CHAPTER 6

TISSUE CULTURE OF WOODY SPECIES

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INTRODUCTION

Woody perennials, comprising a large group of taxonomically diverse species, provide an invaluable natural resource for timber (hardwood and softwood species), paper pulp (bamboos, eucalyptus, poplars), latex and resins (rubber, pines), fruits (apple, mango), nuts (almonds, cashew, pistachio), oil (coconut, oil palm) and feed (mulberry). Because of their long generation cycle and highly allogamous nature, breeding them by conventional methods is difficult. Therefore, maximum gains in the improvement of woody crops has to be realized by selection of elite individuals and their clonal propagation. However, by the time the trees are old enough for assessment of desirable traits, they have crossed the phase amenable to conventional methods of vegetative propagation. Some of these crops, in particularly palms, cannot be propagated by standard horticultural methods of clonal multiplication. Therefore, traditionally these plants are propagated by seeds, which yield a heterogeneous population.

Plant tissue culture has been recognized as an effective method for clonal propagation of plant species and is commercially used for a range of ornamental crops (Doreswamy and Pandiarajan, 2000). However, progress with *in-vitro* clonal propagation (micropropagation) of most woody species has been slow. Some temperate fruit species (e.g., apple) and forest species (e.g., poplars, eucalyptus) are fairly amenable to tissue culture propagation and are commercially micropropagated, but most other woody perennials have proved recalcitrant (Paranjothy *et al.*, 1990). Extensive and intensive efforts during the last 15 years or so have made some breakthroughs and at least laboratory protocols for *in-vitro* vegetative propagation have been developed for several valuable woody species. In

India, two Government-sponsored tissue culture pilot plants are mass multiplying some forest species.

Tissue culture also provides reliable means to raise haploids of highly heterozygous tree species, which can enhance the efficiency of selection of plants with desirable traits. Regeneration of plants from endosperm is a rapid and direct approach to production of triploids. This paper reviews the recent progress in tissue culture of woody perennials, using some tropical tree crops as examples. Progress in tissue culture of softwood species has been extensively reviewed, and many of the problems are common to hardwood and softwood species.

SOME WOODY SPECIES

Palms

The economic importance of palms, such as coconut (*Cocos nucifera*), oil palm (*Elaeis guineensis*) and date palm (*Phoenix dactylifera*), and their recalcitrance to simple horticultural methods for clonal propagation, has provided impetus for tissue culture of these crops, and considerable success has been achieved in this area in recent years. Most of the methods rely on initiation of callus from various explants (young roots, leaves and inflorescence segments) on a medium with high level of 2,4-D followed by somatic embryogenesis and plant regeneration on a medium lacking or having a very low level of auxin.

Coconut

In India over 80% of the annual requirement of planting material of coconut is being met from unselected open-pollinated local cultivars because of low multiplication rates through seed propagation and lack of reliable methods for clonal propagation (Iyer and Parthasarathy, 2000).

Since the initial success of Eeuwens (1976), several laboratories have attempted tissue culture of coconut. During the last two decades considerable progress has been made in the area of coconut tissue culture but a repeatable and commercial protocol has still to be achieved (Iyer and Parthasarathy, 2000).

Raju *et al.* (1984) obtained plant regeneration from tender leaf explants from 1-2-year-old seedlings but the results were non-reproducible. Karunaratne *et al.* (1991) observed embryogenesis in the cultures of leaf explants from 12- to 24-month-old plants but this potential was soon lost. The age of the leaf, even from a juvenile plant, affected the response. The only adult tissue to show embryogenic response in cultures was the immature inflorescence (Verdeil *et al.*, 1994; Areza *et al.*, 1993). Verdeil *et al.* (1994) obtained mature embryos from inflorescence segments which developed into plantlets. Nursery transfer of these plants remains to be achieved. In this plant the callusing process is very slow. Callusing starts after 3-4 months and subculturable callus is formed after 8 months.

Precocious subculture of the callus causes browning and necrosis. Embryogenesis occurs only after 1-4 subcultures of 2 months each on a medium containing 2,4-D ($3/4 \times 10^{-4}$ M). Complete differentiation of embryos occurs only after transfer to a medium with a lower concentration of 2,4-D followed by BAP treatment. Chan *et al.* (1998) made similar observations with plumule cultures of coconut. Embryonic explants showed considerably higher response (50% explants callused and 40% were embryogenic) than the inflorescence segment cultures (5-30% callusing and 10% embryogenic). However, to achieve clonal propagation of elite individuals the protocol using inflorescence segments needs to be refined.

Oil Palm

Progress with oil palm micropropagation has been far ahead of coconut, and commercial protocols have been developed. In this important oil crop, vegetative propagation by natural means is not possible because lateral buds give rise to inflorescence. Clonal propagation of elite trees with exceptional characters such as FFB (Fresh Fruit Bunch) yield, high fruit-set and oil content, superior oil quality and disease and pest resistance, is expected to increase palm oil yield by 30% over seedling propagation, with parents being the same (Iyer and Parthasarathy, 2000).

Staritsky (1970) made the earliest attempt to propagate oil palm *in vitro*. Jones (1974) reported the first successful attempt to clone oil palm using leaf explants. Since then several commercial and institutional laboratories in Europe, Africa and Asia have developed technology for large scale *in-vitro* propagation of oil palm. However, *in vitro* clonal propagation of oil palm suffered a setback when abnormalities in floral development of these plants were observed in early trials (Corley *et al.*, 1986). The main abnormality was feminization of flowers (Paranjothy *et al.*, 1990; Duval *et al.*, 1995). Male flowers exhibited transformation of the stamen into carpel-like structures. In female flowers the staminodes developed into a mantle of fleshy carpels surrounding the fruit, leading to formation of abnormal fruits and partial or complete sterility, depending on the extent of abnormality. Similar feminization of flowers also occurred in some seedling plants but did not persist beyond the first inflorescence bearing. The abnormal micropropagated plants showed no change in ploidy level (Rival *et al.*, 1997a) or in RAPD banding pattern of DNA with 8900 markers compared to controls (Rival *et al.*, 1998a). These observations and the fact that 50% (severely mantled) to 100% (slightly mantled) plants revert back to normal floral phenotypes after 7-9 years suggest an epigenetic basis for this abnormality (Rival *et al.*, 1998b). Recently, Jaligot *et al.* (2000) ascribed the mantled variation to hypomethylation of DNA.

