REVIEW

Breeding for Higher Productivity in Mulberry

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Abstract: Mulberry (*Morus* L.) is an economically important tree being cultivated for its leaves to rear the silkworm *Bombyx mori*. Rearing of silkworm is an art and science popularly known as sericulture; an agrobased cottage industry provides employment to millions in China, India, Korea, Vietnam, etc. Mulberry is a perennial tree that maintains high heterozygosity due to the outbreeding reproductive system. It is recalcitrant to most of the conventional breeding methods, yet considerable improvement has been made in leaf yield and leaf quality. Conventional breeding in mulberry is a tedious, labour intensive and time taking process, which needs to be complemented with modern biotechnological methods to speed up the process. This article enumerates the problems, challenges, constraints and achievements in mulberry breeding along with recent advances in biotechnology and molecular biology to enable mulberry breeders to tackle specific problems more systematically and effectively.

Keywords: association mapping; QTL mapping; *Morus* L.; sericulture

Sericulture is the science of rearing silkworms for the production of silk fibres. Sericulture is one of the major employment providers in India and several other Asian countries (Vijayan 2010). Commercially, four major types of silk fibres, namely mulberry silk produced by *Bombyx mori* L., tasar silk by *Anthereae mylitta* Drury, eri silk by *Samia cynthia ricini* and muga silk *Anthereae assamensis*, are used for textile purposes. India has the distinction of harbouring the silkworms of all these four types of silks, though the quantity of the silk produced varies significantly as the mulberry silk occupies a lion share of the total production. It is also interesting to note that *B. mori* can grow well only on mulberry leaves, hence, to enhance sericulture productivity mulberry leaf production has to be increased, which can be made possible by developing new varieties with higher leaf yield and better adaptability. In order to manipulate the genetic constitution of mulberry, it is essential to have adequate information on the genetics and genomics of the plant. This article, thus, provides major developments in the genetics and breeding aspects of mulberry to enable the breeders to equip with adequate information to further their efforts on enhancing the productivity.

Origin and distribution of mulberry

Evidences gathered from fossils (Collinson 1989), morphology, anatomy (Benavides *et al.* 1994; Hou 1994) and molecular biological (Zerega *et al.* 2005) investigations suggested that mulberry

originated in the foothills of the Himalaya and later spread to major continents including Asia, Europe, North and South America, and Africa (Yokoyama 1962; Machii *et al.* 1999). Cultivation of mulberry and silkworm rearing started in China before 2200 BC (FAO 1990) and currently mulberry is cultivated in almost all Asian countries (Vijayan *et al.* 2011). Taxonomically, mulberry belongs to the genus *Morus* L. and has more than 68 species (Vijayan 2010). Out of which, *M. alba*, *M. indica*, *M. nigra*, *M. latifolia*, *M. multiculis* are cultivated for silkworm rearing, *M. rubra* and *M. nigra* for fruits (Yaltirik 1982) and *M. laevigata* and *M. serrata* for timber (Tikader & Vijayan 2010). It is pertinent to note here that only a small fraction of the total mulberry gene pool is used for developing varieties suitable for silkworm rearing and a great chunk of the gene pool is still left in the wilderness.

Cytogenetics of mulberry

Natural polypoids are common in mulberry, though diploids with 28 chromosomes ($2n = 2x = 28$; Figure 1) or triploids with 42 chromosomes ($2n = 3x = 42$) are more frequent. Tetraploids with 56 chromosomes ($2n = 4x = 56$), hexaploids with

