# Collecting Palm: *In Vitro* Explanting in the Field

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The IBPGR Annual Report 1983 pointed out the need to develop novel, in vitro-based collecting techniques for difficult species. Zygotic embryos and apical meristems, as the explants least prone to somaclonal variation, are perhaps the material of choice. Obtaining explants in the field and introducing them into culture at the collecting site itself offer numerous advantages for the collection of germplasm.

Much space is saved if excised embryos are transported instead of large-sized fruits or seeds (e.g. many palm species, forest trees, and fruit trees), especially when a low risk of contamination permits the transfer of several embryos into a single culture vessel. The germination capacities of many valuable germplasm accessions can be unpredictable and low. Consequently, samples have to be transported in very large numbers with the hope that at least a few of them would germinate successfully on return to the base. However, if embryos are explanted in the field, especially in distant locations, departure from the collecting site may be decided only after ensuring that a sufficient number are already developing in vitro.

The maintenance of seed viability during the course of collection has been reported to be a problem in several cases. Thus, refrigerated transport of seed material may have to be provided for proper conservation in cases of extremely short-lived viability, which could be just for just a few days as in mahogany (Vivekanandan, 1978) or weeks as in palms like Pinanga and Oncosperma (Jones, 1984). Such difficulties are avoided if the embryos of fresh seed are explanted when they are most viable, an operation which is best carried out as soon as they are collected. In other cases, a collecting expedition may not necessarily reach the site at the time when seed is at its optimal maturity. In vitro culture seeks to simulate natural conditions and provide an artificial environment within which maturity as normally attained in the seed can be achieved. Thus, more or less immature embryos may be collected and reared successfully.

Dormancy in collected seed can be a disadvantage when the rapid germination of seed stock is desired as in most reforestation programmes. In addition to dormancy per se, slow germination also contributes to a prolonged and variable delay in many groups. In palms, for example, the germination period may range from ca. 40 to more than 1000 days.

Liberation from the inhibitory influences of the seed-coat or the endosperm by inoculation into in vitro culture often induces immediate germination, provided that nutrients and, if necessary, stimulatory factors are supplied through the culture medium. Collection by explanting embryos in the field can therefore lead to a significant shortening of the breeding cycle of many trees.

Apical meristems can be too fragile to withstand even short periods of transport within collected shoots. Thus inoculation

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in the field may be the only possibility in the case of explants of forest trees from distance sources.

In addition to embryos and shoot apices, other explants may be collected in vitro, for example, anthers. Anther culture is often the best means to obtain homozygous lines quickly for breeding, especially in the majority of trees which are heterozygous and open-pollinated (Radojevic and Kovoor, 1985). The stage of explanting anthers is critical. Anthers of herbaceous annuals may be excised from plants whose development is followed in the glasshouse.

With perennial trees, however, anthers of the right stage can be detected only by a survey of natural stands; when found they are better explanted immediately rather than after transporting them to the laboratory.

## Methodology

Classic laboratory procedure has to be reformulated when the in vitro explanting of germplasm in the field is envisaged (Withers, 1987). Specifically:

(i) Aseptic manipulations should be adapted to practice in the open air;
(ii) Equipment should be redesigned to be portable for utilization on expeditions far away from the base laboratory; and
(iii) Culture vessels used should be small in size yet large enough to hold several explants when appropriate. It is very important that the vessels be designed to withstand rough handling in trsnsit back to base, to maintain asepsis and be capable of providing suitable growth conditions.

We describe here a procedure for initiating in vitro cultures in the field as we have practised it for zygotic embryos of some palm species. The same technique could also be applied to other species and to any primary explant.

The main departures from usual laboratory methods are the use of a portable, collapsible glove-box (Figs. 1 and 3) instead of a laminar air-flow hood,


Fig. 1. Field set-up for explanting zygotic embryos
a: Husking nuts on an iron crowbar stuck in the ground
b: Transferring surface-sterilized embryos into Sassou flasks in a collapsible glove-box
c: Inflating the glove-box with a pedal pump

