Chapter 8
Genomic Designing for Biotic Stress Resistance in Mulberry

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Abstract Mulberry has several economic uses and all parts of the plant have one or another use and the leaves are used as a sole food for monophagous silkworm (*Bombyx mori* L.). Production of premium silk in the sericulture industry is directly associated with quality mulberry leaves production. However, mulberry is affected by various pathogens and pests like fungi, bacteria, virus, nematodes and cause considerable crop loss. Mulberry genetic improvement through traditional breeding relies on the availability of compatible genetic resources carrying the genes of interest. Mulberry being highly heterozygous and with a long generation gap and also due to genetic drag, it is difficult to introgress genes from wild germplasm to cultivars through recurrent back crosses. Nonetheless, significant works have been made to develop disease resistant lines/varieties through germplasm screening and identification of suitable parents for breeding. To speed up the breeding programme, DNA markers tightly linked to the trait of interest are used for early and reliable selection of desirable genotypes through the process of Marker Assisted Selection (MAS). The major limitations MAS include the sparse distribution of markers, large genetic intervals between the markers and the trait genes, many QTLs identified as minor QTLs that show a small effect on phenotypic variations and the low success rate in validating identified QTLs in different genotypes and environments. Further, to develop high resolution maps to identify markers with the tight association, more abundantly available markers like Single nucleotide polymorphisms (SNPs) have to be developed. Such effort is currently in progress at different research organizations

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C. Kole (ed.), *Genomic Designing for Biotic Stress Resistant Technical Crops*,
https://doi.org/10.1007/978-3-031-09293-0_8

across the globe. This chapter deals with current initiatives of genomic designing such as gene editing, and recent concepts and strategies developed for biotic stress resistance in mulberry.

Keywords Mulberry · Biotic stress · Genomics · Transcriptomics · Marker

8.1 Introduction

Mulberry is a fast-growing deciduous, deep rooted, woody perennial plant that originated in the sub-Himalayan tract and afterward spread across the world (Le Houerou 1980; Rodríguez et al. 1994). Currently, mulberry is seen growing in Asia, Europe, Africa, and America (Sanchez 2000a, b). Taxonomically *Morus* L. belonged to the family Moraceae under the order Urticales; molecular phylogenic studies placed Moraceaeunder the order Rosales (Ii 2003). The species delimitations of the genus *Morus* are yet to be solved as the morphological traits vary considerably under different growing conditions and stages of development and also due to uncontrolled natural hybridization among the species most of the accessions being maintained by different institutes are of natural hybrids. The greater success rate with cross-species reproduction indicates that these species have comparable genetic relationships and therefore, 'species' status needs to be studied additionally (Wang and Tanksley 1989). Zeng et al. (2015) used the complete ITS data for reconstruction of a phylogenetic tree and proposed that the *Morus* genus should be classified into eight species, including *M. alba, M. celtidifolia, M. insignis, M. mesozygia, M. nigra, M. notabilis, M. rubra* and *M. serrata.* This observation is further supported by an investigation with genome-wide SNP markers, which shows that utilizing morphological data may not be sufficient to envisage taxonomic inferences in mulberry varieties (Muhonja et al. 2020). Mulberry grows luxuriously in a flat land with fertile, well drained, deep, and clayey to loamy, porous soil with good moisture holding capacity and a pH ranging from 6.5 to 6.8. Mulberry is reported to have a moderate level of tolerance to salinity and drought (abiotic stresses). Since it is a plant of temperate origin, during the winter months it shed the leaves and the buds remain in dormant conditions. Morphologically, the leaves are alternate, simple, petiolate, stipulate, entire or lobed and inflorescences are catkin with drooping or pendent peduncle bearing unisexual flowers develop from axillary dormant buds immediately after the colder months. The long male catkins bear loosely arranged florets but the short female catkin bears compactly arranged florets with bifid feathery stigma and ovary is one-celled. Pollination is anemophilous and the fruit is a sorosis and the colour of the fruit diverges from white to lavender to black depending on the species. Seed is brown or light yellow, at the micropylar region, it is a nearly flat surface with oval shaped. In general, under room temperature seeds keep viability only for a few weeks, but if kept under controlled humidity and temperature, the viability may be extended for 3–6 months. The ideal temperature for germination is 28–30 °C. Since mulberry is propagated mainly through vegetative means, plants in different ploidy

such as haploid (*M. notabilis:* 2n = 14), diploid (*M. indica and M. alba:* 2n = 28), triploid (*M. bombysis:* 2*n* = 42), tetraploid (*M. boninensis, M. cathayana* and *M. laevigata:* 2*n* = 56), hexaploid (*M. serrata* and *M. tilaefolia:* 2*n* = 84), octoploid (*M. cathayana:* 2*n* = 112) and decasoploid (*M. nigra:* 2n = 308) are present in nature (Vijayan et al. 2009). However, diploids and triploids are preferred for cultivation, especially for sericulture purposes.

8.1.1 Economic Significance

Mulberry has several economic uses and all parts of the plant have one or another use. However, the most priced one is the leaf, which is used for feeding the monophagous silkworm *Bombyx mori* to produce silk. Mulberry leaf is also used as cattle feed as it is highly nutritious, palatable and digestible (Uribe and Sanchez 2001). The fruit is a berry that has numerous medicinal compounds like vitamins, minerals, aminoacids, carotenoids, flavonoids, anthocyanins, resveratrol, zeaxanthin, lutein, carotenes are (alpha and beta), apigenin, morin, quercetin, luteolin, caffeic acid, rutin, umbelliferone, gallic acid, chlorogenic acid cyanidin-3-O-β-D-glucopyranoside and kaempferol etc. and is consumed in raw and also processed into jelly, juice, fruit powder, sauce, tea, cakes, Mouro, wine, etc. (Asano et al. 2001; Hassimotto et al. 2007; Ercisli and Orhan 2007; Singhal et al. 2009; Yigit et al. 2010; Yang et al. 2010; Arfan et al. 2012). It is used as a worming agent, as a treatment for dysentery, and as an expectorant, hypoglycaemic, laxative, odontalgic, anthelmintic and emetic (Kang et al. 2006; Chen et al. 2006). Keeping these properties of mulberry fruit is consumed fresh. The barks of the root and stem are a good source of phenolic compounds such as morin, rutin, maclurin, resveratrol, isoquercitrin and also used for anthelmintic, purgative and astringent purposes (Chang et al. 2011). Mulberry wood is very hardy and smooth; hence, it is exploited for manufacturing sports articles, house buildings, turnery items, agricultural implements, spokes, furniture, poles and carts. In Europe and the United States, Mulberry is used for landscaping (Tipton 1994) as it is a perennial crop with good foliage, root-spread it provides green cover and it helps to soil conservation.

8.1.2 Effect of Biotic Stress on Yield and Quality

Mulberry is affected by various pathogens and pests like fungi, bacteria, virus, nematodes and cause considerable crop loss. About 300 pests species of insect and non-insect are known to occur on mulberry (Kotikal 1982). The important fungal diseases of mulberry are the leaf spot caused by *Cercospora moricola*, powdery mildew by *Phyllactinea corylea*, leaf black rust by *Ceroteliumfici*, red rust by *Aecidium mori*, twig blight by *Fusarium pallidoroseum*, root rot by *Resellinianecatrix* and *Helicobasidiummompa*, stem canker and die back by *Botryodiplodia theobromae*, stem rot

by *Polyporushispidus* and *Ganoderma applanatum*, collar rot by *Phomamororum*, stem blight by *Phomaexigua* and bud blight by *Fusarium lateritium* are well known from different parts of the world. These pathogens inflict heavy crop loss in the form of mortality of plants; reduction in the quantity and quality of leaf and fruit yield. Since the quality mulberry leaf is required for better silkworm growth, the leaf with poor nutritive values impairs the cocoon yield and quality of the silk (Vijaya Kumari 2014) as detailed elsewhere.

8.1.3 Increasing Population and Climate Change Scenario

The change in the climate caused by an increase in the average temperature of the atmosphere is defined as global warming due to the increase in the concentration of carbon dioxide (CO_2) because of the industrialization, urbanisation, burning of fossil fuel, deforestation and so on. Global warming affects the ecosystem, agriculture and human beings to a considerable degree. It is estimated that the atmospheric temperature increased at a rate of 0.3% per decade or 5 °C in 170 years but the increase may double by the end of the twenty-first century affecting agricultural production and food security. Global warming can result in severe weather conditions such as drought, unseasonal rain, prolonged winter, the emergence of new pests and diseases etc. and these jointly affect plant growth and development (Jiang et al. 2016). The changes in air temperature can alter the availability of moisture in the soil as well as the atmosphere affecting the growth and reproductive cycle of the crop. The survival, distribution and host preference of pest and other pathogens may also undergo drastic changes necessitating the adoption of new crop management practices (Krupa 2003). Further, the exploding population and loss of arable lands through urbanization and salinization necessitate the utilization of hereto unutilized and underutilized lands for agriculture. Mulberry cultivation, thus, has to be expanded to such areas.

8.1.4 Logical of Genome Designing and Bottlenecks of Traditional Breeding

Mulberry genetic improvement through traditional breeding relies on the availability of compatible genetic resources carrying the genes of interest. Mulberry being highly heterozygous and with a long generation gap and also due to genetic drag, it is difficult to introgress genes from wild germplasm to cultivars through recurrent back crosses. Nonetheless, efforts have been made to develop disease resistant varieties through germplasm screening and identification of suitable parents for breeding. Maji et al. (2009) screened 85 germplasm lines for resistance to powdery mildew and found *M. multicaulis, M. australis*, Italian and Thailand lobed were highly resistant, another nine lines resistant, yet another 43 lines were moderately resistant to

the disease complex. Likewise, Prabhakar et al. (2015) screened 100 accessions and identified 82 genotypes showing immunity to thrips. Among them, three accessions were resistant to mealy bugs *Meconellicoccus hirsutus* Green and 20 were thrips *Pseudodendro thrips mori*. In another effort, twenty mulberry cultivars screened for disease response to *M. phaseolina* and found all are susceptible (Chowdary 2006), though Hongthongdaeng (1987) reported in Thailand, mulberry cultivar Pai and F_1 hybrids Pai × Noi (6, 18, 33 and 36) displayed resistance to root rot disease indicating root rot-resistant genes in mulberry. Therefore, it is necessary to collect as many germplasm as possible from different countries and screen them for resistance. But screening of huge germplasm accessions for biotic resistance is resource demanding and time consuming. Thus, baring a few successes such as the development of "Shimgang" resistance to popcorn disease (Sung et al. 2016) not much success could be obtained in developing disease resistant mulberry through traditional breeding (Vijayan et al. 2010). Further, to develop and release a mulberry variety employing traditional breeding methods take not less than 15 years. Another major bottle neck for the development of desired mulberry varieties is the lack of detail on the genetic control of most of the disease resistance. Therefore, it is intricate to transfer the resistance from other genotypes through introgression (Vijayan et al. 1997, 2008). To resolve these troubles proper understandings of (a) the chromosomal location of these loci, (b) the number of genetic factors (loci) influencing the expression of the traits, (c) pleiotropic effects, (d) the relative size of the contribution of individual loci, (e) variation of expression of individual factors in different environments and (f) epistatic interactions among genetic factors, are essential. Only detailed genomic and transcriptomic analyses can provide such information.

8.2 Description of Different Pathogens Causing Biotic Stress in Mulberry

The biotic stress on plants is caused by living organisms that include mycoplasma, virus, bacteria, fungi, nematodes, insects and other animals that feed on different plant parts causing damage to the plant. The major diseases and pests that cause considerable damage to mulberry are described here under.

