Journal of Pharmacognosy and Phytochemistry

Available online at www.phytojournal.com

E-ISSN: 2278-4136
P-ISSN: 2349-8234
JPP 2019; 8(4): 1126-1129
Received: 16-05-2019
Accepted: 18-06-2019

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Role of biotechnology in mulberry improvement

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Abstract
Mulberry (*Morus* spp.) is one of the economically important trees grown in India and other countries. It is cultivated mainly for leaf, which is used for feeding the mulberry silkworms (*Bombyx mori* L.). Biotechnological approaches for improvement in mulberry promise great impact as conventional breeding is time consuming and found complications due to heterozygous nature of the plant. Recent attempts have been made to complement conventional breeding with modern biotechnological tools such as plant tissue culture, r-DNA technology and molecular markers to make mulberry genetic improvement easier, speedier and more efficient and effective for betterment of the sericulture industry. The techniques of tissue culture has grown considerably in mulberry and encompassed areas including micropropagation, plant regeneration from leaf discs, and screening for stress tolerance. Recently, genetic engineering is adopted to enhance drought and salt tolerance in mulberry. The objective of these techniques lies on the overall improvement of the host plant for exploitation and utilization in sericulture. In this review an attempt has been made to study various biotechnological techniques and their role in mulberry improvement.

Keywords: Mulberry, biotechnological tools, r-DNA technology, molecular markers, micropropagation, genetic engineering

Introduction
Mulberry plant (*Morus* spp.) belongs to the family Moraceae is an economically important tree commonly grown in India, China and several other Asian countries. It is mainly grown for its leaves as they form the sole food material for the silk producing Lepidopteran insect *Bombyx mori* L. Mulberry can be vegetatively propagated through stem cuttings, grafting or budding. However, success of these methods can be altered by a number of factors including genetic makeup of the plant, age, physiological conditions of the plant and climatic conditions etc. On the other hand, biotechnological approaches for mulberry improvement has advanced the areas like tissue culture and molecular biology and also 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.

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 (Banejee, 1998). 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. (Doss *et al.*, 2011) [22]. 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 (Vijayan *et al*., 2011a) [23], or knocking out undesirable genes (RNA interference) (Vijayan *et al*., 2011b) [24]. 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

Off springs through marker assisted selection. Thus, it would be prudent to use biotechnological tools to harness the vast benefit mulberry offers to mankind.

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.

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. Additionally, 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 (Kapur *et al.,* 2001) [16]. 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 as key factors.

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 (Jain and Datta, 1992) [7]. 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.

Direct organogenesis from explants especially from cotyledons and leaf segments has great potential for transgenesis. In mulberry, direct plant regeneration from leaf explants was first reported by Kim *et al.* (1985) [1] and later by Yamanouchi *et al*. (1999) [13]. Vijayan *et al.* (2000) [13] obtained shoots from leaves on MS medium supplemented with BAP 2 mg L-1 and glucose as the sugar source.

Somatic embryogenesis

Somatic embryogenesis provides a valuable tool to enhance the pace of genetic improvement of various plants. Several attempts had been made for induction of somatic embryos in mulberry but the rate of success is less. Shajahan *et al.* (1995) [10] obtained heart shaped embryos from *M.alba* hypocotyls segments cultured on MS medium supplemented with 2,4 D (0.45-4.52 μM) and BAP (2.2 μM). Agarwal (2002) [17] and Agarwal *et al.* (2004) [19] obtained primary and secondary somatic embryoids by culturing zygotic embryos on MS medium containing 0.05 mg L-1 2,4-D + 0.1 mg L-1 BAP and 6 per cent 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.

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. In Mulberry, though anther culture was first attempted by Shoukang *et al.* (1987) [3] and later by Katagiri (1989) [4]. However, no further report on haploidy is available in mulberry, though doubled haploidy is of much use in mulberry breeding.

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 per cent cellulase, 1 per cent macerozyme and 0.5 per cent macerase is found optimal for better isolation of viable protoplast. Protoplast fusion in mulberry was successfully achieved using chemical fusogen (Onishi and Kiyama, 1987) [2] and electro-fusion (Onishi and Tanabe, 1989) [5]. Although protoplast isolation and regeneration was achieved, development of somatic hybrids in mulberry could not be achieved. Hence, efforts on this aspect need to be made.