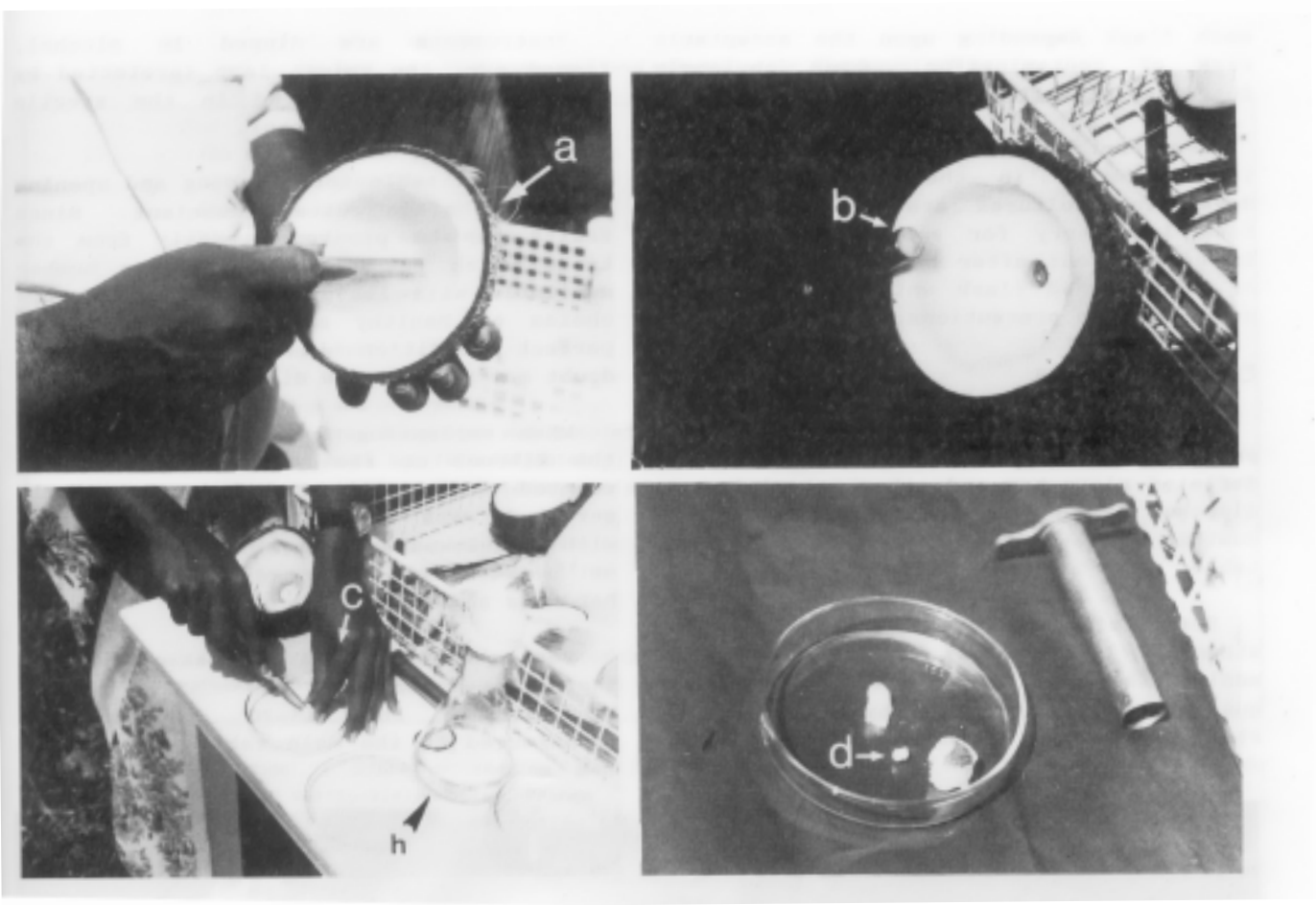


Fig. 2. Excision of coconut embryos
(a) Cracked open half of a husked nut with cork-borer piercing the kernel where the embryo lies (b) Cork-borer with punched out plug of kernel showing the contours of the embedded embryo (c) Plug of kernel dissected in a Petri dish to remove embryo (d) before immersion into a solution of hypochlorite (h)

a sterile metal box instead of sheets of sterile paper in which instruments are cooled after flaming, pre-weighed lots of calcium hypochlorite and sterile water in flasks or screw-capped bottles to prepare surface-sterilizing solutions as required, and special culture vessels (see below) containing the appropriate medium prepared and sterilized in the laboratory. The requirements are listed in Table 1.

Since liquid media have been found to be the most suitable for the embryos of many species, and even essential in some cases, we have devised a sterile plastic disposable vessel (the Sossou flask) whose internal geometry absolutely prevents spilling of the contents during transport in any position (Figs. 3 to 5).

