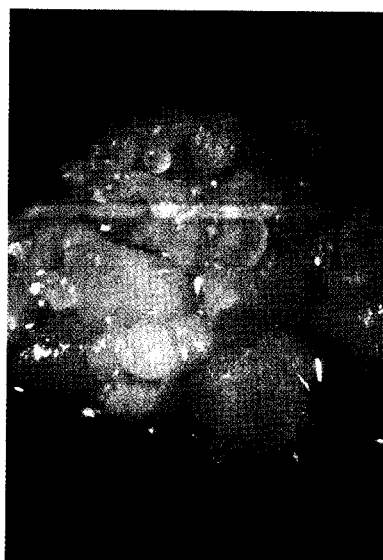


DOREEN K. S. GOH
I.C.S.B.MARIE-CLAUDE BON
CIRAD-ForêtOLIVIER MONTEUUIS
CIRAD-Forêt

PROSPECTS OF BIOTECHNOLOGY FOR A RATTAN IMPROVEMENT PROGRAMME

Innoprise Corporation and CIRAD-Forêt joint project as a case study



↑

Maturation phase of an embryogenic callus obtained from a root tip of *Calamus manan*.

Etape de maturation d'un cal embryogène de Calamus manan obtenu à partir d'une pointe racinaire.



RATTAN IMPROVEMENT

This article reviews the prospects of biotechnology for improving the quality of rattan plantations referring to I.C.S.B. project to which CIRAD-Forêt has been contributing since 1989.

Innoprise Corporation Sdn Bhd, I.C.S.B. for short, is the holding company of Sabah Foundation, the largest timber concessionaire in Sabah (East Malaysia, north of Borneo) with a lease of 1 million hectares (ha). ICSB has long realised the potential of large scale rattan plantations and, in 1987, launched its long-term plan by undertaking to develop 40,000 ha of logged-over forests in the Foundation concession area in Luasong, 100 km west of Tawau, east of Sabah. The rattans produced in the large scale industrial nursery of Luasong have been intensively line-planted during the past few years resulting in up to 10 000 ha composed of various commercial rattan species. The expected rotation for harvesting varies from 7 to 17 years depending on the species planted. These include small cane species like *Calamus caesius* (Rotan sega) and large cane species such as *Calamus manan* (Rotan manau), *Calamus subinermis* (Rotan batu), *Calamus merrillii* (Rotan palasan) and, to a lesser extent due to its less attractive market value, *Calamus ornatus* (Rotan lasun). More information regarding the commercial plantations of rattans developed by ICSB are available from the literature (NASI, MONTEUUIS, 1992).

From 1989 on, CIRAD-Forêt, formerly known as C.T.F.T., has been actively collaborating with ICSB under a formal Memorandum of Understanding which, in terms, includes the Plant Improvement and Seed Production project, P.I.S.P. for short, and the Plant Biotechnology project. Both projects have been initiated to support research and development programmes for rattans. The operations undertaken by the PISP project in that respect have already been presented (NASI, MONTEUUIS, 1992; GARCIA *et al.*, 1994; ALLOYSIUS, BON, 1995). The purpose of this paper is to report, based on our own experience

(BON, MONTEUUIS, 1996; GOH, 1997), what can be realistically expected from the different biotechnologies developed in order to conserve and exploit the natural genetic resources of the most economically important rattan species.

ISOZYMES FOR GENETIC DIVERSITY INVESTIGATIONS

RATIONALE

The scarcity of the wild populations of the most valuable commercial species calls for the development of an *ex situ* conservation program and subsequently, a rattan improvement program, in the context of which understanding the biological dynamics of genetic variation within and between species appears strategic (NAMKOONG *et al.*, 1988).

Two approaches have been used to study the genetic diversity of rattan species of interest for the project: provenances/progenies testing in Luasong Forestry Center and genetic marker screening in the Plant Biotechnology Laboratory.

A genetic marker can be defined as a qualitative, environmentally stable trait whose mode of inheritance is known. The easiest method for obtaining information about genetic diversity of rattan in the context of our laboratory and at the lowest cost was by using isozymes, the advantages of which are:

- Isozymes can be isolated from any organism or any tissue.
- The technical procedures for isozymes are simple and comparatively inexpensive.
- Routine investigation allows determination of genetic structures at many loci.
- Variation is qualitative and usually high within species.

- Variation of many isozymes is controlled exclusively by genetic factors and the relation between phenotypes and genotypes can be established.

Within the framework of the project, the first application of the isozymes was the assessment of the amount and distribution of genetic diversity within and among populations of the target species. In addition, a picture of the population structure for these species will be provided with a priority given to two factors that determine the population structure i.e. gene flow and mating system. In addition, since control pollination programs have already started, isozymes will be used to estimate the purity of the seedlots obtained after controlled crosses.

MAIN ACHIEVEMENTS

For the evaluation of the genetic diversity of *Calamus subinermis* using isozymes, materials have been collected throughout the natural distribution area of the species. In all, 12 natural populations have been sampled throughout Sabah. For *Calamus manan*, on the contrary, 5 out of the 8 origins surveyed are from plantations in Peninsular Malaysia and Sabah.

A proper isozyme procedure suitable for analyses from leaves has been developed in our laboratory (BON, 1996 ; BON, MONTEUUIS, 1996). The results obtained from the study on genetic diversity have already been extensively reported (BON *et al.*, 1995 ; ALLOYSIUS, BON, 1995 ; BON, MONTEUUIS, 1996). They established that both *Calamus manan* and *Calamus subinermis* exhibited a high level of genetic diversity. The diversity of the narrowly distributed *Calamus subinermis* (Sabah only) is of the same order as the diversity of *Calamus manan*, whose distribution is much wider (Sumatra and Peninsular Malaysia). The iso-

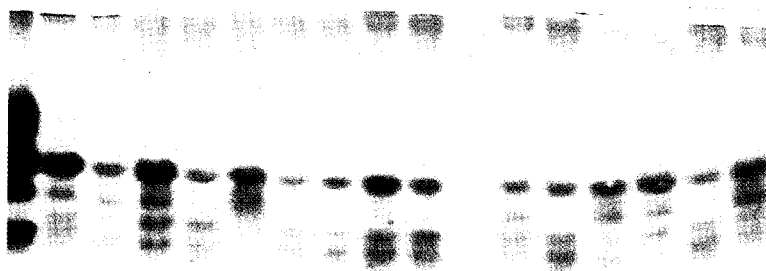


Photo 1.

