5.0 Discussion

Palms, one of the most beautiful as well as economic groups of flowering plants, are essentially distributed in the tropics. Some of them especially the rattan palms form a vital component of forest and agricultural ecosystems and provide raw materials for cottage industries making handicrafts, furniture and other utility items. Rattans being climbing palms and dependent on other components especially the trees and microclimatic conditions of the forest ecosystem, their presence in abundance indicate the health of the forest as a whole. Calamus, the only rattan genus in South India, provides the cane of commerce and ranks next only to coconut (Cocos nucifera) and Arecanut (Areca catechu) in the order of economic importance in India. At the global level, rattans rank forth after coconut palm, oil palm and date palm (Renuka, 1999). Unlike the other three that are domesticated, rattans are still wild types confined to tropical humid forests.

In the state of Kerala, India, many palms are considered to be under threat (Nayar and Sastry, 1990) and rattans figure very prominently among them (Renuka, 1999). Rattans were once a low value product meant for inexpensive and often traditional furniture, used locally. Today, as part of the globalised economy, they are developed into a multi-million dollar rattan industry dominated by the ASEAN countries. Since the indigenous rattan resources are overexploited and their availability in nature is limited and insufficient to meet the vertical increase in demand, the gap between the rate of production and full capacity utilization in the cane processing units has widened remarkably (Venkatesh, 1990). It is not surprising; therefore, that many of the urban units are already closed down owing to shortage of raw material (Bhat et al., 1989). Over the years, the approach of the industry has been one of resource utilization even beyond the level of natural regeneration rather than initiating measures in association with authorities to recover and maintain natural stock of yielding palms in perpetuation. Technological intervention through artificial regeneration of sought-after and over-exploited rattan palms including the endangered ones through application of biotechnological methods and large scale cultivation holds great promise and is the need of the hour. It is quite unrealistic that large scale multiplications of the slow growing rattans are possible through conventional seed and vegetative propagation methods using suckers, cuttings and rhizomes. Since the demand for good quality rattans is growing
rapidly while the converse is true with available wild stocks, cloning through tissue culture may be the only option available now (Rao et al., 1990). Even otherwise when seed availability is scarce in the exploited rattan resources, and vegetative propagation methods are slow and impractical, micropropagation through tissue culture offers an attractive alternative (Wochok, 1981). The high cost of tissue cultured rattan saplings may be offset by the genetic gains possible through efficient use of known—high yielding palms for mass multiplication (Yusoff and Manoharan, 1985).

Contributions of Indian scientists in plant tissue culture are well recognized; however, they are not particularly focused at commercial applications (George and Sherrington, 1984). In the case of rattan palms, even fundamental problems of sex determination, seed viability, conventional propagation systems and cultivation packages and practices have not been addressed properly on scientific footing. Against this background, rapid propagation of the three economic species from low land (C. rotang) and mid ranges (C. thwaitesii, C. travancoricus) and consequent easy availability of planting materials attempted in this rather elaborate study is thought to fill up the void and spur a technology driven agro-forestry activity in Kerala and elsewhere in India and abroad where rattans are an important economic resource. It is also the wish of the author that the investigation reported perhaps for the first time in the Indian context, would make rattans amenable to in vitro handling, thereby enhancing the scope of applied research in these palms for future endeavors.

5.1 Direct multiple shoot formation in embryo cultures of C. thwaitesii, C. rotang and C. travancoricus

Under the climatic and other environmental conditions prevailing in the Western Ghats region, flowering of all the three rattan palms studied namely, C. thwaitesii, C. rotang and C. travancoricus occurs during August to November of the year and mature fruits are formed 10 months after flowering that is in the months of May, June and July. However, for the isolation and culture of embryos, somewhat immature, namely green/greenish-yellow fruits obtained during the second half of November - January (C. thwaitesii) and first half of February - March (C. rotang, C. travancoricus) are more preferred than the mature yellow fruits of the later periods which are difficult to cut open without damaging the embryos. Nearly 20-50% of the embryos dissected out of mature fruits were damaged to varied extent. The embryos were invariably intolerant to
wounding and produced phenolic oxidates which could not be controlled. Very often than not wounding caused death of the embryos and even other embryos that were washed off the oxidates, did not respond favorably during the course of culture.

Isolation of embryos from somewhat immature fruits was easily achieved at 95% success rate by cutting and splitting open the fruits vertically. While doing so, the embryos got disengaged from the mesocarp. The size of the embryos isolated varied depending on the fruit size and ranged from 1.0×0.7 mm in *C. travancoricus*, 1.1×0.2 mm in *C. rotang* and 1.3×0.3 mm in *C. thwaitesii*. The shape of the embryos was more or less the same (triangular, top shaped) in all the species and might show marginal variations depending on the relative maturity of the fruits.

Data presented in Tables 2, 3, 15 and 24 suggest that there was no need to surface sterilizes the embryos after isolation. In fact the fruits could be treated with 0.8% environment friendly surface sterilants like Teepol, Laboline and Savlon at 0.8% (v/v) concentration and washed repeatedly with sterilized water before dissecting out the embryos free of damage. If necessary, the surface sterilized fruits can be further flamed in 70% ethanol, without damaging the inner parts. This method is recommended for isolation of 95-100 % intact and healthy embryos. Instead, if the isolated embryos were further treated with 0.1% *HgCl₂*, more than 30% of the embryos were damaged and in 0.2% *HgCl₂* all the embryos got killed (Table 3). Since as much as 85% embryo germination could be obtained in *C. thwaitesii*, 76% germination in *C. travancoricus*, and 80% in *C. rotang*, the use of noxious *HgCl₂* can be avoided completely. In the reported cases of successful embryo culture in rattan, the surface decontamination of embryos involve the initial wash with a detergent, followed by immersion in commercial bleach or sodium or calcium hypochlorite and thereafter in 0.1% (w/v) *HgCl₂* for 5-10 min depending on the tenderness of the tissue (Sreekumar, 1997). It is well documented that treatment with hypochlorite solution at 5-7% for 5-10min is sufficient to prevent/eliminate bacterial contamination (Rice *et al.*, 1992). However, environmentally toxic, *HgCl₂* is a choice surface sterilant in many plant tissue culture experiments (Muna *et al.*, 1999) as it is efficient to eradicate fungi and bacteria (Zryd 1988; Monier and Ochatt 1995; Puchooa *et al.*, 1998). As expected and reported in many other species (Sreekumar, 1997; Puchooa *et al.*, 1998), prolonged period of treating in surface sterilants helped to reduce the percentage contamination of the explants when
mature tissue was used while tender tissues showed high percentage mortality. The methods and procedures followed in rattan (Barba et al., 1985; Dekkers and Rao, 1989) and coconut (DeGuzman, 1971; De Rosario and de Guzman, 1982) suggest that dissection of infection free embryos directly by surface sterilization of the fruits after immersion in commercial bleach(es) and flaming in alcohol is distinctly possible. Since the method is relatively easy and reproducible to culture and multiply endangered as well as economic species of rattan palms, it was adopted for the investigators reported in the present study. In all the candidate species (C. thwaitesii, C. rotang and C. travancoricus), the embryos were isolated from the immature green fruits without damage and germinated at high frequencies (85-98%) without loss. The method followed is akin to green pod culture in orchids where the fruit bodies (green capsules) are just flamed after a dip in ethanol before dissecting out the embryos for culture (Arditti and Ernst, 1993).

Different nutrient media have been used for embryo culture in palms especially date palm, oil palm and coconut palm. These include Murashige and Skoog (MS) medium and modified MS medium used in date palm (Reynolds and Murashige, 1979; Tisserat, 1981,1983) and oil palm (Rabechault et al., 1970, 1972; Corley et al., 1976; Martin and Rabechault, 1976) and Y3 nutrient medium used in date palm (Beauchesne et al., 1986), oil palm (Teixeire et al., 1995) and coconut palm (Eeuwens, 1976, 1978; Ahee et al., 1981; Blake and Eeuwens, 1982) and Beauchesne medium used in date palm (Beauchesne et al., 1986). In Calamus species, MS (Murashige and Skoog, 1962) medium has been already used for the embryo culture of C. yunnanensis and C. obvoideus, MS, B5 (Gamborg, 1968) and White’s (White, 1973) media for C. gracilis, C. flagellum, and C. nambariensis var. xishuangbannaensis and MS, modified MS, White, and B5 media for C. tetradoxylus, C. simplicifolius and D. margaritae. These published reports indicate that full strength MS medium can be reliably employed compared to its depleted variants (½ MS, ¼ MS) and other nutrient media viz. Y3 (Eeuwens, 1976), WPM (Lloyd and Mc Cown 1981) for embryo culture of rattan palm. Since salt concentration of MS formulation is quite high, it may be assumed that embryos of rattans in culture prefer high concentrations of salts for their growth and differentiation. This was evident from the reduction in the period of embryo culture initiation from 4 weeks in Y3 medium to 2 weeks in MS medium in C. thwaitesii. Under such conditions 3 subcultures of 6 weeks each were only required for the conversion of leafless seedlings into mature seedlings having normal leaves.
The role of activated charcoal in palm tissue culture particularly date palm (Reynolds and Murashige, 1979), oil palm by (Martin and Rabechault 1976) and coconut palm (Eeuwens 1976, 1978) has been variously interpreted. In the present study involving embryo cultures of both C. thwaitesii and C. rotang, supplementation of the medium with 0.1% activated charcoal facilitated rapid germination of the embryos perhaps by adsorbing phenolic oxidates and other small molecular weight toxic substances in the medium. Activated charcoal acted as a growth promoter especially to break dormancy and the large haustoria formed favoured quick germination and development into seedlings. However, as observed in C. rotang, after initial culture in presence of activated charcoal, the embryos should be recultured in charcoal-free medium to accelerate further growth into seedlings. In all the three species, three subcultures of 6 weeks each was required to obtain 9.21±0.48 cm (C. thwaitesii), 8.43±0.45 cm (C. rotang) and 10.57±0.15 cm (C. travancoricus) seedlings with fully functional leaves and in 6 month period, the haustoria were reduced into rudimentary dried up structures having no role in nutrition as the seedlings have already become autotrophic/ photoheterotrophic structures capable of surviving on their own without haustoria. The formation of haustoria in any case mimicked the developmental pattern in nature; however, the difference being that they are short lived in vitro. Haustoria formation is also reported in embryo cultures of Cocos nucifera (Lopez-Villalobos et al., 2001) and Calamus thwaitesii (Ramanayake, 1999). The take home message of these germination studies (Tables 5 and 7) is that use of activated charcoal at 0.1% (w/v) in the nutrient medium facilitates early germination of Calamus embryos presumably by adsorbing low molecular weight inhibitors and breaking physiological dormancy.

There was also evidence that concentration of sucrose used in the medium influenced the developmental pattern of cultured embryos in later stages. While 3% sucrose was optimal for normal shoot development in the seedlings, 5% sucrose promoted maximum root formation. It appears that high concentrations of sucrose (5-6%) induced additional root formation and contributed to enhancement of root-related characters and functions without affecting/contributing to shoot characters and function. The results (Table 6) suggest that root and shoot characters are tightly compartmentalized in seedlings of Calamus developing in vitro. Profuse root differentiation in vitro is an energy intensing process (Hazarika, 2003) which is facilitated by the relatively high concentration of sucrose. The participation of oxidative
metabolism in differentiating tissue cultures promoted by the presence of sucrose in the medium is corroborated by the findings of previous workers (Fowler, 1983; Merillon et al., 1984; Wainwright et al., 1989).