There is no cotton plug and the plastic cover is quite loose-fitting to permit adequate gaseous exchange. The flasks can hold up to 20 ml of liquid medium. They may also be filled with sterilized agar-solidified medium, but in this case their insides should be lined with the medium so that dislodged explants will still lie in contact with the agar whatever the position of the flask during transport.

For this, suitable quantities of hot, sterile medium are poured in three stages with time given for the medium to solidify between each. Flasks are cooled (a) with slow rolling on their sides for the medium to line the cylindrical wall, (b) up-side down and (c) top-side up. For transport to the collecting site, flasks filled with medium in the laboratory may be reinserted in fives into the sterile sleeves in which the manufacturer supplies them (Fig. 4).

Several explants may be inoculated into

each flask depending upon the acceptable risk of contamination, which obviously increases with their number per flask.

Transport back from the collecting site is, likewise, in the sleeves and the developing cultures are removed in the base laboratory for sub-culturing. This is carried out after sawing off the base of an upturned flask while observing the usual aseptic precautions.

Field procedures

The portable table is set up on a level patch of ground in the shade. The deflated glove-box and other materials are disposed as in Fig. 1 with the foot-pump, dustbin and waste bucket underneath the table.

Sterilizing solutions of the desired strength (see below) are prepared by adding sterile water to pre-weighed quantities of calcium hypochlorite in sterile graduated Erlenmeyer flasks.

Instruments are dipped in alcohol, flamed over the spirit lamp (protected by a wind shield), covered in the sterile metal box and left to cool.

A second table for cleaning and opening fruits is often quite convenient. Since fruits can be plucked directly from the trees which bear them, the large number available will leave room for a rigorous choice of healthy material; those whose perfect condition is in the slightest doubt may promptly be discarded.

When explanting from drupes of palms, the fibrous or fleshy mesocarp is first removed and the hard endocarp (nut) is scrubbed with alcohol and cracked open with a nutcracker, saw, crowbar (Fig. 1), or hammer and anvil, depending on its hardness and size.

The endosperm (seed) is taken from the inside, immersed in 5% calcium hypochlorite for 15-20 minutes and transferred to the main table where it is