Esterase zymograms observed for a *Calamus subinermis* progeny from Penampang (West coast of Sabah).

Zymogrammes d'estérasés observés chez une descendance de *Calamus subinermis* d'origine Penampang (côte ouest du Sabah).

zyme study did not reveal any impoverished populations. Hence, the conservation program for genetic resources should take into consideration most of the populations surveyed. Regarding the value of the genetic parameters, an average of 80.4 % and 85 % of the loci investigated were polymorphic for *Calamus subinermis* and *Calamus manan* respectively. For *C. subinermis*, individual population values ranged from 76.5 % to 82.4 %. The average number of alleles per locus was 1.85, ranging from 1.8 to 1.9. Fixation indices with a mean of 0.08 further illustrated conformity to the Hardy-Weinberg equilibrium when sampling was carried out on *in situ* populations.

The degree of genetic differences between two or more populations obviously represents important information for the delineation of provenances. Provenance delineation based on genetic differentiation is

meaningful above all for natural populations. Thus, priority species for comprehensive studies on patterns of genetic differentiation among populations are those whose distribution areas are almost completely surveyed.

Using Nei's genetic distances, the level of genetic differentiation among populations of *Calamus subinermis* was estimated and ranged from 0.005 to 0.188. The relationship of the populations based on cluster applied to genetic distance was portrayed. UPGMA* cluster analysis using Prevosti's genetic distance revealed that the inland areas are the most likely seed sources for the islands. The population from the island of Pulau Banggi located to the north of Sabah is the most genetically distinct from the other populations.

* Unweighted pair-group method with arithmetic averaging (SWOFFORD, SELANDER, 1981).

RATTAN IMPROVEMENT

To sum up, the level of genetic diversity for these species is very high. The isoenzyme study has not revealed any impoverished populations. The conservation program of genetic resources should therefore take into consideration most of the populations surveyed.

POLLEN MANAGEMENT

RATIONALE

Many opportunities for using pollen management technology in plant improvement have been identified (OWENS *et al.*, 1991 ; F.A.O., 1993 ; CHIN, 1994), most of which have applications for *ex situ* conservation and genetic improvement programs, including controlled crosses and hybridization.

Since *Calamus* species are typically recalcitrant, and do not produce orthodox seeds, the most practical and economical methods of conserving these endangered resources *ex situ* are field gene banks and *in vitro* gene banks as a back up. Effective pollen management requires an understanding of pollen development, pollination mechanisms, and methods of pollen storage and, in particular, viability testing (OWENS *et al.*, 1991).

Germplasm preservation strategy using *in vitro* methods will be positively supplemented by the storage of pollen which can be freeze dried, stored in vials, easily shipped and remain viable for more than 10 years (CHIN, 1994). This was evident for special crops, such as the oil palm, for which seed production is carried out by controlled pollination.

The success of collection, drying storage and post-storage application of pollen depends largely on the type of pollen and its initial vigor. A prerequisite for any pollen collection and storage is a sound understand-

ing of the floral biology of the *Calamus* species.

Data on floral biology observations and method of pollen storage will be presented together as they relate to the application of the biotechnologies to a rattan improvement program.

MAIN ACHIEVEMENTS

Although a case of andromonoecy – in other words, the occurrence of separate male and hermaphroditic flowers – has been reported (LEE, personal communication), *Calamus* species have been commonly described as dioecious plants bearing a staminate inflorescence with solitary flower and a pistillate inflorescence with dyads of pistillate and sterile staminate flowers (UHL, DRANSFIELD, 1987).

The longevity of the staminate flower of the male inflorescence after anthesis defined by LEE and JONG (1995) is less than 12 hours, and as anthesis is not synchronous under the environmental conditions of Luasong, only very small quantities of pollen can be easily collected with strong implications for the efficiency of our control pollination program. Pollen yield and quality can be maximized if flower buds are collected as close to anthesis as possible. Moreover, under our conditions and in the course of our study, anther dehiscence and shedding of pollen in the bud occurred before anthesis was recorded. Preliminary investigations showed that the change of color of the staminate petals, from green to yellow, is not necessarily a good indicator of the emergence of pollen maturation.

Abscission of the flower buds before reaching anthesis is a common phenomenon which might affect 3.3 % to 95 % of the flowers with a predominance for those located at the proximal part of the partial inflorescence (64 % on average). Both

the degree of abscission and the rate of flower opening within a rachilla are not correlated with the average degree of pollen maturity and no gradient of pollen maturity has been observed along the rachilla.

Several experiments using flower buds at different stages of development starting from non-opened flower bud to fully opened flower with dehiscent anthers for pollen storage investigations have been carried out. One of the main outcomes is that there is no loss in terms of pollen viability and vigor during the transportation of the non-opened flower buds from the field to the laboratory regardless of the conditioning of the inflorescences. The germination rate was positively enhanced with non-opened flower buds collected just before blooming at 45.8 % vs 33.4 % for fully opened ones ($P < 0.001$ as a result of the Chi-square). Comparatively, the physiological status of the flower had no effect on the length of the pollen tube which averaged 0.38 mm and 0.34 mm for the floral bud and the fully opened flower respectively.

In the course of our study, we observed that above a level of 21 % of germination rate, the maximum length of the pollen tube was 0.48 mm compared to 0.28 mm for a germination rate of lower value, which was most probably the minimum length required for fertilization.

Our results have established that pollen germination rates can reach 39 % when non-opened flower buds are left in silica gel drying conditions for 24 hrs as opposed to 14 % for stamens and 23 % for fully opened flowers. The germination rate was 39 % for both types of flowers before treatment. Similar to the germination rate, the pollen vigour was slightly higher when considering desiccation of non-opened flow-

er buds compared to opened flower buds or stamens.