Zygotic embryos of *C. thwaitesii* cultured in presence of growth regulators showed remarkable increase in size long before bud differentiation from their base in 8 weeks. These early growth responses especially rapid intake of water and nutrients may be dependent on exogenous supply of PGRs as the embryos cultured in basal medium were free of such pronounced growth responses. Among the various morphogenetic differences induced by PGRs, haustorial formation was arrested by the addition of cytokinins. The inhibitory influences of cytokinins on haustoria formation are not particularly reported in any other *in vitro* culture system involving palms. Since the haustorium is a transient structure primarily developed to aid in the nutrition of the maturing embryo and early formation of seedling, the cytokinin's inhibitory influence on haustoria formation may be related to its negative influence on existing organizational structure and functional polarity followed by induction of multiple morphogenetic (caulogenic) centers in the embryos. As a result, under the influence of cytokinins, more than a few shoot initials are formed contributing to enhanced caulogenic responses. Presence of cytokinins may also the obviate need for nutritional assistance to the newly formed shoots rendered by the haustorium. The haustorium being a vestigial structure could be dispensed with when favourable conditions for shoot development, especially in presence of cytokinins, exist.

Among the cytokinins investigated for the shoot bud formation in embryo cultures, TDZ at concentrations 0.05-0.1 mg/l was the best to induce maximum percentage and number of bud formation. All the other cytokinins (BA, 2-iP and kinetin) were weaker than TDZ, and among the weaker ones better responses were achieved in BAP (30%) as in *C. thwaitesii* than in 2-iP and kinetin. In fact, in this species multiple shoot formation did not occur in any of the concentrations of 2-iP and kinetin used (Table 7). So also in *C. travancoricus*, there was high percentage of single shoot formation in these cytokinins. It was also of interest to note the differences in the number of shoots formed between the species. More number of buds (6.2) were formed per embryo in *C. travancoricus* than in *C. thwaitesii* (3.6). However, percentage multiple shoot formation (73.3%) was higher in *C.thwaitesii* compared to bud formation (54.44%)
in *C. travancoricus*. By and large, on a comparative basis, less percentage of embryos formed of shoots in *C. travancoricus* than in *C. thwaitesii* in presence of TDZ or a combination of TDZ and BAP. The differences between the species in their requirement of TDZ also varied, as optimal concentrations for shoot differentiation in embryo culture was 0.05 mg*l*⁻¹ in *C. rotang* to produce as many as 58.2 shoots per embryo through culture initiation and multiplication phases (Table 19) as against 0.1 mg*l*⁻¹ recorded in *C. thwaitesii* and *C. travancoricus*. Percentage fasciated shoot formation increased with increased concentration of TDZ and decreased on supplementation with other cytokinins (BAP/ BAP and 2-iP). In any case, it was not desirable to use more than 0.1 mg*l*⁻¹ TDZ individually in embryo cultures of any of the species. Significant portion of the shoots (66-95%) was fasciated when concentration of TDZ exceeded 0.2 mg*l*⁻¹. Although, combinations of BAP-TDZ-NAA (0.4:0.1:0.1) mg*l*⁻¹ was the best to obtain 2.86±0.27 shoots per embryo at 76.67% efficiency in *C. thwaitesii*, individual concentration (0.1 mg*l*⁻¹) of TDZ yielded maximum number of shoots (*C. travancoricus*). Overall, the results suggest that TDZ at concentrations 0.05-0.1 mg*l*⁻¹ may be indispensable to obtain justifiable number of shoots from embryo cultures. Although in combination with other cytokinins it may produce non-fasciated shoots, sustainable use of this system for consistent production of faithful copies of plants needs to be established. Unlike, many other palms, rattan embryos preferentially responded well in presence of TDZ and this potential can be exploited for commercial purposes. It may be safe to conclude that unlike many plant species where cytokinins other than TDZ are successfully employed, in *Calamus* embryo-cultures, use of TDZ is indispensable for successful culture initiation.

Although combinations of cytokinins including TDZ were not the very best for shoot initiation in embryo cultures of different species of rattans, for multiplication, continued use of TDZ alone is not desirable as it inhibited shoot elongation. Isolated shoots of *C. thwaitesii* subcultured in presence of 0.4 mg*l*⁻¹ BAP, 0.1 mg*l*⁻¹ TDZ and 0.1 mg*l*⁻¹ NAA produced 5.22±0.36 buds in 86.67% of the shoots (Table 8) without seriously inhibiting the growth of the shoots. However TDZ could not be dispensed with even for shoot multiplication in all the species. Multiplication of shoots (8.50±0.63 buds from each shoot in 82.50% of the shoot cultures) in *C. travancoricus* was more rapid than in *C. thwaitesii* (3.75±1.09 shoots in 76.67 % of the shoot cultures). The multiplication rates recorded in *C. rotang* embryo culture were higher than both
C. thwaitesii and C. travancoricus. Again, in vitro response-wise, C. rotang differed from other two species as the responses were rapid in 6-weeks subcultures in relatively low concentration of TDZ (0.05mg/l) compared to 8-week subcultures in 0.1mg/l TDZ required for other species. However, replacement of TDZ by other cytokinins could not be done even in C. rotang as it resulted in poor multiplication of shoots.

Since the shoots initiated and multiplied in presence of TDZ were short and stunted, shoot elongation phase was indispensable. This had to be done by harvesting the individual stunted shoots (1.87 cm) and transferring them to basal agar and liquid media or medium supplemented with cytokinins (BAP, 2-iP) and auxin (NAA). Unlike other two species, two subcultures of the shoots in basal liquid medium were sufficient to get fairly elongated shoots in C. rotang. In C. travancoricus, relief from TDZ was best achieved through 2-3 subcultures of 6 weeks each in MS liquid/agar medium supplemented with 0.5 mg/l each of BAP and 2-iP and 0.1 mg/l NAA to obtain 5.45±0.59 cm shoots. Although use of liquid basal medium was equally good, the use of liquid medium supplemented with cytokinins and NAA had the added advantage of inducing shoot elongation as well as shoot multiplication simultaneously. In C. thwaitesii again liquid basal medium was the best to induce shoot elongation. Medium supplemented with low concentrations of cytokinins (BAP and 2-iP) and NAA induced shoot elongation as well as multiplication of additional shoots as in C. travancoricus but shoot elongation was slow. Since separate shoot elongation phase was unavoidable, it is recommended that use of MS basal liquid medium through two subcultures of 6 weeks each would be sufficient to obtain reasonably long shoots that could be used for rooting. Most of the attempts to have elongated shoots were arrived at in a way, to get rid of the inhibitory influence of TDZ on shoot elongation. This was better achieved in liquid than in agar medium, the former, as expected facilitating easy uptake of nutrient for normal growth. Similar negative influences of TDZ are reported in such other species as Bambusa edulis (Lin and Chang, 1998), apple (Fasolo et al., 1989), and pear (Singha and Bhatia, 1988). The fact remains, however, that this is the first time that TDZ is used for shoot multiplication in embryo cultures in three of the most economically important species of Calamus and in all the three, remedial intervention to obtain elongated normal shoots consisted of transfer of shoots to a secondary medium often lacking TDZ or with a different balance of plant growth regulators. Use of primary and secondary media has been reported in Populus (Russell and Mc Cown, 1986) and Rhododendron (Preece and
Imel, 1991). A primary medium can be used to maximize shoot proliferation, and after a sufficient time, the shoot or bud masses can be transferred to secondary medium with or without other combinations of plant growth regulators for shoot elongation (Huetteman and Preece, 1993). Based on the data, the author presumes that the demonstrated indispensable use of TDZ for multiplication using embryo cultures will go a long way in rapid propagation and conservation of the endangered rattan palms.

Unlike other species of rattan, the shoots of *C. rotang* were rooted (56.67%) even during the elongation phase (8 weeks) in MS basal liquid medium. However, this percentage could be significantly improved (90%) if NAA 1.5 mg{l}^{-1} was present in the medium during the post elongation phase. That the other auxins (IAA, IBA) regularly used for rooting in dicot and monocotyledonous species were relatively less efficient (73-76%), in inducing rooting in this species is a point to be considered. Similar responses were not observed in the shoots of both *C. travancoricus* and *C. thwaitesii* where rooting did not occur in basal liquid medium. Especially in *C. thwaitesii* shoot elongation phase (16 weeks) was long and equally long period (16 weeks) was required to root the shoots with 3-4 mg{l}^{-1} IAA or NAA to induce rooting at 93.33% level. In fact, in *C. thwaitesii*, NAA was more preferred than other auxins for initiating roots in the shoots. Unlike *C. thwaitesii*, elongated shoots of *C. travancoricus* readily formed roots at 100% efficiency in presence of 2-3 mg{l}^{-1} IAA, IBA or NAA. The observed differences in rooting responses between species may be related to genetic/physiological constitution and also the ability to accumulate auxins endogenously in each species. A threshold level of auxins was synthesized endogenously which caused rooting of the *in vitro* formed shoots even without exogenous auxin addition especially in *C. rotang*. It is not uncommon for certain species of dicots to root *in vitro* without exogenous addition of auxins (Sudha and Seenl, 1996; Jose *et al.*, 2007).

The rooted seedlings raised in embryo cultures were established in pure sand medium in polybags or in pots at moderate level (56-63%). If the plants were hardened for 3-4 months in a mist chamber, the establishment rates were increased to 72-96% in different species. The use of pure sand rather than any other mixture in the potting medium may help in easy penetration of roots and also avoid infection encountered in top soil and farmyard manure. The establishment of the seedlings with or without hardening is important as the seedlings had either single or only minimum number (1-2)
of roots. The hardy nature of the roots greatly helped the seedlings to establish in the sand medium. In the published reports on rapid clonal multiplication of Morinda umbellata (Nair and Seeni, 2002) also, sand medium has been successfully used and minimum number of functional roots as reported were sufficient for optimal establishment.

The period required for hardening and green house/net house establishment was long (6 months) due to the slow growth of even the seedlings otherwise raised in vitro under best nutrient milieu. At least 5-6 month intervening period was required before the seedlings could be transferred to the nursery or forest segments for further observation. Similarly observations in the establishment of the reintroduced plants, irrespective of the species were done for a minimum period of six months or more. This long period was required to observe continuous growth of the otherwise slow growing palms. The formation of the first new leaf in the reintroduced/translocated plant itself took nearly two months, after which under the most favourable agro-climate conditions prevailing in the reintroduction areas in the Western Ghats segments of Kerala during the monsoon months of June, July and August, 1-2 new leaves were produced within 2-4 months. This kind of encouraging performance of the tissue cultured plants after reintroduction is also recorded in such species as Blue Vanda (Seeni and Latha 2000) which is attributed to the best health status of the in vitro-derived plants. It is reported that eight weeks after establishment, the micropropagated rooted plants of Plumbago rosea transferred to field produced plumbagin rich tuberous roots much quicker than conventionally propagated plants (Satheeshkumar and Seeni, 2003).

Long term observations on the established plants through 3-year period in C. thwaitesii also confirmed 81-86% success rate of the embryo culture-derived plants. This percentage was even higher in the other two species (90% in C. travancoricus and 95% in C. rotang). The established plants continued to grow uniformly in a given site of reintroduction and was free from morphological and growth defects indicating the desirability of using the embryo culture method for safe handling and revegetation of the degraded and accessible forest areas with these plants. The differences observed in the growth of the plants in different sites were negligible. However, observations need to be continued for 8-10 years when the reintroduced/translocated palms are expected to flower. As of now, there is absolutely no information on gender ratio in the embryo
culture derived plants and their ability to set flower and fruits, possible sterility in them and the quality of canes produced in these palms or any other rattan palm produced in vitro. These basic data need to be included before recommending the embryo culture method for revegetation purposes in toto.

