Journal of Plant Biology, March 2002, 45(1) : 7-21 **REVIEW**

Advances in Mulberry Tissue Culture

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The mulberry (*Morus* spp.) is an important tree in the sericultural industry because its leaves constitute the sole source of food for the Mori silkworm (*Bombyx mori*). Qualitative and quantitative improvements in mulberry varieties play a vital role in industrial advances. However, the perennial nature of the plants, coupled with the species prolonged juvenile period, slows this process. Plant tissue-culture techniques have been used extensively for stock improvement. During the last thirty years, several researchers have reported success in plant regeneration from different explants types. This review describes the major findings in mulberry tissue-culture research.

Keywords: haploids, *Morus*, mulberry, regeneration, tissue culture, triploids

The mulberry silkworm (*Bombyx mori*) eats exclusively the leaves of mulberry (*Morus* sp., family Moraceae). This perennial, deep-rooted tree grows in the tropics and temperate regions, and is believed to have originated from the lower ranges of the Himalayas in either India or China. The earliest use and cultivation of mulberry dates to 2800 BC in China. From there, the trees were exported first to Tibet, then to India. Its widespread sericulture in western countries follows the famous Silk Route or Silk Road that stretched from China to Europe. In China, silk was exchanged for gold; the Venetian explorer Marco Polo travelled along the famous route and brought this treasure from China.

Although mulberry is the prime resource for rearing silkworms, researchers have tried using other species, such as *Cudrantia triloba* (Moraceae), *Ulmus pumilla* (Ulmaceae), and *Brassica oleraceae* (Cruciferae). However, all these attempts to replace mulberry have failed because when the silkworm larvae were reared on those plants, their life spans were prolonged, the spun cocoons were smaller than normal, and metamorphosis sometimes failed to occur.

The possibility for qualitative and quantitative improvements in mulberry varieties is vital to the advancement of the sericultural industry. However, its perennial nature and prolonged juvenile period have slowed this process. Moreover, the dioecious nature of mulberry and the genetic linkage of both desirable and weak traits limit improvements that can be attained through conventional hybridization. Tissue-culture techniques have been used extensively for mass propagation of forest and cultivated trees, including plantation crops (Bajaj, 1986). Over the past thirty years, numerous reports have been published concerning plant regeneration from different explants of mulberry (Table 1). Scientific interest has focused especially on micropropagation, which has been successfully carried out in many woody and crop-plant species. In this review, the major findings in mulberry tissue culture are presented.

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MICROPROPAGATION

Apical and Axillary Bud Culture

Mulberry (*Morus* sp.) has been micropropagated from many explant sources, such as shoot tips and axillary, isolated, or winter buds (Fig. 1). The earliest reports, from Japan, include one from Ohyama (1970), who obtained the first complete plantlets from axillary-bud tissue. The majority of the research has involved the use of MS (Murashige and Skoog, 1962) media for nodal cuttings, and for culture of axillary and apical buds. Sharma and Thrope (1990) observed that an MS medium supplemented with 2.5 µM BA was optimum for in vitro-raised seedlings of *M. alba*. Their explants exhibited significant differ-

Abbreviations: ABA, Abscisic Acid; BA/BAP, 6-Benzylaminopurine; GA3, Gibberellic acid; 2,4-D, 2,4-dichlorophenoxyacetic acid; IAA, Indole-3-acetic acid; IBA, Indole-3-butyric acid; IPA, Indole-3-proponic acid; Kin, Kinetin; LS, Linsmaier and Skoog; MS, Murashige and Skoog; NAA, Naphthalene acetic acid; 4PU, [N^{l}-(2-chloro-4-pyridyl)N^{l}-phenyl-urea]; TDZ, N-phenyl N^{l} 1,2,3-thidiazol-5-yl urea.

Table 1. Summary of important research on tissue culture in mulberry (*Morus* spp).

Species	Explant source	Medium and growth regulators (μM)	Result	Reference
M. alba	Isolated buds	MS + Kin (0.46) + NAA (5.37)	Full plantlets in soil	Ohyama (1970)
Morus sp.	Stem segments	MS + NAA (10.70), IAA (11.42), Kin (0.46), NAA (1.07), Kin (0.09)	Roots from callus	Seki et al. (1971a)
Morus sp.	Stem segments	MS + NAA (10.74)	Roots from callus	Minamizawa and Hirano (1973)
Morus sp.	Isolated buds	MS + BA (4.44), ABA (37.84)	Bud break	Ohyama and Oka (1975)
M. alba	Shoot tips	MS + BA (4.44), GA3 (2.89), NAA (5.37)	Full plantlets in soil	Ohyama and Oka (1976)
M. alba	Leaves	MS + BA (4.44 - 44.00), NAA (0.54)	Full plantlets in soil	Oka and Ohyama (1981)
M. indica	Stem segments, leaves, and petioles	MS + 2,4-D (2.26 - 9.00), IAA (2.85 - 11.42), IBA (2.46 - 9.8), NAA (2.69 - 10.74)	Full plantlets in soil	Patel et al. (1983)
M. alba	Cotyledon, leaf, hypocotyl, shoot	MS + BA (4.44 - 22.20) + NAA (0.0054 - 0.54)	Rooted plantlets in vitro	Ho-Rak Kim et al. (1985)
M. nigra	Dormant buds	MS + BAP (5 - 10)	Shoots with inflorescence	Angrish et al. (1986)
M. indica	Encapsulated buds	MS + BAP (8.88)	Full plantlets in soil	Bapat et al. (1987)
M. acidosa	Axillary and apical buds	MS + BA (4.44)	Full plantlets in soil	Enomoto (1987)
M. nigra	Shoot tips	MS + BAP (2.22), IBA (2.46)	Full plantlets in soil	Ivanicka (1987)
M. bombycis	Nodal explants	MS + BA (4.44), NAA (0.54)	Full plantlets in soil	Yakuwa and Oka (1988)
M. alba	Internodal callus	MS + BA (2.22) 2,4-D (4.52)	Full plantlets in soil	Narayan et al. (1989)
Morus sp.	Male flower	MS + BA (1.78)	Full plantlets in soil	Ogure (1989)
M. indica	Encapsulated buds	No media	Full Plantlets in soil	Bapat and Rao (1990a)
M. alba	Axillary buds	MS + BAP (13.3), NAA (5.4), IAA (5.7)	Full plantlets in soil	Chattopadhyay and Datta (1990)
M. alba	Axillary buds	MS + BAP (8.88), IAA (5.71) NAA (5.37 & 10.74)	Full plantlets in soil	Chattopadhyay et al. (1990)
M. alba	Nodal segments	MS + BA (2.5)	Full plantlets in soil	Sharma and Thrope (1990)
M. alba	Shoot apices	MS + NAA (1.07 -10.74), BA (8.88)	Full plantlets in soil	Tewary and Rao (1990)
Morus sp.	Binucleate pollen grains	B5 + BA (4.44), IBA (4.90)	Division of pollen	Katagiri and Modala (1991)
M. indica	Ovary	MS + BAP (4.4), Kin (4.6)	Full plantlets in soil	Lakshmi Sita and Ravindran (1991)
M. laevigata	Nodal cuttings	MS + BA (10.00), IBA (0.49), NAA (0.54)	Full plantlets in soil	Hossain et al. (1992)
M. bombycis	Internodal segments	LS + 2,4-D (9.05), NAA (2.68), BA (2.20 - 8.90), IBA (2.50)	Full plantlets in soil	Jain and Datta (1992a)
M. laevigata	Cotyledons from seedlings	LS +2,4-D (2.26), NAA (2.69), BAP (0.89), IBA (2.46)	Full plantlets in soil	Jain and Datta (1992b)
Morus sp.	Immature leaf	MS + BA (0.00 - 44.40), NAA (0.00 - 53.71)	Adventitious buds, calli, roots	Machii (1992a)
M. alba	Encapsulated buds	MS medium	100% germination	Machii (1992b)
M. bombycis	Alginate-coated shoot tips	MS medium	Germination of shoots	Niino and Sakai (1992)