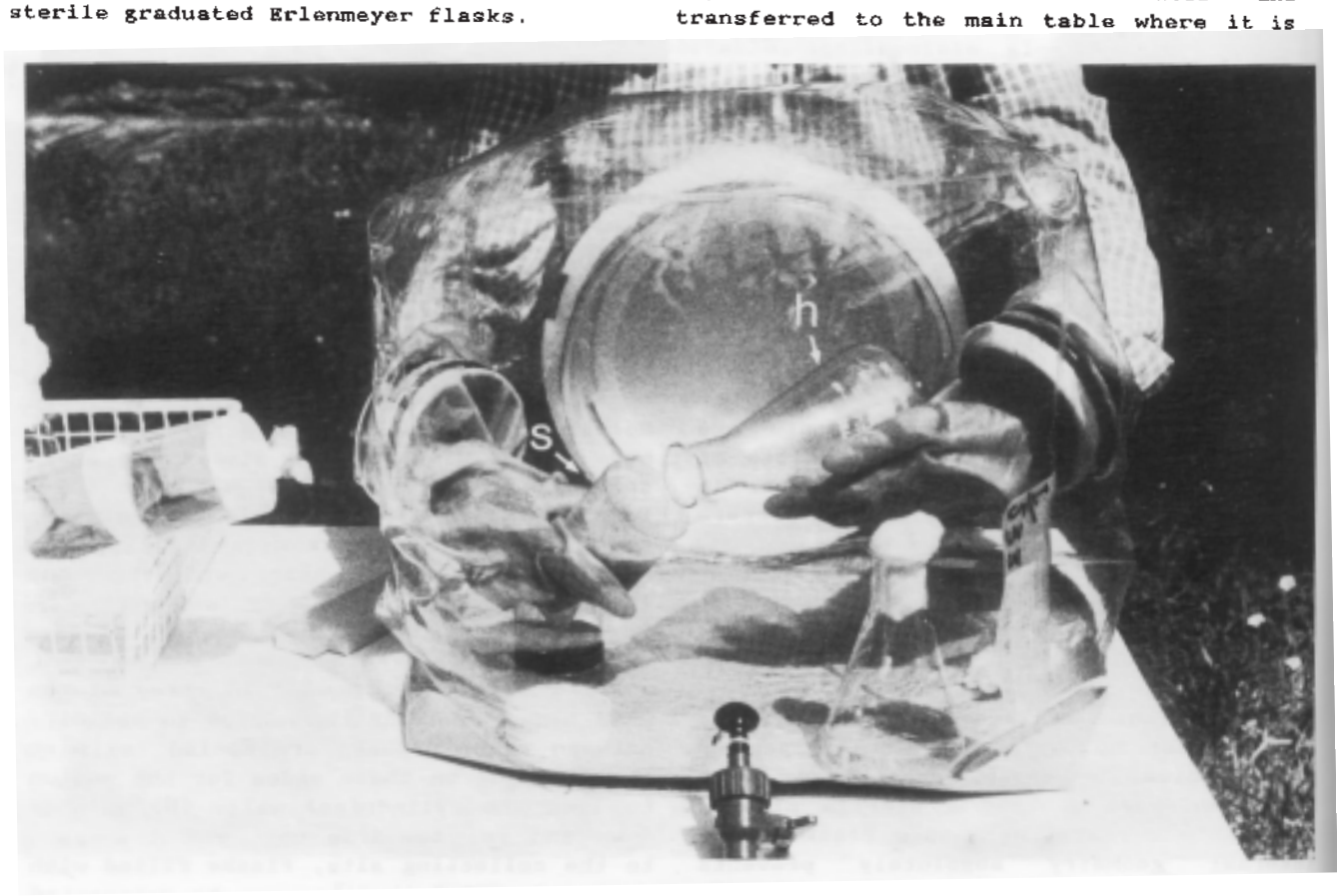


Fig. 3. Transfer of coconut embryos
After washing off the hypochlorite with several changes of sterile water, Erlenmeyer flasks containing surface-sterilized embryos (h) are introduced into the collapsed glove-box along with Sossou flasks (s) containing the appropriate liquid medium. The glove-box is inflated and embryos from the Erlenmeyer flask are tipped into the Sossou flask.

Table 1. List of apparatus and materials for field explanting

- Two folding tables (120 x 60 cm)
- Collapsible glove-box 1/
- Foot-pump or cylinder of compressed air to inflate glove-box
- Bucket for liquid waste
- Bin for solid waste
- Nutcracker
- Saw
- Hammer and anvil
- Crowbar
- Spirit lamp and wind shield
- Instruments for dissection and inoculation
- Metal instrument box (sterilizable)
- Sterile Petri dishes and Erlenmeyer flasks
- Sterile 50 ml plastic syringes
- Sterile graduated Erlenmeyer flasks containing pre-weighed calcium hypochlorite
- Sterile water in screw-capped bottles
- Alcohol and cotton swabs
- Stainless steel beaker
- Culture flasks 2/ containing the appropriate sterile medium
- Packets of sterilized aluminium or tin foil squares

1/ Mini-isolateur (Model I 533) for rearing germ-free animals, supplied by SNE la Calhène, 1 rue du Petit-Clamart, 78140 Vellzy-Villacoublay, France

2/ Sold as 'Sossou Flasks' (autorisation CNRS) by Labo Express Service, 4 rue de Seine, 91171 Viry-Chatillon Cedex, France

dissected in a sterile Petri dish to expose the embryo (Fig. 2).

Excised embryos are treated with 2% calcium hypochlorite for 2-5 minutes. Pockets of air formed in the crevices of explants often prevent total surface contact with the sterilizing agent. This is overcome in the laboratory by applying a moderate vacuum to explants in the solution for a few moments, generally in a desiccator connected to a filter-pump.

A simple substitute procedure (de Fossard, 1976) which can be practised in the field is as follows: a sterile disposable 50 ml plastic syringe (without the needle) is filled to about one-third its volume with sterilizing solution and explants are introduced through the mouth of the barrel; the piston is inserted and pushed; it is then drawn back fully, but this time closing the nozzle air-tight with the forefinger.

The same syringe may be used for expelling the sterilizing solution and for drawing in sterile water in which explants are washed for several changes.

The following items, necessary for the actual transfer of explants into culture flasks, are then placed inside the deflated glove-box, whose inner surfaces have been swabbed with alcohol: surface-sterilized and washed explants in the syringe or a sterile flask whose mouth has been flamed and recapped; culture flasks containing medium; metal box containing flamed instruments; packet of sterilized metal foil squares.

The lid of the wide porthole is closed and the glove-box inflated with the foot-pump after both hands have been inserted into the gloves.

If the water has been well drained off after the last sterile rinse, explants may quite simply be tipped into the culture flask; forceps are needed only rarely (Fig. 3). It is optional, but good practice on long expeditions, to place a square of sterile metal foil over the mouth of the culture flask before pressing down the loosely fitting plastic cover.