For both stamens and flowers, the desiccation process has induced a reduction of the moisture content of 51.45 % and 55.95 % respectively. Taking the practical aspect into consideration, the pollen yield collected from desiccated non-opened buds was higher, because the anthers were more prone to dehiscence and the pollen was less sticky.

Once a sufficient amount of pollen was collected, storing pollen consisted of applying a vacuum for approximately 2 hours in an air conditioned room at 24-26 °C. The pollen was then sealed under vacuum in glass vials and stored at -18 °C. The quality of the pollen lots stored at -18 °C was estimated after dusting pollen onto a Brewbaker's medium containing 0.5 % agar in a petri dish. The pollen viability (whether it germinates or not) was recorded under binocu-

lars at least 24 hours after sowing, as the pollen tube was twice the diameter of the pollen grain. The pollen vigour (rate of pollen tube growth) was based on the measurement of the pollen tube under binoculars.

Pollen could be stored successfully for up to 7 months following vacuum drying and its viability was reduced down to 21 % only.

First attempts at control pollination of *C. subinermis* using fresh pollen resulted in the obtention of fruits of which 60 % germinated. No rehydration of stored pollen was undertaken apart from exposure to humid air while the stigma was covered with pollen grains. The full success of the first control pollination of *C. manan* using frozen pollen experiments has yet to be established pending the completion of fruit maturation although the fertilisation has been achieved and the first signs of fruit sets have appeared.

IN VITRO GERMINATION

RATIONALE

There are several reasons for germinating rattan seeds in *in vitro* conditions, especially bearing in mind two major pieces of information.

First of all, under nursery conditions, the germination rate can be very low and the germination period very long. Secondly, the protocols developed for all the species give rise to higher germination rates than those obtained in nursery conditions. The germination capacity of the seeds, whatever the species, has been observed to decrease rapidly during time course, as reported especially in the case of *Calamus manan* (MORI *et al.*, 1980). From our own observation, the germination of *C. merrillii* decreased from 20 % to less than 5 % within one to three weeks in the nursery following storage of the seeds at 4 °C during those time periods.

Consequently, resorting to *in vitro* germination, despite higher operational cost, can be rationally considered for highly precious seeds available in restricted quantities – endangered species or varieties like « Golden sika » from Palawan (Philippines), or individuals deriving from controlled crosses – and especially to avoid long storage periods likely to lower the germination capacity.

Germinating seeds *in vitro*, either using the whole seed or after the excision of the embryo (RAO *et al.*, 1990), can be useful for storing the genotypes as germplasm (F.A.O., 1993), or for initiating a micropropagation programme avoiding the heavy disinfection procedures needed for explants collected from outdoor growing plants.

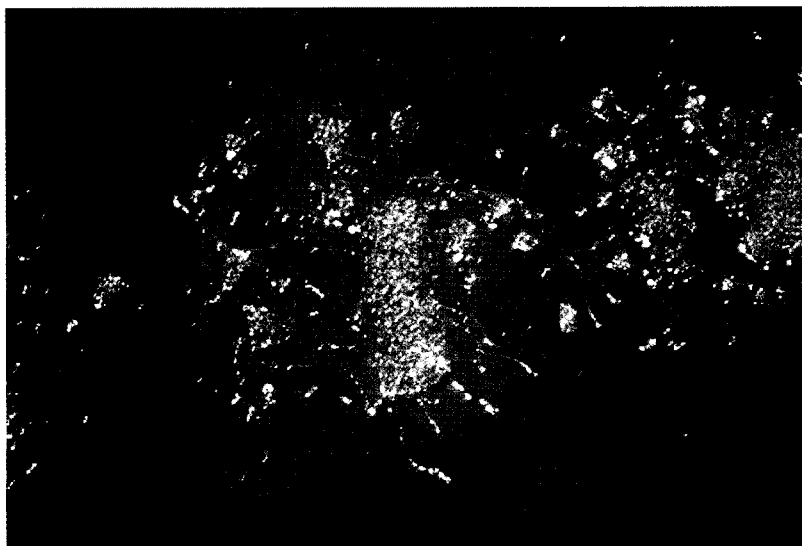


Photo 2.

In vitro germination of *Calamus manan* pollen after 7 months of storage at -18 °C.
Germination *in vitro* de pollen de *Calamus manan* après 7 mois de conservation à -18 °C.

MAIN ACHIEVEMENTS

Calamus manan, *Calamus merrillii* and *Calamus subinermis* were the rattan species of choice owing to their high market value.

C. manan, originating from Peninsular Malaysia and Sumatra is a single-stem species propagated from seeds and suffering from overexploitation. Low overall germination capacity due to storage and limited seed resources prompted us to assess the benefits of *in vitro* germination. The scaly pericarp and the fleshy sarcotesta of the mature fruits were first removed to obtain the seeds. These were then surface sterilized using a preferably aqueous solution of NaOCl (4 to 6 %), before excision of the embryos in contamination-free conditions, that is, under the laminar flow hood. The excised embryos were then inoculated onto a basal culture medium (MURASHIGE, SKOOG, 1962) lacking growth regulators. The particularity of *C. manan* compared to other species is the big size of the embryo (2 to 4 mm) which can be easily located beneath the operculum and can be extracted in good conditions. The cultures were then placed for two months in total darkness before being transferred to a 16/8 : light/dark photoperiod regime. 95 % to 100 % of the embryos inoculated after excision from the endosperm developed normally in the absence of contaminations. 3 to 4 months later, the *in vitro* grown seedlings were tall enough for transfer to outdoor conditions.