The protocol used at ORSTOM-CIRAD, France, to micropropagate oil palm involves culture of leaf explants on high 2,4-D medium to induce

callus, followed by transfer of the callus to a medium with reduced level of auxin for the differentiation of embryogenic structures. Indefinite multiplication of the embryonal structures by adventive embryony is achieved by transferring them to hormone-free medium. On this medium older embryos develop into shoots, which are separated and rooted on NAA-containing medium. After 8 weeks the plantlets are transferred to the nursery. It is felt that the current method of *in vitro* propagation of oil palm is not economical because the frequency of callusing is low (7-30% depending on the genotype), the process is slow (takes 18 months from selection of explant to the production of the first lot of plants for field transfer), high losses during transfer out of culture (*ca* 50%) and less vigour of somatic embryo-derived plants compared to those from zygotic embryos, and loss of 5% plants due to floral abnormalities (Rival *et al.* 1997b). Efforts are underway to overcome these problems and apply molecular markers to detect abnormalities at early stages.

Date Palm

This is a dioecious palm, which produces a limited number of offshoots (20 in a lifetime, of which only half are suitable for producing mature tress; Booij *et al.*, 1993). Since 1977, at least six reports have described successful initiation of embryogenic cultures of date palm (Bhaskaran and Smith, 1995). The cultures for cloning elite individuals of date palm are initiated from immature inflorescence or shoot tips from offshoots. Tissue culture propagation of this palm is being practised on a commercial scale.

The protocol used by Bhaskaran and Smith for clonal propagation of date palm involves callus initiation on a medium containing, besides other things, 2,4-D (100 mg L^{-1}), 2-ip (1 mg L^{-1}) and activated charcoal (3%). The characteristic nodular callus formed on this medium is maintained on hormone-free medium where friable, white embryogenic callus develops. Transfer of the embryogenic calli from hormone-free solid medium to hormone-free liquid medium on a shaker leads to the formation of hundreds of individual embryos, of which 40% germinate. Some success has also been achieved in micropropagation of this palm by axillary budding from offshoot explants.

Al-Wasel (2000) has reported that the micropropagated plants of date palm cv Barhi were more vigorous and uniform and produced significantly more primary, secondary and aerial off shoots than the conventionally propagated plants (*also see* Al Wasel, 1999). However, a number of micropropagated trees planted in 1992 failed to set fruits, and flowers were abnormal, with more than 3 carpels (sometimes >7). Earlier, Booij *et al.* (1993) had noted variation in sugar content of the dates from plants produced by somatic embryogenesis. However, those produced by axillary shoot proliferation from apical and axillary buds from offshoots showed no significant difference in sugar content of the fruits compared to the parent cultivars.

Rubber

Hevea brasiliensis, an arborescent member of the Euphorbiaceae, is the sole source of natural rubber. It can be easily propagated by bud grafting but scarcity of clonal rootstock with taproots from adult material and undesirable stock scion interaction limit the rate of conventional propagation. Tissue culture may help propagate some modern fast-growing and high-yielding clones (1837 kg ha^{-1} y^{-1}) which are over 3 times more productive than the unselected seedlings (512 kg ha^{-1} y^{-1}; Thulaseedharan *et al.*, 2000).

Several attempts have been made to culture rubber tissue from juvenile and adult plants but progress is far from satisfactory, and reliable protocols for micropropagation and/or regeneration have not been achieved. Paranjothy and Ghandimathi (1976) cultured shoot apices from seedlings. These grew and formed a rooted plantlets but did not produce multiple shoots. Mascarenhas *et al.* (1982) reported development of 3 shoots per explant of apical bud from 10-12-year-old plants. Sixty per cent of the shoots rooted but *in-vitro* shoot multiplication did not occur. So far success with micropropagation by axillary shoot proliferation or organogenesis from callus culture is negligible. However, some progress has been achieved in raising embryogenic cultures from anther wall or integument of immature seeds (Paranjothy and Ghandimathi, 1975; Wang *et al.*, 1980; Carron and Enjalric, 1982; Wang and Chen, 1995; Veisseire *et al.*, 1994a,b; Kumari Jayasree *et al.*, 1999). Successful transplantation of plants produced by somatic embryogenesis has been reported (Veisseire *et al.*, 1994; Kumari Jayasree *et al.*, 1999; Carron *et al.*, 1997, 1998) and their genetic uniformity established by cytology and RAPD analysis (Thulaseedharan *et al.*, 2000).This technique can be used to obtain hundreds of plantlets of several clones of industrial interest in a reliable and reproducible manner (Carron *et al.*, 1995), but the procedure is very lengthy, involving several steps and the rate of multiplication extremely slow. These aspects require further research.

Mango

Mangifera indica (Anacardiaceae) is an important tropical fruit tree crop. India is the largest producer of this fruit. Of the world's total production of about 15.7 million tons, India's contribution is 9.5 MT per year. Propagation of this tree species by grafting, layering, budding and rooting of the cuttings has been successful but these methods are extremely slow. Acceleration of clonal multiplication by tissue culture would be very useful in generating the required planting material of highly priced cultivars.

