**BIOTECHNOLOGICAL INTERVENTIONS IN MULBERRY CROP IMPROVEMENT**

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**ABSTRACT**

Biotechnology, in recent years, has created unprecedented opportunities in almost all the sectors. It has become the world’s fastest growing and the most rapidly changing technology. Application of biotechnological methods for crop improvement has significantly contributed to the success of modern day agriculture. Enhancement of yield potential, improvement of quality, resistance to pests and diseases, tolerance to abiotic stresses and resistance to herbicides are the main focus of crop improvement in many agricultural crops through biotechnological approach. Mulberry (Morus spp.) is a crop of economic importance in the sericulture industry. Its foliage forms the sole source of food for the domesticated silkworm, *Bombyx mori* L. Mulberry is a dioecious, heterozygous and perennial tree. In spite of the problems associated with tree crop improvement, considerable progress has been achieved in mulberry breeding through conventional approaches. However, biotechnology application holds a great promise in further improvement of mulberry crop especially in those areas where conventional research has not achieved the desired success. Already considerable progress has been made in this direction. The present chapter attempts to consolidate the important outcome of the biotechnological applications in mulberry and also discusses the need for future research priorities in mulberry improvement, utilization and conservation.

**1.1 INTRODUCTION**

Mulberry (*Morus*) of the family Moraceae is an economically important tree grown commercially in India, China and several other Asian countries to feed the caterpillars of the silk producing Lepidopteron insect *Bombyx mori* L*.* Its leaf is also used for feeding cattle, goat and other animals as it is highly nutritious and palatable to herbivorous animals besides having several medicinal properties including antioxidant (Yen *et al*., 1996) and hypoglycaemial ones. It is also grown for fruit, which is used for human consumption, production of jam, jelly, marmalade, frozen desserts, pulp, juice, paste, ice cream, and wine. Mulberry fruit is a good medicine for dysentery, constipation, hypoglycaemia, and avulsed teeth and it is a good source of phenolic acids and flavonoids. Further, mulberry trees have become an integral part of the landscaping in a number of countries. Traditionally, mulberry (Morus) was placed in the tribe Moreae of the family Moraceae under the order Urticales. However, based on molecular evidence, the angiosperm phylogenetic group (APG II, 2003) placed Moraceae in the order Rosales. More than 68 species of Morus have been widely recognized, of which *M. alba*, *M. latifolia*, *M.  mutlicaulis* are grown for leaves while *M. nigra* is  grown for fruit and *M. serrata* for timber (Vijayan  et al., 2011). Different cytomorphs such as diploids (*Morus Alba*; 2x, 2n=28), triploids (*M.  Alba, M. indica*; 3x, 3n=42), tetraploids (*M.  laevigata, M. cathayana*, and *M. boninensis*; 4x,  4n=56), hexaploids (*M. serrata* and *M. tiliaefolia*; 6x, 6n=84;), octaploids (*M. cathayana*; 8x,  8n=112), and docosaploids (*M. nigra*; 22x,  22n=308) are available in mulberry, though  diploids and triploids dominate mostly. It is believed that mulberry originated in the northern Himalayan foothills and spread to the tropics of southern hemisphere (Benavides et al., 1994). At present, mulberry is growing in all regions between 50oN Lat. and 10oS Lat. from sea level to altitudes as high as 4000 m which include Asia, Europe, North and South America, and Africa.

