Summary

Mulberry (Morus spp.) is the only food plant of the domesticated silkworm (Bombyx mori L.) and its productivity plays an important role in the success of sericulture. One of the major factors limiting the silk productivity in the country is the non-availability of water for irrigation of mulberry gardens. Besides, the uncertainty in the availability of assured irrigation due to increased demand for water for many purposes and consequently over exploitation and depletion of underground water has posed serious threat to sustainable cultivation of agricultural crops including mulberry. There is an urgent need to address the problem by limiting the water dependency of the mulberry crop. This is more so in case of rain-fed areas of traditional states where, poor and marginal farmers practice sericulture. One of the concrete and rewarding effort would be to develop moisture stress tolerant mulberry variety having higher WUE characteristics along with enhanced water mining ability by the roots. The WUE and water mining by the root are complex characteristics and governed by many genes. Development of mulberry varieties tolerant to moisture stress through conventional approach is quite laborious and time taking task. Therefore, there is a need for identification of QTLs governing these traits by linkage mapping and then introgression of these genes into desirable genetic background through marker assisted selection (MAS) for sustainable production of mulberry under water deficit conditions.

In this background, it was envisaged to develop trait specific (WUE and root) mapping populations by identification of contrasting lines from diverse germplasm for construction of genetic linkage maps using DNA markers. The important objective of the study was to locate QTLs controlling traits associated with WUE and rooting in mulberry. Besides, it was also planned to introgress high WUE and high rooting traits in the same genetic background for selection of superior recombinants with improved tolerance to moisture stress.

Identification of parental lines for the development of mapping population for WUE and root traits

A total of 273 accessions in the field gene bank of CSRTI, Mysore were utilized to assess the genetic variability in WUE (measured in terms of $\Delta^{13}C$ value), growth and rooting parameters for identification of suitable parental combination for the development of WUE and root trait specific mapping population. Nine contrasting parental lines (i.e., five for WUE and four for root trait) were selected. $\Delta^{13}C$ value was maximum in case of UP (21.150) and least in case of S-34 (17.669). Dudhia White recorded higher values in root and growth characters and lower values for $\Delta^{13}C$ (17.976) signifying higher WUE characteristics in the germplasm.
Among the selected lines, G-4 recorded maximum for total leaf area and number of leaves in the longest shoot and average shoot length. MS-3, a high WUE type recorded minimum for longest shoot length, average shoot length and average leaf area. Thaimale (low root type) recorded minimum values for total leaf area and number of leaves on the longest shoot. Punjab Local showed a maximum (75.90%) moisture content among the parental genotypes and minimum was seen in case of MS-3 (68.94%). Clustering resulted in 4 groups, of which the Cluster I comprised four parental lines including closely related Himachal Local and Punjab Local. Muki and MS-3 were divergent lines and formed independent group.

Molecular characterization of selected lines were essentially carried out to identify the polymorphic markers for genotyping of mapping population. A total of 919 (79.15%) polymorphic RAPD, 96 ISSR (73.28%) and 25 SSR (96.15%) markers were identified among the parental lines. Cluster analysis of parental lines based on the combined molecular marker data revealed that Himachal Local (low WUE type) and Punjab Local (high root type) were genetically close. Dudhia White (high root type) and MS-3 (high WUE type) were genetically divergent from Muki (low WUE type) with dissimilarity coefficient of 0.2947 between the pairs.

Nine genotypes each belonging to high and low photosynthetic efficiency groups were analyzed for identification of divergent parental combination with contrasting trait. The overall mean photosynthetic rate ranged from 23.74 to 16.65 µmol m⁻² s⁻¹. DNA profiling by RAPD resulted in amplification of 133 markers of which, only 18 markers were monomorphic and rest 115 (86.47%) were polymorphic. Based on the marker similarity values among 18 mulberry genotypes belonging to the two contrasting groups in terms of photosynthetic rates, *M. multicaulis* and English Black were genetically close with a similarity of 88.1% and K-2 and RFS-135 were genetically divergent with least similarity (52.5%). The identification of the parental combination for the development of mapping population for photosynthetic efficiency were primarily based on the contrasting genotype for the trait, reproductive behaviors and genetic distance (GD). Considering these three parameters, the most suitable parental combination appeared to be RFS-135 as low photosynthetic ♂ and either BR-2 (GD = 0.352) or S-1 (GD = 0.413) as high photosynthetic ♀ for crossing programme and development of mapping population.

