

Media for Embryo Culture of Some Tropical Recalcitrant Species

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ABSTRAK

Dalam kajian ini, embrio yang telah diasingkan dari bijibenih 11 spesies "recalcitrant" telah dikaji untuk menentukan media dan keadaan pengkulturan yang sesuai bagi perkembangan menjadi pokok yang lengkap. Media Murashige dan Skoog yang diubah melalui penambahan 0.17 gl^{-1} NaH_2PO_4 telah didapati sesuai sebagai media dasar bagi semua spesies yang dikaji. Media dasar yang ditambah dengan 2 gl^{-1} 'activated charcoal' dan 1.0 mg l^{-1} NAA atau IAA bersama dengan 1 mg l^{-1} Kinetin, BAP atau 2iP didapati sesuai bagi semua 11 spesies di mana embrio yang diasingkan itu dapat berkembang secara normal untuk menjadikan pokok yang lengkap. Bagi beberapa spesies, rendaman embrio yang terasing steril dalam larutan 'antioxidant' (10 mg l^{-1} asid askorbik dan 5 mg l^{-1} asid sitrik) selama 10-45 minit sebelum dikulturkan didapati menggalakkan perkembangan normal bagi embrio-embrio.

ABSTRACT

In this study, the excised embryos of 11 recalcitrant species were studied to determine suitable media and cultural conditions for their development into whole plants. Murashige and Skoog's medium formulation modified slightly by the addition of 0.17 gl^{-1} NaH_2PO_4 was found to be a suitable basal medium for all the species studied. The basal medium supplemented with 2 gl^{-1} activated charcoal and 1.0 mg l^{-1} NAA or IAA in combination with 1 mg l^{-1} Kinetin, BAP or 2iP was found to be suitable for all the 11 species where the embryos developed normally to form whole plants. For some of the species, soaking the sterile excised embryos in an antioxidant (10 mg l^{-1} ascorbic acid and 5 mg l^{-1} citric acid) solution for 10-45 minutes prior to culture enhanced the normal development of embryos.

INTRODUCTION

The storage of seeds is the traditional means of conservation and international exchange of germ-plasm. For many crops, including most of the major world food resources, conservation can be carried out very efficiently in seed banks (Ellis *et al.*, 1985a, 1985b; Williams, 1984). The orthodox (i.e. desiccation tolerant) nature of these

seeds enables them to be dried to very low moisture levels, making them very amenable to long-term storage. However, in a number of tree species and plantation crops found especially in the tropics and subtropics, the seeds produced are recalcitrant (Roberts and King, 1980). Such seeds are sensitive to desiccation and temperature and thus cannot be preserved by conventional

seed storage methods for long periods due to degeneration of the embryos (Bajaj, 1985). Their maximum longevity varies from a few days to a few months.

Orthodox seeds are relatively simple to store for long periods, in addition their excised embryos can be stored and cryopreserved. Culture of excised embryos of soybeans (Chin *et al.*, 1974, 1977), frenchbeans (1975), pea and wheat (Chin and Atiken, 1975) were easily cultured on sterilized soil. The general effect of cotyledon excision is slower growth and delay in senescence. However with the excised embryos of recalcitrant species which cannot tolerate desiccation, there was great difficulty raising seedlings from excised embryos in particular after a period of stress in storage at extremely low temperatures. These low vigour embryos need aseptic conditions and enriched media for their culture. In this paper, the objective is to find suitable media that support normal growth of excised embryos of recalcitrant species.

This paper reports the findings of the first phase of the study carried out over a one year period, on the screening for suitable media for the culture of excised embryos of some of the recalcitrant species found commonly in this region. The carrying out of this part of the study prior to the actual work on finding storage techniques was necessary because a literature search revealed that very little work had been done to establish suitable media and cultural conditions for the normal development of excised embryos of the tropical recalcitrant species to be studied for long-term storage.

MATERIALS AND METHODS

The excised embryos of 11 recalcitrant species were screened for suitable medium and cultural conditions. They were: jackfruit (*Artocarpus heterophyllus*), cempedak (*Artocarpus Champeden*), rambutan (*Nephelium lappaceum*), langsung (*Lansium domesticum*), durian (*Durio zibethinus*), mango (*Mangifera indica*), kundang (*Bouea ganadaria*), avocado (*Persea americana*), cocoa (*Theobroma cacao*), rubber (*Hevea brasiliensis*) and dryobalanops (*Dryobalanops aromatica*).

