

Embryo and Tissue Culture for Crop Improvement, Especially of Perennials, Germplasm Conservation and Exchange – Relevance to Developing Countries*

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ABSTRACT

Plant tissue and organ culture techniques are increasingly applied for the improvement of economically important crops in the developing countries. Work done at this Institute on coconut spathe tissue and embryo culture, as well as turmeric tissue culture for rapid multiplication of superior clones, is briefly described. The application of tissue and organ culture in other crops, especially the perennials, like agricultural plantation crops, forest trees and horticultural plants is briefly reviewed with regard to (a) the conservation and international exchange of germplasm, especially of bulky seeded materials like coconut and vegetatively propagated ones like tapioca, potato and certain fruit trees; (b) clonal multiplication of elite materials; (c) induction of haploids; (d) induction of mutations and rare recombinations and (e) embryo culture. The rapid advances made in tissue, cell and organ culture hold great promise for their immediate application to problems in the developing countries where bulk of the farm holdings for most crops fall below one hectare. Hence, any advance towards improving the yield or disease resistance of the planting materials, made through the use of tissue culture techniques is sure to benefit the farmers.

INTRODUCTION

It is customary to begin every discussion on plant tissue and organ culture with Haberlandt (1902) who was the first to conceive of the totipotency concept for plant cells (see Krikorian and Berquam, 1969) and Hannig (1904) who first demonstrated the embryo culture technique. Clonal multiplication has been achieved in several higher plants, where tissue and organ culture has got established as a vital tool of biotechnological research as well as applied horticulture and agriculture. One of the main areas of vital importance where tissue and organ culture can be useful is in the conservation of germplasm, particularly of the endangered species, the native flora, and wild relatives of crop plants, which are facing elimination owing to expanding urbanisation and the rapid advent of improved crop varieties and hybrids. In the monotypic coconut (*Cocos nucifera* L.), massive replanting programmes are threatening to destroy native genetic resources in Philippines, Indonesia, India and Malaysia, and responsible scientists like Carlos (1979) have cautioned the Governments and over-enthusiastic scientists, to go slow on replanting large areas with the same source of hybrids which certain business-minded agencies are trying to push into every country. Embryo culture technique for transport of such bulky materials like coconut would also greatly aid in germplasm collection and conservation in a limited space, free from quarantine problems. Screening for disease and pest reaction using tissue and embryo cultures would also become feasible with advances in technology and with more knowledge on the toxins released by the pathogen/pest. This would facilitate evaluation of germplasm in early stages without having to raise a plantation which would require a lot of space, time and manpower.

The other application of embryo culture for overcoming the barrier of incompatible crosses in crop plants is quite well recognised, and has been documented in several reviews. Some of the recent work will be dealt with here briefly. Work on coconut and other perennial plantation crops carried out at the Central Plantation Crops Research Institute (CPCRI) and elsewhere, is also described in this

paper, which is of direct relevance to the economic uplift of the developing countries.

MATERIALS AND METHODS

Coconut: At the CPCRI, we have the largest collection of world germplasm in coconut consisting of 62 exotics and 32 indigenous cultivars. The most common local Tall variety, namely, West Coast Tall has been used for the tissue culture studies. Meristematic tissues excised from one year old seedlings as well as adult palms were used for standardizing the techniques of callus induction and morphogenesis. For embryo culture, both mature, 12-month old nuts, as well as immature (7–10 months) ones were selected. The fibrous husk at the 'eye' region was carefully chopped off and the embryo along with surrounding endosperm tissue was scooped out of the soft eye with the help of a double-edged sharp knife. This was then surface-sterilized in 20% chlorine water for 10 minutes and after thorough rinsing with water placed in a mixture of ascorbic acid and citric acid (150 ppm each) to prevent oxidation and release of polyphenols before transfer to the culture medium.

From one year old seedlings, the apical bud region was carefully dissected out and surface-sterilized as described. So also, the immature spathe excised from the first or second open leaf axils of an adult palm, was surface-sterilized and the rachilla explants were cultured. In an earlier experiment, it was found that older spathes were unsuitable, as they gave rise to only normal flower morphogenesis. The stage of the spathe (preferably from first open leaf axil after the central spindle) was critical for effecting the conversion of floral primordia into vegetative shoot-primordia. Two expert climbers were trained to excise the first spathe, without damaging the single shoot bud of an adult palm. After giving a swab with 70% ethyl alcohol the spathes were dissected inside a Laminar-flow chamber. The rachilla explants measuring 1–1.5 cm were excised and cultured either on MS (Murashige & Skoog, 1962) basal medium supplemented with Nitsch's (1969) vitamins and 4–8% sucrose, or on Eeuwens's (1976, 1978) Y-3 medium containing 8% sucrose. Initially the correct stage of spathe was determined by

* This paper is humbly dedicated to the memory of the late Dr Emerita V. de Guzman of the Philippines, for her pioneering contributions to coconut embryo and tissue culture.

planting the entire range of developing spathes taken from trees that were either felled for other reasons or had fallen due to wind. As a result of the screening studies, it was found that the spathe subtended in the axil of the first open leaf after the central spindle, was the ideal stage for effecting the conversion of floral primordia into vegetative shoot-like out-growths.

Root tissues both from primary as well as secondary laterals, excised from adult palms, as well as young seedlings, and from embryo cultures, were grown both on agar as well as liquid media after surface-sterilization with chlorine water. Leaf bases as well as tender leaf primordia around the apical meristem of seedlings were also cultured in a similar way. All cultures were incubated initially in darkness at $28 \pm 1^\circ\text{C}$ in a BOD incubator at 70% relative humidity, for 4–6 weeks. The addition of activated carbon (5–10 g/l) was beneficial in arresting browning of coconut explants on agar media. Among hormones, NAA (0.5–1 mg/l) or 2,4-D (0.2–2 mg/l) was used individually in conjunction with a cytokinin like Kinetin (0.1 mg/l), 6-Benzylaminopurine (BAP) 2–10 mg/l, and 6-(γ - γ -dimethyl allylamino) purine riboside (PR 2–10 mg/l) particularly for floral tissues.

