Tree and Forestry Science and Biotechnology ©2011 Global Science Books

Application of Tissue Culture Techniques for Propagation and Crop Improvement in Mulberry (*Morus* spp.)

Kunjupillai Vijayan[1*] • Amalendu Tikader[2] • Jaime A. Teixeira da Silva[3]

[1] Research Coordination Section, Central Silk Board, BTM Layout, Madiwala, Bangalore-560068, India
[2] Central Sericultural Germplasm Resources Centre, Central Silk Board, Thally Road, Hosur, Tamil Nadu-635 109, India
[3] Faculty of Agriculture and Graduate School of Agriculture, Kagawa University, Miki-cho, Ikenobe 2393, Kagawa-ken, 761-0795, Japan
Corresponding author: * **kvijayan01@yahoo.com**

ABSTRACT

Mulberry (*Morus* spp.) is an economically important tree grown widely in China, India and several other countries in Asia. The major economic product of mulberry is its leaf which is used for feeding the monophagous silkworm *Bombyx mori* L. Mulberry leaf is also used as fodder for livestock. Mulberry fruit is good for human consumption. Although mulberry is amenable to both sexual and asexual methods of reproduction, due to the high heterozygosity of the parental lines and long juvenile periods, propagation is mainly through stem cuttings or bud grafting. However, success of propagation through stem cuttings is greatly dependent on the genotype, environment, and age of the planting materials. Besides, most of the temperate species are hard to root from the stem cuttings. Micropropagation is seen as a cost effective method for propagation of these species, though a number of factors affect the success. In this article, we summarize these factors along with the causes and remedies for them. Other applications of tissue culture such as germplasm conservation, screening for stress tolerance, triploid developments, genetic transformation, and their impacts on the sericulture industry have also been detailed and discussed.

Keywords: cryopreservation, gene transfer, haploids, micropropagation, triploids

CONTENTS

INTRODUCTION

Mulberry (*Morus*) of the family Moraceae is one of the most economically important tree crops in Asia, as the leaf of mulberry is the sole food material available for rearing the silkworm *Bombyx mori* L. It is estimated that the cost of mulberry leaf production alone covers more than 60% of the cocoon production cost (Das and Krishnaswami 1965).

Received: 4 June, 2009. Accepted: 23 September, 2010.

Invited Review

Therefore, high leaf productivity is essential for sustaining profitability in sericulture. Additionally, in several countries where sericulture is not popular, mulberry leaf is used as fodder for livestock (Castro 1989; Benavides *et al.* 1994). Since mulberry fruit is good for human consumption, many commercial products such as jam, marmalade, frozen desserts, pulp, juice, paste, ice cream, and wine are produced from it (Koyuncu 2004). Mulberry trees are good for landscaping as well (Tipton 1994). Although mulberry has its origin in the Himalayan foothills (Vavilov 1951; Benavides *et al.* 1994; Hou 1994), currently it is present in both old and new world regions (Yokoyama 1962). Morphologically, mulberry is a fast growing deciduous woody perennial tree with a deep root system; alternate, stipulate, petiolate, entire or lobed leaves; catkins with pendent or drooping peduncle bearing unisexual flowers; and the fruit as a sorosis composed of a collection of individual achenes. Out of the 68 species that have been reported from mulberry (Katsumata 1979; Sanjappa 1989), a few important among them in terms of leaf, fruit and timber production are *M. alba*, *M. indica*, *M. serrata*, *M. laevigata*, *M. multicaulis*, *M. tartarica*, *M. nigra*, *M. australia*, *M. cathyana*, *M. mierovra M. atropurpurea*, *M. mizuho*, *M. rubra*, *M. insgnis*, *M. mesozygia*, and *M. macroura* (Tikader *et al*. 2010).

CONVENTIONAL PROPAGATION TECHNIQUES IN MULBERRY

Mulberry is amenable to both sexual and asexual modes of reproduction. Owing to the heterozygosity of the parents, propagation through seeds is not practiced commercially as seed-grown plants show a high degree of variability. Developing inbred lines in mulberry is not easy due to a longer juvenile period, high inbreeding depression and the dioecious nature of the plant (Vijayan 2010). Therefore, commercially, mulberry is propagated primarily by planting stem cuttings directly in the field or raising saplings in the nursery and later transplanting them into the field. One of the advantages of propagation through stem cuttings is the ability of perpetuating the good characteristics of the mother plant without any alteration. Likewise, triploids can only be propagated vegetatively. However, the degree of success of propagation through stem cuttings depends on a number of factors such as the origin of the genotype (most of the genotypes originating from temperate regions do to root from stem cuttings), the physiological condition of the plant, the age of the stem and environmental conditions (Ohyama and Oka 1987; Narayan *et al.* 1989). Nonetheless, 60-80% success can be obtained from tropical genotypes if appropriate cultural practices are assumed (Sau *et al*. 1995; Tikader *et al*. 1995a). Grafting of desirable scions into established seedlings is also practiced in some areas where plating with stem cuttings is not possible or the genotype is not suitable for propagation through stem cuttings. Spring budding is the most common method used in mulberry, wherein a 'T-cut' is made in the rootstock and a smooth, sloping cut is made on the lower end of the scion. The scion is then inserted into the 'T-cut', wrapped, and sealed with grafting wax.

MICROPROPAGATION FROM AXILLARY AND ADVENTITIOUS SHOOTS

Although propagation through stem cuttings is easy and economical, its success depends on a number of factors as mentioned above. Furthermore, newly developed mulberry varieties cannot be immediately propagated through stem cuttings into large areas. Additional problems in developing saplings in a nursery such as maintenance and management costs for 6-12 months (Kapur *et al.* 2001), seasonal influence (during winter period no propagation can be achieved) and the loss of vigor of plants grown from stem cuttings as compared to those from seedlings (Bapat *et al.* 1987; Zaman *et al.* 1997) requires a viable alternative with which mulberry can be propagated rapidly in a cost-effective way. Micropropagation provides such an alternative tool for the rapid and cost-effective multiplication of mulberry, as a large number of clones can be produced in a relatively short time and space.

Importance of explants

Explants have key roles in the success of micropropagation as the initial establishment and the speed of subsequent multiplication steps under *in vitro* conditions are greatly dependent on the genetic make up, age and origin, physiological and pathological conditions of the explants. Ohyama (1970), using axillary buds of *M. alba*, demonstrated for the first time that complete plants could be regenerated if explants are cultured on Murashige and Skoog (1962) medium supplemented with growth regulators. Later, Oka and Ohyama (1974, 1975, 1978, 1982, 1986, 1987) demonstrated that adventitious buds could directly be induced from nodal, shoot-tip and leaf explants without the formation of intermediate callus in mulberry (**Table 1**). Generally for mulberry micropropagation, shoot tips and nodal segments of juvenile or adult shoots of current year growth with dormant axillary buds are found to be best, as evident from **Table 1** (Mhatre *et al.* 1985; Enomoto 1987; Ivanica 1987; Chattopadhyay and Datta 1990; Chattopadhyay *et al.* 1990; Jain *et al.* 1990; Sharma and Thorpe 1990; Tiwary and Rao 1990; Yadav *et al.* 1990; Hossain *et al.* 1992; Pattnaik *et al.* 1996; Pattnaik and Chand 1997; Chitra *et al.* 2002; Bahu and Wakhlu 2003). Embryos, hypocotyls, cotyledons and leaf tips were also found to be suitable for micropropagation (Kim *et al.* 1985; Thomas 2003).

Effect of media on shoot proliferation

Media composition is another key factor that affects the success of micropropagation of any plant (**Table 1**). Therefore, much effort has been made to standardize the culture medium. The basal medium found best suited for mulberry shoot multiplication is full-strength MS (Murashige and Skoog 1962) medium, although other media like AE (Von Arnold and Eriksson 1981), B5 (Gambhorg *et al.* 1968) and woody plant medium (WPM) (Lloyd and McCown 1980) also showed varying degrees of success (Kim *et al.* 1985; Narayan *et al.* 1989; Chitra *et al.* 2002; Bahu and Wakhlu 2003). Kim *et al.* (1985) compared zygotic embryos grown in media with and without plant growth regulators (PGRs) as initial explants for shoot initiation and multiplication. They found that explants such as young leaf tips (10-15 days old) from embryos grown in media containing 2 mg L^{-1} 6-benzylamino purine (BAP) formed buds within 4 weeks but no buds formed on PGR-free media; rather, only callus formed. This difference in the response of explants from different growing conditions could be due to the effect of PGRs on the physiological condition of the explant. In another study, Anis *et al.* (2003) used explants from field-grown plants on MS basal medium containing different combinations of BAP, α-naphthaleneacetic acid (NAA), asparagine and glutamine, the latter two being amino acids. They found that MS medium containing 2 mg L^{-1} BAP + 0.2 mg L^{-1} NAA + 25 mg L^{-1} asparagine + 1 mg L^{-1} glutamine was the best among all the media combinations for shoot formation. Yadav *et al.* (1990), Patnaik and Chand (1997) and Chitra and Padmaja (1999) also observed that BAP was more effective than Kinetin (Kn) for shoot induction from both shoot tip and nodal explants. Chitra and Padmaja (2002) found that the addition of 0.5 mg L^{-1} BAP was suitable for shoot multiplication inducing 6–8 shoots per culture in all cultivars after 30 days. However, Bhau and Wakhlu (2003) found that an increase of 6-benzyladenine (BA) above 2 mg L^{-1} inhibited shoot initiation and multiplication in *M. alba* and *M. multicaulis* genotypes. Although sucrose was the most commonly used carbohydrate for most micropropagation studies, Oka and Ohyama (1982) found fructose (3%) to be a better carbon source than sucrose (3%) for *in vitro* plant induction from bud cultures of

Table 1 Explants, media and plant growth regulators (PGR) used for micropropagation of mulberry (*Morus* spp.).

