**SUMMARY**

6.1 Selection of potentially antagonistic fluorescent pseudomonads from rhizosphere soils.

- A total of 10 carnation samples were collected from different locations of Himachal Pradesh (H.P.). *Fusarium oxysporum* infecting carnation was consistently isolated on Potato Dextrose Agar (PDA) or Carnation Leaf Agar (CLA) from the discoloured stem vascular tissue of all the samples. In pathogenicity testing, 6 isolates manifested the vascular wilt symptoms as chlorosis, necrosis and plant death on artificially infected carnation and was similar to those in commercial glasshouses.

- Characterisation by morphological and molecular means identified a highly pathogenic isolate, CAFO-IHBT as *Fusarium oxysporum* f.sp. *dianthi* inciting vascular wilt of carnation.

- A total of 134 fluorescent pseudomonad strains were isolated from the rhizosphere soils (150 nos.) collected from various locations of Trans-Himalayan region located in H.P by soil dilution plating or enrichment culture on KB agar medium.

- In dual culture assays for antifungal activity, among 134 isolates of fluorescent pseudomonads, 9 isolates significantly inhibited the mycelial growth of the pathogen with inhibition zones ranging from 5 to 19 mm. Among the antagonistic isolates, P3(4) showed the maximum mycelial growth inhibition of 19 mm on PDA both in presence and absence of FeCl₃.

- In assessing the morphological, physiological and biochemical characteristics of P3(4), the colonies on KB agar were observed to be irregular, raised, and greenish yellow coloured. The cells were gram-negative, rod-shaped and aerobic. Growth occurred at temperatures ranging from 24 to 41°C, pH levels of 4.5 to 11 and NaCl levels of 0% to 10%. The isolate was tested positive for oxidase, catalase, starch, citrate, gelatin, and casein hydrolysis, and negative for MR- VP test, indole formation, levan formation and urea hydrolysis. Acid was produced from dextrose, inulin, xylose, glycerol, glucosamine, ONPG, esculin, D-arabinose, citrate and galactose. No acid was produced from sucrose, salicin, dulcitol, raffinose, sorbitol, melibiose, adonitol, cellobiose, fructose, inositol, maltose, mannose, rhamnose, trehalose, mannitol,
lactose, L-arabinose, sodium gluconate, alpha-methyl D-glucoside, ribose, melezitose, alpha-methyl D-mannoside, xylitol, malonate and sorbose.

- In 16S rRNA homology analysis of 1528-bp sequence (GenBank accession no. AM268040) and phylogenetic relationship, the isolate was closely associated (99%) to *Pseudomonas putida* in the similarity search.

### 6.2 Elucidation of mechanisms of antagonism in selected fluorescent pseudomonads

- In plate assays, none of the antagonistic rhizobacteria produced the deleterious biocontrol traits cellulase and pectinase. In screening for plant growth promoting traits, while all the fluorescent pseudomonads showed high “P” solubilisation efficiency (>100) and IAA production, three isolates *viz.*., TEPF-Sungal, ROPF-Chandpur and BBPF-Holta lacked the production. Among the antifungal traits, XXPF-MDU2 was the only strain producing HCN. Though siderophore production was displayed by all the strains, P3(4) and G4(2) showed the maximum clearing zone of > 12 mm. Similarly, P3(4) displayed maximum lytic activity on chitin and chitosan with 28 and 23 mm zone of clearance, respectively.
- The role of extracellular chitinases in antagonism of fluorescent pseudomonads against the vascular wilt pathogen was assessed using soil reaction as an indicator.
- The diversity and antifungal activity of fluorescent pseudomonads isolated from rhizospheres of tea, gladiolus, carnation and black-gram grown in acidic soils with similar texture under similar climatic conditions were studied.
- Biochemical characterisation including antibiotic resistance assay, RAPD and PCR–RFLP studies revealed a largely homogenous population.
- At soil pH (5.2), the isolates exhibited growth with varying levels of siderophore production, irrespective of crop rhizospheres. Two isolates with maximum chitinase production showed antagonism. However, increased pH levels beyond 5.2 caused reduction in metabolite production with reduced antifungal activity.
- The homogeneity of the bacterial population irrespective of crop rhizospheres together with decreased secondary metabolite production at higher pH levels reinstated the importance of soil over host plant in influencing rhizosphere populations. The studies also yielded acid tolerant chitinase producing antagonistic fluorescent pseudomonads.
6.3 Identification of chitinase(s) from selected fluorescent pseudomonad