5.2 Embryogenesis in embryo callus cultures of *C. thwaitesii*

In order to augment planting material production and supply in rattan palms, embryos as opposed to vegetative tissues of mature palms are the explants of choice for in vitro culture of both clustering (Dekkers and Rao, 1989; Padmanabhan and Ilangovan, 1993) and solitary (Yusoff and Manokaran, 1985; Yusoff, 1989) types. With a few exceptions, the embryos are often released from the mature fruits by cutting open the hard endosperm and cultured invariably in Murashige and Skoog (1962) medium to produce single seedlings (Padmanabhan and Ilangovan, 1989; Chuthamas *et al.*, 1989) or callus in which caulogenesis was induced later (Gunawan and Yani, 1986; Yusoff, 1989; Padmanabhan and Ilangovan, 1993). In the present study, the use of somewhat immature green fruits of *C. thwaitesii* collected in the month of November was found most suitable to release with ease the embryos more than 95% of which were free from damage. The endosperm of the ripe fruits collected in the month of February being stony, was broken with difficulty to yield embryos more than 70% of which were damaged and could not be used for culture. As already demonstrated in the previous section, nutrient formulation of MS without supplementation was sufficient to induce germination of all the immature embryos into typical seedlings with normal haustorium, root and shoot formations as in mature embryos (Padmanabhan and Ilangovan, 1989) while auxin supplementation promoted callus proliferation. Therefore, we recommend the use of relatively younger fruits for easy isolation and culture of intact embryos in rattan palms.

The establishment of embryogenic callus cultures in *C. thwaitesii* offers yet another system characteristic of some species and not others of *Calamus*. So far embryogenesis is reported only in a few tissue culture studies of coconut (Eeuwens 1976, 1978; Apavatjrut and Blake, 1977; Pannetier and Buffard-Morel, 1982), oil palm (Martin and Rabechault, 1976) and rattan viz. *C. tetradactylus, C. simplicifolius* (Zhang Fangqiu, 1993), *C. egregius, C. rhabdocladin, C. guangxiensis, C. merrillii* (Zeng Bingshan, 1997, 1999, 2000). In this context, the observed ability of the embryo derived callus to produce embryoids through repeated subcultures (Plate 4 e, f, g and h) makes the system
interesting as there is a distinct possibility of scaling up the production of embryos (embryoids). The independent vasculature of the differentiated embryoids (Plate 4i) validates the true nature of somatic embryo formation and the conversion of 65% of the embryoids into plantlets further confirm the potential use of the system for mass propagation of this economically important rattan palm. It appears that embryoids differentiated in cultures need to attain certain level of maturity and remain committed to the developmental process; otherwise as observed, the young and mature embryoids will normally develop into calloid structures rather than plantlets.

In both embryo and tissue cultures of rattan palms (Dekkers and Rao, 1989; Yusoff, 1989; Padmanabhan and Ilangovan, 1993; Kundu and Sett, 1999; Sett et al., 2002), date palm (Reynolds and Murashige, 1979), coconut (Gupta et al., 1984) and oil palm (deTouchet et al., 1991; Teixera et al., 1994), 2,4-D was by far the most frequently used auxin to produce calli which were subsequently used for plant regeneration through indirect organogenesis. Though the concentration (7 mg l\(^{-1}\)) of 2, 4-D optimised for callus formation in embryo culture of *C. thwaitesii* is in broad consensus with the concentrations used by other workers in different *Calamus* species, the results obtained (Table 13) suggest that 2,4,5-T is far more superior auxin than 2,4-D to induce consistent formation of semi-friable/friable and embryogenic calli in 92% of the embryos. The normal developmental path of the excised embryo to produce shoot and roots had to be reversed and this was better achieved with 2,4,5-T at lower concentration (3 mg l\(^{-1}\)) than with 2,4-D at high (7 mg l\(^{-1}\)) concentrations. Consequently, even at the suggested optimal concentration of 7 mg l\(^{-1}\) 2,4-D, apical dominance of the shoot tip of the emerging seedling could not be completely reversed and 20% of the embryos still formed roots and shoots with calloid outgrowth. In addition to the tendency to form organs to varied extent through the entire range of concentrations tested, the 2,4-D induced calli differed significantly in morphology, texture and physiology. After 16 weeks of culture, the compact and translucent segment(s) occupied significant part of the callus, and the subcultured semi-friable portion produced relatively few (10.13±0.07) embryoids in medium containing NAA and BA. On the contrary, the 2,4,5-T induced calli were essentially semi-friable, tended to be amorphous during subculture and were very much embryogenic with the formation of large number (21.14±0.06) of embryoids as discrete loose structures that could be easily separated. Although somewhat compact type callus was recorded in low concentrations of this auxin, the translucent callus was never formed
in any of the concentrations tested. These results (Fig 2) together with the unpublished data obtained in embryo cultures of other species of Calamus (C. rotang, C. hookerianus, C. travancoricus and C. nagabetai) suggest that there are obvious differences in callus inducing ability of 2,4-D and 2,4,5-T. Certainly, 2,4,5-T (92%) was more potent than 2,4-D (65%) in inducing calluses at high frequency. The better quality and discrete embryoids formed in presence of 3 mg/l or more of 2,4,5-T confirmed the desirability of using this auxin regularly for embryogenic callus induction in zygotic embryo cultures. Continued proliferation was possible by division and regular subculture of embryogenic callus in medium containing 7 mg/l, 4-D or 9 mg/l 2,4,5-T. In presence of 2,4-D, new embryoids continued to grow from the growing portions of the callus, while those from the relatively old portion of the callus could be separated and germinated in basal medium to develop into normal plantlets. The amorphous callus with the loosely held embryoids formed during subculturing of the essentially semi-friable callus may indicate the utility of such a callus for establishing suspension cultures of embryoids to achieve mass multiplication of the species. This is not possible with the 2,4-D induced semi-friable callus which seldom became amorphous. Both the auxins proved to be toxic at concentrations exceeding 12 mg/l, when 15-30% of the embryos were lost due to browning and necrosis. The loss of 8-12% of the non-responding embryos due to eventual yellowing or browning during culture in presence of the optimal concentrations of the auxins, is a matter for further investigation as possible irreversible damage of the embryos occurring during isolation could not be ruled out. The diversity of responses observed in the embryos subjected to a given treatment viz. organogenesis, callusing and browning at best indicated their physiological heterogeneity.

In most of the published works on rattans, the embryos or tissues were first cultured in a high auxin medium to produce callus and then the callus subcultured in medium containing high cytokinin - auxin ratio to induce multiple shoot formation. This may involve a number of subcultures of embryo callus before organogenetic competence could be induced as in C. manan (Rao et al., 1990). The shoots were then separated and rooted to obtain complete plantlets. The rapid multiplication achieved in the present study with initial callusing of embryos on media containing different concentrations of 2,4-D and 2,4,5-T in 16 weeks followed by division of the callus into ca 500 mg pieces, subculture of each callus piece in medium amended with BA and NAA to produce 10-20 embryoids in 6-8 weeks and germination of mature embryoids into plantlets in basal
nutrient medium is qualitatively different and confirms the higher propagation potential of *C. thwaitesii* through the embryogenic rather than organogenetic route. It is particularly so in the calli raised on 2, 4, 5-T which induced enhanced friability and embryogenic potential of the callus tissues. In fact, the demonstrated ability to produce up to 116 plantlets from a single zygotic embryo within a year in *C. thwaitesii* confirms the desirability of using embryogenic rather than indirect organogenetic route otherwise reported in general, and in *C. flagellum* (Kundu and Sett, 1999) and *C. tenuis* (Sett et al., 2002) in particular. The proliferation of embryogenic callus in presence of 2,4-D or 2,4,5-T, concomitant formation of embryoids from the callus in medium supplemented with low concentrations of BA and NAA, repeated harvest of embryoids through 2-3 subculture cycles and development of embryoids into plantlets in basal medium together form a novel plant regeneration system unrecorded earlier in *Calamus* species. The embryogenic competence of the tissues was so high that 15% of the plants raised from the 2,4-D induced embryogenic calli formed secondary embryoids precociously on the petiole and lamina parts which were separated and cultured in basal medium to germinate into individual plants at 62% success rate. The mature embryoids (2.0-3.0 mm in size) derived from the zygotic embryo callus, being organized bipolar structures, on culture in basal nutrient medium individually or as part of the callus were converted into rooted plantlets at 65% efficiency. However, the immature embryoids were callogenetic and probably not differentiated or determined to the extent of developing into mature embryos or plantlets in an organized/programmed fashion as the case may be. The results suggest that further refinement is particularly needed to convert all the embryoids obtained from different sources into plantlets.

The precocious formation of embryoids in clusters on leaf sheath and lamina of plants raised from embryoid-bearing calli served as an additional source of regeneration and multiplication of *C. thwaitesii*. There is as yet perhaps no precedence of somatic embryogenesis on *in vitro*-raised rattan plants. In date palm however, Sudhersan et al. (1993) reported the formation of somatic embryos on the young leaf lamina of *in vitro* plants. Since production of embryoids ceases after 2-3 subcultures of the embryo callus, it would be worth investigating the establishment of embryogenic suspension cultures from the zygotic amorphous embryo callus obtained during subculture in presence of 2,4,5-T or from the precocious proembryogenic cell masses proliferated upon the leaves of *in vitro* plants for continuous production of embryoids. Differences between 2, 4-D
and 2,4,5-T was further evident when precocious embryoid formation was observed in the leaves of the embryoid and embryogenic callus-derived plants of 2,4-D treatment. These results are qualitatively different from the results reported in embryo cultures of other rattan palms and have a match only with leaf tissue culture of oil palm by Martin and Rabechault (1976) and Ahee et al. (1981). Precocious embryoid formation was particularly absent in 2, 4, 5-T mediated embryogenic callus induction. Precociously formed embryoids were also converted into plantlets at more or less equal efficiency (62% in 8-16 weeks).

In the initial trials, more than 60% of the rooted plantlets were lost during the hardening and post-hardening phases in the nursery. That the method of coating of the plantlets with 50% glycerin originally developed for other species (Selvapandyan and Subramani, 1988) helped to prevent evapotranspiration and under such conditions of extra care, achieve 78% establishment indicated lack of epicuticular wax formation and possibly ineffective stomata on the leaves of in vitro derived plants which may be caused by high humidity and also by low levels of illumination (Sutter and Langhans, 1982) employed during culture. Perhaps, the dark respiration of the micropropagated plants as suggested by Yusoff (1988) also plays certain role in plant establishment after weaning. Finally, the high rate of survival and continued performance of 78-82% of the plants transplanted into the forests without morphological and growth abnormalities, and formation of 5-7 new leaves and spines all over the plant body during the three years after planting confirmed the uniformity of the plants. Apart from direct shoot regeneration, the results suggest that embryogenesis in zygotic embryo callus cultures is yet another option available to agro-industry for production and supply of planting materials in selected rattan palms.

5.3 Axenic seedling shoot cultures of *C. thwaitesii*, *C. rotang* and *C. travancoricus*

*In vitro* propagation of rattan has been tried more with seedlings than with adult explants. Success reported in seedling explants are: *C. yumnanensis*, *C. obovoideus* (Zhuang Chengii, 1991) *C. gracilis*, *C. nambariensis* Var. *Xishuangbannaensis*, *C. flagellum* (Zhiying et al., 1995) *C. manan* (Yusoff and Manokaran, 1985; Yusoff, 1989; Aziah Mohd.Yusoff and Darus Haji Ahmed 1989; Aziah Mohd Yusoff and Nur Supardi Md. Noor, 1993; Goh et al., 1999). Seedling explants unlike those from the adult palms are
developmentally in a juvenile state, are more meristematic than the adult ones and therefore are expected to respond better under in vitro conditions. The favorable responses of seedling explants compared to adult ones are reported in a number of plant species including the endangered Blue vanda (Seeini and Latha, 2000) and Quercus robur (Vieitez et al., 1985). Therefore, it is not surprising that in vitro multiplication of all the three rattan palms was possible with seedling explants. It is particularly so with axenic seedlings raised in vitro that are grown under controlled conditions of nutrition, temperature, illumination, etc. and are even otherwise expected to have better health status than garden/nursery grown seedlings. Besides, aseptic conditions employed for germinating the seeds/embryos make the surface decontamination of seedling explants unnecessary, thereby saving the explants from the damaging influences of surface sterilizing agents.