Species	Cultivar	Explants	Shoot initiation-Medium and PGR (mgL^{-1})	Shoot multiplication-Medium and PGR (mgL^{-1})	Rooting- Medium and PGR (mgL^{-1})	Remarks	References
M. alba	Kenmochi	Winter buds	MS + BA (1)	--	---	Only shoot initiation	Oka and Ohyama 1974
M. alba	Kenmochi Ichinose	Axillary buds	MS + BA (1)+ NAA (1)	MS + BA (1) + NAA (1)	---	Shoot formation	Oka and Ohyama 1975
M. spp.	---	Shoot tips	MS + BA (1) + GA_3 (1)	MS + BA (1) + GA_3 (1)	MS + NAA (1)	Plants transferred to soil	Ohyyama and Oka 1976
M. alba	--	Axillary buds	SDS + BA (1)	MS + BA (1) + NAA (1)	---	Shoot formation	Oka and Ohyama 1978
M. alba	Yanagima Kenmochi	Winter buds. Young leaves	MS + BA (1-10)	MS + BA (1-10)	MS + NAA (0.1)	Plants transferred to soil	Oka and Ohyama 1981
M. alba	--	Hypocotyl, Cotyledon, Eipcotyledon	MS + BA (1)/ 4PU (1)/ NAA (1)	MS + BA (1)/ 4PU (1)/ NAA(1)	---	Shoot formation	Oka and Ohyama 1982
M. indica	--	Axillary buds	MS + IBA (0.5) + 2,4D (1) + NAA (0.5)	MS + IBA (0.5) + 2,4D (0.5) + NAA (0.5)	MS + Kn (1) + NAA (0.5)	---	Patel *et al*. 1983
M. alba	--	Mature embryos, cotyledons, leaves	MS/ AE + BA (1.0-5.0) + NAA (0.001-0.1)	MS/AE + BA (1.0-5.0) + NAA (0.001-0.1)	MS + IBA (0.1-5.0)	Plants transferred to soil	Kim *et al*. 1985
M. nigra	--	Shoot tips	Snirs media with Knop's micro + BA (1)	Snirs media with Knop's micro+ BA (1)	MS + IBA (0.2) + NAA (0.2)	Plants transferred to soil	Ivanicka 1987
M. nigra	--	Shoot tips Nodal segments	MS + BA (1)	MS + BA (1)	MS	Plants transferred to soil	Enomoto 1987
M. alba	---	Nodal segments	MS + BA (0.5)	MS + BA (0.5)	MS + BA (0.5) + Activated charcoal)	Plants transferred to soil	Sharma and Thrope 1990
M. bombycis	Schimanochi, Mizusawa	Axillary buds	MS+ BAP (0.25-2.0); GA_3 (0.5-1.0)	MS + BAP (0.25-2.0); GA_3 (0.5-1.0)	MS + IBA (0.5)	Plants transferred to soil	Jain *et al*. 1990
M. alba	S1	Axillary buds	MS + BA (.75) + NAA (0.2)	MS + BA (3) + NAA (0.2)	½ MS + NAA (0.2)	Plants transferred to soil	Chattopadyay *et al*. 1990
M. laevigata	---	Nodal segment	MS + BA (0.5)	MS + BA (2.5)	MS + IBA (0.2) + NAA (0.2)	Plants transferred to soil	Hossain *etal*. 1992
M. spp.	10 hybrids	Axillary buds	MS + BA (1)	MS + BA (1)	MS + NAA (0.5)	Plants transferred to soil	Raghunath *et al.* 1992
M. laevigata	---	Nodal segment	MS + BA (2.25)	MS+ BA (2.25)	MS + IBA (0.1)	Plants transferred to soil	Islam *et al.* 1993
M. alba	S1	Nodal segment	MS + BAP (2.0)	MS+ BAP (2.0)	MS + IBA (0.1)	Plants transferred to soil	Islam *et al*. 1994
M. alba	----	Shoot tips	Liquid MS + 4PU (4) + BA (1)	Liquid MS + 4PU (4) + BA (1)	MS + NAA (1)	Plants transferred to soil	Hayashi and Oka 1995
M. australis	---	Shoot tips , Nodal segments	MS + BA (1)+ GA_3 (0.5)	MS + BA (1)+ GA_3 (0.5)	1/2MS + IAA/ IPA/ IBA (1)	Plants transferred to soil	Pattnaik *et al.* 1996
M. alba	V1, S34	Apical buds	MS + BA (2)	MS + BA (2.0)	MS + NAA (0.1) IBA (1)	Plants transferred to soil	Tewary *et al.* 1995
M. alba	---	Nodal explant	MS + BAP (2) + IAA (0.5)	MS + BAP (2) + IAA (0.5)	1/2MS+ IBA (0.5)	Plants transferred to soil	Verma *et al.* 1996
M. cathyana, M. lohu M. serrata	--	Apical buds, Nodal segments	MS + BA (0.5-1.0), GA_3 (0.4)	MS + BA (0.5-1.0), GA_3 (0.4)	½ MS + IAA (1)	Plants transferred to soil	Pattnaik and Chand 1997
M. alba	S1	Apical buds	MS + BAP (2)	MS + BAP (2)	MS + NAA (0.5)	Plants transferred to soil	Chakraborti *et al.* 1998
M. indica	---	Shoot apex, Nodal segment	MS + CPP (2)	MS+ CPP (2)	MS + CPP (0.5)	Plants transferred to soil	Tewary and Oka 1999
M. indica M. alba	---	M-5	MS + 2,4D (0.3)	MS + BA (0.5-1.0)/ BA (4.0) + GA_3 (0.05)	MS + 2,4 D (1)	Plants transferred to soil	Chitra and Padmaja 1999
M. alba	M5, S36, S13	Nodal segment	MS + 2,4D (0.3)	MS + BA (0.5)	IAA (0.1 and 1)/ IBA (0.1 and 1)/ NAA (0.1 and 1)/ 2,4-D (0.1and 1)	Plant transferred to soil	Chitra and Padmaja 2002
	Chinawhite		MS + Kn (2)				
M. latifolia	---	Nodal segments	MS + BA (2)	MS + BA (2)	MS + IBA (1)	Plants transferred to soil	Lu 2002
M. alba	---	Shoot tips, Nodal segments	MS + BA (2) + NAA (0.2)	MS + BA (2) + NAA (0.2)	MS + NAA (1)	Plants transferred to soil	Anis *et al.* 2003
M alba	China white, Kokuso-27, Ichinose	Nodal segment, Shoot tips	MS + BA (1.0-4.0)	MS + BA (1.5)	MS + NAA (0.5-1.0)/ IBA (0.5-1.0)	Plants transferred to soil	Bhau and Wakhlu 2003
M. alba	S36, S1, K2	Cotyledons	MS + TDZ (1.5)	MS+ BA (1.2)	IBA / NAA (0.25)	Plant transferred to soil	Thomas 2003

Table 1 (Cont.)

Species	Cultivar	Explants	Shoot initiation-Medium and PGR (mgL^{-1})	Shoot multiplication-Medium and PGR (mgL^{-1})	Rooting- Medium and PGR (mgL^{-1})	Remarks	References
M. alaba	M5, S36, S13	Leaves	MS + BAP (0.5 and 1) or TDZ (0.5 and 1)	MS + BAP (0.5 and 1) or TDZ (.5 and 1)	IAA (0.1 and 1), IBA (0.1 and 1, NAA (0.1 and 1) or 2,4-D (0.1 and 1)	Plant transferred to soil	Chitra and Padmaja 2005
	Chinawhite						
M.alba	Sujanpur	Nodal segments	MS + BA (2.5) + GA_3 (0.3)	MS + BA (2.5)+ GA_3 (0.3)	MS + IBA (1.0)	Plants transferred to soil	Kashyap and Sharma 2006
M. alba	Sujanpur		MS + BA (2.5) + GA_3 (0.3)	MS + BA (2.5)+ GA_3 (0.3)	MS + IBA (1.0)	Plant transferred to soil	Ahmed *et al.* 2007

AE: von Arnold and Eriksson (1981) medium, MS: Murashige and Skoog (1962) medium, 4PU: [NL(2-chloro-4-pyridyl)NLphenyl-urea], 2,4-D: 2,4-dichlorophenoxyacetic acid, BA: 6-benzyladenine, BAP: 6-benzylamino purine, IAA: indole-3-acetic acid, IBA: indole-3-butyric acid, IPA: indole-3-proponic acid, GA_3: gibberellic acid, Kn: kinetin, NAA: α-naphthaleneacetic acid, TDZ: thidiazuron.

M. alba, which was later confirmed by Chitra and Padmaja (2002) and Lu (2002) as the rate of shoot multiplication was increased by a factor of 6.5 shoots per culture in four cultivars such as M5, S-13, S36 and *M. alba* cv. 'China White'. Vijayan *et al.* (2000) tested the effect of sucrose (3%), glucose (3%), fructose (3%) and maltose (3%) on shoot formation from leaf explants and found glucose to be best. However, Chitra *et al.* (2002) observed that shoot tips cultured on medium supplemented with glucose turned pale green whereas those cultured on medium supplemented with maltose induced buds with no further growth. Furthermore, they found 2,4-dichlorophenoxy acetic acid (2,4-D) to be the best PGR for sprouting and growth in cultivars like 'M-5', 'S-13' and 'S-36' whereas Kn was best for 'China White', which is a cultivar with poor rooting from stem cuttings. Among the range of pH levels tested, a pH of 5.6-5.8 was best for shoot multiplication in mulberry (Oka and Ohyama 1978; Enomoto 1987). Similarly, the optimum concentration of agar was 0.8% (Patel *et al.* 1983; Mhatre *et al.* 1985; Enomoto 1987; Pattnaik and Chand 1997; Thomas 2003). Shoot growth was inhibited by 1.0% agar than by 0.8% agar (Oka and Ohyama 1978).

Effect of media on rooting

Production of plantlets with profuse rooting *in vitro* is important for successful field establishment of *in vitro* regenerated plants (Ohyama 1970). Therefore, the effect of genotype, media composition and PGRs on root growth and development in mulberry was investigated in detail. Narayan *et al.* (1989) reported 95% success in rooting from *M. alba* cultivar 'S1' in half-strength MS medium supplemented with 0.5 mg L^{-1} NAA. Later, Yadav *et al.* (1990) found indole-3-butyric acid (IBA) to be the best auxin for induction of rooting from *M. nigra*. However, Anuradha and Pullaiah (1992), Hossain *et al.* (1992) and Rao and Bapat (1993) confirmed the superiority of NAA for root initiation and growth in mulberry. Recently, several auxins such as NAA, indole-3-acetic acid (IAA), IBA and 2,4-D were used singly or in combination to induce rooting from *in vitro* raised shoots of many cultivars (**Table 1**). Chitra and Padmaja (2002) got better root induction with 2,4-D. However, Vijayan *et al.* (2003) found NAA to be better for root initiation in 63 genotypes of mulberry from different species. Similarly, Vijayan *et al.* (1998) observed that higher concentrations of auxins (> 1.0 mg L^{-1}) inhibited root induction. Thus, overall, it is evident that in mulberry *in vitro* rooting greatly depends on genotype and the nature and concentration of PGRs. Although NAA at 0.1-0.2 mg L^{-1} is capable of inducing roots from most species, other mild auxins like IBA or IAA are also effective in certain genotypes. In general, rooting initiates within 12-18 days of explant transfer as seen with *M. laevigata* (Hossain *et al.* 1992; Islam *et al.* 1993) and *M. cathayana* and *M. lhou* (Pattnaik and Chand 1997).

Hardening and transfer of plants into the field

Hardening of plants developed *in vitro* is one of the critical steps in the field establishment of micropropagated plants. Achievement of uniform plant growth and high survival rate not only demand good greenhouse conditions, but also modifications of the internal microclimate to match the local environment (Mondal *et al.* 2004). Patnaik and Chand (1997) transferred plantlets with well-developed roots to plastic pots (5 cm diameter) containing autoclaved vermicompost (Ranjan's Agrotech, Bhubaneswar, India). The potted plantlets were maintained inside a plant growth chamber (SICO, India) with 80–85% relative humidity, 25 ± 1°C and 50 μE m^{-2} s^{-1} light intensity, provided by cool white fluorescent tubes. Plantlets were watered every 4 days for a period of 2 weeks. They were then transferred to earthenware pots (12 cm diameter) containing garden soil (50% soil+ 50% compost) and kept under shade for 3 weeks before transferring to an experimental garden. Chakraborti *et al.* (1998) transferred the rooted plants to small plastic cups (5 cm diameter) containing autoclaved fine soil and maintained them in a culture room with 80–85% relative humidity, 25 ± 1°C and 50 μE m^{-2} s^{-1} light intensity, provided by cool white fluorescent tubes. The plants were covered with polythene bags to maintain better humidity. For the first week plantlets were moistened with ½-strength MS basal liquid. After two weeks, the plants were transferred to earthen pots containing sand, soil and farmyard manure (1: 1: 1; w/w/w) and kept under shade in the greenhouse for 60 days. Thereafter, the plants were transferred to normal growing conditions in the greenhouse and kept there for 6 months. After 6 months growth the plants were transferred to the field and planted at 60 cm × 60 cm spacing. Zaman *et al.* (1997) transferred the plantlets after thorough washing to remove the remaining media, into polythene bags containing non–sterile garden soil and compost (1: 1) and kept for the first 2 weeks inside a growth chamber at 26 ± 2°C with 16-h photoperiod provided by 60–70 μmol m^{-2} S^{-1}. Later the plants were transferred to the field. Bhau and Wakhlu (2001) transferred the rooted plants to plastic cups containing a mixture of vermiculite and sand (1: 1). The plantlets were covered with polythene bags to maintain high humidity and were placed in a shaded place. Plantlets were watered every 2 days with Knop's solution for a period of 4 weeks, and then transplanted to polythene bags containing garden soil, sand and farmyard manure (1: 1: 1) and kept in the shade for 2 weeks.

Biological hardening

Biological hardening is another effective method for hardening micropropagated plants. The major advantage of this technique is the earlier conditioning of the micropropagated plants to the challenges of biotic stress caused by soils microbes. Since micropropagated plants are developed under sterile conditions, their sudden exposure to soil pathogens make them highly vulnerable to many diseases like dam-

ping off, bacterial canker, etc. Therefore, it is desirable to expose the micropropagated plants gradually to microbial attack by co-cultivating them with known microbial cultures (Mathur *et al*. 2008). Many fungi like vesicular arbuscular mycorrhiza (VAM), *Trichoderma* and *Piriformospora indica* have been found to be suitable for biological hardening of several woody plants (Singh *et al.* 2000). In mulberry, Kasyap and Sharma (2006) used arbuscular mycorrhiza (AM) and *Azotobactor chroococcum* during acclimatization and they obtained more than 50% survival rate in the field.