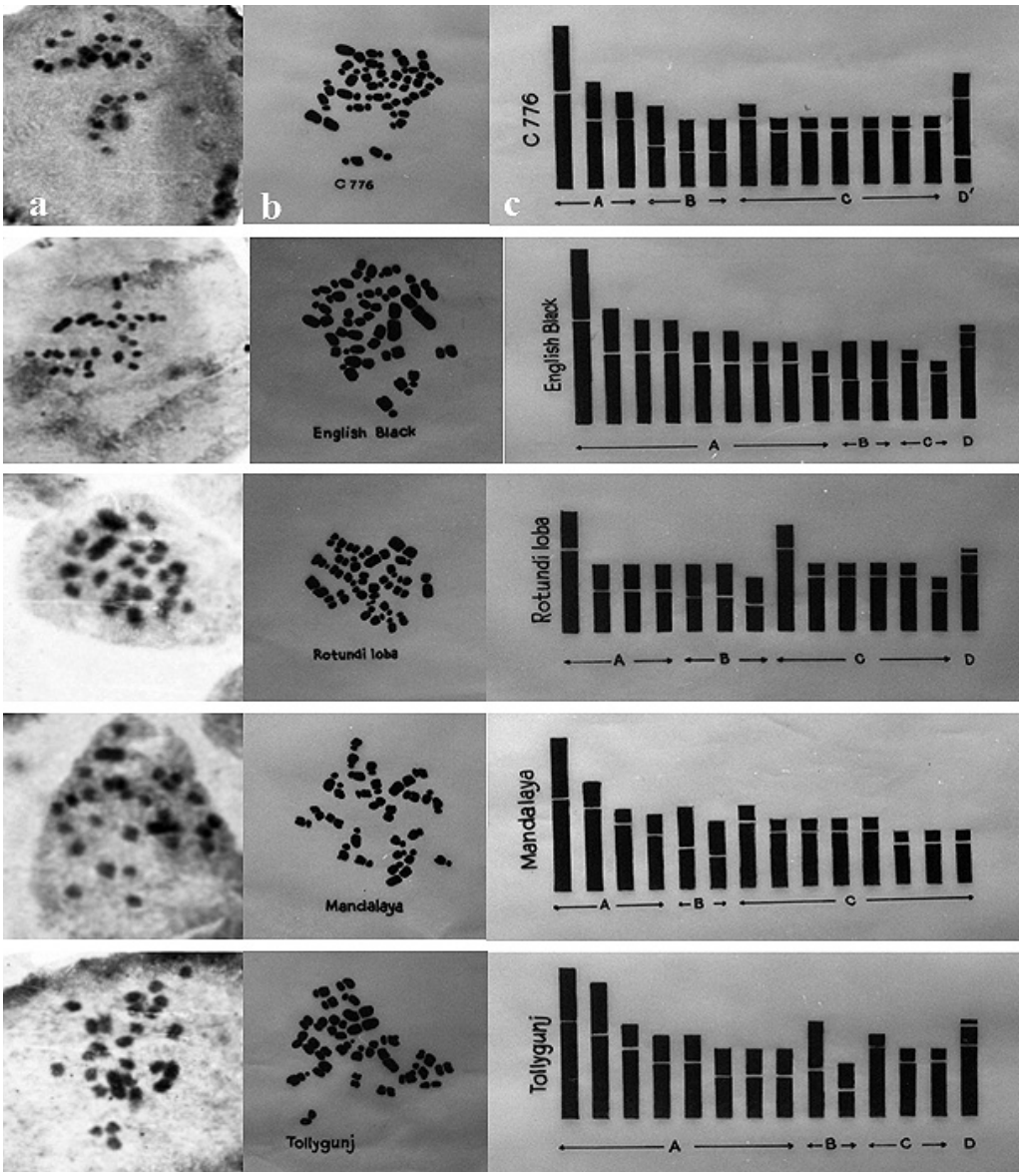

Figure 1. Chromosomes and karyotypes of a few cultivars of mulberry: a – metaphase chromosomes, b – camera lucida drawings of chromosomes, c – idiograms

84 chromosomes ($2n = 6x = 84$) and octaploids with 112 chromosomes ($2n = 8x = 112$) are also found in nature (Basavaiah *et al.* 1989). Chromosomes of mulberry are small as the length varies from 1.17 µm to 5.23 µm (Chakraborti *et al.* 1999).

Availability of genetic resources

Large numbers of germplasm accessions are available in China, India, Japan, Korea and Vietnam (Table 1). China has more than 1860 germplasm accessions (Pan 2000), Japan has 1375 germplasm accessions (Kazutoshi *et al.* 2004), Korea has 614 accessions while India has more than 1120 germplasm accessions (www.silkgermplasm.com).