**1.2 NEED OF BIOTECHNOLOGICAL TOOLS IN MULBERRY**

The main focus of mulberry breeding is to improve leaf productivity as it alone contributes more than 38.2 per cent to the sericulture productivity. However, it is not easy to improve the leaf productivity as it is a multifactorial trait determined by a number of associated characters such as plant height, number of branches, leaf retention capacity, nodal length, leaf size and weight, total biomass etc. High heterozygosity and inbreeding depression hinder the development of inbreeds, hence, directional breeding failed to make much progress. Therefore, the heterozygous parents are used to generate F1 progenies, which are then subjected to different evaluation and selection procedures to identify the best one. This type of breeding system bears the possibility of introgressing genes of desirable traits from wild relatives or species due to genetic drag and subsequent difficulty in eliminating the undesirable traits that come along with it. Under such circumstances, the feasible means of improving specific traits without disturbing the current trait combinations is adoption of biotechnological tools like transgenesis, which enable introduction over expression of desirable genes or knocking out undesirable genes (RNA interference). Mulberry, being a tree with high heterozygosity, poses difficulties on improving traits of economic importance through conventional breeding and selection. Environmentally less influenced and developmentally stable molecular markers provide reliable tools for the breeders to characterize the germplasm and to select parents and offsprings through marker assisted selection. Thus, it would be prudent to use biotechnological tools to harness the vast benefit mulberry offers to mankind

**1.3 TISSUE CULTURE IN MULBERRY**

Tissue culture technique in mulberry has developed and ramified into different areas such as micropropagation, callus culture, organogenesis, screening of genotypes for stress tolerance, induction of polyploids, cryopreservation, transgenesis and others. Following are some of the significant contributions tissue culture made in mulberry.

**1.4** **MICROPROPAGATION**

Mulberry can be vegetatively propagated through stem cuttings, grafting or budding.  However, success of these methods depends on a number of factors such as genetic makeup of the plant, age and physiological conditions of the parental cutting, climatic conditions and others. Newly developed mulberry varieties cannot immediately be propagated through stem cuttings as at least 6-7 month maturity is required to make the cuttings from the parental plant. Micropropagation, on the other hand, allows multiplication of the plant in a short period under the controlled conditions. Further, in conventional method of propagation through stem cuttings, each stem cutting produces only one plant, whereas in micropropagation thousands of plants can be produced from a single plant piece. Moreover micropropagation can provide plantlets throughout the year irrespective of seasonal variations. It is thus an efficient and cost effective tool for rapid multiplication of mulberry in a relatively shorter time and space. Micropropagation also facilitates production of virus-free plants from the apical meristematic tissues. However, success of micropropagation is dependent on a number of factors among them genetic makeup, age and origin, physiological and pathological conditions of the explants, media composition and culture conditions are considered key factors. Ohyama (1970) initiated mulberry micropropagation by regenerating whole plants from axillary buds of *M. alba*. Shoot tips and dormant axillary buds were found suitable for mulberry micropropagation.

**1.5 ORGANOGENESIS IN MULBERRY**

Organogenesis is a complex phenomenon involving de novo formation of organs. Successful organogenesis depends on a number of factors which include appropriate selection of explants, age of the explants, media compositions, specific growth regulators, genotype, sources of carbohydrate, gelling agent, and other physical factors including light, temperature, humidity and other factors. Depending on these factors plant regeneration may occur either directly or indirectly. In direct organogenesis, plants develop directly from the explants without formation of intermediate callus while in indirect organogenesis plant develops via callus formation.  Again, callus induction depends on a number of factors such as nature of explants, genotype, medium and its composition. A variety of explants has been tested to initiate callus formation in mulberry.

**1.6** **SOMATIC EMBRYOGENESIS**

Somatic embryogenesis provides a valuable tool to enhance the pace of genetic improvement of commercial crop species. Several investigating groups attempted induction of somatic embryos in mulberry but the rate of success is less. Shajahan *et al*., (1995)  obtained heart shaped embryos from *M. alba* hypocotyl segments cultured on MS medium  supplemented with 2,4 D (0.45-4.52 µM) and BAP  (2.2 µM ). Primary and secondary somatic embryoids can be obtained by culturing zygotic embryos on MS medium containing 0.05 mg L-12,4-D + 0.1 mg L-1  BAP and 6% sucrose. However, due to the difficulty in hormonally controlling the formation of adventitious shoots and roots in mulberry, somatic embryogenesis has not been developed as it is in many other crop plants. Thus, concerted efforts are needed to make somatic embryogenesis successful in mulberry.