Field performance of micropropagated plants

Although a number of reports are available on micropropagation of mulberry, only one or two studies have been conducted to assess the field performance of micropropagated plants. Zaman *et al.* (1997) using three cultivars demonstrated that *in vitro* raised mulberry plants were ore vigorous in growth than those raised from stem cuttings. However, unlike morphological traits, the biochemical characters did not show any significant differences. Since, in micropropagation only proliferation of plants from axillary buds or apical buds is taking place, significant genetic changes cannot be expected (Murashige 1974).

Microbial contaminations in micropropagation and their control

The major problem that is often encountered in mulberry micropropagation is the high rate of contamination, especially when mature nodal explants are taken from field-grown plants. Field-grown plants are often heavily contaminated with various epiphytic and endophytic organisms. The occurrence of microbial contamination also depends on genotype, maturity of the explants and seasons. During the rainy season, heavy bacterial infection is observed. Patnaik and Chand (1997) reported that an initial thorough washing of field-grown nodal explants with running water for 1-2 h followed by a treatment with 5% (v/v) liquid detergent and 7% sodium hyphochlorite for 8-10 min are essential before surface sterilization with 0.1% aqueous (w/v) mercuric chloride ($HgCl_2$) to contain contamination. Narayan *et al.* (1989) washed field-grown nodal explants under running tap water, immersed them in 0.1% (w/v) carbendazim, an antibiotic, for 20 min and washed them thoroughly with distilled water before treating with 0.2% (w/v) cetavlon, an antiseptic detergent, for 10 min, and with 0.1% (w/v) aqueous $HgCl_2$ solution for 5 min for effective surface sterilization. Chitra and Padmaja (2002) washed nodal explants first in running tap water for 30 min and subsequently surface sterilized them with 70% alcohol for 1 min followed by 0.1% $HgCl_2$ for 15 min under sterile conditions. Our personal experience is that when mature nodal plants are used for micropropagation, fungal growth takes place from the lenticels and scale leaves after 20-30 days of culture. In order to avoid this contamination, it is better to subculture newly sprouted young shoots as soon as possible.

ORGANOGENESIS IN MULBERRY

Plant regeneration from somatic cells has a high impact in woody tree improvement as it facilitates the development of transgenic plants, isolation of somaclonal variants, development of stress-tolerant plants through cell line selection and multiplication of desirable genotypes (Bajaj 1986; Gupta 1988). Therefore, attempts have been made in mulberry to develop protocols for direct plant regeneration from explants like leaves, cotyledons, and embryos and also for indirect plant development via callus culture (**Table 2**). Direct organogenesis from explants has the advantage of inducing the least genetic variation among regenerated plants, i.e. clonal propagation whereas organogenesis from callus has the advantage of higher plant productivity; moreover, plants developed from callus serve as a significant source of variation for isolation of somaclonal variants with desirable traits.

Explants suitable for induction of callus

A variety of explants have been used to initiate callus in mulberry. The first attempt was with cambial regions (Narasimhan *et al*. 1970), which was followed by efforts with hypocotyls segments (Kathiravan *et al.* 1995, 1997; Shajahan *et al.* 1997), cotyledons (Thomas 2003), stem segments (Oka and Ohyama 1976; Mhatre *et al*. 1985; Narayan *et al.* 1989; Jain and Datta 1992; Sahoo *et al*. 1997; Vijayan *et al.* 1998; Bhatnagar *et al.* 2001; Bhau and Wakhlu 2001), and young leaves (Susheelamma *et al.* 1996; Bahu and Wakhlu 2001; Chitra and Padmaja 2005). Callus formation was obtained from all these explants, though a strong genotype × PGR interaction was observed in most cases (Mhatre *et al.* 1985; Rao *et al.* 1989; Susheelamma *et al.* 1996). From these attempts, it became clear that internodal segments from young shoots are the best explants for callus initiation (Oka and Ohyama 1976; Mhatre *et al.* 1985; Narayan *et al.* 1989; Jain and Datta 1992; Sahoo *et al.* 1997; Vijayan *et al.* 1998; Bhatnagar *et al.* 2001; Bhau and Wakhlu 2001).

Effect of medium composition on callus induction and growth

As in the case of micropropagation, the most common basal medium used for callus induction and organogenesis in mulberry has been MS medium (Murashige and Skoog 1962), although some other media like LS medium (Linsmaier and Skoog 1965) were also tested (Jain and Datta 1992). Similarly, 2,4-D was the most widely used PGR for callus induction. The addition of Kn, IAA and NAA into medium together with 2,4-D was found to enhance the proliferation capability of the callus in some cases. It has also been noticed that by adding abscisic acid (ABA), viability of the callus in the culture medium can be sustained up to 8 weeks (Onishi *et al.* 1986). The addition of coconut water (CW; 150 ml L^{-1}) and casein acid hydrolysate (CH; 100 mg L^{-1}) was found to be essential for callus induction from foliar explants cultured on MS medium containing 2 mg L^{-1} 2,4-D (Susheelamma *et al.* 1996). Sahoo *et al.* (1997) found that the combination of NAA (2.0 mg L^{-1}) and BA (0.2 mg L^{-1}) was essential for callus induction from hypocotyls. They also observed that the addition of BA (0.5 mg L^{-1}), CH (100 mg L^{-1}) and 15% fresh CW had a positive effect on callus induction from internodal segments. Pre-soaking of internodal explants in a low concentration of BA (1.0 mg L^{-1}) for 48 to 72 h was essential to induce a good amount of greenish, friable and nodular callus in *M. bombycis* (Jain and Datta 1992) and the untreated explants developed only numerous roots without any callus formation. However, Kathiravan *et al.* (1995) and Bhau and Wakhlu (2001) later found that this pretreatment of explants with BA was not a prerequisite for callus initiation from explants of other genotypes. Thus, overall, it is clear that MS basal medium is the best for induction of callus, and the type of explant, parental genotype, PGRs and other supplements in the medium highly influence induction and development of callus in mulberry. Similarly, multiplication of callus is essential for organogenesis as subculturing makes the callus more friable and responsive to changes in PGRs (Vijayan *et al.* 1998; Bahu and Wakhlu 2001). Subculturing at an interval of 4 weeks is better for callus growth and for preventing blackening of the callus.

Shoot induction from callus

Shoot buds from callus can be induced by growing the callus on a medium supplemented with a higher concentration of cytokinin and less auxin. BA was the most commonly used cytokinin. Narayan *et al.* (1989) reported successful regeneration of plantlets from the callus of *M. alba* in MS medium supplemented with BA (2 mg L^{-1}). In *M. bombycis* shoot buds were induced from callus on LS medium sup-

Table 2 Experiments on oraganogenesis in mulberry (*Morus* spp.) and the results.

Species	Cultivar	Explant	Medium and PGR (mgL^{-1})			Remark	References
			Induction	Organogenesis	Rhizogenesis		
M. alba	---	Cambial region	MS + 2,4D (0.06)	---	---	Up to callus formation	Narasimhan *et al*. 1970
M. alba	Kenmochi, Yoso, Ichinose	Roots, Hypocotyl, Isolated buds	MS + 2,4D (1); IAA (1.0)/ NAA (1)	MS + Kn (0.1) + NAA (1.0)	MS	Plant to soil	Ohyama 1970
M. alba	---	Young twigs	MS + 2,4D/ IAA/ IPA/IAA/ NAA/ 2,4,5 T/GA_3 (1^1)	---	---	Only rooting from callus	Ghugale *et al*. 1971
M. alba	Ichinose	Stem segments	MS+2,4D/ IAA/ IBA/ Kn/ BA (1)	--	--	Only callus	Oka and Ohyama 1973
M. alba	Kenmochi	Winter buds	MS+ BA (1)	---	---	Up to shoot formation	Oka and Ohyama 1974
M. alba	---	Young stem pieces	MS + BA)/ NAA/ 2,4D (1)	---	---	Up to callus	Oka and Ohyama 1976
M. indica	---	Axillary buds	---	MS + IBA (0.5)_+ 2,4D (0.5) + NAA (0.5)	MS + Kn (1) + NAA(0.5)	---	Patel *et al.* 1983
M. alba	---	Mature embryos, cotyledons, leaves	---	MS/AE + BA (1.0-5.0) + NAA (0.001-0.1)	MS + IBA(0.1-5)	Plants transferred to soil	Kim *et al*. 1985
M. indica		Leaves, internodal segments and axillary buds	MS-macro + micro + B5-vitamins + Coconut milk + 2,4-D (2)	MS + BA (2)	MS (No hormone)	Presoaked in BA. Plants transferred to soil	Mhatre *et al.* 1985
M. alba	S1	Leaves	MS (salts) + B5 (vitamins) + BA (2)+ 2,4D (0.5)	---	---	Callus initiated	Tewary *et al.* 1989
M. alba	S1	Internodal segments	MS + 2,4D (1) + BA (05)	MS + NAA(0.5) + BA (2)	MS + NAA (0.5) + BA (1)	Plants transferred to soil	Narayan *et al*. 1989
M. alba	S1	Shoot apices	MS + NAA (0.5-2.0)+ BA (2)	--	MS + NAA (1-2)	Plant transferred to soil	Tewary and Rao 1990
M. bombycis		Internodal segments	LS + 2,4D (2) + NAA (.5) + BA (0.5)	LS + BA (2)	LS + IBA (.2-0.5)	Plant transferred to soil	Jain and Datta 1992
M. alba	MR2	Hypocotyl segments	MS/ LS + IAA (0.5) + BA (0.75)	LS + IAA (0.5) + BA (0.75)	IBA (0.75)	Plant transferred to soil	Kathiravan *et al.* 1995
M. alba	MR2	Hypocotyl segments	MS + 2,4D (1) + BA (0.5)	Liquid MS + 2,4D (0.5)	---	Embryoids only	Shajahan *et al*. 1995
M. indica		Embryos	---	MS salt + B5 vitamins + TDZ (1)		Embryoids only	Thin and Katagiri 1995
Morus spp.	25 genotypes	Young leaves	MS + 2,4D (2) + Casein acid hydrolysate (100) + coconut water (150 ml L^{-1})	MS + BAP (2) + NAA (1)	---	Plant transferred to soil	Susheelamma *et al.* 1996
M. alba	MR2	Hypocotyl segments	MS + 2,4-D (1.5) + BA (0.5)	LS + NAA (0.5) + BA (0.75)	IBA (0. 5)	Plant transferred to soil	Kathiravan *et al.* 1997
M. alba	S1	Hypocotyl segments	MS + 2,4D (2)	MS + BA (2)	NAA (0.1)	Plant transferred to soil	Vijayan *et al.* 1998
M. indica	S13	Internodal segment	MS + 2,4D (2) + BA (0.5)	MS + BA (0.5)	MS + IBA (1)	Plant transferred to soil	Sahoo *et al.* 1997
Morus ssp.	Shin-ichinose, Hayatesakari, Kokuso-21, Unryu, Kyukyokuso, Kibanjumonji, Garyu, Shidareguwa, Jikunashi, Ryomensou, Shinjuro, Keikansou, Turugisanso	Leaf explants	---	MS + BA (1.02)/ B5 + BA (1.02) + fructose	---	Only shoot induction from leaf explants. Nitrate: amonica ration was optimized as 1:1 or 3:1.	Yamanouchi *et al*. 1999
M. alba	S1	Leaf	---	MS + BA (2) + glucose as the carbohydrate	MS + NAA (0.1)	Plant transferred to soil	Vijayan *et al*. 2000
M. alba	Chinesewhite, Kokuso, Ichinose	Leaves, petioles, internodal segments	MS (1) + 2,4-D (1) + BA (0.5)	MS + BA (1) + TIBA (0.1)	MS + IBA/ NAA (0.5)	Plant transferred to field	Bhau and Wakhlu 2001

Table 2 (Cont.)