Table 1. Species-wise distribution of mulberry germplasm accessions available in Japan, China, India and Korea

Species	Japan	China	India	Korea
M. bombycis Koidz.	583	22	15	97
M. latifolia Poir.	349	750	19	128
M. alba L.	259	762	93	105
M. acidosa Griff.	44	–	–	1
M. wittorium Hand-Mazz.	–	8	–	–
M. indica L.	30	–	350	5
M. mizuho Hotta	–	17		–
M. rotundiloba Koidz.	24	4	2	–
M. kagayamae Koidz.	23	–	–	1
M. australis Poir.	–	37	2	–
M. notabilis C.K. Schn.	14	–	–	–
M. mongolica Schneider		55	–	–
M. boninensis Koidz.	11	–	–	–
M. nigriformis Koidz.	3	–	–	–
M. atropurpurea Roxb.	3	120	–	–
M. serrata Roxb.	3	–	18	–
M. laevigata Wall.	3	19	32	1
M. nigra L.	2	1	2	3
M. formosensis Hotta.	2	–	–	–
M. rubra L.	1	–	1	–
M. mesozygia Stapf.	1	–	–	–
M. celtifolia Kunth.	1	–	–	–
M. cathayana Hemsl.	1	65	1	–
M. tiliaefolia Makino	1	–	1	14
M. microphylla Bickl.	1	–	–	–
M. macroura Miq.	1	–	–	–
M. multicaulis s Perr.	–	–	15	–
Morus spp. (unknown)	15	–	106	259
Total	1375	1860		614

Conservation of genetic resources

In India, mulberry genetic resources are conserved in four different ways such as *in situ* conservation, *ex situ* conservation, *in vitro* conservation and DNA banking (Figure 2). The merits and demerits of these techniques are summarized in Table 2.

In situ conservation

Conserving plants at their original habitat is known as *in situ* conservation and it allows evolutionary forces such as natural selection, mutation, population structuring, etc. to act continuously to promote further evolution of the species. Out of the 14 biosphere reserves identified by the National Committee on Environmental Planning and Coordination (NCEPL) and man and biosphere (UNESCO), eight locations, viz. Uttarkhand, Nandadevi, Namdapha, Kaziranga, Manas, Nokrek, North Andaman and Great Nicobar, conserve mulberry (Rao 2002).

Ex situ conservation

Conservation of seeds at a low temperature is not preferred for germplasm conservation in mulberry due to the high heterozygosity of the parental plants. Since mulberry is capable of being propagated through stem cuttings, the common methods of conservation are field germplasm banks and/or preserving vegetative buds in the laboratory. Germplasm banks are maintained in different ways depending on their longevity and utility. Active collections of germplasm are used for evaluation of accessions for economic traits and distribution of genetic resources to breeders and other research groups whereas base collections are used for long-term preservation without much disturbances. Duplicates of base collections are usually developed in geographically distant places as a safety measure against loss due to natural disasters and biotic destructions.

