Propagation of Bamboo and Rattan Through Tissue Culture

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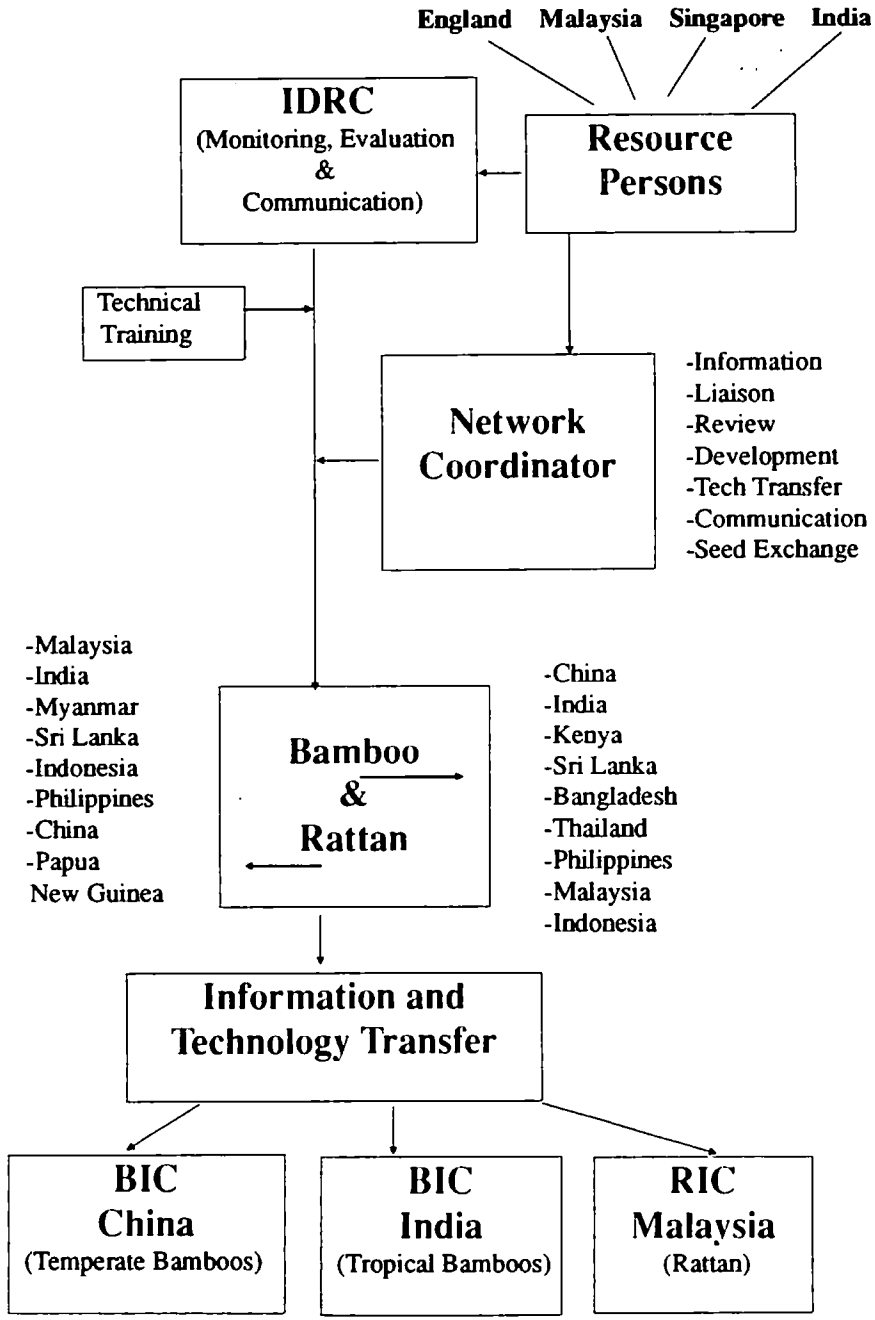

PROPAGATION OF BAMBOO AND RATTAN THROUGH TISSUE CULTURE

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PREFACE

We are currently in the midst of a biological revolution. The application of biotechnology, or the utilization of biological processes on an industrial scale, promises to improve the quality of life of people both in the developed as well as the developing countries. Of the various aspects of biotechnology, tissue culture is one technique that has found ready application in several areas, the principal one being in the propagation of plants. In the developing countries, the impact of tissue culture is beginning to be felt only now with advances being made in the tropical tree species.

Realising the value of this technique in forestry, IDRC organised an international seminar on the tissue culture of forest species in 1987. IDRC has also funded several tissue culture projects on bamboo and rattan under the Network, some of which have succeeded in developing useful methodologies. The interest in bamboos and rattans stems from the fact that these groups of plants not only provide livelihood but are crucial to the well-being of millions of rural poor. Tissue culture is one method that can help stem the tide of deforestation and improve the quality of life in the developing countries. This state-of-the-art report on propagation of bamboo and rattan through tissue culture includes details of the methods involved. We are sure that the information contained here will not only bring to your notice current achievements in this area but also help those embarking on similar research and development programmes to achieve quicker success in their efforts.

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Introduction

One of the most unique and versatile groups of plants known to mankind, the bamboos occupy a special place in the lives of the rural poor, especially in Asia. Often called the giant grass, the graceful, weaving stalks give it an enchanting appearance. The plant is symbolic of the wise man who, shaken violently by the storm, bends but never breaks. There is a certain amount of mysticism associated with this tall grass, its age-old association in human life giving rise to respect and exaltedness for the bamboos. Indeed, there seems to be a certain degree of inseparableness between man and bamboo and the degree of its acceptance by the people in many developing countries can scarcely be gauged. From the sliver of bamboo that severs the umbilical cord of the infant, to the bier that carries man on his last journey, there is a close interweaving of the two. Thus, in Indonesia, the term "sedular nunggal welat" means "kin of the same bamboo umbilical knife", and when someone was born as brother or sister, the same bamboo knife was used again to cut the umbilical cord.

For almost half the human race in the world, life would be quite different without bamboo. It is used for food, as a weapon or as a tool, as a cooking instrument or a container, as a musical instrument or as an object of beauty, as shelter and even as medicine. To the Asian, it provides him food and the wood for the home he lives in, the mat he sleeps on, the chopsticks he eats with, and the cup he drinks from. It provides a hat to shade his eyes from the sun and an umbrella to shelter him from the rain. He irrigates his paddy fields with a length of bamboo piping. He hangs his rice over a bamboo pole to dry and then sifts it through a bamboo sieve and carries it home in a bamboo basket. Then he has a good meal of rice and tender bamboo shoots and finishes his day with some music from a bamboo flute. If, as a boy, he is bad, he is

punished with a bamboo cane, and perhaps, if he is unlucky, tortured with bamboo stakes and finally strangled with a bamboo rope!

Bamboos are the most important items of forest produce used by the rural communities in Asia and the Pacific. It is reported that over 75 genera and 1250 species of bamboos occur in the world (Anonymous, 1978) with the majority in the tropics. About 300 species are so far reported from China, 130 species from India, 55 species from the Philippines, 50 species from Thailand, 33 species from Bangladesh, 31 species from Indonesia, 26 species from Papua New Guinea and 12 species from Malaysia (Sharma, 1987). The largest forest area under bamboos is in India with 9.57 million hectares of bamboo forests or 12.8% of the total forest area. The principal bamboos are *Dendrocalamus strictus* and *Bambusa arundinacea* with an overall annual production of 5 million tons.

In China 3.4 million hectares are under bamboos (3% of total forest area), again with a production of 5 million tons. Two thirds of the area is under *Phyllostachys pubescens.*

Scarcity of Bamboo Resources

One of the most important contributions of bamboo to modern-day man is in the production of paper. Though once called poor man's timber, it is no longer cheap. Its use as a long-fibre raw material in the pulp and paper industry is well known and it is one of the most sought after raw materials in the tropics. Its use for making paper, however, dates back to the Western Jin Dynasty, or more than 1,700 years ago (256 AD). Such bamboo-made paper became world famous during the Tang Dynasty. Said Su Dongpo, the famous poet of the Song Dynasty in China, "There are bamboo tiles for shelter, bamboo hats for shading, *bamboo paper for writing,* bamboo rafts for carrying, bamboo skin for clothing, bamboo shoes for wearing, bamboo shoots for eating and bamboo fuel for fires. Indeed we cannot live without bamboos for a single day."

In India, industrial production of paper was started in the 1930s. Since independence, however, there has been a rapid expansion of paper factories, with 35 of them using bamboo as a source of long-fibre (Liese, 1985). After the Second World War, Sri Lanka and Indonesia also set up paper and pulp factories. China recently set up several small-scale paper factories using bamboo pulp (Jifan, 1987).

At one time the supply of bamboo was thought to be perpetual. Bamboo was often viewed by foresters in many countries as a weed species and a nuisance due to its rapid growth, and therefore its mass-utilization in the paper industries was welcome. This impression soon proved to be a mirage. The

rapidly increasing population with associated demands for fuel and farmland, resulted in a significant decrease of land area under forest in Asia (including that under bamboo). Between 1960-80, one-half of the increase in food or plantation production in Southeast Asia was achieved by extending plantation areas under cultivation by clearing forest land. In addition to the decrease in forest cover, overexploitation, bad management, lack of adequate state control over natural forest stands and vested interests have resulted in a situation where the forest stock of bamboo and the total annual net growth has decreased and will continue to decrease significantly in Asia (Barney, 1980).

The shortage of supply has been compounded by absence of or inadequate replantation. Also flowering (bamboo being monocarpic) results in the death of the entire clump. Populations of a given bamboo species belonging to the same provenance have been known to flower simultaneously irrespective of their planting locations. Seeds of *Thyrsostachys oliveri* that flowered in Burma (in 1891) were planted in Calcutta and at Dehra Dun (India) which are 1500 km apart. Yet, at both places, the clumps flowered synchronously in 1940. The synchronous flowering of *Melocanna baccifera* was observed in Garo Hills (Assam, India) and Dehra Dun. Thus the period between gregarious flowering of a species over the same area seems to be constant and cyclic. Such a phenomenon has also been recorded in other countries (Clement, 1956; Anantachote, 1987; Zhang & Chen, 1987). In many states in India, assessments of bamboo wealth have been upset by subsequent gregarious flowering and the death of bamboo clumps following seeding. The resultant regeneration takes time to establish and has to face factors such as intense grazing pressure and forest fires.

Principally, however, it is the mass-utilization of bamboo by the paper and pulp industry that is quickly taking the plant out of reach of the common man such that it no longer remains the poor man's timber. On the other hand, it is fast becoming a high-value crop. In certain countries growing bamboos for shoots has proved to be more profitable than rice cultivation. These facts warrant reconsideration of the classification of bamboos as a 'minor forest produce' in some countries and in others as 'non-commercial species' (Sharma, 1987). This in itself would serve to bring a new focus on the bamboos and help in their conservation and replantation. As of now, many areas in countries where bamboos grew in dense thickets a decade ago now lie barren, save for a few isolated clumps, or the land has been reserved for other purposes after clearing bamboos.

Replanting of Bamboo

There is a growing realization that to combat the rapidly dwindling natural

resource, large-scale replanting needs to be done. Replanting efforts using offsets or culm cuttings are slow and expensive. Only propagation with seeds is cheaper and easier with the seedlings being raised in nurseries and transplanted to the forest. This, however, offers only a limited answer to the problem since most of the larger, economic bamboos flower only once every 30-60 years. The sporadic flowering that takes place annually in isolated clumps yields few viable seeds from a large mass of empty florets. Viable seeds obtained from gregarious flowering also suffer much damage due to rodents, insect attack and rapid loss of viability due to poor storage. Clearly, research into newer and more rapid methods of propagation is urgently called for.