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 (Vijayan *et al*., 2011c) [25]. Maintenance of uniformity of salinity across the field and seasons is difficult, therefore, screening of the plants under *in vitro* is considered as an ideal option, where most of the environmental conditions can be controlled. Vijayan *et al.* (2003) [18] using auxiliary buds of 63 mulberry germplasm accessions maintained at the Central Sericultural Research and Training Institute, Berhampore, West Bengal, India isolated salt tolerant genotypes by surface sterilizing the nodal explants and culturing on MS medium. This method is more economical, efficient and less time consuming for screening large number of mulberry accessions for salt tolerance.

Induction of tetraploidy

In general the mulberry is propagated through vegetative means. 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) [6]. Considering these advantages, tetraploids are developed from diploids by colchicine treatment of the growing shoots. Application of colchicine *in vitro* solves most of these problems and also makes the system more economic. Additionally, *in vitro* application of colchicine is more cost effective as the same medium can be used for at least 4 repeated treatments without reducing the efficiency of the colchicine to induce tetraploidy. Another method of getting triploidy in mulberry is to culture the endosperm because in angiosperm, endosperm is a triploid tissue formed via double fertilization (Bhojwani and Razdan, 1996) [11]. In mulberry, Thomas *et al.* (2000) [14], for the first time, successfully developed triploids from endosperm of the variety S-36.

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 or auxiliary buds or somatic embryos with 3-5 per cent sodium alginate and 100mM calcium chloride solution as complexing agent (Kamareddi *et al.,* 2013) [27]. Sodium alginate solution is mixed with culture medium containing all necessary ingredients essential for proper growth. Patnaik *et al*. (1995) successfully developed this technology for artificial seed synthesis in mulberry. Although somatic embryos could not be developed easily in mulberry, dormant auxiliary buds proved to be an ideal material for the synthesis of synthetic seed, it can be used for cryopreservation of germplasm accessions.

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 as *ex situ* germplasm, which is laborious, needs huge resources, and is in a risk of destruction by natural calamities, pests and diseases (Vijayan *et al*., 2011d) [26]. 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. Cryopreservation techniques have been standardized for *M. indica, M. alba, M. latfolia, M. cathayana, M. laevigata, M. nigra, M. australis, M. bombycis, M. sinensis, M multicaulis* and *M. rotundiloba* species (Rao *et al*., 2007, 2009) [20]. The general procedure for cryopreservation of shoot tips is that the shoot segments are pre-frozen at -3°C for 10 days, -5°C for three days, -10°C for one day and -20°C for one day and immersed into liquid nitrogen. The cryopreserved material is brought back to the ambient temperature via thawing.

Genetic engineering in mulberry

Although conventional plant breeding has contributed significantly by developing several high yielding mulberry varieties, high heterozygosity and long generation cycles and inbreeding depression prevent introgression of traits from wild species and relatives. Transgenesis is one such technique that enables direct transfer of genes of interest. Out of the two popular gene transfer techniques viz., particle bombardment and *Agrobacterium tumefaciens*-mediated transformation, the latter received much attention in mulberry due to the easiness and efficacy. Major diseases of mulberry like powdery mildew (*Phyllactinia corylea*) and bacterial blight (*Xanthomona campestris* cv. *Mori)* are causing crop loss of 20-30 per cent in the field (Philip *et al*., 1994) [8]. Therefore, sincere and serious efforts should be made to develop disease resistant transgenic plants against these diseases.

Molecular markers

Advancement of molecular biology techniques in the last two decades has aided in the concise classification of individual plants and species. Molecular markers have been used in screening of germplasm, genetic diversity, identifying redundancies in collections, testing accession stability and integrity as well as resolving taxonomic relationships. Molecular markers such as RPAD, ISSR and SSR have also been used in mulberry for molecular characterization of germplasm, biodiversity analysis and genetic mapping etc. but SSRs are advantageous over many other markers as they are highly polymorphic, robust, can be automated; only very small DNA is required, highly abundant, analytically simple, readily transferable and have a co-dominant inheritance.

Conclusions

Biotechnology of mulberry has advanced far and wide in areas like tissue culture and molecular biology and also contributed in the fields like micropropagation, 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 are still a number of issues such as resistance to fungal and bacterial diseases, infestation of pests and insects, and tolerance to drought and salinity, which are to be sorted out to make mulberry cultivation sustainable to meet the demand of the growing silk industry.

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