**1.7 HAPLOID PRODUCTION**

Haploid plants being gametophytic in origin possess only half the normal number of chromosomes as present in the parent. They can be used to produce homozygous lines, which are invaluable for any breeding programmes especially for tree crops with longer generation cycle and high heterozygosity. Since the first successful report on regeneration of haploid plants from pollen grains of the cultured anthers of datura this technique has been extensively used in most of the agriculturally important plant species. However, only limited success could be obtained on tree species. In Mulberry, though anther culture was first attempted, till date no plants could be regenerated.  Nonetheless, the plants regenerated gynogenic haploids by culturing immature female catkins on MS medium. However, no further report on haploidy is available in mulberry, though doubled haploidy is of much use in mulberry breeding.

**1.8** **PROTOPLAST ISOLATION, CULTURE AND REGENERATION OF  PLANTLETS**

Somatic hybridization through protoplast fusion has opened a new avenue for developing new characteristics, which are not possible through conventional breeding. There are only a few reports dealing with plant regeneration from protoplasts in mulberry. A combination of 2% cellulase, 1% macerozyme and 0.5% macerase is found optimal for better isolation of viable protoplast. Protoplast fusion in mulberry was successfully achieved using chemical fusogen and electro-fusion. Although protoplast isolation and regeneration was achieved, development of somatic hybrids in mulberry could not be achieved. Hence, efforts in this end need to be continued.

**OTHER APPLICATIONS OF TISSUE CULTURE IN MULBERRY**

 **1.9 SCREENING FOR STRESS TOLERANCE**

Since salt tolerance in plants is a complex phenomenon involving morphological, physiological and biochemical processes, screening of genotypes for salt tolerance need to be done in such conditions where the influence of external factors is minimal. India isolated salt tolerant genotypes by surface sterilizing the nodal explants and culturing on MS medium supplemented with 2 mg L-1 BAP, 30 mg L-1 sucrose and 0.0% to 1.0% NaCl.

**1.10** **INDUCTION OF TETRAPLOIDY**

In general the mulberry is propagated through vegetative means. Hence, sterile high yielding varieties/cultivars do not pose any problems for their true to type multiplication. Triploidy in mulberry is considered as the optimum level of ploidy because triploids show several advantages over plants of other ploids. Triploids are superior in leaf yield, stress resistance and chemical components of the leaf (Yang and Yang, 1989).  Considering these advantages, tetraploids are developed from diploids by colchicine treatment of the growing shoots. In this method, small cotton pads soaked with 1.0-2.0% colchicine solution is applied over the growing buds for 2-3 consecutive days. Though this method is easier to apply, it suffers from quick drying of the cotton pad, excessive loss of colchicines and difficulty in maintaining the uniform concentration of the colchicines solution.

Another method of getting triploidy in mulberry is to culture the endosperm because in angiosperm, endosperm is a triploid tissue formed via double fertilization. In mulberry, the first time, successfully developed triploids from endosperm of the variety S36 were developed.

**1**.**11 SYNTHETIC SEEDS**

Synthetic seeds are the encapsulated somatic embryos, which functionally mimic zygotic seeds and can develop into seedlings under sterile conditions. In a broader sense, it would also refer to encapsulated buds or any other form of meristems, which can develop into plants. In mulberry, synthetic seeds are produced mostly by encapsulating the apical/axillary buds or somatic embryos with 3-5% sodium alginate and 100mM calcium chloride solution as complexing agent. Sodium alginate solution is mixed with culture medium containing all necessary ingredients essential for proper growth.  Successfully developed this technology for artificial seed sysnthesis in mulberry. However, adoption of this technology for mulberry propagation was limited to a few species of *M. indica.* Researchers have explored the possibility of using *in vitro* derived vegetative propagules for synthetic seed production since it was found difficult to develop somatic embryos in mulberry.