The prospects for *in vitro* germination were also assessed for *Calamus subinermis*, endemic in Sabah, and *Calamus merrillii*, from the Philippines for which seed supply can be problematic. Both are large diameter cane and multiple stem species. The embryos were observed to be noticeably smaller (about 1 mm in overall size) and barely visible compared to *Calamus manan*. Inoc-

ulation of the whole seed of *C. subinermis* after disinfection by applying the same protocols as for *C. manan*, and to a lesser extent, of the excised embryos resulted in *in vitro* developed seedlings. However, the cultures showed higher contamination rates, due especially to bacteria, and more pronounced within sample variation with regard to further development including some cases of abnormal growth than those from excised embryos of *C. manan*. The contaminations could have originated from the inner tissues of the seed (endosperm) when inoculated whole, or from numerous and tedious manipulations to excise the small embryos, with possible damages responsible for the observed abnormal growth pattern. As they are not suitable for long-term cultures in axenic conditions, the bacteria-contaminated *in vitro* seedlings were successfully transferred to outdoor conditions as soon as they had reached the appropriate height – 5 to 10 cm – for acclimatization. They can be used for instance, for establishing *ex situ* conservation plots.

In the laboratory, removal of the hilar cover from the seeds of *C. merrillii* increased the germination rate significantly ; within four weeks, about 80 % of seeds germinated. On the contrary, removal of the hilar cover from seeds sown in the nursery was not practical owing to the disappearance of the embryos. This was perhaps a result of feeding by insect pests and warranted further investigations. Germination of whole intact seeds, by contrast, took more than six weeks in the laboratory and nursery. Nonetheless, from an economic standpoint, removal of the hilar cover, which is labour intensive, would only be worth considering when the seeds concerned are scarce or precious and need to be germinated within a short period.

MICROPROPAGATION

The advantages of vegetative propagation for plant improvement programmes have been extensively developed (BURDON, 1982 ; ZOBEL, TALBERT, 1984 ; AHUJA, LIBBY, 1993).

As far as rattans are concerned, micropropagation can be considered as a useful alternative to more conventional nursery techniques which have been proven to be rather inefficient especially the vegetative propagation of the single stem species (AZIAH, MANOKARAN, 1985).

Keeping this in mind, micropropagation of rattan species can be useful for :

□ **Mass propagating** a mixture of unselected genotypes without maintaining any individual identification, also referred to as « Bulk propagation ». The purpose is to increase the quantity of planting material to overcome for example, uncertainty about seed availability or a very restricted number of germinants due to low germination rates caused by long-term seed storage.

□ **Clonal propagation** with a view to :

- Gather information about genetic parameters involved in the deployment of clonal plantation strategies, such as broad sense heritability, genotype X environment interactions, « C effects » (AHUJA, LIBBY, 1993).

- Produce clones for large scale operational planting programs providing this is economically viable.

- Establish (bi)clonal seed orchards taking advantage of the strict allogamy of the rattan species to produce genetically improved seedlings in large quantities. This option, which requires more information on rattan breeding, and es-

SUCCESSIVE STEPS OF MICROPROPAGATION THROUGH MULTIPLE SHOOT PRODUCTION
OF YOUNG *CALAMUS MANAN* GENOTYPES GERMINATED *IN VITRO*
ÉTAPES SUCCESSIVES DE LA MICROPROPAGATION PAR BOURGEONNEMENT
DE JEUNES GÉNOTYPES DE *CALAMUS MANAN* GERMÉS *IN VITRO*

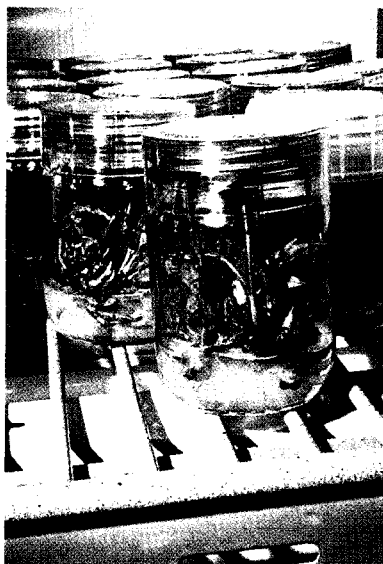


Photo 3.
Elongation of the multiple shoots induced.
Allongement des pousses initiées.

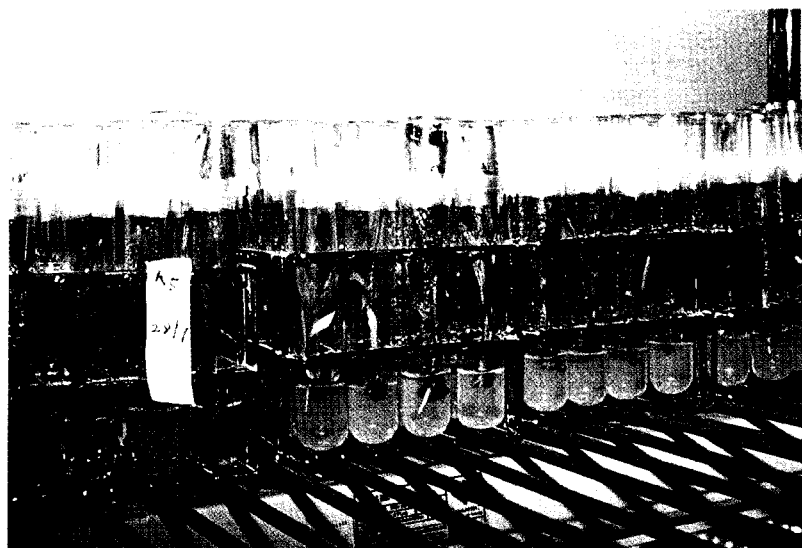


Photo 4.
Adventitious rooting of the microshoots individually in test tubes.
Enracinement adventif des tiges individuellement en tubes de culture.



Photo 5.
Transfer of the *in vitro* rooted microshoots under mist system for acclimatization to *ex vitro* conditions.
Transfert des tiges enracinées in vitro sous « mist-system » en vue de l'acclimatation aux conditions ex vitro.



Photo 6.
In vitro vegetatively propagated offspring of *Calamus manan* established outdoors.
Plants de Calamus manan issus de micropropagation établis en conditions naturelles.

pecially on what can be practically expected from specific combining ability, will dilute the initial cost of the clonal offspring used as seed producers.