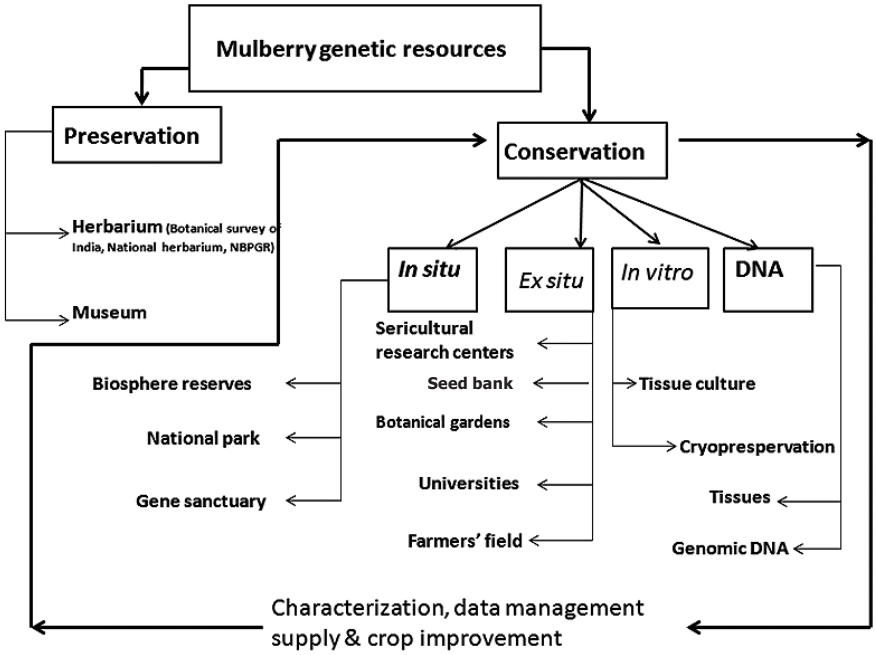

Figure 2. Conservation strategies for mulberry genetic resources

Cryopreservation

Tissue culture with its distinct advantages is used for short-term preservation (Withers & Engelmann 1997). However, tissue culture does not serve for long-term preservation. Hence, cryopreservation is adopted for long-term preservation. Under cryopreservation, plant materials are stored at ultra-low temperatures in liquid nitrogen (–196°C). At this temperature, cell division and metabolic activities remain suspended and the material remains unchanged for a long period. Thus, cryopreservation ensures genetic stability of the mulberry germplasm besides requiring only limited space and protecting material from contamination. Further, it requires little maintenance, hence it is considered a cost-effective method for the conservation of mulberry germplasm. In fact, cryopreservation is the only economically viable method for the long-term conservation of mulberry. Two different techniques are available

Table 2. Merits and demerits of different methods used for conserving the genetic resources of mulberry

In situ	*Ex situ*	*In vitro*	DNA banking
Apt for forest species and wild crop relatives	only option for the asexually reproducing plants	suitable for both sexually and asexually reproducing plants	
Field oriented, laborious and expensive	field oriented, laborious and expensive	laboratory oriented minimum space and less laborious	
Allows evolution to continue	evolution restricted	no chance of evolution	
Increases genetic diversity	less prone to genetic variability	no genetic variation	
Vulnerable to disease and other natural calamities	vulnerable to disease and other natural calamities	well protected against disease and other natural calamities	
Strengthens the link between conservationists and local people who traditionally maintain the plant	minimum interactions	no interactions	
Exchange of materials is difficult	exchange of materials possible but needs extra care	easy exchange of materials	

for cryopreservation, i.e. classical freeze-induced dehydration technique and vitrification technique (Engelmann 2000). Classical cryopreservation technique involves the slow cooling of materials at a controlled rate (usually 0.1–4°C/min), down to about –40°C and subsequent rapid immersion of samples into the liquid nitrogen (–196°C). This method is generally operationally complex and requires the use of expensive, sophisticated programmable freezers. In the vitrification procedures, cell dehydration is performed prior to freezing by physical or osmotic dehydration of explants, which 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. These techniques are less complex and do not require any sophisticated programmable freezers. In mulberry, the most appropriate material for cryopreservation is the winter bud, though embryonic axes, pollen, synthetic seeds can also be used (Niino 1995). Generally, for cryopreservation, 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 before their immersion into the liquid nitrogen. Prior to pre-freezing at –20°C, partial dehydration of the bud up to 38.5% was found to improve the recovery rates. The survival rates of winter buds stored in liquid nitrogen up to 3–5 years did not change significantly (Rao *et al*. 2009). The 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 mulberry (*M. bombycis*) attached to shoot segments by pre-freezing and storing in liquid nitrogen. The buds were later thawed, and the meristems were excised for culture on MS medium supplemented with 1 mg/l BA to regenerate plants. Either pre-freezing at –10°C or –20°C along with rapid thawing at 37°C or pre-freezing at –20°C or –30°C along with slow thawing at 0°C was a suitable treatment for high percentages of survival and shoot regeneration (Rao *et al*. 2007).

DNA banking

Preservation of genomic DNA is another safe method of conservation of genetic information in mulberry. Genomic DNA can be extracted easily from leaves using standard protocols (Vijayan 2004) and can be stored in alcohol (Mandal 1999) or in lyophilized conditions at room temperature in small vials (Ford-Lloyd 1990). A novel method of DNA distribution has recently been in vogue wherein DNA clones or PCR products are pasted on pages of books and distributed to users. Although a well-established DNA banking system is yet to be established for mulberry in India, small-scale preservation has already been started at the Central Sericultural Germplasm Resources Centre, Hosur, Tamil Nadu.