Species	Cultivar	Explant	Medium and PGR (mgL^{-1})			Remark	References
			Induction	Organogenesis	Rhizogenesis		
M. indica	K2	Hypocotyl, cotyledon, leaves, petioles, internodal segments and roots	---	MS + TDZ (.5-1)	NAA (1) + 0.1 % activated charcoal	Plant transferred to field	Bhatnagar *et al*. 2001
M. alba		Mature zygotic embryo	---	MS + 2,4-D (2) + BAP (0.5)	---	Primary zygotic embryos	Agarwal *et al*. 2002
M. alba		Primary somatic embryos		MS + 2,4-D (0.05) + BAP (0.1) and 6% sucrose	---	Secondary embryoids	Agarwal *et al*. 2004.
M. spp.	DD, K2, AR-12, MR-2	Leaf, epicotyl, hypocotyl		MS+TDZ+IAA+AgNO$_3$		Organogenesis	Raghunath *et al*. 2009

AE: von Arnold and Eriksson (1981) medium, MS: Murashige and Skoog (1962) medium, 4PU: [NL(2-chloro-4-pyridyl)NLphenyl-urea], 2,4-D: 2,4-dichlorophenoxyacetic acid, 2,4,5 T: 2,4,5-trichlorophenoxyacetic acid, BA: 6-benzyladenine, BAP: 6-benzylamino purine, IAA: indole-3-acetic acid, IBA: indole-3-butyric acid, IPA: indole-3-proponic acid, GA$_3$: gibberellic acid, Kn: kinetin, NAA: α-naphthaleneacetic acid, TDZ: thidiazuron.

plemented with BAP 0.5 mg L^{-1} (Jain *et al.* 1992). The addition of gibberellic acid (GA$_3$) and dithiothreitol (DTT) to the culture medium breaks pseudo-dormancy and initiates the regeneration capacity of stored calli (Yasukura and Onishi 1990). Susheelamma *et al.* (1996) formed shoots by transferring callus into MS medium supplemented with 1 mg L^{-1} BA. They found that the addition of 0.1 mg L^{-1} 2,3,5-triiodobenzoic acid (TIBA) to the medium enhanced shoot formation. Further, it was reported that TIBA enhances the long-term organogenic potential of the callus. Susheelamma *et al.* (1996) also observed that there was a strong genotypic and hormonal influence on all developmental phases of tissue culture in mulberry. This conforms with the findings of Mhatre *et al.* (1985) and Rao *et al.* (1989) that under the same cultural conditions genotypes differ greatly in their response. In addition, the existence of a strong genotype × PGR or medium interaction for callus initiation from leaf meristems and regeneration ability in 25 genotypes was also noticed (Susheelamma *et al*. 1996).

Direct shoot induction from explants

Direct shoot induction from explants like leaves and cotyledons has the advantage of generating genetically homogenous populations. Direct organogenesis is also important for the development of transgenic plants. In mulberry, direct plant formation from leaf explants was first reported by Kim *et al.* (1985) and later by Yamanouchi *et al.* (1999). Vijayan *et al*. (2000) also obtained shoots from leaves on MS medium supplemented with 2 mgL^{-1} BAP and glucose as the carbon source. Subsequently, Bhatnagar *et al*. (2001) used hypocotyls, cotyledons, leaves, internodal segments, roots and petiole explants to induce direct shoot regeneration on MS medium supplemented with thidiazuron (TDZ). They achieved 50% regeneration from hypocotyls and 70% regeneration from cotyledons of *M. indica* cv. 'K2'. Leaf explants produced adventitious buds after 30 days of culture on 0.5 mg L^{-1} TDZ. 7-day old leaf explants excised from *in vitro* cultured axillary buds were more efficient in terms of regeneration percentage than leaf explants derived from seedlings or from plants growing in an experimental field. The effectiveness of TDZ on direct shoot induction from leaf explants was later confirmed by Chitra and Padmaja (2005) using four cultivars such as M-5, S36, S13 and China white. They obtained high shoot regeneration (77.6-89.2%) by culturing the explants on media containing 18.17 µM TDZ for the initial 10 days and later on media containing 8.88 µM BAP. Yamanoushi *et al.* (1999) observed that ratio of nitrate ions and ammonium ions in the medium plays a crucial role in the induction of shoot buds directly from leaves. A 1: 1 or 3: 1 ratio was optimal for direct shoot formation. Induction of somatic embryos in mulberry was also attempted, though not much success has yet been reported. Nevertheless, Shajahan *et al.* (1995) reported the formation of embryo-like structures from liquid cultures of hypocotyls-derived callus. Agarwal *et al.* (2002, 2004) could obtain primary and secondary somatic embryoids by culturing zygotic embryos. These reports point to the possibility of developing suitable protocols for somatic embryogenesis in mulberry.

Development of haploids

Although many years of intense research has led to the development of inbred lines through conventional methods, no success has been reported to date due to inbreeding depression, a long juvenile period, high heterozygosity and the dioecious nature of mulberry plants. Inbred lines are extremely important for genetic analysis of many important traits in mulberry. In fact, the genetic basis of almost all agronomically important traits in mulberry is not yet well understood. Since doubled haploidy is another way of developing homozygous lines, efforts have been made to develop haploid plants through andro- or gynogenesis. The first attempt to develop haploids through anther culture was made by Lin *et al.* (1987) and Katagiri (1989). They could get only divisions in the cultured pollen. Katagiri and Venkateshwaralu (1991), Venkateshwaralu and Katagiri (1991), and Sethi *et al.* (1992), Tewary *et al*. (1994) and Chakraborti *et al*. (1999a) reported the formation of globular and heart-shaped embryoids from pollen isolated from anthers cultured on MS media supplemented with glutamine, CW and 2,4-D. Jain *et al.* (1996) reported the induction of callus from microspores cultured at the uninucleate stage on MS medium containing 0.5 mg L^{-1} NAA and 1.0 mg L^{-1} BA. Transferring the callus to MS medium supplemented with NAA and BA (each at 0.5 mg L^{-1}), 1.0 mg L^{-1} 2,4-D and 600 mg L^{-1} polyvinylpyrrolidone (PVP) promoted the differentiation of callus into different types of embryoids. These embryoids later developed shoots and roots when 2,4-D was removed from the medium. However, reproducibility of this protocol was later found to be difficult. Subsequently, Thomas *et al.* (1999) developed protocols for gynogenic haploids. They used MS medium containing 2.0 mg L^{-1} BA and 1.0 mg L^{-1} 2,4-D for the first 3 weeks, then used MS medium with 1.0 mg L^{-1} 2,4-D, 88.8 mg L^{-1} glycine and 15 mg L^{-1} proline for subsequent culture. Using this technique, they developed gynogenic plants which were later transferred to soil. However, despite of all these efforts no report is available where haploid plant was used for development of double haploids or for any other breeding or genetic studies. Thus, it is essential to make intense efforts to fine tune these protocols to develop a large number of haploids from desired parental lines for breeding as well as genetic

studies.

Development of triploids through endosperm culture

Triploids forms of mulberry are superior to diploids and tetraploids in growth, biotic and abiotic stresses, leaf yield and nutritive qualities of leaves (Seki and Oshikane 1959; Hamada 1963). Good characters of mulberry triploids can be perpetuated through clonal propagation with out any loss. The conventional method of triploid production, by crossing a diploid with artificially induced tetraploids, is lengthy and tedious. The endosperm in angiosperms is a triploid tissue formed via double fertilization. Therefore, regeneration of plants from endosperm tissue provides an easy and direct approach to develop triploids (Bhojwani and Razdan 1996). Keeping this in view, Thomas *et al.* (2000) developed triploids from the endosperms of mulberry variety S36. Endosperm cells isolated from young fruits were cultured on MS medium supplemented with 5 µM 2,4-D and different concentrations of BA, Kn, TDZ, IBA, NAA, GA_3, along with tomato juice (TJ), yeast extract (YE), CH and coconut water (CW). Shoot buds developed when the callus was subcultured on a medium containing a cytokinin or the combination of a cytokinin and NAA. The best result for callus induction (70–72%) was obtained on MS medium with 5 mM BAP, 1 µM NAA and 15% CM or 1000 mg/l YE. The maximum number of shoots formed on the medium containing 1 µM TDZ, or 5 µM BA and 1 µM NAA. Cytological investigation indicated that all 10 plants examined cytologically were triploids with 42 chromosomes. This clearly proved the efficiency of the protocol and it could be used for developing triploids from other desirable diploid lines in mulberry.

Protoplast isolation and culture

Non-synchronization of flowering between tropical and temperate mulberry genotypes is a big impediment for hybridizing genotypes from these two groups. Tropical mulberry genotypes normally flower at the end of winter and the beginning of spring whereas temperate mulberry flowers in the middle of spring to the first half of summer (Tikader *et al.* 1995b). In order to overcome this barrier, protoplast isolation and subsequent somatic hybridization can be adopted. Efforts have been made to isolate protoplasts from callus (Onishi and Kiyama 1987) and leaf mesophyll cells (Katagiri 1988; Tewary and Sita 1992; Tewary *et al.* 1995; Umate *et al.* 2005). Ohnishi and Kiyama (1987) demonstrated that primary callus culture gives better protoplast yield than secondary callus cultures. Tewary and Sita (1992) and Umate *et al.* (2005) found a combination of 2% cellulase, 1% macerozyme and 0.5% macerase to be the optimal enzyme concentrations for higher protoplast yields. Katagiri (1989) and Umate *et al*. (2005) found that the first division of protoplsts occurred on the 4^{th} day of plating on a medium containing 2.3 µM zeatin and 2.3 µM 2,4-D. Only medium supplemented with 2.3 µM zeatin and 13.5 µM 2-methoxy-3,6-dichlorobenzoic acid (dicamba) supported subsequent divisions. Micro colonies reached approximately 50 cells after 40-42 days of culture. The cells of these colonies continued growth leading to the formation of micro calli. Whole plants were obtained by culturing the micro-calli on MS medium containing 4.5 µM TDZ and 1.7 µM IAA. Earlier, Ming *et al.* (1992) also demonstrated the regeneration of complete plants from callus derived from mesophyll protoplasts of mulberry through organogenesis. Similarly, protoplast fusion in mulberry was successfully carried out using a chemical fusogen (Onishi and Kiyama 1987) and electro-fusion (Onishi and Tanabe 1989). However, further reports on the success of somatic hybridization and plant regeneration are lacking. Therefore, efforts in this direction are urgently needed.

Somaclonal variants in mulberry

The potential of somaclonal variation is enormous in asexually propagated plants like mulberry due to the ease by which such variations can be maintained. The first result on development of a somaclonal variant (SV1) in mulberry was from *M. alba* var. S1 (Narayan *et al.* 1993). The leaf yield and number of branches of SV1 were superior to those of the mother plant S1 (Narayan *et al.* 1993). Field testing at different locations and cultural practices like irrigated, rainfed and saline soils revealed that SV1 was better than S1 in several agronomic traits, especially leaf yield. SV1 in irrigated condition yielded 34,938 kg/ha/year, which was 26% higher than S1 (28,048 kg/ha/year). Similarly, the number of branches and plant height were also significantly higher in SV1. In rain-fed condition leaf yield of SV1 (13,105 kg/ha/year) was slightly higher (6.0%) than S1 (12, 356kg/ha/year). In saline soil, the leaf yield of SV1 (17,557 kg/ha/year) was 13.27% higher than S1 (15, 449 kg/ha/year) (Chakraborti *et al.* 1999b). The same trend was observed in other leaf yield-contributing characters. The somaclonal variant was phenotypically stable after repeated multiplication through stem cuttings and also under different growing conditions. Based on the result, SV1 has been recommended for commercial exploitation under irrigated conditions (Chakroborti *et al.* 1999b). Similarly, Susheelamma *et al.* (1996) also isolated another somaclonal variant from plantlets developed through callus culture of var. S-14. The somaclonal variant showed beneficial variations like shorter internodal distance (2.90 cm), thicker leaves (150.45 µm), higher chlorophyll content (3.8 mg/g fresh wt), higher moisture content (79.50%) and better moisture retention (67.80% after 12 hr) compared with the same traits in parent plants. These results clearly suggest that generation of beneficial somaclonal variants in mulberry is quite possible if large-scale attempts are made.