**1.12 CRYOPRESERVATION OF GERMPLASM**

The high heterozygosity hinders conservation of mulberry germplasm through seeds as the progenies from such seeds are heterogenous in nature and getting true to the parental type is difficult. Thus, mulberry germplasm is conserved an *ex situ* germplasms, which is laborious, needs huge resources, and is in a risk of destruction by natural calamities, pests and diseases. Thus, safe alternative methods with economically viability need to be explored.  Cryopreservation is one such alternative wherein plant materials are stored at ultra-low temperatures (-196°C) in liquid nitrogen. At this temperature all the metabolic activities of the cell including divisions remain arrested; hence, the material remains unaltered for long period. Two different cryopreservation techniques in vogue are the classical one and the modern vitrification. In classical cryopreservation technique, the plant material is cool down slowly at a controlled rate of 0.1-4°C/ min to about -40ºC and rapidly immersed in liquid nitrogen. In vitrification, plant material is physically or osmotically dehydrated and is subsequently subjected to ultra-rapid freezing resulting in vitrification of intracellular solutes, i.e. formation  of an amorphous glassy structure without  occurrence of ice crystals. Although different plant materials are used for cryopreservation, the most appropriate material for cryopreservation of mulberry is winter buds (Niino, 1995). It is concluded from different experiments that dormant buds of mulberry can be cryopreserved for 11 years without reducing the viability of the bud.

**1.13 GENETIC ENGINEERING IN MULBERRY**

*1.13.1 GENOME CHARACTERIZATION*

Understanding of genetic structure of the plant is very important for crop improvement, utilization and conservation. Mulberry being a perennial, heterozygous tree, traditional methods of analysis have not provided sufficient insight into the genetic architecture. Compared to the phenotypic characters, molecular markers are highly heritable, consistent, fast and easy to measure and evaluate. Among the molecular markers, isozyme and DNA markers are widely employed for genome characterization and analysis of plants and animals.

*1.13.2 ISOZYME MARKERS*

Hunter and Markert were first to introduce isozymes as genetic markers in plants. Hirano used peroxidase isozyme technique to evaluate the affinities in mulberry and its relatives and showed that the results supported the conventional view. The study of inheritance of peroxidase isozyme of mulberry was initiated in Japan and established that particular isozyme banding type was significantly correlated with leaf stalk length. Hirano also used isozyme technique to analyze 284 mulberry varieties. He used seven enzyme systems and a sap protein to characterize these varieties. Based on the electrophoretic pattern he categorized 131 varieties into seven groups and established the gentic relationship among them. The study also demonstrated the correlation between amino acid content and peroxidase enzyme in the leaf. Katagiri and co-workers successfully utilized peroxidase isozyme technique to differentiate hexaploid mulberry strains collected from Mexico. In India, peroxidase isozyme studies were reported on introduced species from Indonesia triploids and aneuploids of mulberry. Even though isozyme analysis is comparatively easy, less costly and the markers are codominant in expression, they are less attractive compared to the DNA markers because of lack of sufficient polymorphism.

*1.13.3 DNA MARKERS*

Studies on mulberry genome were first initiated in Japan. Katagiri and coworkers successfully isolated chloroplast DNA from mulberry. Later Machii reported the isolation of total DNA by ultracentrifugation method.The detected DNA marker variation using RAPD technique in 12 mulberry varieties with 24 primers. Relationships among the operational taxonomical units (12 species and 2 varieties) of Morus were examined with 20 random decamer primers, generating 238 polymorphic markers. However, the study also concluded that the genetic base of cultivated mulberry is narrow. A recent study showed that as many as five RAPD and one DAMD primers generated profiles can together differentiate all the nine mulberry varieties in terms of unique bands. Central Sericultural Germplasm Resources Centre, Hosur in collaboration with Seribiotech Research Laboratory, Bangalore has characterized number of mulberry germplasm using DNA fingerprinting techniques. RAPD analysis of 15 mulberry species revealed few species diagnostic markers indicating the usefulness of the technique in identification. Phylogenetic analysis of RAPD and ISSR markers showed the separation of wild and cultivated mulberry species into a different cluster. Study of 44 cultivated mulberry varieties and 27 *M. laevigata* collections with RAPD marker data has resulted in generation of useful information on genetic diversity and identity. The results indicate that RAPD can be effectively used to DNA fingerprint mulberry cultivars and also can be successfully employed to study the inheritance pattern and for the development of molecular linkage map.