Strategies in Bamboo Tissue Culture

Plant tissue culture includes several techniques that together enable the aseptic nurturing of a plant organ, tissue, cells or even cells without walls (protoplasts) in a controlled nutrient medium. The constituents of the medium can be manipulated to keep the explanted tissue in alive, active state and make it dedifferentiate and divide to give rise to an undifferentiated mass of cells (callus). Haberlandt (1902) predicted that each of the millions of cells that form a plant body, has the innate potential to recapitulate and duplicate the developmental events that occur after a zygote is formed in the plant, a property referred to as cellular totipotency. A zygote, which is the diploid single cell formed after sexual fusion, has the inherent capability to undergo programmed development leading to the formation of the embryo which, when the seed is sown, germinates to give rise to a new plant. Thus under appropriate conditions, an individual cell can give rise to an embryo.

The dream of Haberlandt was realised in 1958 when Steward and his coworkers demonstrated the formation of numerous embryos from tissues taken from carrot. These are called "somatic embryos" or "embryoids" (somatic=vegetative - to distinguish them from normal zygotic embryos which arise from sexual fusion). These somatic embryos are identical to zygotic embryos, and are equipped with both the shoot and the root pole and only need to be germinated to obtain complete plants. An alternative method to somatic embryogenesis, as the above process is called, is the method of micropropagation. In a broad sense, it means vegetative propagation in vitro or under sterile conditions. For example, the growth controls that operate in an intact plant can be broken down or eliminated under in vitro conditions, leading to profuse production of several shoots from a single shoot that was the initial explant. These shoots can be separated and rooted to give rise to entire plants.

There has been active research in India, China, Taiwan and the ASEAN countries on experimental methods of in vitro plantlet production of bamboos. Micropropagation is being attempted using nodal cuttings from the minor branches of bamboo. This method has the distinct advantage in that an identified bamboo plant with superior characteristics can be rapidly and clonally propagated using nodal cuttings so that plants identical to the parent elite plant are produced in large numbers and used to develop plantations. However, early flowering of such micropropagated bamboo plants cannot be ruled out. Bamboos which are monocarpic and have a fixed vegetative life span are known to determine the time elapsed from initial germination by an as-yet-unknown internal mechanism.

Somatic embryogenesis offers another easy method for mass-propagation. It is now possible to mass-produce embryos in the laboratory which follow a similar path of development as in zygotic embryogenesis. The juvenile period and growth habit of plantlets from somatic embryos are similar to those of in vivo seedlings. Thus, bamboo plants from somatic embryos can be relied upon to last for their full vegetative life-span.

But one limitation of this method at present is that somatic embryogenesis is possible only from juvenile tissues. Active research is, however, going on to determine methods for the induction of somatic embryogenesis from mature tissues of an identified elite bamboo and the results are promising. When accomplished, this will satisfy the objective of mass-propagation of elite bamboo clones while ensuring their full vegetative life-span. The bamboo farmer would also be doubly pleased to grow plants with superior qualities and an assured vegetative growth period.

BAMBOO TISSUE CULTURE - A REVIEW[1]

Introduction

Research on the tissue culture of bamboos is fairly recent except for the report by Alexander and Rao (1968) on the culture of bamboo embryos and their germination into plants in vitro, and by Tseng et al. (1975) on the isolation of protoplasts from leaves. Intensive research began with the work of Mehta et al. (1982) on the production of plantlets of *Bambusa arundinacea* through somatic embryogenesis. This was followed by several reports on a present total of about 15 species from 7 genera of the bamboos.

Much of the advances in the tissue culture of monocots has been by using embryogenic, meristematic and reproductive tissues. These have been shown to be more amenable to callus proliferation and morphogenesis. This is especially evident in the grass family, Poaceae, where a good response is only obtained from immature embryos and much less from other tissues.

It has been generally observed that callus initiation and outgrowth requires an auxin in the medium, and a reduction or complete auxin elimination is often necessary to obtain morphogenesis and production of organs (chiefly, embryogenesis). Various species also have specific requirements for cytokinin with respect to callus induction and regeneration.

On the other hand reports of bud, shoot-tip or meristem culture followed by direct shoot development are so far limited in perennial monocotyledons. It is generally accepted that breaking of apical dominance, if these buds are cultured, can be achieved by the application of cytokinins. This overcomes the effect of the terminal (apical) bud and the axillary buds start to grows out. In the bamboos, however, success with this technique of 'micropropagation' has been very limited.

The work done so far is summarized in Table 1 which gives a bird's eye view of the progress on the in vitro propagation of bamboos.

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1 Based on a review by Dekkers (1989)

Embryo Rescue

In a preliminary study to investigate the possibility of rescuing non-viable hybrids between *Bambusa* and *Saccharum*, embryos were germinated in vitro (Alexander & Rao, 1968; not included in Table 1).

Micropropagation

Embryo shoot-tips can be induced to produce several shoots. This strategy was adopted for *Dendrocalamus strictus* by Nadgir et al. (1984). 6-Benzyl-aminopurine (BAP) was used to induce axillary shoots. The best results were obtained in shaking liquid cultures and it was projected that 10,000 plantlets per year could be obtained from one embryo. In the same plant, Dekkers (1989) also obtained multiple shoots from the embryo. However, only few of these shoots could be rooted on subsequent transfer to a rooting medium.

Multiple shoots can also be obtained from axillary buds. Culm buds (Banik, 1987) and branch buds (Nadgir et al., 1984; Prutpongse & Gavinlertvatana, 1987) obtained from mature plants of various bamboos treated with a wide range of concentrations of cytokinins (1-25 mg/l of BAP) could be induced to form multiple shoots in liquid as well as on agar medium.

According to Banik (1987), rooting of the culm-bud derived shoots did not pose any serious problems as the addition of α -naphthalene acetic acid (NAA) to shoots on BAP - containing agar medium induced profuse rooting of plantlets after $3\ ^{1}/_{2}$ months. Seedling-derived shoots could be rooted in liquid medium with the application of indolebutyric acid (IBA) (Nadgir et al., 1984). However, rooting problems remained for the branch bud-derived cultures. Although proliferating multiple-shoots of *Dendrocalamus strictus* needed cytokinins for their maintenance, it was not possible to induce roots in the presence of these cytokinins. A boost application of IBA alone for 96h resulted in root induction. Also the immediate transfer to half-strength Murashige and Skoog (1962; MS) medium with activated charcoal (AC) was found to be necessary to protect the shoots from the 'negative effects of auxin' (Nadgir et al., 1984). Two other species investigated in the report by Nadgir et al. (1984), namely *Bambusa vulgaris* and *Bambusa arundinacea*, could not be rooted. Chaturvedi and Sharma (1988) incorporated phloroglucinol in the medium and kept the nodal explants in an inverted position to induce rooting in *D. strictus*. Recently Dekkers (1989) cultured nodal explants of *Bambusa ventricosa* where sprouting of the axillary shoot bud and multiple shoot formation took place but continued growth of the shoots and rooting were not obtained.

Table 1 - Summary of the Literature on In Vitro Propagation of Bamboos**

Species	Explant	Medium	Results	References
Bambusa arundinacea	embryo	N6+2,4-D(7)*	callus	Mehta et al., 1982
	callus#	N6+2,4-D(7)+BAP(1)	somatic embryos	"
	node	MS+CW(10%)+BAP(0.5)+Kn(0.2)	multiple shoots	Nadgir et al., 1984
B. beecheyana var. beecheyana	inflorescence	MS+2,4-D(3)+Kn(2)	callus/embryoids	Yeh & Chang, 1986b
	embryoid#	MS	embryoid germination	"
	root#	MS+2,4-D(3)+Kn(2)	callus/albino plantlets	"
B. glaucescens	culm bud	MS+BAP(1)+AC(3g/l)	shoots	Banik, 1987
	shoot#	MS+BAP(5)+AC(3g/l)+NAA(1)	rooting	"
B. multiplex	shoot-tip	MS+2,4-D(3)	callus/cell suspension	Huang, 1988+
	shoot-tip	MS+NAA+BAP	callus/shoots/roots	"
B. oldhamii	shoot-tip	MS+2,4-D(3)	callus	Huang, 1988+

Table 1 - Continued

Species	Explant	Medium	Results	References
B. oldhamii	shoot-tip	MS+NAA+BAP	callus/shoots/roots	Huang, 1988
	inflorescence	MS+2,4-D(3)+Kn(2)	callus/embryoids	Yeh and Chang, 1986a
	embryoid#	MS+Kn(2)	embryoid germination	" "
	root#	MS+2,4-D(3)+Kn(2)	albino plantlets	" "
B. ventricosa	node	MS+BAP(3-40), BAP(5)+NAA(0.1-10) ±AC(0.3%)	shoots	Dekkers, 1989
	culm sheath base	MS+2,4-D(0.5-25), NAA(3-10), IBA(10), IBA+NAA(5+5)	callus	Dekkers, 1989
	internode	MS+2,4-D(5)	callus	Dekkers, 1989
B. vulgaris	node	MS+CW(10%) +BAP(0.5)+Kn(0.2)	multiple shoots	Nadgir et al., 1984
Dendrocalamus latiflorus	internode	MS+2,4-D(1-25)	callus	Zamora et al., 1989
	callus#	MS+BAP(1)+ 2,4-D(1)	shoots/plantlets	"

Table 1 - Continued

Species	Explant	Medium	Results	References
D. latiflorus	shoot-tip	MS+NAA+BAP	callus/shoots/roots	Huang, 1988
D. strictus	embryo	B5+2,4-D(2 and 6)	callus/embryoids	Rao et al., 1985
	embryoid#	B5+IBA(1)+NAA(0.2)	germination	"
	embryo	MS+2,4-D(1 and 10) +CW(10%)	callus/embryoids	Dekkers, 1989
	node	MS+CW(10%) +BAP(0.5)+Kn(0.2)	multiple shoots	Nadgir et al., 1984
	shoot#	MS+IBA(1;4 days)	root induction	"
	shoot#	MS(1/2)+AC(0.25%)	rooting	"
	seedling	MS+CW(5%)+BAP(0.2)	multiple shoots	"
	shoot#	MS+IBA(0.1;2 days)	root induction	"
	shoot#	MS(1/2)+AC(0.25%)	rooting	"
	node	MS+NAA(1)+1BA(1) + 2,4-D(0.5) + Phloroglucinol	shoot growth and rooting	Chaturvedi and Sharma, 1988
Phyllostachys aurea	shoot-tip	MS+2,4-D(3)	callus/cell suspension	Huang, 1988+
	shoot-tip	MS+NAA+BAP	callus/shoots/roots	"

Table 1 - Continued

Species	Explant	Medium	Results	References
P. viridis	leaf	MS+2,4-D(2)	callus/embryoids/	Hassan and Debergh,
	embryoid#	MS	germination	1987
Sasa pygmaea	shoot-tip	MS+2,4-D(3)	callus/cell suspension	Huang, 1988+
	shoot-tip	MS+NAA+BAP	callus/shoots/roots	"
Sinocalamus latiflora	embryo	MS+2,4-D(6)+Kn(3)	callus,embryoids	Yeh and Chang, 1987
	embryoid#	MS+2,4-D(3)+Kn(2)	plantlets	" "
Schizostachyum brachycladum	culm sheath base	MS+2,4-D(1,5,10), NAA(3,5)	callus	Dekkers, 1989
	internode	MS+2,4-D(10)	callus	"
	root#	MS+2,4-D(1)	callus	
Thyrsostachys siamensis	culm sheath base	MS+2,4-D(0.5,1,20)	callus	Dekkers, 1989
	internode	1/2MS+2,4-D(5)	callus	

Table 1 - Continued

**	:	Table based on Dekkers (1989)
*	:	growth regulator concentrations in mg/l
#	:	regenerated in culture
+	:	including the results of Huang and Murashige, 1983
N6	:	Chu, 1976
MS	:	Murashige & Skoog (1962)
B5	:	Gamborg et al. (1968)

AC	:	Activated charcoal
BAP	:	6-benzylaminopurine
CW	:	Coconut water
2,4-D	:	2, 4-dichlorophenoxyacetic acid
IBA	:	Indolebutyric acid
Kn	:	6-furfurylaminopurine
NAA	:	α-naphthaleneacetic acid

Somatic Embryogenesis and Shoot-bud Induction through Callus Formation

Explants

In general, the explant type and its stage of development can be crucial to the success of embryogenic callus initiation. The availability and the presence of active meristematic tissue have been the primary considerations for the selection of explant materials. The explants which have been used for callus induction in bamboo are mature embryos, young florets, internodal sections (including the intercalary meristem), leaves, leaf bases, shoot tips and regenerated roots. As detailed in the following sections, immature embryos, most parts of the seedling (including rhizomes) as well as all parts of the plantlets regenerated from somatic embryos (including the somatic embryos themselves) can also be used for induction of embryogenic callus. Explants and media used by different workers are given in Table 1.