Two ways of micropropagation can be envisaged: micropropagation via shoot production, and micropropagation via somatic embryogenesis.

MICROPROPAGATION VIA SHOOT PRODUCTION

RATIONALE

Micropropagation via shoot production is dependent firstly, upon the capacity of the explant to produce shoots, and secondly, upon the possibility of getting these shoots rooted once they have been individually separated. These are usually shoots of an adventitious origin, especially in the case of juvenile structures like the collar region of young germinants (AZIAH, personal communication). Basically, micropropagation of *in vitro* germinated seeds or excised embryos can be differentiated from micropropagation of shoot apices collected from outdoor plants.

The advantages of initiating micropropagation from seeds or excised embryos lie in the possibility of obtaining contamination-free cultures more readily. These usually display a great potential for organogenesis, including shoot production, due to the juvenile physiological status of the tissues, and have so far been the most widely used source of explants (CALINAWAN, HALOS, 1988; AZIAH, 1989; RAO *et al.*, 1990).

Although these genotypes are usually micropropagated as a « Bulk » since they are too young to be clonally micropropagated based on field performance, clonal micro-

propagation from *in vitro* germination can be used to assess:

- Inter- and intra-clonal differences in terms of micropropagation responsiveness in relation to the relatedness of the genotypes (coming from the same provenance, half-sib, full-sib).
- The impact of environmental factors on traits of major economical importance, such as the duration of the « rosette » stage. Whether this rosette stage, which may last a few years and is liable to seriously affect the overall growth rate of the plantations, is mostly influenced by environmental factors or by the genotype remains a basic issue. A possible answer can perhaps be found from the setting up of monoclonal field plots.

Micropropagation from explants collected from outdoors offers the possibility of being more selective as regards the genetic value, at least from a phenotypic evaluation, of the donor plant. On the other hand, micropropagation during the initial stages of culture can be seriously hindered by contamination problems and a limited capacity for organogenesis, particularly shoot production, due to the maturity of the tissues manipulated. This may be responsible for noticeable within-clone variability.

MAIN ACHIEVEMENTS

Once the *in vitro* germinated and contamination-free seedlings have reached 3 to 5 cm in height, usually after 2 to 4 months, they can be induced to produce shoots. The origin of these shoots is still uncertain and requires histological studies in relation to the anatomical and morphological features of the species. It is most probable that multiple stem species like *C. subinermis* and *C. merrillii* produce axillary shoots, whereas in the case of *C. manan*, known to be naturally single-

stemmed, the supernumerary shoots may be derived from an adventitious origin.

The induction of the multiple shoots consisted in placing the *in vitro* seedling onto a basal culture medium including a cytokinin (BA) and eventually the addition of an auxin (NAA). The seedling roots were removed and used for somatic embryogenesis induction (see hereinafter). Separate inoculation of the collar region and the shoot apex was done to increase the number of explants available, although this required more manipulations. The records showed no clear effect of decapitating the seedlings – which from a physiological point of view consists in suppressing the apical dominance – on the number of shoots produced.

For *C. manan* and *C. subinermis*, the average number of shoots long enough to be separated and further subcultured ranged from 2 to 4 per explant although scores as high as 60 potential shoots were recorded for a few explants; these however did not elongate further. The capacity of the shoots to further develop was observed to vary a lot from one explant to another within the same sample, and also between species. Applying the protocols established for multiple shoot production of *C. manan* to *C. subinermis* resulted in higher scores overall. This was similarly observed for *C. merrillii* but the further development of these shoots appeared inhibited, perhaps as a result of high concentrations of cytokinins.

The elongated shoots obtained for *C. manan* and *C. subinermis* were further subcultured in alternating multiplication and elongation media, with the alternative of getting them rooted on an auxin-enriched (addition of 15 mg/l IAA) medium. Rooting rates of 75% were obtained for shoots of 10 cm

or above in height, while shorter shoots displayed a much more reduced potential for adventitious rooting. About 1,000 shoots of *C. manan* and *C. subinermis* rooted *in vitro* were sent to adapted nursery facilities consisting mainly of a mist-system for the successful acclimatization to outdoor conditions, then raised until they reached a size suitable for field planting. From a practical and economical point of view, the method of choice consists in getting the *in vitro* elongated shoots rooted in *ex vitro* conditions. This attractive option has yet to be tested.

The shoot apices introduced into tissue culture from outdoor collections gave rise more readily to vigorous multiple shoot clusters than when starting from *in vitro* germinated seedlings. The main problem for cultures initiated from explants coming from outdoor growing plants is the amount of contamination, which usually appears, especially in the case of bacterial origin, only after several cycles of manipulations. Despite various disinfection protocols tested so far, these contaminations still have to be considered as a severe impediment for mass producing shoots from outdoor plants.

As an attempt to sum up, the experiments conducted so far in our laboratory have shown that *C. manan*, *C. subinermis* and *C. merrillii* can be micropropagated through multiple shoot production from *in vitro* germinations or even from outdoor plants, although in the latter case, the contaminations remain a serious handicap. With a view to maximizing the yield, the noticeable differences observed among these three species in terms of potential for multiple shoot production accounts for the development of protocols specific to each species, notwithstanding the obvious explant to explant variation within the same species.

MICROPROPAGATION VIA SOMATIC EMBRYOGENESIS

RATIONALE

Somatic embryogenesis has been extensively described in the literature (BRACKPOOL *et al.*, 1986), and from an ontogenetical point of view, the development of somatic embryos displays many similarities with zygotic embryos, except that they are all genotypically identical to the mother plant they derive from, theoretically speaking. Somatic embryogenesis is in fact a clonal technique that involves ontogenetical rejuvenation. This is of paramount importance for the basic study of phase change in relation to the potential for true-to-type cloning from mature donor plants. Somatic embryogenesis, especially in the absence of somaclonal variation, is likely to produce very uniform clones, compared to those issued from axillary or even adventitious shoot production. In addition, the most widely recognized practical outcomes resulting from somatic embryogenesis is the enormous multiplication rates (HAINES, 1994).