For anther cultures spathes on the 6th to 7th leaf-axil had suitable stage of uninucleate microspores. The spathes were opened after wiping with ethanol and the anthers were surface-sterilized with mild chlorine water and inoculated both on agar and liquid media of Y-3, B-5 (Gamborg *et al.*, 1968) and N-6 (Chu *et al.*, 1975) compositions, and the cultures were incubated in dark. Anthers were periodically examined in acetocarmine squashes for pollen development into embryoids. Responding anthers were transferred to fresh media.

Turmeric: Sprouting buds from storage rhizomes of a high-yielding, high curcumin clone 15B, obtained from the All India coordinated Spices and Cashewnut Improvement Project, were used for culture. After surface-sterilisation in chlorine water, individual buds measuring 1 cm were cultured on MS agar medium containing 4% sucrose, Kinetin (0.2 mg/l), BAP (0.4 mg/l) and GA_3 (0.01 mg/l) and incubated at $28^\circ\text{C} \pm 1^\circ\text{C}$ under fluorescent light (alternating 12 hrs light and dark period) of 3000 lux (4300 K). After the production of multiple shoots, individual shoots along with some roots were separated carefully and transferred to fresh media with or without auxin and GA_3 . This operation was repeated several times in order to obtain a high rate of multiplication. Multiple shoot production and rate of growth of shoots and roots was considerably enhanced in liquid cultures kept on a platform flask shaker. Rooted plants were then transferred to sterilized soil in poly-bags and kept in a glasshouse for further growth. When the plants had grown fully in about eight months, and leaves started drying, the rhizomes were harvested and weighed separately for each individual plant.

RESULTS

Coconut: Rachilla explants taken from spathe primordia at the 1st leaf axil (Fig. 2) showed the maximum response in the conversion of floral primordia into vegetative shoot-like outgrowths (Fig. 3). As the explants became older, the frequency of such conversions decreased and after the 3rd leaf axil, the tissues of rachillae gave rise to normal or abnormal floral primordia, which showed either (a) the

male flower with a trilobed pistillode that developed to a flattened column or (b) a trifid structure or (c) an indeterminate columnar structure full of scale leaves, which could be subcultured individually (Fig. 4). So far, only one of them has rooted but no true leaves are discernible yet. However, nearly 50% of the floral primordia developed into shoot-like structures on Y3 medium of Eeuwens (1978) supplemented with 6-BAP (2 mg/l) and 6-(γ , γ -dimethyl-allylamino) purine riboside (2 mg/l) with 80 g/l sucrose.

Embryos from mature coconuts grew well on MS medium without any growth adjuncts. Addition of activated carbon was essential to prevent rapid browning of tissues which otherwise arrested further growth. The haustorium (= cotyledon) which in nature enlarges into a spongy, sweet, tissue drawing nourishment from the endosperm, for the growing embryo, degenerated in culture and turned dark brown (Fig. 5). When the seedling (Fig. 6) was transplanted to soil, the haustorium portion was infected, resulting in high mortality of the plants. Root growth *in vitro* was stimulated by decapitating the first large root that emerged out of the sprouted embryo with development of many laterals and sublaterals (Fig. 6). Liquid cultures, with filter paper platforms for supporting the embryos, showed better rooting response, with less problems of tissue browning.

Anthers cultured at uninucleate microspore stage, after cold pretreatment at 4°C for 48 hrs, on Y3 medium containing 6 per cent sucrose, 10 mg/l 2,4-D, 1 mg/l Kinetin and 1% activated carbon, in dark at $28^\circ\text{C} \pm 1^\circ\text{C}$, showed multicelled pro-embryoids within the exine (Fig. 7). Responding anthers have been subcultured on fresh media.

Meristematic tissues excised from one year old seedlings grew rapidly especially on liquid media placed in darkness, in petridishes in a thin layer and from the cut ends a slow growing, friable callus was observed. Leaf primordia also grew and expanded rapidly, turning green on exposure to light.

Root tip segments excised from sprouted embryos, and from seedlings or germinated nuts, cultured on MS medium supplemented with 40 g/l sucrose, 0.5 mg/l NAA, 0.2 mg/l 2,4-D, and 5–10 g/l activated charcoal, grew rapidly producing fresh growth in 4–6 weeks. However, without root tip, the segments showed no noticeable growth. The laterals and sublaterals showed elongation, and those on the surface of medium produced typical pneumatophores, containing loose, granular and spongy tissue, growing upwards, apogeotropically (Fig. 8). Roots excised from embryo cultures grew in better way and several fresh roots arose from the base of the embryonal tissue which had callused on 2,4-D medium (Fig. 9). On the other hand, roots excised from adult palms have not responded so far in culture, due to rapid browning.

Turmeric: On a simple MS medium devoid of growth regulators, the cultured buds sprouted only single plantlets. However, when supplemented with Kinetin (0.2 mg/l), BAP (0.4 mg/l) and GA_3 (0.01 mg/l), the buds gave rise to a large number of adventitious multiple shoots (Fig. 10), with or without a preceding callus phase (Fig. 11). When the shoots attained sufficient height and rooting, individual shoots were separated and cultured on a medium with or without IAA and GA_3 . At the end of 60 days a large number of rooted plantlets were produced from a single initial bud,

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