Many mango cultivars exhibit nucellar adventive embryony (polyembryonate) while others are monoembryonate. Most of the popular Indian cultivars of mango are monoembryonate (Jaiswal, 1999). Vegetative

explants, such as cotyledons and leaves, have not yielded regenerative cultures in mango (Kendurkar *et al.*, 1995). The only maternal tissue from adult trees that has given promising results is the nucellus. Litz *et al.* (1982, 1984) reported embryogenesis in ovule and nucellus cultures of some monoembryonate and polyembryonate Floridean mango cultivars on MS medium, with 6% sucrose, and supplemented with 400 mgL^{-1} glutamine, 100 mg L^{-1} ascorbic acid, and 20% coconut milk or 4.4/8.8 µM BAP. The embryogenic response was better in liquid medium than in solid medium.

Considerable success has recently been achieved in regenerating plants *via* somatic embryogenesis, from nucellar tissue of several popular Indian cultivars, *viz* Amrapali, Chausa (Jaiswal, 1999; Ara *et al.*, 2000a), Langra, Malika (Pandey and Jaiswal, unpublished), Alphonso, Mundan and Baneshan (Jana *et al.*, 1994). All these cultivars are monoembryonate. In nucellus cultures of polyembryonate cultivars embryos develop directly from the pre-existing embryonically competent cells, but in monoembryonate cultivars, this involves a transient callus phase. The callus is rapidly consumed in embryo formation and further proliferation occurs by embryo cloning (adventive embryony from preformed embryos) as also in polyembryonate cultivars (Litz *et al.*, 1995).

Ara *et al.* (1999, 2000a) cultured longitudinal halves of ovules (without the zygotic embryo) from immature fruits (1-3.5 cm long) so that the nucellus was in contact with the medium (Fig. 6.1A-D). On MS (1/2 strength major salts and iron) containing 6% sucrose, 400 mg L^{-1} glutamine, 100 mg L^{-1} ascorbic acid and 4.5 µM 2,4-D, the nucellus proliferated and differentiated somatic embryos. Initially, the cultures were frequently transferred to fresh medium and stored in darkness to overcome the browning problem. On this induction medium the nucellus callused and formed a dark brown or black callus which after about 8 weeks differentiated pale yellow or cream-coloured, shiny and translucent proembryogenic calli (PEC; Fig. 6.1E-G). On the induction medium the embryogenic callus proliferated and differentiated globular, heart-shape and cotyledonary stage embryos (Fig. 6.1H). The frequency of dicotyledonous embryos considerably increased on substituting the major salts of MS with those of the B_5 medium. Ara *et al.* (2000b) established fast-growing fine embryogenic suspension cultures by transferring the embryogenic callus to liquid medium in darkness. Embryogenic calli and fully differentiated embryo could be obtained by plating the suspension or their protoplasts (Ara *et al.*, 2000b,c). Germination of the somatic embryos was better in liquid medium than on semi-solid medium (Fig. 6.2A,B). Ara *et al.* (1999) reported that somatic embryos encapsulated in Ca-alginate exhibited higher frequency and faster germination (73%) and conversion (46%) than the naked embryos. Exposure of the plantlets to high CO_2 (20,000 ppm) in nitrogen gas, in light (180-200 µM m^{-1} s^{-1}; 16 h photoperiod) induced thick leaves and reduced losses due to tip necroses,