8.2.1 Major Diseases in Mulberry

8.2.1.1 Fungal Diseases

Leaf Spot

This is one of the major foliar diseases established during the rainy and winter seasons. It affects considerable losses in vegetative yield and degrades leaf quality (Philip et al. 1991; Peris et al. 2012). The disease causes a direct leaf yield loss due to defoliation is 5–10% and the destruction of leaf area is of 20–25% as additional loss (Sukumar and Ramalingam 1989; Srikantaswamy et al. 1996). The disease adversely affects moisture, protein, chlorophyll and total sugar contents in the leaves (Srikantaswamy et al. 1996). Hence, the economy of sericulture is severely affected by foliar diseases. Further, feeding the silkworm with diseased leaves affects the commercial characters of the cocoons (Nomani et al. 1970; Sullia and Padma 1987). There are many fungal pathogens causing leaf spot of mulberry and they are discussed below.

Cercospora moricola

The most common symptoms of leaf spot caused by *C. moricola* are the appearance of small brownish, irregular spots on the leaves in the initial stages which gradually increase in size and turn dark brown. As in severe cases, the dead tissues from the spot fall off resulting in the formation of a shot hole lining yellow circle around it. Severely affected leaves become yellowish and fall off prematurely (Fig. 8.1a). The disease is very common in the rainy season (Siddaramaiah et al. 1978) and causes a leaf yield loss of 10–20% besides making the leaf nutritionally poor due to less moisture, proteins and sugars. Rearing with infected leaves affects the health of silkworms and in turn quality and quantity of cocoons (Sikdar and Krishnaswami 1980).

Setosphaeria rostrata

Setosphaeria rostrata caused by *S. rostrata* appearing as specks at initial stages and the spot enlarges into irregular shape with brownish center surrounded by a yellow halo as the disease becomes severe. The spot size ranges from 0.4 to 1.5 cm. These spots are inter-veinal in character and a few of the spots starts from the leaf margins, expand and combine, leading to blightened appearance (Fig. 8.1b). Rigorously affected leaves turn out to be yellowish and fall prematurely (Arunakumar et al. 2019a).

Fig. 8.1 Different types of leaf spot in mulberry. **a** Leaf spot caused by *Cercospora moricola* (arrows). **b** Leaf spot caused by *Setosphaeria rostrata* (arrows). **c** The shot hole symptoms caused by *Nigrospora sphaerica*

Nigrospora sphaerica

The shot hole symptoms caused by *N. sphaerica* appears specks initially (2 to 4 mm), characterized by circular or irregular shape with a brownish center surrounded by a yellow halo. These spots began from the leaf margin and gradually enlarged and coalesced to form large lesions (Fig. 8.1c). Later, the larger lesion dries and fell out and appears as a shot hole (Arunakumar et al. 2019b).

Powdery Mildew

Powdery mildew disease caused by *Phyllactinia corylea* is the most common and widespread disease, causes a direct leaf yield loss due to defoliation is 5–10% and by the destruction of leaf area is of 20–25% as additional loss (Sukumar and Ramalingam 1989; Teotia and Sen 1994). It is an obligate, biotrophic parasite of the phylum Ascomycota of Kingdom Fungi and belongs to the order Erysiphales. The conidia of fungus spread through wind, when it lands on the leaf surface, it germinates and mycelium is observed as a white mat on the abaxial surface of the leaf. (Fig. 8.2a). In addition to the loss of leaf yield, the quality of the mulberry leaf is also affected, when such low quality leaves are fed to silkworms, cocoon productivity and quality reduces. (Manimegalai and Chandramohan 2007). It is also observed that feeding of mildew affected leaves to silkworm adversely affects silkworm growth and development resulting in poor cocoon yield and silk quality. Infection of disease reduces leaf yield qualitatively and quantitatively and feeding of infected leaves to silkworm prolongs larval duration (Qadri et al. 1999). Spraying of Dianocap 0.2% can control the disease with a safe period of 10 days for silkworm feeding (Gunasekhar and Govindaiah Datta 1994).

Leaf Rust

It is caused by *Ceroteliumfici* (Cast). Arthur and also known as *Peridiospora mori* Barclay (Prasad et al. 1993). Leaf rust is also called black rust belongs to the family Uredinaceae under the order Uredinales in the class Imperfect fungi. The pathogen produces numerous pin-head-sized circular to oval, brownish to black eruptive lesions/spots on the surface of the leaves (Fig. 8.2b). The affected leaves turn yellowish, under severe disease conditions, the leaves wither off prematurely. The disease appears on mature leaves and can cause a crop loss of 5–10% and also affects the quality of the leaf reducing moisture, crude protein, sugars and total sugars in the infected leaves (Sengupta et al. 1990; Philip and Govindaiah 1994). Out of fourteen fungicides evaluated, Ametoctradin 27% + Dimethomorph 20.27% SC, a combi-product was found highly effective at all the concentrations tested and showed the least spore germination (0.62%) at 0.1% concentration. Similarly, chlorothalonil at 0.3%, a non-systemic fungicide that is currently recommended for management of leaf rust of mulberry also found on par with other effective fungicides and showed

Fig. 8.2 Different types of foliar diseases in mulberry. **a** Powdery mildew caused by *Phyllactinia corylea.* **b** Leaf rust caused by *Cerotelium fici*. **c** Twig blight caused by *Fusarium lateritium*

2.06% spore germination. The effective novel fungicide molecule could be used for the management of leaf rust after evaluation in field condition and bio-assay with silkworm. It is the combination of both systemic and contact mode of action, that becomes an alternative to the existing fungicide in leaf rust disease management (Poojashree et al. 2021).

Red Rust

Red rust is caused by *Aecidium mori* Barclay. This disease causes up to 15% leaf loss (Teotia and Sen 1994; Prateesh Kumar et al. 2000). It belongs to a family Pucciniaceae under the order Puccinales in the class Pucciniomycetes. Upon infection, numerous round shiny spots appear on both surfaces of the leaf which later protrude gradually into yellow. The affected young shoots become swollen and curl up abnormally with densely and slightly protruded yellow spots on the malformed buds. The disease can be controlled by applying 0.25% wettable powder of 5% zinc or 0.75 solutions of 5% wettable powder of nitrite are reported effective for controlling the disease with a safe period of 7 days (Maji 2003).

Twig Blight

It is caused by *Fusarium lateritium* that belongs to the order Moniliales of Class Deuteromycetes. The diseased plants show bushy appearance with profuse growth of auxiliary branches, leaves show marginal browning/blackening at the beginning and complete burning in the later stages resulting in severe defoliation. Affected, branches have black longitudinal lesions which later lead to the splitting and drying of branches (Fig. 8.2c). The disease management can be taken by application of Foltaf 80 W and Dithane M-45 as a foliar spray.

Root Rot

It is a major limitation in mulberry farming due to its epidemic nature and potential to kill plants completely, resulting in leaf yield loss of up to 31.5% (Chowdary and Govindaiah 2009). A range of root rot such as dry root rot caused by *Fusarium solani* (Mart.) Sacc., *F. oxysporum* Schlecht. (Manomohan and Govindaiah 2012), black root rot—*Botryodiplodia theobromae* Pat. [syn. *Lasiodiplodia theobromae* (Pat.) Griffon and Maubl.] (Sowmya et al. 2018) and charcoal root rot—*Macrophomina phaseolina* (Tassi.) Goid. [syn. *Rhizoctonia bataticola* (Taub.) Butler] (Muthuswami et al. 2011) have been reported (Sharma et al. 2003). All the root rot diseases can be controlled by the application of *Rot-fix* a target specific plant based formulation (Pratheesh Kumar 2019). The various types of root rot symptoms are discussed below (Fig. 8.3a–d).

Black Root Rot

It is caused by *Lasiodiplodia theobromae* Pat. (Syn: *Botryodiplodia theobromae* (Pat.) Griff. and Maubl.). These fungi cause black root rot disease and incidences were reported in the southern Indian states like Tamil Nadu and Karnataka (Radhakrishnan et al. 1995; Sukumar and Padma 1999). Once the mulberry plants are weak

Fig. 8.3 Symptoms of root rot in mulberry. **a** Partial wilting of mulberry shoots. **b** Root rot affected dried shoots. **c** Complete dried plants affected. **d** Rotted roots

to infection, fungus control inside the roots rapidly multiplies in the cortical tissues and reaches up to the pith. It enters the xylem vessels and causes the death of the plants (Radhakrishnan et al. 1995; Sukumar and Padma 1999; Sharma and Gupta 2005). As the disease advances, it is observed that vascular browning and subsequent death of the plants (Sharma and Gupta 2005). The survey reports the incidence of black root rot disease in major southern sericulture states of India. Disease incidence maximum recorded in the districts of Tamil Nadu followed by Andhra Pradesh and Karnataka. The root dip method of inoculation was highly effective for germplasm screening against *L. theobromae* (Arunakumar and Gnanesh 2022).

Rhizopus Rot

It is caused by *Rhizopus oryzae,* initial symptoms of Rhizopus rot in mulberry are the roots turning to black colour and tissues becoming fragile. Infested plant leaves are characterized by yellowing wilting and defoliation, followed by decaying of roots. (Yoshida et al. 2001; Fang et al. 2011; Gnanesh et al. 2020). In severe conditions, white and cottony fungal hyphae were observed on the affected root tissues.

Dry Root Rot

In India, earlier dry root rot has been reported as violet and white root rot in mulberry (Rangaswami et al. 1976) but various surveys indicate that the disease is dry root rot caused by *Fusarium solani* and *F. oxysporum* (Philip et al. 1995c; 1997). There are reports on *Fusarium* species causing root rot disease in China and Philipines which is restricted to certain areas and occurring occasionally (Luo and Zhaoxuan 1989; Telan and Gonzales 1999). The dry root rot causing *F. solani* and *F. oxysporum* is considered to be one of the most devastating fungal pathogens, which causes root rot disease on more than 500 plant species worldwide. It is difficult to control the fungus due to its thick-walled resistant sclerotia which persist in the soil and plant debris. The initial symptoms can be seen as root bark turn black in colour due to the presence of fungal spores/mycelium and decaying of root cortex. On severity, the entire root system gets decayed and plants die. The affected plants after pruning, either fail to sprout or plant sprouted bears small and pale yellow leaves with a rough surface. Colonized sorghum grain was found to be the best method for germplasm screening against *F. solani,* and *F. oxysporum* (Arunakumar and Gnanesh 2022).

Charcoal Root Rot

Macrophomina phaseolina is the most widespread pathogen in the South Indian sericulture belt (Sowmya et al. 2018; Yadav et al. 2011). Most mulberry cultivars are prone to charcoal rot disease and can cause up to 35% leaf yield loss, reduction in leaf size, deterioration of leaf quality, and plant mortality (Chowdary 2006). These in turn adversely affect profitability in sericulture (Philip et al. 2009).

8.2.1.2 Bacterial Diseases

Bacterial Blight

It is caused by *Pseudomonas syringae* pv. *mori* that accounts for 5–10% leaf yield loss during the rainy season (Sinha and Saxena 1966). Numerous irregular water-soaked patches appear on the lower surface of leaves. In severe conditions, the leaves become curled, rotten and turn brownish black. The disease can be controlled by

uprooting and burning of the affected plants and also by application of phytobacteriomycin (0.05 to 0.1%) containing glycerine, streptomycin sulphate, streptocycline and streptopenicillin.