During the respective fruiting seasons, fresh ripe fruits were brought back to the laboratory and processed for their seeds. Embryos

from the seeds were excised and cultured within two days of the arrival of fruits in the laboratory.

Prior to aseptic excision of the embryos, the seeds were either surface-sterilized with 15% Clorox solution or 80% alcohol, depending on the seed types. For mango and kundang, in which the flesh could not be completely removed from the testa, surface-sterilization was carried out using 15% Clorox containing 1 – 2 drops of 7-x detergent for 20 minutes followed by 3–4 rinses with sterile distilled water. For jackfruit, cempedak, rambutan, langsung, durian, avocado, cocoa, rubber and dryobalanops, individual seeds were swabbed with 80% alcohol and allowed to dry in a laminar flow cabinet for about 20 minutes before aseptically excising the embryos.

Embryos from those species that exhibited browning during excision were immersed in a filter-sterilized antioxidant solution (10 mg⁻¹ ascorbic acid and 5 mg⁻¹ citric acid) until they were cultured. This reduced browning of the cut ends, excessive secretion of polyphenols into the medium and increased the survival rate of the embryos in culture.

Murashige and Skoog's (1962) medium formulation modified slightly by the addition of 0.17 gl⁻¹ of NaH₂PO₄ was used as the basal medium for the entire study. Various growth hormones at different concentration and combination were added to this medium. The auxins tested were NAA (α -naphthalene acetic acid) and IAA (indole-3-acetic acid) at concentrations of 0, 0.1, 0.5 and 1.0 mg⁻¹ while the cytokinins examined were Kinetin, BAP (benzylamino-purine) and 2iP (2-isopentyladenine) each at concentrations of 0, 0.1, 0.5 and 1.0 mg⁻¹. The effect of activated charcoal at concentrations of 0 and 2 gl⁻¹, on normal development of the excised embryos was also studied. Difco Bacto agar at 6 gl⁻¹ was used to solidify the medium. For each treatment three replicates of ten excised embryos were employed. Each experiment was repeated twice over the fruiting season, once at the beginning of the season and the other towards the end.

The number of normally-developing embryos (ie with a good shoot and root system) was scored as a percentage over the total number of cultures in each treatment. Evaluations were carried out at the end of six weeks of culture.

TABLE 1
 Mode of embryo development on a non-charcoal and charcoal medium
 supplement with various auxins and cytokinins of suitable levels

Species	Modified Murashige & Skoog's Medium	
	Without Charcoal + 0.1 mg l ⁻¹ NAA or IAA and 0.1 mg l ⁻¹ Kinetin, BAP or 2iP	With Charcoal (2 gl ⁻¹) + 1.0 mg l ⁻¹ NAA or IAA and 1.0 mg l ⁻¹ Kinetin BAP or 2iP
<i>Artocarpus heterophyllus</i>	Normal Development	Normal Development
<i>Artocarpus champeden</i>	Normal Development	Normal Development
<i>Nephelium lappaceum</i>	Normal Development	Normal Development
<i>Lansium domesticum</i>	Normal Development	Normal Development
<i>Durio zibethinus</i>	Slow Growth	Enhanced Growth
<i>Mangifera indica</i>	Inhibited Growth	Normal Development
<i>Bouea ganadaria</i>	Inhibited Growth	Normal Development
<i>Persea americana</i>	Inhibited Growth	Normal Development
<i>Theobroma cacao</i>	Normal Development	Normal Development
<i>Hevea brasiliensis</i>	Slow Growth	Enhanced Growth
<i>Dryobalanops aromatica</i>	No development	Normal Development

All cultures were maintained at 28°C ± 1°C in a temperature-controlled room with a 12-hour photoperiod of 8,000 to 10,000 lux intensity, provided by fluorescent tubes.

RESULTS

The best results obtained for the various embryo types are summarised in Table 1. The optimum cultural conditions and problems encountered for individual species are reported below:

(a) *Artocarpus heterophyllus* and *Artocarpus champeden*: The embryos in these two species could easily be excised and showed no browning during excision or in culture. Normal development was obtained both on a non-charcoal and charcoal added medium. On the non-charcoal medium, the best development was seen when 0.1 mg l⁻¹ NAA or IAA was used with 0.1 mg l⁻¹ Kinetin, BAP or 2iP. On charcoal medium, 1.0 mg l⁻¹ NAA or IAA when used with 1.0 mg l⁻¹ Kinetin, BAP or 2iP appeared optimum. Within six weeks, robust plants with good shoot and root system were obtained.

