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## SOMATIC EMBRYOGENESIS IN THREE RATTAN SPECIES OF MAJOR ECONOMIC VALUE



**Figure 1a.** *Calamus merrillii* growing naturally in Leyte (Eastern Visayas, Philippines).

**Figure 1b.** Somatic embryogenesis-derived *Calamus merrillii* produced by the PBL.

This paper reports the main achievements of the Plant Biotechnology Laboratory on somatic embryogenesis in three rattan species of major economic value, i.e. *Calamus manan*, *Calamus merrillii* and *Calamus subinermis*.

Rattans are climbing spiny palms (figure 1) belonging to the large subfamily *Calamoideae*. World wide, rattans are represented by some 600 species in 13 genera (DRANSFIELD, MANOKARAN, 1993). *Calamus* is the largest genus encompassing 370-400 species distributed from tropical Africa, the Indian subcontinent, southern China and east through the Malaysian region to Fiji, Vanuatu and eastern Australia.

As a non-wood forest product, rattan canes are an attractive source of raw material for numerous and varied uses. This is particularly true for *Calamus* species which are particularly prized for furniture-making due to their aesthetic features, strength, length (up to 100 m for *Calamus manan*), flexibility and uniformity. In Southeast Asia, the huge market demand for raw material has accounted for their overexploitation during the past decades. This context prompted Innoprise Corporation Sdn Bhd in Sabah, East Malaysia, to implement a comprehensive rattan plantation program including a plant improvement component with the involvement of CIRAD-Forêt (NASI, MONTEUUIS, 1992). The Plant Biotechnology Laboratory (PBL), set up within the same collaborative program (BON, MONTEUUIS, 1996), provided access to biodiversity investigations using molecular markers to enable wiser management of the genetic resources of economically valuable rattan species (GOH *et al.*, 1997). In addition, PBL's main objective was to develop suitable propagation protocols for mass producing genetically superior rattan planting stock. Priority was given to three large-caned species that have the highest market demand and, as such, are exposed to serious risks of depletion. These were:

(i) *Calamus manan*, single-stemmed species, found naturally in Sumatra, Southern Kalimantan, Peninsular Malaysia and southern Thailand;

(ii) *Calamus subinermis*, mostly multi-stemmed, but also observed to be solitary, and endemic to Sabah (Malaysia), Palawan (the Philippines) and part of Sulawesi (Indonesia);

(iii) *Calamus merrillii* Becc., multi-stemmed species, endemic to the Philippines.

The rationale of opting for *in vitro* propagation techniques, and more specifically somatic embryogenesis, to meet this goal was warranted for different reasons.

Conventionally, rattans are propagated by seeds that are abundantly available during each fruiting season. However, more and more, immature rattans from the wild are extracted before fruiting, which drastically affects the production of seeds whose viability decreases in a short span of time. The harvesting time and post-harvest treatments or conditions, especially temperature and humidity, can also dramatically affect the germination process.

Vegetative propagation can be another option, notwithstanding its interest for germplasm conservation, or clone banks, as well as for plant improvement programs with a view to establishing plantations of higher quality and yield (GOH *et al.*, 1997). However, nursery vegetative propagation methods based on the use of offsets are only applicable to multiple-stemmed species like *C. merrillii* and to a certain extent *C. subinermis*, but their success has been quite limited.