Bacterial Leaf Spot

This disease is caused by *Xanthomonas campestris* pv. *Mori* (Choi et al. 1989). Numerous water soaked spots initially appear on the lower surface of the leaf as soon as the rainy season starts. These spots grow gradually into brown to brownish black surrounded by a yellow halo around the spot. The application of agromycin may control the disease to a certain extent.

8.2.1.3 Nematode Disease

Root-Knot Nematode

The primary root-knot nematode parasitizing mulberry is *Meloidogyne incognita* (Kofoid and White) Chitwood which belongs to class Nematoda, order Tylenchida of family Heteroderidaeand it was reported on mulberry for the first time by Narayanan et al. (1966) from Mysuru, Karnataka, India. In India, RKN infestation is widespread and more prevalent in red sandy soils followed by red loamy soils. The severity of RKN increases with the age of the garden and the estimated leaf yield loss is up to 20%, besides affecting leaf quality (Devi and Kumari 2014). The RKN control is very difficult, because of its wide host range and its ability to survive in the soil for several years. Four races of *M. incognita* have been identified and reported across the world (Hartman and Sasser 1985). Among them, race-2 has been reported to infect the mulberry in India (Govindaiah Sharma et al. 1993). The stunted growth with marginal chlorosis and necrosis of leaves is the common symptoms of severely infected mulberry plants (Fig. 8.4a). In the root system of susceptible plants, the formation of knots/galls which are spherical or vary in size, younger galls are small and yellowish, while older galls are big and blackish brown (Fig. 8.4b, c) (Arunakumar et al. 2018). It can be managed by the application of Neemahari—a plant based product for the management of root-knot nematode in mulberry (Sharma et al. 2013).

8.2.1.4 Molecular Characterization

Classifying the fungal species just based on morphological features is not sufficient and for this purpose DNA sequence-based approaches have been widely recommended (Crous and Groenewald 2005; Bautista-Cruz et al. 2019). Precise identification of *L. theobromae* can be achieved by using the combination of two or more

Fig. 8.4 Root-knot nematode symptoms of mulberry. **a** Above ground symptoms of interveinal chlorosis. **b**, **c** Below ground symptoms showing galls on the roots of mulberry

genes, like internal transcribed spacer (ITS), β-tubulin (TUB), and translation elongation factor 1-α (TEF1) genes (Chen et al. 2013, 2021; Marques et al. 2013; Rosado et al. 2016).

For many years, *L. theobromae* was treated as a monotypic genus within the Botryosphaeriaceae (Larignon et al. 2001; Slippers et al. 2013). However, the application of DNA meta barcoding for phylogenetic analysis evidenced the existence of many additional species (de Silva et al. 2019; Rosado et al. 2016; Santos et al. 2020). Hence, it is likely that many previous findings might be inappropriately classified, in

addition, literature suggests the occurrence of hybridization between various species of *Lasiodiplodia* spp. (Cruywagen et al. 2017). For example, *L. viticola* is described as a hybrid species produced by hybridization of *L. theobromae* and *L. mediterranea* (Úrbez-Torres 2011). Transport of plant material that hosts *Lasiodiplodia* spp. to new fields/soil with autochthonous strains can stimulate the formation of new hybrid species. This implies that the taxa reported so far are not stable and highlights the need of considering multiple genes in analyzing phylogeny, along with referencing the type strains directly. This strategy can avoid misidentifications (Cruywagen et al. 2017; de Silva et al. 2019).

Several research investigations reported new species of *Lasiodiplodia* and an increasing number of first reports (Rosado et al. 2016; de Silva et al. 2019) indicating the extension of its host range highlights the need of prospecting novel *Lasiodiplodia* species associated with mulberry. There are limited data on molecular characterization of mulberry pathogens in India, and the previous characterization was based on morphology. Sowmya et al. (2018) used RAPD and SSRs to study the genetic variability, among the ten isolates of *L. theobromae* causing Black root rot of mulberry, similarly, Pappachan et al. (2020) characterized only one isolate of *L. theobromae* (Table 8.1).

8.2.2 Major Insect and Pests in Mulberry

8.2.2.1 Sap Suckers

Pink Mealybug

Tukra is the name of the malformation of the leaves and shoots of mulberry caused by the Mealybug (*Maconellicoccus hirsutus* (Green)). The main symptoms of the disease are retardation of the growth of shoot shows and appearance of dark green wrinkled leaves initially and later turn into pale yellow. Due to the stunted growth, the shoot and leaf form a hard and compact structure that cannot be opened without breaking away the crisp leaves (Fig. 8.5a, b). Because of the stunted growth, the leaf yield is tremendously reduced and the leaves become nutritionally very poor. The pink mealy bugs are one of the major pests of mulberry, causing severe damage and recurring loss in leaf yield of about 3000–6000 kg/ha/year (Kumar et al. 1989). Tukra appears mostly during the summer months and can be controlled to a certain extent by removing and burning the affected shoots, spraying chemicals like DDVP (Nuvan) prepared in detergent solution and releasing of the natural enemies like *Cryptolaemus montrouzieri* @ 250 beetles/acre and *Scymnuscoccivora* @ 500 beetles/acre (Table 8.2).

Table 8.1 List of mulberry fungal pathogens identified using gene specific markers

Pathogen	Disease	Marker	Geographic region	References
Setosphaeria rostrata	Leaf spot	ITS	CSRTI-Mysore, Karnataka, India	Arunakumar et al. (2019a)
Nigrospora sphaerica	Shot hole Disease	ITS	Santai County, Sichuan Province, China	Chen et al. (2018)
Nigrospora sphaerica	Shot hole leaf spot	ITS	CSRTI-Mysore, Karnataka, India	Arunakumar et al. (2019b)
Rhizopus oryzae	Rhizopus root rot	ITS, ACT, TEF	South India	Gnanesh et al. (2020)
Lasiodiplodia theobromae	Root Rot	ITS, β-tubulin	Kolasib, Mizoram, India	Pappachan et al. (2020); Gnanesh et al. (Unpublished)
Lasiodiplodia theobromae	Root Rot	ITS, EF1-α	Guangxi Province, China	Xie et al. 2014
Phyllactinia corylea	Powdery mildew	ITS	CSRTI-Mysore, Karnataka, India	Arunakumar et al. (Unpublished)
Ceroteliumfici	Leaf rust	LSU & ITS		
Lasiodiplodia theobromae	Leaf spot/blight	ITS		
Fusarium equiseti	Leaf blight	ITS, TEF, β-tubulin		
Meloidogyne incognita	Root-knot Nematode	SCAR	Karnataka, Andhra Pradesh and Tamil Nadu, India	Manojkumar et al. (Unpublished)
Meloidogyne enterolobii	Root-knot Nematode	rDNA-IGS2	Hainan provinces of China	Sun et al. (2019)

Thrips

Pseudodendrothrips mori Niwa, (Thysanoptera: Tripiidae) are tiny, slender insects that feed on mulberry causing deformation and quality deterioration of leaves (Ye and Gu 1990). The infested leaves gradually become brittle, dry and assume a stippled or silver flecked appearance (Lewis 1997). The infested leaf generally records a loss 8.0–10% of leaf moisture, 10–15% of protein content and 5–10% of total sugar content, which makes the leaf qualitatively poor for silkworm rearing (Fig. 8.5c). The sprinkling of water on the infested leaf, spraying of 0.02% DDVP twice at weekly interval with a safe period of 7 days and application of Quinalphos (0.2%) with no adverse effect on the rearing of silkworms, may reduce the infestation (Misra 2003).

Fig. 8.5 Pests of mulberry. **a** Lower leaf affected by mealy bugs. **b** Mealybug infestation on stems. **c** Thrips infestation on young twigs. **d** Adult white flies on lower surface of the leaf

Whitefly

The white flies attacking mulberry plants belong to the species *Dialeuropora decempuncta* (Quaintanceand Baker) and *Aleurodicus dispersus* Russell (David 1993). It is a highly polyphagous pest causing heavy leaf damage to the plant by sucking the plant sap and secreting honeydew which acts as a substrate for the growth of the sooty mold *Capnodium* sp. to interfere with the process of photosynthesis (Fig. 8.5d).

Table 8.2 Important predators and parasitoids used to control mulberry pests

Name of the insect pest	Name of the biocontrol agent	Numbers to be released/acre/crop
Pink mealy bug *Maconellicoccus hirsutus*	Predators A. *Cryptolaemus montrouzieri* B. *Scymnus coccivora*	250 adults 500 adults
Thrips *Pseudodendrothrips mori*	Predator *Chrysoperla* spp.	4000–8000 eggs
Spiraling whitefly *Aleurodicus dispersus*	Predators A. *Axinoscymnus puttarudriahi* B. *Scymnus coccivora*	250 adults 250 adults
Papaya mealy bug *Paracoccus marginatus*	Parasitoids A. *Acerophagus papayae* B. *Pseudleptomastix mexicana* C. *Anagyrus loecki*	50–100 adults
Leaf Webber *Diaphania pulverulentalis*	Parasitoids A. *Trichogramma dchilonis*—egg B. *Bracon brevicomis*—larval C. *Tetrastichus howardii*—pupal	4 tricho cards per acre 200 adults 1 lakh adults in 3 splits

Source Sakthivel et al. (2019)

Both nymphs and adults remain in colonies under the surface of leaves and suck the sap which results in chlorosis, falling of leaf and reduction of plant growth sooty mold fungus. When whitefly infestation is >50% of leaf area, loss of leaf yield goes up to 28.09%, cocoon yield reduces by 48.09%. Use of triazophos 40EC at 0.06%, dimethoate 30 EC at 0.05%, neem oil and cotton seed oil at 0.01% is found effective in controlling the pest. Further, several parasitoids like the aphelinid parasitoids *Encarsiahaitiensis* Dozier and *E. meritoria* Gahan are also effective in managing the pests.

Jassids

Jassid, *Empoasca flavescens* F. (Homoptera: Cicadellidae) called leaf hoppers is the major sucking pest of mulberry. Both adult and nymphs suck the sap of the leaf giving rise to "hopper burn" symptoms (Fig. 8.6a, b). Initially, symptoms appear as triangular brown spots at the tip of the leaf and gradually, the affected leaves become brick red or brown, crinkled, curled and ultimately the plant shows stunted growth. Attack of jassids not only affects the leaf yield quantitatively but also reduces the quality. The infestation occurs more during February (20.36%) and September (21.80%) months in India. Mahadeva and Shree (2007) reported that free amino acids, total soluble proteins, reducing sugars, soluble sugar, phenol and photosynthetic pigments (total chlorophyll, chlorophyll-a, chlorophyll-b, chlorophyll-a/b ratio and carotenoids) affected considerably the jassid attack. Setting light traps for attracting

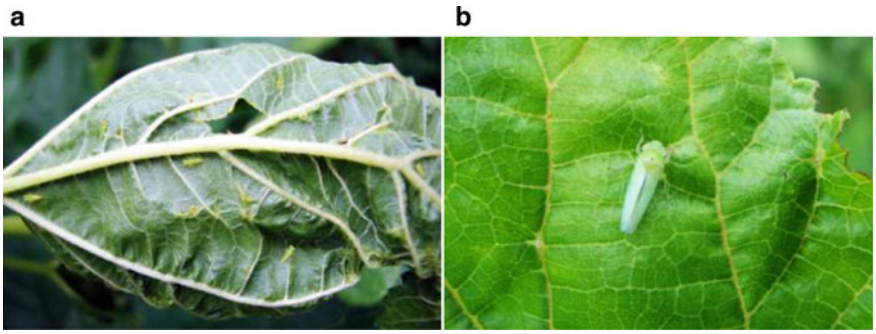

Fig. 8.6 Jassid **a** Nymph on a lower surface of the leaf. **b** Adult on a lower surface of the leaf (Sakthivel et al. 2019)

Fig. 8.7 Leaf roller infestation of mulberry. **a** Webbing of tender leaves. **b** Young larvae on tender leaves

and trapping adults and spraying 0.1% Dimethoate (Rogar) or 0.05% DDVP (Nuvan) with a safe period of 11 days can be adopted to manage Jassid infestation.

