

CONTENTS

1. Introduction	1-7
2. Review of Literature	8-45
3. Materials and methods	46-83
3.1 Plant Material	46
3.1.1 Collection	46
3.1.2 Maintenance in glass house condition	46
3.1.3 Maintenance in field condition	46
3.2 Fungal culture	49
3.2.1 Isolation and maintenance	49
3.2.2 Morphological and microscopic observation	49
3.2.3 Completion of Koch's postulate	49
3.2.4 Assessment of mycelia growth	49
3.2.4.1 Solid media	50
3.2.4.2 Liquid media	50
3.3 Soluble protein	50
3.3.1 Extraction of soluble protein	50
3.3.1.1 Fungal mycelia	50
3.3.1.2 Leaf	50
3.3.2 Estimation of soluble protein content	50
3.4 SDS-PAGE analysis of soluble protein	51
3.4.1 Preparation of stock solution	52
3.4.2 Preparation of gel	53
3.4.3 Sample preparation	53
3.4.4 Electrophoresis	53
3.4.5 Fixing and staining	53
3.5 Preparation of antigen	54
3.5.1 Fungal Antigen	54
3.5.2 Leaf antigen	54
3.5.2.1 Healthy leaf	54
3.5.2.2 Artificially inoculated leaf	55
3.5.2.3 Naturally infected leaf	55
3.5.3 AMF antigen	55
3.6 Serology	55
3.6.1 Rabbits and their maintenance	55
3.6.2 Immunization	55
3.6.3 Bleeding	56
3.6.4 Purification of IgG	56
3.6.4.1 Precipitation	56
3.6.4.2 Column preparation	56
3.6.4.3 Fraction collection	57
3.7 Immunological assays	57
3.7.1 Agar gel double diffusion	57

3.7.1.1	Preparation of agarose slides	57
3.7.1.2	Diffusion	57
3.7.1.3	Washing, staining and drying of slides	57
3.7.2	Plate –Trapped antigen coated (PTA) ELISA	58
3.7.3	Dot Immunobinding assay	58
3.7.4	Western blot analysis	59
3.7.5	Fluorescence antibody staining and microscopy	59
3.7.5.1	Fungal mycelia	59
3.7.5.2	Conidia	60
3.7.5.3	Cross section of som leaves	60
3.7.5.4	AMF in som roots	61
3.8	Isolation of genomic DNA	61
3.8.1	Preparation of extraction buffer	61
3.8.2	Genomic DNA extraction	61
3.8.3	Purification of genomic DNA	61
3.8.4	Agarose gel electrophoresis to check DNA quality	62
3.8.4.1	Preparation of sample for DNA electrophoresis	62
3.8.4.2	Run gel for electrophoresis for DNA fraction	62
3.9	ITS-PCR Analysis	62
3.9.1	ITS-PCR primers	63
3.9.2	Amplification conditions	63
3.9.3	Sequencing of rDNA gene	63
3.10	BLAST analysis of sequence	63
3.11	Submission of rDNA gene to NCBI gene bank	63
3.12	Multiple sequence alignment and Phylogenetic analysis	63
3.13	RAPD PCR analysis	64
3.13.1	RAPD-PCR primers	64
3.13.2	Amplification conditions	65
3.13.3	Analysis of RAPD band patterns	65
3.13.4	Scoring of individual bands	65
3.13.5	Reconstruction of the phylogenetic tree	65
3.13.5.1	UPGMA method	65
3.14	Denaturing Gradient Gel Electrophoresis (DGGE)	65
3.14.1	PCR amplification of genomic DNA of the isolates for DGGE analysis	65
3.14.2	DGGE of the PCR products	66
3.14.2.1	Reagents and solutions required for DGGE analysis	66
3.14.2.2	Creating the gel sandwich	67
3.14.2.3	Preparation of the gel	67
3.14.2.4	Running the gel	67
3.14.2.5	Staining gels and photography	68
3.14.2.6	Data analysis	68
3.14.2.6.1	Scoring of individual bands	68