Table 1. Continued

Species	Explant source	Medium and growth regulators (μM)	Result	Reference
Morus sp.	Axillary buds	MS + BAP (4.44), NAA (2.69)	Plantlets in soil	Raghunath et al. (1992)
M. multicaulis	Anthers	MS + IBA (5 - 10), BAP (5 - 15)	Embryo emergence	Sethi et al. (1992)
M. alba	Leaves and callus	MS + 2,4-D (9.05), BAP (0.44 - 2.22)	Protoplast division	Tewary and Lakshmi Sita (1992)
M. laevigata	Nodal cuttings	MS + BA (2.5 - 15.0), Kin (2.5 - 15.0), NAA (2.5 - 15.0)	Full plantlets in soil	Islam et al. (1993b)
M. alba	Nodal cuttings	MS + BA (10.0), IBA (0.5)	Full plantlets in soil	Islam et al. (1993a)
M. australis	Pollen grains	MS & B5 + IBA (2.46 - 4.90), BA (4.44)	Embryoids	Katagiri and Modala (1993)
M. alba	Leaves	MS + BAP (8.88), IBA (0.46)	Adventitious buds	Katagiri and Thinh (1993)
Morus sp.	Immature leaf	MS + BAP (4.44)	Flower bud	Machii and Yamanouchi (1993b)
M. alba	Internodal segments	MS + BAP (2.22)	Female flowers from calli	Narayan et al. (1993)
M. alba	Nodal cuttings	MS + BAP (8.88), IBA (0.46)	Full plantlets in soil	Islam et al. (1994)
M. alba *M. indica* *M. latifolia*	Male inflorescence	MS + 2,4-D (2.26), BAP (8.88)	Embryoids	Tewary et al. (1994)
Morus sp.	Leaves	MS + TDZ (0 - 20)	Callus, buds, and organ-like structures	Thinh and Katagiri (1994)
M. alba	Nodal cuttings	MS + BA (4.44) + Tyrosine (50 - 200 mg/L)	Full plantlets in soil	Zaman et al. (1994)
M. alba	Shoot tips	Solid and liquid MS + 4PU (9.42), BA (4.44), NAA (0.05)	Full plantlets in soil	Hayashi and Oka (1995)
M. alba	Hypocotyl	MS + BAP (0.44 -13.30) + tyrosine (50 mg/L)	Plantlets with roots in vitro	Islam et al. (1995)
M. australis	Zygotic embryos	MS salt & Vit B5 + TDZ (0.5 - 10.0)	Embryoids	Katagiri and Thinh (1995)
M. indica *M. multicaulis* *M. bombycis*	Encapsulated axillary buds	MS medium	Germination	Pattnaik et al. (1995)
M. alba	Hypocotyl callus	Solid and liquid MS + 2,4-D (0.45 - 4.52), BAP (2.22)	Heart-shaped embryos	Shajahan et al. (1995)
M. alba	Apical shoots	MS + BA (8.88), NAA (0.54), IBA (4.90)	Full plantlets in soil	Tewary et al. (1995)
Morus sp.	Zygotic embryos	MS + TDZ (0 - 9)	Somatic embryos	Thinh and Katagiri (1995)
M. indica	Anthers	MB + NAA (2.69), BA (4.44), NAA (2.69), 2,4-D (2.26)	Embryoids	Jain et al. (1996)
M. australis	Nodal cuttings and shoot apices	MS + BA, GA3, IBA, IPA	Full plantlets in soil	Pattnaik et al. (1996)
M. alba	Hypocotyl, petioles, and leaves	MS & LS + 2,4-D (6.78), BAP (2.22), NAA (2.69), BAP (3.33), IBA (2.46)	Full plantlets in soil	Kathiravan et al. (1997)
M. ihou *M. serrata* *M. cathayana*	Apical buds and nodal cuttings	MS + BAP (4.44), GA3 (1.15) IBA (4.9), IAA (5.71)	Full plantlets in soil	Pattnaik and Chand (1997)
M. indica	Internodal segments and leaves	MS & LS + 2,4-D (9.05), BA (2.22)	Full plants in soil	Sahoo et al. (1997)
M. indica	Nodal cuttings	MS + 2,4-D (1.36), BAP (2.22 - 17.80), GA3 (0.14)	Full plantlets in soil	Chitra and Padmaja (1999)