Somatic embryogenesis so far remains the only means of obtaining clonal offspring from single stem rattan species, like the most prized *Calamus manan*, without the risk of losing the genotype resulting from the micropropagation of the sole shoot apex of the selected plant after its excision.

MAIN ACHIEVEMENTS

Prospects for somatic embryogenesis were assessed for *C. manan*, *C. merrillii* and more recently, *C. subinermis*, starting from root tips and leaf portions excised from *in vitro* grown seedlings. The intention was to adapt the protocols to more mature field donor plants with a view to cloning.

By virtue of the argument outlined above, *C. manan* is the priority rattan species whose potential for somatic embryogenesis has been assessed. Picloram (1 to 10 mg/l) was observed to be more efficient than 2,4-D (2.2 to 22 mg/l) for inducing the formation of organogenic calli (35 to 40 % vs 10 to 15 %, out of the total number inoculated) from root tips excised from *in vitro* seedlings. These calli maintained in total darkness occurred after a time period varying from a few weeks to a few months. 4 to 6 months are usually required before being able to visually distinguish between the embryogenic and the non-embryogenic calli. The prospective calli needed to be frequently transferred onto a less and less Picloram-concentrated medium to stimulate the maturation and the differentiation of the pro-embryogenic structures into somatic embryos capable of further development. The determining step upon which the efficiency of this technology depends has yet to be optimized, although several somatic embryo-issued plantlets have already been obtained. It has to be noted that leaf portions placed in the same conditions have not responded at all. The greater potential of root tip explants compared to leaf portions was also observed for *C. subinermis*, in contrast to *C. merrillii* for which the somatic embryos obtained so far were derived from leaf explants which had undergone a callus stage induced by picloram.

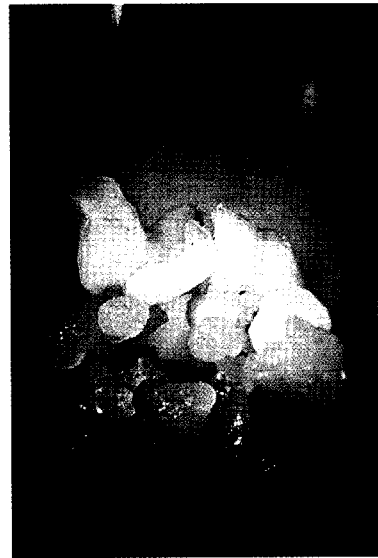
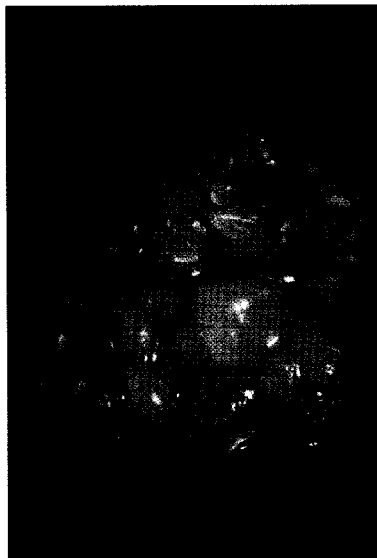
The origin of somatic embryogenesis of the plantlets regenerated so far from these experiments was attested by histo-cytological investigations which revealed the characteristic bipolar structure observed in zygotic embryos.

For the three species, attempts to induce somatic embryogenesis from root tips and leaves from outdoor plants, with special reference to mature *C. manan* individuals, have so

SUCCESSIVE STEPS OF SOMATIC EMBRYOGENESIS IN *CALAMUS MANAN*
 ÉTAPES SUCCESSIVES DE L'EMBRYOGENÈSE SOMATIQUE CHEZ *CALAMUS MANAN*



Photo 7.
 Production of potentially embryogenic calli from roots.
Obtention de cals potentiellement embryogènes à partir de racines.



Photos 8 and 9.
 Successive steps of maturation of the somatic embryos.
Étapes successives de la maturation des embryons somatiques.

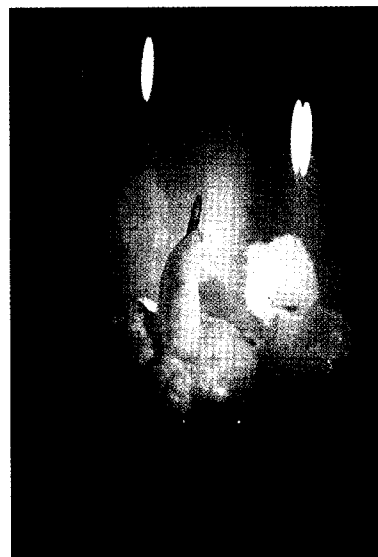


Photo 10.
 Germinating somatic embryo.
« Germination » d'un embryon somatique.

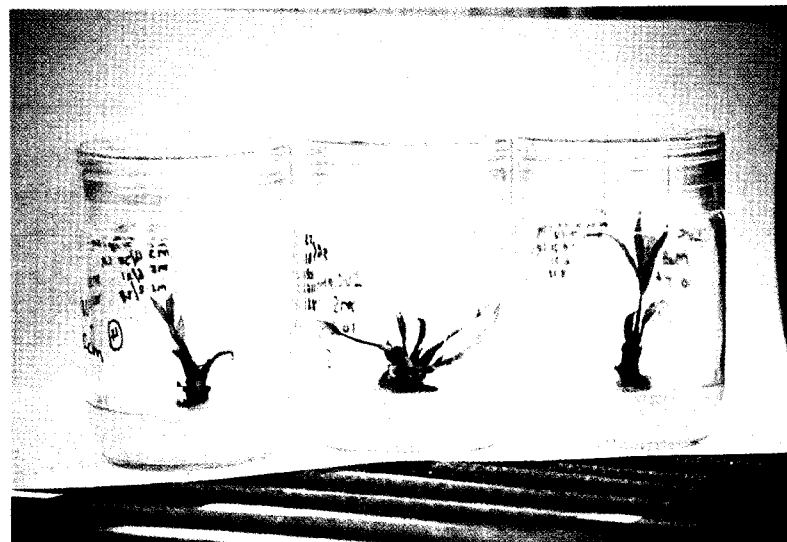


Photo 11.
In vitro development of somatic embryogenesis-derived plantlets.
Développement in vitro de plants issus d'embryogenèse somatique.

far failed due to high contamination rates or too drastic disinfection procedures.