Problems associated with callus culture and organogenesis in mulberry

The high phenolic contents often exuded from explants and callus makes them brownish due to enzymatic oxidation. The oxidative products of phenolic compounds also lower the pH of the tissue culture medium, thereby inhibiting further growth and development. Several measures like the addition of activated charcoal (Mhatre *et al.* 1985), silver nitrate (Chakraborti *et al.* 1999a), and regular subculturing at an interval of 20-25 days (Narayan *et al.* 1989; Vijayan *et al.* 1998) have been found to be effective to ward off such problems to a certain extent. The genotype of the plant also plays a major role in the phenol exudation. For instance, calli of *M. laevigata* and *M. serrata* were found blackening much faster than that of *M. alba* and *M. indica*. Hence, it is difficult to pin-point a single factor and suggests a solution for it. The best preventive measure can be decided only through trial and error.

OTHER APPLICATIONS OF TISSUE CULTURE TECHNIQUES IN MULBERRY

Screening for stress tolerance

Salt tolerance in plants is a complex phenomenon involving morphological and developmental changes as well as physiological and biochemical processes. Screening of genotypes for salt tolerance in field conditions is not a suitable procedure as the levels of salinity in the field varies depending on season and soil depth. Plants also interact with many other environmental factors which interfere with the expression of salinity tolerance. Therefore, screening of plants has to be conducted under controlled environmental conditions to facilitate true expression of the plant's innate ability to tolerate the stress caused by salinity. *In vitro* screening of axillary buds and shoot tips for salt tolerance was found to be an efficient, rapid and cost effective

method for early detection of salt tolerance in mulberry. Hossain *et al.* (1991), Twewary *et al*. (2000) and Vijayan *et al.* (2003) used this technique to isolate salt-tolerant genotypes from several germplasm accessions. Testing of the selected genotypes in pot culture further confirmed the efficacy of *in vitro* screening for salt tolerance in mulberry. Seed germination under *in vitro* saline conditions was also used as a criterion for identification of salt-tolerant maternal parents (Vijayan *et al.* 2004). Ahamad *et al*. (2007) used *in vitro* screening to study the effect of $NaHCO_3$ on various characters of mulberry. They found that 59 mM $NaHCO_3$ reduces the shoot length by 49.1 and 43.2% and the fresh shoot weight by 83.8 and 70.2% in 'Sujanpuri' and the local cultivar, respectively. Cell line cultures were also used to develop salt-tolerant plants in mulberry. Kathiravan *et al.* (1995) developed salt tolerant plants of the cultivar MR2 by subjecting the callus continuously to 0.5% NaCl for 8 months. Regarding drought tolerance, Tewary *et al.* (2000) screened five selected mulberry genotypes such as G2, G3, G4, S13 and S34 for osmotic stress using 1.0-10% polyethylene glycol (PEG) to simulate osmotic stress using 14 different media combinations. Out of the 14 media combinations, the optimum responses were observed on 1 mg/l Kn, in the case of G3 genotype, on 2 mg/l Kn with G2 genotype, on 1 mg/l BAP with G4 genotype and on 2 mg/l BAP with S34 and S13 genotypes. S13 itself exhibited the highest sprouting percentage and shoot growth compared to the other genotypes. The genotype G3 has been identified as suitable for drought prone areas.

Induction of tetraploidy

Triploids in mulberry have better leaf yield, stress resistance and leaf quality. In mulberry, leaf is the main produce, thus, sterility of triploids does not have any influence either on the primary product or the propagation of the plant as triploids can be propagated through stem cuttings. Therefore, efforts have been made to develop triploids through crossing between diploids and tetraploids with desirable agronomic traits. Tetraploids from desirable diploids are developed by treating growing apical buds with colchicine. Conventionally, colchicine is applied by maintaining soaked cotton pads on actively growing axillary buds for 1-2 days. However, in this method cotton pads dry quickly, there is excessive loss of colchicines, etc. In order to circumvent these problems, *in vitro* application of colchicine was adopted in mulberry (Chakraborti *et al.* 1998). Apical buds were cultured on MS medium supplemented with 2 mg L^{-1} BAP and four concentrations of colchicine (0.0, 0.05, 0.1 and 0.2% w/v). Tetraploidy at a frequency of 39.4 ± 4.8% was obtained from 0.1% colchicine. A further increase in the concentration of colchicine significantly reduced the percentage of tetraploids. Morphological, histological and cytological evidence showed phenotypic and genomic similarities in tetraploids induced *in vitro* and (conventional method) *ex vitro*. A comparison of both *in vitro* and *ex vitro* techniques indicated that the *in vitro* technique was 80.8% more efficient than the *ex vitro* method. The repeated use of the same colchicine medium for at least 4 repeated treatments made the *in vitro* technique more cost effective than the *ex vitro* method.

Synthetic seeds

The acclimatization of tissue cultured plants and their delivery to the field is one of the major bottlenecks for wider adoption of *in vitro* technology on a commercial basis. Synthetic seed technology has great potential to overcome the problems in acclimatization and transportation of *in vitro* developed plants. Synthetic seeds are basically defined as "encapsulated somatic embryos which functionally mimic seeds and can develop into seedlings under sterile conditions" (Bapat 1993). In a broader sense, it would also refer to encapsulated buds or any other form of meristems which can develop into plants. An encapsulable unit is a necessary component of a synthetic seed. In mulberry, synthetic seeds are produced by encapsulating the apical/axillary buds or somatic embryos with 3-5% sodium alginate and 100 mM calcium chloride ($CaCl_2$) solution as the complexing agent (Bapat *et al.* 1987). Sodium alginate solution is mixed with tissue culture medium containing all necessary ingredients essential for proper growth. Chand *et al.* (1994) successfully developed a technology for artificial seeds in mulberry. The use of synthetic seed technology for mulberry propagation is limited to a few species of *M. indica* (Bapat and Rao 1990). The possibility of using *in vitro*-derived vegetative propagules for synthetic seed production has been explored by researchers since it is difficult to develop somatic embryos in mulberry (Pattnaik and Chand 2000; Kavyashree *et al.* 2004). Shoot cultures established from axillary buds on Linsmaier and Skoog's basal medium supplemented with 4 mg L^{-1} BAP and 1 mg L^{-1} TIBA used for encapsulation. Sodium alginate and carboxy methyl cellulose were added at 2, 4, 6 and 8% (w/v) to liquid initiation medium separately. For complexation, 25×10^3, 5×10^4, 75×10^3 and 10×10^4 µM $CaCl_2$ solutions were prepared using distilled water. Gel was complexed by mixing the axillary buds with hydrogels, dropping these into different concentrations of $CaCl_2$ solution and incubated in an orbital shaker for different time intervals (20, 30, 40, 50 and 60 min) to obtain uniform beads. The sodium alginate and carboxy methyl cellulose embedded axillary buds were collected using a sterilized tea strainer and rinsed 2–3 times in sterile water to remove traces of $CaCl_2$. The synthetic seeds were tested for their conversion potential under *in vitro* and *ex vitro* conditions. Although different concentrations of sodium alginate were used, the best results were obtained at 4% alginate (Bapat *et al.* 1987).

Cryopreservation of germplasm

Owing to their high heterozygosity, conservation of mulberry germplasm through seeds is not practiced. Therefore, mulberry genetic resources are conserved through in *ex situ* germplasms located in different research institutes and botanical gardens. Maintenance of plants in the field is simple, technically less demanding and provides easy access to the conserved material for divergent uses. However, it is in a risk of destruction by natural calamities, pests and diseases. Cryopreservation of plant materials has proven to be a potentially ideal method for long-term preservation of tree crops, because it requires minimum space, labor, medium and maintenance cost. Divergent cryopreservation techniques are currently used (Bajaj 2008). The classical technique was based on freeze-induced dehydration of cells while the newer technique was based on vitrification (Engelmann 2000). Classical cryopreservation techniques involve slow cooling down at a controlled rate (usually 0.1-4°C/min) down to about -40°C, followed by rapid immersion of samples in liquid nitrogen ($N_{2\ (l)}$). They are generally operationally complex, as they require the use of sophisticated and expensive programmable freezers. In the new vitrification-based procedures, cell dehydration is performed prior to freezing by physical or osmotic dehydration of explants. This is followed by ultra-rapid freezing which results in vitrification of intracellular solutes, i.e. formation of an amorphous glassy structure without occurrence of ice crystals, which are detrimental to cellular structural integrity. These techniques are less complex and do not require a programmable freezer, hence are suited for use in any laboratory with basic facilities for tissue culture. Cryopreservation involves storage of plant material at ultra-low temperatures in $N_{2\ (l)}$ (-196°C). At this temperature, cell division and metabolic activities remain suspended and the material can be stored without changes for long periods. Thus, cryopreservation method ensures genetic stability of mulberry germplasm and requires limited space, protects material from contamination, involves very little maintenance and is considered a cost-effective method for conservation of mulberry germplasm. In fact, cryopreservation is the only

available method for long-term conservation of vegetatively propagated plants like mulberry. In mulberry the most appropriate material for cryopreservation is winter buds, though embryonic axes, pollen, synthetic seeds can also be used (Niino and Sakai 1992; Niino *et al.* 1992a, 1992b, 1993; Niino 1995) Keeping this in mind, many laboratories across the world has established cryopreservation laboratories. For instance, the cryopreservation facilities at the Central Sericutiral Germplasm Resources Centre (CSGRC), Hosur are actively involved in preservation of 908 mulberry germplasm accessions (Rao *et al.* 2007). Success has been achieved in the cryopreservation of several accessions belonging to *M. indica*, *M. alba*, *M. latfo1ia*, *M. cathayana*, *M. laevigata*, *M. nigra*, *M. australis*, *M. bombycis*, *M. sinensis*, *M multicaulis* and *M. rotundioba*. Likewise, following the cryopreservation research by Sakai (1960), Japan has undertaken cryopreservation of mulberry at a large scale. About 450 germplasm accessions within several species have been cryopreserved in $N_{2\ (l)}$ tanks in a mulberry gene bank (Okuno *et al.* 2004). Shoot tips of pre-frozen winter buds of *M. bombycis* were able to withstand long-term storage in $N_{2\ (l)}$. The general procedure for cryopreservation of shoot tips is that the shoot segments are first pre-frozen at -3°C for 10 days, -5°C for 3 days, -10°C for 1 day and -20°C for 1 day before immersion in $N_{2\ (l)}$. Buds were cultured on MS medium after thawing in air at 0.0 to 20.0°C. Survival rate was 55 to 90% (Rao *et al*. 2007). Prior to pre-freezing at -20°C partial dehydration of the bud up to 38.5% improves the recovery rate. The survival rates of winter buds stored in $N_{2\ (l)}$ up to 3-5 years did not change significantly. Encapsulation of winter hardened shoot tips of many mulberry species with calcium alginate coating was also tested successfully. In addition, Yakua and Oka (1988) conducted experiments on cryopreservation of intact vegetative buds of *M. bombycis* attached to shoot segments by prefreezing and storing in $N_{2\ (l)}$. The buds were later thawed, and the meristems were excised for culture on MS medium supplemented with 1 mg L^{-1} BA to regenerate plants. Either prefreezing at -10 or -20°C along with rapid thawing at 37°C or prefreezing at -20 or -30°C along with slow thawing at 0°C was a suitable condition for high percentage survival and shoot regeneration.

Development of transgenic plants

Genetic improvement through gene transfer has not been very successful in mulberry due to the recalcitrant nature of the plant. Initial attempts to transform mulberry callus through *Agrobacterium tumefaciens* and particle bombardment by Machii (1990), Machii *et al.* (1996), Nozue *et al.* (2000) and Oka and Tewary (2000) resulted only in transformation of callus but no transgenic plants could be developed. Later, Bhatnagar *et al.* (2002, 2003) succeeded in regenerating transgenic plants with stable incorporation of the β-glucuronidase (GUS) gene. Subsequently, transgenic plants with glycine gene *AlaBlb* and oryzacystatin gene *OC* were developed in China (Wang *et al*. 2003). Recently, using *Agrobacterium*-mediated transformation a transgenic plant overexpressing the barley *HVA1* gene was developed in India (Lal *et al.* 2008a). Thus, a protocol for transgenic plant development is now well established in mulberry. However, genetically modified plants in mulberry should be developed with great care as mulberry is anemophilous and cross pollinating, hence the transgene can easily spread into wild species.