Tissue culture is an alternative vegetative propagation option, especially for single-stemmed species such as *C. manan* (GOH *et al.*, 1997). Consistently with other reports (UMALI-GARCIA, 1985; AZIAH, 1989; UMALI-GARCIA, CANLAS-MENDOZA, 1996), we demonstrated the possibility of micropropagating *C. manan*, *C. merrillii* and *C. subinermis* through multiple shoot production, either from *in vitro* germination or from outdoor seedlings. However, the multiplication rates were shown to vary considerably from one explant to another within the same species (GOH *et al.*, 1997). This resulted in low overall efficiency, which is not compatible with large-scale production in economically viable conditions. Moreover, this technology is not applicable to mature selected individuals from single-stemmed rattan species as the only shoot apical meristem is used as primary explant with highly questionable chances of success. This may lead to the death of the selected genotype. In contrast, *in vitro* somatic embryogenesis can be initiated from other tissues than shoot meristems for mass cloning genotypes selected for outstanding traits (CHELIAK, ROGERS, 1990; RIVAL, in press). Somatic embryogenesis potentially offers a number of other prospects like cryoconservation, genetic engineering, etc. (CHELIAK,

ROGERS, 1990; THORPE, 1995; VERDEIL *et al.*, 1994; RIVAL, in press).

These aspects therefore prompted us to apply somatic embryogenesis to the three priority rattan species mentioned above.

## MATERIAL AND METHODS

### INITIAL EXPLANTS

Very young leaves and root tips from 3-4 month-old *in vitro* germinated seedlings as well as zygotic embryos were preferably used as primary explants at this stage, with the ultimate aim of adapting the protocol to mature selected field plants. Zygotic embryos, whose availability depends upon seasonal fresh fruit production, were excised from mature seeds after scrubbing the fleshy sarcotesta off the fruit with a stiff brush. The seeds were then disinfected with 10% commercial grade sodium hypochlorite for 10 min, followed by three washes in sterile water. Embryo-issued primary cultures were easier to establish for *C. manan* (95% of success on average) than for *C. subinermis* characterized by tiny embryos.

### CULTURE CONDITIONS

For callus induction, root tip fragments of about 1-2 cm in length, zygotic embryos or leaf explants (1 cm square) were inoculated onto a basal Murashige and Skoog derived culture medium (GOH *et al.*, 1999) supplemented with 2.5, 5 or 7.5 mg.l<sup>-1</sup> (10.4, 20.7, 31.1 µM respectively) picloram. After pH adjustment to 5.6-5.8 with 1N KOH and the addition of 7 g l<sup>-1</sup> "high gel strength" Sigma agar, 12.5 ml of the medium were dispensed into 21 × 150 mm glass test tubes prior to sterilization by autoclaving at 120°C and 95 kPa for 20 min. This constituted the primary culture medium for the induction of embryogenic cells. The maturation medium used for further development of embryogenic cells differed from the previous callus induction medium by lower picloram concentrations, i.e. 1, 2.5 or 5 mg.l<sup>-1</sup> (4.1, 10.4 or 20.7 µM, respectively). All cultures in glass tubes were maintained in total darkness at 26 ± 2°C and 90-95% relative humidity (RH) for the induction process, and under a 16 h photoperiod (50-60 µmol m<sup>-2</sup>s<sup>-1</sup>, "TLD 36W/84 Philips" fluorescent lamps) at 28 ± 2°C, and RH 70% for the germination of the somatic embryos.

### HISTOLOGICAL OBSERVATIONS

Histological examinations of the various structures obtained in the course of the *in vitro* somatic embryogene-

sis process were undertaken using the procedure described in GOH *et al.* (1999).

## MAIN RESULTS

### MORPHOLOGICAL ASPECTS

From a morphological standpoint, callus induction was observed to vary according to the types of explant and species studied (table I).

In *C. manan* and *C. subinermis*, root explants appeared to be the most suitable for inducing calli with potential for somatic embryogenesis, in contrast to zygotic embryos or leaves that rapidly became oxidized even when placed in darkness. The zygotic embryo-derived calli failed to differentiate upon transfer to the maturation medium, becoming fuzzy before vitrification or oxidation occurred. The primary calli produced by 78% and 68% of root explants 3-6 and 6-8 months after inoculation for *C. manan* and *C. subinermis* respectively occurred on apices of primary or secondary roots or along the cut surface of the root as protuberances of the central cylindrical zone (figure 2a). Among the three rat-tan species, *C. merrillii* demonstrated the highest potential for regeneration via somatic embryogenesis (table I). In contrast with the two other species, the three types of *C. merrillii* explants started to swell before they underwent callus formation within only 6 weeks after inoculation. Although callogenesis was induced in 74% of the introduced root explants, no embryogenic structures evolved from the calli during the course of our study. The calli eventually became translucent or brownish due to phenolic oxidation after subsequent transfers. Conversely, zygotic embryos and young leaves (figure 2b) from *in vitro* seedlings used as explants were equally