8.2.2.2 Defoliators

Leaf Roller

Diaphania pulverulentalis (Lepidoptera: Pyralidae) infestation on mulberry apical shoot after webbing the tender leaves (Fig. 8.7a, b) together and inhibit the growth of plants and causes a leaf loss of 12.8% with an average incidence of 21.77%

Fig. 8.8 May June beetle *Holotrichia serrate* Fabricius infestation on mulberry during the night time

resulting in economic loss to sericulturists (Siddegowda et al. 1995). Infestation also affects biochemically as protein percentage reduces up to 29.08%, carbohydrates were 24.92%, phenols 13.7% and chlorophyll 13.6%. Being seasonal, its infestation starts with the onset of monsoon, remains up to February, but the maximum infestation is observed from September to November. Spraying of 0.076% DDVP (76% EC) for 12–15 days after pruning (1 ml in 1 L water) with a safe period of 10 days can control the pest.

May–June Beetle

Holotrichia serrate Fabricius (Coleoptera: Scarabaeidae) infestation coincides with the onset of monsoon and an occasional pest to mulberry crop in south India. During night time, adult beetles enter into mulberry fields in swarms and feed voraciously on the foliage, leaving only the stem (Fig. 8.8a, b). After the first monsoon keep a vigil for adult beetles in mulberry field (Sakthivel et al. 2019). Collect adult beetles and destroy them by keeping them in Kerosene solution. Spray 0.2% DDVP 76% EC (2.5 ml/lit) with a safe period of 15 days preferably during evening hours. Drench the soil with 0.2% Chloropyriphos 20% EC to kill the grubs. Plowing just before the monsoon helps in the exposure of various stages of the pest to natural enemies.

8.3 Germplasm Resources for Disease Resistance

The complete alleles present in a population are called gene pool. The conservation strategies used for mulberry plants encompass a wide spectrum of activities ranging from the establishment of protected areas to building of DNA libraries (Tikader et al. 2009). However, the most widely adopted one is the ex situ field gene bank established by planting saplings/stem cuttings/bud grafting on ideal rootstocks. Considering the importance of collecting and conserving as many genetic resources to have a good gene pool, countries across the world have collected and being maintained a good number of accessions in the ex situ germplasm. These germplasm resources have been well characterized for different traits including responses to different pests and diseases (Akioet al. 2002; Tzenov 2002; Pan 2003; Tikader and Dandin 2006). Accurate detection of pathogens is very essential for the development of proper management approaches, moreover the use of highly pathogenic or aggressive isolates is necessary for inoculation trials for selecting genotypes with broader resistance (Oliveira et al. 2016, 2021). Also, there is an immediate need to identify resistant sources to transfer resistance genes into elite backgrounds of mulberry. In this direction, there are different germplasm resources resistant to different diseases have been identified in mulberry (Table 8.3).

8.3.1 Primary Gene Pool

As the primary gene pool (GP-1) is the gene reservoir for crop improvement mulberry genetic resources containing commercial stocks and landraces, wild gene pool comprising possible ancestors and closely connected species with a reasonable percent of fertile relationships with domesticated ones (Allem et al. 2001). The primary gene pool of mulberry has been evaluated for biotic stress caused by pests and diseases. For instance, Maji et al. (2009) screened 85 mulberry accession collected from different countries were screened for powdery mildew caused by *Phyllactinia corylea* (Pers), Myrithecium leaf spot caused by *Myrothecium roridum* Tode Ex. Fr. Pseudocercospora leaf spot caused by *Pseudocercospora moricola* (Hara), bacterial leaf spot caused by *Xanthomonas campestris* pv. *mori* and sooty mold caused by Acetomycetes and Deutromycetes fungi and identified eight accessions resistant to powdery mildew, 78 to *Myrothecium*, six to *Pseudocercospora* leaf spot, and 44 to sooty mold. Later, Banerjee et al. (2009) screened another 82 mulberry germplasm accessions for stable resistance to Bacterial leaf spot (BLS) pathogen *Xanthomonas campestris pv.mori* and identified the accessions *M. rotundiloba* and *MS-8* resistant, which could be utilized for breeding. Likewise, the accession Surat is highly susceptible to the disease. Bacterial leaf spot caused by *Xanthomonas campestris Pv. Mori* is a major foliar disease in mulberry causing foliage loss of up to 15%. It was observed that resistant and susceptible germplasm showed a high positive correlation between disease severity index and stomata frequency and a

Table 8.3 Resistant sources identified for different diseases of mulberry

Disease	Resistant sources	References
Leaf spot	Kanva-2, S-54, C-799, Shrim-2, Shrim-8, MR-2, Assambola, AB × Phill, Mouli, Mizusuwa, English black, K2 × Kosen, China peaking, Cattaneo, Sujanpur × Kokuso-13, Calabresa, Miruso, Acc-153, Acc-152, Acc-151, Acc-150, Acc-128, Acc-135, Acc-106, Acc-109, Acc-112, Acc-114, Acc-115, Acc-116, Acc-117, Acc-119, Acc-121, Acc-123, Acc-124, OPH-1, OPH-3, MS-2, MS-5, Acc-210, Paraguay, RFS-135, RFS-175, S-146, S-523, MS-7, MS-8, Acc-125, Acc-128, K2 × Kokuso, S-1531, LF-1, Almora local, S-31 and S-1096	Govindaiah Sharma et al. (1989)
	S 54, MR 2, S 36, Dharwar, Kajali, VS Z, Kosen, Prutppine, Itaiiai, papua, J,4l lenlbang	Philip et al. (1995a)
	Kajali, MS2	Philip et al. (1995b)
	Belidevalaya, Kaliakuttai	Philip et al. (1996)
	S-30 × ber C 776, K 2 × Kosen, Miz x RFS 135, Miz × S41, ACC 155 × Ber S 799, S-30 × ber S 799, S54	Philip et al. (1996)
	Kajali, Jatinuni. *Morus cathayana*, Almora local, Bogura-1, Meergund-6, Fernodias, Punjab local, *M. tiliaefolia*, Sultanpur, Golaghat, Bush malda-A and Sujanpur	Pratheesh Kumar et al. (2003)
	M. rotundiloba, MS-8	Banerjee et al. (2009)
	BM-4, BM-11	Ahmed et al. (2016)
	Morus multicaulis	Arunakumar et al. (2019a)
Brown leaf spot	Kanva-2	Peris et al. (2012)
Die-back	Fukayuk, Yakshinogi	Philip et al. (1995a)
Bacterial blight	Bulgaria-24, Butgaria-3, Galicea, Tbilisuri, Minarnisakar, Hatesakari, Husang, Chine, Shine, Ichinose, Gumji, Ichihie, Mysore Local, Berhamporelocaj, S-36, S-41, S-54, Goshoerami and Kosen	Philip et al. (1995a)

(continued)

Table 8.3 (continued)

Disease	Resistant sources	References
Leaf spot and powdery mildew	Goshoerami, Ichinose, Rokokuyaso	Mir et al. (2013)
Powdery mildew	Mandalaya, Cattaneo, Chinawhite, Jodhpur, Calabresa, Mizsuwa, Acc-123, OPH-3, S-523, Punjab Local, Shrim-2, Himachal Local, S-796, S-1531, S-1096, S-31, Almora local	Govindaiah et al. (1989)
	Buriram-60, Betidevalaya, kaliakutta, S54, K2	Philip et al. (1995a)
	M. laevigata and *M. serrata*	Babu et al. (2002)
	BM-11, BM-8, Black	Ahmed et al. (2016)
	Morus multicaulis and Kalimpong	Arunakumar et al. (Unpublished)
Leaf rust	MS-6, MS-2, Cattaneo	Philip et al. (1991)
	China Peking, Cattaneo	Philip and Govindaiah (1994)
	Kokuso-27, Kajryonezumigaesh, Cattaneo, China Peking	Philip et al. (1995a)
	Acc. 12, Rajouri, Acc. 148 and Acc. 9	Arunakumar et al. (2022)
Root-rot	Negumigaesi, Russkaya, Adrcnelli 03 and 02, Griyaus, Ghrusnl l, Sh-2 and Grusia X 020	Philip et al. (1995a)
	M. cathayana, M. multicualis (ME-0006 and ME-0168), Thai Pecah, Hazzaz, S-799, RFS-135, Acc. 106, T-36, UP-22, ERRC-103, ERRC-73, Acc. 8, Seekupari, Moulai, Pillighat, Kollihills-1, Kota-4, Jalalgarah-3, G2	Pinto et al. (2018)
Black root rot	*M. multicualis* (ME-0006 and ME-0168), Philippines, Australia, LF-1, C-18, Vadapuram and Meghamalai-1	Gnanesh et al. (Unpublished)
Root-knot nematode	Calabresa	Campos et al. (1974)
	S30, MR2 and RFS 135, Shrim 5, AB x Phillippines and K2 x Kokuso, Himachal Local	Philip et al. (1995a)
	RFS-135	Gnanaprakash et al. (2016)

(continued)

Table 8.3 (continued)

Disease	Resistant sources	References
	BR-8, Karanjtoli-1, Hosur-C8, Nagalur Estate, Tippu, Calabresa, Thai Pecah and SRDC-3	Arunakumar et al. (2021)

negative correlation of DSI with leaf thickness. Two unique RAPD primers with the fragment of 500 bp and 450 bp for the resistant and susceptible progenies respectively were identified (Banerjee et al. 2011). Under temperate conditions, Mir et al. (2013) screened seven germplasm resources for resistance to powdery mildew caused by *P. corylea* and leaf spot caused by *Cercospora moricola* (Cooke), found that the mulberry accession Kairyo-nezumigaeshi (KNG) is moderately resistant to both the pathogens. Pinto et al. (2018) screened 214 mulberry accessions for resistance to *M. phaseolina* charcoal root rot through simulated inoculation and identified twenty accessions with <26% root rot. Some accessions like *M. cathayana* (Hybrid) with 9.85%, *M. multicualis* Perr., with 12.03% G-4, with 35.91% infection were selected as resistant to root rot. Evaluation of worldwide collection of mulberry germplasm accessions for leaf spot (235), powdery mildew (14) and leaf rust (235) was undertaken during 2015–19 under natural epiphytotics. It was found that none of the accessions found resistant and twenty accessions showed moderate resistance to leaf spot. Four accessions namely Acc. 12, Rajouri, Acc. 148 and Acc. 9 were found resistant to leaf rust. *Morus multicaulis* and Kalimpong were identified as resistant to powdery mildew at CSR&TI, Mysuru, Karnataka (Arunakumar et al. 2021). A total of 415 different indigenous and exotic germplasm accessions were screened under glasshouse conditions and found mulberry accessions with 48 resistant and 21 immune. Further, 30 accessions were screened at 4 locations based on rooting ability with infested soil. Finally, 8 germplasm accessions viz., BR-8, Hosur-C8, Karanjtoli-1, Tippu, Nagalur Estate, Thai Pecah, Calabresa and SRDC-3 were identified as possible genetic resources for root knot resistance breeding programme and rootstock establishment of mulberry garden (Arunakumar et al. 2021).