3.14.2.6.2	UPGMA analysis of the DGGE bands	68
3.15	Assessment of disease caused by fungal pathogens on som plants	68
3.15.1	Detached leaf inoculation	68
3.15.2	Whole plant inoculation	69
3.16	<i>In vitro</i> testing for antagonism to fungal pathogens	69
3.16.1	Antifungal test of PGPR	69
3.16.2	Antifungal test of PGPF	70
3.17	Mass multiplication and application of bioinoculants and pathogen	
3.17.1	Arbuscular Mycorrhizal Fungi (AMF)	70
3.17.1.1	Isolation of AMF spores	70
3.17.1.2	Histopathology of som roots	71
3.17.1.3	Mass multiplication of AMF	71
3.17.2	Plant growth promoting fungi (PGPF)	72
3.17.2.1	Selection of PGPF	72
3.17.2.2	Mass multiplication	72
3.17.2.2.1	Wheat bran culture	72
3.17.2.2.2	Tricho-compost	72
3.17.3	Plant growth promoting rhizobacteria (PGPR)	72
3.17.3.1	Selection of PGPR	73
3.17.3.2	Mass multiplication	73
3.17.3.2.1	Soil drench	73
3.17.3.2.2	Foliar Spray	73
3.17.3.2.3	Talc based formulation	73
3.17.4	Vermicompost preparation	74
3.17.5	Application of different bioinoculants under pot and field condition	74
3.17.6	Inoculum preparation of pathogen	74
3.18	<i>In vivo</i> assessment of plant growth promotion	75
3.18.1	Assessment of plant growth following application of bioinoculants	75
3.18.2	Assessment of disease severity	75
3.18.3	Assessment of soil phosphate	75
3.18.3.1	Extraction of soil phosphate	75
3.18.3.2	Estimation of soil phosphate	75
3.19	Extraction and assay of defense enzyme activities after application of bioinoculants	75
3.19.1	β -1,3 Glucanase	75
3.19.2	Chitinase	76
3.19.3	Phenylalanine Ammonia lyase	77
3.19.4	Peroxidase	77
3.19.5	Isozyme analysis of peroxidase	77
3.19.5.1	Preparation of stock solution	77
3.19.5.2	Preparation of gel	78
3.19.5.3	Sample preparation	79

3.19.5.4	Electrophoresis	79
3.19.5.5	Fixing and Staining	79
3.19.6	Extraction and estimation of Phenol content	79
3.19.6.1	Extraction of phenol	79
3.19.6.2	Estimation of Total phenol	80
3.19.6.3	Estimation of Ortho-phenol	80
3.20	Analysis of antifungal compounds	80
3.20.1	Collection of leaf diffusates and their bioassay	80
3.20.2	High Performance Liquid Chromatography (HPLC)	81
3.21	Scanning Electron Microscopy (SEM)	81
3.22	Transmission Electron Microscopy (TEM)	81
3.22.1	Specimen preparation	81
3.22.1.1	Fixation	81
3.22.1.2	Dehydration	81
3.22.1.3	Infiltration	82
3.22.1.4	Embedding	82
3.22.2	View preparation	82
3.22.2.1	Trimming	82
3.22.2.2	Sectioning	82
3.22.3	Immunogold labeling	82
3.22.3.1	Primary antibody	82
3.22.3.2	Secondary antibody	82
3.22.4	Staining	82
3.22.5	Viewing	83
4.	Results	84-187
4.1	Foliar Fungal Disease incidence of som plants (<i>Persea bombycina</i> Kost)	84
4.1.1	Leaf Blight	84
4.1.2	Grey Blight	84
4.1.3	Completion of Koch postulate	87
4.2	Growth and spore characters of <i>Pestalotiopsis disseminata</i>	
4.2.1	Growth	87
4.2.2	Sporulation	88
4.2.3	Spore morphology	88
4.3	Growth and spore characters of <i>Colletotrichum gloeosporioides</i>	91
4.3.1	Growth	91
4.3.2	Sporulation	92
4.3.3	Spore morphology	92
4.4	Screening of resistance of som plants towards foliar fungal pathogens	
4.4.1	Detached leaf inoculation	95
4.4.1.1	<i>P. disseminata</i>	95
4.4.1.2	<i>C. gloeosporioides</i>	95
4.4.2	Whole plant inoculation	99
4.4.2.1	<i>P. disseminata</i>	99