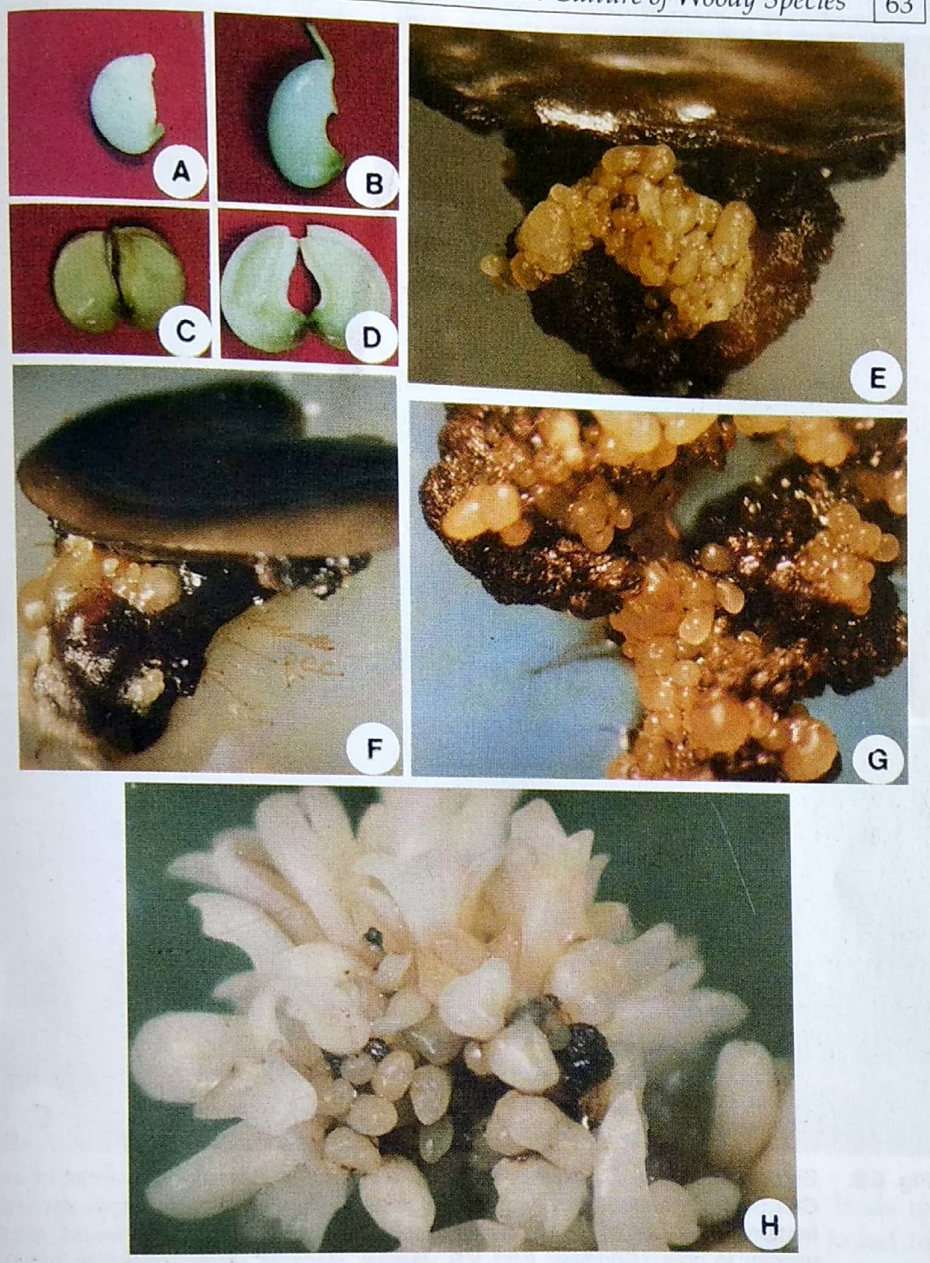

Fig. 6.1. Somatic embryogenesis in nucellus cultures of two monoembryonate cultivars of mango. **A,B**. Excised ovules of cvs Amrapali and Chausa, respectively. **C,D**. Longitudinal halves of ovules of cvs Amrapali and Chausa, respectively, showing nucellus and zygotic embryo. **E,F**. Culture of ovular halves of cvs Amrapali (**E**) and Chausa (**F**), showing light yellow, proembryogenic callus (PEC) arising from dark brown nucellar callus. **G**. Differentiation of globular embryos from PEC of cv Chausa. **H**. Globular and early heart-shape embryos formed by PEC of cv Amrapali. (*Courtesy:* Prof. V.S. Jaiswal, Banaras Hindu University, India)

resulting in high recovery of plants (Litz *et al.*, 1995). The plants were then transferred to soil (Fig. 6.2C).

Fig. 6.2. Embryogenesis in nucellus cultures of mango. **A.** A 6-week-old plantlet of cv Chausa regenerated from nucellar callus. **B.** A plantlet of cv Amrapali derived from nucellar callus. **C.** Hardened plants of cv Amrapali, 8-12 weeks after transfer to pots. (*Courtesy*: Prof. V.S. Jaiswal, Banaras Hindu University, India)

Although it is difficult to root cuttings from field-grown trees, microshoots derived from nucellar embryos could be rooted *in vitro* with high frequency (89%) by pulse-treating them with 5 mg L^{-1} IBA for 24 h followed by transfer to auxin-free medium in darkness (Ara *et al.*, 1998). Apparently, a passage through the embryogenic phase induced juvenility.

Several problems remain to be solved before these protocols can be commercialized. Prolonged embryogenic cultures may suffer loss of

morphogenic potential and lead to the appearance of morphological abnormalities, such as fasciation and fusion of cotyledons, loss of bipolarity of the embryos, reversible hyperhydration and the occurrence of somaclonal variation (Litz *et al.*, 1993). Poor germination of the embryos and extremely slow or no post-transplantation growth of the micro-propagated plantlets are some other problems requiring further research (Jaiswal, 1999).

Jayasankar and Litz (1998) recovered somaclonal variants of mango that had greater resistance to anthracnose, the most serious mango disease worldwide.

Cashew

Anacardium occidentale (Anacardiaceae), commonly called cashew, is an evergreen tree grown throughout the tropics, especially along the coastal regions. Cashew kernels are an important export commodity of India. The kernel is a prized dry fruit and its oil is used in paint and pharmaceutical industries. The cashew apple is used to prepare jam, juice and alcohol. The average yield of a cashew tree in India is 7 kg per year. High-yielding elite clones have been identified. Their cloning for replanting and planting new areas can considerably increase the cashew yield. However, conventional methods of vegetative propagation, *viz.* air layering and grafting, are very slow and cumbersome (Cardoza *et al.*, 1997).

D'Silva and D'Souza (1992a,b) reported multiple-shoot formation in cotyledonary node cultures on MS + 44.4 µM BAP. Addition of 14.6 mM maltose along with 117 mM sucrose enhanced the rate of shoot multiplication from 18-fold to 40-fold in 3 weeks. However, it required transfer to a medium with lower level of BAP (4.4 µM) and addition of 100 ml L^{-1} of CM to the aforesaid medium to promote elongation of shoots suitable for rooting. The 6-year-old micropropagated plants attained a height similar to the seedling-raised plants and produced fruits with colour and size similar to the parent plants. Moreover, the girth of the micropropagated trees was 40% more than the seedling plants of the same age and, unlike the cutting-raised plants, the micropropagated plants did not form plagiotropic shoots (Hegde *et al.*, 1997).

D'Souza *et al.* (1997) observed differentiation of 3-4 axillary buds in the cultures of nodal cuttings from adult plants but the buds failed to grow. Browning and delayed infection were serious problems in the cultures of field-grown adult plants. Bogetti *et al.* (1999) made some progress in regenerating full plants by *in vitro* culture of cuttings from 1-5-year-old trees but the protocol is far from satisfactory. More recently, Cardoza and D'Souza (2000) have reported somatic embryogenesis from nucellus cultures of cashew. Germinable embryos were obtained through four stages: induction, development, maturation and germination of somatic embryos. This approach appears promising for cloning elite trees.

Cocoa

Theobroma cacao, family Sterculiaceae, is a major tropical crop grown for its oil-rich seeds, unique source of cocoa solids and cocoa butter. It is a short understorey tree, showing dimorphic growth. The orthotropic branch tip stops growing after the shoot attains a height of 1-2 m and thereafter lateral plagiotropic shoots develop. The dimorphic growth pattern, lack of efficient propagation techniques and high cost of individual plants hamper the commercial use of asexual propagation of this plant. The plagiotropic shoots are available in large numbers but the plants derived from them by budding or cuttings form a low spreading canopy, which is difficult to manage. On the other hand, the availability of orthotropic shoots is limited. If their number could be increased by tissue culture, this would reduce the cost of individual plants.