8.3.2 Secondary Gene Pool

The species differentiation in mulberry is very thin as natural cross hybridization is very common among these species. However, there are a few species of mulberry that show very poor hybridization with other species. Prominent among them are *M. serrate* Roxb, *M. cathayana* Hemsl., *M. laevigata* Wall., *M. nigra* Linn., and *M. mongolica* Schneid., *M. wittiorum* Hand-Mazz., and these species are considered to be the secondary gene (GP-2) pool of mulberry as, though, the species can cross with other species but produce fewer seeds and sterile hybrids as most of them are polyploids (Weiguo et al. 2007). Most of these secondary gene pools have several unique traits which can be exploited effectively for crop improvement. For instance,

M. serrata is known to have several agronomical importance traits such as greater leaf moisture content, moisture retention, higher leaf thickness and resistance to biotic and abiotic stress. The highest numbers of root-knot nematode resistant accessions were found in *M. alba* (Arunakumar et al. 2021).

8.3.3 Tertiary Gene Pool

The tertiary gene pool (GP-3) consists of distantly related species of the primary gene pool and the crossing between these two is difficult and gets only sterile hybrids. Paper mulberry (*Broussonetiapapyrifera*) may be one of the geniuses which could be considered as the tertiary gene pool of mulberry as it belongs to the family Moraceae and has variable-shaped leaves that are rough to touch the plant looks like a hybrid that originated from a cross between mulberry and Osage-orange.

8.3.4 Artificially Induced/Incorporated Traits/Genes

Plants use different strategies and mechanisms to overcome numerous beneficial and harmful organisms (pathogens) in the environment (Kozjak and Meglič 2012). Initially, plants employ physical and mechanical barriers to prevent the pathogen entry into the plants through structural and anatomical modifications. If the pathogens overcome these barriers, plant receptors initiate the expression of the resistance genes (R genes). In a specific gene-for-gene fashion, *R* genes code for proteins that recognize specific pathogen effectors known as avirulence proteins. For a century, plant breeders have genetically characterized and used *R* genes to manage the loss due to diseases. However, recently to provide broader spectrum control and improved durability transgenic approaches have been adopted. Although mulberry gene pool has enough genetic variation and mulberry is highly heterozygous, efforts have been made to generate variations through various means such as plant tissue culture, mutation, polyploidy, and genetic engineering to explore the possibility of creating *de nov* variations. In genotype RFS-135, induction of mutations with EMS resulted in the isolation of varieties with wide economic importance in sericulture (Anil Kumar et al. 2012, 2013); it was brought to the notice that 0.1% and 0.3% of EMS treatment effective for changing the morphometric characters, phytochemical constituents such as proteins, minerals, reducing sugars, biomass yield and moisture content. Mutation induced through gamma rays irradiation, two mutants resistant to die-back disease. Similarly, the somaclonal variant (SV1) developed from S1 (*M. alba*) gave increased branching, higher leaf yield and tolerance to drought (Chakraborti et al. 1999). New plant varieties have also been developed through the induction of polyploidy (Chakraborti et al. 1998) to develop triploids as triploids are known to be more resistant than diploids.

8.4 Overview on Classical Genetics and Traditional Breeding

Traditionally, mulberry is developed through hybridization and selection. The whole breeding process in mulberry starts with the evaluation of germplasm using morphological, biochemical, physiological characters, and suitable parental germplasm are chosen and controlled hybridization is imposed. Ripened fruits from those formed by natural hybridization and controlled hybridization in selected mother plants are collected to take out seeds, further seeds will be sown in nursery beds to raise seedlings.

8.4.1 Traditional Breeding Methods

Screening and selection of hybrids initially based on a few important characters like growth, leaf texture, branching and disease susceptibility are done in progeny row trials (PRT). Due to more or less all mulberry accessions being highly heterozygous and poses a longer gestation period, conventional breeding methodologies mainly carried on the production of the F1 hybrid (Das 1984). Hybrids with advantageous characters, identified through the PRT, are additionally evaluated in primary yield trial (PYT) for agronomic, biochemical and silkworm bioassay. From the PYT, 5–10% of top performing hybrids are further chosen through the final yield trial (FYT) for the detailed assessment using 25–49 plants per replication with 3–5 replication. In FYT, plants are put to thorough assessment for rooting ability, leaf quality, leaf yield, response to agronomic practices, adaptation, susceptibility to pests and diseases, and silkworm bioassay. Once a hybrid is found to have almost all the desired traits, hybrid is chosen and vegetatively mass multiplied and tested under multilocation trials (MLT) at various Seri regions. In general, 8–9 hybrids are selected for MLT studies. Hybrids that exhibit consistently good in all the locations, seasons and years are further selected tested in All India Coordinated Experimental Trial (AICEM) to assess hybrids performance in different agro-climatic conditions across India, AICEM will be carried at least for four years. The current AICEM test is carried out at 24 test centers lying from south to north and west to east of India. The best performing hybrids under the AICEM are authorized and released for commercial utilization by the Seri-farmers.

8.4.2 Breeding Objectives: Positive and Negative Selection

As mulberry leaves are the primary plant part for silkworm rearing in most of the Asian nations, the breeding was aimed at the development of varieties with wider adaptive and higher leaf yield potential. Mulberry growth and leaf production depend

on several factors and associated traits, thus, the breeding process always relies on certain markers/traits which contribute considerably to the growth and development of the plant. The section process may be of two types, positive and negative. The selection based on traits or markers that confer a selective advantage for the plant is called positive selection while those confer a disadvantage is called negative selection. Positive (Darwinian) selection is in which genes/traits/variants that have a selective advantage increase in number and spread until they fix in the relevant population. On the other hand, the negative selection also called purifying selection, is a purging process wherein disadvantageous or deleterious alleles/genes/traits get eliminated from the population. The strength of selection varies between locus/genes. In the case of strong negative selection on a locus, the purging of deleterious variants will result in the occasional removal of linked variation, producing a decrease in the level of variation surrounding the locus under selection. In mulberry, a number of traits have been identified which have a strong and positive correlation with the survival and leaf yield of mulberry under stress conditions and also a set of characters that have negative correlations (Vijayan et al. 2010). It has been found that the character association changes with the intensity of the stress imparted by salinity. Under normal condition, leaf yield is significantly and positively correlated with leaf size, root length, shoot length, protein content of the leaf and the photosynthesis of the plant. However, under a stress caused by 1.00% NaCl (ECe 19 dSm^{-1}) the leaf yield has highly significant correlation with plant height, leaf size, shoot weight, root weight, root length, protein, NRase activity and WUE of the plant. Likewise, under normal cultural conditions the leaf fresh, leaf moisture and dry weights showed a non-significant negative correlation with leaf yield. Thus, the selection strategy for different traits is made based on the breeding objectives.

8.4.3 Achievements of Conventional Breeding (Quality, Stress Resistance, Yield etc.)

Through conventional breeding, several mulberry lines/varieties have been developed across the world. For instance, India has developed 27 mulberry varieties and China has developed 31 mulberry varieties. The leaf productivity of these varieties increased considerably from 8–10 MT/ha/yr in traditional varieties to 60–65 MT/ha/year in the newly developed varieties. Similarly, a few varieties with stress resistance have been developed. AR-12 for alkalinity tolerance and C776 for salinity stress are examples of it.

8.4.4 Constrains of Conventional Breeding and Basis for Molecular Breeding

The biggest limitation of mulberry breeding is the long periods required for the development of varieties. It takes almost 15–20 years to develop varieties as mulberry has a long juvenile period as it takes a minimum of 2–3 years to get the plant ready for developing the next generation. Further, the lack of inbreds and the high heterozygosity associated with the accessions make the genetic improvement through conventional breeding is highly laborious. Hence, in most of the breeding plans, crosses between selected parents are made to develop F_1 hybrids and the F_1 hybrid is further used for screening and selection of promising ones to put into evaluation for variety development. Thus, trait specific improvement does not have much scope in this type of breeding programme as recurrent hybridization and selection take decades to be completed. Additionally, stress tolerance is a difficult phenomenon including morphological, physiological, biochemical, and developmental changes in plants (Hirayama and Shinozaki 2010; Gill and Tuteja 2010). Stress tolerance selection in the field is not a suitable method as the intensity of stress imposed by both drought and salinity in the field can differ depending on soil depth and season. Plants also interact with other numerous environmental factors which involve the intensity of stress tolerance. Thus, screening of plants has to be under controlled environmental conditions to assist the true appearance of their natural capacity to tolerate biotic and abiotic stresses. However, a large number of F_1 hybrids screening for stress tolerance by imparting stress is near prohibitive. Therefore, it is highly desirable to use molecular biology tools such as Marker-Assisted Selection and genetic modification through genetic engineering.

8.4.5 Classical Mapping Efforts and Its Limitations and Utility of Molecular Mapping If Any

As mulberry is a highly heterozygous plant with an extended juvenile period, high genetic load, no inbred lines could be developed to work out the genetic basis of traits. Since it is not easy to develop segregation populations no efforts have so far been made to construct a genetic map of mulberry. Further, the expression of most of the important traits is highly influenced by environmental factors and stages of development. A certain set of characters would appear under a given set of climatic conditions in a particular stage of development and another set under another set of conditions and growth stages. Thus, no systematic efforts have been made to use the classical genetical approach to elucidate the genetic base of the character of mulberry. The molecular markers on the other hand are present in abundance, stable across the developmental stages, least influenced by environmental factors, devoid of the pleiotropic and epistatic effects. Thus, molecular markers were found much better than the phenotypic markers in assessing the genetic diversity, identification of

parents, and evaluation and selection of hybrids. Depending on the techniques used, these markers can be broadly classified as hybridization based markers and polymerase chain reaction (PCR) based markers. In hybridization based marker systems like restriction fragment polymorphism (RFLP), the DNA profiles are visualized by hybridizing the DNA with restriction enzyme digestion and blotted against a solid membrane with a labeled probe. PCR based marker method, amplification of desired DNA sequences are carried out with the help of arbitrarily or specifically chosen primers using thermostable enzyme, called *Taq* polymerase under in vitro condition. The amplified fragments are aligned using an electrophoresis system on agarose or polyacrylamide gels and banding patterns (amplicons) are identified either by autoradiography or staining. Some of the important PCR based marker systems are amplified fragment polymorphism (AFLP), random amplified polymorphic DNA (RAPD), inter simple sequence repeats (ISSR), simple sequence repeats (SSR), expressed sequence tag (EST). Each of these marker system has its own merits and demerits. Markers like SSR, RFLP and EST are co-dominant, therefore, have ability to detect genetic variability at allelic level. Though, the development and utilization of these marker systems are costly, laborious and time taking. Thus, in mulberry, Random Amplified Polymorphic DNA (RAPD), Amplified Fragment Length Polymorphism (AFLP), and Inter Simple Sequence Repeat markers were used for genetic divergence and molecular characterization of germplasm (Vijayan et al. 2004a, 2005, 2006; Zhao et al. 2006; Vijayan 2010; Sangannavar and Vijayan 2020). However, all the above stated markers had several inherent limitations including, dominant nature, reproducibility, anonymity etc. The advent of next-generation sequencing and the drastic reduction in their cost of sequencing have enabled designing and utilization of more robust, reproducible and informative molecular markers such as SSR and SNPs. Using SSR markers, many QTL maps have been developed in mulberry for different traits (Sarkar et al. 2017). With the advent of NGS, efforts are underway to develop SNP panels for their utilization in mulberry.