Nutrient Medium

The medium used for bamboo tissue culture has mostly been the Murashige and Skoog (1962) medium and only embryo cultures were induced on other media such as B5 (Gamborg et al., 1968). Huang and Murashige (1983) provided a detailed study of the organic requirements of the bamboo callus tissue. Inositol (100 mg/l), thiamine (1 mg/l) and nicotinic acid (0.5 mg/l) were found to be beneficial for callus growth. Sucrose could be replaced with glucose but this did not improve the growth of cultures (Huang & Murashige, 1983). The optimum concentration of sucrose that is commonly used for callus growth is 20-30 g/l. Other authors reported much higher levels of 50 g/l (Mehta et al., 1982) or 60 g/l (Yeh & Chang, 1986a,b).

Growth Regulators

As in most grasses, callus could be induced in bamboos with the auxins picloram and NAA and, with less success, indoleacetic acid (IAA) and IBA (Huang & Murashige, 1983). However, the most commonly used auxin and the one that has largely given rise to embryogenic callus is 2,4-dichlorophenoxyacetic acid (2,4-D), with concentrations ranging from 1 to 25 mg/l and more frequently at 2 and 3 mg/l.

Huang and Murashige (1983) reported that the cytokinins BAP (also reported by Zamora et al., 1989), 2-isopentenyladenine (2-iP), 6-furfurylaminopurine (kinetin; Kn) and zeatin (Zn) were repressive at relatively low concentrations. However, combinations of auxins and cytokinins were effective for callus development of other explant types.

Browning

In order to prevent browning of callusing stem explants of *Dendrocalamus latiflorus*, several antioxidants and adsorbing agents were tested. Most effective was the application of activated charcoal (2 g/l) and incubation in the dark. The former, however, inhibited callus development (Zamora et al., 1988) and higher levels of growth regulators were needed to compensate for the inactivating properties of activated charcoal.

Physical Environment

Incubation in continuous darkness has been found to be effective for callus induction of some bamboos (Huang & Murashige, 1983). Yeh and Chang (1986a, b) reported no difference between continuous darkness and a 16/8 h day/night regime. A day/night regime was effectively used by Mehta et al. (1982), whereas Rao et al. (1985) and Hassan and Debergh (1987) used continuous light.

Morphogenesis

The different calli that were produced in bamboo cultures fall into three categories, namely (1) a mucilaginous callus, (2) a soft, friable, non-embryogenic callus and (3) an off-white to pale yellow, smooth, nodular and compact callus. The mucilaginous and the compact calli have the potential to regenerate embryoids whereas the friable callus has been shown in one instance to produce roots. It is, however, largely non-morphogenetic. In several instances it has been observed that the callus obtained from explants taken from mature bamboos failed to differentiate or only gave rise to roots although it was not truly of the friable type. Dekkers (1989) reported that when the culm internodes of *Bambusa ventricosa* and *Thyrsostachys siamensis* were cultured, callusing took place followed by rhizogenesis (rooting). In *Schizostachyum brachycladum* only callusing of the explant took place. Culturing the culm sheath base in all the three bamboos also gave rise to callus followed by rhizogenesis.

The callus that is compact and nodular was in most cases responsible for regeneration of embryoids and is commonly referred to as the embryogenic callus (Rao et al., 1985; Yeh & Chang, 1986a,b). Mehta et al. (1982) found embryoid formation in the mucilaginous callus. Hassan and Debergh (1987) explained the occasional regeneration of plantlets from the friable callus by suggesting the presence of embryogenic cells in this largely friable and non-embryogenic callus.

Two different regeneration pathways (from callus) can be identified in the published literature. The first is somatic embryogenesis occurring in callus in-

duced from embryos, young inflorescences and leaves. The other is an unspecified or adventitious shoot and root formation from callus or callus derived from roots, shoot tips, internodal and rhizome sections of mature plants.

Regeneration in bamboos, in most cases, took place in the same medium as callus induction and growth. Only Mehta et al. (1982) added cytokinin and PVP (polyvinylpyrolidone) before somatic embryogenesis took place. Zamora et al. (1989) obtained shoot formation only on media with BAP after initial culture on 2,4-D, but the incidence was too low for conclusions to be drawn about the necessity of this step.

Somatic embryogenesis from the scutella of regenerated embryoids took place both in *Dendrocalamus strictus* (Rao et al. 1985) and two *Bambusa* species (Yeh & Chang, 1986a,b). Thus embryoids can be made to undergo further cycles of somatic embryogenesis in vitro.

Cell-Suspension Culture

The application of this culture system to bamboos was only reported by Huang (1988) and Huang et al. (1988). Shoot-tip derived callus transferred to liquid medium with continuous shaking at 150 rpm produced a suspension of small aggregates and free cells. Cell suspensions subcultured every 3 weeks and plated in gelled medium reinitiated callus growth. No regeneration was observed. Dekkers (1989) attempted to raise suspension cultures but was not successful.

Protoplasts

Protoplasts from *Bambusa* sp. were obtained by Tseng et al. (1975) who used leaves in an enzyme screening test. It was recently reported by Huang (1988) that protoplasts could be obtained from shoot tip-derived callus or cell-suspensions after enzyme digestion with Cellulysin* (2%), Drieselase* (1%) and Pectolyase Y-23* (0.5%) at 12^0C for 16h under continuous agitation. Protoplasts formed callus when cultivated in agarose and bathed in feeder cells. The culture medium included MS nutrients plus bovine serum albumin (0.1%), malt extract (0.05%) and arginine-HCl (50 µM). The protoplast source was non-embryogenic callus induced with 2,4-D. Dekkers (1989) was able to obtain low yields of protoplasts chiefly from the stem tissue of *Bambusa ventricosa* and *Schizostachyum brachycladum*. However, when cultured, the protoplasts collapsed after 3 to 11 days.

* *Commercial enzyme preparations.*

BAMBOO MASS-PROPAGATION THROUGH TISSUE CULTURE[2]

Introduction

Plant tissue culture offers the unique opportunity not only for realizing the totipotency of cells into whole plants but also for providing conditions under which physiological manipulations can be carried out with the objective of overcoming endogenous controls inherent in the intact plant. Micropropagation through nodal segments and somatic embryogenesis are methods which could help the regeneration of large numbers of plants in a relatively short time.

At the Department of Botany, University of Delhi, there is active research in progress using both the above mentioned methods for mass-producing bamboo plantlets[3]. By using embryogenic cultures and the embryoids resulting therefrom, any number of plantlets can be produced. The application of the method of micropropagation is, however, still limited, since there has been partial success using nodes or other materials from mature, adult bamboo plants by which 'cloning' of these (making multiple, identical copies of the parent plant) could be achieved. So far only about 4-10% of the shoots obtained from mature nodes (depending on the species) in culture can be induced to root. Better success has been obtained with seedling material (Nadgir et al., 1984; Mascarenhas, 1989) but this does not satisfy the objective of cloning mature, elite bamboos.

In comparison, using the method of somatic embryogenesis large-scale propagation of bamboos is possible at minimal cost and with the lowest labour input. Whereas this is also possible at present from juvenile tissues, recent research in the laboratory at the University of Delhi is showing that the barrier posed by mature tissues may be eliminated. The work described below outlines the method followed to mass-produce bamboos in tissue culture through somatic embryogenesis and the successful planting of these tissue-culture raised bamboos in several forest areas of India.

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2 Developed by Drs I.V. Ramanuja Rao and I. Usha Rao, Department of Botany, University of Delhi, Delhi, India.
3 Mehta et al., 1982; Kakkar, 1983; Tikiya, 1984; Rao et al., 1985, 1986; Jerath, 1986; Rao et al., 1987; Rao & Rao, 1988; Rao et al., 1988

Explants Used to Initiate Embryogenic Cultures

In this laboratory, somatic embryogenesis has been achieved in three bamboos, namely, *Dendrocalamus strictus, Bambusa arundinacea* and *Bambusa balcoa*. Embryogenic cultures can be initiated from a wide range of juvenile explants. Immature embryos, mature embryos, and mesocotyl, node, leaf sheath, leaf and root of the young seedling can be used. Newly initiated rhizomes can also be utilized for raising embryogenic cultures.

Sterilization of Explants

Whereas seeds are commonly sterilized with a chlorine-releasing agent or with chlorine water, explants taken from the field are sterilized with mercuric chloride. Seedling explants are ordinarily taken from in vitro raised seedlings.

After dehusking, the mature seeds are washed in 2% Teepol solution (Shell, India) on a magnetic stirrer for 5 min. The Teepol solution is removed by washing in running tap water for 15-20 min followed by a rinse in distilled water. Sterilization of the material is effected by a 5-min immersion of seeds in chlorine water (saturated chlorine water diluted five times with distilled water) followed by thorough washing in sterile distilled water. Surface moisture is removed with a sterile filter paper and the seeds implanted in tubes containing the inductive medium under aseptic conditions (Rao et al., 1985).

Induction and Multiplication of Embryogenic Callus

The inductive medium consists of salts and vitamins of B5 basal medium (Gamborg et al., 1968), 2% sucrose, 0.8% agar (w/v) supplemented with 2,4-dichlorophenoxyacetic acid (2,4-D) at 10 and 30 µM concentration. The pH of the medium is adjusted to 5.8 prior to autoclaving at 15 psi for 15 min. The cultures are maintained at 27 $\pm$ 2°C under continuous illumination (2,500 lux) provided by cool white daylight fluorescent tubes.

Callusing starts soon after germination of the zygotic embryo. Both friable and compact calli are formed (Fig.1). The compact callus was whitish to creamish in colour. Even when mature nodes are used, similar embryogenic compact callus is induced (Fig.1-4). Callus origin was traced to the vascular bundles (Rao et al., 1986). Cells giving rise to the embryogenic compact callus are first recognizable by the densely packed starch contents and thick walls. These cells become meristematic and initially undergo transverse divisions. Starch breakdown accompanies further cell divisions and continued divisions lead to compact callus formation. Sometimes, the cells adjoining the bundles are also induced. The somatic embryos arise as protuberances on the compact callus (Figs. 2-4). Subsequently, two humps form on top (Fig. 5)

and the tissue at the back grows up into a green hood-like scutellum (Fig. 6). The enclosed central region forms the shoot pole whereas the root pole differentiates below it (Fig. 6). The general appearance of the callus at this point of time varies from white to cream with green areas where the embryoids are maturing and becoming chlorophyllous (Figs. 7,8,15).

Prior to the differentiation of embryoids the embryogenic compact callus can be maintained by subculturing on a 2,4-D medium. Alternatively, the plantlets obtained from the germinating embryoids can be explanted back into the inductive medium to obtain fresh embryogenic compact callus.

Multiplication of Embryoids

In comparison to multiplication of embryogenic compact callus followed by differentiation of embryoids, secondary somatic embryogenesis has been found to be more rapid. In this process the primary embryoids proliferate further and give rise to a new crop of secondary embryoids. These arise by the proliferation of the scutellar epidermis (Fig. 9). Numerous embryoids develop further from the proliferated epidermis (Fig.10). A further crop of embryoids can be obtained from these secondary embryoids and the process continued indefinitely until removed from the permissive medium (Figs.11,12).

Germination of Embryoids and Rearing of Plantlets

If left on the initial inductive medium, the mature embryoids germinate and produce both shoot and root (Fig.16). The plantlets thus produced can be separated and transferred to pots after an in vitro growth period of 1 month. At present the method followed is that the surplus embryoids from a round of embryoid multiplication are transferred for maturation and germination. Here the embryoids are given an equal opportunity to mature and germinate into plantlets. The plantlets formed are removed to a medium that permits them to grow further (Fig. 17) or precocious rhizome induction is carried out. The ungerminated embryoids are then re-transferred for another round of embryoid maturation and germination. About 30% of the mature embryoids germinate in one round of subculture. At the present stage it has not been possible to evolve a protocol that permits simultaneous maturation of all embryoids on semi-solid medium and their subsequent simultaneous germination. Several experiments are, however, underway for this purpose.