CONCLUSION AND FUTURE PROSPECTS

It is evident from the foregoing discussions that considerable achievement has been made on micropropagation in mulberry. Now it is easily possible to propagate mulberry from nodal explants and shoot tips following the schemes shown in **Fig. 1**. However, transferring this technology to a large-scale commercial purpose has not been achieved till to date (Tewary and Oka 1999). Thus, to make micropropagation of mulberry commercially viable and feasible, further effort in this direction is required urgently. Similarly, a protocol for organogenesis in mulberry through both callus culture and direct plantlet formation is also well established

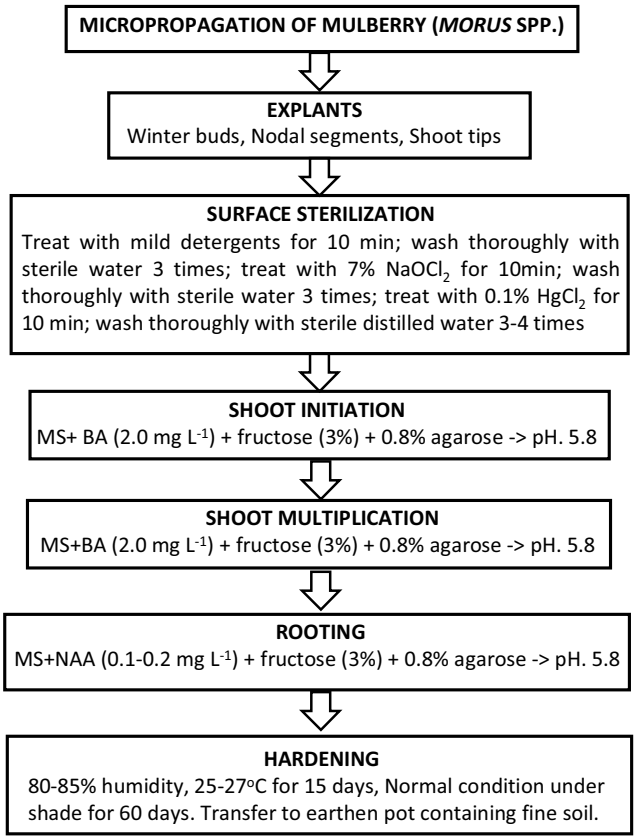

Fig. 1 Schematic representation of the procedures of micropropagation in mulberry.

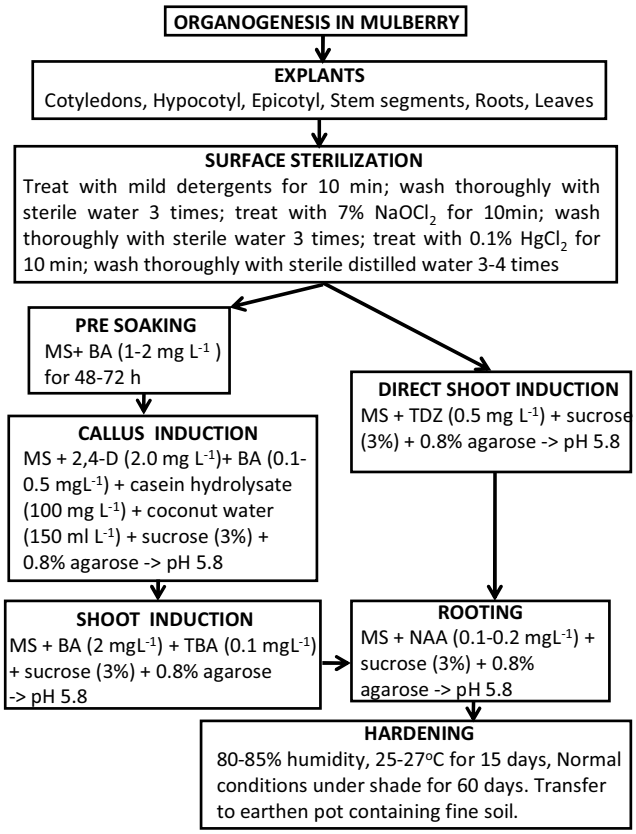

Fig. 2 Schematic representation of the procedures of organogenesis in mulberry.

as depicted in **Fig. 2**. However, not much progress has been made on somatic embryogenesis compared to many other tree crops. Since somatic embrygenesis has a key role in gene transfer technologies, emphasis must be given to develop a reproducible protocol for somatic embryogenesis in mulberry. Although there is tremendous scope for transgenic plants with higher leaf productivity and better leaf quality in mulberry, not much progress has been made in mulberry. Although a few isolated efforts to transfer reporter genes like GUS through *A. tumefaciens* and particle bombardment, no intense research has been made to develop mulberry with desired genes transferred. Finally, it is essential to develop protocols for easy and rapid development of haploids through andro- and gynogenesis. Homozygous lines through doubled haploids can be of much use in deducing the genetic basis of many highly important agronomic traits in mulberry and also to develop genetic maps and QTLs for important traits like leaf yields, leaf quality and stress resistance. These would be essential techniques for the long-term preservation of important mulberry germplasm and genetic resources (Vijayan *et al*. 2011).

ACKNOWLEDGEMENTS

The authors express their deep gratitude to Dr. S. Gandhi Doss and C. Venugopalan Nair for their help in the preparation of this manuscript.

REFERENCES

Agarwal S, Kanwar K, Saini N, Jain RK (2002) *Agrobacterium tumefaciense* mediated genetic transformation and regeneration of *Morus alba* L. *Scientia Horticulturae* **100**, 1-4

Agarwal S, Kanwar K, Sharma DR (2004) Factors affecting secondary somatic embryogenesis and embryo maturation in *Morus alba* L. *Scientia Horticulturae* **102**, 359-368

Ahmad P, Sharma S, Srivastava PS (2007) *In vitro* selection of $NaHCO_3$-tolerant cultivars of *Morus alba* (Local and Sujanpuri) in response to morphological and biochemical parameters. *Horticultural Science (Prague)* **34**, 114-122

Anis M, Faisal M, Singh SK (2003) Micropropagation of mulberry (*Morus alaba* L) through *in vitro* culture of shoot tip and nodal explants. *Plant Tissue Culture* **13**, 47-51

Anuradha M, Pullaiah T (1992) Micropropagation of mulberry (*Morus alba* L.). *Annali di Botanica* **15**, 35-41

Bajaj YPS (1986) Biotechnology of tree improvement for rapid propagation and biomass energy production. In: YPS Bajaj (Ed) *Biotechnology in Agriculture and Forestry (Vol 1) Trees*, Springer Verlag, Berlin, pp 1-22

Bajaj YPS (2008) Cryopreservation of plant germplasm-1. In: Nagata T, Lorz H, Widholm JM (Eds) *Biotechnology in Agriculture and Forestry 32*, Springer-Verlag, Berlin, 54 pp

Bapat VA, Mhatre M, Rao PS (1987) Propagation of *Morus indica* L. (mulberry) by encapsulated shoot buds. *Plant Cell Reports* **6**, 393-395

Bapat VA, Rao PS (1990) *In vivo* growth of encapsulated axillary buds of mulberry (*Morus indica* L.). *Plant Cell, Tissue Organ Culture* **20**, 69-70

Bapat VA (1993) Studies on synthetic seeds of sandalwood (*Santalum album* L.) and mulberry (*Morus indica* L.). In: Redenbaugh K (Ed) *Synseeds: Applications of Synthetic Seeds to Crop Improvement*, CRC Press Inc., Boca Raton, FL, pp 381-407

Benavides J, Lachaux M, Fuentes M (1994) Efecto de la aplicación de estiércol de cabra en el suelo sobre la calidad y producción de biomasa de morera (*Morus* sp.). *Árboles y arbustos forrajeros en América Central*, Technical Report N^o 236, Vol 2, Turrialba, Costa Rica, CATIE, pp 495-514

Bhatnagar S, Kapur A, Khurana P (2001) TDZ-mediated differentiation in commercially valuable Indian mulberry, *Morus indica* cultivars K2 and DD. *Plant Biotechnology* **18**, 61-65

Bhatnagar S, Kapur A, Khurana P (2002) Evaluation of parameters for high efficiency gene transfer via particle bombardment in Indian mulberry, *Morus indica* cv. K2. *Indian Journal of Experimental Biology* **40**, 1387-1392

Bhatnagar S, Kapur A, Khurana P (2003) Evaluation of parameters for high efficiency gene transfer via *Agrobacterium tumefaciens* and production of transformants in Indian mulberry, *Morus indica* cv. K2. *Plant Biotechnology* **21**, 1-8

Bhau BS, Wakhlu AK (2001) Effect of genotype, explant type and growth regulators on organogenesis in *Morus alba*. *Plant Cell, Tissue and Organ Culture* **66**, 25-29

Bhau BS, Wakhlu AK (2003) Rapid micropropagation of five cultivars of mulberry. *Biologia Plantarum* **46**, 349-355

Bhojwani SS, Razdan MK (1996) *Plant Tissue Culture: Theory and Practice* (Revised Edn), Elsevier, Amsterdam

Chakraborti SP, Vijayan K, Roy BN, Quadri SMH (1998) *In vitro* induction in tetraploidy in mulberry (*Morus alba* L). *Plant Cell Reports* **17**, 794-803

Chakraborti SP, Vijayan K, Roy BN (1999a) Isolated microspore culture in mulberry (*Morus* spp.). *Sericologia* **39**, 541-549

Chakraborti SP, Das C, Vijayan K, Misra AK, Raghunath MK, Narayan P, Roy BN (1999b) Field evaluation of somaclonal variant (SV1) developed from *Morus alba* L. var. S1. *Indian Journal of Sericulture* **38**, 69-71

Chand PK, Sahoo Y, Pattnaik SK (1994) Artificial seeds: a novel approach to mulberry propagation. *Indian Silk* **September**, 33-38

Chattopadhay S, Datta SK (1990) A rapid clonal propagation of mulberry tree (*Morus alba*) through tissue culture. *Proceedings of Indian Science Congress* **Part 3**, 208-209

Chattopadhyay S, Chattopadhyay S, Datta SK (1990) Quick *in vitro* production of mulberry (*Morus alba*) plant lets for commercial purpose. *Indian Journal of Experimental Biology* **28**, 522-525

Chitra VDS, Padmaja G (1999) Clonal propagation of mulberry (*Morus indica* L. cultivar M-5) trough *in vitro* culture of nodal explants. *Scientia Horticulturae* **80**, 289-298

Chitra DSV, Padmaja G (2002) Seasonal influence on axillary bud sprouting and micropropagation of elite cultivars of mulberry. *Scientia Horticulturae* **92**, 55-68

Chitra DSV, Padmaja G (2005) Shoot regeneration via direct organogenesis from *in vitro* derived leaves of mulberry using thidiazuron and 6-benzylaminopurine. *Scientia Horticulturae* **106**, 593-602

Castro A (1989) *Producción de leche de cabras alimentadas con King grass (Pennisetum purpureum x P. typloides), suplementadas con diferentes niveles de follaje de Poró (E. poeppigrama) y de fruto de plátano (Musa sp. var. Pelipita).* PhD thesis, University of Costa Rica, CATIE. 58 pp

Das BC, Krishnaswami S (1965) Some observations on interspecific hybridization in mulberry. *Indian Journal of Sericulture* **4**, 1-8

Engelmann F (2000) Importance of cryopreservation for the conservation of plant genetic resources. In: Engelmann F, Takagi H (Eds) *Cryopreservation of Tropical Plant Germplasm. Current Research Progress and Application*, IPGRI, Rome, Italy, pp 8-20

Enomoto S (1987) Preservation of genetic resource of mulberry by means of tissue culture. *Japanese Agriculture Research Quarterly* **21**, 205-210