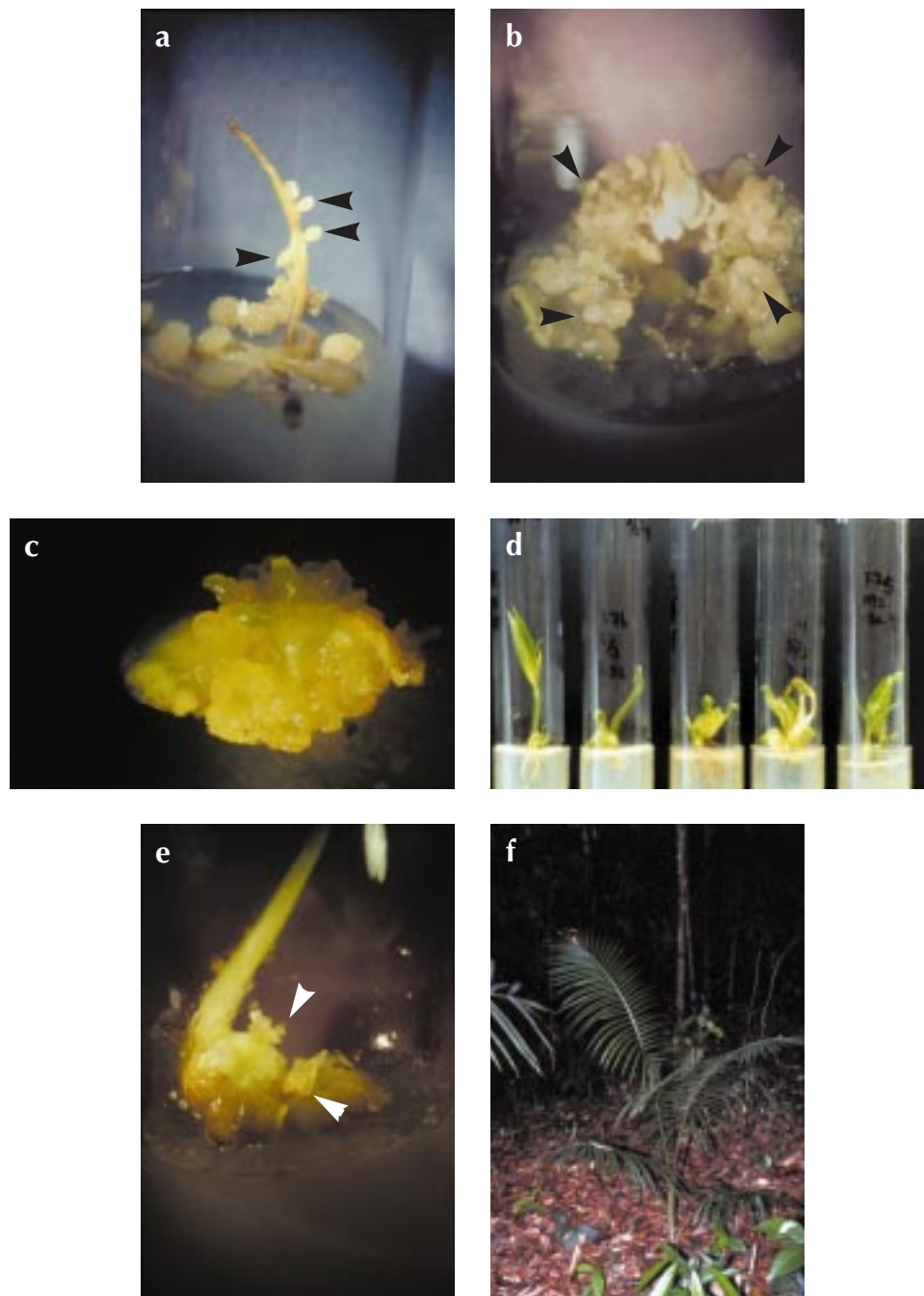
and highly responsive at 90% and 84%, respectively, of the samples introduced.