Callus and shoot cultures of cocoa have been raised from a variety of juvenile and adult explants but it has not been possible to achieve establishment of proliferating shoot cultures and, consequently, this method of micropropagation of cocoa has not been practised to date.

Several reports on somatic embryogenesis from zygotic embryos (Esan, 1977; Pence *et al.*, 1979), nucellus (Söndahl *et al.*, 1989; Figueira and Janick, 1993), young petals (Söndahl *et al.*, 1989; Lopez-Baez *et al.*, 1993), leaves (Litz, 1986) and stamen and staminodes (Alemanno *et al.*, 1996) have been published. Lopez-Baez *et al.* (1993) reported embryogenesis in inflorescence segment cultures of cocoa. According to Alemanno *et al.* (1996), of the various floral organs, only stamens and staminodes are capable of yielding embryogenic calli, which are yellowish and nodular. The white and compact calli formed by other floral organs are non-embryogenic. Alemanno *et al.* (1996) also observed various abnormalities in the somatic embryos, such as pluricotyledony, fusion of cotyledons, absence of apical meristems and secondary embryogenesis.

The somatic embryos exhibited accumulation of anthocyanins, lipids, alkaloids and proteins similar to zygotic embryos when treated with increased concentrations of sucrose from 9-27% (Figueira and Janick, 1995), but they failed to mature and could not be converted into plants. However, exposure of torpedo-shape somatic embryos to high CO_2 concentration (20,000 ppm) induced high frequency conversion into plantlets (Figueria and Janick, 1993). Some of the plants raised from nucellus of this crop have been established in the field (Figueira and Janick, 1995).

Sandalwood

Santalum album, popularly called the 'fragrant gold' of Indian forests, is a root parasitic member of the Santalaceae. The essential oil obtained from the heartwood and roots of 30-60-year-old trees is in great demand by the perfumery, cosmetic and soap industries. The fragrant, close-grained wood is ideal material for carving idols and making furniture. Clonal

propagation of this tree by grafting and cuttings has not been successful and hence the need for tissue culture.

Rangaswamy and Rao (1963) were the first to establish unorganised callus cultures from mature endosperm of sandalwood. Plant regeneration by organogenesis from seedling and adult explants has been reported but rooting the shoots has been difficult (Rao and Bapat, 1978). The embryogenic pathway of regeneration seems more feasible for cloning this tree species.

In 1965, Rao reported somatic embryogenesis in the cultures of zygotic embryos of sandalwood. Since then somatic embryogenesis has been observed in the cultures of hypocotyl (Bapat and Rao, 1979), internodal segments (Lakshmi Sita *et al.*, 1979), endosperm (Lakshmi Sita *et al.*, 1980) and protoplasts (Bapat *et al.*, 1985) of this tree species.

According to Rao and Bapat (1995), internodal segments from 20-year-old trees produced embryogenic callus on MS containing 4.52 µM 2,4-D. Highly embryogenic cultures were established on MS supplemented with 2.85 µM IAA and 2.22 µM BAP. Bapat *et al.* (1990; *also see* Bapat, 1993) used a 7-litre stirred tank bioreactor (MS + 0.5 mgL^{-1} IAA + 0.5 mgL^{-1} BAP) for large-scale conversion of non-embryogenic cells into embryogenic cells and their proliferation, and a 1 litre bioreactor (MS + 0.5 mgL^{-1} each of IAA, BAP and GA_3) for conversion of preglobular embryos to mature embryos with well developed cotyledons. Extract of the cyanobacterium *Plectonema boryanum* induced and stimulated somatic embryogenesis in the absence of a growth regulator (Bapat, 1993). Full plants regenerated from somatic embryos and established in soil. However, somatic embryogenesis was asynchronous and the embryos exhibited considerable morphological abnormalities, resulting in very poor germination (Bapat, 1993). Moreover, the plants regenerated from stem segments *via* somatic embryogenesis exhibited somaclonal variation with high frequency (Rao *et al.*, 1984).

Mulberry

Morus alba (Moraceae) is an indispensable tree for the sericulture industry. The leaves of this plant constitute the exclusive feed for the elite mori silkworm, *Bombyx mori*. Improvement of this tree crop is hampered by its perennial, dioecious and obligate outbreeding nature.

Micropropagation of mulberry by shoot proliferation has been reported by several authors but somatic embryogenesis has not been very successful. Even shoot proliferation has not been entirely satisfactory. The *in vitro* developed microshoots are easy to root *in vitro*. Cuttings from adult plants exhibit excellent bud-break and develop multiple shoots on a cytokinin medium. Regeneration from leaf explants has also been observed. However, with successive cycles of shoot multiplication, shoot growth and rate of multiplication decline drastically.

Anther/pollen culture to produce haploids of male clones has not been very successful (Katagiri and Modala, 1991; Jain *et al.*, 1996; Sethi *et al.*, 1992). Production of gynogenic haploids of female clones of *Morus alba* has been achieved (Thomas *et al.*, 1999; Fig. 6.3). Nodal segments of female clones of *M. alba* produce normal inflorescence on a variety of culture media which can be used to produce gynogenic plants. *In vitro* developed inflorescences are not only aseptic but also protected from chance pollination. The number of inflorescences per node may vary from 2-6. In the cultures of inflorescence segments the ovaries enlarge and turn pink but do not produce a gynogenic plant. However, if the ovaries are individualised at this stage and transferred to another medium (development medium), they develop a gynogenic plant with no sign of callus formation. Histological studies revealed that in inflorescence segment cultures gynogenesis is induced but the parthenogenic embryo does not develop beyond the preglobular stage (Fig. 6.4A, B). It requires individual ovaries to be transferred to the development medium, on which the embryo develops and germinates normally and forms a seedling. Sixty per cent of the gynogenic plants were haploid (Fig. 6.3E).