(b) *Nephelium lappaceum*: The tiny embryo in this species was difficult to excise

because it is tightly held between the cotyledons at one end of the seed. It was impossible to excise it without causing serious damage. In this case, a small block 2 x 4 x 4 mm of the cotyledonary tissue enclosing the embryo was treated as the explant material. This tissue exhibited no browning and was easy to handle in culture. Growth of the embryo was observed in all the treatments both on a non-charcoal and charcoal added medium.

However, the best development was observed with a combination of 0.1 mg l⁻¹ NAA or IAA and 0.1 mg l⁻¹ Kinetin, BAP or 2iP on a non charcoal medium and with a combination of 1.0 mg l⁻¹ NAA or IAA and 1.0 mg l⁻¹ Kinetin, BAP or 2iP on a charcoal added medium.

(c) *Lansium domesticum*: The seeds of this species are polyembryonic. The seed could be fragmented into 2 or 3 pieces, each piece carrying an embryo embedded between the cotyledons. If the cotyledons were split open, the embryo embedded between them was torn into two. To avoid damage to the embryo, small blocks measuring 2 x 4 x 4 mm of the cotyledonary tissue enclosing the embryo was taken as the culture material. The excised tissues showed

no browning and grew satisfactorily to form plants. Best development was obtained in either a non-charcoal medium containing 0.1 mg l^{-1} NAA or IAA in combination with 0.1 mg l^{-1} Kinetin, BAP or 2iP or on a charcoal added medium containing 1.0 mg l^{-1} NAA or IAA in combination with 1.0 mg l^{-1} Kinetin, BAP or 2iP.

(d) *Durio zibethinus*: The embryos in this species were large measuring on the average $4 \times 4 \times 12 \text{ mm}$. The plumular end of the embryo is attached at the centre of the seed and when excised, exuded a mucous type secretion which was highly oxidative. Slight injury to the surface of the embryo caused strong browning reactions of the injured parts. Direct culture of these embryos on culture medium resulted in excessive secretion of phenols into the medium that slowed the growth and development of the embryo. However, when the embryos were soaked in antioxidant solution for 30–45 minutes, both the browning reaction and the secretion of phenols into the medium was greatly reduced and development was enhanced. Best development was observed in a charcoal medium containing 1.0 mg l^{-1} NAA or IAA in combination with 1.0 mg l^{-1} Kinetin, BAP or 2iP. The charcoal in the medium appeared to enhance normal and early development, compared to the non-charcoal medium in which growth was slow and plantlet development appeared weak and abnormal.

(e) *Mangifera indica* and *Bouea ganadaria*: These two species have a fibrous mesocarp. The embryos excised from both species exhibited intense browning and phenols were secreted into the medium within hours of culture. Soaking the embryos in sterile antioxidant solution for about 30–45 minutes reduced the oxidative process of the polyphenols. Best growth and development was observed on a charcoal medium containing 1.0 mg l^{-1} NAA or IAA in combination with 1.0 mg l^{-1} Kinetin, BAP or 2iP. On a non-charcoal medium, inhibition of either the shoot or root system was commonly observed.

(f) *Persea americana*: In this species, the embryo is located in the centre of the seed attached to both the cotyledons. Attempts to split the cotyledons to expose the embryo resulted in tearing the embryo into two. Hence, a small block of cotyledonary tissue measuring $4 \times 4 \times 4 \text{ mm}$ and enclosing the embryo was excised and

treated as the culture explant. During excision, browning of the cotyledonary tissue was intense. Soaking the excised blocks in sterile antioxidant solution for 25–30 minutes prior to culture was found to be beneficial in reducing the oxidative effects of the polyphenols. For this species, charcoal medium containing 1.0 mg l^{-1} NAA or IAA in combination with 1.0 mg l^{-1} Kinetin, BAP or 2iP was found to be most promotive for normal development of the embryos. On a non-charcoal medium, inhibition of normal development was clearly evident.

