Chapter 6

Summary and Conclusion

6.1 Summary

The aim of the present study was to observe genotypic differences between the male and female trees of *P. ciliata* at molecular or biochemical level. Morphological differences were observed from the leaves of mature male and female trees which could be used for sex identification purposes in the field at the mature stage of their growth. For differentiating the gender of the trees at seedling stage, RAPD and isozymes were used as molecular markers. Phytochemical screening was done using simple chemical tests from the leaves, buds and bark of the tree. In addition to these, efforts were also made to investigate the monthly variation in the level or concentration of two phenolic glycosides (salicin and populin) and their role in flowering (if any) from the bark and buds of the both sexes. The findings of the study are summarized below:

6.1.1 Leaf morphology of male and female adult trees

The morphological variations (qualitative and quantitative) were observed between four male and four female trees of *P. ciliata* during the study in the month of August from Shimla district. Qualitative study dealt with six morphological characters viz., shape of base and tip of leaf blade, sinus with petiole, pubescence on the lower surface of the leaf blade, leaf margin and color of the leaf lamina. Among all these characters, only three characters (shape of the leaf lamina, shape of sinus and leaf blade margin) showed variation between female and male trees. The major differentiating character was the shape of the leaf blade, deltoid-cordate in females and deltoid-ovate in males. In quantitative study, different characters studied were LA, L/W %, P/N %, perimeter, aspect ratio and shape factor, etc. In all these characters males excelled the females except the lamina length/width percent. Among all used parameters statistically significant (t-test; P < 0.0001) result was obtained with only shape factor. Only the qualitative traits were able to differentiate the sex of the trees at vegetative stage.

6.1.2 Markers for gender identification at seedling stage

Genetic differences among four male and four female genotypes were investigated using ten RAPD primers (OPA-10, OPC-19, OPE-04, OPG-02, OPH-05, OPK-20, OPG-06, OPC-11, OPG-05 and OPG-17) and seven enzyme systems viz., PER, EST, CAT, MDH, ADH, APH and RUBISCO.
In RAPD analysis, genomic DNA was isolated by the modified CTAB method from young, fresh green leaves, quantified by spectrophotometer and then amplified using PCR with random primers. All primers showed successful amplification in both male and female trees. Out of ten random primers, OPK-20 gave significant difference between male and female trees and produced female specific three amplification products of different sizes, i.e., 400 bp, 500 bp and 800 bp from female trees, therefore identified as female specific marker. For isozyme analysis, proteins/ enzymes were extracted from the young leaves in the month of March. Extracted protein/ enzyme samples were examined with separate enzyme systems with standard methods. Out of all, only two enzymes (PER and EST) showed differentiation between male and female trees. Peroxidase (PER) enzyme system produced three bands PER-I, PER-II and PER-III at the position of Rm values 0.10, 0.20 and 0.29, respectively. The band at Rm value 0.29 was found only in female trees, therefore identified as female specific band. On the other hand, esterase enzyme (EST) system produced five differentiating bands EST-I, EST-II, EST-III, EST-IV and EST-V at the anodal position of Rm value 0.06, 0.11, 0.15, 0.27 and 0.30, respectively. Out of five bands, two were found to be tightly linked to gender of the trees. The band EST-V at the position of Rm value 0.30 was observed as a male sex- linked band, whereas band EST-II at the position of Rm value 0.11 was identified as female specific.

6.1.3 Phytochemical analysis of buds and bark

Preliminary phytochemical screening of methanolic and aqueous extracts from the dried tree parts (bark, buds and leaves) of *P. ciliata* was carried out by simple chemical tests. Result showed that the tree was rich in phenolic compounds, tannins, glycosides, carbohydrates, proteins, amino acids, gum and mucilage.

To see the monthly variation in phenolic glycosides concentration and their role in flowering, two phenolic glycosides (salicin and populin) were used as standard in TLC and HPLC. Salicin was purchased from the market, whereas populin was chemically synthesized in the laboratory from salicin. The confirmation of synthesized populin was done with simple physical and chemical tests, whereas physical characterization was proceeded with FTIR, $^1$H NMR and $^{13}$C NMR.

Seasonal variation of salicin and populin and their quantification was done by HPLC from the methanolic extract of bark and buds. Every month methanolic extracts of bark of
both trees showed an almost similar pattern of two peaks. Salicin content was found to be increased starting from September to April and then declined from April to August. The trend was almost similar in both the sexes. Both sexes produced maximum content of salicin in April, whereas minimum was observed in September in female and in August in the male tree. Salicin content showed the major difference between the two sexes in the month of October, whereas in females it was maximum ca. 3.90 mgg\(^{-1}\) DW, and in males it was minimum ca. 0.19 mgg\(^{-1}\)DW. Populin content in both sexes revealed the similar pattern only from January to April while in other months the pattern was dissimilar. In male tree it was highest from September to April (0.050 mgg\(^{-1}\)DW) and lowest in June, while in the remaining it was totally absent. On the other hand, in the female tree maximum concentration of populin was observed in March (0.012 mgg\(^{-1}\)DW) and from January to May, populin showed its presence while in other months it was absent. This difference in populin content was found to be very much prominent in both the sexes. However, in male tree, populin content was observed from September onward till December and also in June.

The comparison of both salicin and populin content exhibited that the concentration of both phenolic glycosides was higher in males than in females. In general, salicin production was higher in both trees than populin production. Similar to bark samples, male buds have a higher concentration of salicin and populin than females. The concentration of both phenolic glycosides was higher in buds as compared to bark of both male and female trees. One highest peak in the buds and bark of both trees chromatogram was identified as cinnamoyl- salicin by LC-MS studies. Its presence was found to be significant and constant in the flowering season.

### 6.2 Conclusion

The study concludes that in *P. ciliata* the gender is determined by the genotype of the tree. Although morphological and phytochemical differences exist between the male and female trees, they could not be used for the identification of gender of trees at seedling stage. However, molecular markers (RAPD and isozymes) can be used for this purpose before flowering. In this study, we have identified, for the first time, the female specific sex- linked DNA markers from *P. ciliata*. The multiple RAPD bands observed during the study also creates the possibility of heteromorphic sex chromosomes (ZW) in females and shows that *P. ciliata* female could be a heterogametic gender. Cinnamoyl- salicin is identified for the first time during the study, which was yet not reported in the literature.
Sex related differences help to identify the gender influenced region in the genome, which further helps the researchers, breeders, molecular biologists for further studies.

The main points of conclusion are:

1. The sex markers developed here could also be used as a starting material towards sequence characterization of sex-linked genes for better understanding the developmental as well as evolutionary pathways in sexual dimorphism.

2. Female linked OPK-20400, OPK-20500, OPK-20800 RAPD markers and isozymes (peroxidase and esterase) markers are found to be reliable enough for detecting sex of *P. ciliata* genotypes from different geographical regions. This will facilitate screening of plants at the seedling stage and maintain an optimum sex ratio in plantations, as well as save time and costs in ongoing poplar breeding and clonal propagation programs.

3. Sexual dimorphism is reported to be linked to many economically important traits (e.g., wood quality, disease resistance, biomass, ornamental purpose, nurse crop, etc.) and ecologically important traits (e.g., better adaptability to xeric sites and competitive areas for nutrition and light, phytoremediation purposes, regeneration of fragile hills, soil fertility, etc.) exploring the relationship between sex-linked markers and desirable traits in future will assist targeted genetic improvement.

4. The phenolic glycosides study only confirms their accumulation in flowering season, but could not identify a significant marker having a specific role in gender determination.