Flow Chart of the Propagation Method

A flow chart that summarises the tissue culture protocol currently employed is depicted in Figure 13. In the box is given the flow chart of the method ini-

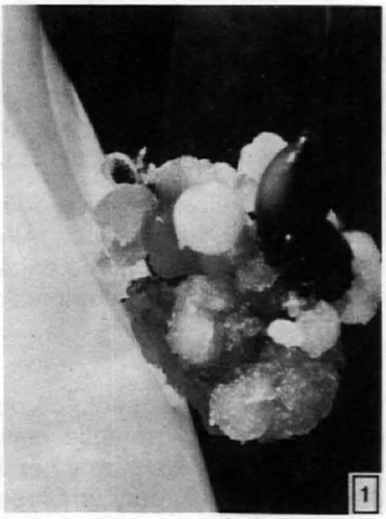
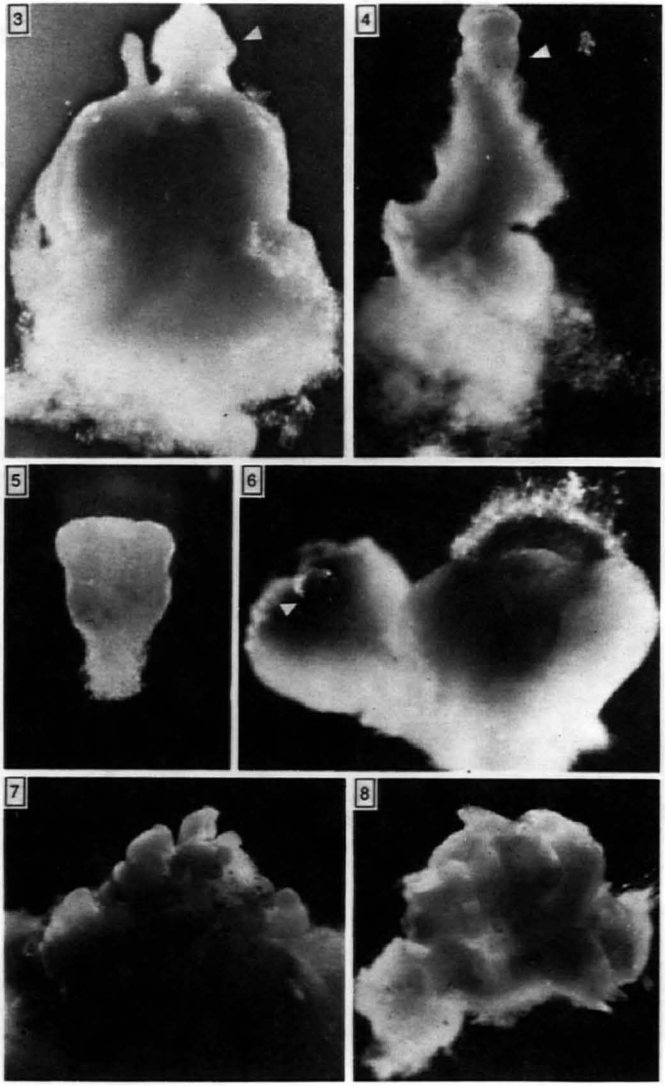


Fig. 1: Compact callus from zygotic embryo

Fig. 2: Somatic embryos differentiating from compact callus

Note: Fig 1-12 — Dendrocalamus strictus



Figs. 3, 4: Formation of somatic embryos (arrow) from compact callus.

Fig. 5-7 : Maturing somatic embryos. Arrow indicates shoot apex.

Fig. 8: Mature embryos.

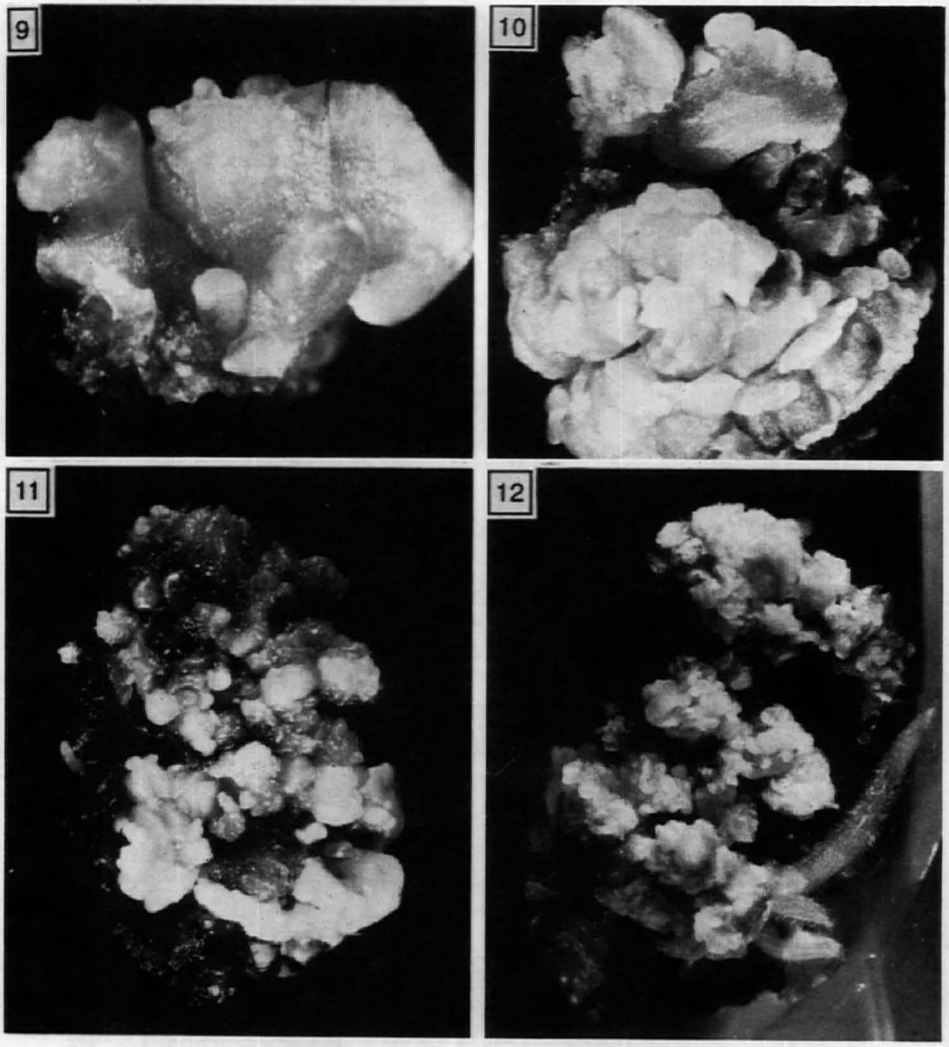


Fig. 9 : Proliferating scutellum of somatic embryos.

Fig. 10 : Development of secondary embryos from scutellar margins.

Figs. 11, 12 : Proliferating embryonal masses.

tially devised (Rao et al., 1985). It will be noticed that the method now being followed is more elaborate with a greater number of steps in between, although in both situations, bamboo plantlets are obtained through somatic embryogenesis. The current protocol, however, allows great flexibility in large-scale operations and provides for the self-generation of inoculum, with two multiplication steps built in through which amplification of the output can be achieved.

The initial explants are inoculated on a 2,4-D-supplemented inductive medium (Rao et al., 1985) which helps to produce both a compact, embryogenic callus as well as a friable non-embryogenic callus in 5 weeks. Sometimes, inoculation on a GA_3-containing medium prior to 2,4-D treatment may promote callusing. The formation of embryoids can be induced in another 3 weeks' time. Several of these embryoids if left as such will germinate into plantlets on the same medium. Alternatively, the immature embryoids are put through a multiplication cycle on medium with 2,4-D in which secondary somatic embryogenesis is induced. This process can be repeated to increase and maintain the stock of embryoids. The embryoids are then removed out of multiplication cycle and allowed to mature and germinate by going through an embryoid maturation and germination cycle either by reduction of 2,4-D levels or supplementation with Kn, that allows maximum recovery of somatic embryos into plantlets. The plantlets are potted out directly after removing from the medium. These are maintained in the acclimatization chamber for 2-3 weeks and then removed to the greenhouse for further growth. The plantlets are subsequently transferred to polyethylene bags, maintained in the open and handed over to forestry agencies when they are about 4-12 months old.

Potting of Plantlets and Acclimatization

Mature plantlets which are about 8-10 cm tall are ordinarily potted out (Fig.17). Methods have, however, been developed to pot out smaller (younger) plantlets. The plantlets are removed from the agar medium and are potted directly into a soil:fine sand:farmyard manure (1:1:1) potting mix in 10-cm diameter earthenware pots (Fig.19). These are then maintained in the acclimatization chamber in which temperature, humidity and light conditions are controlled (Figs.18,19). After an acclimatization period of 2-3 weeks, the plantlets are transferred out to the glasshouse or to PVC greenhouses where they are maintained for another 2 months (Figs. 20-22). Subsequently they are repotted into polyethylene bags with several holes for drainage and maintained until they are taken by the foresters (Figs. 23-24).

Ordinarily the in vitro produced bamboo plantlets are handed over to the foresters when they are at least 4 months old. Normally, planting in the forest

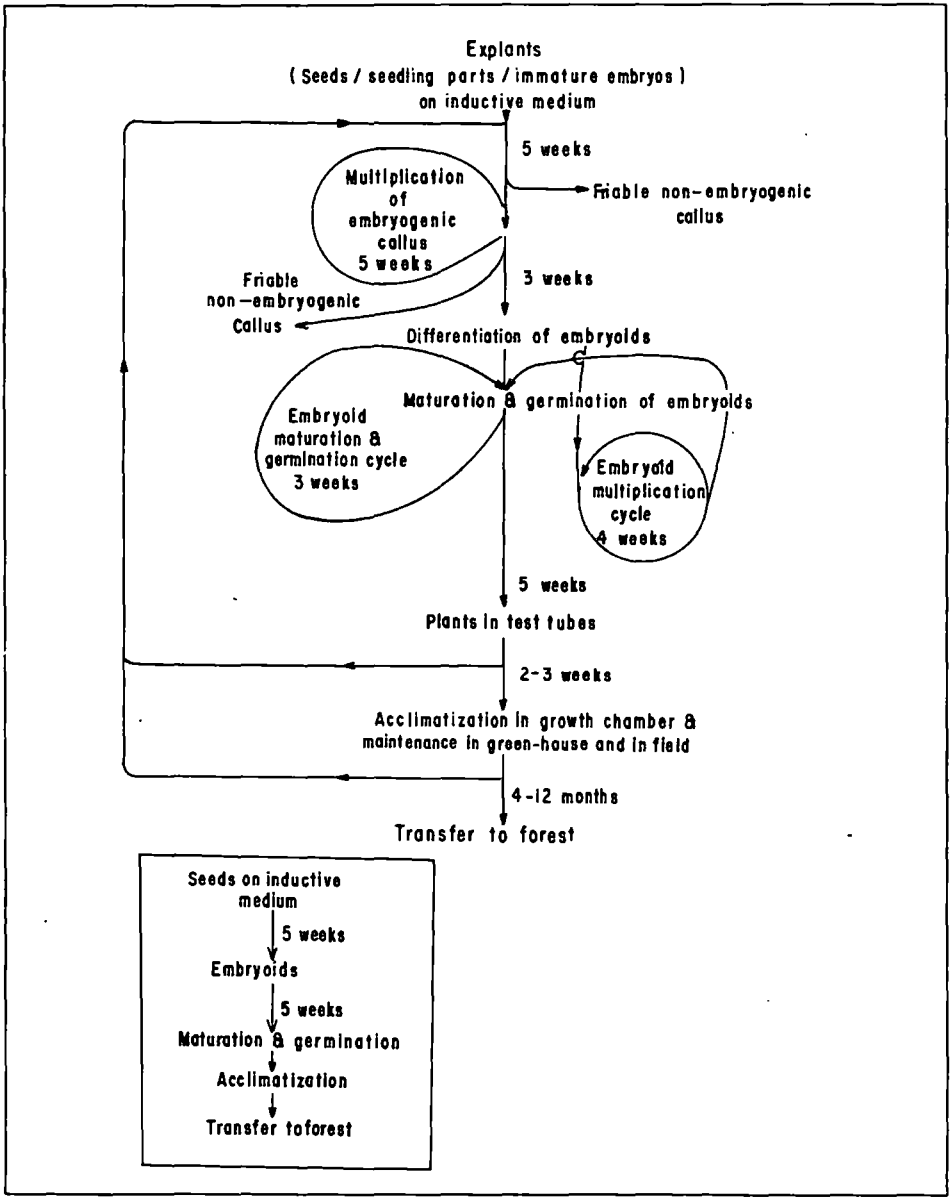


Fig. 13 : Flow chart of the propagation method for bamboo.

is not done before 8 months and is generally carried out when the plants are 12 months old. The rhizome system is well developed by this time and the plants establish easily.