Gamborg OL, Miller RA, Ojima K (1968) Nutrient requirements of suspension cultures of soybean root cells. *Experimental Cell Research* **50**, 151-158

Ghugale DD, Kulkarni DD, Narasimhan R (1971) Effect of auxins and gibberellic acid on growth and differentiation of *Morus alba* and *Populus nigra* tissues *in vitro*. *Indian Journal of Experimental Biology* **9**, 381-384

Gupta PK (1988) Advances in biotechnology of conifers. *Current Science* **20**, 629-637

Hamada S (1963) Polyploid mulberry trees in practice. *Indian Journal of Sericulture* **1**, 3-6

Hayashi K, Oka S (1995) Formation of multiple bud bodies and plant regeneration in mulberry, *Morus alba. Journal of Sericultural Science Japan* **64**, 117-123

Hossain M, Rahaman SM, Joarder OI (1991) Isolation of sodium chloride-resistant genotypes in some mulberry cultivars. *Bulletin of Sericulture Research* **2**, 67-73

Hossain M, Rahman SM, Zaman A, Joarder OI, Islam R (1992) Micropropagtion of *M. laevigata* Wall. from matured trees. *Plant Cell Reports* **11**, 522-524

Hou YJ (1994) *Mulberry Breeding*, Sericulture Department, Zhejiang Agriculture University, Hangzhou, China, 4 pp

Islam R, Zaman A, Jorder OI, Barman AC (1993) *In vitro* propagation as an aid for cloning of *Morus laevigata* Wall. *Plant Cell, Tissue and Organ Culture* **33**, 339-341

Islam R, Jorder OI, Zaman A, Jorder OI, Barman AC (1994) Clonal propagation of *Morus alba* L. cv S1, from nodal explants of mature trees: maintenance of proliferating cultures. *Sericologia* **34**, 343-345

Ivanicka J (1987) *In vitro* micropropagation of mulberry *Morus nigra* L. *Scientia Horticulturae* **32**, 33-39

Jain AK, Datta RK (1992) Shoot organogenesis and plant regeneration in mulberry (*Morus bombycis* Koidz.): Factors influencing morphogenetic potential in callus cultures. *Plant Cell, Tissue and Organ Culture* **29**, 43-50

Jain AK, Dandin SB, Sengupta K (1990) *In vitro* micropropagation through axillary bud multiplication in different mulberry genotypes. *Plant Cell Reports* **8**, 737-740

Jain AK, Datta RK (1992) Shoot organogenesis and plant regeneration in mulberry (*Morus bombycis* Koidz.): Factors influencing morphogenetic potential in callus cultures. *Plant Cell, Tissue and Organ Culture* **29**, 43-50

Jain AK, Sarkar A Datta RK (1996) Induction of haploid callus and embryogenesis in *in vitro* cultured anthers of mulberry (*Morus indica*). *Plant Cell, Tissue and Organ Culture* **44**, 143-147

Kapur A, Bhatnagar S, Khurana P (2001) Efficient regeneration from mature leaf explants of Indian mulberry via organogenesis. *Sericologia* **41**, 207-214

Karthiravan K, Shajahan A, Ganapathi A (1995) Regeneration of plantlets from hypocotyls derived callus of *Morus alba. Israel Journal of Plant Science* **43**, 259-262

Kathiravan K, Ganapathi A, Shajahan A (1997) Adventitious shoot forma-

tion and plant regeneration from callus cultures of mulberry (*Morus alba* L.) *Sericologia* **37**, 727-733

Kashyap S, Sharma S (2006) *In vitro* selection of salt tolerant *Morus alba* and its field performance with bioinoculants. *Horticulture Science (Prague)* **33**, 77-86

Katagiri K (1980) Callus induction in cultures of mulberry pollen. *Journal of Sericultural Science Japan* **58**, 267-268

Katagiri K (1988) Difference in cell division in mulberry mesophyll protoplast culture due to species and culture conditions. *Journal of Sericultural Science Japan* **57**, 445-446

Katagiri K (1989) Colony formation in culture of mulberry mesophyll protoplasts. *Journal of Sericultural Science Japan* **58**, 267-268

Katagiri K, Venkateswarulu M (1991) Induction of callus, embryo like and organ like division in pollen cultures of Indian mulberry variety Behrampur, *Proceedings of the Japanese Sericulture Association*, Nov. 28-30, Mito, Japan, p 30

Katagiri K, Venkateswaralu M (1993) Induction and calli and organ-like structures in isolated pollen culture of mulberry, *Morus australis* Poiret. *Journal of Sericultural Science Japan* **62**, 1-6

Katagiri K, Venkateswaralu M (1991b) Effects of sugars and sugar alcohols in the division of mulberry pollen in tissue culture. *Journal of Sericultural Science Japan* **60**, 514-516

Katsumata F (1979) Chromosomes of *Morus nigra* L. from Java and hybridization affinity between this species and some mulberry species in Japan. *Journal of Sericultural Science Japan* **48**, 418-422

Kavyashree R, Gayatri MC, Revanasiddaiah HM (2006) Propagation of mulberry variety – S54 by synthetic seeds of axillary bud. *Plant Cell, Tissue and Organ Culture* **84**, 245-249

Kim HR, Patel KR, Thorpe TA (1985) Regeneration of mulberry plantlets through tissue culture. *Botany Gazette* **146**, 335-340

Koyuncu F (2004) Organic acid composition of black mulberry. *Chemistry of Natural Compounds* **40**, 368-369

Sita LG, Ravindran S (1991) Gynogenic plants from ovary cultures of mulberry (*Morus indica*). In: Prakash J, Pierik RLM (Eds) *Horticulture: New Technologies and Applications*, Kluwer Academic Publishers, Dordrecht, pp 225-229

Lal S, Gulyani V, Khurana P (2008) Overexpression of HVA1 gene from barley generates tolerance to salinity and water stress in transgenic mulberry (*Morus indica*). *Transgenic Research* **17**, 651-663

Lin S, JI D, Qiu J (1987) *In vitro* production of haploid plants from mulberry (*Morus*) anther culture. *Scientia Sinica* **30**, 853-863

Linsmaier EM, Skoog F (1965) Organic growth factor requirement for tobacco tissue cultures. *Physiologia Plantarum* **18**, 100-127

Lloyd G, McCown B (1980) Commercially feasible micropropagation of mountain laurel *Kalmia latifolia* by use of shoot tip culture. *International Plant Propagators Society Proceedings* **30**, 421-427

Lu MC (2002) Micropropagation of *Morus latifolia* Poilet using axillary buds from mature trees. *Scientia Horticulturae* **96**, 329-341

Machii M (1990) Leaf disc transformation of mulberry plant (*Morus alba* L.) by *Agrobacterium* Ti plasmid. *Journal of Sericultural Science Japan* **59**, 105-110

Machii M, Sung G-B, Yamanuchi H, Koyama A (1996) Transient expression of GUS gene introduced into mulberry plant by particle bombardment. *Journal of Sericultural Science Japan* **65**, 503-506

Mathur A, Mathur AK, Verma P, Yadav S, Gupta ML, Darokar MP (2008) Biological hardening and genetic fidelity testing of micro-cloned progeny of *Chlorophytum borivilianum* Sant. et Fernand. *African Journal of Biotechnology* **7**, 1046-1053

Mhatre M, Bapat VA, Rao PS (1985) Regeneration of plants from the culture of leaves and axillary buds in mulberry (*Morus indica* L.). *Plant Cell Reports* **4**, 78-80

Ming WZ, Xu ZH, Xu N, Ren HM (1992) Plant regeneration from leaf mesophyll protoplasts of *Morus alba*. *Plant Physiology Communications* **28**, 248-249

Mondal TK, Bhattacharya A, Laxmikumaran M, Ahuja PS (2004) Recent advances of tea (*Camellia sinensis*) biotechnology. *Plant Cell, Tissue and Organ Culture* **76**, 195-254

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plantarum* **15**, 473-497

Murashige T (1974) Plant propagation through tissue cultures. *Annual Review of Plant Physiology* **25**, 135-166

Narasimhan R, Dhruva B, Paranjpe SV, Kulkarni DD, Mascarenhas AF (1970) Tissue culture of some woody species. *Proceedings of the Indian National Science Academy* **LXXI**, 204-212

Narayan P, Chakraborty S, Subba Rao G (1989) Regeneration of plantlets from the callus of stem segments of mature plants of *Morus alba* L. *Proceedings of the Indian National Science Academy* **55**, 469-472

Narayan P, Chakroborti SP, Roy BN, Sinha SS (1993) *In vitro* regeneration of plant from internodal callus of *Morus alba* L. and isolation of genetic variant. *Seminar on Plant Cytogenetics in India, University of Calcutta*, March 4-5, 1993, pp 188-192

Niino T (1995) Cryopreservation of germplasm of mulberry (*Morus* spp.). In: Bajaj YPS (Ed) *Biotechnology in Agriculture and Forestry*, Springer-Verlag, Berlin, pp 102-113

Niino T, Sakai A (1992a) Cryopreservation of alginate-coated *in-vitro* grown shoot tips of apple, pear and mulberry. *Plant Science* **87**, 199-206

Niino T, Sakai A, Yakuwa H (1992b) Cryopreservation of dried shoot tips of mulberry winter buds and subsequent plant regeneration. *Cryo-Letters* **13**, 51-58

Niino T Sakai A, Enomoto S, Magoshi J, Kato S (1992c) Cryopreservation of *in-vitro* grown shoot tips of mulberry by vitrification. *Cryo-Letters* **13**, 303-312

Niino T, Koyaman A, Shirata K, Ohuchi S, Suguli M, Sakai A (1993) Long term storage of mulberry winter buds by cryopreservation. *Journal of Sericultural Science Japan* **62**, 431-434

Nozue M, Cai W, Li L, Xu W, Shioiri H, Kojima M, Saito H (2000) Development of a reliable method for *Agrobacterium tumefaciens*-mediated transformation of mulberry callus. *Journal of Sericultural Science Japan* **69**, 345-352

Ohyama K (1970) Tissue culture in mulberry tree. *Japanese Agriculture Research Quarterly* **5**, 30-34

Ohyama K, Oka S (1976). Regeneration of whole plants from isolated shoot tips of mulberry tree. *Journal of Sericultural Science Japan* **45**, 115-120

Ohyama K, Oka S (1987) Mulberry. In: Bonga JM, Durjan DJ (Eds) *Cell and Tissue Culture in Forestry* (Vol 3), Martinus-Nijhoff Publishers, Leiden, the Netherlands, pp 272-284

Oka S, Ohyama K (1973) Induction of callus and effect of constituents of medium on callus formation of mulberry trees. *Journal of Sericultural Science Japan* **42**, 317-324

Oka S, Ohyama K (1974). Studies on *in vitro* cultures of excised buds in mulberry trees I. Effect of growth substance on the development of shoots and organ formation from winter buds. *Journal of Sericultural Science Japan* **43**, 230-235

Oka S, Ohyama K (1975) Studies on *in vitro* cultures of excised buds in mulberry trees II. Effect of growth substance on the development of shoots from buds. *Journal of Sericultural Science Japan* **44**, 444-450

Oka S, Ohyama K (1976) A study on the medium of subculture of mulberry callus. *Journal of Sericultural Science Japan* **45**, 385-391

Oka S, Ohyama K (1978) Studies on *in vitro* cultures of excised buds in mulberry trees III. Effects of agar concentration, pH and sugar of medium on the development of shoot from winter buds. *Journal of Sericultural Science Japan* **47**, 15–20

Oka S, Ohyama K (1981) *In vitro* initiation of adventitious buds and its modification by high concentration of benzyladenine in leaf tissue of mulberry (*Morus alba*). *Canadian Journal of Botany* **59**, 68-74

Oka S, Ohyama K (1982) Sugar utilization in mulberry (*Morus alba* L.) bud culture. *Proceedings of the 5th International Congress on Plant Tissue and Cell Culture*, pp 67-68

Oka S, Ohyama K (1986) Mulberry (*Morus alba* L.). In: Bajaj YPS (Ed) *Biotechnology in Agriculture and Forestry* (Vol 1), Springer-Verlag, Berlin, pp 384-392