Conventional breeding

The conventional breeding technique that has been used for mulberry genetic improvement follows a very specific procedure as depicted in Figure 3 (Vijayan 2010). Prior to parental selection, the characterization of germplasm accessions is carried out using morphological, biochemical and physiological characters, rooting ability of stem cuttings, leaf yield, leaf moisture, protein and sugar contents, photosynthetic efficiency, physiological water use efficiency etc. Based on a statistical assessment, parents with desired traits are selected and control hybridization is effected. Ripe fruits from controlled hybridization as well as those formed by natural hybridization of selected mother plants are collected to extract seeds. Seedlings raised in a nursery are transplanted to the field in progeny row trials (PRT) for initial screening based on selected traits like growth, branching, leaf texture, and disease susceptibility. Since almost all mulberry accessions are highly heterozygous and have a long gestation period, traditional breeding methodologies mostly rely on the production of F_1 hybrids (Das 1984). Hybrids with desirable traits, identified through the progeny row trial, are further evaluated in primary yield trial (PYT) for important agronomic, biochemical and silkworm feeding qualities. From the PYT, the top 5–10% hybrids are selected for detail assessment in final yield trial (FYT) using 3–5 replications and 25–49 plants per replication. Here, the plants are subjected to thorough assessment for leaf yield, leaf quality, adaptation, susceptibility to pest and diseases, rooting ability, response to agronomic practices, and silkworm feeding qualities. The best hybrid is selected and mass multiplied vegetatively for further testing at different regions (MLT). Usually 8–9 hybrids are