Intact shoots of 2-3 month old seedlings, with/without leaves dissected out and cultured in MS agar nutrient formulation responded well for organogenesis under the influence of plant growth regulators especially the cytokinins. Browning of the leaf sheath and leaves may occur as in C. thwaitesii but it did not interfere with the morphogenetic expression of the whole shoots. The collar segments i.e., the portion comprising the shoot apex and meristematic region of the developing seedlings may also be dissected out and cultured with accompanying browning which again did not affect the morphogenetic potential of the explants as demonstrated in C. rotang. In all the shoot cultures, the basal part comprising the shoot tip was the most responding, apart from the isolated collar segment. In order to raise start up shoot cultures, it is better to use defoliated and rootless whole shoot cultures. Invariably, whole shoot cultures responded with differentiation of buds from basal part (resident meristem of the shoot tip) in 8-16 weeks. Most of the cytokinins tested individually except TDZ were weak and in the order of priority TDZ alone (0.2 mg l⁻¹) and TDZ in combination with BAP (0.1 mg l⁻¹ TDZ and 0.4 mg l⁻¹ BAP) were preferred to obtain maximum shoot initiation in terms of frequency and number of shoots formed. Among the three species, the whole shoots and collar explants in C. rotang uniformly responded to these concentrations and combinations of cytokinins. However, unlike the other two species, in addition to TDZ and BAP, 0.1 mg l⁻¹ NAA was also needed for optimal responses of shoot proliferation and to some what shoot growth in C. thwaitesii. This species somehow differed from other two species as in presence of BAP, TDZ and NAA, a maximum of 56.67%
explants responded with the formation of fewer shoots. Although only 75% collar segments responded in *C. rotang*, the number of shoots (5.44±0.48) formed was quite high compared to other two species viz. *C. thwaitesii* and *C. travancoricus*. TDZ was indispensable and it can be combined with BAP at an approximate ratio to aid multiplication and shoot elongation in *C. rotang* and *C. travancoricus* and combination of BAP and NAA as in *C. thwaitesii*. Extensive studies in seedling explants with TDZ or combination of TDZ with other hormones as in the present case have not been made in any other rattan palm. Explants of *C. rotang* responded quicker (8 weeks) than *C. travancoricus* (12 weeks) and *C. thwaitesii* (16 weeks). These variations in response between species could not be attributed to reasons other than the genetic constitution of the species. Among the three species, *C. thwaitesii* was more robust, growing in evergreen, semi-evergreen and moist deciduous forests between 75 to 300 m throughout the Western Ghats areas than the other two. However, other reasons are not ruled out to explain the differences between the species.

Though shoot buds of *C. thwaitesii* were formed en masse in presence of 0.2 mg l\(^{-1}\) TDZ, the shoot buds remained more as a proliferating mass than as individual, elongated shoots. The use of TDZ alone inhibited shoot elongation and later rooting of the shoots as evidenced from the known influences of this synthetic cytokinin (Ellis *et al.*, 1991; Pijut *et al.*, 1991; Huetteman and Preece, 1993). Such masses of shoot buds formed especially around the base of the cultured whole shoots usually lacked rhizogenic activity and hence ratio of BAP-TDZ was crucial to induce the formation of somewhat elongated normal shoots. Addition of NAA (0.1 mg l\(^{-1}\)) to 0.4 mg l\(^{-1}\) BAP and 0.1 mg l\(^{-1}\) TDZ greatly helped to obtain normal shoot formation. Unlike *C. thwaitesii* (56.67%), the combination of 0.4 mg l\(^{-1}\) BAP and 0.1 mg l\(^{-1}\) TDZ induced maximum number of shoot formation (5.00±0.51) in 91.67% of the whole shoot cultures (comparable to 0.2 mg l\(^{-1}\) TDZ) and also collar segment cultures (75%; 5.44±0.48 shoots) in *C. rotang*. This kind of response also observed in *C. travancoricus* was not as much marked as in *C. rotang* and instead 0.2 mg l\(^{-1}\) TDZ (83.33%; 6.25±0.29 shoots) was the best for shoot initiation. The buds formed in *C. rotang* and *C. travancoricus* in presence of 0.2 mg l\(^{-1}\) TDZ were not proliferative as in *C. thwaitesii*, thereby indicating the suitable differences between the responses of the shoot cultures of different species. Although combinations of BAP and NAA also induced shoot bud formation in whole shoot cultures, the frequencies and number of shoot buds formed were less and could not be recommended. Therefore, it is
justified that 0.2 mg l\(^{-1}\) TDZ is used for mass shoot initiation in *C. thwaitesii* and *C. travancoricus* and a combination of 0.4 mg l\(^{-1}\) BAP, 0.1 mg l\(^{-1}\) TDZ used for shoot initiation in *C. rotang*. Hitherto, such individual hormonal concentrations and combinations have been used with certain success for shoot initiation in seedling explant cultures of *Nothapodites foetida* (Satheeshkumar and Seeni, 2000), Mangosteen (Sompong Te-chato and Mangkol Lim, 1999) and *Areca catechu* (Hsiang-chih Wang *et al.*, 2003) and certainly not reported for *Calamus* species.

The indispensability of TDZ for *in vitro* propagation of rattan tissues was evident during the multiplication stage as well. Each shoot bud formed in shoot cultures of *C. thwaitesii* and recultured in presence of 0.2 mg l\(^{-1}\) TDZ produced 4.89±0.31 buds at 90% efficiency in 6 weeks. The second best regime to induce the multiplication of 4.56±0.35 shoots of 2.36±0.29 cm shoots at 93.3% efficiency consisted of 0.4 mg l\(^{-1}\) BAP, 0.1 mg l\(^{-1}\) TDZ and 0.1 mg l\(^{-1}\) NAA. Other than these two, no other concentration/combination were preferred as both the frequency and shoot number was reduced dramatically. As also observed during culture initiation, combinations devoid of TDZ were no good for shoot multiplication, though shoot elongation was somewhat improved in them. However, a combination of 0.4 mg l\(^{-1}\) BAP, 0.1 mg l\(^{-1}\) TDZ and 0.1 mg l\(^{-1}\) NAA was adjudged the best due to some what elongated shoots in significant number obtained compared to 0.2 mg l\(^{-1}\) TDZ. In *C. travancoricus* also, combinations devoid of TDZ promoted less number and percentage of shoot multiplication. Shoot buds cultured in presence of 0.2 mg l\(^{-1}\) TDZ produced 7.00±0.35 and 7.25±0.37 shoots at 100% efficiency during 1\(^{st}\) and 2\(^{nd}\) subcultures each of 6 week duration. In *C. rotang*, different explant types (shoot, shoot tip, leaf) used for multiplication responded remarkably with 4-9 shoots/ explant in 6 weeks. Comparative performance revealed that shoot tip produced more shoot buds (8.40±0.18) than isolated leaves (6.80±0.43) and whole shoot (7.40±0.46) preferably due to immediately responding resident apical and axillary meristem present in it. A combination of 0.4 mg l\(^{-1}\) BAP and 0.1 mg l\(^{-1}\) TDZ proved the best to multiply elongated shoot types through at least 3 subcultures at high frequencies. As the number of subcultures increased, marginal increase in the number of shoot buds perhaps due to better adaptation occurred. It was evident that the activity of apical meristem present in shoot tip cultures slowly got arrested while buds proliferated from the base due to axillary/ accessory meristem formation. In any case, the availability of more than a few leaves and shoot tip in addition to whole shoot enabled better harvest of
19, 23 and 21 shoots from different explants viz. leaf base, shoot tip and whole shoot respectively. Each shoot having 3-4 leaves can produce up to 32 shoot buds in 6 weeks period and thus made mass multiplication distinctly possible. Since all the leaves of in vitro-derived shoots were meristematically active, leaves contributed to better multiplication rate than other single explants put together. However no difference in shoot bud multiplication was observed between liquid and agar media. By and large, explants of seedlings in *C. rotang* are more regenerative and responded with ease compared to other two species (*C. thwaitesii, C. travancoricus*).

Thidiazuron has been shown to induce higher shoot/bud proliferation rate than other purine-based cytokinins in a number of woody species, including *Fagus sylvatica* (Vieitez and San-Jose 1996). *F. orientalis* (Cuenca et al., 2000) and *Salix nigra*, although in many species TDZ-induced buds failed to elongate into shoots (Lyrya et al., 2006). There have been many reports on poor elongation of TDZ induced shoots, which may be consistent with its high cytokinin activity; the concentration and duration of exposure to this compound is critical in this respect (Lu 1993; Murthy et al., 1998; Debnath 2005). In the present study also shoot elongation did not occur during enhanced shoot multiplication in presence of TDZ or combination of TDZ, BAP and NAA as the case may be. Shoot elongation was better achieved using liquid or agar medium free of hormones (Russell and Mcown, 1986; Singha and Bhatia, 1988; Fasolo et al., 1989) or medium containing 0.5 mg/l each of BAP and 2-iP and 0.1 mg/l NAA. Irrespective of differential origin of buds from source tissue (shoot tip, leaf, whole shoot), they responded alike during the elongation phase. As such, the characteristic feature of this phase are absence of TDZ in the nutrient medium and marginal multiplication of shoots along with shoot elongation through two (*C. thwaitesii* and *C. travancoricus*) and three (*C. rotang*) subcultures of 6 weeks each. Though both agar and liquid media were not tested in all the species, detailed studies carried out with the shoot buds (2-3 cm) revealed best results were achieved with hormone free liquid medium where up to 6.49±0.84 cm long shoots could be harvested after 2 subcultures. For both elongation and multiplication, however, agar medium supplemented with 0.5 mg/l each of BAP and 2-iP and 0.1 mg/l NAA is preferred. Similarly, in the case of shoots either single or groups of 2-3 shoots drawn from the multiplication phase may be used with the same efficiency. It appears that inclusion of NAA in the nutrient medium contributed to some extent to the elongation process. It is possible that simultaneous additional formation of
1.5±0.11 to 2.20±0.17 shoots during shoot elongation is due to the residual effects of TDZ from the multiplication medium.

The multiple shoots obtained in *C. rotang* behaved somewhat differently during shoot elongation phase. The buds/shoots (1-2 cm) subcultured in liquid medium in presence of 0.5 mg/l BAP, 0.5 mg/l 2-iP and 0.1 mg/l NAA not only developed into 7-10 cm long shoots but also they formed 1-2 roots at 66.67% success rate (Table 40). The combined multiplication, elongation and rooting was not observed in other species. This may be due to the differences in endogenous concentration of hormones present in this species compared to other two species of rattan. Such observations are not specifically reported in seedling explant derived shoot cultures of any other rattan palm. Critical analysis of the results indicate that for continued elongation together with multiplication, elongation of shoots and rooting, use of a combination of 0.5 mg/l each of BA and 2-iP and 0.1 mg/l NAA is desirable. Rooting of the shoots was achieved with ease by supplementation of the medium with auxin (NAA, IBA). Although the rooting frequencies were more than 90% in all the three species, most of the shoots were single rooted in *C. rotang*, 1-2 rooted in *C. thwaitesii* and 2-4 rooted in *C. travancoricus*. In general, IAA, the natural auxin was a poor substitution to NAA for rooting. Similar results are also reported in other species of rattans viz. *C. simplicifolius* (Zhang Fangqiu, 1993) *C. egregius* (Zeng Bingshan, 1997). The process of rooting was slow, often taking 2-3 months before the plants could be deflasked. The fact that nearly 67% of the shoots got rooted even during shoot elongation could in no way be correlated with an increase in the number of roots. Since most of the plants were single rooted, the ability to form only 1-2 roots may be genetically controlled in certain species of *Calamus* including *C. simplicifolius* (Zhang Fangqiu, 1993) and *C. egregious* (Zeng Bingshan, 1997).