**Development of mapping population for WUE trait, construction of linkage map and QTL analysis**

About 250 progeny of the cross Himachal Local (low WUE) and MS-3 (high WUE) were established in an experimental plot and, out of which 200 progeny were randomly selected and used as mapping population for WUE trait. Morpho-physiological data were recorded for 13 important
associated traits in the mapping population in the years 2007 and 2008. In general the traits showed normal frequency distribution. The magnitude of coefficient of variation was maximum (55 \%) in case of total stem weight followed by shoot biomass (50 \%). The CV was minimum (4 \%) in case of moisture content among the segregating progeny in 2007 and similar trend in 2008.

A total of 82 RAPD primers were used for profiling of WUE mapping population, which amplified 194 polymorphic. Chi square test (\( \alpha = 0.05 \)) was performed to test the null hypothesis of 1:1 and 3:1 segregation of all markers scored. The marker loci not indicating any segregation distortion from the expected 1:1 ratio were further utilized for map construction and thereby eliminating the spurious linkages. A total of 20 linkage groups of Himachal Local (♀ parent) and 16 of MS-3 (♂ parent) were established. Approximately 75\% of the markers used for linkage analysis could be placed on Himachal Local map covering 2374.7 cM of total map distance. Similarly, on MS-3 map, 75\% of the markers could be placed with 60 loci or loci clusters covering a map distance of 1327.2 cM.

Single marker analysis showed the association of different markers with the specific traits at the significance of P<0.05 level. Analysis of data recorded in September 2007, showed association of markers with 13 traits in female parent and 11 traits in male. In April 2008, maximum number of traits were associated with makers were in the male parent. Composite interval mapping plotted three QTLs (LLS, IND and ASL) on LG7 and one QTL SPAD chlorophyll value on LG13 in female and on male map two QTLs for IND (R^2 \% =0.16.86) and ALA (R^2 \% = 0.19.62) on LG3 were identified based on the phenotypic expression during September, 2007. QTL analysis based on the phenotypic data of April 2008 showed 6 QTLs on female map. Two QTLS for IND and ASL on LG7, three for SPAD, MRC6 and ML6 on LG8 and one for SPAD on LG14 were identified. The male also showed a total of six QTLs in the same year.

Development of mapping population for root trait, construction of linkage map and QTL analysis

About 550 segregating progeny were raised from the cross of Dudhia White (high root) and U P (low root) and 200 progeny were randomly selected for mapping. Morpho-physiological data were recorded for 13 parameters in the year 2007 and 2008. Majority of the traits showed normal distribution among the segregating progeny. In 2007, large variability in phenotypic expression among segregating progeny was observed. For example, total shoot weight (TSW) ranged from 10.00 -1400.00 g/plant and total leaf yield (TLY) ranged from a minimum of 17.98 to a maximum of 1158.98 g/plant. The standard deviation was least (0.81) in case of IND. Similar trend was also observed in the phenotypic expression during April 2008. A special root structure study was
conducted for this population, where the progenies were grown in cemented block structure for root studies. In root structure studies 6 root parameters were recorded. Considerable variation of all the root trait parameters were observed among the mapping population. The maximum root-shoot ratio was 0.62 with a minimum of 0.04 was observed. The root weight ranged from 0.33 to 33.33 g with an average of 12.64 g. Coefficient of variation was maximum in shoot as well as root weight (0.484). Average longest root length was 69.33 cm.

Segregation of 94 RAPD and 6 ISSR primers was scored which showed a total of 101 RAPD and 10 ISSR markers were specific to female parent whereas, 73 RAPD and 6 ISSR markers were specific to the male parent. \( \chi^2 \) test was performed to test the null hypothesis of 1:1 and 3:1 segregation of all markers scored. The marker loci without any segregation distortion from the expected 1:1 ratio were further utilized for map construction. A total of 17 linkage groups of Dudhia white (♀ parent) and 14 of U P (♂ parent) were established. Approximately 55% of the markers used for linkage mapping for Dudhia White could be placed on the framework defining a total of 58 loci or loci clusters and covering 1150.8 cM of total map distance. In U P, 59% of the markers could be placed on a framework of 42 loci or loci clusters covering 856.7 cM.