More recently, zygotic embryos were induced for embryogenic callus formation. These calli evolved subsequently into promising pro-embryogenic structures. The main purpose intended starting from zygotic embryos was to maximize the number of offspring with identical genetic make-up (clones) deriving from one genotype, bearing in mind the limitations of micropropagation through shoot production from the same kind of initial explant. Efforts along this line are being pursued.

Similar to multiple shoot production, the experiments carried out so far indicate specific differences in the potential for somatic embryogenesis; this means that the protocols have to be optimized according to the species studied, in accordance with previous observations (UMALI-GARCIA, 1985).

SYNTHESIS AND PROSPECTS

The choice of the rattan species selected by I.C.S.B. for large-scale plantations has been based mainly on their commercial value. The wish to improve the overall returns explains why adapted plant improvement programs have been embarked upon for each of these species.

The availability of isozyme techniques allowed to screen the genetic diversity of each species in their natural environment, with the possibility of obtaining the relevant information from any individual, regardless of its age and in a non-destructive manner. The richness of the genetic resources within a species is an essential parameter when it comes to assessing the expectations from a genetic improvement program. The information gathered can be useful for establish-

ing *ex situ* conservation plots or seed stands with sufficient genetic variability. Seed collections from natural populations of *C. manan* and *C. subinermis* undertaken by the project have been achieved accordingly. It might also be envisaged to use this kind of molecular marker to differentiate precocious male and female plants to optimize the sex ratio and the gene flows of the seed production areas set up.

The technologies developed within the Plant Biotechnology Unit to store pollen from *C. subinermis* and *C. manan* have been useful for the breeding programs under way in the PISP Unit in Luasong. The benefits of these pollen management technologies are likely to become more and more important for artificial hybridization within and among species, especially when involving individuals that do not flower at the same time or grow in different locations. Promising results have already been obtained in this field, with a view to progress in the knowledge of the genetics of rattans, and more practically, to improve economically important traits.

Longer term applications of these pollen management technologies may include germplasm conservation in very restricted spaces, especially if the conservation period can be extended for up to several years, and androgenic haploid cultures from which true breeding homozygous diploid lines can be expected (F.A.O., 1993; HAINES, 1994).

The rationale for germinating seeds *in vitro* was initially to establish the right technology and then to provide enough tissue culture plant material to develop suitable micropropagation protocols. Special attention was devoted to the acclimatization process, and to the field behaviour of the shoots produced *in vitro* and adventitiously rooted. A normal development in outdoor conditions has been shown for both

Calamus manan and *Calamus subinermis*, comparable to the seedlings used as control. Losses during the acclimatization process, and the delay observed before the acclimatized shoot developed further after potting in the nursery have to be seriously considered. This could warrant *ex vitro* testing of rooting under suitable mist system. Our experience so far urges us to consider that the time required through the successive *in vitro* steps, and the overall cost of such technologies warrant their utilization only in the case of very precious and quantitatively limited seed lots – for instance, to increase the number of offspring resulting from controlled pollination. Such an application is particularly pertinent for *Calamus manan* considering the advances of the project in this field.

In contrast to young seedlings germinated *in vitro*, micropropagation from outdoor explants has been severely handicapped by contaminations. Bacteria which were observed to appear sometimes only after several transfers and which interfere with explant development have proven to be the most difficult to remove. In addition, although shoot tip cultures obtained from outdoor plants may produce earlier and more vigorous-looking multiple shoots than *in vitro* germinations, the ability to maintain this capacity for micropropagation over time has to be assessed. The negative effect of the maturation process on the potential for vegetative propagation often reported for ligneous plants (BONGA, 1982) still has to be evaluated for the rattan species under micropropagation. This is especially true when starting clonal propagation from mature selected individuals.

Somatic embryogenesis offers a possibility of overcoming inhibitory effects of ageing on the *in vitro* potential for clonal propagation of ma-

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ture rattan genotypes. This clonal propagation technique has been developed on *Calamus manan* because of the risks of losing the selected genotype from any attempts to micropropagate the only shoot tip available after collection. This is always the risk with single-stemmed palm species. Hence, vegetative propagation methods based on the production of organogenic calli from different parts of the plant which are abundantly available, such as leaf portions or root tips, allow to preserve the donor plant. In this respect and referring to other palm species (BRACKPOOL *et al.*, 1986), inflorescences also seem worthy of being tested as a source of explants without causing any damage to the donor plant. The main suspected drawback connected with micropropagation from organogenic calli, either through adventitious shoot production or somatic embryogenesis, is the risk of somaclonal variations (F.A.O., 1993) although the practical impacts will have to be objectively checked.

Based on the information gathered so far from other laboratories and from our own experience, it does not seem practical to mass micropropa-

gate rattan species only to be used as planting material for large scale industrial plantations. Cost and time are the two main constraints supporting this view, in spite of AZIAH's speculations (1995). And it is most unlikely that the returns from clonal plantations can justify such an investment, compared to plantations established from nursery-produced seedlings. Bearing this in mind, the most rational way to make use of micropropagation for genetically improved quality planting stock of rattans may be through the vegetative propagation of genotypes to be utilized as seed producers within vegetative orchards. The cost of the *in vitro* produced individuals will be diluted through the large number of seeds produced every year. These may be biclonal orchards, taking advantage of the strict dioecy of the rattan species. At this point in time however, more information is needed, especially in the field of genetics, to justify this option and to figure out experimentally on what can be practically expected from such clonal orchards.