Design of the Plant Acclimatization Chamber (Growth Chamber)

Several sophisticated growth chambers are commercially available and are being commonly used for the acclimatization of plantlets. But these are relatively expensive and beyond the reach of ordinary establishments. We have graduated from a similar sophisticated chamber to a simple but functional one that serves the same purpose and in an equally efficient way. Also, any effort for mass-production of tissue-culture raised plantlets would necessarily require a large facility for acclimatization of the plantlets. A facility of two chambers has been designed and set up in our laboratory with 100 m^2 of shelf area for keeping the plantlets under lighted conditions (Figs. 18,19). Up to 12,000 plantlets can be acclimatized at a time. Considering that bamboo plantlets need hardening for 3 weeks (for *Dendrocalamus strictus*; 2 weeks for *Bambusa arundinacea)*, around 0.2 million plantlets can be acclimatized in both chambers in 1 year's time.

Each chamber has two floors, with four shelves each. There is an internal staircase to service both floors. Delhi has extremes of cold and hot conditions and hence the chamber walls and roof are made of Isowall^{TM} panels. These are PVC-coated galvanised steel sheets that are sandwiched to a central 10-cm thick styrofoam layer. The pre-fabricated panels are joined together at site by riveting and are designed for a life span of 20 years in the open. The whole structure is riveted on to a steel channel structure that is grouted with bolts in cement-concrete. The structure can thus be dismantled and refixed elsewhere if so desired at a later date. The internal structure is of PVC-coated steel slotted angles and sheets and is strong enough to take the weight of the numerous potted plantlets.

The air-conditioning is through two 1.5 TR capacity room air-conditioners which also take care of dehumidification. Humidification is done through two aerosol humidifiers. The use of humidifiers of this design not only increases humidity but also serves to markedly reduce the heat load through evaporative cooling. Lighting is provided by four 40-W capacity tubelights of the cool-white daylight or daylight type fixed at a distance of 30-cm from the plantlets. All fittings are of water-resistant materials. The fluorescent tube ballasts are located outside the chamber to reduce the heat load.

Two fans are provided to allow for adequate air circulation. There is also

provision for fresh air intake. The internal conditions of temperature and humidity are centrally monitored and controlled by digital controllers. Safety thermostats are provided which, in the event of a failure in cooling, switch off the lighting, that there is no further heating and the plantlets are protected. An alarm is simultaneously sounded.

Clonal Numbering

A system of clonal numbering of the cultures and plantlets raised from the cultures has been evolved to permit the tracing of a group of plantlets/cultures to the initial culture or explant. This is done by a combination of an alpha-numeric and a numeral code that identifies the plant and the original culture and its history sheet. Thus, once field performance trials are completed, the original cultures of the superior plants as identified in the field can be selectively multiplied and that clone mass-propagated.

Packing and Transport

Initially the hardened plantlets used to be potted into large 25-cm diameter earthenware pots and transported as such. This has now been changed to 15-cm diameter polyethylene bags with holes in them, although our experience has shown that the plantlets perform much better in 25-cm diameter polyethylene bags. These changes have been made in deference to the requirements of the foresters as also keeping in mind the easier transport with polyethylene bags. Alternatively, the plantlets are removed from the bags, the earth balled up around the roots, and the plantlets put together and tied in bundles of 25 each. This bundle is then wrapped in a layer of moss and newspaper, and put into a large polyethylene bag. This method is suitable for air-transporting plants and is relatively inexpensive as 500 plants required for planting 1 hectare weigh only 50kg and can be transported easily in cardboard cartons. However, such plants require replanting and care in a nursery before planting in the forest.

Assessment of Field Performance of Plantlets

The tissue-culture raised bamboo plantlets are being assessed by the foresters in terms of the height of the plant, diameter and number of culms produced. The performance data are being compared with those from seed-raised check plants of the same age.

Cost of Production

The cost of production of the plantlets has been steadily decreasing over time due to improvements in the in vitro procedure. At present, a mature bamboo

plantlet costs Rs 5.00 (US$1 = Rs 16.40) to produce in culture and Rs 2.50 to acclimatize and rear in the field for 8 months, based on recurring expenses alone. We expect to reduce the culture cost to half in 1 year's time, resulting in an overall plantlet cost of Rs 5.00 at 1988 prices. Even if the overall cost of production can be made to remain static, it will mean a reduction in plantlet cost in real terms.

Future Outlook

We have often been asked about the utility of raising polyclonal populations of bamboos through tissue culture as the initial explant largely remains the zygotic embryo (in the seed) or seedling parts. It has also been argued that seeds are always available through sporadic flowering and that the problems of limited viability can be overcome by establishing seed-rhizome banks in the field. The latter are estimated to have a useful life of 8-10 years during which they can be dug up and planted out. However, the problems of bulkiness, transportation, expense and labour involved in maintaining and working of the seed-rhizome banks as also the poor efficiency, productivity and output of the system cannot be wished away (Stapleton, 1987).

Seed supplies also remain short and seed set from sporadic flowering remains very low and unreliable. The problem of sifting large quantities of empty florets and recovering the few well-formed seeds in sporadic flowering remains. Large quantities of viable seeds are obtained only when gregarious flowering takes place. Also bamboo seeds remain viable only for short periods. Seed storage methods are also currently primitive and result in futher loss of seed viability. To overcome this, foresters raise seedling nurseries from which seedlings emerging from the few viable seeds are picked up and transplanted into polyethylene bags for further growth.

At present, in a situation where there is severe paucity of planting materials, plantlets regenerated through somatic embryogenesis from juvenile tissues can satisfy this requirement to an extent identical to that obtainable with seeds. The use of seeds or seedings only in the initial step of culture establishment with subsequent autonomy being attained in the generation of plants (using somatic embryo-raised plantlets for culture establishment) makes this method very attractive. Embryogenic cultures are also very rapid in growth and facilitate the production of large numbers of plantlets fairly easily.

It needs to be remembered that, conventionally, one seed ordinarily gives rise to only one plant whereas numerous plantlets can be obtained through the method of somatic embryogenesis. By this method, one seed can "plant" a whole and perhaps several hectares. If the plants prove to be of good quality,

a whole hectare of monoclonal, superior plants will be available for further propagation by conventional methods. Besides, the original cultures can be maintained and can give rise to several more plants. Tissue culturing of bamboos would also lead to the isolation of superior somaclonal variants, induction of in vitro flowering and an understanding of rhizome physiology. Above all, it will lead to the restoration of our bamboo forests to their earlier green glory.

Legend for pg 33 & 34 (D. strictus)

Fig. 14 : Compact embryogenic callus from nodal explant from mature plants.

Fig. 15 : Differentiating compact callus showing the presence of young (white) and mature (green) embryoids.

Fig. 16 : Young plantlets from germinating embryoids.

Fig. 17 : Mature plantlets ready for potting.

Fig. 18 : Front view of the plant acclimatization chambers. The ballasts of the tubelights can be seen mounted outside.

Fig. 19 : Inside view of part of one chamber showing the arrangement of shelves with several hundred potted plantlets undergoing hardening.

Figs. 20,21 : Plantlets after acclimatization in the fibre-glass greenhouse.

Fig. 22 : A view of the PVC greenhouse in which the plantlets are maintained after repotting into large polythylene bags.

Fig. 23 : Eight-month-old plantlets inside a PVC greenhouse.

Fig. 24 : A truck-load of tissue-culture raised bamboo plantlets being sent from the University to a forest area.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CURRENT STATUS OF RATTAN CULTIVATION AND TISSUE CULTURE

Introduction

Rattans are spiny, climbing plants belonging to the subfamily Calmoideae of the palm family. They are represented by 600 species in 13 genera (Uhl & Dransfield, 1987). The most widespread as well as the most commercially important genus is *Calamus* which is distributed from West Africa to Fiji and from South China to Queensland (Australia). It is the largest genus of the rattans (370 species) and indeed of all the palms.

Although rattan is classified as a minor forest product, its commercial and social importance is second only to timber in some countries. Indeed, in his study of the natural history of palms, Corner (1966) states that long before the Portugese brought rattan commerce to Europe with the opening of the Orient, "rattans were so invaluable to village-life that one can speak of the rattan civilization of Southeast Asia as one can speak of the tree-palm civilization of India and the bamboo civilization of Indo-China, China and Japan". Rattan forms the raw material principally for making furniture, although several other articles such as baskets are made from it. The properties of rattan worth mentioning are its strength, durability, bending ability which allows the formation of a wide variety of shapes and the aesthetic value of the canes themselves.

Rattan products bring in a considerable amount of income to many countries. It is estimated that over half a million people are involved in the rattan industry in Southeast Asia (Menon, 1980). According to 1979 statistics, trade in raw rattan amounted to US$50 million and by the time the manufactured product reached the consumer, its value increased to about US$1.2 billion. Currently, it is estimated that the world trade in rattan and rattan products exceeds US$2 billion.

Indonesia is the world's largest supplier of raw materials accounting for about 90% of the trade (Menon, 1980). Although it is estimated that there are about 300 species in Indonesia, the principal rattan species of the export trade are: *Calamus manan, C. trachycoleus, C. caesius, C. inops* and *C. scipionum.* Rattan harvest has been about 120,000 tonnes annually for several years and, based on a preliminary inventory of rattans conducted recently in 16 provinces, it is estimated that production levels could be increased to nearly 575,000 tonnes annually (Silitonga, 1985).

The Malay Peninsula, which is considered to be the centre of diversity of rattans has 104 species in 9 genera (Dransfield, 1979).Rattans are harvested from the natural forests, and constitute the most important commercial minor forest product (Wong & Manokaran, 1985), amounting to 4% of the total world trade in raw material.

In China, recent records show that there are about 35 species belonging to 3 genera (Huangcan, 1989). The annual value of the rattan trade in China totals 60-70 million US$. Rattan is the second most important minor forest product to bamboo in the southern provinces of China and an important source of income for the rural population. Here also, rattan production is almost entirely from the wild and is insufficient for the rattan industry.

In Thailand, there are about 50 species of rattan and in the Philippines, there are over 60 species. The Sri Lankan flora is represented by 10 species, all belonging to the genus *Calamus*. The resource in Sri Lanka is seriously depleted and some species show signs of being critically overexploited. Even as early as 1898, Trimen classified 7 out of the 10 species of rattans as 'rather rare' and rare.

In India, there are 46 species represented by 4 genera. Here also the resource is very limited. Some of the rattan-growing states have imposed a ban on the interstate trade of rattan.

In the Philippines, the export of raw rattan is banned. In March 1978, Thailand followed suit. Indonesia has also imposed a ban on the export of raw and processed rattan from 1989.