*1.13.4 RANDOM AMPLIFIED POLYMORPHIC DNA (RAPD)*

Random amplified polymorphic DNA markers uses arbitrary short oligomers (usually 10 bases) to PCR amplify the genomic DNA and the variation in the band pattern is due to base pair substitutions modifying the primer binding site. In mulberry, RAPD was the first molecular marker used for genetic diversity analysis. RAPD has been used by many investigators to work out genetic diversity among the cultivars to identify molecular markers associated with sex characteristics in mulberry to identify mutants and to develop linkage maps in mulberry. The advantages of RAPD are requirement of small amount of template DNA, low cost of development, easiness in use and the major disadvantages are the poor reproducibility under estimation of genetic distances between distantly related individuals (Powell et al., 1996) and are unable to distinguish homozygous from heterozygous ones.

*1.13.5 INTER SIMPLE SEQUENCE REPEATS (ISSR)*

Inter simple sequence repeat (ISSR) markers  amplify DNA segments between two identical  microsatellite repeat regions oriented in opposite  direction by primers of 16-25 bp long designed  from the microsatellite core regions bordering  them. The primers either anchore at 3′ or 5′ end with 1 to 4 degenerate bases extended into the flanking sequences or to remain unanchored usually di nucleotide repeats anchored either at 3′ or 5′ end reveal high polymorphism (Joshi et al., 2000).  Polymorphism occurs whenever one genome misses the sequence repeat or has a deletion or insertion or translocation between the repeats. ISSR markers are generally dominant markers following Mendelian inheritance however, incidence of segregation as co-dominant markers also have been reported. ISSR markers have higher reproducibility than RAPD markers. ISSR has also been used to estimate the biodiversity of wild populations of mulberry. Populations of *M. serrata* present in Uttaranchal (29o22′- 30o45′ N latitude and 75o52′- 80o12′ E longitude) and Himachal  Pradesh (30o30′ - 30o54′ N latitude and 77o06′ - 77o40′ E longitude) were assessed and conservation  strategies were formulated. Likewise, the phylogenetic relationship among nineteen genotypes belonging to five mulberry species viz., *M. latifolia, M. bombycis,* *M. alba, M. laevigata* and *M. indica* were also worked out using both  RAPD and ISSR markers.

 The study revealed that *M. laevigata* can be considered as a separate species while the other four species may be grouped together and treated as sub-species. Subsequently the admixture of the mulberry genetic pool of the eastern India and the southern India using 34 mulberry cultivars collected from different regions of India. In China, ISSR along with RAPD to estimate the genetic diversity of 27 mulberry accessions. The ISSR along with RAPD to estimate the genetic diversity of 20 mulberry varieties. Along with RAPD, it has also been used to develop linkage map of mulberry.

