning. Also, these protocols had been tested with a limited number of coconut varieties. In this workshop, which was participated by seven countries, the embryo culture techniques of country participants were compared and good features were adopted to develop an upgraded protocol to be further tested. The results of this workshop were published to guide embryo culture researchers (Batugal and Engelmann 1998). Another IPGRI/COGENT-coordinated international project, funded by the UK Department for International Development (DFID), was thus implemented to address two main objectives: (1) to improve the maturation and germination of embryos, and their development into plantlets; and (2) to determine and select the most efficient *in vitro* culture protocol and to test it with a large number of varieties (Batugal and Engelmann 1998). At the end of this project, the success of coconut embryo *in vitro* culture was significantly improved, with 31 to 81% of inoculated embryos developing into plantlets *in vivo* (Engelmann and Batugal 2002). A large diversity of coconut germplasm was employed since the tissue

culture protocols have been tested with over 20 varieties. These experiments also revealed a very strong genotypic effect in response to *in vitro* culture. No optimal protocol was identified due to the high variability of the responses obtained in the different laboratories involved in the project. However, the 'hybrid protocol' proposed by one laboratory, which combines the most efficient steps of the four protocols tested, seems to hold good promises for further improving the performances of coconut embryo *in vitro* culture.

Zygotic embryos have also been employed as starting material for large-scale propagation of coconut genotypes through somatic embryogenesis (Verdeil *et al.* 1999). However, the reactivity of coconut tissues to *in vitro* manipulation is very low, and only few plantlets have been obtained from a limited number of coconut accessions. Additional research is therefore required before large-scale propagation of coconut through somatic embryogenesis can be undertaken.

Conclusion and prospects

The various examples of *in vitro* collecting protocols developed for coconut embryos range from extreme simplicity to a relatively high level of sophistication and illustrate the flexibility and adaptability of the basic concepts of the procedure. It is with coconut that the largest amount of research has been directed towards the establishment of *in vitro* collecting protocols because of the particular difficulties encountered with germplasm collecting and exchange for this species. *In vitro* collecting is currently used on a routine basis for coconut more than with any other species. The utilization of this technique is expected to increase with the establishment of COGENT's multi-site International Coconut Genebank, thus making coconut one of the best models for the application of *in vitro* collecting.

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