(g) *Theobroma cacao*: Excision of the embryonic axis in this species was easily effected, however browning of the embryo after excision occurred very quickly. Treatment of the embryos in an antioxidant solution for 10–15 minutes prior to culture appeared beneficial but not essential. On a non-charcoal medium, a hormone combination of 0.1 mg l^{-1} NAA or IAA and 0.1 mg l^{-1} Kinetin, BAP or 2iP was optimum for normal development. On charcoal medium, a hormone combination of 1.0 mg l^{-1} NAA or IAA and 1.0 mg l^{-1} Kinetin, BAP or 2iP was best for normal shoot and root development.

(h) *Hevea brasiliensis*: Embryonic axes of this species was easily excised and grown on medium. They exhibited no browning and were amenable in culture. Normal growth and development was much slower on a non-charcoal medium compared to a charcoal medium. On a charcoal medium containing 1.0 mg l^{-1} NAA or IAA in combination with 1.0 mg l^{-1} Kinetin, BAP or 2iP, a well developed shoot and root system was obtained after 6 weeks in culture.

(i) *Dryobalanops aromatica*: This forest species set seeds only once in two or three years. The embryonic axes were easily excised from the seeds and emitted a sweet smell of camphor. No browning of the embryonic axes occurred during excision. However, after 2–3 days in culture, the embryo developed necrotic spots either at the plumular or radical region and failed to develop on any of the medium tested. When the excised embryos were dipped in an antioxidant solution for 10–15 minutes prior to culture, those embryos on the charcoal medium started to develop while none of the embryos on the non-charcoal medium developed normally. For best development of these embryos in a charcoal

medium, a hormone combination of 1.0 mg l^{-1} NAA or IAA and 1.0 mg l^{-1} Kinetin, BAP or 2iP was found to be suitable.

DISCUSSION

A vital prerequisite to *in vitro* storage of embryonic axes for long term conservation is the elucidation of a reliable method for normal development of embryonic axes into seedlings under *in vitro* conditions when required. In the past, embryo culture techniques were developed principally for use in plant breeding, horticulture and for basic studies on embryo physiology and biochemistry (Collin and Grosser, 1984; Dunwell, 1986). Moreover, most of the studies on embryo culture have been confined to temperate crops and very little work has been carried out for tropical species.

With the advent of the concept that excised embryos have the potential to be used as genetic material for difficult-to-store (recalcitrant) seeds, there now arises an urgent need to establish suitable *in vitro* practices for the regeneration of the embryos of recalcitrant species into whole plants. This study therefore aimed to fulfill this need and suitable media and cultural conditions for 11 recalcitrant species have been elucidated.

From the study, it was found that Murashige & Skoog's (1962) basal medium modified slightly with the addition of sodium dihydrogen phosphate was found to be suitable for all the species screened. Monnier (1978) states that embryos that have their own endogenous hormones can be considered as plant initials. However, in several cases embryo culture has been facilitated by exogenously supplied hormones. In our study too we have found that in media without hormones, the embryos began to grow but development was either very slow or abnormal. However, growth and normal development was enhanced in medium containing low levels of growth hormones. There appeared to be no significant differences between the types of hormone combination used. Either NAA in combination with Kinetin, BAP or 2iP or IAA in combination with Kinetin, BAP or 2iP was found to promote normal development. However, the concentration of the hormones used was critical. On media without charcoal, 0.1 mg l^{-1} of both auxin and cytokinin was found to be optimum while a charcoal media

containing 1.0 mg l^{-1} was found to be ideal for normal development.

The use of activated charcoal at 2.0 g l^{-1} was found to be promotive for the embryo development for all the species screened. Fridborg and Erickson (1975) suggested that the presence of activated-charcoal in the medium tends to remove those substances produced in the medium that promote unorganized growth, inhibit embryogenesis, root formation and elongation. In our study, we have observed a similar effect. Embryos developing on a charcoal-medium showed well-organized and robust growth for all the species. In the case of *Durio zibethinus* and *Hevea brasiliensis* growth and development was better in a charcoal medium as compared to a non-charcoal medium. Moreover, for *Dryobalanos aromatica*, only the charcoal medium appeared to enhance normal development. This suggest that some of the species tend to release some toxic substances into the medium owing to injury during excision and these substances, if not removed, tend to be inhibitive to development of the embryos. Figures 1 (a)–(i) show some of the developing embryos in culture.