Oka S, Tewary PK (2000) Induction of hairy roots from hypocotyls of mulberry (*Morus indica* L.) by Japanese wild strains of *Agrobacterium rhizogenes*. *Journal of Sericultural Science Japan* **69**, 13-19

Okuno K, Shirata K, Niino T, Kawase M (2004) Plant genetic resources in Japan: Platforms and destinations to conserve and utilize plant genetic diversity. *Japanese Agriculture Research Quarterly* **39**, 231- 237

Ohnishi T, Yasukura F, Tan J (1986) Preservation of mulberry callus by the addition of both abscisic acid (ABA) and p-aminobenzoate (PABA) in culture medium. *Journal of Sericultural Science Japan* **55**, 252-255

Ohnishi T, Kiyama S (1987) Effects of change in temperature, pH, Ca ion concentration in the solution used for protoplast fusion on the improvement of the fusion ability of mulberry protoplasts. *Journal of Sericultural Science Japan* **56**, 418-421

Ohnishi T, Tanabe K (1989) On the protoplast fusion of mulberry and paper mulberry by electrofusion method. *Journal of Sericultural Science Japan* **58**, 353-354

Ohnishi T, Kobayashi Y (1991a) Separation of free cells from the hypocotyl of mulberry. *Journal of Sericultural Science Japan* **60**, 117-124

Ohnishi T, Kobayashi Y (1991b) Suspension culture of cells from hypocotyl of mulberry. *Journal of Sericultural Science Japan* **60**, 320-323

Oshigane K, Zhuang D (1990) Effect of *in vitro* colchicine treatment of culture isolated buds in mulberry. *Journal of Sericultural Science Japan* **59**, 187-195

Patel GK, Bapat VA, Rao PS (1983) *In vitro* culture of organ explants of *Morus indica*: Plant regeneration and fruit formation in axillary bud culture. *Zeitschrift für Pflanzenphysiologie* **111**, 465-468

Pattnaik SK, Sahoo Y, Chand PK (1996) Micropropagation of *Morus nigra* L. from shoot tip and nodal explants of mature trees. *Scientia Horticulturae* **42**, 61-67

Pattnaik SK, Chand PK (1997) Rapid clonal propagation of three mulberries, *Morus cathayana* Hemsl., *M. lhou* Koiz. and *M. serrata* Roxb., through *in vitro* culture of apical shoot buds and nodal explants from mature trees. *Plant Cell Reports* **16**, 503-508

Rao PS, Bapat VA, Mhatre M, Patel GK (1989) Application of plant cell, tissue and organ culture in mulberry improvement programme. In: Sengupta K, Dandin SB (Eds) *Genetic Resources of Mulberry and Utilization*, Central Sericultural Research and Training Institute, India, pp 125-131

Rao PS. Bapat VA (1993) Micropropagation of sandalwood (*Santalum album* L.) and mulberry (*Morus indica* L.). In: Ahuja MR (Ed) *Micropropagation of Woody Plants*, Kluwer Academic Publishers, Dordrecht, pp 317-345

Rao AA, Chaudhury R, Kumar S, Velu D, Saraswat RP, Kamble CK (2007) Cryopreservation of mulberry germplasm core collection and assessment of genetic stability through ISSR markers. *International Journal of Industrial Entomology* **15**, 23-33

Raghunath MK, Chakraborthy SP, Roy B, Sen SK (1992) Micropropagation of superior hybrids through axillary bud culture in mulberry (*Morus* L.). *Cell and Chromosome Research* **15**, 12

Raghunath MK, Lal S, Khurana P (2009) *In vitro* plant regeneration from different explants of elite mulberry (*Morus* sp.) genotypes. *Bangladesh Journal of Sericulture* **3**, 31-39

Sahoo Y, Pattnaik SK, Chand PK (1997) Plant regeneration from callus cultures of *Morus indica* L. derived from seedlings and mature plants. *Scientia Horticulturae* **69**, 85-90

Sanjappa M (1989) Geographical distribution and exploration of the genus *Morus* L. (Moraceae). In: Sengupta K, Dandin SB (Eds) *Genetic Resources of Mulberry and Utilization*, Jwalamukhi Job Press, Bangalore, India, pp 4-7

Sakai A (1960) Survival of twigs of woody plants at –196°C. *Nature* **185**, 392-394

Sau H, Sahu PK, Yadav BRD, Saratchandra B (1995) Evaluation of mulberry (*Morus* spp.) genetic resources – sprouting, survival and rooting ability. *Journal of Environmenatal Research* **3**, 11-13

Seki H, Oshikane K (1959) Studies in polyploid mulberry trees III. The valuation of breeded polyploid mulberry leaves and the results of feeding silkworms on them. *Research Report of the Faculty of Text Sericulture Shinshu University* **9**, 6-15

Sethi M, Bose S, Kapur A, Rangaswamy NS (1992) Embryo differentiation in anther culture of mulberry. *Indian Journal of Experimental Biology* **30**, 1146-1148

Shajahan A, Kathiravan K, Ganapathi A (1995) Induction of embryo-like structures by liquid culture in mulberry (*Morus alba* L.). *Breeding Science* **45 (4)**, 413-417

Shajahan A, Kathiravan K, Ganapathi A (1997) Selection of salt tolerant mulberry callus tissue culture from cultured hypocotyl segments. In: Khan IA (Ed) *Frontiers in Plant Science*, The Book Syndicate, Hyderabad, India, pp 311-313

Sharma KK, Thorpe TA (1990) *In vitro* propagation of mulberry (*Morus alba* L.) through nodal segments. *Scientia Horticulturae* **42**, 307-320

Singh A, Sharma J, Rexer KH, Varma A (2000) Plant productivity determinants beyond minerals, water and light: *Piriformospora indica* - A revolutionary plant growth promoting fungus. *Current Science* **79**, 1548-1554

Susheelamma BN, Shekhar KR, Sarkar A, Rao MR, Datta RK (1996) Genotypes and hormonal effects on callus formation and regeneration in mulberry. *Euphytica* **90**, 25-29

Tipton J (1994) Relative drought resistance among selected southwestern landscape plants. *Journal of Arboriculture* **20**, 151-155

Thomas TD, Bhatnagar AK, Razdan MK, Bhojwani SS (1999) A reproducible protocol for the production of gynogenic haploids of mulberry, *Morus alba* L. *Euphytica* **110**, 169-173

Thomas TD, Bhatnagar AK, Bhojwani SS (2000) Production of triploid plants of mulberry (*Morus alba* L.) by endosperm culture. *Plant Cell Reports* **19**, 395-399

Thomas TD (2003) Thidiazuron induced multiple shoot induction and plant regeneration from cotyledonary explants of mulberry. *Biologia Plantarum* **46**, 529-533

Tewary PK, Gupta BK, Rao GS (1989) *In vitro* studies on the growth rate of callus of mulberry (*Morus alba* L.). *Indian Journal of Forestry* **12**, 34-35

Tewary PK, Rao GS (1990) Multiple shoot formation through shoot apex culture of mulberry. *Indian Journal of Forestry* **13**, 109-111

Tewary PK, Sita LG (1992) Protoplast isolation, purification and cultures in mulberry (*Morus* spp.). *Sericologia* **32**, 651-657

Tewary PK, Sarkar A, Kumar V, Chakraborthy S (1995) Rapid *in vitro* multiplication of high yielding mulberry (*Morus* spp.) genotypes V1 and S-34. *Indian Journal of Sericulture* **34**, 133-136

Tewary PK, Oka S (1999) Simplified clonal multiplication of mulberry using liquid shake culture. *Plant Cell, Tissue and Organ Culture* **59**, 223-226

Tewary PK, Sharma A, Raghunath MK, Sarkar A (2000) *In vitro* response of promising mulberry (*Morus* sp.) genotypes for tolerance to salt and osmotic stresses. *Plant Growth Regulation* **30**, 17-21

Tikader A, Shamsudin M, Vijayan K, Pavankumar T (1995a) Survival potential in different varieties of mulberry (*Morus* spp.). *Indian Journal of Agriculture Science* **65**, 33-35

Tikader A, Vijayan K, Raghunath MK, Chakraborti SP, Roy BN, Pavankumar T (1995b) Studies on sexual variations in mulberry (*Morus s*pp.). *Euphytica* **84**, 115-120

Tikader A, Vijayan K (2010) Assessment of biodiversity and strategies for conservation of genetic resources in mulberry (*Morus* spp.). In: Nageswara-Rao M, Soneji JR (Eds) *Tree and Forest Biodiversity*. *Bioremediation, Biodiversity and Bioavailability* **4 (Special Issue 1),** 15-27

Tojyo I (1985) Research of polyploidy and its application in *Morus*. *Japanese Agriculture Research Quarterly* **18**, 222-227

Umate P, Rao VK, Kiranmayee K, Jayasree T, Sadanandam A (2005) Plant regeneration of mulberry (*Morus indica*) from mesophyll-derived protoplasts. *Plant Cell, Tissue and Organ Culture* **82**, 289-293

Wang H, Lou C, Zhang Y, Tan J, Jiao F (2003) Prelimarily report on oryzacystatin gene transferring into mulberry and production of transgenic plants. *Acta Sericologica Sinica* **29**, 291-294

Vavilov NI (1951) The origin, immunity and breeding of cultivated plants. Translated from Russian by K. S. Chester. *Chronica Botanica* **13**, 1-366

Venkateswaralu M, Katagiri K (1991) Induction of greenish status and callus in anther culture of mulberry. In: *Proceedings of Japanese Sericulture Association*, Mito, Japan, Nov 28–30, p 11

Verma B, Patni V, Kant U, Arya HC (1996) Direct plantlet regeneration from axillary buds of *Morus alba* L. (grafted var White). *Journal of the Indian Botanical Society* **75**, 45-47

Vijayan K (2010) The emerging role of genomics tools in mulberry (*Morus*) genetic improvement. *Tree Genetics and Genomics* **6**, 613-625

Vijayan K, Chakraborti SP, Roy BN (1998) Regeneration of plantlets through callus culture in mulberry. *Indian Journal of Plant Physiology* **3**, 310-313

Vijayan K, Chakraborti SP, Roy BN (2000) Plant regeneration form leaf explants of mulberry: Influence of sugar, genotype and 6-benzyladenine. *Indian Journal of Experimental Biology* **38**, 504-508

Vijayan K, Chakraborti SP, Ghosh PD (2003) *In vitro* screening of mulberry for salinity tolerance. *Plant Cell Reports* **22**, 350-357

Vijayan K, Chakraborti SP, Ghosh PD (2004) Screening of mulberry (*Morus* spp.) for salinity tolerance through *in vitro* seed germination. *Indian Journal of Biotechnology* **3**, 47-51

Vijayan K, Saratchandra B, Teixeira da Silva JA (2011) Germplasm conservation in mulberry (*Morus* spp.). *Scientia Horticulturae* in press

von Arnold S, Eriksson T (1981) *In vitro* studies on adventitious shoot formation in *Pinus contorta*. *Canadian Journal of Botany* **59**, 870-874

Yadav V, Madan L, Jaiswal VS (1990) Micropropagation of *Morus nigra* L. from shoot tip and nodal explants of mature trees. *Scientia Horticulturae* **44**, 61-67

Yakua H, Oka S (1988) Plant regeneration through meristem cultures from vegetative buds of mulberry (*Morus bombycis* Koidz.) stored in liquid nitrogen. *Annals of Botany* **62**, 79-82

Yamanouchi H, Koyama A, Machii H (1999) Effect of medium conditions on adventitious bud formation in immature mulberry leaves. *Japanese Agriculture Research Quarterly* **33**, 267-274

Yang JH, Yang XH (1989) Breeding of artificial triploids in mulberry. *Journal of Sericultural Science Japan* **15**, 65-70

Yasukura F, Ohnishi T (1990) Effects of regeneration period of mulberry cultures on their growth after their storage and after the pseudo dormancy induced by storage. *Journal of Sericultural Science Japan* **59**, 255-258

Zaman A, Islam R, Joarder OI (1997) Field performance and biochemical evaluation of micropropagated mulberry plants. *Plant Cell, Tissue and Organ Culture* **51**, 61-64