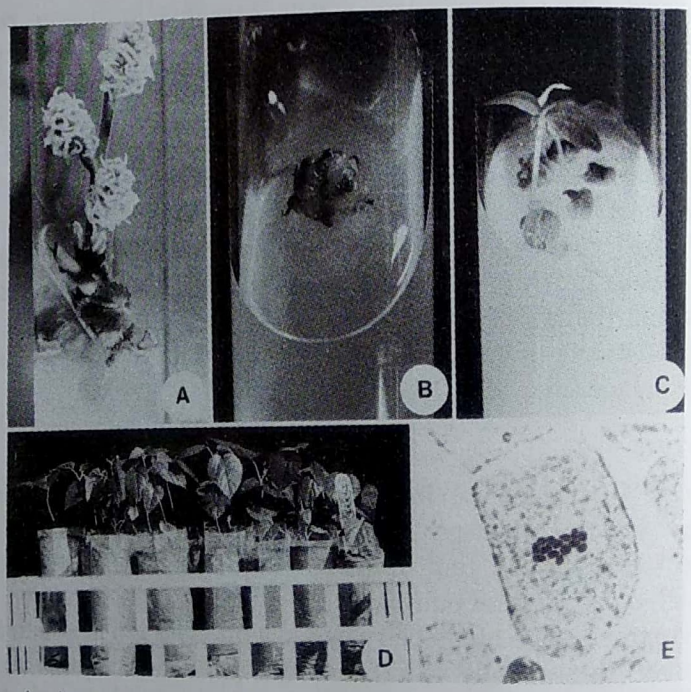

Fig. 6.3. *In vitro* gynogenesis in *Morus alba*. **A**. A single node segment cultured on MS + 5 µM BAP. The axillary shoot has developed several female inflorescences. **B**. A piece of catkin cultured on MS + 4.5 µM each of BAP and 2,4-D. The ovaries have enlarged and turned red. **C**. Individual ovaries cultured on MS + 5.5 µM IAA + 2.3 µM kinetin; two of the five ovaries have developed gynogenic plantlets with no sign of callusing. **D**. Hardened gynogenic plants. **E**. A root tip cell from a gynogenic plant showing haploid chromosome complement (*after* Thomas *et al.*, 1999).

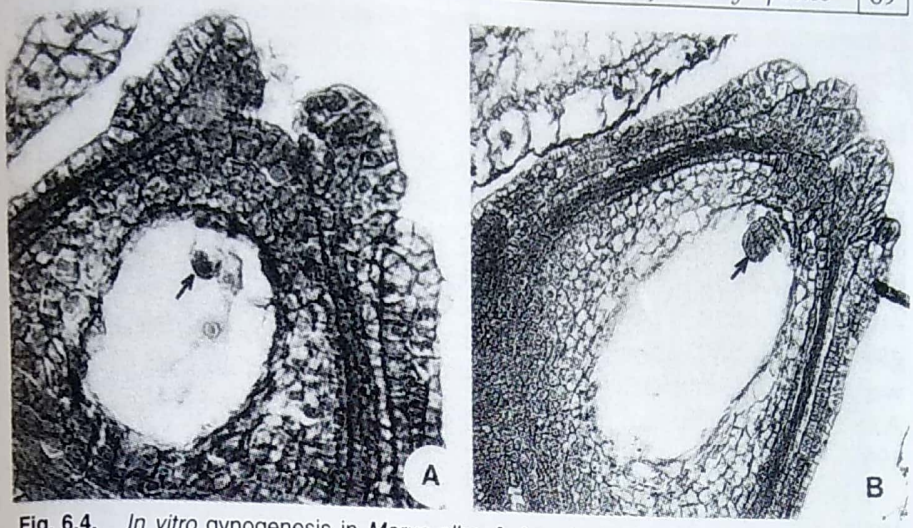

Fig. 6.4. *In vitro* gynogenesis in *Morus alba*. **A**. Longitudinal section of an ovule at the time of inflorescence segment culture; a well-organized egg and polar nuclei are seen. **B**. Longitudinal section of an ovule at the end of inflorescence segment culture, showing a proembryo. Endosperm is absent (*unpubl. work of* Thomas and Bhojwani, University of Delhi, India)

In mulberry, sex expression is very weak and a considerable degree of heterosexuality occurs. It is possible to induce female flowers on genetically male plants. We are now essaying feminization of male plants and production of gynogeneic haploids of genetically male plants, because androgenesis has not been successful with this tree crop.

Mulberry is an asexually propagated crop, making ploidy breeding feasible. Indeed, it is one of the methods presently used to produce high-yielding varieties of mulberry (Sikdar and Jolly, 1995; Chakraborty *et al.*, 1998). A large number of triploids of mulberry are available and some of them are under cultivation in the northern part of Japan for their superior quality of leaves and cold and disease resistance (Hamada, 1963). However, most of the triploids produce leaves of poor quality. Therefore, efforts are being made to evolve desirable triploids (Sikdar and Jolly, 1995).

The traditional method of producing triploids by crossing tetraploids and diploids is very tedious and lengthy. On the other hand, triploids can be regenerated in a single step by endosperm culture. Endosperm-derived triploids of several tree species are known (*Acacia, Citrus, Malus, Putranjiva*). We recently reported regeneration of plants from immature endosperm of mulberry (Thomas *et al.*, 2000). All the regenerants were triploid. An initial presence of the embryo was beneficiary for the proliferation of the endosperm in darkness. After 7 days the embryo was discarded. Best callusing of the endosperm (58%) occurred on MS + 5 µM BAP + 1 µM NAA. Continuously growing callus was established on MS + 5 µM 2,4-D. On this medium the callus was generally brown, friable and unorganized. Over 75% of the calli transferred to MS + 5 µM BAP + 1 µM NAA

differentiated shoot buds after 4 weeks. All the regenerants were triploid (2n=3x=42). The endosperm-derived plants are under field trials.