In the early phase of the process, the promising calli appeared white and friable for *C. manan* and *C. merrillii*, but underwent a soft watery phase in *C. subinermis*. Regardless of the species, when transferred onto fresh media or media with lower picloram levels, these calli proliferated, evolving towards a mixture of creamy and yellowish nodular calli which were considered as potentially embryogenic.

The subsequent development of calli into somatic embryos was similar in all three species and as such will be described without specific reference to any species unless otherwise noted. When transferred onto media containing lower picloram levels (1 to 5 mg. l<sup>-1</sup>), the nodular calli became glossy, eventually giving rise to globular, rounded or elongated structures that could co-exist within the same callogenic clump (figure 2c). Discrete structures with a dome-shaped appearance, usually glossy and translucent, were observed to be the most prone to differentiate into somatic embryos. Over time, the pointed apex of such structures developed into a greenish shoot, becoming leaf-like soon thereafter. Development of the first leaf from the shoot apex together with elongation of the radicle then ensued (figure 2d). This marked the completion of the germination process, occurring only at a low percentage in *C. manan* and even worse for *C. subinermis*. In this latter species, most of the prospective emblings appeared to be stunted with no elongation of the shoot-like structures despite exposure to various treatments (GOH *et al.*, in press). In contrast, *C. merrillii* embryogenic calli developed more quickly and in greater proportions into germinating somatic embryos exhibiting bipolar development with the appearance of the plumule and radicle. Overall, the

**TABLE I**  
COMPARATIVE PROPORTIONS OF CALLI OBTAINED FOR DIFFERENT TYPES OF PRIMARY EXPLANTS EXPOSED TO THE SAME EXPERIMENTAL CONDITIONS FOR EACH OF THE THREE *CALAMUS* SP. WITHIN EACH SPECIES, LETTERS DISTINGUISH PROPORTIONS WHICH ARE SIGNIFICANTLY DIFFERENT AT P = 0.05 ( $\chi^2$ -PEARSON'S TEST)

| Species              | Type of primary explant |                |               |
|----------------------|-------------------------|----------------|---------------|
|                      | Zygotic embryo          | Leaf portion   | Root tip      |
| <i>C. manan</i>      | 103/200 = 51.5% b       | 1/50 = 2% a    | 39/50 = 78% c |
| <i>C. merrillii</i>  | 180/200 = 90% b         | 42/50 = 84% ab | 37/50 = 74% a |
| <i>C. subinermis</i> | 96/200 = 48% c          | 0/50 = 0% a    | 34/50 = 68% b |



**Figures 2. Somatic embryogenesis in rattans: morphological aspects.**

**a)** Primary callus formation (arrows) from root tips of *C. manan*; **b)** Embryogenic callus produced by a leaf explant from *C. merrillii* and showing embryogenic structures at different stages of development (arrows); **c)** Different types of glossy and translucent (pro)embryo-like structures emerging from the same callogenic clump; **d)** Emblings developing *in vitro*; **e)** Germinating embling with secondary embryos (arrows); **f)** *C. merrillii* somatic embryogenesis-derived plantlet growing outdoors.

whole process from callogenesis to the formation of the first leaf and elongation of the primary radicle remained quite slow, especially for *C. manan* and *C. subinermis* which required more than 12 months (GOH *et al.*, 1999). Regardless of the species, germination included significant proportions of abnormal developmental patterns such as multiple shoot apices and profuse rooting. In a few cases, it was interesting to note at the surface of primary somatic embryos of *C. manan* and *C. merrillii* more specifically, the presence of secondary or supernumerary embryos (figure 2e).