Table 1. Continued

Species	Explant source	Medium and growth regulators (μM)	Result	Reference
M. alba	Unfertilized ovaries	MS + 2,4-D (4.5), glycine (500 mg/L), proline (200 mg/L)	Haploid plantlets in soil	Thomas et al. (1999)
M. alba	Endosperm	MS + BAP (5) + NAA (1)	Triploid plantlets in soil	Thomas et al. (2000)
M. indica	Axillary buds	MS + 2,4-D (1.4 - 9.0), Kin (1.4 - 9.3), BAP (1.3 - 8.8)	Full plantlets in soil	Chitra and Padmaja (2001)
M. alba	Nodal cuttings	MS + BAP (0 - 7)	Full plantlets in soil	Paul et al. (2001)

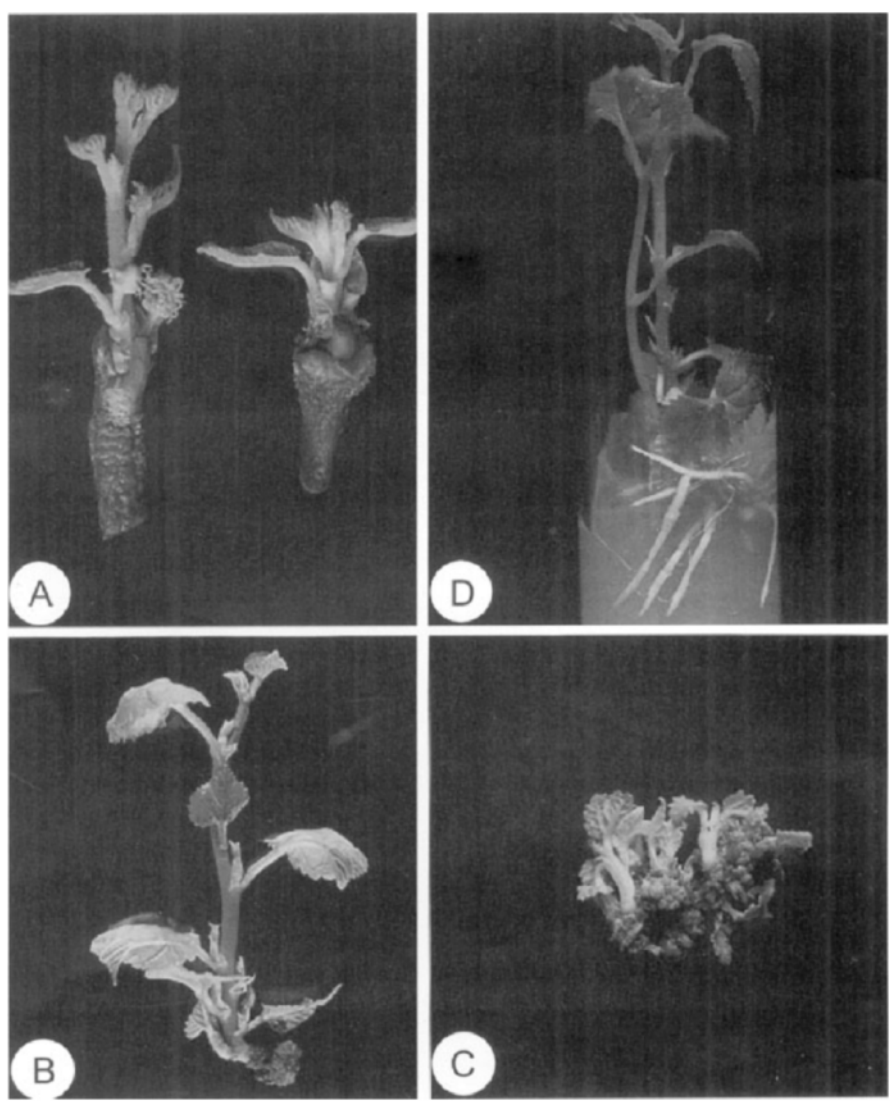

Figure 1. Micropropagation in mulberry (*M. alba* L). **A.** Two single-node cuttings cultured on MS medium supplemented with 5 μM BAP at four (left) and two (right) weeks after culture. Emergence of several inflorescences can also be observed from the nodes of the emerging shoot. **B.** Early stage for induction of multiple shoots on MS + 7 μM BAP. Note the emergence of a number of axillary buds. **C.** Endosperm callus regeneration in mulberry on MS + TDZ (1 μM) showing distinct, multiple shoots. Photograph was taken 45 d after culture. **D.** 30-d-old culture on ½ MS + 7 μM IBA, showing the rooting of micropropagated shoots. A number of roots have already emerged from the basal cut end.

ences in shoot-multiplication rates, and could be stored at 4°C for at least six months without any decrease in vigor. Rooting was enhanced by activated charcoal (0.05 - 0.10%). Transplantation was 100% successful when the shoots were grown in a 1:1 sand-vermiculite mix in the greenhouse.

The combination of GA_3 and BAP sometimes increases the frequency of bud break in mulberry. Pattnaik and Chand (1997) reported rapid clonal propagation of three species through in vitro culture of apical shoot buds and nodal explants from mature trees. Their MS medium was supplemented with 2.2 to 4.4 µM BAP, in an attempt to induce bud break and multiple-shoot formation. Adding GA_3 along with the BAP enhanced bud-break frequency.

The success of shoot-culture experiments is highly influenced by the source and age of the explant, as well as the conditions under which it is cultured. Positioning of the explant also plays a crucial role in bud induction. Hossain et al. (1991) showed that, in the ten genotypes they studied, the maximum response of multiple-shoot induction was obtained from subterminal buds (Hossain et al., 1991). Chattopadhyay et al. (1990) cultured single axillary buds of *M. alba* and achieved high rates of multiple-shoot production when using an MS medium and BAP as the growth regulator. Rooted plantlets were then successfully established in soil. Paul et al. (2001) have described a rapid in vitro-propagation procedure, in which very young axillary buds can be cultured, established, multiplied, rooted, and transplanted to soil within three months.

Enomoto (1987) developed an efficient in vitro culturing method for germplasm preservation, taking isolated buds from axillary tissue and culturing them on a modified MS medium supplemented with 4.4 µM BA. However, Enomoto found that adding fructose made the media somewhat soft so that the cultured buds were prone to sinking. This new culture method resulted in the preservation of 24 mulberry strains (both Shimaguwa and foreign), and was most successful when the media were hormone-free. Rooting of the explants was also observed on the medium without supplemental hormones.