Rooted plantlets were either separated or potted as individuals or in clumps of rooted plantlets in pure sand medium. The pure sand medium under irrigation permitted profuse rooting and growth of the plantlets as in the embryo culture derived plants. Hardening was essential and under the high humid conditions maintained in the mist chamber, 81-93% of the plants survived after hardening. Since all the rattan palms employed for cultivation are tropical humid species, the high humidity levels (80±5% RH) maintained in the mist house are very much needed for optimal survival and establishment of the plantlets. This was evident from the loss of significant number of
plants transferred directly to the shade net house. The period of hardening may vary from 2-4 months depending on the species. However, in order to assure optimal growth of the hardened plants, it was necessary to transfer them to different nutrient rich potting medium consisting of sand: top soil: FYM (3:1:1) to permit continued growth of the hardened plants in the shade net house. Procedures followed for the establishment of the plants vary. In some such species as *Trichopus zeylanicus* (Krishnan *et al.*, 1995) and *Decalepis arayalpathra* (Gangaprasad *et al.*, 2005), the percentage survival of the plantlets developed in IAA and IBA were 71.8% and 75% respectively. NAA induced maximum amount of basal callus, which ultimately led to poor rates of establishment (48.8%) in community pot. The percentage establishment of the plants varied from species to species based on the supplementation of different hormonal regimes during culture initiation, multiplication and rooting stages.

The results obtained in all the three palms suggest that seedling explants are somewhat easy to culture and produce large number of plants which could be successfully used for conservation and sustainable economic utilization through reintroduction into forests to raise rattan plantations. Therefore in the light of the achieved rates of establishment reported in other *Calamus* species, the high percentage establishment recorded in all the three species is significant. The results vouch for the use of seedling explants for mass multiplication, rehabilitation and sustainable utilization of the rattan resources of India and elsewhere.

Plants established in the green house/ shade net house with 50% sunlight could be successfully transferred to the forest segments after 6-12 months. Transfer during the onset of south-west monsoon rains in June-August ensured nearly 90% establishment in the forest during reintroduction trials. It should be noted, however, transfer of relatively larger plants >25 cm (grown) in the pots/ polybags resulted in 20-25% loss due to drying of leaves and possible damage to the roots. Therefore, relatively smaller (8-20 cm) plants would be better to get more than 90% establishment as in the case of *C. travancoricus*. In any case, as observed, damage to the roots and shoots should be avoided and the leaves should not be dried due to exposure to direct sunlight during summer months. Actually, the tissue plants were more fragile than the natural seedlings and suckers and their mortality rate can be high, if not properly handled. In case the monsoon rains fail, the reintroduced plants will have to be irrigated in nature. Otherwise, substantial loss of
plants may be incurred in the post-transplantation period. Rattans being shade lovers have to be reintroduced into sites where the shade of adjacent trees is available always so that they can climb on the tree support later. The tropical forests of the Western ghats including the TBGRI campus at Palode provide some of the best conditions for the survival and establishment of the reintroduced palms. The continued growth and performance of the seedling explant-derived plantlets free of morphological abnormalities through 3 year period indicated the stability of the characters in the mass propagated shoots. However, the different locations used for reintroduction experiments are all part of the same Western Ghats forest ecosystem without significant variations in terms of vegetative, climatic and other parameters. Therefore, future reintroduction trials should be carried out in different montane and coastal ecosystems to ensure applicability and credibility of such practices across the country.

The schematic representations of the in vitro propagation protocols for all the three species (Figs 7, 8 & 9.) suggest that 18-20 months period is required for different species to reach the field after culture initiation, multiplication, elongation, rooting and hardening phases. This period is reasonable considering the wild nature of the palms and difficulties in working with the thorny palms where natural propagation rates are low. The multiplication rates could be further improved by using all the foliar explants, shoot tips, collar segments and whole shoots of the axenic seedlings in all the three species as it is now demonstrated as part of this study only in the case of C. rotang. It would also be worthwhile to try mass cultivation of shoot buds using liquid nutrient media in shake cultures as has been well demonstrated in orchids (Seeni and Latha, 2000) and oil palm (de Touchet et al., 1991; Teixeira et al., 1995). There is no reason why with the help of the methods developed, commercial multiplication and processing of rattan palms in India should not be attempted.

5.4 In vitro cloning of rattan palms viz. C. thwaitesii, C. rotang and C. travancoricus

Rattans are an economic group of plants, often perennial in nature but seldom propagated artificially. Since all palms including coconut palm, oil palm, date palm and rattan palm are liberally permissive of open pollination, seed progeny varies so much and no seedling derived mature palm is identical to the other. The problem is further compounded by the fact that some of the economic palms (oil palm, rattan palm) are
dioecious, making it necessary to maintain both male and female palms for successful pollination and seed production. Vegetative propagation is the most difficult as the palms are monopodial in growth as in coconut, Palmyra, date palm and oil palm. Fortunately, most of the rattan palms like the ones used in the present investigation are sympodial as they produce offsets or suckers that can be separated and used for vegetative propagation and multiplication. In practice, however, the rate of multiplication and consequently bulking of propagules using such methods is remarkably slow. The availability of the suckers in sympodial types naturally provides an ample source of explants (shoot tips, adjacent condensed nodes) that could be gainfully used for mass cloning of the palms through tissue culture. This plus attribute of the palms has been gainfully employed for the present investigations. The ability to isolate shoot tip with basal rhizome part from the suckers enabled the standardization of in vitro vegetative propagation protocols for all the candidate species (C. thwaitesii, C. travancoricus and C. rotang) selected for the thesis work. It is a different matter that unlike other palms, the rattans are extremely thorny making it difficult to extract the shoot tips from the growing palm or offset. Again in an actively growing offshoot, the shoot tip with the basal rhizomatous part is so deeply located amidst thorny leaves that it has to be fished out, making the decontamination procedures rigorous and difficult to obtain meaningful number of surface decontaminated, responding shoot tips often collected from accessible forests.

The shoot tip with basal rhizome part obtained and subjected to vigorous decontamination had to be thinned with the removal of young/ primordial leaves and leaf sheath one by one during the disinfection process. This had to be done due to the under ground, soiled nature of the explants which are expectedly loaded with micro organisms of all sorts. Underground plant parts harboring different micro organisms and the need to surface decontaminate them using rigorous procedures are reported in anthurium (Pierik et al., 1974; Kunisaki, 1980; Kuehnle et al., 1992; Teng, 1997), taro (Yam et al., 1990; Yongwei Li et al., 2002) and Zantedeschia aethiopica (Krilzinger et al., 1998). In such cases, multistep disinfection process is usually followed viz. the initial wash with a detergent, followed by immersion in commercial bleach or sodium or calcium hypochlorite and thereafter in 0.1% (w/v) HgCl₂ for 5-10 min depending on the tenderness of the tissue. Even then, only 70-80% of the shoot tips could be saved from infection. Based on the results obtained (Tables 52, 59 and 66), it is safe to infer that at least the shoot tip explants of the rattan palms are free from endophytic bacteria and
fungi that infest explants of Piper nigrum (Purseglove et al., 1981 and Kulkarni et al., 2007). The contamination free explants obtained compare well with the reported success rate of decontamination in anthurium, (Geier, 1986; Teng et al., 1997), Taro (Tim et al., 1990) and mango (Thomas and Ravedra, 1997). However, the observed rate of bacterial contamination higher than fungal ones indicates certain level of bacterial colonization in the outer parts of the rhizome are still possible, even if such associations are not strictly endophytic. If some of these microbes have co-evolved along with the plants, their decontamination process _per se_ may be difficult (Cassells, 1991; George, 1993). Whatever may be the nature of the plant-microbe interaction in rattan palms, it is evident that microbes associated with the explants grow faster than the plant cells and if allowed to continue, the tissue may not grow and eventually get plagued with bacterial colonies, phenolic oxidants and necrotic responses. The two step (5% sodium hypochlorite with and 0.5% Teepol) treatment with bleaching agent, fairly long treatment (40-80 minutes) of the downsized explant with 0.1% streptomycin sulphate and the last treatment with 0.1% HgCl₂ for 4-5 minutes together offered a reasonable decontamination protocol for different species, the differences in size of the explants between the species not withstanding. The shoot tip explants of _C. thwaitesii_ were always larger (0.7 mm) than those of _C. travancoricus_ (0.4 mm). To be successful with the decontamination procedure, the explants had to be collected during summer months (February-March) and those collected during rainy season obviously showed higher percentage contamination due to possible penetration of microbes along with rain water and subsequent colonization of parts including crown and leaf sheath making the decontamination process difficult and cumbersome.

The infection-free shoot tip explant together with the basal rhizomatous part cultured in MS basal medium invariably responded with the elongation of the existing shoot tip to varied length, but stopped short of additional shoot formation and proliferation of shoot buds. Exogenous supply of cytokinins was essential to arrest longitudinal growth of shoot tip but also to keep the apical bud alive and active in different species of Calamus. Cytokinins other than TDZ (BAP, 2-iP, kinetin) were just sufficient to reverse the longitudinal growth of the apical bud (apical dominance) but were short of inducing high percentage axillary bud proliferation. In _C. rotang_, BAP and 2-iP could not reverse apical dominance completely and in _C. thwaitesii_, high concentration (3 mgL⁻¹) of BAP was required to induce minimal additional bud
formation. Poor caulogenic response was noticed in combinations of cytokinins without TDZ. The inability of conventional cytokinins (BAP, 2-iP) in isolation or in combination with auxin to induce multiple shoot formation at reasonable levels made it compulsory to try TDZ individually and in combinations. TDZ was very effective to induce multiple shoot formation though its concentration required for optimum bud release and frequencies varied between species. Lowest concentration of TDZ (0.5 mg l\(^{-1}\)) was required to induce the formation of 3.00±0.20 shoots in 66.67% of the explants in *C. rotang* and 3.25±0.11 shoots in 46.67% of the cultures in *C. travancoricus* within 12 weeks. In *C. thwaitesii*, however, relatively high concentration (1.0 mg l\(^{-1}\)) of TDZ was required to initiate 2.25±0.23 shoots in 83.33% of the explants in 16 weeks. The differential frequencies, number of buds formed and the period required for shoot bud initiation may indicate the genotypic differences between the species (Mullins *et al.*, 1997; Lyyra *et al.*, 2006). However, in all the species, in presence of TDZ apical dominance was completely arrested, the shoots initiated did not elongate beyond 1.5 cm. Additional shoot bud initiation was axillary as it occurred from the base of the shoot tip particularly in the rhizomatous portion. The inverse relationship between apical bud growth and axillary bud release as observed in all the species and stunted growth of even the newly formed axillary shoots could be only related to the known inhibitory influence of TDZ on the longitudinal growth of existing and newly formed shoots (Huetteman and Preece, 1993).