Due to differences in the quantity of *in vitro* emblings available for the three species, conversion ability was more particularly tested on *C. merrillii* and *C. manan* plant material, for which successfully acclimatized somatic embryogenesis-derived plantlets were ultimately established in field trials (figure 2f).

### HISTOLOGICAL ASPECTS

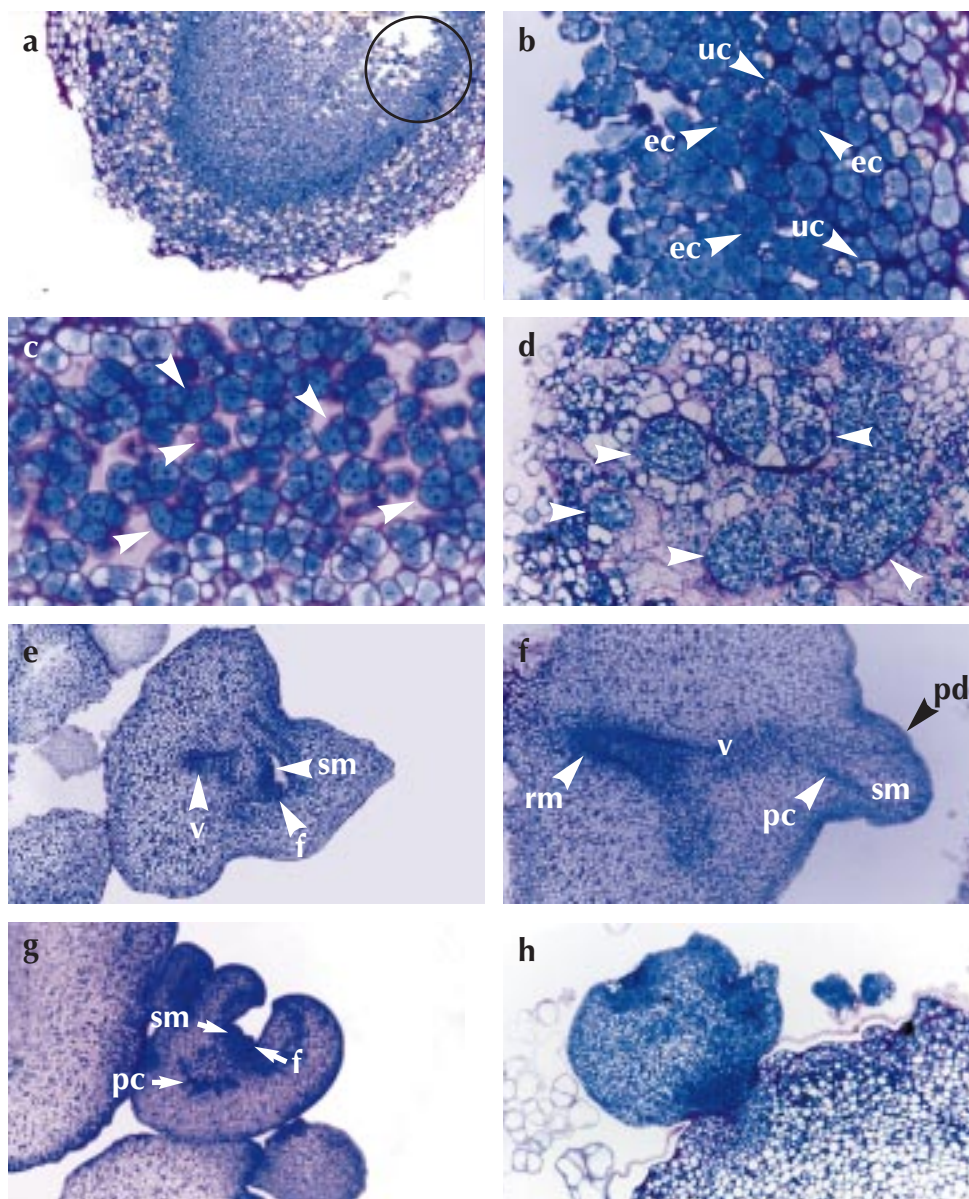
Histological observations showed that primary calli proliferated mainly from the perivascular zone of root explants for *C. manan* and *C. subinermis* (figure 3a), and of leaf and zygotic embryos for *C. merrillii* (GOH *et al.*, 1999; in press). Friable and yellowish calli originated from a mixture of undifferentiated cells—with a small nucleolus and highly vacuolated—and actively dividing embryogenic cells characterized by enlarged nucleolus, high nucleoplasmic ratio, dense vacuolated cytoplasm and starch reserves in the form of granules (figure 3b). These embryogenic cells gradually became isolated from each other and often appeared to be surrounded by a polysaccharide mucilage, stained pink by periodic acid Schiff (PAS), which originated from modification of the median lamella and the primary cell walls (figure 3c). This, more specifically observed in *C. subinermis* and *C. merrillii*, is generally an indication of healthy cells with prospects for further development. These isolated embryogenic cells actively divided to form clusters (figure 3d). When transferred onto media with lower concentrations of picloram or totally lacking it, these became progressively embryonic and could be easily distinguished by the intense staining of abundant soluble proteins in the cytoplasm and the presence of enlarged nucleoli. Concomitantly, starch granules disappeared. These clusters of embryonic cells delimited into zones that later evolved into proembryos. No protein reserves could be seen using naphtol blue-black (NBB). At this point, the proembryos appeared to develop quite heterogeneously, ranging from a degenerating process to different stages of maturation, independently from the rest of the callus. Maturation corresponded to the formation of a protoderm through the establishment of a peripheral zone consisting of one and then several layers