Yamanouchi et al., (1993) studied the effect of inorganic nitrogen sources on adventitious-bud formation. Winter buds were cultured on media containing different concentrations of ammonium and nitrate ions. The growth and shape of the leaves varied according to the level of inorganic nitrogen. However, bud formation depended not only on particular concentrations, but also on the ratio of those concentrations.

Season also plays an important role in the control of bud sprouting. Ponchia and Gardiman (1992) found that MS media supplemented with BA and NAA induced shoot proliferation in mulberry ('Florio' and 'Morettiana') at various times throughout the growing season. They noted that auxin was essential for promoting root induction. Proliferation rates did not differ between the cultivars. Likewise, they found that the best period for collecting explants was during active shoot growth. For this study, the percent success was better for shoot-tip sprouting than for lateral buds in both cultivars, but both types had higher sprouting percentages in the spring than in summer or autumn. In contrast, an investigation of the seasonal influence on axillary-bud sprouting by Chitra and Padmaja (2001) showed that the frequency was higher in the summer than during any other season.

Explants from older plants can also be employed for in vitro multiplication. Using an MS medium supplemented with 2.2 to 22.0 µM benzyl adenine (BA), Hossain et al. (1992) was able to produce multiple shoots from nodal explants of a 10-year-old *M. laevigata* tree. Shoot proliferation was higher when the BA level was raised to as much as 11 µM, but further increases suppressed development. The highest percentage of explants giving rise to shoots, and the maximum length of those shoots, was observed on MS media supplemented with 4.4 µM BA, whereas the greatest number of shoots (7.5) per explant was gained from 11 µM BA. The average shoot multiplication rate was quite high, being six-fold per subculture (i.e., from subculturing once after five weeks). With older explant sources, shoots can also be induced on an LS medium (Linsmaier and Skoog, 1965), as was reported by Jain and Datta (1992a). However, its use is very rare.

Exudation from the cut end of the tissue can sometimes cause problems in the medium when certain species are being tested. Islam et al. (1992) found that in vitro establishment of explants from mature trees of *M. laevigata* was made more difficult because of the excessive latex-like milky substance in its exudate. Changing the media two to three times during the initial stages of culture was effective.

Growth regulators play an important role in explant multiplication. Several cytokinins especially BA is routinely used for shoot propagation from nodal cuttings (Oka and Ohyama, 1974; Hossain et al., 1990; Jain et al., 1990; Yadav et al., 1990); the combination of cytokinin and auxin is less effective. The addition of BAP also may enhance the pace of bud break. However, Pattnaik et al. (1996) studied micropropagation techniques with *M. australis*, and found that an MS

medium supplemented with 4.4 μM BAP and 0.6 μM GA_3 accelerated bud break from nodal explants and apical shoot buds, and enhanced its frequency, compared with the results when using BAP alone.

The addition of certain amino acids to the growth regulators in the media may be beneficial in promoting shoot multiplication. Zaman et al. (1994) found proline was absolutely necessary for in vitro shoot proliferation in *M. alba*, and tyrosine was even superior to proline. However, glutamine had some inhibitory effect. In another study, Zaman et al. (1996) reported on the clonal propagation of *M. alba* 'S-799' from shoot apices and nodal explants. They supplemented the MS media with 9 μM each of BA, Kin, 2iP, and Zeatin, all with or without 50 mg/L tyrosine. Culture on MS media supplemented with 8.8 μM BA and 50 mg/L tyrosine, using tissues from the shoot apex, axillary bud, or cotyledon, yielded the best response in terms of percentage shoot-formation. Explants from axillary buds produced the maximum number of shoots (92.3%), compared with 72.4 and 20.3% from apices and cotyledons, respectively. This promoter effect with tyrosine was also reported by Islam et al. (1993a).

Sucrose was the best carbon source for micropropagation of shoots in *M. alba* (Enomoto, 1987) and *M. laevigata* (Zaman et al., 1992a), but Ohyama (1970) and Ohyama and Oka (1976) reported fructose as being more favorable. Oka and Ohyama (1982) studied the effect of different sugars on in vitro shoot elongation from bud culture, and determined that both fructose and glucose were better carbon sources than sucrose. Fructose also had a promoting effect during the initial stages of shoot-axis elongation.

Zaman et al. (1992b) evaluated the role of agar type and pH on shoot proliferation in *M. alba*, and noted that the percentage of shoot-proliferating explants as well as the number of shoots per explant increased when the level of agar was raised up to 0.6%; further increases decreased both measured parameters. The optimum pH for bud break ranged from 4.0- to 5.5; less acidic levels, in fact, decreased its rate. For producing multiple shoots, pH 5.5 was the best, and variations either up or down caused this frequency to decrease.

Survival rates for acclimatized plants varied from 75% in *M. nigra* (Yadav et al., 1990) up to 90 or 95% in *M. nigra* (Jain et al., 1992a, 1992b); *M. ihou*, *M. Cathayana*, and *M. serrata* (Pattnaik and Chand, 1997); and *M. australis* (Pattnaik et al., 1996). Sharma and Thrope (1990) transplanted *M. alba* plants with 100% success.

Ma et al. (1996) standardized a technique for in vitro shoot-apex grafting of *M. alba*. De-coated seeds were cultured on an MS medium supplemented with 0.2% gelrite, and were kept in the dark for 10 d. The bases of excised shoots were then soaked in 500 μM IBA for 30 min, and transferred to a vermiculite support medium moistened with half-strength MS salts. After again being held in the dark for 10 to 12 d, the seedling stems were used as cutting sources. Incisions were made at the top of the rootstock, the base of which was then inserted into the culture medium. Scions of each shoot apex were inserted into the incision, yielding a success rate of approximately 75 to 80%.

When micropropagated plants are compared with those generated through cuttings, the former have significantly higher vigor. Zaman et al. (1997) have assessed the field performance and biochemical analyses of both tissue culture-derived plants and plants raised according to conventional methods. They also confirmed that micropropagated plants had significantly greater morphogenic vigor. However, the biochemical analysis of the leaves revealed no significant nutritional difference between the two types. Tewary et al. (1995) standardized a technique for multiple-shoot induction from cultured shoot buds. After establishing them in the field, they analyzed them quantitatively and found that the tissue culture-derived plants were true to type compared with the mother stock.