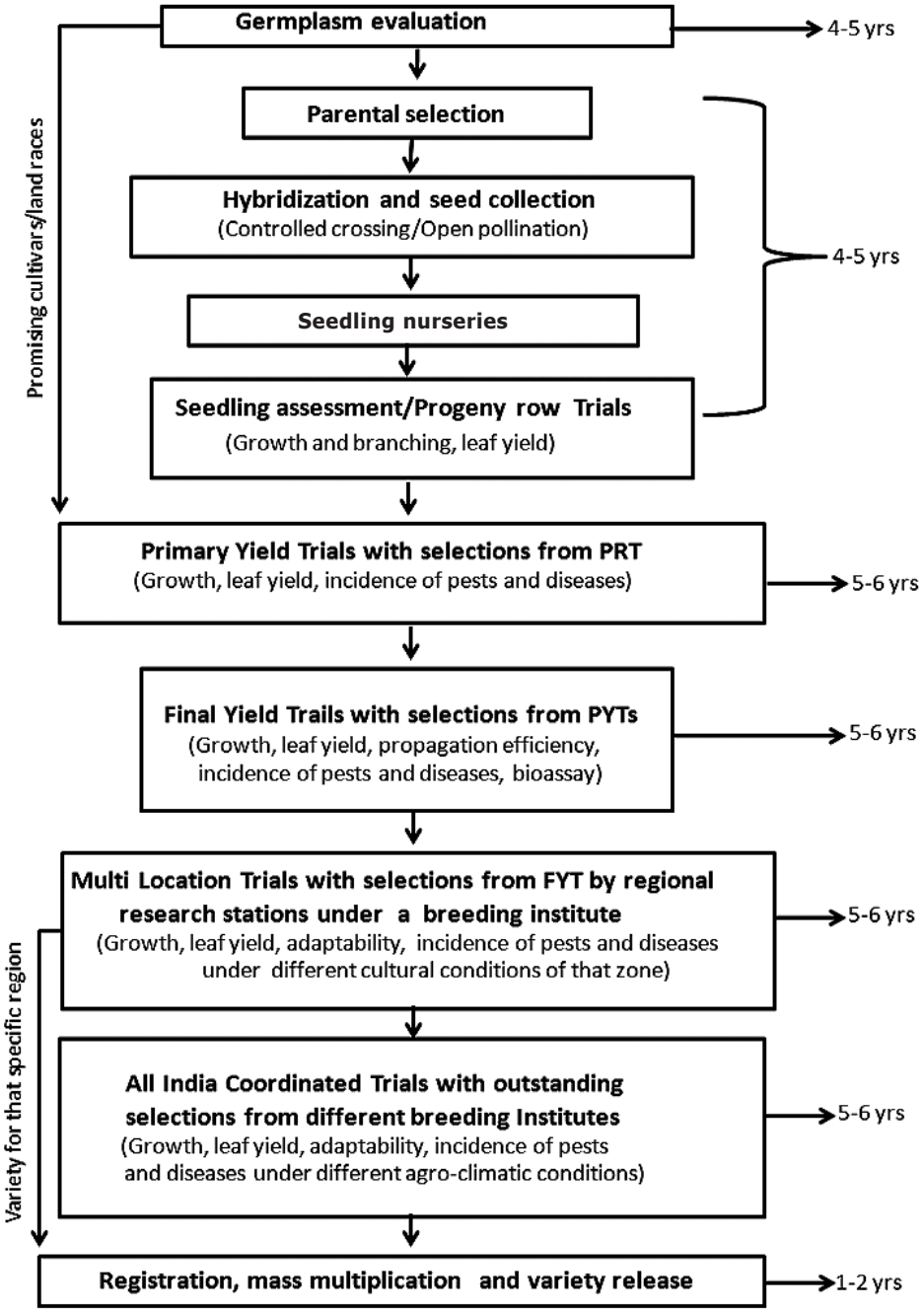

Figure 3. General breeding strategy in mulberry

used for regional multi-location studies. Those hybrids which perform consistently well in all the seasons, locations and years are selected and further tested in All India Coordinated Experiments on Mulberry (AICEM) along with hybrids selected in the same way from other regions to evaluate their performance in different agro-climatic conditions in India for a minimum of four years. The best performers of the AICEM are released for commercial exploitation. By this way, a number of high-yielding mulberry varieties have been developed and released for commercial cultivation in India (Table 3; Saratchandra *et al.* 2011).

Recently, nine selected hybrids developed through systematic breeding were tested to identify high-yielding ones during the colder months in the state of West Bengal (Gandhi Doss *et al* 2011). Two hybrids, CT-44 with 47.94 Mt/ha/year and CT-11 with 43.99 Mt/ha/year, were selected for their commercial exploitation.

Table 3. High-yielding mulberry varieties developed in India

Variety	Region	Developing Institute	Origin
Victory–1	South India, irrigated	CSRTI, Mysore	hybrid from S30 × C776
Vishala	South India, irrigated	KSSRDI, Thalaghattapura	clonal selection
Anantha	South India, rainfed	APSSRDI	clonal selection
DD	South India, irrigated	KSSRDI, Thalaghattapura	clonal selection
S–13	South India, rainfed	CSRTI, Mysore	selection from polycross (mixed pollen) progeny
S–34	South India, rainfed	CSRTI, Mysore	selection from polycross (mixed pollen) progeny
S–1	Eastern and NE India, irrigated	CSRTI, Berhampore	introduction from (Mandalaya, Myanmar)
S–7999	Eastern and NE India, irrigated	CSRTI, Berhampore	selection from open pollinated hybrids
S–1635	Eastern and NE India, irrigated	CSRTI, Berhampore	triploid selected from an open pollinated hybrid population
C776	Saline soils	CSRTI, Berhampor	hybrid from English balck and Multiculis
S–146	North India and hills of Jammu and Kashmir, irrigated	CSRTI, Berhampore	selection from open pollinated hybrids
Tr–10	hills of Eastern India	CSRTI, Berhampore	triploid developed from the cultivar S1
BC259	hills of Eastern India	CSRTI, Berhampore	back crossing of hybrid of Matigare local × Kosen with Kosen twice
Goshoerami	temperate	CSRTI, Pampore	introduction from Japan
Chak Majra	sub temperate	RSRS, Jammu	selection from natural variability

Biotechnological methods for mulberry breeding

Screening for stress tolerance

Screening of a large number of mulberry germplasm accessions under field conditions requires a large space and incurs huge expenditure. Additionally, stress tolerance is a complex trait under the control of several genes and their interactions among themselves and with environmental factors. Therefore, screening of genotypes under soil conditions is a complicated process. *In vitro* screening of axillary buds and shoot tips was found to be an effective and efficient method to select salt and drought tolerant genotypes in mulberry (Hossain *et al.* 1991; Tewary *et al*. 2000; Vijayan *et al.* 2003). Vijayan *et al.* (2003) screened 63 mulberry accessions for salt tolerance and five mulberry accessions, namely Rotundiloba, English Black, Kolitha-3, BC259 and C776, were found to have better tolerance as they could develop roots in 0.3% NaCl and the survivability of axillary buds at 1.0% NaCl in *ex vitro* conditions varied from 11.1 ± 7.9% to 50.0 ± 13.6. From these accessions, English Black, Rotundiloba (females) and C776 (male) were used for breeding and three hybrids, viz. SR1, SR2 and SR3, with higher salt tolerance capacities were developed. Among these hybrids, SR3 was much superior to the other two hybrids in survival and growth (Vijayan *et al.* 2009).

