

Contents

1	Detection and Diversity of Fungi from Environmental Samples:	
	Traditional Versus Molecular Approaches	1
	<i>R. Jeewon and K.D. Hyde</i>	
1.1	Introduction	1
1.2	Microscopy and Culture-Based Methods	2
1.3	Molecular-Based Methods	4
1.4	The Nuclear-Encoded Ribosomal DNA Gene: Phylogenetic and Systematic Value	5
1.5	Denaturing Gradient Gel Electrophoresis: Applicability, Usefulness and Bias	7
1.6	Conclusions and Future Directions	11
	References	11
2	Functional Genomic Approaches for Mycorrhizal Research	17
	<i>A. K. Pandey, H. White, and G.K. Podila</i>	
2.1	Introduction	17
2.2	Yeast Two Hybrid: An Approach for Understanding Signaling Pathways	18
2.3	<i>Agrobacterium</i> -Mediated Transformation in <i>Laccaria bicolor</i> ...	22
2.4	Materials and Methods	24
2.4.1	Interaction Studies of <i>Laccaria bicolor</i> with Aspen (<i>Populus tremuloides</i>) Seedlings	24
2.4.2	Yeast Two-Hybrid Protocol	26
2.4.3	<i>Agrobacterium</i> -Mediated Transformation in <i>Laccaria bicolor</i>	28
	References	30
3	Automated Fluorescence Sequencing and Troubleshooting	35
	<i>S. Gochhait, D. Malhotra, E. Rai, and R.N.K. Bamezai</i>	
3.1	Introduction	35
3.2	Evolution of the Method	36
3.2.1	Manual Sequencing	36

3.2.2	Automated Sequencing	37
3.2.3	Pyrosequencing	39
3.3	Methods	39
3.3.1	Template Preparation	39
3.3.2	Reaction Setup (BigDye Terminator Cycle Sequencing)	40
3.3.3	Performing Cycle Sequencing	41
3.3.4	Preparing Extension Products for Electrophoresis	42
3.4	Trouble Shooting	43
3.4.1	Problem: Flat Line or “Dead On Analysis”	43
3.4.2	Problem: Noisy Data (Background)	45
3.4.3	Problem: Reading Near the Primer	46
3.4.4	Problem: Strong Terminator Peaks	46
3.4.5	Problem: Low Intensity of Shorter Products	48
3.4.6	Problem: Longer Fragments Missing	49
3.4.7	Problem: Presence of Spikes	49
3.4.8	Problem: Weaker Signals	49
	References	50
4	mRNA Quantitation Using Real Time PCR	53
	<i>S. Gochhait, S.I. Bukhari, and R.N.K. Bamezai</i>	
4.1	Introduction	53
4.2	Methods	54
4.2.1	Chemistry and Primer/Probe Design	54
4.2.2	RNA Isolation from the Sample	57
4.2.3	Reverse Transcription	58
4.2.4	Real Time PCR Set Up	59
4.2.5	Instrumentation	61
4.2.6	Data Analysis	67
4.3	Notes	68
	References	71
5	Laboratory Practice for the Production of Polyclonal and Monoclonal Antibodies	73
	<i>S. Khurana, S. Bhaskar, and A. Mukhopadhyay</i>	
5.1	Introduction	73
5.2	Production of Polyclonal Antibodies	74
5.2.1	Choice of Animal and Method of Immunization	74
5.2.2	Preparation for Immunization	75
5.2.3	Production of Polyclonal Antibodies	76
5.2.4	Materials and Equipment	77
5.3	Production of Monoclonal Antibodies	77
5.3.1	Immunization of Mice or Rats	77
5.3.2	Myeloma Cell Culture	78
5.3.3	Setup for Fusion of Myeloma with Spleen Cells	79
5.3.4	Selection and Cloning of Hybridoma	79

5.4	5.3.5 Production of Monoclonal Antibodies	80
	5.3.6 Materials and Equipment	81
	5.4 Purification of Antibody	82
	5.4.1 Purification of IgG by Precipitation with Ammonium Sulfate	83
	5.4.2 Purification of IgG by DEAE-Sepharose Chromatography	83
	5.4.3 Purification of IgG Using Immobilized Protein A	84
5.5	5.5 Analysis of Purity of IgG by Electrophoresis	86
	5.5.1 Materials and Equipment	88
5.6	5.6 Enzyme-Linked Immunosorbent Assay	88
	5.6.1 Materials and Equipment	90
5.7	5.7 Conclusion	90
	References	90
6	6 Modern Techniques for Analyzing Immunological Responses	93
	<i>Satish Khurana, Sangeeta Bhaskar, and Asok Mukhopdhyay</i>	
6.1	6.1 Introduction	93
6.2	6.2 Type of Immune Responses	93
	6.2.1 Innate Immune Response	94
	6.2.2 Adaptive Immune Response	94
6.3	6.3 Adaptive Immune System	94
	6.3.1 Humoral Immune System	95
	6.3.2 Cellular Immune System	96
6.4	6.4 Different Assay Systems to Study the Adaptive Immune Response	96
	6.4.1 Mixed Lymphocyte Proliferation Assays	96
	6.4.2 Detection of Type of T Helper Responses (Th1/Th2) ..	98
	6.4.3 Cytotoxic T Lymphocyte Activity	99
6.5	6.5 Flow Cytometric Analysis of Immune Cells	102
	6.5.1 Materials and Equipment	105
6.6	6.6 Magnetic Activated Cell Sorting	105
	6.6.1 Materials and Equipment	107
6.7	6.7 Isolation of Mononuclear Cells from Peripheral Blood	107
	6.7.1 Materials and Equipment	108
6.8	6.8 Conclusions	108
	References	108
7	7 Transcriptome Analysis	111
	<i>S.K. Yadav, S.L. Singla-Pareek, and A. Pareek</i>	
7.1	7.1 Introduction	111
7.2	7.2 RNA Preparation	113
7.3	7.3 Northern Analysis	113
	7.3.1 Principle	113
	7.3.2 Procedure	114