Leaf, Cotyledon, and Hypocotyls Culture

Shoot regeneration from explants of leaves, cotyledons, and hypocotyls could prove useful for gene transfer via *Agrobacterium* or particle bombardment. Because the rate of regeneration is generally reduced when explants are treated with *Agrobacterium*, a good regeneration system is required for higher transformation frequencies. Oka and Ohyama (1981) studied the effect of BA on adventitious-bud formation from excised leaves. Primary leaves from both aseptically maintained shoots and seedlings were cultured on MS media in which the ferric component was replaced with 32 mg/L Na-Fe-EDTA. NAA or BA was supplied to the medium either separately or in combination. The rates of shoot-bud formation did not differ significantly for either source of tissue. However, removing the petioles remarkably increased success. This result demonstrates that the transitional zone (i.e., the area between the petioles and the midribs) has specific capacity for producing shoot buds.

Furthermore, histological observations of the cultured leaves revealed that, at the time of excision, the

veins contained no meristematic tissue. Bud formation, therefore, was of peripheral origin. At the initial stage of culture, the adaxial epidermal cells of the midribs (near the cut end) enlarged unequally, leaving behind depressions. Later, some of the deeply stained cells along the depressions divided anticlinally, followed by division in the sub-epidermal cells. After four to six days of culture, dome-shaped tissue appeared, from which leaf primordia developed within 14 to 21 d. Ho-Rak Kim et al. (1985) also observed shoot regeneration from cotyledon and leaf explants when they used a medium supplemented with high levels of BA, with or without low levels of NAA.

Machii (1992a) analyzed organogenesis from immature leaf cultures, using two types of media -- MS and B5 (Gamborg et al., 1968) -- both of which comprised 20 mg/L fructose and 13 g/L agar. Four concentrations of BA (0.00, 0.44, 4.40, and 44.00 µM) and NAA (0.00, 0.54, 5.37, and 53.71 µM) were tested, under light and dark conditions. Adventitious buds formed only on the MS medium supplemented with 4.40 µM BA and 0.54 µM NAA; none formed on the B5 medium. Callus development was maximized under light conditions. In contrast, the number of roots forming in the dark on the B5 medium was higher than that on MS media under either light or darkness.

Katagiri and Thinh (1993) observed that the efficiency of adventitious-bud induction was about twice as great from leaves cultured with rotary shaking in a liquid MS medium supplemented with 8.8 µM BAP, compared with those cultured on a solid MS agar medium containing the same amount of BAP. The ability of the juvenile leaves to produce shoot buds was lost after 10 d of culture on a hormone-free liquid medium. More buds were produced from the basal than from the apical portion. Low levels (i.e., 100 mg/L) of some amino acids, e.g., L-alanine, also promoted adventitious-bud formation. However, rotary-shaking the leaves in a liquid MS medium supplemented with 5 µM thidiazuron (TDZ) gave better results than did BAP when culturing continued on an MS solid medium supplemented with 5 µM TDZ, two weeks after incubation in the liquid medium (Thomas, unpublished).

No differences were seen between leaves from field-grown or seedling plants when TDZ was used to induce adventitious buds (Thinh and Katagiri, 1994). Immature leaves were isolated from actively growing axillary buds of dormant field-grown plants and seedlings. In both cases, the response ranged from formation only of callus to the induction of buds and/or organ-like structures. TDZ concentrations varied from 0.1 to 30.0 mg/L. Higher levels considerably reduced bud formation, but increased the rate of organ-like structures. However, those structures failed to develop into plantlets.

Machii and Yamanouchi (1993b) observed female-flower formation from immature leaves that were cultured on an MS medium supplemented with 4.4 µM BA, 20.0 g/L fructose, and 4.0 g/L gelrite. The highest percentages of flower-bud formation were observed in genotypes 'Kanadasanso' (6.1%) and 'Shin-ichinose' (2.4%). The ovaries of these flowers were swollen 70 d after culture initiation.

Soaking the cotyledons in liquid medium is most effective in efforts for their regeneration. Jain and Datta (1992b) studied the effects of cytokinin, auxin, and the combination of both in *M. laevigata*. After collecting 7- to 10-day-old seedlings, they soaked the cotyledons for 24 h in a liquid basal LS medium that was supplemented with 3% sucrose and 8.8 µM BAP. These cotyledons were subsequently cultured on an LS medium supplemented with 2.46 or 4.9 µM IBA). Multiple shoots emerged after six weeks of culture. The shoot buds were then transferred to an LS medium fortified with 2.46 µM IBA to promote rooting, and the resulting plantlets were successfully transferred to soil. Likewise, Ohyama and Oka (1982) studied the effect of 4PU, a cytokinin-active urea [N^{l}- (2-chloro-4-pyridyl) N^{l}-phenyl-urea], on cultured hypocotyl segments, and found that multiple shoots were produced only at the apical ends.

Callus Induction and Regeneration

Both MS and LS media perform equally well for inducing callus development. Although auxin is necessary for induction, both auxin and cytokinin are necessary for promoting callus multiplication (Oka and Ohyama, 1973). Ohyama (1970) has also reported on suspension cultures of calli derived from hypocotyl explants. Earlier research on callus regeneration was limited to root induction only. Ghugale et al. (1971) reported successful root formation from calli in the presence of 1 µM each of IAA, IBA, and IPA, supplemented individually. However, these calli gave rise to roots only on the medium containing auxin and kinetin, which suggests synergistic action. Nevertheless, no shoots were differentiated over a 30-month test period. Seki et al. (1971a) obtained similar results.

Jain and Datta (1992a) observed the formation of shoots from pretreated callus. Internodal cuttings were presoaked under various periods of darkness in LS liquid media comprising 4.4, 8.9, 17.8, or 35.5

μM BA. The pretreated internodes were then cultured on LS media variously supplemented with 2.2 to 8.9 μM BA, 4.5 to 13.5 μM 2,4-D, or 2.6 to 10.7 μM NAA. Calli were best formed when the explants were soaked in low levels of BA (4.4 to 8.9 μM), then cultured on the LS medium supplemented with 8.9 μM BA. Only the greenish calli were used for regeneration experiments involving de-novo formation of adventitious-shoot buds. There, the optimum treatment was with 8.9 μM BA and a 72-h pretreatment. Regeneration potentials declined when 10- and 15-week-old calli were cultured. Histological studies of the regenerating callus revealed five to six layers of cells below the surface that showed significantly high levels of division occurring in both the dermal and the epidermal layers. In another experiment, LS media were superior to MS media in promoting shoot regeneration from hypocotyl-derived calli (Oshigane, 1989, 1990).