8.4.6 Use of Morphological Markers

Plenty of research has been done on characterization and evaluation of parental materials, screening of hybrids and evaluation of selected genotypes using morphological markers such as leaf shape and size, lenticels ferequency, stem colour, flower color, stigma length and nature, fruit shape and colour, seed colour and size, plant height, stem length, leaf retention capacity, number of branches, moisture content and retention capacity, nodal length, leaf yield, biomass production, etc. (Bindroo et al. 1990; Sahu et al. 1995; Vijayan et al. 1997; Tikader et al. 2009) along with others traits like adaptability to different climatic conditions, resistance to biotic stresses like diseases and pests, tolerance to abiotic stresses like salinity, cold and drought, other abilities like higher vegetative propagation, better leaf quality, and better coppicing (Vijayan et al. 2009). Plant defense mechanism against pathogen attack is not simple, with many local and systemic aspects (Felle et al. 2004). Further, resistance to biotic

stress has been correlated with a few morphological markers such as leaf cuticular thickness, quality of wax and cuticle that cover the epidermal cells, frequency of leaf hairs, cystolith, structure of epidermal cell walls, shape, size and location of lenticels and stomata. Other than these, thick walled cells tissues also avoid the progress of the pathogen. Morphological characteristics (significantly higher thickness of epidermis cum cuticle, more number of Palisade layers, nature of palisade and cuticle tissue, relatively thinner spongy parenchyma and significantly higher palisade proportion) are ideal to act as physical or structural barriers against the diffusion and incursion by different pathogens (Sonibare et al. 2006). However, with the high heterozygosity, long juvenile period of the plant, multigenic and multifarious nature of these morphological markers, their application often becomes difficult (Vijayan et al. 2006). Further, testing of a large number of progenies for pest and disease resistance requires huge space and resources, which often act as the major impediments.

8.4.7 Limitations and Prospect of Genomic Designing

For the identification of genetic variability in mulberry, several molecular marker techniques have been successfully utilized in mulberry crop improvement. These markers are RAPD (Xiang et al.1995; Bhattacharya and Ranade 2001; Chatterjee et al. 2004; Srivastava et al. 2004; Zhao et al. 2009; Orhan et al. 2007), AFLP (Sharma et al. 2000; Wang and Yu 2001; Huang et al. 2009; Pinto et al. 2018), ISSR (Vijayan and Chatterjee 2003; Awasthi et al. 2004; Vijayan 2004; Vijayan et al. 2004a, b, c, 2005, 2006; Zhao et al. 2006, 2007; Kar et al. 2008) and SRAP (Zhao et al. 2009) and DAMD (Bhattacharya and Ranade 2001; Bhattacharya et al. 2005). However, the dominant markers could not yield much desired information. Thus, genetic variation among the mulberry genotypes was measured by using codominant markers such as SSR markers extensively for identification of potential parents and progenies (Aggarwal and Udaykumar 2004; Zhao et al. 2005; Pinto et al. 2018; Garcia-Gómez et al. 2019; Orhan et al. 2020). A total of 247 mulberry specific SSR markers have been deposited in NCBI (www.ncbi.nlm.nih.gov) by various groups and are available for molecular genetic analysis. Consequent to genome sequencing of *M. notabilis* (He et al. 2013), over 2.17 lakh SSR motifs have been mined and hosted in an online mulberry microsatellite marker database (MulSatDB, Krishnan et al. 2014). The evaluation of molecular variation using polymorphic SSRs among the resistant and susceptible genetic resources is crucial for their effective and efficient utilization in plant breeding (Arunakumar et al. 2021; Shinde et al. 2021). Recent developments in next-generation sequencing, bioinformatics and consequent attempt to sequence the whole genome of mulberry and annotate the sequences have opened a plethora of opportunities to develop more densely distributed SNP markers for their application in mulberry genome analysis.

8.5 Diversity Analysis in Brief

8.5.1 Diversity Analysis Based on Phenotype

Evaluation of germplasm and grouping of the accessions based on diversity among them is required for selecting suitable parents for breeding. In the earlier days in the absence of molecular markers, germplasm was evaluated using phenotypic traits such as morphological traits and agronomical traits such as drought tolerance, alkaline and saline stresses, winter hardiness, or early sprouting, pests and diseases resistance (Tikader and Kamble 2007). Tikader et al. (1995) explored the variability in the expression of sex in mulberry in 301 genotypes from diverse geographical origins, and found that nearly 16% was male, 53% female, 17% monoecious and 13% were bisexual. Significant variation was noted in the flowering time, anthesis and floral characteristics. Variability was also observed in pollen grain viability, fruit morphology and seed setting %. Vijayan et al. (1999) estimated the genetic diversity among the 62 mulberry accessions indigenous to India, irrespective of their species and ploidy status. Significant genetic divergence was observed among these indigenous mulberry accessions based on the leaf yield traits. Tikader and Kamble (2008) evaluated the genetic diversity of 50 mulberry germplasm accessions using eight agronomically important traits and found a high amount of genetic diversity among the accessions. Banerjee et al. (2011) evaluated twenty two morphological traits in twenty-five Indian mulberry accessions belonging to the five species of *Morus*. Six principal components were identified explaining >88% of the total variation. Among the 18 major variables included for the analysis, shoot and root traits such as longest shoot length, leaf area, intermodal distance, green and dry leaf weight, lamina length, lamina weight, root volume, and fresh and dry root weight were identified as important variables. Chang et al. (2014) from seven *Morus* spp., assessed the genetic diversity of 27 mulberry accessions using 20 vegetative traits, chilling requirements, and reproductive traits. Based on the study, a classification system was suggested with three clusters: (1) *M. laevigata*; (2) *M. atropurpurea*, *M. bombycis*, *M. australis* and *M. formosensis*; (3) *M. alba* and *M. latifolia*. The study also showed that *M. atropurpurea*, often regarded as a member of *M. alba*, is closed to *M. bombycis*, *M. australis* and *M. formosensis*. Peris et al. (2014) assessed the genetic diversity among five mulberry accessions being maintained in Kenya which include the accessions Embu, Thika, Thailand (*M. alba*), Kanva-2 and S41 (*M. indica*) using twelve phenotypic traits recorded from two localities (Nairobi and Eldoret). Leaf lamina width and petiole length, petiole width and growth height, internodes distance and the number of branches showed significantly and using Duncan's Multiple Range Test (DMRT) the accessions were clustered into four groups. Efforts were also made to evaluate the germplasm for variability in stress tolerance. Hossain et al. (1991) evaluated 10 mulberry genotypes under tissue culture conditions to screen out the tolerant genotypes. Vijayan and Chatterjee (2003) under in vitro conditions evaluated 63 mulberry genotypes and selected 5 genotypes with a higher tolerance level. Likewise, Tewary et al. (2000) evaluated mulberry genotypes for osmotic stress tolerance by using

the medium with 1.0–10% polyethylene glycol (PEG) and observed considerable genetic diversity among the genotypes. These studies clearly showed that considerable genetic diversity is present in mulberry for stress tolerance. However, incorporation of the specific trait through conventional breeding has several bottlenecks important among them is the difficulty in introgressing a trait to a recurrent parent from a donor parent through repeated backcrossing and selection, because of the dioecy of the plant and prolonged juvenile period (Vijayan 2010).

8.5.2 Diversity Analysis Based on Genotype, Molecular Markers Applied

Since diversity analysis using morphological characters in plants are not very reliable due to the evolutionary dynamics, influenced by the growing conditions and development stage, information from non-morphological characters such as biochemical molecules and nucleic acids is increasingly being used for genetic resource management and utilization. Among all non-morphological markers, molecular based markers are suitable for genetic characterization of mulberry germplasm resources as they are widely polymorphic, multiallelic, codominant, non-epistatic, insensitive and neutral to environment control (Xiang et al. 1995; Vijayan et al. 2004a, b, c). Although several DNA markers such as Amplified fragment length polymorphism (AFLP) (Sharma et al. 2000), Random Amplified Polymorphic DNA (Zhou et al. 2014), Inter simple sequence repeats (ISSR) (Vijayan and Chatterjee 2003; Vijayan 2004; Vijayan et al. 2004a, b, c, 2005, 2006; Zhao et al. 2006, 2007; Sangannavar and Vijayan 2020) have been developed and used for genetic diversity analysis of mulberry. However, considering the reproducibility, robustness, and information generating ability, simple sequence repeats (SSR), and single nucleotide polymorphism (SNP) markers are considered the most suitable molecular markers for genetic diversity analysis in mulberry. Simple sequence repeats (SSR) or microsatellite or short tandem repeat (STR) or simple sequence length polymorphism (SSLP) are tandem repeats of short (2–6 base pair) DNA fragments scattered throughout the genome that lies between conserved sequences (Litt and Luty 1989). The three mechanisms that create a new allele at SSR loci are (a) replication slippage (b) unequal crossing-over and (c) genetic recombination. Replication slippage is considered to be a major factor affecting the repeat number for STR sequences, whereas unequal crossing-over is thought to result in a very large number of alleles for long tandem repeat arrays (Huang et al. 2002). However, the major disadvantage of SSR was the need genomic information to develop primers, which was expensive and time consuming, but with the introduction of Next generation sequencing technique, the cost has come down heavily and now it is possible to sequence any plant genome at a reasonable cost. Regarding SNPs, they are the most abundantly present DNA marker in any genome with a frequency of >1% in a population (Collins et al. 1997; Halushka et al. 1999). The frequency of SNPs is roughly estimated to be one in

every thousand nucleotides in the human genome, and one in 60–120 bp in maize (Ching et al. 2002). However, to date, no attempt was made to discover SNPs in mulberry. Nevertheless, considering the tremendous progress made on low-cost and high-throughput SNP genotyping in other crops, and the current pace of genomic research in mulberry genome, it is certain that within a short time SNPs become the commonly used molecular markers in mulberry. A large number of ESTs from mulberry genome have been deposited in the data bank (Lal et al. 2009; Zhao 2008). Attempts should, therefore, be made to identify potential SNPs from these ESTs, which can also be used for identifying causal polymorphism. Likewise, SNPs can also be developed through locus specific amplification (LSA) and comparative re-sequencing from multiple individuals (Rieder et al. 1998) by utilizing the information available from the genomic sequences deposited from markers like RAPD and ISSR that are linked with important phenotypic traits.

8.5.3 Relationship with Other Cultivated Species and Wild Relatives

Usage of crop wild relatives (CWRs) in cultivation and breeding is the best way to harness natural trait variation in genetic improvement programs. Wild relatives often have unique alleles for specific traits like resistance to biotic and abiotic stresses. Thus, it is desirable to understand the relationship between domestic and wild species to execute the crop improvement programs effectively. The phylogenetic relationship among different genera of the family Moraceae was generated with information from nuclear and chloroplast DNA sequence variations of thirteen species of *Morus* distributed in Asia, Africa, Europe, and North, Central, and South America. The study revealed that the genus *Morus*, as currently circumscribed, is non-monophyletic as the species *M. mesozygia* and *M. insignis* are placed outside the other domestic species. Thus, a further detailed investigation is required to clarify natural generic relationships of the family Moraceae (Nepal and Ferguson 2012). Vijayan et al. (2004d) used inter-simple sequence repeat (ISSR) and random amplified polymorphic DNA (RAPD) markers to find out the relationship among five species viz., *M. latifolia*, *M. bombycis M. alba* and *M. laevigata* and found that *M. laevigata*is different from other species. Population analysis further stressed the wild nature of *M. laevigata* as it showed considerably low gene flow (Nm) with other species. Likewise, Weiguo et al. (2007) using ISSR and SSR markers investigated the genetic diversity among 27 mulberry accessions including nineteen cultivated accessions (three *M. alba*, two *M. bombycis*, six *M. multicaulis*, two *M. atropurpurea*, two *M. rotundiloba*, one *M. australis*, one *M. alba* var. *pendula*, one *M. alba* var. *venose* and one *M. alba* var. *macrophylla*) and eight wild accessions (two *M. laevigata*, two *M. cathayana*, two *M. wittiorum*, one *M. mongolica* and one *M. nigra*). It has been found that prolonged cultivation caused loss of genetic diversity in domestic species.