Thidiazuron is capable of fulfilling both the cytokinin and auxin requirements of various regeneration responses (Jones *et al.*, 2007). It can induce new meristem formation, promote shoot development from pre-existing meristems and induce adventitious bud regeneration in a number of species including recalcitrant woody plants (Murthy *et al.*, 1998; Cuenca *et al.*, 2000; Bunn *et al.*, 2005). One of the most effective uses of TDZ has been in the regeneration of woody plant species in which organogenesis was only possible with high concentrations of adenine-type cytokinins, or in those species in which these compounds were ineffective (Murthy *et al.*, 1998). The results present in this study are consistent with these observations, as TDZ has played an important role in inducing multiple shoot formation from shoot tips derived from offshoots/suckers with greater efficiency than other cytokinins (BAP, 2-iP).
The indispensability of TDZ for shoot initiation was again confirmed when combinations of cytokinins on shoot initiation were tested. Only combinations involving TDZ proved good for bud initiation in 53.33% of the shoot tip in *C. thwaitesii*. Among the various concentrations and combinations of PGRs tested in this species, maximum of 2.75±0.23 shoots with some what elongated shoots (2.54±0.48 cm) were initiated at 80% rate after 16 weeks of culture in presence of 0.4 mg l⁻¹ BAP and 0.1 mg l⁻¹ each of TDZ and NAA. The beneficial effect of NAA for obtaining somewhat elongated shoots even in presence of the cytokinin was evident. In certain species, combinations involving cytokinins and auxins act synergistically to obtain normal lengthy shoots during culture initiation/multiplication. This kind of observation was noticed in *Citrus grandis* (Paudyal and Haq, 2000), Culture of *Robinia pseudoacacia* L., *Sorbus aucuparia* L., *Tilia cordata* Mill. (Chalupa, 1987) and *Yucca aloifolia* (Atta-Alla and Van Standon, 1997). In the other two species, however, combinations of PGRs were less desirable than optimized individual concentrations of TDZ for multiple shoot initiation. Overall analyses of the results on shoot initiation reveal that meaningful initiation of shoot buds in culture initiation experiments were possible only with TDZ in isolation or in combination with other PGRs. Shoots induced by higher 2.0 mg l⁻¹ concentrations of TDZ were always distorted or fasciated as observed in *C. travancoricus* and *C. rotang*. Other cytokinins are too weak to initiate buds in shoot tip cultures of *Calamus*. TDZ has been used with great success in such species as *Malus domestica* (Fasolo *et al.*, 1988a, 1988b, 1989; Elobeidy and Korban, 1988) *Pyrus* sp. (Chevreau *et al.*, 1989), *Rhododendron* (Imel and Preece, 1988; Preece and Imel, 1991), *Rubus* (Fiola *et al.*, 1990; Cousinean and Donnelly, 1991) and *Populus* sp. (Russell and Mc Cown, 1988) wherein the shoots initiated remained stunted. Since the number of buds formed even in presence of TDZ was relatively small, that too, after prolonged period of incubation, the rattans form a specialized group of palms, where even repeated trials with strong cytokinins like TDZ do not yield good number of shoot initiation required of a mass multiplication scheme. There is no wonder why previous workers have not met with great success *in vitro* mass multiplication of shoots in *Calamus* species (Yusoff *et al.*, 1985).This is also true with other workers including Patena *et al.* (1984), Umeli Garcia (1985), Barba *et al.* (1985) Yusoff *et al.* (1985), Gunawan and Yani (1986), Yusoff (1989), Dekkers and Rao (1989) and Padmanabhan and Illangovan who worked on different species of rattans (1989, 1993). This work has established that it is possible to clone rattan palm through tissue culture using TDZ as the principal cytokinin; however,
initiation of large number of shoot buds required of mass multiplication schemes is a distant reality.

Multiplication of the buds initiated was achieved through at least two subculture passages of 6 to 8 weeks each in media supplemented with 0.2 mg/l (C. travancoricus) or 0.1 mg/l (C. thwaitesii, C. rotang) TDZ, 0.1 mg/l NAA and 0.4 mg/l BAP. Combinations of hormones tried in the latter two species were more desirable as the shoots obtained were some what elongated and negative influence of TDZ in inducing fasciated shoot development and poor rooting could be avoided to some extent. In C. travancoricus, however, the use of this combination resulted in poor multiplication compared to 0.2 mg/l TDZ. Invariably in all the species, the multiplication responses in terms of percent shoot multiplication and the number of shoots formed was less in the first subculture passage compared to the second subculture. This might be due to the better acclimatization and consequent increase in the caulogenic responses of the shoots in the second subculture. Since repeated subculture in presence of 1.0 mg/l TDZ (C. thwaitesii) or 0.01-0.2 mg/l TDZ (C. rotang) beyond 3-4 subcultures led to formation of abnormal/distorted shoots, combinations of hormones including TDZ were better desired than the individual concentration of TDZ for the in vitro clonal multiplication of these species. Although the combinations of hormones was also tested for multiplication of C. travancoricus shoots, the concentration of TDZ, reduced from 0.5 mg/l to 0.2 mg/l gave satisfactory results without distortion of shoots for multiplication in this species. Thus responses of the shoots used for multiplication again indicated the differences between the species in their responses to TDZ. The inhibitory influence of TDZ on shoot elongation was further confirmed when its replacement by 2-iP in C. thwaitesii led to significant increase in shoot length and not so in shoot number. Both percentage response and number of multiplied shoots were reduced if TDZ was replaced by 2-iP in a combination of BAP-2-iP-NAA (instead of BAP-TDZ-NAA).

Shoot buds multiplied through subculture two-four times had to be necessarily transferred to full/half strength MS medium with/without hormones for shoot elongation. Although half strength culture medium devoid of hormones also favored shoot elongation, the number of shoots harvested was relatively less presumably due to the dependence of the young shoots on hormones for multiplication. The inhibitory effect of high salts present in full strength MS medium on shoot elongation and consequent choice
of half strength nutrient medium for shoot elongation is also reported by earlier workers in different systems viz. *Eucalyptus* F₁ hybrids (Bisht et al., 2000; Joshi et al., 2003), *Syzygium alternifolium* (ShaValli Khan et al., 1999) and *Calophyllum apetalum* (Nair and Seeni, 2003). For shoot elongation, the medium has to be devoid of TDZ (Russell and MC Cown, 1986; Singha and Bhatia, 1988; Fasolo et al., 1989; Preece and Imel, 1991; Neuman et al., 1993; Huetteman and Preece, 1993, Tiwari et al., 2001; Thengane et al., 2006) but supplemented with relatively higher concentrations of other cytokinins (0.5 mg l⁻¹ each of BAP and 2-iP) in half strength medium. Though reduced concentrations of relatively weaker cytokinins (BAP and 2-iP) were indispensable for shoot elongation, use of full strength MS medium contributed to increase in number of shoots each of reduced length compared to half strength salts which promoted lengthy, but reduced number of shoots as in *C. rotang*. Again, at least two transfers of the shoots to fresh medium were required for optimal shoot elongation, though periods of such transfers varied between the species: 8+8=16 weeks in *C. thwaitesii* and *C. travancoricus* and 6+6=12 weeks in *C. rotang*. Invariably in the cultures, mainly due to the spill over effect from the multiplication stage, a marginal number of shoots were also added during the elongation phase. Such observations have been recorded mostly in Bamboo (Singh et al., 2001), *Ixia flexuosa* (Mayer and Van, 1988), *Vitis rotundifolia* and *Rhododendron* (Huetteman and Preece, 1993).

Supplementation of the medium with auxins was essential to get rooting of the shoots. However, despite the presence of auxins, shoots that were multiplied in higher concentration of TDZ (2.0 mg l⁻¹) never rooted presumably due to the antirhizogenic influence of this synthetic cytokinin. Percentage of rooting (83-90%) was significant if the shoots were treated with NAA (4.0 mg l⁻¹) or IBA (3.0 mg l⁻¹) in two transfers totaling 16 and 12 weeks in *C. thwaitesii* and *C. rotang* respectively. The natural auxin IAA was a poor substitute for synthetic auxins, though all the auxins induced only 1-2 healthy roots with branching. Shoots of *C. travancoricus* responded with 83.33% rooting in 3 mg l⁻¹ NAA compared to 66.77% rooting recorded with IAA and IBA. The remarkable similarity of 1-2 root formation in all the species tested indicate that root formation may be a predetermined character in presence of an inducer auxin like NAA. By and large, NAA was more desirable than other auxins for safe and efficient root induction in all the species. In such other species of *Calamus*, as *C. simplicifolius* (Zhang Fangqiu, 1993)
and *C. egregius* (Zeng Bingshan, 1997) also the *in vitro* shoots formed only 1-2 roots which are in agreement with the present results.

Rooted plants hardened in the mist house for 4-6 months got established at significant frequencies (79-88%) in pure sand medium under irrigated conditions. Micropropagated plants of *C. rotang* transferred directly to shade net house showed poor percentage (60%) establishment under constant irrigation. These results indicated the need for hardening for optimal establishment. This is not surprising due to the fact that rattan palms are mostly distributed in tropical humid forests under the shade of other trees and therefore provision of high humidity in the mist house favored high percentage establishment of the hardened plants. However, the establishment of up to 60% of the plants transferred directly to the shade net house under 50% shade and manual irrigation is still significant and indicates the feasibility of obtaining reasonable frequencies of establishment of even the rattan palms under less sophisticated conditions. It is possible that further refinement of the improvised methods improves the establishment frequencies so that without the costly mist house and associated facilities, high percentage establishment of the microplants could be achieved. The transfer of the hardened plants to a different potting medium (sand, topsoil and FYM 3:1:1) for nursery establishment is to provide enough nutrients to the plants so that they will start growing. The fine sand medium used during hardening is practically devoid of nutrients. The use of only sand during hardening or as major part of the potting mixture during nursery establishment is to allow profusion of rooting and easy penetration of the roots into the sand medium apart from easy evaporation of water and consequent enhanced humidity in the surroundings at the post transplantation stage. Besides, when sand is the predominant ingredient of the potting media no stagnation of water contributing to the rotting of the roots occurs in the micropropagated plants.

The microclonal plants of all the three species of rattan palms already established in the nursery for 6-8 months showed conspicuous growth as evidenced from emergence of new leaves. In exceptional cases, emergence of new leaves may occur from second month onwards. These plants were successfully reintroduced into specific sites in the forest segments in the windward side of the Western Ghats with appreciable rates (mean values 75%-77%) of establishment. Care should be taken to introduce the plants during the pre monsoon showers in late May or monsoon rains in early June which facilitated
easy establishment of the plants back in nature without getting desiccated. The uniformly high establishment of the plants recorded in different experimental sites further strengthened possible utility of the methods followed for reintroduction in other parts of India as well where again rattan forms a natural resource. It should be noted that hitherto no successful studies on in vitro cloning and reintroduction of the propagated rattan plants have ever been attempted. The methods described make certain for the first time successful cloning and restoration of all the three rattan palms. The observation that the cloned plants reintroduced into the forest segments showed uniform growth and were free from morphological and growth abnormalities further confirmed the utility of the methods for conservation and revegetation purposes. Hopefully, this kind of reintroduction practices are not a cause for objection from the point of environment, as tissue culture mediated propagation of plants is widely accepted in horticulture and micropropagation itself is a specialized form of vegetative propagation.

As a result of the investigations carried out and results reported for the first time, it has become amply clear that selected species of tropical rattan palms could be clonally multiplied through in vitro means. Multiplication was achieved through direct organogenesis (multiplication of axillary buds). In the other successfully cloned oil palm, multiplication was achieved through indirect organogenesis from root callus (Rabechault et al., 1972; Smith and Thomas, 1973;) while in date palm cloning was achieved using offshoots (Sudhersan and Abo Et-Nil, 2004). Direct organogenesis free of callus is more desirable for cloning purposes as it is free of possible somaclonal variations and also maintains cytological stability of the regenerated plants (Seeini and Latha, 2000). However, the use of TDZ is indispensable for achieving direct organogenesis in the shoot tip cultures. Prolonged subculture of the initiated shoot buds in presence of TDZ should be avoided as it interferes with shoot elongation and rooting of the multiplied shoots. As the rates of multiplication were not appreciable even in presence of TDZ, and successful multiplication, root initiation, hardening and establishment of the plants in the natural forest segments took nearly 20 (C. rotang) to 25 months (C. thwaitesii, C. travancoricus), the entire process should be considered as slow despite the tissue culture-based intervention. The results suggest that rattan palms are exceptional plants not easily amenable to in vitro manipulation. In order to make the methods developed as part of the study acceptable to the industry, further refinement is certainly needed to improve the multiplication rate. Future workers can certainly do this job by building on
the protocols proposed as part of the present investigations. Multiplication protocols
developed using zygotic embryos, seedling explants and shoot tips of offshoots should
certainly serve as models for future innovations to improve upon. Then only, practical
applications of the methods in agro forestry for revegetation of the denuded forests may
become possible.