The role of the medium in callus regeneration is very critical. A pretreatment is sometimes required to ensure successful regeneration on LS media. Narayan et al. (1989) standardized a technique for this. Using internodal segments from *M. alba*, they first pretreated the tissue on an MS liquid medium supplemented with 2.2 μM BAP in 250-mL Erlenmeyer flasks. The flasks were kept on a gyratory shaker for 36 h at 25 ± 2°C. Afterward, the internodes were transferred to an MS solid medium supplemented variously with 4.52 μM 2,4-D or 2.2 μM BAP. The calli were then transferred to 2.2, 4.4, 8.8, or 13.2 μM BAP for regeneration. Treatment with 8.8 μM BAP was most beneficial, producing the maximum number of cultures that showed shoot regeneration. These callus-derived shoots were then rooted on 2.69 μM NAA.

In some experiments, the MS medium was used for callus induction while further development occurred on LS media. Kathiravan et al. (1995) described the regeneration of plants from hypocotyl-derived calli of *M. alba* cv. MR-2. The original MS medium was supplemented with 2.85 IAA and 2.2 μM BA. Four-week-old cultures were transferred to fresh LS media supplemented with NAA and BA to promote shoot development, with the best results coming from the combination of 2.69 μM NAA and 3.69 μM BA.

By adding GA3 and dithiothrietol (DTT) to the culture media, Yasukura and Ohnishi (1990) were able to break a pseudodormancy and regenerate calli that had been stored at low temperatures.

Shajahan et al. (1995) have reported on the effect of 2,4-D on somatic embryogenesis in *M. alba*. They studied various stages of development in embryo-like structures that arose from hypocotyl-derived calli that were not pretreated. Transverse hypocotyl sections were cultured on an MS medium supplemented with 4.52 μM 2,4-D and 2.2 μM BAP. After 40 d of culture, the yellowish-green, friable calli were subcultured onto an MS liquid suspension-culture medium with 200 mg/L glutamine and various concentrations of 2,4-D, ranging from 0.45 - 4.52 μM. These suspension cultures were then subcultured every 7 d on the same liquid medium. After four weeks, they observed a number of embryo-like structures. Induction of those structures was greatly influenced by the particular concentration of 2,4-D, with 0.45 μM being the optimum. Any decrease or increase from that level promoted either rooting or callusing, respectively. At more advanced stages, the somatic embryos showed globular or heart-shaped structures. These results demonstrated that, although 2,4-D was indispensable for induction of those structures, it inhibited further development. Ohnishi and Kobayashi (1991) also observed the formation of embryo-like structures from calli grown on a hormone-free LS medium.

Thinh and Katagiri (1995) studied the formation of somatic embryos from cultured zygotic embryos. Embryonic tissues were excised at different stages from green fruits, then cultured on a medium of MS mineral salts and B5 vitamins (Gamborg and Wetter, 1975) supplemented with various combinations of TDZ and 2,4-D. They identified 1 mg/L TDZ as the optimum for somatic embryo production. When isolated zygotic embryos harvested at the torpedo stage were cultured on the media, they produced somatic embryos in all the explants examined. Secondary somatic embryos were also induced on some primary embryos. This process of complete somatic embryogenesis was observed only on the TDZ-containing media. In contrast, the combination of TDZ and 2,4-D suppressed the formation of somatic embryos, and instead promoted callus formation (see also Katagiri and Thinh, 1995).

Narayan et al. (1993) were able to obtain female flowers from 35% of their long-term (two-year-old) callus cultures of pretreated internodal segments in *M. alba*. The light-green, internodal calli also induced a few shoot buds on an MS medium supplemented with 2.2 μM BAP. Solitary flowers as well as flowers in catkins were observed on the callus surface. The regenerated carpels were healthy and perianths were red at maturity. These researchers had originally doubted that a floral stimulus was involved. However, in some of the older tissues that were also associated with the newly formed material, a floral stimulus was indeed synthesized and transported to the vegetative buds for

conversion to flower buds. Only female flowers were obtained in this manner.

HAPLOID PRODUCTION

Androgenesis

Because the mulberry is a highly heterozygous perennial plant, it is not readily amenable to conventional methods for plant improvement. Inbreeding to produce homozygous lines is impossible because of the dioecious nature, and male and female lines are genetically diverse. Therefore, production of haploid plants is the easiest and most rapid method for producing those homozygous lines. This technique, involving anther culture, has been successful in numerous plant species, including some crop trees (Bhojwani and Razdan, 1996). Its use in *Morus* was first reported by Shoukang et al. (1987). They tested three media - full-strength MS, half-strength MS, and White's media (White, 1963), but had success only with the full-strength MS.

The supplemental hormones in the media play a very important role in determining the success of culturing. When BA (4.4 or 8.9 µM) and IBA (4.9 or 9.8 µM) were added simultaneously, the maximum rate of induction was 13.6%. Anthers harvested at the mid-uninucleate stage showed better frequencies of induction (22 to 24%) for embryoids than did those taken at the early- and late-uninucleate stages. Treatment with low temperatures did not prompt any induction. Differentiation in the embryoids was best when the concentration of auxin was fairly reduced and that of cytokinin was slightly increased. A cytological analysis of the androgenic plants revealed haploid, diploid, and hetroploid chromosome numbers, with haploid cells accounting for the majority in young plants. However, those haploid cells tended to change to diploid as the pollen developed further in to plantlets.

Katagiri and Modala (1991) studied the effect of sugars on the division of mulberry pollen in vitro. Grains at the binucleate stage were given a starvation treatment in 0.4 M mannitol. Afterward, they were resuspended in the culture medium. Disaccharides were more effective than monosaccharides for promoting divisions. Compact and rough calli were observed in all cases, and embryo- and organ-like structures were common.