This maintains the cover tightly, though not hermetically, allowing the

passage of air between the folds of the tin-foil, which is otherwise quite gas-permeable. Inoculated flasks may be replaced in their sleeves before opening the glove-box to remove them for transport.

## Results

The technique has been tested on several palm species. It has turned out to be very successful for coconut embryos, in terms of the practical advantages offered in collecting elite material.

The kernel (endosperm), which is the principal seat of the coconut's economic value, is a post-fertilization tissue. Its ontogenic status is that of the daughter (embryo) and not the mother-tree, although its gene-dosage is triploid (2♀ + 1♂) and not diploid (1♀ + 1♂) as in the embryo.

Therefore, the best criterion by which to judge seed as germplasm is to consider the very kernel which accompanies the embryo. But it is hardly practicable to open a nut first and appreciate its kernel before sowing it. Using excised embryos, however, each kernel may even be weighed before choosing only those embryos associated with elite kernels for distribution to growers.

Apart from its application for collecting accessions for germplasm banks, the technique may also be used to obtain planting material from an abundant source for distribution to growers for selection such as in a copra-factory. The top one or two percentiles by weight of kernels which are exposed upon shelling nuts before processing may be retrieved and their embryos excised.

Since ambient conditions in a factory rarely proved sufficient asepsis for tissue culture operations as practised in the laboratory, the field set-up as described here should easily resolve that difficulty.

The 'makapuno' nature of many kernels is also perceived only when their nuts are opened in the factory; all such embryos could hence be saved for culture leading to a crop of exclusively 'makapuno' bearing progeny.

Suitable culture conditions for explanting coconut embryos *in vitro* have been described in initial reports (Cutter and Wilson, 1954; Abraham and Thomas, 1962) and later in the vast study of de Guzman and her collaborators leading to the successful rearing of homozygous 'makapuno' trees which cannot otherwise be germinated normally from seed (de Guzman and del Rosario, 1964; 1974; 1975).

Thus, a liquid medium is considered best for primary explants, while the induction of rooting in cultures (de Guzman, 1969) is increased by subsequent transfer to agar solidified medium (Balaga and de Guzman, 1971) with higher sugar levels (de Guzman *et al.*, 1971; del Rosario and de Guzman, 1976) auxin in the form of α-naphthalene-acetic acid (NAA) (Sajise and de Guzman, 1972) and, especially, activated charcoal (de Guzman and Manuel, 1977).

The inorganic constituents of the culture medium have also been studied (Balaga and de Guzman, 1971; del Rosario and de Guzman, 1976; Miniano and de Guzman, 1978) and the important step of transplanting to soil and subsequent survival has been worked out in some detail (de Guzman and del Rosario, 1974). Certain phytohormonal formulations produce a callus type of growth (Balaga *et al.*, 1973; del Rosario and de Guzman, 1976; Fisher and Tsai, 1978), showing distinct histological deviations (de Guzman *et al.*, 1978). Such media are better avoided in view of the risk of somaclonal variation (see below).

In the present trial, we used a charcoal-containing liquid medium whose composition was based on the principal findings of the above authors and, notably, had a higher concentration of inorganic salts. The successive steps of the explanting procedure are illustrated in Figs. 1 to 3 and described in the corresponding captions.

Fig. 5 gives an idea of the volume attained by growing embryos some days after explanting. It indicates that the number of coconut embryos which may be inoculated into each flask should be restricted to about five or six, unless of course a slow-growth medium were to be formulated which would allow the transfer of larger numbers and when a long expedition is foreseen.