5.5 Reintroduction and assessment of post-transplantation
Performance

Reintroduction and strengthening of existing depleted populations of the selected
species in nature represents a novel and a powerful approach to achieve conservation and
sustainable utilisation of plant genetic resources, duly recognized by the Convention of
Biological Diversity. In fact, reintroduction can be considered as an ideal follow up
activity of ex situ conservation programme as it is an important component of Global
strategy for Plant conservation (Bramwell et al., 1987; Cheney et al., 1999; CBD
Secretariate, 2002; Planta Europa, 2002). Among the methods followed, clonal
propagation using shoot tip may lead to increased uniformity of plants in the natural
landscape. However embryo cultures followed by seedling explant cultures as followed
will certainly help to build genetically diverse population which is in confirmation with
accepted conservation needs. As of now, very few documented cases of ex situ
conservation based on in vitro multiplication are available (Erica E Benson et al., 2000;
Lal et al., 2003; Patil et al., 2004 and Avani et al., 2006) and rattan palms do not figure
in such models. The results presented in this study should stimulate further research in
these lines so that conservation of endangered monopodial rattan palms including
C.vattayila, C.gamblei, C.dransfieldei and C.andamanicus also becomes a distinct
possibility. In addition, rattans being an economic resource with industrial applications,
large scale planting of in vitro cloned elite genotypes may enhance forest productivity
with significant employment generation in-built into the system. Thus, the in vitro
enabled plant multiplication may help achieve the twin objectives of conservation and
sustainable utilization of rattan palms.

It should be noted that plant reintroduction in the Indian context is quite new and
not many examples are available (Abraham and Vatsala, 1981; Singh, 1985;
Anonymous, 1987; Seeni and Latha, 1992; Krishnan et al., 1995). Further, reintroduction
based on in vitro propagated planting materials is seldom attempted. Micropropagation
can be best adopted as a crisis management tool especially in the case of RET (Rare, Endangered and Threatened) species. However, reintroduction practice today is built on a sound scientific basis, and this solid foundation cleared the way from many methodological doubts and perplexities that were often raised in the past on its feasibility and ethics. Attempts to compile databases and directories on reintroduction projects were made in the past (Soorae and Seddon, 1998) and a new one has recently been launched by the IUCN (International Union for conservation of Nature) in 2007. Listing and documenting reintroduction is important to provide examples and case studies to clarify aims, operating procedures and expected results. It is particularly so with in vitro derived plants where it is possible to debate, define and set standards and methodologies for full scale integration into existing agro-forestry practices. Still, at global level, many examples of reintroduction including in vitro based ones are not sufficiently illustrated to the plant conservation community; case studies, best practice and experience are not sufficiently circulated and known by the actual conservationists working in the field (Vignali et al., 1998; Morgan, 1999; Rich et al., 1999; Bonafede et al., 1999; Sgarbi et al., 2001; Bonafede et al., 2002; Forte et al., 2002; Cerabolini et al., 2004; Kepart, 2004; Dominione et al., 2005; Raimondo and Schicchi, 2005; Rinaldi e Rossi, 2005; Wagner et al., 2005; Accogli et al., 2006; Aplin, 2006; Del Prete et al., 2006; Rossi et al., 2006; Waldren, 2006; Wenham, 2007). From a global perspective reintroduction can be summarized with a specific plant conservation cycle where in situ and ex situ techniques integrate in a complementary way. It is more so in the processes described in the present thesis work, where artificially (ex situ) propagated plants are used for strengthening the population of selected Calamus species in situ. Thus, ex situ activities can play a pivotal role if they are used to support in situ conservation, contributing to the long term survival of the natural populations of threatened plants, reinforcing the diminishing ones and reintroducing the endangered ones.

The possibility of using tissue culture to help solve the problem of species depletion was recognised by several authors in 1970s who noted that only specific refinements were required (Thompson, 1975; Raven, 1976). Wochok (1981) opined that where seed availability is scarce or traditional vegetative propagation methods impractical, micropropagation through tissue culture is an attractive alternative. In India, Mohan Ram (1983) compared the depreciation of tropical forest to 'a house on fire' where only a few precious could be saved with the application of tissue culture
technology. However, even in 1980s, use of tissue culture for the micropropagation of native species was found to be a relatively new application of a sophisticated technology though it dealt with only a part of the complex issue of threatened and endangered species and the benefits derived and scope for germplasm preservation were rather vast. Globally, the recommendations of Convention on Biological Diversity (CBD) are both conservation and sustainable utilization of plants, as in CBD's view without an utilization component, there is no incentive for conservation. Therefore, the present investigation is very much in tune with the current thinking on conservation and sustainable utilization of the otherwise depleted economic species of rattans. Besides, against a mass extinction spasm and loss of one-third of Indian biodiversity predicted for the year 2030 (Nayar, 1996), policy and technological interventions are urgently needed to guarantee the survival of the remaining rare and useful taxa including the rattan palms. As a natural sequel to this thinking, improvement of conservation status in situ complemented by ex situ germplasm collection and multiplication are indispensable to achieve practical conservation and sustainable economic utilization. Micropropagated plantlets obtained through embryo, axenic seedling explant culture and offshoot tip culture of all the three species (C. thwaitesii, C. rotang and C. travancoricus) were hardened and successfully reintroduced to raise pilot level plantation through five years to realize the twin objectives of conservation and sustainable utilization. The data on growth, suckering habit, leaf production and other biomass attributes of (Table 75, Plate13) rattan plantation confined the feasibility of raising mini-plantations using in vitro derived plantlets. Similar kind of reintroduction and plantation activities of rattan palm limited to embryo culture work has been reported only from China (Zeng Bingshan et al., 1997).

Published reports related to reintroduction of tissue cultured plants other than rattans are also scanty. The first demonstration of in vitro multiplication of an endemic orchid, Epidendrum ilense and reintroduction of the species back into its native habitats in Ecuador was made in 1981 (Dodson, 1981). Subsequently, seedlings of Paphiopedilum rothschildianum (Grell et al., 1988), tissue cultured plants of Cacti (Vazquez and Rublou,1989), seed and tissue cultured plants of the endemic orchid Ipsea malababarica (Gangaprasad et al., 1999), Blue vanda (Seeni and Latha, 2000), Vanda spathulata (Decruse et al., 2003) Nepenthes khasiana (Tandon et al., 1990), Decalepis arayalpathra (Gangaprasad et al., 2005) and a few trees such as Pinus torreyana (Ledig,
1996) and *Conradina glabra* (Gordon, 1996) were reported to be successfully recovered through reintroduction. In perennials such as trees and rattans, due to their long life and large size, the cultivation and improvement practices are time consuming, usually taking years to achieve the results in culture and even then reproduction of results is not guaranteed (Vieitez et al., 1985; Dhawan, 1993). Therefore, most workers have for long ignored woody plant species including the rattan palms. Successes on micropropagation and mass production of plantlets are reported in common economic trees of Neem, Teak, Eucalyptus, Bamboo (Department of biotechnology, Annual Report, 1999). Hence, the present investigation is significant from the point of micropropagation, successful reintroduction and reestablishment of the spiny climbing palms viz. *C. thwaitesii*, *C. rotang* and *C. travancoricus* back in selected forest habitats that are not yet attempted in Indian or elsewhere in South East Asia. It should be noted that even in conservation programmes, the economic species are accorded priority (Nayar, 1996) and therefore, the data reported herein may go a long way in furthering research on conservation as well as sustainable economic utilization of equally important other rattans. The economic importance of rattans are unique and non-conventional as a recent report suggests that processed rattans are a potential replacement for fractured bones and have been successfully used for replacing the broken bones in sheep (Online Tech News Daily, January 2010; Green Fertility, January 2010; Jaymi Heimbuch, 6th January 2010).

The reintroduced plants at Palode site developed into a pilot-level plantation through the five year period free of disturbance from any quarters. Although excessive growth of weeds especially grasses was noticed particularly during the summer months (March – May), no attempt was made to remove them as part of sylvicultural management. The plantation was practically free of diseases and pests. The only perceivable disturbance was from wild boars and elephants which fortunately had not uprooted the plants through the period of observation. As such, there was no expenditure on maintenance. The plants can easily tide over the 2-3 month drought experienced during summer; however, if the drought conditions persisted for long in the first year of the planting, certain level of mortality can not be ruled out. Although this is not a problem in Kerala forests otherwise enjoying both southwest and north-east monsoon rains, there may be difficulties in extending such plantations to areas having less rainfall and long summer. The luxuriantly growing pilot-level plantation at Palode is presented in Plate13. The exemplary performance of the plantation, as evidenced from continued
Plate 13

Reintroduction and pilot-level plantation through the five year period of *in vitro* propagated rattan palms

a. Plantation activities of three species of rattan palms at same forest segment (Palode) under Thiruvananthapuram forest range of Kerala Forest Department.

b. Reintroduced plants of *Calamus thwaitesii*

c. Reintroduced plants of *Calamus rotang*

d. Reintroduced plants of *Calamus travancoricus*
growth and biomass production vouched for by the quantitative characters viz. height of the plant, number of leaves, sucker production, girth of the cane, nature of the plant etc. (Table 75).

**Table 75** Field performance of micropropagated plantlets. Data collected from 15 plants of each species reintroduced into the same forest segments (Palode, Thiruvananthapuram Forest Range). At the time of planting, each species of *Calamus* viz. *C. thwaitesii*, *C. rotang*, *C. travancoricus* had a mean height of 15.15±0.60cm, 10.53±0.31cm, 15.59±1.17cm respectively, 2-3 leaves and no suckers.

<table>
<thead>
<tr>
<th>Indicators</th>
<th>Observations were made after five years of reintroduction (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>C. thwaitesii</em></td>
</tr>
<tr>
<td>Average height of the plant</td>
<td>149.20±9.36</td>
</tr>
<tr>
<td>Average number of leaves / clump</td>
<td>34.25±4.35</td>
</tr>
<tr>
<td>Average number of suckers/plant</td>
<td>5.20±0.84</td>
</tr>
<tr>
<td>Girth of the cane, whether the cane is apparent</td>
<td>Not apparent</td>
</tr>
<tr>
<td>Nature of the plant (whether it is climbing or not)</td>
<td>Not climbing</td>
</tr>
</tbody>
</table>
6.0 SUMMARY

Rattans are a special group of spiny climbing palms waning from the forests of the Western Ghats in India largely due to anthropogenic pressures of unsustainable harvest, habitat degradation and consequent decline in self perpetuation. Despite their global recognition as the fourth economically important palm after coconut, oil palm and date palm, and as an important raw material of the cottage industrial sector which plays a significant role in rural economy, available information on conservation, multiplication and sustainable utilization of rattans is scanty and they remain a sadly neglected subject of scientific investigations. This being the case, the present investigation was mainly aimed at the development of appropriate non-conventional propagation systems leading to reintroduction and restoration of three of the economic and endemic rattan palms of the Western Ghats namely Calamus thwaitesii, C. rotang and C. travancoricus.

The observations and findings of this investigation are:

EMBRYO CULTURE

1a. Direct multiple shoot formation in embryo cultures of Calamus thwaitesii, C. rotang and C. travancoricus

- Immature green fruits of C. thwaitesii (ca 2.16×1.74 cm), C. rotang (ca 1.42×0.82 cm) and C. travancoricus (ca 0.9×0.68 cm) surface decontaminated, embryos dissected out and cultured to obtain 85-100% contamination free embryos in C. thwaitesii (1.3×0.3 mm), 90-100% in C. rotang (1.1×0.2 mm) and 98% in C. travancoricus (1.0×0.7 mm) were germinated in MS basal medium. Reinforcement of the nutrient medium with 0.1% activated charcoal facilitated rapid germination of the embryos within one week without intervening dormancy.