Anther pretreatments can also help determine androgenic responses. Katagiri and Modala (1993) investigated the effect of pretreatment hormones on pollen division, as well as how the particular composition of minerals and organics influenced the induction of organ-like structures in *M. australis*. They had their greatest success when, following a starvation treatment, they pretreated the tissue for 5 d with quarter-strength minerals in a B5 medium comprising 0.4 M mannitol and 1 mg/L IBA. In contrast, the combination of BA and IBA had an inhibitory role in the medium. Organ-like structures also formed from induced calli on the modified B5 and MS medium. The rate of pollen divisions in this species was very low compared with that reported by Katagiri (1989b) for *M. latifolia*. This difference may have been due to variations in the genetic constitution of the materials.

Another important factor in androgenesis is the use of a dark period. Sethi et al. (1992) showed embryo differentiation from anther cultures of three cultivars of *Morus*. Anthers containing microspores were cultured on an MS medium containing 5 to 10 µM IBA and5 to 15 µM BAP. The cultures were kept in the dark at 26 ± 1°C for 15 d. Cv. RFS-175 had a 1.82% response whereas the success rates with 'Goshoerami' and 'RFS-135' were 0.39 and 0.23%, respectively.

Jain et al. (1996) studied the effect of temperature-pretreatment on the induction of androgenesis, haploid-callus formation, and subsequent development in *M. indica*. The catkins were pretreated in the following manner:

1. In vitro cold shock of catkins at 4°C for 0, 24, 48, 72, or 96 h.

2. In vitro high-temperature treatments at 36°C for 0.5, 1.0, 2.0, 4.0, or 6.0 h, followed by treatment at 4°C.

Of all the treatment combinations, cold shock at 4°C for 24 h was most effective, whereas the high-temperature treatments were deleterious. The pretreated anthers were cultured on a Modified Bourgin (MB) medium (Qian et al., 1982) and gave rise to friable and compact embryogenic calli. Although root formation was common from these calli, no shoots were regenerated.

Tewary et al. (1994) also cultured *Morus* pollen in vitro. Grains were isolated from inflorescences that were starved for periods ranging from 24 h to 10 d. Afterward, the pollen suspensions were cultured in drops on MS media. All six genotypes showed pollen enlargement, increased cytoplasmic contents, and large nuclei, with cv. S-1 responding the best.

Ogure (1989) obtained plantlets from chopped male inflorescences that were cultured in the dark on an MS medium. These inflorescences were carrying pollen grains harvested at the mid-binucleate stage.

However, this report failed to confirm whether the plantlets had originated from pollen or from somatic cells. The ploidy level of the originating plants was 72.4% diploid and 27.6% triploid.

Gynogenesis

Despite its tremendous progress, haploid production via anther culture has numerous drawbacks that impede its widespread application. Many of the limitations inherent to androgenesis could be overcome by culturing unfertilized ovule/ovary/flower buds, then inducing a haploid cell of the female gamete (egg or synergid) to develop into an apogamous sporophyte (Fig. 2). For example, Lakshmi Sita and Ravindran (1991) were able to produce gynogenic plants from ovary cultures of *M. indica*. Individual ovaries, harvested either before or after fusion to form sorosis, were cultured on MS media supplemented with growth regulators. They obtained 65 plants without any callusing phase being necessary. In some treatments, e.g., using a combination of 4.4 μM BAP and 4.6 μM Kin, four plants developed from a single ovary within three weeks of culture.

Gynogenic haploids were also reported by Thomas et al. (1999). They adopted an approach of obtaining haploid plants by culturing unpollinated ovaries. Segments from in vitro-developed inflorescences were cultured. After three weeks, individual ovaries were transferred to the same medium supplemented with various growth regulators. On an MS medium fortified with 4.5 μM 2,4-D, 500 mg/L glycine, and 200 mg/L proline, 16% of the ovaries developed gynogenic seedlings. Out of the 20 plantlets analyzed cytologically for ploidy, 12 were haploids, the other 8, aneuploids. The gynogenic plants were then established in soil (Thomas, 1999).

Triploid Production

Many of the triploid lines in mulberry are superior to the diploids, especially in terms of yield and nutritive qualities of the leaves (Seki and Oshikane, 1959). Both natural and in vivo-induced triploids have been reported (Das et al., 1970; Katagiri et al., 1982; Dwivedi et al., 1989). The conventional method of triploid production is lengthy and tedious. Endosperm tissue offers an excellent system for experimental morphogenic studies and for plant regeneration (Bhojwani and Razdan, 1996).

Seki et al. (1971b) studied the effect of different growth regulators on endosperm-callus proliferation

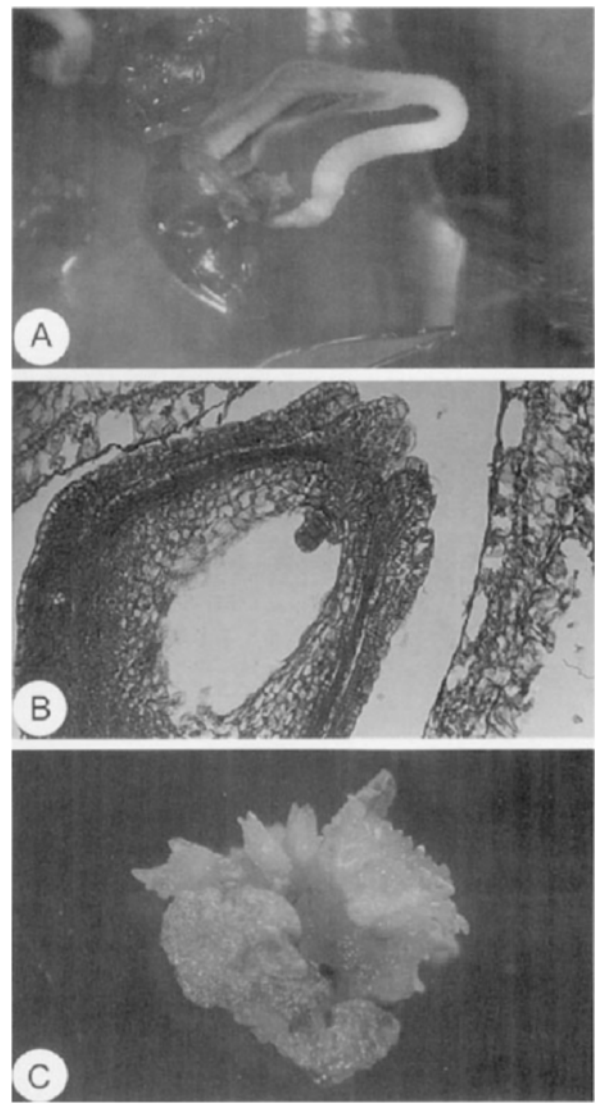

Figure 2. Gynogenesis and cotyledon regeneration in mulberry (*M. alba* L). **A.** Ovary showing development of gynogenic plantlet on MS + 4.5 μM 2,4-D + 500 mg/L glycine + 200 mg/L proline. Note the dried ovary wall at the base of the gynogenic plant. **B.** Section of an ovule from three-week-old culture, showing a gynogenic embryo. **C.** Emergence of several shoots from a 14-d-old cotyledon on MS medium containing 7 μM TDZ, 45 d after culture.