Earlier, rattan supply and availability were adequate in nearly all rattan-growing countries. However, the supply of rattan has decreased considerably in recent years, because of destruction of tropical forests, the home of the rattans. A major cause of forest decline is the increase in population in this region which requires more land and forest resources for shelter construction, personal consumption and food production. The illegal clearing of forests for agricultural purposes and for the timber trade is another cause worth mentioning. Rattans, which are unique climbing palms and which require trees for support are the first to be affected by deforestation. About 35% of the rattan species recorded in Peninsular Malaysia, about 25% of those recorded in Sabah and about 30% of those recorded in Sarawak are thought to be under threat. All species in Java are severely under threat.

Such is the present nature of the rattan production and trade that problems affecting the local rattan trade of one country have tended to affect other countries in the region. Indeed, the rattan trade in East and Southeast Asia

and the Malaysian Archipelago has become increasingly interdependent and the actions of one country affect the rattan trade in another. Indonesia, which has the largest wild stocks of rattan, has put into effect a ban on the export of rattan except as finished products. This policy in trade will help increase employment in the local rattan industry and greatly improve the foreign exchange earned by the rattan trade, and also helps in the conservation and protection of Indonesian rattan from potential overexploitation. But much greater pressure will be brought to bear on the wild stocks in Thailand, China and the Philippines which depend mostly or partially on raw rattan imported from Indonesia. Already wild rattan is almost entirely depleted from Thai forests and survives only in national parks from where rattan is sometimes illegally collected. The most likely result of the ban will be initially the harvesting of the remaining wild stocks of cane in the countries concerned, followed by total depletion (Dransfield, 1989).

In addition, we should also be very concerned about the social consequences of disappearing wild rattan supplies. Siebert and Belsky (1985) have clearly shown how rural people living on the edge of poverty in the Philippines depend very heavily on rattan in order to survive. The loss of income generated by rattan collecting by such rural people due to disappearance of harvestable wild rattan, may have serious social consequences.

Many countries have realised the necessity of rattan replanting and have established programmes for rattan cultivation on plantation scale in different areas. However, most of these programmes are relatively new. Some of the larger programmes are in Malaysia, Indonesia and China (Shim, 1989; Zhang et al., 1989). This has received an added impetus because many of the rattan-growing areas are difficult to reach, and rattan collectors are becoming increasingly difficult to find and to persuade to collect mature canes deeper in the forests. Nevertheless, the demand for supplies of good quality rattan is growing rather rapidly while the converse is true of available wild stocks. Therefore, it has become necessary to find ways and means to cultivate sufficient quantities of good quality rattan in areas accessible to the rural people because of the largely rural nature of the industry.

Propagation of Rattan

While rattan can be propagated through offsets (Yusoff & Manokaran, 1985), seed propagation is a more common method since there is difficulty in gathering enough suckers or rhizomes for extensive propagation. In addition, the suckers and rhizomes are bulky, expensive, difficult to separate, handle and transport and show a low survival rate after transplanting in the field. However, vegetative propagation by suckers is of value in the clonal propagation of su-

perior genotypes and the establishment of seed orchards of economically valuable species.

All rattans ordinarily produce abundant fruits during the season. While *C. laevigatus* produces a relatively lower number (fewer than 500) from a single plant, *C. caesius* can bear a total of 4000 or more fruits with half that number maturing at one time. *C. manan* can bear as many as 5000 fruits that can all mature at the same time. There is, however, difficulty in supplying high quality seeds of the desired species to the cultivator. Besides, in some areas, there is uncertainty of seed availability, insufficient quantity and problems involved in seed storage. The mass-scale harvest of rattans has also led to a situation where there is an inadequate number of mature plants resulting in the scarcity of seeds. Also as rattan is becoming scarce, seed resources are increasingly becoming tightly controlled by governments.

The Role of Tissue Culture

In the present scenario, the propagation of rattan by tissue culture is a potentially useful method for raising large-scale plantations, especially when the available seeds (seed = fruit) become limited in the near future. Conventional vegetative propagation is also difficult for some rattans and is almost impossible in the solitary, single-stemmed rattan species such as *Calamus manan* which is commercially the most important. It has been observed that it is almost impossible to vegetatively propagate such solitary palms by conventional vegetative propagation methods.

Unlike *C. manan, C. caesius* and *C. trachycoleus* can be propagated vegetatively. Besides initial orthotropic branching, these rattans form rhizomes *(C. caesius)* or stolons *(C. trachycoleus).* However, tissue culture of these species could speed up propagation, being a potential means to mass-propagate elite clones. The establishment of genebanks, which was one of the strategies recommended by Wong and Manokaran (1985) to ensure conservation of relatively rare rattan species threatened with extinction, can also be aided by tissue culture.

Whereas tissue culture work on the palms started about three decades ago, work on rattan has been taken up only in the past few years. A few reports have been published including that of Patena et al. (1984), Umali-Garcia (1985), Barba et al. (1985), Yusoff et al. (1985), Gunawan and Yani (1986), Yusoff (1989) and Dekkers and Rao (1989) (Table 2). This work has established that it is possible to propagate rattan through tissue culture. Whereas the large-scale production of plantlets has not as yet been achieved as in the bamboos, it is now only a question of time and sufficient effort in realising the

objective of establishing a protocol for mass-production. The work done to date on the tissue culture of rattans is summarized below.

Tissue Culture of Rattans

In an initial investigation Umali-Garcia (1985) examined 11 species of *Calamus* and two of *Daemonorops*. The explants used were from the shoot apex region (cabbage). In three species of *Calamus*, the explants developed callus which subsequently gave rise to shoots. These were isolated and rooted in a different medium to obtain complete plantlets.

On the other hand Padmanabhan and Krishnan (1989) and Padmanabhan and Sudhersan (1989) only obtained an increase in size of the leaf explants and formation of a wrinkled mass of laminate tissue which they termed as 'laminoid'.

The embryo has been used more commonly in tissue culture investigations on rattan. Barba et al. (1985) cultured the embryos of *Calamus manillensis* and obtained shoots. These could be excised and rooted to obtain complete plantlets. Dekkers and Rao (1989) cultured embryos of *C. trachycoleus* on MS supplemented with 2,4-D or NAA (5 mg/l). An amorphous swelling of the embryo was obtained in 2 weeks. By about 8 weeks after inoculation, the collar region was covered with callus and a green shoot appeared. The callus could be subcultured onto medium with a lower concentration of 2,4-D. However, no morphogenetic responses were recorded.

When immature embryos of *Calamus manan* were cultured on MS (1962) and Y3 (Eeuwens, 1976) media, it was observed that these were able to grow into complete plantlets. This work was done to overcome the difficulty of finding mature fruits in the forest as most of them are eaten by monkeys etc., and the seeds dispersed (Chuthamas et al., 1989). In comparison, young fruits (having immature embryos) could be obtained on the plants. In other work on the same species, the embryo was induced to produce callus which subsequently could differentiate several plantlets. The plantlets were separated and transferred to soil and then to the field (Gunawan & Yani, 1986).

Earlier, Yusoff and Manokaran (1985) were able to obtain callus from cultured embryos of *C. manan*. In a subsequent study (Yusoff, 1989) it was found that the initial friable callus became nodular, creamy and opaque on subsequent transfers. Later greenish protuberances were formed which developed into shoots. It was easy to separate and root these shoots and the plantlets were successfully transferred to the soil. Yusoff (1989) also succeeded in inducing multiple shoots from the collar region of the in vitro raised seedlings on MS + BAP or Kn (10^{-6} to 10^{-4} M). The multiple shoot structure could be subdivided

Table 2. Tissue Culture of Rattans

Species	Explant	Medium	Results	References
Calamus sp.	Stem and petiole explants from "cabbage"	MS, HB, W, NN media with 2-6% sucrose + auxin and cytokinin + 0.3% AC	Callus induction MS + 4% sucrose identified as best medium	Umali-Garcia, 1985
C. discolor	"			"
C. filispadix	"			"
C. halconensis	"			"
C. merillii	"	"	"	"
C. microcarpa	"	"	"	"
C. mindorensis	"	"	"	"
C. ornatus var. *philippinensis*	"	"	"	"
C. ramulosus	"	"	"	"
C. reyesianus	"	"	"	"
C. hookerianus	Leaf primordia	MS basal	Increase in size	Padmanabhan & Krishnan, 1989
C. manan	Root and shoot including leaf blade from nursery and aseptically-raised seedlings	MS + auxin(?) + BAP (10^{-7} to $5x10^{-7}$M)	White friable callus Better callusing from aseptically-raised seedlings	Yusoff & Manokaran, 1985

Table 2 - Continued

Species	Explant	Medium	Results	References
C. manan	Young leaves Embryo	MS + auxin(?) + BAP (10^{-7} to $5x10^{-7}$ M)	No callusing White friable callus and seedling development	Yusoff & Manokaran, 1985
" "	Embryo	-	Callus and plantlets	Gunawan & Yani, 1986
" "	Embryo	Modified MS and Y3 media	Seedling	Chuthamas et al., 1989
" "	Collar region of seedling	MS + BAP/Kn at 10^{-6} to 10^{-4}M	Multiple shoots	Yusoff, 1989
	Multiple shoots from culture	MS (reduced strength) + IBA (10^{-6}M)	Rooting (plantlets)	"

Table 2 - Continued

Species	Explant	Medium	Results	References
C. manan	Embryo	MS + auxin + AC	Morphogenic callus	Yusoff, 1989
	Morphogenic callus from embryo culture	-	Shoots	"
	Shoots from embryo culture	-	Rooting (plantlets)	"
C. manillensis	Stem and petiole explants from "cabbage"	MS, HB, W, NN with 2-6% sucrose+auxin +cytokinin+0.3% AC	Callus induction MS + 4% sucrose identified as best medium	Umali-Garcia, 1985
" "	Embryo	-	Shoots	Barba et al. 1985
	Shoots from culture	-	Rooting (plantlets)	" "
" "	Seed (?)	-	-	Patena et al. 1984
C. thwaitesii	Leaf	MS + 2,4-D (5 mg/l) + 500 mg/l CH	Crescent-shaped wrinkled green outgrowths	Padmanabhan & Sudhersan, 1989

Table 2 - Continued

Species	Explant	Medium	Results	References
C. trachycoleus	Embryo Embryo Callus from embryo culture	MS+BAP (0.3 & 1 mg/l) MS+2,4-D or NAA (5 mg/l) MS+2,4-D (1 mg/l)	Shoot and root Callusing of collar region Callusing	Dekkers & Rao, 1989 " "
Daemonorops mollis *D. ochrolepis*	Stem and petiole explants from "cabbage"	MS, HB, W, NN with 2-6% sucrose + auxin and cytokinin + 0.3% AC	Callus induction MS+4% sucrose identified as best medium	Umali-Garcia, 1985

MS : Murashige & Skoog (1962)
HB : Hartney & Barker (1980)
W : White (1973)
NN : Nitsch & Nitsch (1969)
Y3 : Eeuwens (1976)
A : Activated charcoal

BAP : 6-benzylaminopurine
CH : Casein hydrolysate
2,4-D: 2,4 - dichlorophenoxyacetic acid
Kn : 6-furfurylaminopurine (kinetin)
IBA : Indolebutyric acid
NAA : α-naphthaleneacetic acid

and subcultured onto a medium with a lower cytokinin concentration. The rooted plantlets were subsequently transferred to soil.

The above results indicate that it is possible to establish rattan cultures either by using embryos or tissues from the shoot apex region. Complete plantlets can be obtained. It will be necessary, however, to refine and scale up the procedures, so that tissue culture becomes a viable method of mass-production of the rattans.

RATTAN PROPAGATION THROUGH TISSUE CULTURE[4]

Introduction

Rattans are an important forest produce in the South East Asian countries and are used principally for making furniture. The increase in world demand has led to an overexploitation of this forest resource as discussed in the earlier section. The most seriously affected species is *Calamus manan* due to its high economic value. It is being harvested indiscriminately resulting in a rapidly decreasing natural resource. The removal of mature plants is also causing scarcity of seeds, leading to serious regeneration problems. Besides, the solitary nature of this species precludes the use of conventional vegetative methods.