Neem

Azadirachta indica (Meliaceae) is a majestic forest tree, avenue tree and a tree valuable for soil reclamation of poor and wastelands. It is of great commercial value because of its pesticidal, medicinal and pharmacological properties. With the isolation and characterization of azadirachtin and a number of other bioactive chemicals from its seeds, neem is receiving global attention (Venkateswarlu and Mukhopadhyay, 1999). Margosan-O was the first neem pesticide to be registered (Eeswara *et al.*, 1998). Recently, Azatin has been approved as a biopesticide for food crops (Johnson *et al.*, 1994).

Venkateswarlu and Mukhopadhyay (1999) selected 5+ year-old neem trees with clean bole of 2 m and above, high fruit yield (air-dried fruit yield >75 kg), high oil content (>25%) and high azadirachtin content (0.6% and above). Since conventional methods of vegetative propagation have not been very successful for clonal propagation of this tree species, their propagation is normally done by seeds.

Several papers have described plant regeneration from seedling explants of neem (Joshi and Thengane, 1996; Sarker *et al.*, 1997; Eeswara *et al.*, 1998). Joshi and Thengane remarked that the response of nodal explants from adult plants was negligible. However, Islam *et al.* (1997) reported propagation of 25-year-old trees by axillary shoot proliferation. We have observed recurrent shoot multiplication in nodal segment cultures of adult trees (Fig. 6.5A). The response of nodal explants varied with the season. Whereas in the flowering season the explants developed several small shoot buds, requiring an additional treatment for elongation, the cultures initiated during active vegetative growth period produced a single, long (8-9 cm), multinodal (6-7 nodes) shoot on MS +1 µM BAP + 250 mg L^{-1} CH. The shoots could be rooted on 1/2 MS + 0.5 µM IBA and transferred to soil (Fig. 6.5B,C).

Recently, several people have reported somatic embryogenesis in tissue cultures of neem from embryonic explants (Su *et al.*, 1997; Murthy and Saxena, 1998; Thengane *et al.*, 1995). The somatic embryos exhibited various abnormalities and were difficult to germinate (authors' unpubl. observations). Plant regeneration from nucellus tissue of neem occurs via organogenesis (Islam *et al.*, 1997).

Venkateswarlu and Mukhopadhyay (1999) reported that the neem plants micropropagated via axillary shoot proliferation and field planted in 1996 flowered and set fruits after 25 months. These plants were comparable to mother plants phenotypically and in azadirachtin content of their seeds.

Recently, androgenic haploids and endosperm-derived triploids of neem have been raised (Chaturvedi *et al.*, 2003 a, b).

Fig. 6.5. Micropropagation of mature plants of *Azadirachta indica*. **A**. Nodal segments after 8-week culture on MS + 1 μM BAP + 0.025% CH. Both explants developed a long, multinodal, unbranched shoot. **B**. Three-week culture of shoot on 1/4 MS + 0.5 μM IBA, showing healthy roots developed directly at the basal end. **C**. Hardened micropropagated plants 8 months after transfer to pots. (Chaturvedi and Bhojwani, unpublished).

Jojoba

Simondsia chinensis (Euphorbiaceae), commonly called jojoba, is a dioecious shrub of the arid zone. The seeds of this plant produce high quantities (40-60% of DW) of liquid wax, similar to the sperm whale oil used exclusively in high-pressure lubricants in heavy machinery. Production of large quantities of high-quality seeds can be ensured by planting selected male and female individuals in the correct proportion.

Since 1977, several reports have dealt with callusing and rhizogenesis from juvenile explants (Agarwal *et al.*, 2000). Chaturvedi and Sharma (1989) succeeded in raising full plants of jojoba by shoot proliferation in long-term cultures of nodal segments from adult plants. Agarwal *et al.* (1999) reported a complete protocol for micropropagation of identified male and female trees.

Somatic embryogenesis in the cultures of embryonic explants of jojoba was reported by Lee and Thomas (1985) and Wang and Janick (1986), but full plants were not regenerated. Recently, Agarwal *et al.* (2000) reported embryogenesis in cultures of leaves from *in-vitro* developed microshoots of a female tree. The embryos showed morphological abnormalities and did not germinate.

Acacia

Acacia nilotica, a multipurpose leguminous tree species, is popular for afforestation of arid zones. It has two morphologically distinct varieties, *viz.* indica and cupressiformis. The latter is preferred for planting in agricultural fields. Seed propagation of this cv shows segregation into indica and cupressiformis types, with ratio in favour of the former. Therefore, vegetative propagation of the cupressiformis type is desirable but difficult by natural means.

Micropropagation of this plant from seedling explants is not very difficult (Bhojwani, unpublished). Nodal explants from aseptic seedlings, cultured on ½ MS + 5 µM BAP, developed a single, rarely two, multinodal shoots, which could be further multiplied by nodal cuttings at a rate of 3-4-fold every 5 weeks. Multiple shoot formation was very rare. After 7 passages, the explant did not show much growth or collapsed after precocious leaf drop. Rooting of these shoots was erratic. Whereas in some experiments 87% shoots rooted on MS medium following a pulse treatment of 125 µM IAA for 4 h, in others it was extremely poor. No other treatment improved the rooting response.