- The role of extracellular chitinases in antagonism of fluorescent pseudomonads was also assessed using chitin amended media as an indicator (substrate specificity assay). The bacteria were studied for their ability to grow and produce chitinases on different substrates.
- Bacterial cells grown on chitin-containing media showed enhanced growth and enzyme production with increased anti-fungal activity against the pathogen. Furthermore, the cell-free bacterial culture filtrate from chitin-containing media also significantly inhibited the mycelial growth.
- Both the strains and their cell-free culture filtrate from chitin-amended media showed the formation of lytic zones on chitin agar, indicating chitinolytic ability.
- Extracellular proteins of highly antagonistic bacterial strain were isolated from cell-free extracts of media amended with chitin and fungal cell wall. Western blot or in-gel assays detected multiple isoforms of chitinases in these cell-free conditioned media.

6.4 Overexpression and characterisation of chitinase from selected fluorescent pseudomonad

- The chitinolytic and antagonistic *P. putida* strain P3(4) of pea rhizosphere soil was used to clone the glycosyl hydrolase (GH5) gene specific for chitin and chitosan and overexpress in *Escherichia coli* for biochemical and functional characterisation of the recombinant protein.
- Standard bacteriological tests and sequencing of the 16S rRNA indicated the taxonomic affiliation of the isolate to *Pseudomonas putida*, a member of fluorescent pseudomonads.
- PCR primers specific for glycosyl hydrolase family 5 (GH5) of *Pseudomonas putida* isolate KT2440 amplified a 947 bp fragment of the GH5 gene from P3(4).
- Cloning of this gene into *Escherichia coli* M15 using an expression vector pQE-30UA and screening on chitin and chitosan detection agar identified one positive clone (Pchі\(^+\)).
Sequence analysis of the cloned insert revealed an open reading frame of 947 nucleotides corresponding to a protein of 315 amino acids with a predicted molecular mass of 38.0 kDa.

The deduced amino acid sequence of the open reading frame (gene product/GH) showed 83-84% homology to the GH5 of *P. putida* strains F1 and KT2440, respectively.

The purified enzyme was homogenous, as examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and was visualized as single fluorescent band in native gel assay with 4-methylumbelliferyl-N-acetyl-β-D-glucosaminide and glycol chitosan, respectively.

For hydrolysis of 4-nitrophenyl-N-acetyl-β-D-glucosaminide (pNP-(GlcNAc) and colloidal chitosan, the enzyme had an optimal temperature of 40°C, and was stable within the temperature range of 10°C to 40°C. The enzyme showed an optimal pH of 3.5, with maximum stabilities at 5.0 and 5.5 for hydrolysis of pNP-(GlcNAc) and colloidal chitosan, respectively. Fe$^{3+}$ and Cu$^{2+}$ stimulated chitinase and chitosanase activities by 74.20 and 51.38%, respectively.

The purified glycosyl hydrolase displayed 70 and 45% inhibition of spore germination of the pathogenic fungi, *Fusarium oxysporum* f.sp. *dianthi* and *Alternaria solani*.

In conclusion, this study indicates that the bifunctional 38.0 kDa GH5 is a hitherto unexplored bifunctional enzyme exhibiting antifungal activity. Therefore, this enzyme is a good candidate for biotechnological application to produce biopesticide and generate oligosaccharide elicitors from chitin or chitosan. Further, GH5 gene can be utilized (candidate) to design appropriate strategies for transgenic resistance in crop plants to combat fungal pathogens and to improve biocontrol strains producing secondary metabolites, although further characterisation in this regard remains to be done.