For rattans as for other forest species, there is an obvious need for good complementarity between la-

boratory and field activities, involving synergically research disciplines such as plant improvement, physiology, and biology in order to draw the best benefits from biotechnology. The ongoing joint project initiated between Innoprise Corporation and CIRAD-Forêt has been conceived with this in mind aiming at a better overall efficiency, with possible backup from the French research teams who are quite well-experienced in the field of biotechnology applied to palm species. This can be particularly useful for further investigations into the areas of somatic embryogenesis and germplasm cryoconservation. □

► Doreen K. S. GOH
I.C.S.B.
Po. Box 60793
91017 TAWAU
Sabah
Malaysia

► Marie-Claude BON
Olivier MONTEUUIS
CIRAD-Forêt/Baillarguet
B.P. 5035
34032 MONTPELLIER CEDEX 1
France

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A B S T R A C T

PROSPECTS OF BIOTECHNOLOGY FOR A RATTAN IMPROVEMENT PROGRAMME
Innoprise Corporation and CIRAD-Forêt joint project as a case study

The prospects of biotechnology for a rattan improvement programme are presented based on the experience developed in this field within the ongoing collaboration between CIRAD-Forêt and Innoprise Corporation Sdn Bhd, the holding company of Sabah Foundation which is the largest timber concessionaire in Sabah (East Malaysia).

The isozyme techniques developed have made it possible to screen the genetic diversity of the priority rattan species, when necessary in a non-destructive manner from individuals of any age.

The possibility of storing pollen for long periods while maintaining its germination capacity is useful for breeding programmes, especially in view of interspecific hybridization, and also for germplasm conservation.

Compared to what can be expected from nursery, the established tissue culture protocols resulted in higher germination rates of all the selected rattan species. These species can also be vegetatively propagated *in vitro*, either as a mixture of genotypes (« Bulk propagation ») or clonally. This was achieved through multiple shoot production and somatic embryogenesis. The latter option appears to be the most appropriate technology for clonally propagating mature selected genotypes of single-stem species.

In practical terms, it seems that the most rational way to make use of micropropagation techniques for mass producing genetically improved quality planting stock of rattans may be through the vegetative propagation of genotypes to be utilized as seed producers within vegetative orchards.

Key words : *Calamus*. Genetic improvement. Biotechnology. Somatic embryogenesis. Isoenzymes. Pollen. Micropropagation.

R É S U M É

INTÉRÊT DES BIOTECHNOLOGIES POUR L'AMÉLIORATION DES ROTINS
Cas du projet Innoprise Corporation et CIRAD-Forêt

L'intérêt des biotechnologies pour l'amélioration génétique des rotins est présenté sur la base de l'expérience acquise dans ce domaine au sein de la collaboration entre le CIRAD-Forêt et Innoprise Corporation Sdn Bhd, la filiale commerciale de la Sabah Foundation qui est le plus gros concessionnaire forestier du Sabah (Malaisie orientale).

Les techniques d'électrophorèses isoenzymatiques mises au point, notamment à partir de portions de limbe foliaire d'individu de n'importe quel âge, ont permis d'appréhender la diversité génétique des espèces de rotins prioritaires pour le projet.

La possibilité de conserver le pollen pendant de longues périodes tout en maintenant sa faculté germinative est un réel atout pour l'amélioration génétique des espèces, plus spécialement pour les hybridations interspécifiques et les « banques de gènes ».

La faculté germinative des espèces de rotin travaillées s'est révélée bien supérieure en culture *in vitro* qu'en pépinière ou autres conditions plus naturelles. Ces espèces peuvent être aussi propagées végétativement *in vitro* soit sous forme de mélange de génotypes (propagation « en vrac » ou « Bulk propagation »), soit sous forme de clones. Des protocoles de micropropagation par bourgeonnement et par embryogenèse somatique, qui semble la technologie la plus adaptée au clonage de génotypes âgés d'espèces monocaulées, ont été mis au point à cette fin.

Pratiquement, il semble que l'utilisation la plus rationnelle de la micropropagation pour produire massivement des plants de qualité supérieure pour des plantations industrielles soit la multiplication végétative de génotypes en vue de constituer des vergers à graines.

Mots-clés : *Calamus*. Amélioration génétique. Biotechnologie. Embryogenèse somatique. Isoenzyme. Pollen. Micropropagation.

R E S U M E N

INTERÉS DE LAS BIOTECNOLOGÍAS PARA LA MEJORA DE LAS ROTAS
Caso del proyecto Innoprise Corporation y CIRAD-Forêt

El interés de las biotecnologías para la mejora genética de las rotas se presenta según los fundamentos de las experiencias conseguidas en este área con motivo de la colaboración entre el CIRAD-Forêt e Innoprise Corporation Sdn Bhd, que es la filial comercial de la Sabah Foundation, que a su vez, es el principal concesionario forestal del Sabah (Malasia oriental).

Las técnicas de electroforesis isoenzimáticas desarrolladas, fundamentalmente a partir de porciones de limbo foliar de individuos de cualquier edad, han permitido comprender de mejor modo la diversidad genética de las especies de rotas prioritarias para el proyecto. La posibilidad de conservar el polen durante largos periodos sin perder en nada su facultad germinativa constituye una ventaja efectiva para la mejora genética de las especies, y con mayor énfasis, para las hibridaciones interespecíficas y los « bancos de genes ».

La facultad germinativa de las especies de rotas trabajadas ha manifestado ser muy superior en cultivo *in vitro* que en vivero u otras condiciones más naturales. Estas especies se pueden también propagar *in vitro*, ya sea en forma de mezcla de genotipos (propagación « a granel » o « Bulk propagation »), o bien, en forma de clones. Se han desarrollado a este respecto diversos protocolos de micropropagación por brotes y por embriogénesis somática que, según parece, constituyen la tecnología más adaptada para el clonado de genotipos de edad madura de especies monocaulées, que se han desarrollado para esta finalidad.

Prácticamente, parece ser que la utilización más racional de la micropropagación para producir masivamente plantas de calidad superior para las plantaciones industriales consiste en la multiplicación vegetativa de genotipos, para, de este modo, constituir viveros semilleros.

Palabras clave : *Calamus*. Fitomejoramiento. Biotecnología. Embrión somático. Isoenzimas. Polen. Micropropagación.