5.1 SUMMARY

The various infected leaves samples were collected from different places of H.P. in order to check *Colletotrichum gloeosporioides* infection present in nature. *C. gloeosporioides* was successfully isolated from collected samples and finally cultured on PDA plates. All isolates had different morphology. The standard strain of guava from IMTECH Chandigarh showed similar morphology to guava isolates of Dhaul Kuan. Mango and kiwi isolates also showed similar morphology with each other. But apple isolate showed different morphology from all collected isolates. In order to confirm isolates as *C. gloeosporioides* by ITS region amplification and sequencing. DNA was isolated by Choi et al., 1992 method. All the isolates were confirmed by amplification of their ITS regions. The product size of 450bp was obtained and sequenced. The final sequence when analysed with DNA sequence available in the database using BLASTn algorithm, it has been confirmed that the isolated fungi were *Colletotrichum gloeosporioides* as they showed 99% homology with the already known and reported *C. gloeosporioides* isolates. Further experiments were done using standard strain of guava. The sporulation study of *Colletotrichum gloeosporioides* was done using Potato dextrose agar (PDA), malt extract agar (MEA), limabean agar (LBA) and oat meal agar (OMA). The best growth and sporulation of *Colletotrichum gloeosporioides* was obtained on oat meal agar plates as compared to PDA, MEA and LBA. Random hygromycin resistant mutants of *Colletotrichum gloeosporioides* were generated using REMI (restriction enzyme mediated integration) method (Redman and Rodriguez, 1994). Approximately 4000 mutants we generated after repeated transformation and 121 mutants were further used. The selection of mutants was done on PDA containing 50µg/ml hygromycin and all the mutants (121 selected) were single spored to get pure colony. All the mutants were first confirmed by amplification of their ITS regions by ITS region specific primers in order to check whether mutants were of *Colletotrichum gloeosporioides* origin or not. Further confirmation of mutants was done with hygromycin specific primers in order to check whether hygromycin resistance gene was incorporated in the genome of *Colletotrichum gloeosporioides* mutants or not. All the tested mutants gave positive amplification with both set of primers. The PCR
product of hygromycin primer of one of the mutant was sequenced. Obtained sequence was analysed with DNA sequence available in the database using BLASTn algorithm. It has been confirmed that the obtained sequence of *Colletotrichum gloeosporioides* mutant showed 99% similarity to hygromycin resistance region of cloning vector pBSVirHvgGW’s complete sequence. The mutants were then checked for type of integration. Only 9 mutants showed single integration out of 20 mutants selected. The morphological studies of mutants was done on PDA and PDA containing 50µg/ml hygromycin. All mutants showed morphological variations in term of growth, colony color and sporulation. Spore germination and penetration experiment were also performed with wild and mutant type in triplet in order to check holistic view/ any effect of mutation by using $10^4$/ml spore suspension. The mutants 2.3, C80, B27, H6 showed more spore germination but less spore penetration as compared to the wild type. The spore germination was less in C7 as compared to wild type and other mutants. The spore penetration was less in all the mutants as compared to the wild type. For pathogenesis, all mutants were subcultured on OMA plates and different morphological variations were obtained in all the cases. The mutants 2.2, 2.3, C28, C55 showed same morphology in term of growth and sporulation to the wild type. The mutants C7, B27 showed similar morphology in term of growth and sporulation. The mutants H7, H8, H9, H13, H14 showed less growth and sporulation as compared to wild type and other mutants. Pathogenesis assay was done on various detached leaves, fruits and vegetables using $10^6$ spores/ml suspension. The inoculated samples were checked periodically for infection and photographs were taken after 5-7 days of inoculation. In all the cases, mutant 2.2 showed more infection as compared to other mutants but less infection than the wild type and no growth was observed in control. After pathogenesis all mutants were checked for identification of tagged gene by inverse PCR using three set of nested primers. Out of all tested mutants only mutant H4 showed amplification of 3500bp with 3rd set of nested primers and mutant H7 showed amplification of 3000bp with 1st set of nested primers. The amplified product of mutants H4 and H7 were sequenced. Obtained sequence showed similarity to some hypothetical protein whose function is not known. Even after repeated sequencing attempts, satisfactory results were not obtained.
5.2 CONCLUSIONS

- *Colletotrichum gloeosporioides* was successfully isolated from all collected samples from various places of H.P.

- Morphological study of all isolated samples showed that, standard strain from guava showed similar morphology to guava isolate of Dhaul Kuan. Mango and kiwi isolates were also showed same morphology with each other but apple isolate showed different morphology when grown on PDA plates.

- All the isolates were confirmed by amplification of their ITS regions and sequencing of amplified product was done.

- The maximum sporulation of *Colletotrichum gloeosporioides* was observed on Oat meal agar media as compared to potato dextrose agar, malt extract, lima bean agar.

- 4000 mutants were obtained after repeated transformation using REMI and 121 mutants were selected on PDA plates containing 50µg/ml hygromycin.

- All 121 mutants were confirmed by amplification of their ITS regions by ITS region specific primers and Hygromycin gene specific primers. Around 450 bp product was obtained.

- Single integration was observed in 9 mutants out of 20 mutants selected by southern hybridization.

- Morphological variations were observed when mutants were grown on PDA and PDA containing 50µg/ml hygromycin.

- Spore germination was more in 2.3, C80, B27, H6 but spore penetration was less as compared to wild type. Least spore germination was obtained in mutant C7.

- Spore penetration was less in all the mutants as compared to wild type.

- Pathogenesis assay was performed on various detached leaves and fruits. The mutants showed less infection as compare to wild but 2.2 mutant showed more infection in
comparison to other mutants but less infection as compare to wild and no growth was observed on control.

- Approximately 3500 and 3000bp fragments were amplified corresponding to tagged genes in mutant H4 and H7 respectively in Inverse PCR and sequenced.

- The obtained sequence after sequencing showed similarity to hypothetical protein. These needs to be confirmed as the sequencing results were not very conclusive.