Recently, Jiao et al. (2020) stated a high-quality, chromosome level domesticated mulberry genome (*M. alba*) and they confirmed with 28 chromosomes with *M. alba* and it is a diploid (2n = 2x = 28).

8.5.4 Association with Geographical Distribution

Although not much information is available of mulberry on the relationship between genetic diversity and geographic distribution, Vijayan and Chatterjee (2003) analysed the relationship between geographic distribution and genetic diversity among 11 cultivars, which are being widely cultivated in different parts of India. The study revealed that geographic groupings of the cultivar were based on their geographic origin. Chatterjee et al. (2004) found a strong relationship between geographic distribution and genetic diversity of *M. laevigata* (Vijayan et al. 2005) assessed the genetic diversity among 34 Indian mulberry accession and found that the accessions resolved into groups based on their geographic relationships. Efforts to find out the relationship among cultivars originated in India and Japan also showed clear and distinct grouping based on their geographic origin (Vijayan 2004). Jiao et al. (2020) used population genomic analysis by resequencing 132 mulberry accessions split into three geographical groups, namely, northern and southwestern China, Taihu Basin of southeastern China (Hu mulberry) and Japan. Among these Hu, mulberry exhibits the lowest nucleotide diversity and established apparent signatures of selection, representing environmental adaptation. Thus, mulberry varieties and cultivars from different geographic regions show high genetic diversity.

8.5.5 Scope of Genetic Diversity

The scope of genetic diversity within a species was investigated in *M. alba* and *M. serrata* with RAPD and ISSR markers. It is found that the genetic similarity among 11 mulberry genotypes of *M. albaviz*., Limoncina, Schinichinose, Kattaneo, Obawasa, Rangoon, China white, China black, Canton china, Almora local, Punjab local and Sujanpur-2 estimated based on Nei and Li (1979) varied from 0.644 between Rangoon and Punjab local to 0.943, between Sujanpur-2 and Almora local, with an average genetic similarity of 0.793 for data generated by ISSR markers. The same was in the range of 0.738 for China white and Kattaneo to 0.909 for Sujanpur-2 and Punjab local with an average of 0.834 in RAPD analysis. When the RAPD and ISSR data were combined to analyse with more markers the genetic similarity among the genotypes varied from 0.733 between China white and Kattaneo to 0.888 between Sujanpur-2 and Punjab local with a mean coefficient of 0.819. Genotypes collected from different climatic conditions such as temperate, tropical and sub-tropical from various countries show substantial amount of genetic similarity.

The genetic similarity coefficients revealed substantial amount of genetic similarity among the genotypes, though the genotypes were collected from different countries of much varied climatic conditions such as tropical, temperate and subtropical. Genotypes from similar geographic regions showed closer genetic similarity than those from geo graphically distant region. The correlation coefficient among the matrices as tested by Mantel's (1967) Z-statistics, revealed high correlations ($r = 0.4$; $p = 0.000$ between ISSR and RAPD; $r = 0.976$, $p = 0.000$ between ISSR and Pooled matrices, $r = 0.982$; $p = 0.000$ between RAPD and Pooled data matrices) (Srivastava et al. 2004). The genetic diversity evaluation of 16 populations of *M. serrata* Roxb., revealed presence of significant genetic diversity among the populations on morphological and anatomical as well as DNA markers. The average genetic distance, estimated from the ISSR markers was 0.165 (Vijayan et al. 2006). Thus, there is a great amount of inter and intra species genetic diversity in mulberry which can be used for crop development.

8.6 Association Mapping Studies

8.6.1 Genome Wide LD Studies

Gene scan surveys and genome wide association (GWA) mapping helps in identifying the genetic variation existed in the whole genome to locate genes or narrow regions that have important statistical connections with numerous complex traits. Since to conduct a genome wide association analysis, an enormous number of densely distributed markers is required, whole genome scan is usually carried out using the most frequent genetic variants available in the genome is SNPs. Generally, thousands of SNP markers are required for a whole genome scan for crops with high haplotype diversity and low LD. Recently, Pinto et al. (2018) used 214 accessions germplasm panels to spot out markers associated with root rot resistance (charcoal) and identified 5 AFLP markers allied with root rot resistance. These markers accounted for allele frequency of 0.132–0.401 and 9.6–12.7% of the total phenotypic variation in the trait (R^2). Similalry, Zhang et al. (2016) used a germplasm panel of 93 mulberry accessions of diverse origin was to identify markers for a few important fruit traits. A total of 24 markers associated with fruit traits were identified. Thus, very scanty work only has been done in mulberry on LD mapping.

8.6.2 Future Potential for the Application of Association Studies for Germplasm Enhancement

LD mapping is very useful for identification trait-marker associations in species where biparental mapping has limitations, especially crops like mulberry where

inbreds are hardly available. Mulberry being a perennial species, association mapping is very appealing as it is a reservoir of natural genetic variations in the form of wild species, weedy species, land races and evolved cultivars, which are originated from a number of historical genetic recombination events in response to different climatic conditions. (Tikader and Vijayan 2017). Exploitation of these genetic variability in the ex situ conserved genetic resources is vital to overcome future problems associated with narrowness of genetic base of modern cultivars as strong genetic diversity means diverse morphological traits and a higher potential to develop varieties for varied cultural and agronomic conditions (Abdurakhmonov and Abdukarimov 2008). Since LD analysis has the potential to identify a single polymorphic locus within a gene that is responsible for a difference in phenotype and to predict the best haplotype across one or multiple genes for optimum expression of the target trait, it can be used to determine the best donor parents for crop improvement programs. The current efforts to sequence the genome of diploid mulberry species in India and to identify SNPs would help perform more association mapping as biallelic codominant type of markers like single nucleotide polymorphisms (SNPs) is perfectly suitable for the quantification methodology of LD. LD quantification using dominant markers such as RAPD, AFLP, ISSR is poorly explored and usually subject to wrong perception and interpretation. Another important factor that determines the success of LD mapping is the choice of germplasm or population (Yu et al. 2006) as the false positives generated by population structure may make a marker allele that occurs at high frequency in a preferentially sampled subpopulation associated with a trait of interest even though it is not linked to a real QTL (Pritchard et al. 2000). In sort to overcome these interruptions, several methods such as structured association, mixed model approach, genomic control and principle component approach have been developed (Devlin and Roeder 1999; Pritchard et al. 2000; Yu et al. 2006). Thus, the true potential of LD mapping is yet to be harnessed in mulberry.

8.7 Map-Based Cloning of Resistance/Tolerance Genes

8.7.1 Traits and Genes

Genomic technologies such as genome sequencing and transcriptome analysis generated valuable information on functional and structural aspects of genes involved in various processes of stress responses, growth, and development, in a variety of plant species. Mulberry has the potential features to consider as a perennial tree model system. Limited genomic studies have been conducted in mulberry with transcriptomes, proteome, metabolome, and degradome approaches to elucidate comparative gene expression in response to stresses, variation between tissues and genotypes in *Morus* species. (Dhanyalakshmi and Nataraja 2018). Phytoplasma is a devastating pathogen causing yellow dwarf disease in mulberry. The molecular mechanism of phytoplasm pathogenicity is poorly understood due to the inability to culture In-vitro

(Wei et al. 2013). To understand the molecular mechanism of pathogenicity, differentially expressed miRNAs from phloem sap were analyzed. A total of 30 conserved miRNA and 13 novel miRNAs were differentially expressed upon phytoplasm infections were identified. It was suggested that Mul-miR482a-5p might negatively regulate resistance to phytoplasm infection in mulberry. Mul-miR482a-5p predicted to target the RCC1 gene, which is the guanine nucleotide exchange factor for the nuclear GTP binding Ran and it may act as a positive regulator of defense responses. Therefore, upon phytoplasm infection, Mul-miR482a-5p expression level increases that may repress the RCC1 gene and reduce host resistance to phytoplasma (Gai et al. 2018).

8.8 Genomics-Assisted Breeding for Resistance/Tolerance Traits

8.8.1 Functional and Structural Genomic Resources Developed

Some work has been done in identifying and characterizing downstream genes involved in defense response such as Pathogen-related proteins (PR), lectins, phenylpropanoid and Proanthocyanidins biosynthetic pathways. Pathogen-related proteins are a group of family genes that are induced in response to pathogen attack. The transcription and translation level of PR1 significantly increased in response to pathogens and therefore considered as marker proteins for the establishment of systemic acquired resistance (SAR) in plants (Ali et al. 2018). To explore the possibility of utilizing PR1 in mulberry breeding through genetic engineering, muPR1 was isolated from *M. multicaulis*. The isolated muPR1 expressed constitutively in selected tissues and induced by pathogens, methyl jasmonate, salicylic acid and GA3 phytohormones. Further, the involvement of muPR1 in disease resistance was shown by overexpression in transgenic Arabidopsis that ensued in increased resistance to *Botrytis Cinerea* and Pst. DC3000. It was also shown that muPR1 may have roles in the rate of reactive oxygen species formation and detoxification (Fang et al. 2019). Lectins are proteins that contain at least one non-catalytic sugar-binding domain and are synthesized in response to abiotic and defense response (Lannoo and Van Damme 2010). In *M. notabilis* 197 genes belonging to 12 distinct gene families have been identified. Expression analysis identified 4 lectin genes as upregulated under Jasmonic acid and salicylic acid treatments reminiscent of biotic stress conditions (Saeed et al. 2015). Proanthocyanidins are abundant polyphenolic compounds and have a role in disease resistance in plants additionally can improve human health. Proanthocyanidins are polymers of flavan-3-ols, primary catechin, and epicatechin where leucoanthocyanidin reductase (LAR) and anthocyanidin reductase (ANR) catalyzes the synthesis of catechin and epicatechin respectively.TheMnANR and MnLAR transcripts increased in response to *B. cinerea* infection and methyl

Jasmonate stress conditions. The ectopic expression of MNANR and MnLAR in tobacco increased the disease resistance against the *B. cinerea* infection (Xin et al. 2020).