In the present study, in addition to

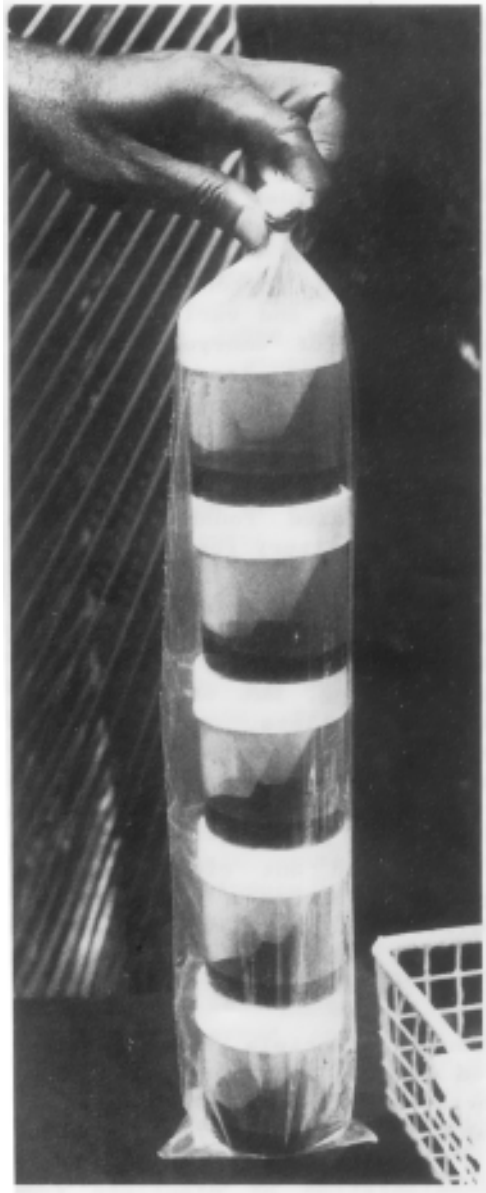

Fig. 4

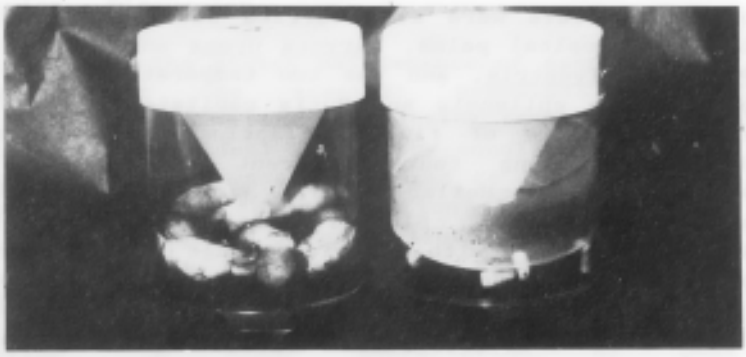

Fig. 5

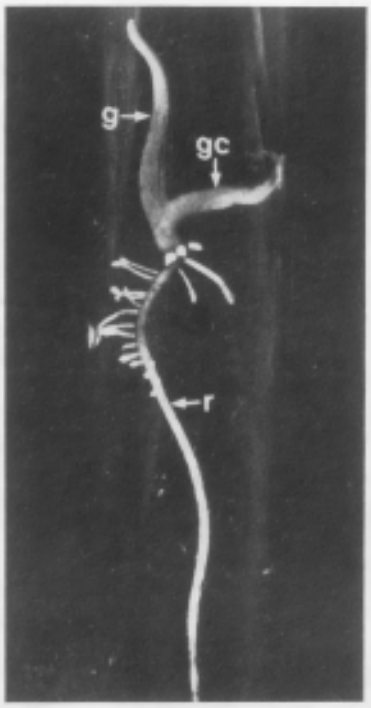


Fig. 6

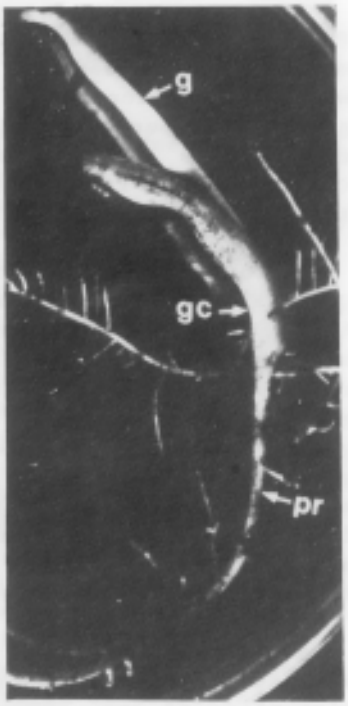


Fig. 7

Fig. 4. Sleeve of five culture flasks containing liquid medium with charcoal

Fig. 5. Growth of embryos *in vitro*: Freshly transferred coconut embryos (right) in Sossou flasks with liquid medium containing charcoal and developing embryos (left) as they would appear at the end of a 2- or 3-week expedition

Fig. 6. Embryos of *Caryota* 5 weeks after transfer to Blaydes' (1986) medium containing $GA_3$ (5 mg/l)
g: Plumule
gc: Cotyledonary sheath
pr: Primary radicle

Fig. 7. Embryo of *Phoenix* 2 months after culture in agitated liquid medium of Eeuwens (1976) containing 2,4-D (2 mg/l) and kinetin (1 mg/l)
g: Plumule
gc: Cotyledonary sheath
pr: Primary radicle

coconut, trials were also carried out on the two tropical palms, Caryota urens and Phoenix sylvestris, and the two temperate palms Jubea chilensis and Butia capitata. The feasibility of field explanting was determined using four different basal media, those of Heller (1953), Murashige and Skoog (1962), Blaydes (1966) and Eeuwens (1976), with a wide range of phytohormonal and other culture conditions.