of cells. Vascular tissues then developed between shoot and root apices formed at each extremity, giving rise to a bipolar embryo (figure 3e, f, g). In *C. manan*, the somatic embryos did not show any starch or protein reserves in contrast to the zygotic embryos. Several somatic embryos displayed structural abnormalities such as several shoot apices or multiple foliar primordia, consistently with the morphological observations. Histological examination of the secondary embryo cases observed in *C. manan* and *C. merrillii* did not reveal any vascular connection with the primary embryos from which they arose (figure 3h).

## DISCUSSION

Although somatic embryogenesis of *C. manan* and *C. merrillii* was already attempted by several researchers during the late 1980's, early 1990's (PARANJOTHY, 1993), this is the first time as far as we are aware that the phenomenon was histologically demonstrated, also including *C. subinermis*. These histological observations revealed that for the three rattan species the proembryos derived unambiguously from segmenting single cells, similar to observations in coconut by VERDEIL *et al.* (1994). This unicellular origin constitutes a striking illustration of the cell totipotency concept, in addition to the prospects for genetic engineering (THORPE, 1995). This differs radically from many palm species, including oil palm, where somatic embryogenesis was reported to be of multi-cellular origin (SCHWENDIMAN *et al.*, 1988). Tissue culture conditions may have a determining influence on the uni- or multi-cellular origin of *in vitro* somatic embryos (MICHAUX-FERRIÈRE *et al.*, 1992), as well as on the occurrence of supernumerary embryos (HACCIUS, 1978). The lack of vascular connection with the tissues of the primary embryo supports the somatic embryo origin of these supernumerary structures, more specifically observed in our study in *C. manan* and in *C. merrillii* (HACCIUS, 1978).