Single marker analysis showed the association of different markers with the specific traits at the significance of P<0.05 level. In the year 2007, maximum number of markers (10) associated with ALA in female, whereas in male two traits (TSW and SPAD) were associated with 4 markers each. In 2008, the maximum number of markers (7) associated with TSW and in case of male, LLS and NLLS were associated with 5 markers each. In root studies, the maximum number of marker (7) were associated with SW in female and 5 markers each with NPR and LRL in male.

Composite interval mapping plotted seven QTLs on female map in the year September 2007 of which four QTLs (for LLS, TSW, TLY and SBM) on LG9. On male map, one QTL (for SPAD) was located on LG10. In the year 2008, the female map showed fourteen QTL in total of which ten QTLs (LLS, NLLS, IND, TSW, TLY ALA, SBM and ASL) on LG9. The male showed same results as IM, four QTLs (2-LLS, IND and ASL) in the year 2008 all of which were on 11th linkage group. CIM analysis of root parameters showed that both SW and LLR were mapped on female LG5 as in IM with LOD score > 3.0 in concurrence with IM analysis. Genomic region for root-shoot ratio (RSR) was also identified with LOD score > 3 on the same linkage group.

**Introgression of water use efficiency and root traits in mulberry and identification of improved lines**

Introgressed lines for WUE and root traits were developed by crossing Dudhia White (high rooting) and MS-3 (high WUE). The fully-grown seedlings were transferred to the main field (1104 lines).
Based on the visual observation of different parameters (branching nature, leaf color, leaf nature, leaf lobation, leaf size, leaf appearance, inter-nodal distances and number of branches) along with shoot biomass were recorded which showed wide variability. Maximum lines recorded low number of branches and unlobed leaves, whereas the leaf size was medium in maximum number of lines. The shoot biomass of introgressed lines ranged from minimum of 20 g to a maximum of 3180 g with an average of 1125 g and the coefficient of variation was 0.347. The data was analyzed and 63 lines were short-listed.

Gas exchange parameters viz., photosynthetic rate, conductance, intercellular CO₂ concentration, transpiration rate, leaf vapor pressure deficit, leaf temperature, ratio of assimilation rate and stomatal conductance and ratio of intercellular carbon concentration and stomatal conductance were recorded in the short-listed lines. Analysis of gas exchange parameters among the short-listed lines showed that the coefficient of variation (%) ranged from 2.94 to 32.61. Based on the shoot biomass (surrogate for root trait), gas exchange parameters and Δ¹³C (surrogate for WUE) the lines were finally short-listed from 63 to 5 lines. These lines were, IL - 126, 280, 309, 311 and 314.

Conclusions

The study is one of the first of its kind in mulberry, where construction of linkage maps of mulberry using a segregating population for both WUE and root traits have been achieved and located QTLs governing above traits and associated characters on the map. So far, the mulberry crop improvement relied mainly on random chance factor of obtaining superior gene combination by hybridization. Non-availability of homozygous lines, high heterozygosity due to out breeding coupled with problems associated with tree crops has retarded the progress in mulberry improvement. Virtually, no information on inheritance of important agronomical traits of mulberry has been recorded. In this background, the present study is of immense importance in mulberry genetics and breeding. Linkage map of mulberry and the identification of QTLs governing important traits give's an insight into the genetics of this poorly studied crop. Even though, deep investigation is needed to understand mulberry genome, the present study is one of the stepping-stones for further unraveling of genetic control of phenotypic traits. Identification of genomic regions controlling WUE and allied traits in mulberry is one of the crucial steps towards directional crop improvement for drought tolerance in mulberry. A relatively sparse ‘framework’ of two parental lines has been constructed. From this point there is need for saturation of maps consisting of evenly spaced markers, which is adequate enough for most of the genetic analysis including QTL
identification. The framework map developed in the study required to be further saturated specifically using co-dominant markers like SSRs, SNPs, SCARs etc., to find markers tightly linked to the genomic regions of interest. This will help in a long way in understanding the phenotypic expressions of mulberry towards crop improvement. Five superior performing introgressed lines (IL - 126, 280, 309, 311 and 314) were identified. It is recommended that these lines could be further subjected to evaluation trial under moisture stress condition towards better utilization. The identified introgressed lines may be also used in crop improvement as pre-breeding resource for transfer of high WUE and root traits to elite cultivars for improving the targeted trait. Lastly, the mapping resources developed in under the study is virtually immortal (because of clonal propagation) and available for genome mapping by different group investigators across the country.