Having established a system for normal development of plants from the embryos of these species, we have now moved on to working out a protocol for the long term storage of these species by cryopreservation of aseptic embryos in liquid nitrogen, (-196°C). Withers (1987) made a cautioned statement that after cryopreservation, tissues tend to undergo a considerable amount of damage and hence the composition of the initially-established medium for non-cryopreserved embryos may not be sufficient as a recovery medium and may have to be further enriched with other growth promoting substances. It is therefore anticipated that the above established media for the different species will probably need to be modified in the course of the study to cater for the need of the recovering embryos after cryopreservation.

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Fig. 1a: Jackfruit

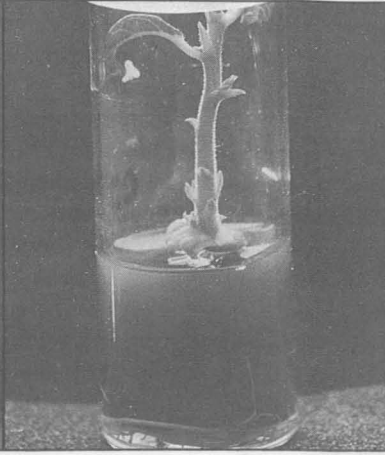


Fig. 1b: Cempedak

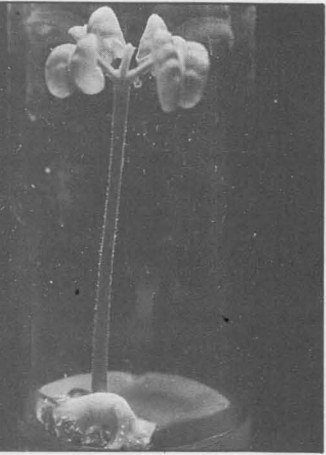


Fig. 1c: Rambutan

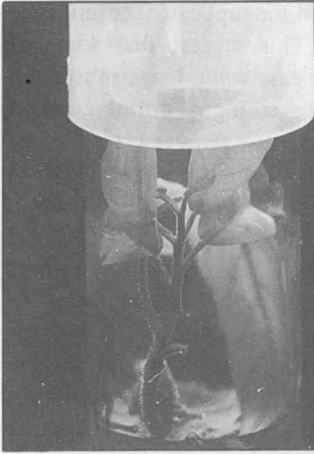


Fig. 1d: Langsat



Fig. 1e: Mango



Fig. 1f: Kundang

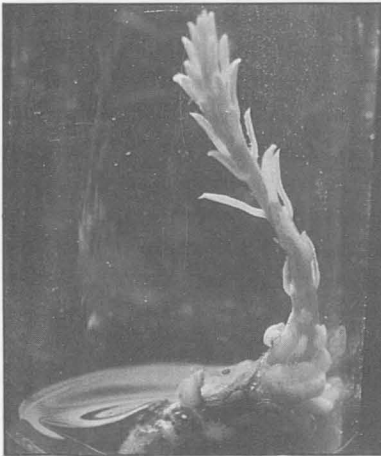


Fig. 1g: Avocado

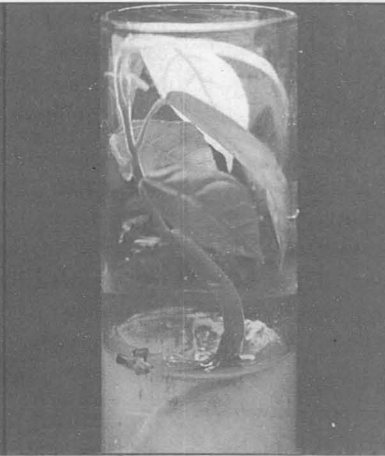


Fig. 1h: Cocoa

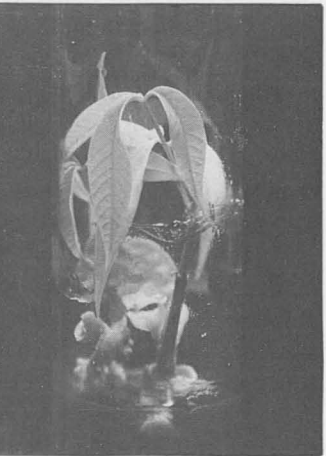


Fig. 1i: Rubber

FIGURE 1: Embryo culture of some of the recalcitrant species

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