Plant tissue culture offers alternative methods for the propagation of plants. At the Forest Research Institute Malaysia, work in an IDRC-funded project has been in progress since 1984 for developing techniques for the mass-propagation of commercial rattans, principally *C. manan*. Two methods of propagation have been developed, the multiple shoot formation method and shoot formation from callus.These are described below to serve as models for similar work that might be undertaken elsewhere. In both the methods, the initial explant used is the mature embryo from the seed.

Excision of Embryo

The scaly pericarp and the fleshy sarcotesta of mature fruits are first removed to obtain the hard seeds. These are washed several times with water and surface sterilized in 20% Clorox to which a few drops of Tween 20 have been added as a surfactant (wetting agent). After 10 minutes in the sterilizing solution, the seeds are washed several times with sterile distilled water to remove all traces of chlorine.

The embryo is located in a cavity immediately below the operculum (Fig. 25). It is first exposed by carefully teasing away the hard endosperm surrounding

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4 Developed by Aziah Mohd. Yusoff, Forest Research Institute Malaysia, Kepong, Malaysia.

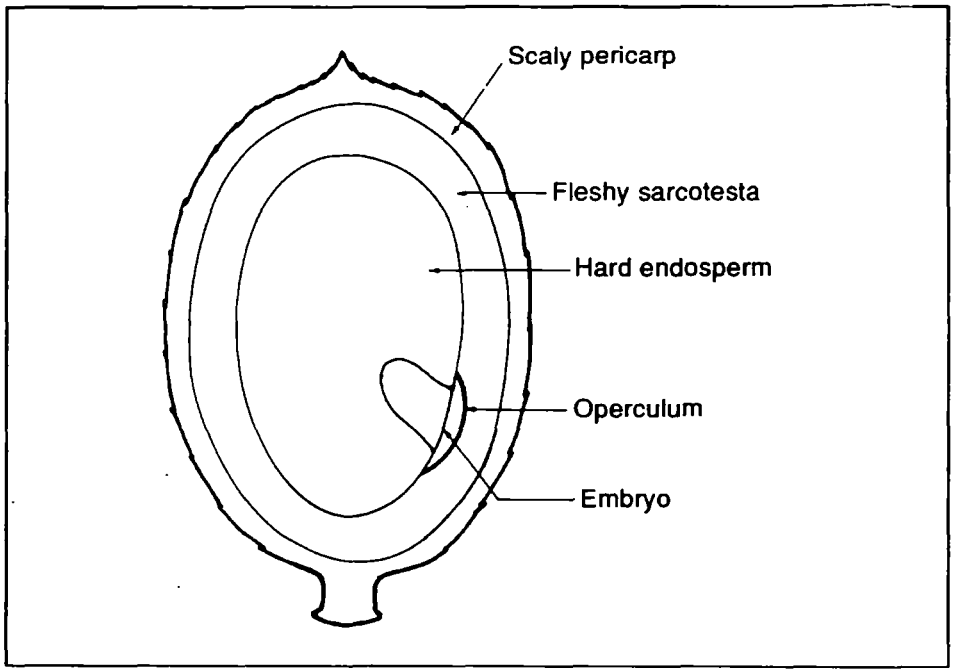


Fig. 25: Vertical section of fruit of Calamus manan showing position of the embryo.

the embryo (Fig.28). The embryo is then removed using a pair of forceps (Fig.29) and inoculated onto the solid medium.

Multiple Shoot Formation Method

Raising of Seedling and Excision of the Explant

The multiple shoot formation method is outlined in Figure 26.The embryos were inoculated in the Murashige and Skoog (1962) basal medium supplemented with 0.5% agar, 3% sucrose, α-naphthalene acetic acid and 6-benzylaminopurine. The pH of the medium was adjusted to 5.7 before autoclaving at 121°C for 15 min. Cultures were maintained at 28°C and at high humidity (ca. 80% RH) with a 16-h photoperiod of 400-600 lux.

After germination, seedlings (about 90%) having distinct collar regions are selected (Fig. 30) which contains the shoot apex region within. This collar region is excised out and used as the explant.

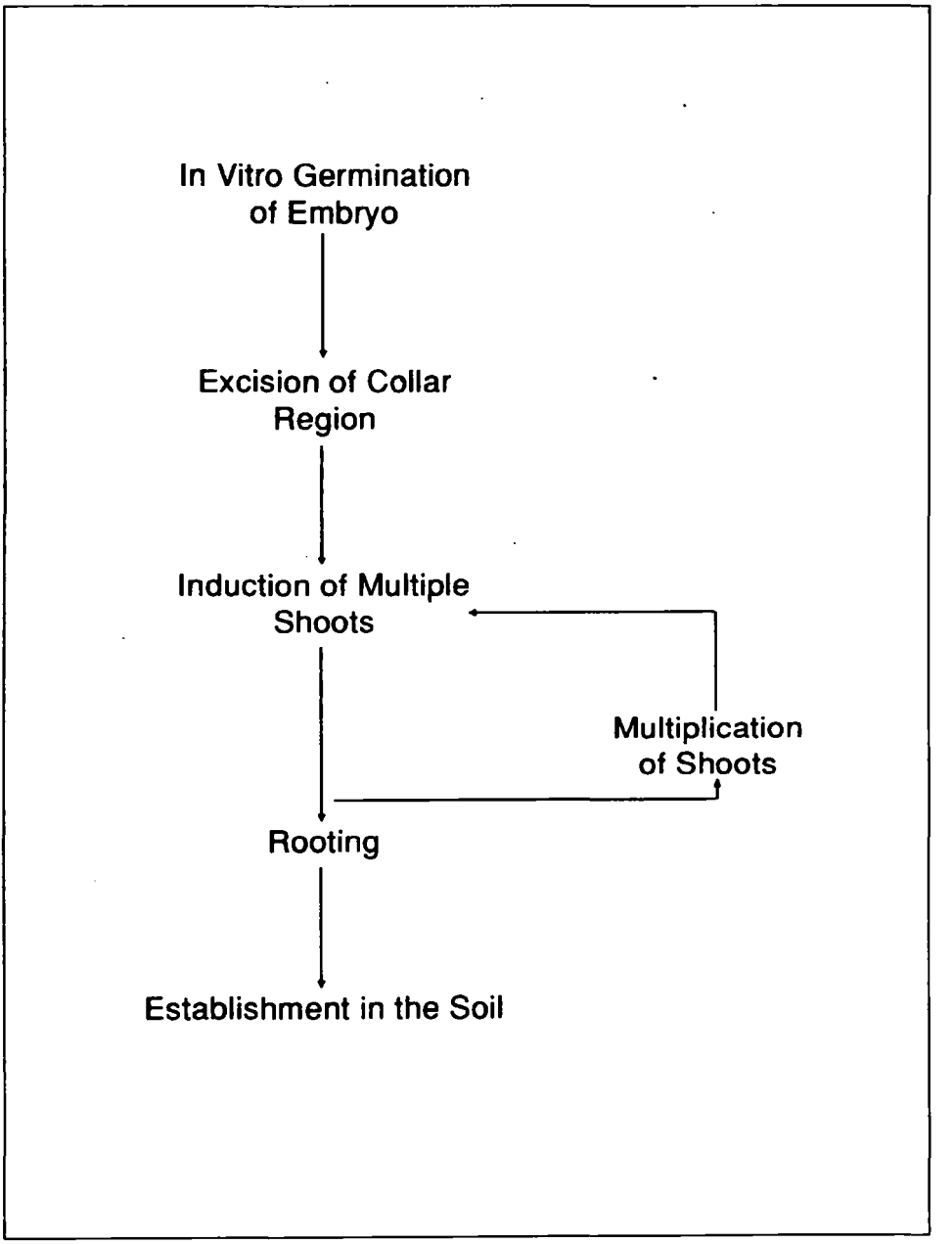


Fig. 26 : Outline of the multiple shoot formation method.

Induction of Multiple Shoots

The collar region explant is inoculated in MS basal medium supplemented with 0.5% agar, 0.3% sucrose and a combination of NAA and BAP. The cultures are maintained in semi-darkness. After about 1 month of culture, whitish nodular structures develop from the basal end of the explant (Figs. 31,32). The explant is subcultured into the same medium at monthly intervals until the nodular structures develop into greenish conical multiple shoots from which leaves emerge (Fig. 33).

Rooting of Shoots

Shoots 3 to 4 cm in height are excised from the multiple shoot structure and rooted in vivo. The shoots are treated with a commercial rooting powder and 0.05% Benlate and planted into rooting boxes containing a 1:1 mixture of sand and forest topsoil (Fig. 34). The shoots produced vigorous root systems in 2 months time.

Establishment in the Field

The rooted plantlets are transplanted after 2 months from the rooting boxes to polybags (Fig. 43). The potted plants are maintained under 50% sarlon-netting shade for 4 to 5 months to prevent drying and scorching and are planted in the field when 30-35 cm tall (Fig. 44).

Shoot Formation from Callus

The method of shoot formation from callus is outlined in Figure 27. Embryos isolated from seeds are used as explants. MS medium is used, supplemented with thiamine-HCl (1 mg/l), pyridoxine-HCl (1 mg/l) and nicotinic acid (1 mg/l). Other components of the medium are m-inositol (0.1 g/l) and casein hydrolysate (0.5 g/l). Sucrose is added at 3% and the medium gelled with 0.5% of Difco Bacto-agar.

For induction and multiplication of callus, 2,4-D or NAA is added at 5×10^{-6} to 10^{-4} M. BAP and Kn are used to induce shoot formation at 10^{-4} to 10^{-8} M. GA_3 at 10^{-7} to 10^{-8} M is added to the medium to promote shoot elongation. The cultures are incubated at $28 \pm 2°C$ with a light intensity of 400-600 lux and a 16-h photoperiod.

Induction of Callus

A flow-chart of the method is given in Figure 27. The embryos are isolated in the manner illustrated in Figures 28 and 29.

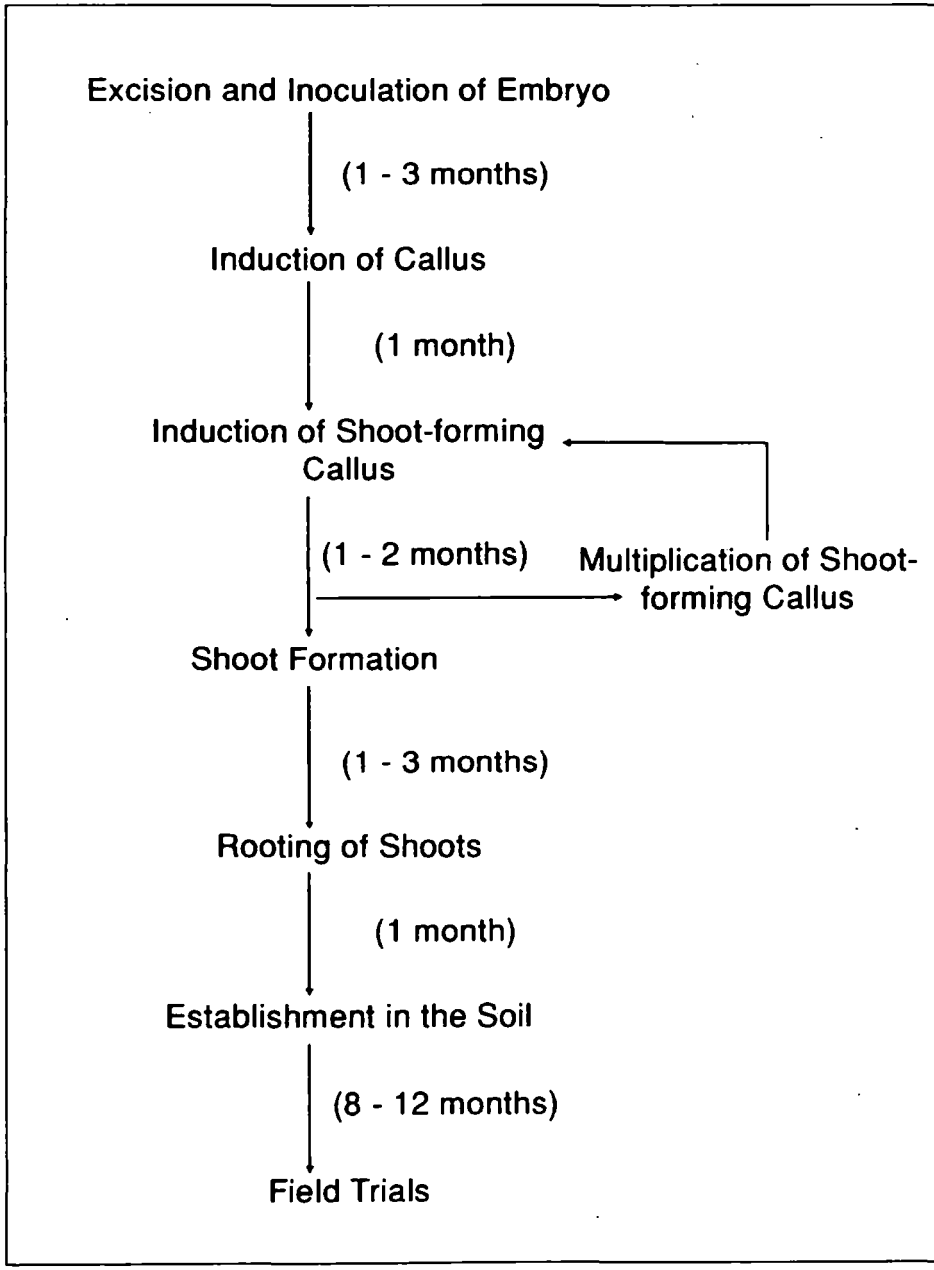


Fig. 27 : Outline of the method of shoot formation from callus.