*1.13.6 AMPLIFIED FRAGMENT LENGTH POLYMORPHISM (AFLP)*

Amplified fragment length polymorphism (AFLP) is a combination of RFLP and polymerase chain reaction (PCR) techniques wherein the speed of PCR combines with the precision of RFLP though it requires only a small amount of DNA, it can be readily automatable. AFLP is more robust, reliable and reproducible than RAPD and ISSR. AFLP was first used in mulberry to study the genetic diversity of 45 mulberry accessions from different eco-geographic regions of Japan and other parts of the world. Five primer combinations were used and an average of 110 AFLP markers was generated by each primer pair. The size of the bands varied from 35bp to 500 bp. The polymorphism ranged from 69.7 to 82.3% across all the genotypes. From the study it is concluded that since spontaneous and artificial hybridization is possible, and due to continuous variation of most phenotypic characteristics, the taxonomy of the genus *Morus*, especially for *M. alba*, *M. latifolia* and *M. bombycis* species, is not well defined. The 43 mulberry accessions from different regions of Turkey using fluorescent dye amplified fragment length polymorphism (AFLP) markers and capillary electrophoresis. Unweighted pair group method of arithmetic mean (UPGMA) clustering grouped the accessions according to the species they belong. Furthermore, the study also clearly brought out the ability of AFLP markers to identify the accessions of *M. nigra*, *M. rubra*, and *M. alba* without any ambiguity. These studies demonstrated, the resolving power of  AFLP, which can be used for identifying genotypes  for conserving genetic resources, eliminating  duplicate accessions from germplasm collections  and monitoring erosion of genetic diversity within  the populations.

*1.13.7 SIMPLE SEQUENCE REPEATS (SSR)*

Simple sequence repeats (SSR) or microsatellites or short tandem repeats (STR) or simple sequence length polymorphism (SSLP) are tandem repeats of short (2-6 base pair) DNA fragments present throughout the genome (Litt and Luty, 1989). Variations at SSR loci are generated through (a) replication slippage (b) unequal crossing-over and (c) genetic recombination.  Among them, replication slippage is considered to be a major factor affecting the repeat number for short tandem repeat sequences, whereas unequal crossing-over is thought to result in a very large number of alleles for long tandem repeat arrays. SSR markers are co-dominant, stable, robust and are highly reproducible.  However, the major disadvantage of SSR is the need for prior information on the target genome to develop suitable primer sets. Using these SSR markers the genetic diversity among mulberry genotypes present in Kenya and India respectively. Likewise, these SSR markers have also been used in constructing a linkage map along with RAPD and ISSR markers. Similarly, used SSR primers along with RAPD and ISSR to test the quality of mulberry genomic DNA extracted with a new protocol. Although SSR markers have several advantages over other dominant marker systems, they have not yet been exploited widely in mulberry. Hence, attempts should be made to develop more number of SSR markers so as to utilize them in identification of QTLs for enabling marker assisted selection breeding in mulberry.

**1.14 FUTURE PERSPECTIVES**

Biotechnology of mulberry has advanced far  and wide in areas like tissue culture and molecular  biology and contributed to micropropagation of  hard to root genotypes, isolation of somaclonal  variants, screening of germplasm for tolerance to  abiotic stresses, induction of polyploids,  production of synthetic seeds, and cryopreservation  of genetic resources, development of transgenic  plants, characterization of germplasm accessions  and identification of markers associated with  economically important traits. However, there is much more to do than what has been accomplished.  Inbred lines are urgently required for elucidating the genetic basis of most of the economically important characters in mulberry. Considering the difficulty to develop inbreds through conventional breeding, developing the same through doubled haploidy should be attempted. The development of a reproducible system for the production of doubled haploids, either using anther cultures, microspore cultures and/or cultivation of ovary segments containing unfertilized ovules, need to be developed. Deeper insight into each particular step in the process of haploid plant production can help to develop more sophisticated and more successful protocols for rapid application of the gametic embryogenesis. Regarding the molecular marker systems, only a few SSR primers are still available for use. These few primers are not enough to make saturated linkage maps to identify QTLs tightly linked to economically important traits. Thus, it is important to develop large numbers of SSR and SNP (Single Nucleotide Polymorphism) markers for wider use of these marker systems. In this context it is heartening to note that the first draft sequence of mulberry genome has just been published which will facilitate development of more information on mulberry genome to enable fast improvement of this very important crop plants of Asia.

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