Out of the large mass of data gathered, we briefly describe here only those conclusions relevant to the technique of germplasm collecting.

The importance of explanting embryos of fresh material, facilitated by the present method, was evident in the case of Jubea where we recorded a four-fold higher germination percentage in vitro of embryos from fruits of the same year's harvest (1984) than from those harvested in 1981-1983 and stored until 1984.

A further observation with Jubea and Caryota was the remarkable shortening of the germination time when excised embryos are cultured in vitro. Jubea seeds are reported to take up to 15 months for germination (Koebernik, 1971), whereas in vitro embryos explanted in the field germinated within three weeks. Similarly, the germination of Caryota was reduced from seven months to three, which is still a significant saving of time when it comes to reforestation programmes.

A liquid medium, especially with activated charcoal suspended in it, seems virtually to be essential for Caryota embryos since germination was noted in all formulations tried out (Fig. 6), while many of the same media solidified with agar led to no development whatsoever.

Phoenix embryos developed on both solid and liquid media, but agar was held to be clearly preferable since the type of growth on it resembled that of normal germination in soil (see later), while in liquid (Fig. 7), the cotyledonary sheath showed undue linear growth and a long delay before it was pierced by the plumule.

Agitation of the liquid medium also hastened the emergence of the leaves through the sheath, an expedient which it must be noted is inherent during the transport of explants. It would also appear that when the field explanting of a species is being attempted for the first time, a sequential change from liquid to solid medium may have to be considered.

The most suitable basal medium, was found to differ for each species. For example, that of Blaydes (1966) was the best for Jubea, whilst embryos of Phoenix performed better in the medium of Eeuwens (1976). As expected, phytohormones exert the most far-reaching and varied influence on the development of embryos in vitro. Gibberellin ($GA_3$) increased the percentage germination in Jubea and Caryota but not in Phoenix. Auxin in the form of indole-3-acetic acid (IAA) is a widely used phytohormone in tissue culture, but we have found it to be generally quite inhibitory for embryos.

The cytokinin 6-benzylaminopurine (BAP) was found to reduce germination percentages and retard the sprouting of the plumule in Jubea, but otherwise, BAP led to sturdier plantlets. In addition to interactions between different classes of phytohormone, they also interact with the inorganic constituents of the basal medium to produce specific effects. Thus coconut water, an undefined but rich source of growth regulators, inhibits the emergence of the radicle in Jubea embryos when in Heller's (1953) medium, but not in Blaydes' (1966) medium.

As a general principle, the behaviour of explanted embryos should be judged from two perspectives: ultimate viability and genetic stability.

The transplantation of plantlets from in vitro culture to the soil is often accompanied by a fairly high mortality. Many authors have pointed out that the principal cause could be the transition from a mode of absorption involving the whole surface of the culture to one which is restricted to the root system alone. Hence, due attention should be paid to root development in explanted embryos. Culture conditions favouring a profuse root system may be retained, even at the expense of other qualities. For example, we found that transfer from liquid to solid medium resulted in an abundant production of root by Phoenix embryos. The use of specific rhizogenic factors may also be considered. $GA_3$ added to Blaydes' (1966) medium induced roots in Caryota (Fig. 6) but not when included in Murashige and Skoog's (1962) medium. In

spite of some resultant loss of greening in shoots, use of $GA_3$ was still a key factor for the successful survival of plantlets upon transfer to soil in pots.

On the other hand, indole-3-butyric acid (IBA), which is reputed to be a powerful rooting hormone for many species had no such effect on Jubea embryos; this led to considerable difficulty in our trials. The best root development that we obtained with Jubea was with $GA_3$. Even then, roots stopped growth after elongation to about 20 mm, which was quite insufficient to withstand the dehydrative shock of entry into soil.

The retention of genetic integrity is a prime requirement of any germplasm collecting and conservation exercise. However, in vitro culture, in spite of its many advantages can introduce a risk of somaclonal variation (Scowcroft, 1984). This phenomenon is believed to be most prominent during prolonged in vitro culture involving unorganized culture systems such as callus.