When the embryos (Fig. 35) are cultured on solid MS medium supplemented with 2,4-D, most of the embryos enlarge, become irregular in shape and later callus (Fig. 36). The embryo callus is isolated from the original explant and later multiplied on either a 2,4-D or NAA-supplemented medium. Once the primary callus produces the secondary callus, subsequent multiplication is easy. The secondary callus is whitish, watery and translucent (Fig. 37). Upon further subculture, it turns opaque, yellowish and nodular. Sometimes the callus turns brown and does not grow further (Fig. 38).

Induction of Shoots

The callus derived from embryos develops shoots when BAP or Kn is included in the medium after the seventh subculture. It first becomes more opaque and the nodules enlarge (Fig. 39). Later greenish protuberances develop from the nodular structures (Figs. 40,41).

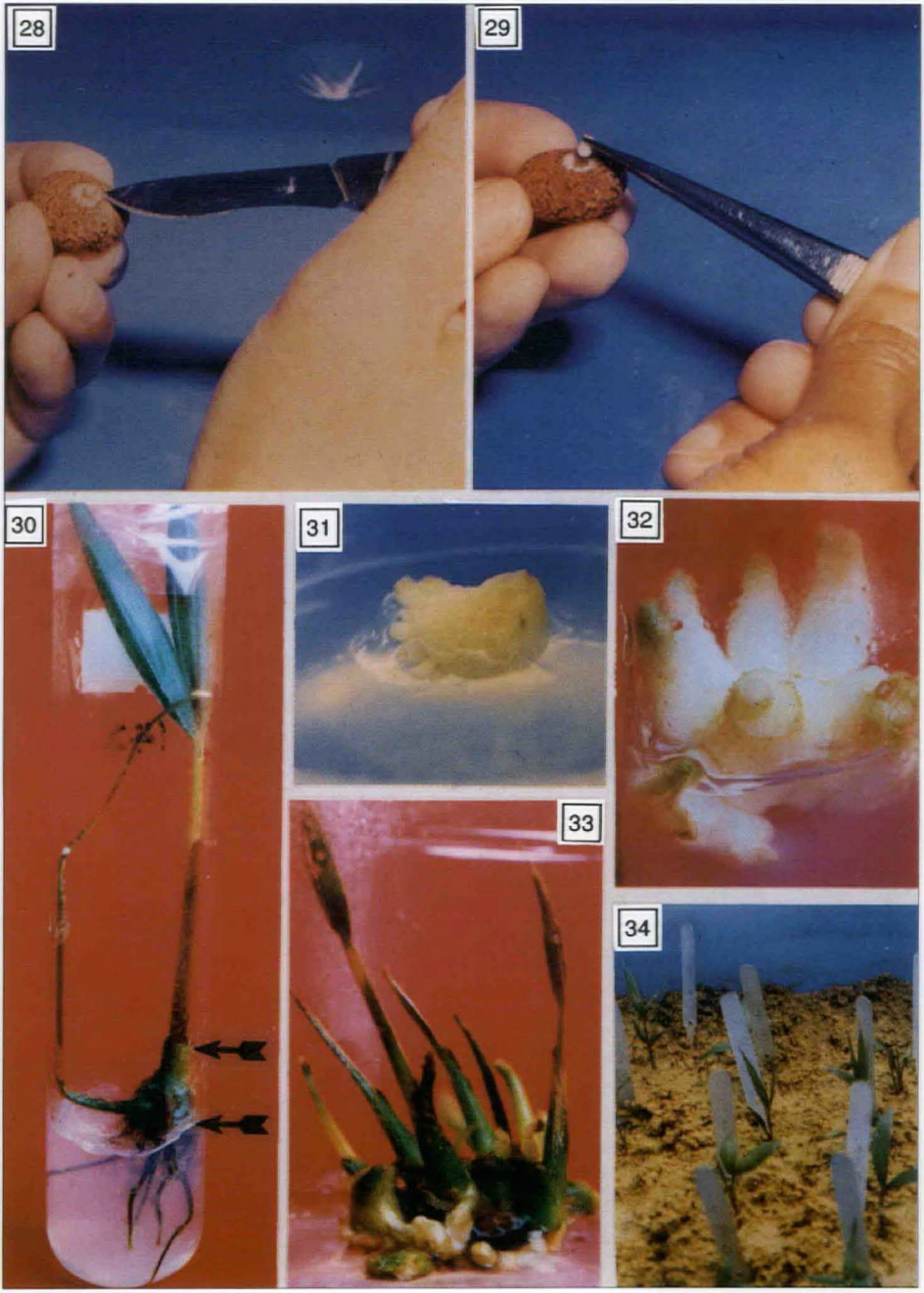
Shoot Elongation, Rooting and Establishment in Soil

Shoot development and elongation takes place when the callus with green protuberances is transferred to solid MS medium supplemented with GA_3. When shoots 4-5 cm high are isolated and transferred to 25% MS medium supplemented with IBA, rooting is induced (Fig. 42). The method followed for potting-out of the tissue culture-raised plantlets is similar to that described for plantlets produced through the multiple shoot method (Figs. 43,44).

Conclusions

Intensive shoot production has been obtained by the multiple shoot formation method using the collar region as the explant in comparison to that from callus. In both methods the embryo is the initial explant. Although plantlets have been produced, potted and even transferred to forest sites through the method of shoot formation from callus, this method needs further refinement before it will be useful for mass-propagation. Establishment of an efficient protocol for somatic embryogenesis from callus will facilitate the production of thousands of plantlets from an initial explant, i.e. embryo.

On the other hand, progress with the multiple shoot formation method has been more rapid and has resulted in the production of a greater number of plantlets in a shorter period. The oldest plantlets transferred to the forest sites are more than 1 year old and their growth rates are comparable with those of the seed-raised plants.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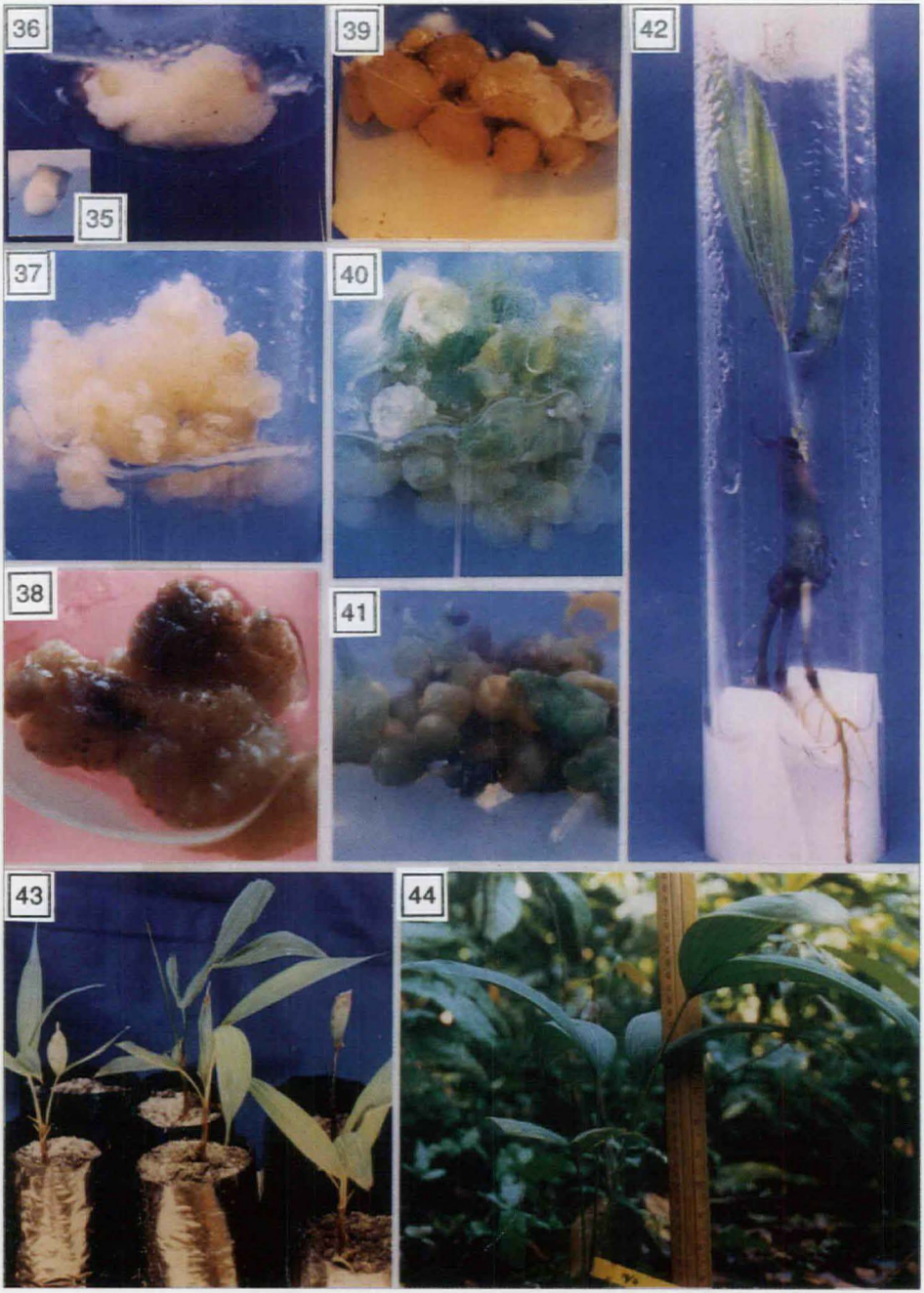
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Figs. 28, 29: Excision of embryo.

Fig. 30 : In vitro germinated seedlings. Arrows indicate the collar region of explant.

Fig. 31 : Whitish shoot buds developed on the basal end of collar region of explant.

Fig. 32 : Further development of shoot buds into whitish conical structures.

Fig. 33 : Formation of multiple shoot structures.

Fig. 34 : In vivo rooting of regenerated shoots in rooting box.

Fig. 35 : Newly inoculated embryo.

Fig. 36 : Initial callusing of *C. manan* embryo.

Fig. 37 : Multiplication of primary callus into secondary callus.

Fig. 38 : Browning of callus.

Fig. 39 : Development of embryogenic callus which is nodular and opaque.

Figs. 40, 41: Greening of embryogenic callus and development of greenish protuberances.

Fig. 42 : Rooted plantlet.

Fig. 43 : Establishment of plantlet in soil.

Fig. 44 : Establishment of plantlet in the field.

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Bamboo - The Miracle Grass - Film/Video

Rattan - Film/Video

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