Adult material from the field posed serious problems of infection and seasonal variation in their response. April-May was the best time of the year, when maximum bud-break and shoot development (80%) and least infection (10%) occurred. In this season 80% of the cultures of the nodal cuttings on MS basal medium showed bud-break but only half developed a distinct shoot. Addition of BAP slightly improved shoot growth and in some cultures multiple shoots developed. However, most

of the cuttings available for the next cycle of shoot multiplication were the same as on basal medium. On MS + 2.5 µM BAP + 2.5 µM Kinetin + 2 µM IAA , which was the best medium for bud break and shoot growth, the average shoot length was 4.3 cm, with 6.6 nodes per explant. Over 3 nodal cuttings could be taken from such cultures. In subcultures on MS + 5 µM BAP + 0.5 µM IAA + 10% CM, 30% of the nodal cuttings survived and some multiplied (1.5-fold/6 weeks). Mortality increased in subsequent cultures and surviving cultures showed very poor growth. Interestingly, many cultures formed multiple shoot buds (20-40 per explant) but the shoots failed to elongate into rootable shoots. One could take several harvests of elongated shoots from the parent nodal explants although sustained multiplication of shoots was not possible, suggesting the presence of some unknown factor in the parent cuttings which is necessary for shoot growth.

We obtained somatic embryogenesis in endosperm cultures of *A. nilotica* but the embryos did not develop into full plantlets (Garg *et al.*, 1996). The embryos, which were triploid as expected, turned green, elongated and developed a long taproot but did not form plantlets.

SOMATIC EMBRYOGENESIS

Somatic embryogenesis is being seriously considered as the method of choice for mass propagation of superior genotypes of woody species (Jain *et al.*, 1995; Merkle, 1995; Thorpe and Stasolla, 2001). It offers several potential advantages over other methods of *in-vitro* propagation (axillary and adventitious shoot proliferation): (i) very high rate of multiplication, (ii) possibility of scaling up production in bioreactor and (iii) direct delivery in the greenhouse and soil as artificial seeds. Consequently, there has been dramatic progress in the induction of embryogenesis in tissue cultures of woody species during the last decade or so. According to a recent survey, to date somatic embryogenesis has been reported in 186 woody species belonging to 77 genera and 50 families of angiosperms (Thorpe and Stasolla, 2001). However, despite this impressive development, not a single tree species, other than possibly palms, is being propagated by this method. This is because there are many limitations in commercialization of somatic embryogenesis for clonal propagation.

Most of the reports on somatic embryogenesis in tree species, including gymnosperms, are based on zygotic embryo culture, whereas for tree improvement tissue cultures from mature trees are needed if the mature phenotype is to be reproduced. To bypass the problem of recalcitrance of adult tissues for somatic embryogenesis, it has been suggested that the embryogenic cultures from zygotic embryos may be maintained as cryopreserved material while the plants derived from them are under field trials. The source cultures of the desirable trees may be retrieved and further multiplied. This may require storage of cultures for 5-7 years before desirability of the genotype is established. The feasibility of this

approach, in terms of survival rates and genetic stability during storage, is yet to be demonstrated. A more attractive alternative is to select juvenile tissue(s) from mature plants suitable for somatic embryogenesis. There are several examples wherein nucellus (cashew, mango, cocoa, grape, loquat) and floral tissues (cocoa, palms, rubber tree, sweet gum) of woody species have yielded embryogenic cultures. In sweet gum and cocoa the staminate parts responded better than other floral organs (Ellis and Strabala, 2000).

A major concern in applying embryogenic systems for clonal propagation is the danger of introducing genetic variation, especially when indirect embryogenesis is involved. It is well known that oil palm and date palm propagated by somatic embryogenesis from leaf or inflorescence tissues suffered problems of floral sterility and poor fruit-set. The nature of the callus may also affect the genetic stability of the regenerants. In oil palm, leaves from adult plants yielded two types of callus, *viz.*, slow-growing nodular and compact callus and fast-growing friable callus. Only the former is regarded as suitable for establishing embryogenic lines expressing clonal fidelity, suitable for use by industry. The plants derived from the other type of callus exhibit considerable variability (Durand-Gasselin *et al.*, 1990). Furthermore, the culture period should be kept as short as possible because abnormalities increase with time in culture.

It may be possible to initiate proliferating shoot cultures of elite trees from plants rejuvenated by an *in vitro* regeneration cycle through embryogenesis. A cycle through *in vitro* multiplication is known to yield shoots more amenable to *in vitro* rooting (mango—Ara *et al.*, 1998) and proliferation (jojoba—Agarwal *et al.*, 2000) than those from field-grown plants.

A large number of papers on somatic embryogenesis deal only with the formation of a large number of embryos. However, for application of this system to clonal propagation or plant production for any other purpose, it is not enough to mass produce embryos. It requires generation of a large number of normal, germinable embryos. Unfortunately, this happens to be a serious limitation of embryogenic systems in general. For a long time the maturation aspect of embryo, so important for normal germination, was ignored by scientists. Of late, several treatments, such as desiccation, osmotic stress, cold treatment and ABA application, have been shown to promote embryo maturation and subsequent germination.

Until these problems are solved and the art of somatic embryogenesis attains the sophistication enabling induction of genotype-independent somatic embryogenesis, its application will remain limited and unreliable. It is premature to conclude whether somatic embryogenesis, a multistep process (induction, multiplication, development, maturation and germination) would actually prove an economical approach to clonal propagation.

CONCLUSIONS

Commercialization of plant tissue culture techniques for the improvement and propagation of woody species has been hampered by the recalcitrance of their tissues *in vitro*. The number of papers published on tissue culture progress. The problems of infection and browning are no longer serious limitations. However, regeneration in tissue cultures of woody species is still largely restricted to embryonic and seedling explants. From adult plants only floral parts and nucellus have yielded regenerative cultures and the mode of regeneration is mainly through somatic embryogenesis. The latter is regarded as the most practical approach to clonal propagation of woody species at a reasonable price, as it may allow automation at least at the production (in bioreactor) and field planting (as synthetic seeds) stages. However, much more research is needed to achieve normal differentiation, maturation and germination of somatic embryos, and a high degree of genetic stability in embryogenic cultures.

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