Unfortunately, the most efficacious media by certain criteria such as fast growth tend to induce profuse but somewhat unorganized development. Thus, potent auxins like naphthoxyacetic acid (NOA) and NAA induce callus in explanted embryos of Jubea, Butia and Phoenix. Adventitious buds were observed on these calli and although this type of behaviour may appear to be a tempting basis for vegetative propagation, it should be remembered that from the point of view of genetic stability it may be unsafe. Multiple bud formation on callus of Phoenix was caused by high levels of 2,4-dichlorophenoxy-acetic acid (2,4-D) but histological examination showed most buds to possess only procambial strands with no sign of foliar initials. We have also noted that immature embryos generally show a greater tendency to form callus and adventitious buds, confirming earlier observation in date palms (Mater, 1983) and Acrocomia (Teixeira, 1985).

It would seem that the best practical approach is to ensure that the medium finally adopted for explanting the embryos of a given species is one on which development shows the closest resemblance to the normal germination pattern of embryos planted in soil.

Discussion

The strict maintenance of asepsis is by far the greatest practical problem besetting this collecting technique. Even under ideal laboratory conditions, primary explants are recognized to be a source of much more contamination than sub-cultures of callus or cell lines. Rigour in the choice of material cannot thus be overemphasized.

A clear appreciation of what constitutes really healthy tissue is possible only after acquiring considerable experience with the particular species in question. Furthermore, except during actual manipulations inside the glove-box, there is a heavy and constant shower of contaminants from all sides.

The atmospheres of forests and plantations possess the highest known densities of spores and micro-organisms distributed in stray air currents. It would, therefore, be very helpful if a mobile van, modified and equipped for the purpose, could be brought to the site and used to house the main table and glove-box. Due attention should then be paid to the fire hazard while flaming with alcohol inside the van.

A number of other practical points require examination in relation to collecting itself. Culture flasks may be transported in specially constructed cases of transparent plastic-ware if light is required, or of reinforced cardboard (in which they are packed by the manufacturer) if darkness is acceptable or preferred.

A case measuring 70 x 45 x 15 cm, which a traveller can carry by hand, will hold 100 flasks. Relatively large embryos like those of coconut may be transferred into a flask in lots of five or six, while 20 or more embryos of Jubea, which are much smaller, may go into the same flask.

Large numbers of small embryos developing vigorously could soon outgrow the volume of a flask and the nutrients therein before the end of an expedition. When this likelihood becomes apparent, aliquots of solutions of suitable growth retardants (abscisic acid, anti-auxins, etc.) previously tested in the laboratory for the species in question could be added from sterile ampoules.

For each new species whose collection is proposed, a fairly exhaustive study should first be carried out in the laboratory to determine the optimal culture conditions such as basal medium composition, phytohormonal balance conducive to normal development, requirement for liquid or solid medium with or without charcoal, lighting requirements, etc.

The most suitable procedure worked out for negotiating the delicate transfer from culture to the glasshouse should be available by the time that cultures reach the base laboratory. Alternatively, if subsequent conservation in vitro is intended, the proper conditions for that too will have to be determined (environmental conditions and media for slow growth, cryopreservation conditions).

Alternatively, if subsequent conservation in vitro is intended, the proper conditions for that too will have to be determined.

In conclusion, the technique described here could be of immense practical value for reforestation or the distribution of stock to growers of big trees like Jubea: a single case carrying 2000 developing embryos would suffice to replenish a sizeable plantation.

Another potential application would be in the case of Mangifera. Forty hitherto little-known species have been described with some remarkable genetic characteristics (Mukherjee, 1985). Their inclusion in future breeding programmes of cultivated mango is awaited with interest.

However, a centralized germplasm bank of these species has to be established first. Since practically all of them are large-fruited and found in wild locations, the present technique seems to be the most promising, and perhaps the only possible, method for collecting them. Indeed, the same reasoning also holds for the long list of forest fruit trees (Anon., 1982; 1983; 1984; 1986) which are sorely under-exploited at present.

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RESUME

Il est nécessaire de développer de nouvelles techniques de collectes à partir de la culture in vitro pour les espèces qui sont normalement difficiles à collecter. L'introduction en culture in vitro sur le lieu d'echantillonage offre de nombreux avantages pour la collection de souches génétiques.

RESUMEN

Existe una necesidad de desarrollar nuevas técnicas de recolección in vitro para especies que son difíciles normalmente de cultivar. Introducirlas en el lugar de recolección ofrece numerosas ventajas para la recolección de germoplasma.