8.8.2 Genome Sequencing, Assembly and Annotation

The first whole genome sequencing of any mulberry species was done by He et al. (2013), they used *M. notabilis* with seven distinct pairs of chromosomes (2n = 14) for genome sequencing. The genome size of *M. notabilis* was estimated to be 357.4 Mb predicted to code for 29,338 genes and contains 128 Mb repetitive sequences. It is found that nearly half of the mulberry genome is composed of repetitive elements and nearly 50% of these sequences were belonging to *Gypsy*-like (6.58%) and *Copia*-like (6.84%) long-terminal repeat retrotransposons. A total of 27,085 protein-coding loci with complete gene structures were predicted using 21 Gb RNA-seq data from five tissues and unique ESTs. In another effort, Jiao et al. (2020) sequenced the genome of a diploid mulberry species *M. alba* L 28 chromosome (2n = 2x = 28) using combined three different technologies, the Oxford Nanopore, Illumina HiSeq, and high-throughput chromatin conformation capture (Hi-C) platforms and found the genome size as 328.3 Mb. A total of 180.11 Mb of non-redundant repetitive sequences by a combination of homology-based approaches and de novo, accounted for 52.85% of the assembled genome. A total of 22,767 protein coding genes were explained with an average gene length of 3209 base pairs (bp). In addition to these, the chloroplast of mulberry has been sequenced using a combination of long PCR and shotgun approaches. The chloroplast genome has circular double-stranded DNA of size of 158,484 bp containing two identical inverted repeats of 25,678 bp each, separated by a large (87,386 bp) and small (19,742 bp) single copy regions. From this sequence, 83 protein-coding genes, eight ribosomal RNA genes and 37 tRNA genes were identified. In another attempt, Chen et al. (2016) sequenced the chloroplast of *M. notabilis* and found that the circular genome is 158,680 bp in size, and comprises a pair of inverted repeat (IR) regions of 25,717 bp each, a large single-copy (LSC) region of 87,470 bp and a small single-copy (SSC) region of 19,776 bp. The chloroplast genome contains 129 genes, including 84 protein-coding genes (PCGs), eight ribosomal RNA (rRNA) genes and 37 transfer RNA (tRNA) genes. The maximum likelihood (ML) phylogenetic analysis revealed that *M. notabilis* was more related to its congeners than to the others. Later, chloroplast sequences from five other species of mulberry were generated (Kong and Yang 2016, 2017).

8.8.3 Impact on Gene Discovery and Germplasm Characterization

The whole genome sequencing has a significant impact on the characterization of germplasm as illustrated with 134 mulberry accessions by Jiao et al. (2020). Using the newly identified 14,273,912 high-quality SNPs, the phylogenetic relationship among 132 cultivars using 2 wild mulberry genotypes was assessed and found that the phylogenetic tree was based on whole-genomic SNPs was not good with consistent with the traditional delimitations of mulberry species. The cultivars from Chinese grouped into two viz., Hu mulberry (HU), from Taihu Basin, and non-Hu mulberry (NH), from the rest of China. This latter group could be further divided into two subgroups, East and West. Further, it was noticed a lower level of heterozygosity with high linkage disequilibrium decay. Likewise, Muhonja et al. (2020) worked out the genetic relationship among 54 mulberry accessions from seven species (*M. indica, M. alba, M. bombycis, M. latifolia, M. acidosa, M. rotundiloba and M. kagayamae*) using genome-wide 2229 SNPs. The phylogenetic analysis resulted in the construction of only 3 clear monophyletic clades viz, *M. acidosa* and *M. kagayamae* from different geographically isolated islands, two Japanese native species and a Thai species, *M. rotundiloba*, and other species were found non-monophyletic. It is also interesting to note that no clear monophyletic clades could form by varieties from *M. alba* and *M. latifolia* indicating admixture among them through natural hybridizations. These studies suggest the classification of the genus *Morus* is not an easy task even with genome-wide DNA markers. A similar type of results was obtained earlier with ISSR and ITS markers (Muhonja et al. 2020; Zhao 2005). Besides the inefficiency of the current species delimitations of the genus *Morus,* these studies also brought out the usefulness of the whole genome sequencing and DNA markers for germplasm characterization and crop improvement. The SNPs markers developed in the studies can be used for making SNP panels for automation of the mulberry germplasm to identify suitable parents for breeding programs. The genes identified from these studies can be used for further studies by gene knock out, overexpression and gene editing to develop varieties with desirable traits. Mulberry fruit is of economic value because of its high nutrition and presence of potential pharmacological active compounds beneficial to human health. Mulberry Sclerotiniose is caused by *Ciboria shiraiana*, which affects the quality of the fruit. To gain insight into the molecular mechanisms to provide direction to molecular breeding, diseased fruit was investigated using a transcriptome and metabolome approach. Differential expression analysis between healthy and diseased fruits revealed, genes related to plant hormone signal transduction, transcription factors and phenylpropanoid biosynthesis may play an essential role in response to Sclerotiniose pathogen infection (Bao et al. 2020).

8.9 Recent Concepts and Strategies Developed

8.9.1 Gene Editing

Abiotic stress is a complex trait controlled by many genes and their products that involved in signaling, regulatory and metabolic pathways, thus, just a modificationin a single gene may not produce any desired results. Therefore, more advanced and effective techniques that affect several genes simultaneously need to be applied. Gene manipulation with CRISPR-Cas 9 is one such technique. CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) and CRISPR-associated (Cas) genes are essential components of a bacterial adaptive immune system to acquire resistance against invading virus. CRISPR/Cas9 uses a protein-RNA complex to target and cleavage the target sequence using a short guide RNA (Pennisi 2013). Scientists have found a great many utilities for this system in gene manipulation such as the introduction of single point mutations, deletions, insertions, inversions, translocations etc. in a particular target gene. In general, gene manipulation of monogenic traits is always easy than those controlled by many genes. However, abiotic stress is a trait controlled by many genes involved in gene regulations, signaling and several metabolic pathways. Thus, simultaneous manipulation of many of these genes is required to get the desired results. CRISPR-Case 9 can target several genes simultaneously due to the easiness of designing the high efficiency of sgRNAs. Multiplex genome editing has been successfully implemented in model and crop plants (Li et al. 2013; Mao et al. 2013; Zhou et al. 2014). CRISPR system is a powerful tool for genetic screen to identify gene function at genome wide level through generating point mutations and gene knock out. Guide RNA can be targeted to almost all the genes present in the genome in almost all the plant species, where genome sequence information is available. In addition, potential multiplexing CRISPR system help to investigate function of the gene families resulting from gene duplication. The screening of CRISPR induced mutants displaying altered biotic stress response aid in identifying genes involved in the process and also to develop disease tolerant crop plants. The use of CRISPR-Cas9 system in genotyping natural variations to distinguish homozygous biallelic mutants from wild-type has been demonstrated (Kim et al. 2014). However, in mulberry CRISPR based technology has been used yet. Thus, with the advancement of the genomics of mulberry, it is expected that gene editing with CRISPR-Cas9 technology would be applied in mulberry soon.

8.10 Brief on Genetic Engineering for Resistance/Tolerance Traits

Genetic engineering consists of isolation of a gene of interest, ligating it on a vector to transfer it into a plant genome to meet a purpose. The most important advantage of genetic engineering is the ability to manipulate gene expression as desired. In plant

breeding, the breeders can work only with plants that are cross-fertile but with genetic engineering genes from any organism including micro organisms can be inserted into the plant. However, the biggest challenges are the development of a robust, reproducible plant regeneration protocol and a genetic transformation method. In mulberry, such an efficient protocol for direct plant regeneration from leaf explants is still to be developed, though direct plant regeneration from hypocotyls has become an easy task (Vijayan et al. 2011).

8.10.1 Target Traits and Alien Genes

In mulberry, the most important trait is the leaf yield which is under the cumulative contribution of a number of associated traits such as plant height, leaf weight, number of branches, leaf retention capacity, nodal length, root length (Vijayan et al. 1997). However, under saline conditions, a change in correction was observed and the leaf yield had a significant correlation with plant height, leaf size, shoot weight, root weight, root length, protein, NRase activity and WUE of the plant. Similarly, the plant height was also found changing its correlation with most of the characters studied. This clearly shows that under different salinity levels the selection criteria for plants should be changed (Vijayan et al. 2009). It has also been observed that mulberry possess certain traits to confer higher tolerance to stress conditions. Some of these traits are elongated roots, thicker epicuticular wax, synthesis and accumulation of osmolytes like Proline, glycine beatain, etc. (Vijayan et al. 2005). Plants have progressed several mechanisms like thicker epicuticular wax, para heliotropic movements, salt-secreting hairs, elongated roots, synthesis and accumulation of osmolytes, etc. to tolerate the stress to facilitate retention and/or acquisition of water, protect chloroplast functions and maintain ion homeostasis (Vijayan et al. 2008). The genes involved in these pathways and mechanisms need to be incorporated either through conventional breeding or through genetic engineering. Since transfer of genes and traits into mulberry is very difficult due to breeding behaviour of the plant the easiest method is through genetic engineering. In genetic engineering, genes may be knocked out, over expressed, or modify the expression and product through gene editing. Over expression of *DREB*s (dehydration responsive elements binding proteins), *ERF* (Ets-2 Repressor Factor), *MYB* (myeloblastosis), *bZIP* and *WRKY* transcription factor families have shown promising results in several plant species (Jung et al. 2007). Further, Lu et al. (2008) have identified a low temperature encourage gene *WAP25* from Mongol mulberry, one of the wild species of genus *Morus* that cloned the gene and grows in cold regions (GenBank accession N0. DQ104333) into expression vector pIG121/*Wap*25 and transformed *Petunia hybrid* Vilm via *Agrobacterium.* This study, possesses the scope of genetic improvement of other mulberry species such as *M. indica, M. alba, M. latifolia* which are being used for silkworm rearing and are highly susceptible to cold and other stresses. Disease arises from compatible interaction between the host plant and pathogen. There are some genes in the plant which facilitate infection and further proliferation of pathogen upon entry, they

are referred to as susceptibility (S) genes. Therefore, mutation or loss of function of the S gene can provide resistance to different strains of the pathogen and long lasting protection. The best example for utilization of S genes in providing field resistance is Mildew Resistance Locus O (MLO) genes involved in powdery mildew resistance in barley (Acevedo-Garcia et al. 2014). To identify a susceptible gene, MLO was involved in powdery mildew in mulberry bioinformatics analysis using Arabidopsis MLO genes and MLO domain search was undertaken in *M. notabilis* genome. A total of 16 MLO genes were identified and their characteristic motifs were also determined. To identify MLO gene involved in powdery mildew susceptibility in mulberry, various criteria were applied such as phylogenetic analysis to identify clade V specific genes, protein motifs that are exclusively present in the functionally characterized MLO proteins and MLOs gene induction in response to powdery mildew infection identified MLO2 and MLO6A as candidate genes (Ramesha et al. 2020). For future work, the identified candidate genes may be screened for presence of non-functional mutants in the resistant germplasm or employ novel genome editing technologies to knock down the genes to impart powdery mildew resistance in mulberry.

8.11 Future Perspectives

Mulberry host plant is a perennial tree with prolonged generation times, high heterozygosity, out crossing breeding behavior, poor juvenile-mature trait correlations, polygenic nature of most of the vital traits posed many challenges to conventional breeders. Thus, marker-assisted selection (MAS) is considered a tool to accelerate breeding through early selection, especially for abiotic stresses. MAS depends on identifying DNA markers that are tightly linked to the trait of interest. As stated above, although a few genetic linkage maps have been developed using biparental mating with pseudo-test cross strategy, non of these genetic maps and subsequent efforts were able to identify validated QTLs to be used in breeding program in mulberry. The major limitations of the above efforts include the sparse distribution of markers, lack of tightly linked markers to the traits, minor QTLs and the low success rate in validating QTL in different genetic backgrounds and environments. Further, to develop high resolution maps to identify markers with a tight association, more abundantly available markers like Single nucleotide polymorphisms (SNPs) have to be developed. Such effort is currently in progress at different research organizations across the globe.

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