The wide diversity of cell types observed within proembryogenic calli, from degenerating ones to proembryonic clusters, may result from unsuitable culture medium composition. This could also account for the overall low efficiency of the protocols developed so far for producing somatic embryos. This aspect, as well as the influence of the genotype, the type of primary explant and its physiological state of competency with regard to proembryo formation undoubtedly deserves further investigation, similar to the efforts needed for improving the process in other species (TISSERAT, 1987; RIVAL, in press). This is particularly true when starting from mature selected plants whose interest for plant improvement is obvious (GOH *et al.*, 1997). In this respect, the possibility of



**Figures 3. Somatic embryogenesis in rattans: histological aspects.**

**a)** Callus originating from root tip perivascular cells; box magnified in **b)**; **b)** Magnification of box **a)** showing undifferentiated cells (uc)—with a small nucleolus and highly vacuolated—and actively dividing embryogenic cells (ec)—with enlarged nucleolus, high nucleoplasmic ratio, dense vacuolated cytoplasm and starch reserves in the form of granules; **c)** Actively dividing embryogenic cells (arrows) gradually becoming isolated from each other to form clusters, and often surrounded by a polysaccharidic mucilage—stained pink by PAS—which originated from the modification of the median lamella and the primary cell walls; **d)** Pro-embryos at different stages of development (arrows); **e)** Longitudinal section of a developing somatic embryo of *C. subinermis* showing the shoot apical meristem (sm), a foliar primordium (f) and the vascular axis (v); **f)** Longitudinal section of a somatic embryo of *C. subinermis* exhibiting its bipolarity with the shoot (sm) and root (rm) apical meristems, the protoderm (pd), the procambium (pc) and the vascular tissues (v); **g)** Longitudinal section of a *C. merrillii* somatic embryo showing the shoot apical meristem (sm), a foliar initium (f) and the procambium (pc); **h)** Longitudinal section of a secondary embryo which did not show any vascular connection with the *C. manan* primary embryo from which it emerges.

using inflorescences as primary explants, successfully applied in date palm and coconut (VERDEIL *et al.*, 1994), seems particularly attractive for rattan species, especially for the single-stemmed species. The other option could be to use root tips, as undertaken on oil palm (JONES, 1983), but all attempts tested so far, on *C. manan* more specifically, have failed, mainly due to unsuitable disinfection protocols. In addition, root tips are fewer and less accessible in rattans than in oil palm.

Another concern is whether the regeneration system is sustainable, i.e. can the callus be indefinitely sub-cultured and retain its regeneration potential. Further assessment of the culture conditions would be necessary and useful to determine this. True-to-typeness, in other words whether the plants originating from the callus are identical to the originally selected mother plant, i.e. are not affected by any somaclonal variation, also remains a crucial issue considering the problems encountered with oil palm (PARANJOTHY, 1993; RIVAL, in press). Ultimately, an evaluation of the performance of somatic embryogenesis-derived plants in the field will be important to detect any abnormality. This is currently underway.

Cost and time can still be serious impediments to the utilization of this technology for mass-producing the planting stock needed for large-scale rattan plantations. The returns may simply not warrant such an investment compared to plantations from low cost nursery-produced seedlings. An interesting option could be to apply somatic embryogenesis to a limited number of selected genotypes to be utilized as seed producers within vegetative orchards. The cost of the somatic embryogenesis-derived individuals would be diluted through the

large number of seeds produced every year. These may be biclonal orchards, taking advantage of the dioecy of the rattan species. However, more information is still needed, especially in the field of genetics, to justify this option and experimentally determine what could be practically expected from such clonal orchards.

Another promising option is to use somatic embryogenesis for cryoconservation, as successfully developed for oil palms (ENGELMANN *et al.*, 1985) for the storage of endangered rattan genotypes and even species. This could protect them from total extinction resulting from wild harvesting operations which are increasing dramatically.

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