Molecular marker technology

In order to acquire thorough knowledge of the total genetic make-up of the germplasm bank, environmentally insensitive, developmentally stable, reproducible, easy to define, unbiased, numerous, and ubiquitous molecular markers have been used for germplasm characterization in mulberry (Vijayan *et al.* 2006). The most commonly used marker systems

Table 4. Transgenesis in mulberry for abiotic stress tolerance (adopted from Vijayan *et al*. 2011)

Gene	Expression profile	Reference
WAP21	cold tolerance	Ukaji *et al*. (1999)
COR	cold tolerance	Ukaji *et al*. (2001)
AlaBlb	salinity tolerence	Wang *et al*. (2003)
OC	insect resistance	Wang *et al*. (2003)
SHN 1	drought tolerance	Ahroni *et al*. (2004)
HVA1	drought and salinity stress	Lal *et al*. (2008)
bch	drought and salinity stress	Khurana (2010)
NHX	drought and salinity stress	Khurana (2010)
Osmotin	drought and salinity stress	Das *et al*. (2011)

WAP21 – water allocation plan; *COR* – cold on regulation; *AlaBlb* – soybean glycine gene; *OC* – osteocalcin; *SHN 1* – schnurri from *Drosophila melanogaster*; *HVA1* – *Hevea braziliensis* abiotic stress gene; *bch* – *L* inhibitor 2-aminobicyclo-(2,2,1)-heptane-2-carboxylic acid; *NHX* – Na+/H+ exchanger; *Osmotin* – osmotic stress induced gene

are random amplified polymorphic DNA (RAPD) (Srivastava *et al*. 2004), amplified fragment length polymorphism (AFLP) (Wang & Yu 2001), and inter-simple sequence repeat (ISSR) (Vijayan *et al*. 2005, 2006; Zhao *et al*. 2006). Using ISSR markers, genetic divergence among 34 indigenous mulberry accessions has been worked out and the genetically distant parents with better economic traits are being used for breeding purposes (Vijayan *et al.* 2005). Similarly, genetic divergence among 16 populations of the Himalayan mulberry species (*M. serrta*) has been worked out for better utilization in breeding as well as for the formulation of conservation strategies (Vijayan *et al.* 2004). Since, RAPD and ISSR marker systems are reported to have problems of reproducibility, simple sequence repeat (ISSR) (explain the abbreviation) marker system is being tested now in mulberry (Vijayan 2010).

Genetic engineering

Genetic engineering has recently made some interventions into mulberry research. Efficient protocols have been developed for direct plant regeneration from explants and insertion of desired genes into the plant genome via *Agrobacterium tumefaciens* and particle bombardment mediated methods (Bhatnagar *et al.* 2002, 2003). Transgenic mulberry plants with several desired genes (Table 4) have been developed (Lal *et al.* 2008). Among them, the transgenic plant overexpressing HVA1, a group-3 LEA protein isolated and characterized from barley, showed increased cell membrane stability, higher relative water use efficiency and growth under salt stress (200mM NaCl) in mulberry (Lal *et al*. 2008). Physiological, biochemical and molecular studies revealed that this transgenic mulberry plant performed much better than the non-transgenic plant when subjected to salinity (200mM NaCl) and drought (2% PEG, MW 6000) induced stresses. Transgenic plants showed better cell membrane stability, photosynthetic yield, less photooxidative damage and high relative water content under salinity and water stress. Initial evaluation of the suitability of transgenic plants for silkworm rearing also showed promising results. Another transgenic plant overexpressing a tobacco osmotin gene under the constitutive expression of the CaMV 35S promoter and stress-inducible promoter rd29A also showed higher salt tolerance (Das *et al*. 2011)

Conclusions and prospects

Over the years, conventional breeding has made considerable achievements in mulberry by developing varieties with high leaf yield, wider adaptability, better leaf quality, and suitable to specific cultural practices. Nonetheless, due to high heterozygosity and outbreeding reproductive system, genetics of mulberry remains an enigma to the breeders. Lack of adequate information on the genetics and breeding behaviour of important traits makes mulberry breeding quite uncertain. Recent advancements in biological tools and techniques have armed the geneticists and breeders with new tools to tackle

some of the recalcitrant problems. Characterization of germplasm by molecular markers enables the selection of parents with wider genetic differences and desirable traits. DNA markers tightly associated with desirable traits are handy for earlier identification of hybrids with desirable gene combinations. Genetic engineering is another potential tool that can be used for mulberry genetic improvement. Thus, concerted efforts are to be made to integrate conventional breeding with advanced technological developments to accelerate varietal development in mulberry for better sustainability and profitability of the silk industry in India.

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Received for publication November 15, 2011
Accepted after corrections September 4, 2012

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