- TDZ rather than BAP and 2-iP or combinations of the latter two was essential to induce direct multiple shoot initiation and high frequency shoot multiplication in embryo cultures. In C. thwaitesii either TDZ (0.1 mg l⁻¹) or a combination of BAP (0.4 mg l⁻¹), TDZ (0.1 mg l⁻¹) and NAA (0.1 mg l⁻¹) was required to induce the formation of 3.57±0.23 (73.33%) or 2.86±0.27 (76.67%) shoot buds in eight weeks; however, in C. rotang maximum number (13.76±0.33) of shoots at 83.33% and in C. travancoricus 6.20±0.29 shoots at 54.44% rates were produced in media containing 0.05-0.1 mg l⁻¹ TDZ in eight weeks.
Shoots proliferated upon the embryos were divided and subcultured to have maximum caulogenic response in *C. thwaitesii* and *C. travancoricus*. Altogether in two subculture passages of eight weeks each up to 20.15 shoots were amassed in presence of 0.4 ml^{-1} BAP, 0.1 ml^{-1} each of TDZ and NAA (*C. thwaitesii*) and up to 36.12 shoots were amassed in presence 0.1 ml^{-1} TDZ (*C. travancoricus*). In *C. rotang* embryo derived multiple shoots were transferred as such without division into the same medium (0.05 ml^{-1} TDZ) to obtain maximum 58.17±3.84 shoots/shoot buds at 96.67% efficiency after two subculture passages.

Continuous use of TDZ was inhibitory to shoot elongation. Therefore, rather stunted shoots so obtained after 20 weeks of culture were passed through two subculture cycles of 8 weeks each in hormone free liquid medium to obtain 5.90±0.46 cm and 4.88±0.86 cm shoots in *C. thwaitesii* and *C. rotang*. In *C. travancoricus*, maximum shoot elongation (5.55±0.62 cm) was observed after two subculture passages of 6 weeks each in liquid medium free of hormones. Invariably, the spill over influence of TDZ in these cultures was evident from the formation of 2-5 additional shoots during shoot elongation phase.

During shoot elongation in basal liquid medium, shoots of *C. rotang* rooted at 56.67% rate especially during the second subculture passage. Rooting in the other two species was exclusively dependent on exogenous supply of auxin(s). Among the three auxins viz. IAA, IBA and NAA tested individually, NAA at 1.5-3 mgl^{-1} proved best to induce 90-100% rooting.

Single root formed in most plantlets or 2-3 roots formed exceptionally were sufficient to achieve plantlet establishment at 82.5%, 96.36% and 72% rates in *C. thwaitesii*, *C. rotang* and *C. travancoricus* respectively under mist house condition (28±2°C; 80±5% RH).

**1b. Embryogenesis in embryo callus cultures of Calamus thwaitesii**

Zygotic embryo cultures of *C. thwaitesii* were successfully established in MS medium supplemented with 2,4-D (7 mgl^{-1})/ 2, 4, 5-T (9 mgl^{-1}) and BAP (0.5 mgl^{-1})-NAA (0.2 mgl^{-1}) involving such stages as semifriable callus formation, embryoid formation, embryoid multiplication through subcultures followed by embryoid maturation and germination into plantlets in hormone free basal medium. The percentage callusing and friability of the embryo-derived callus varied between the
two auxins (2,4-D and 2,4,5-T) with 65% of the embryos producing semi-friable, proliferating calli at 7 mg/l 2,4-D and 92% of the embryos even more rapidly proliferating into friable calli at 9 mg/l 2,4,5-T in Murashige and Skoog (1962) medium after 16 weeks of culture.

- Pieces of semi-friable/friable calli (500 mg fresh weight) subcultured to MS medium fortified with 0.5 mg/l BA and 0.2 mg/l NAA showed less growth but differentiated into 10-21 discrete white colored bipolar embryos in 6-8 weeks.

- Majority (65%) of the mature bipolar embryos (2.0-3.0 mm) developed into shoots and roots in 4-8 weeks and finally got converted into plantlets in 12-16 weeks. The younger embryos (0.5-1.0 mm) devoid of distinct shoot pole invariably produced calloid structures devoid of morphogenetic response.

- Approximately 15% of the embryoid-derived, in vitro plantlets formed secondary somatic embryos precociously on the sheath and lamina parts of leaves, which were separated and cultured in basal nutrient medium for 8-16 weeks to develop into plantlets at 62% success rate. Altogether, maximum of 116 rooted plantlets were harvested in a year from a single zygotic embryo cultured in 2, 4-D supplemented nutrient medium. Plantlets raised from 2,4,5-T-induced embryogenic callus cultures were, however, free from precocious embryogenic responses.

- Plantlets derived from direct embryo callus regeneration, embryoid and secondary embryoid germination were transferred to the nursery. Conditions of potting and hardening were optimized to achieve >78% establishment. Twelve weeks after hardening, the plants reintroduced into natural forest segments showed signs of growth with the emergence of a new leaf in 12 weeks. The plants established at 78-82% rate showed uniform growth and were free from morphological and growth abnormalities during the three year period (2004-2007) of observations in the field.

2. Axenic seedling shoot cultures of Calamus thwaitesii, C. rotang and C. travancoricus

- Whole shoots free of roots from 8-12 weeks old axenic seedlings were cultured in MS nutrient medium supplemented with cytokinins. In Calamus thwaitesii (90%, 3.89±0.27) and C. travancoricus (83.33%; 6.25±0.29), maximum percentage and number of shoot buds were induced by 0.2 mg/l TDZ in 12 weeks. In C. rotang, a combination of 0.4 mg/l BAP and 0.1 mg/l TDZ was required to obtain 5.00±0.51 buds in 91.67% of shoots. Calamus rotang differed from the other two species in all
the three explant types viz. whole shoot, shoot tip and leaves of axenic seedlings responding well with the formation of 4-9 shoot buds from the base of each explant at 75% level in 6 weeks. The shoot tip in culture produced more number of shoots (8.40±0.18) than the leaves (6.80±0.43) and whole shoot (7.40±0.46) at the end of the third subculture passage in MS liquid medium supplemented with the same combination of cytokinins used for culture initiation (0.4 mgl⁻¹ BAP and 0.1 mgl⁻¹ TDZ). In other two species, the number of shoots so formed from the shoot tip culture were relatively small (in C. thwaitesii 4.56±0.35 shoots at 93.33% efficiency in media supplemented with 0.4 mgl⁻¹ BAP and 0.1 mgl⁻¹ each of TDZ and NAA; in C. travancoricus 7.25±0.37 shoots at 100% efficiency in media supplemented with 0.2 mgl⁻¹ TDZ)

- A combination of 0.5 mgl⁻¹ each of BAP and 2-iP and 0.1 mgl⁻¹ NAA was adjudged the best for shoot elongation in whole shoot cultures of C. thwaitesii and C. rotang. Shoots of C. thwaitesii, C. rotang and C. travancoricus attained a mean length of 7.76±0.23 cm, 8.48±0.03 cm and 6.49±0.84 cm respectively even while 2-3 new buds continued to form due to the carry over effect of TDZ.

- Again, shoots of C. rotang differed from others in showing multiplication, elongation and rooting (66.67%) in a combination of 0.5 mgl⁻¹ each of BAP and 2-iP and 0.1 mgl⁻¹ NAA. Otherwise, isolated shoots of all the species rooted at 91.67-96.7% efficiency in presence of 2-4 mgl⁻¹ NAA. Rooted plants got established at 81-93% only under mist house (80±5% RH; 28±2 °C) conditions. Differences in establishment rates between species were insignificant.

- One year old nursery established plants reintroduced into four different forest segments (Palode, Peppara, Kulathupuzha, Aryankavu forest range) in the Western Ghats region in south-west monsoon months of late May, June and July 2004 got recovered at 72-89% rate. Differences between the species were marginal. Nursery established plants of 14-16 cm length showed better field establishment than the larger plants (>25 cm) presumably due to no damage to the rooting system of the poly bag plants.

3. In vitro cloning of Rattan palms viz. Calamus thwaitesii, Calamus rotang and Calamus travancoricus

- Shoot tips (0.5-0.7 cm) of all the three candidate species dissected out of 3-6 year old offshoots (suckers) and decontaminated at 70-80% success rate responded slowly and
were often plagued with exudates. Incorporation of relatively higher concentration (0.5 mg l\(^{-1}\)) of TDZ than those (0.1 mg l\(^{-1}\), 0.2 mg l\(^{-1}\)) used for embryo and seedling explant cultures was essential to induce maximum number (3.20-3.25) and frequency (40-80%) of shoot formation in 12-16 weeks.

- Concentration and combinations of PGRs required for optimal caulogenic response varied between species. In *C. thwaitesii*, shoot bud initiation was particularly induced by 0.4 mg l\(^{-1}\) BAP and 0.1 mg l\(^{-1}\) each of TDZ and NAA. Shoot tip elongation was arrested and was soon followed by emergence of 2.75±0.23 buds that developed into 2.54±0.48 cm shoots after 16 weeks of culture.

- *Calamus rotang* required 0.2 mg l\(^{-1}\) TDZ to arrest shoot tip elongation and to differentiate 3.0±0.20 axillary shoots/buds in 66.67% of explants in 12 weeks. In *C. travancoricus*, 3.25±0.11 shoots were formed in 46.67% of shoot tips in medium fortified with 0.5 mg l\(^{-1}\) TDZ.

- Separation and subculture of the buds/shoots so formed in presence of 0.2 mg l\(^{-1}\) TDZ or a combination of BAP-TDZ-NAA enabled the harvest of up to 5-9 shoots in two subcultures of six weeks each.

- The pace of development of these shoots in subcultures and the number of shoots formed per bud, however, varied: 5.11±0.30 shoots in 93.33% of cultures after two subculture passages of 8 weeks each in *C. thwaitesii*, 3.75±0.20 and 2.67±0.13 shoots in 80% and 90% of the cultures in *C. rotang* and *C. travancoricus* respectively after two subculture passages of 6-8 week intervals. The rate of shoot multiplication even in the optimum nutrient milieu was slow and the number of shoots accrued was limited.

- A shoot elongation phase in half MS nutrient medium devoid of hormones was essential to obtain 2.5-7.0 cm shoots in 12-16 weeks in different species.

- Shoots so formed were rooted rather slowly at 83-90% success rate in presence of 3.0 mg l\(^{-1}\) IBA or 4 mg l\(^{-1}\) NAA after 16 weeks (*C. thwaitesii*), 3.0 mg l\(^{-1}\) NAA after 12 weeks (*C. rotang*, *C. travancoricus*). Shoots with 1-2 roots got established in pure sand potting medium in polybags at 79-85% rates under misting in the greenhouse in 4-6 months.

- Though multiplication of shoots through the clonal propagation route was slow, when compared to embryo and seedling explant cultures, it certainly demonstrated the feasibility to produce at least 25 rooted plants from a single shoot tip in one year. A reliable clonal multiplication scheme thus became a reality. The importance of the
clonal multiplication scheme as compared to the embryo and seedling explant culture is that it is free from callusing and presumably somaclonal variations as well.

- Reintroduction of clonal plants into selected forest segments of Thiruvananthapuram and Kollam districts in June-July 2005 resulting in 71.43-79.31% establishment in two year period in C. thwaitesi, 73.91-76.19% establishment in C. rotang and 61.5-88.9% establishment in C. travancoricus confirmed the utility of the plants, free of abnormalities for conservation as well as future commercial planting. However, for successful establishment in the open areas, the clonal plants ought to be reared in shade house (50% sunlight) for a minimum of 6 months to make them hardy to withstand natural conditions. The results confirmed the utility of clonal plants for reintroduction and possible revegetation programmes and the plants so grown in the natural forest habits were free of growth and morphological abnormalities.

- Data on increase in height, leaf and sucker production, girth of the cane and other related biomass attributes obtained on a five year old pilot plantation of some sort raised from embryo cultures at Palode site are presented to confirm the direct use of the micropropagation method for field level applications. At least some of the findings of the investigations are new, novel and reported for the first time in rattan palms.