4.4.2.2 <i>C. gloeosporioides</i>	99
4.5 Immunoassays for detection of <i>Pestalotiopsis disseminata</i> and <i>Colletotrichum gloeosporioides</i>	101
4.5.1 Soluble protein	101
4.5.2 Immunological assays	101
4.5.2.1 Optimization of PTA-ELISA	102
4.5.2.2 PTA-ELISA	102
4.5.2.3 Dot-immunobinding Assay	104
4.5.2.4 Western blot	104
4.5.2.5 Indirect Immunofluorescence	105
4.5.2.5.1 Mycelia	105
4.5.2.5.2 Spore	105
4.6 Detection of major cross reactive antigens shared by <i>Persea bombycina</i> and foliar fungal pathogens	110
4.6.1 PTA-ELISA	110
4.6.2 Cellular localization of CRA in som leaf tissue using indirect immunofluorescence	111
4.6.3 Cellular localization of CRA in som leaf tissue following immunogold labeling	111
4.7 Serological detection of <i>P. disseminata</i> and <i>C. gloeosporioides</i> in som leaf tissue	116
4.7.1 Natural infection	116
4.7.1.1 PTA-ELISA	116
4.7.1.2 Dot blot	116
4.7.2 Detection of foliar fungal pathogens in leaf tissues following artificial inoculation	119
4.7.2.1 PTA-ELISA	119
4.7.2.2 Dot-blot	119
4.7.3 Immunolocalization of pathogen in infected leaf tissues	121
4.7.3.1 Indirect immunofluorescent antibody staining of infected leaves	121
4.7.3.2 Immunogold labeling of blight infected leaves	121
4.8 Mycorrhizal association of som plants and immunolocalization of Arbuscular Mycorrhizal Fungi in root tissues	126
4.8.1 Mycorrhizal association in roots of som plants	126
4.8.2 Histopathology and colonization of roots with AMF	126
4.8.3 Immunolocalization of AMF in som root tissue	131
4.8.3.1 Indirect immunofluorescence	131
4.8.3.2 Immunogold labeling	131
4.9 Molecular detection of foliar fungal pathogens of som plants	134
4.9.1 <i>Colletotrichum gloeosporioides</i>	134
4.9.1.1 18S rDNA sequence analysis	134
4.9.1.2 Multiple sequence alignment	134
4.9.1.3 Phylogenetic analysis	134

4.9.1.4	Species specific primer for identification of <i>C. gloeosporioides</i>	137
4.9.2	<i>Pestalotiopsis disseminata</i>	139
4.9.2.1	18S rDNA sequence analysis	139
4.9.2.2	Multiple sequence alignment	139
4.9.2.3	Phylogenetic analysis	139
4.10	Molecular characterization of isolates of foliar fungal pathogens	
4.10.1	RAPD analysis	144
4.10.2	DGGE analysis	144
4.11	Determination of activity of defense enzymes in som leaves infected naturally with foliar fungal pathogens	147
4.11.1	Peroxidase	147
4.11.1.1	Peroxyzyme analysis	147
4.11.2	Phenylalanine ammonia lyase	150
4.11.3	Chitinase	150
4.11.4	β 1,3 Glucanase	150
4.12	Analysis of diffusable compounds in som following natural infection with pathogens	150
4.12.1	Changes in levels of phenolics in healthy and infected som leaves	
4.12.1.1	Total phenol	150
4.12.1.2	Ortho-dihydroxy phenol	150
4.12.2	Studies on biological activities of leaf diffusates of som	151
4.12.3	High Performance Liquid Chromatography	152
4.13	Antagonistic activity of selected bioinoculants against fungal pathogens	155
4.13.1	Plant Growth Promoting Rhizobacteria (PGPR)	155
4.13.2	Plant Growth Promoting Fungi (PGPF)	157
4.14	Growth promotion and biochemical changes in som plants following application of bioinoculants	159
4.14.1	Application of PGPR and AMF	159
4.14.1.1	Growth promotion	159
4.14.1.2	Biochemical changes	159
4.14.2	Application of Vermicompost with value addition (PGPR, AMF)	
4.14.2.1	Growth promotion	163
4.14.2.2	Biochemical changes	163
4.14.3	Application of PGPR, PGPF and AMF	168
4.14.3.1	Growth promotion	168
4.14.3.2	Biochemical changes	168
4.15	Activation of defense response of som plants against phytopathogens following application of bioinoculants	168
4.15.1	Effect of PGPR, PGPF and AMF against <i>Colletotrichum gloeosporioides</i>	168

4.15.1.1	Disease suppression	172
4.15.1.2	Changes in defense enzymes	172
4.15.2	Effect of Vermicompost, PGPR and AMF against <i>Pestalotiopsis disseminata</i>	172
4.15.2.1	Disease suppression	174
4.15.2.2	Changes in defense enzymes	174
4.16	Induction of resistance in field grown som plants against <i>Colletotrichum gloeosporioides</i>	175
4.16.1	Growth promotion	175
4.16.2	Biochemical changes	177
4.16.3	HPLC analysis of phenolics	177
4.16.4	Changes in defense enzyme after artificial inoculation	179
4.17	Cellular localization of glucanase and chitinase in leaf and root tissues of som plant following induction of resistance using bioinoculants	182
4.17.1	Cellular localization of glucanase	182
4.17.1.1	Indirect immunofluorescence	182
4.17.1.2	Immunogold labelling	182
4.17.2	Cellular localization of chitinase	187
4.17.2.1	Indirect immunofluorescence	187
4.17.2.2	Immunogold labeling	187
5.	Discussion	192-204
6.	Conclusion	205-208
7.	Bibliography	209-238