in mulberry. Growth rates did not show much variation when auxins, such as 2,4-D, IAA, or NAA, were added to the media and the cultures were maintained under either dark or light conditions. However, the addition of casein hydrolysate significantly improved the growth rate of the callus, but did not induce its regeneration. The first report of endosperm-derived callus regeneration in mulberry was by Thomas et al. (2000).

A continuously growing callus was obtained from immature endosperm cultures of *M. alba*. Shoot buds were produced when the callus was subcultured on a medium containing either cytokinin alone or in combination with NAA. Thidiazuron was also very effective for inducing callus regeneration. After their triploid nature was confirmed, the plants were established in soil (Thomas, 1999).

Genetic Engineering

Genetic engineering has become a vital thrust in current plant research. Machii (1989) isolated total DNA from *M. acidosa* leaves, and suggested a protocol for preparing a genomic DNA library. However, Hirano and Murata (1985) reported that mulberry plants were susceptible to *Agrobacterium tumefaciens*. Nevertheless, a culture system has been established for regenerating whole plants from adventitious buds induced on leaf discs (Oka, 1985). Machii (1990) was able to achieve genetic transformation in *M. alba*, using *A. tumefaciens* LBA 4404 as the vector. The kanamycin-resistant gene and the β-glucuronidase (GUS) gene were transferred through Ti-plasmid PB1121 to the leaf discs, and their expressions were confirmed in the regenerated plants. Kanamycin-resistant adventitious buds were induced from 34% of the discs, 30 d after infection. Five of the induced adventitious buds then developed into shooted plantlets.

Protoplast Culture and Somatic Hybridization

Somatic hybridization and transformation via protoplast manipulation is a novel approach for crop improvement. Using enzyme solutions, Ohnishi and Kiyama (1987a) and Katagiri (1988, 1989a) have isolated mulberry protoplasts from mesophyll and callus tissues. Isolated, viable protoplasts from several genotypes were cultured on MS media, and 50-celled microclones were obtained. Optimized concentrations of cellulase, macerozyme, and macerase enzymes resulted in the formation of a high density of protoplasts from the mesophyll and callus tissue of *M. alba* Cv K-2. Rao et al. (1989) studied the various parameters required for establishing protoplast cultures and found that the protoplasts from mesophyll cells and calli responded better than those from axillary buds. In addition, occasional and spontaneous fusion of protoplasts was followed by multinucleation and wall regeneration. Ming et al. (1992) also obtained regeneration of complete plantlets from mesophyll protoplasts cultured on MS media.

Somatic hybridization in mulberry can be achieved through protoplast fusion that incorporates chemical fusogens (Ohnishi and Kiyama, 1987b) or electrofusion (Ohnishi and Tanabe, 1989). Ohnishi et al. (1989) have also investigated the effects of polyethylene glycol, calcium ions, temperature, and pH on protoplast fusion.

Micropropagation through Encapsulation

Since Kitto and Janick (1982) first reported on synthetic seed production, this technique has been used in several annual crops. Bapat et al. (1987) encapsulated the shoot buds and axillary buds of mulberry (*M. indica*) in alginate and agar, and produced individual beads. These beads could then be germinated on filter paper, vermiculite, or agar, with tap water serving as the sole supply of nutrients. Bapat and Rao (1990a, 1990b) succeeded in germinating plantlets from the beads, both in vitro and in vivo.

This approach provides a simple method for propagating the difficult-to root-germplasm inherent to mulberry. Pattnaik et al. (1995) used 4% sodium alginate with 75 mM calcium chloride to encapsulate the axillary buds of three indigenous and two exotic mulberry varieties. In addition, Machii (1992b) encapsulated adventitious buds from immature leaf cultures. In a study of media preferences by synthetic seeds, the highest rate of germination, shoot growth, and rooting was on a soil-based medium, followed by those containing either sand or vermiculite (Machii and Yamanouchi, 1993a).

Cryopreservation

Collecting and preserving mulberry germplasm is the backbone of successful programs of breeding and genetic improvement. Large areas of land traditionally have been required, but not always readily available, for producing crop trees. With the new technological developments, large stocks of mulberry germplasm can now be preserved ex situ. Cryopreservation efforts have been successful in vegetative buds (Yakuwa and Oka, 1988), shoot tips, and the lateral buds of in vitro shoots (Niino, 1990; Niino and Oka, 1990; Takano, 1990; Takano and Oka, 1990; Niino et al., 1992a; Niino et al., 1993). Similarly, for a number of genotypes, researchers have used liquid nitrogen to preserve winter buds attached to shoot pieces (Niino et al., 1991) or shoot tips (Niino et al., 1992b), and have been able to regenerate plantlets from those buds. Cryopreservation of synthetic seeds has also been reported (Niino and Sakai, 1992). In separate experiments, Ohnishi et al. (1991a, 1991b) studied cryo-

preservation of callus tissue and the effect of different refrigeration periods on its regeneration.

Conclusions

During the last thirty years, tremendous progress has been made in mulberry improvement through tissue-culture techniques. Micropropagation procedures are already standardized in various *Morus* species. It is estimated that several thousand plantlets are now being regenerated from different explant sources each year. Despite these advancements, much research is still needed in the areas of haploid and triploid plant production, somatic embryogenesis, etc. Likewise, more immediate attention should be focused on genetic engineering in mulberry. Judicious choice of the explant source, coupled with some refinements in media composition, should increase the success rate for recalcitrant genotypes. Improvements in regeneration ability and control of somaclonal variation are crucial for maximum exploitation of this genus. When more emphasis is paid to these research areas, the future sericulture industry will be revolutionized.

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