7.3.3 Applications	116
7.4 In Situ Hybridization	116
7.4.1 Principle	116
7.4.2 Procedure	117
7.4.3 Applications	119
7.5 Dot Blot and Slot Blot	120
7.5.1 Principle	120
7.5.2 Procedure	120
7.5.3 Applications	123
7.6 Reverse Transcriptase–Polymerase Chain Reaction	123
7.6.1 Principle	123
7.6.2 Procedure	124
7.6.3 Application of RT-PCR	125
7.7 DNA Microarray	126
7.7.1 Principle	126
7.7.2 Procedure	127
7.7.3 Applications	129
7.8 Conclusions	129
References	130
8 RNAi Technology: a Tool for Functional Validation of Novel Genes <i>R. Karan, S. Kumari, S.K. Yadav, and A. Pareek</i>	133
8.1 Introduction	133
8.2 Machinery Involved in RNAi	134
8.2.1 Inducer	135
8.2.2 Dicer	135
8.2.3 RNA-Dependent RNA Polymerase	135
8.2.4 RNA-Induced Silencing Complex	135
8.2.5 miRNA and siRNA	136
8.3 RNAi as a Tool of Functional Genomics	136
8.3.1 Production of dsRNA	137
8.3.2 Constitutive and Inducible RNAi	138
8.3.3 Antisense RNA and RNAi	140
8.4 Potential Areas of Application	140
8.5 Conclusions	142
References	142
9 Molecular Matchmaking: Techniques for Biomolecular Interactions <i>R. Oberoi, P. Kumar, and S.K. Lal</i>	145
9.1 Introduction	145
9.2 Tools for the Study of Protein–Protein Interactions	145
9.2.1 The Two-Hybrid System	147
9.2.2 The Split-Ubiquitin System	148

9.2.3	Reverse Two-Hybrid System	148
9.2.4	Sos Recruitment System (Cyto Trap Yeast Two-Hybrid System)	148
9.2.5	Yeast One-Hybrid System	149
9.2.6	Double Interaction Screen	149
9.2.7	Yeast Three-Hybrid or Tri-Hybrid System	150
9.3	Procedure	150
9.3.1	Reagents, Materials, and Equipment	151
9.3.2	Notes and Points to Watch	152
	References	152
10	Environmental Proteomics: Extraction and Identification of Protein in Soil	155
	<i>Z. Solaiman, M. A. Kashem and I. Matsumoto</i>	
10.1	Introduction	155
10.2	Sample Preparations	156
10.3	Protocols for Protein Extraction from Soil	157
10.3.1	Extraction of Extracellular Protein	157
10.3.2	Extraction of Whole-Cell Protein	157
10.4	Protein Loading	158
10.5	Protein Expression Analyses	158
10.5.1	SDS-PAGE	158
10.5.2	Two-Dimension SDS-PAGE Analysis	158
10.6.	Gel Staining	161
10.6.1	Coomassie Brilliant Blue Staining Protocol (For Mini Gels)	162
10.6.2	Silver Staining Protocol	162
10.7	Image Analysis	163
10.8	Spot Cut	163
10.9	Protein Digestion	163
10.10	Mass Spectrometry Analysis	164
10.11	Spectral Analysis	164
10.12	N-Terminal Amino Acid Sequencing	165
10.13	Conclusions	165
	References	166
11	DGGE and RISA Protocols for Microbial Community Analysis in Soil	167
	<i>Z. Solaiman and P. Marschner</i>	
11.1	Introduction	167
11.2	Soil DNA Extraction	168
11.2.1	Equipment	168
11.2.2	Chemicals	168
11.2.3	DNA Extraction Protocol	168

11.3	Polymerase Chain Reaction Protocol for DGGE	170
11.3.1	First Round PCR	170
11.3.2	GC Clamp 16S PCR (Second Round PCR)	171
11.4	DGGE Techniques	172
11.4.1	Equipment	172
11.4.2	Chemicals	173
11.4.3	Assembling the Gel Chamber	173
11.4.4	Casting the Gel	174
11.4.5	Loading of the Samples	175
11.4.6	Staining and Imaging of the Gels	175
11.5	Ribosomal Intergenic Spacer Analysis	175
11.5.1	Equipment	176
11.5.2	Chemicals	176
11.5.3	PCR Protocol	176
11.5.4	Gel Preparation and Loading	177
11.5.5	Gel Running	178
11.5.6	Staining and Imaging of the Gels	178
11.6	Data Analysis	178
11.7	Conclusions	179
	References	179
12	Soil Microbial Community Structure and Function Assessed by FAME, PLFA and DGGE – Advantages and Limitations	181
	<i>P. Marschner</i>	
12.1	Introduction	181
12.2	Microbial Community Structure Based on Fatty Acid Patterns	182
12.2.1	FAME Extraction and Data Analysis	183
12.2.2	PLFA Analysis	185
12.2.3	Advantages and Limitations of Fatty Acid Patterns	188
12.3	Denaturing Gradient Gel Electrophoresis	189
12.3.1	DNA Extraction from Soil	189
12.3.2	Polymerase Chain Reaction	190
12.3.3	DGGE Procedures	192
12.4	Conclusions	196
	References	197
13	Measurement of Microbial Biomass and Activity in Soil	201
	<i>Z. Solaiman</i>	
13.1	Introduction	201
13.2	Protocols for Microbial Biomass Determination	202
13.2.1	Chloroform Fumigation–Extraction Method for Microbial Biomass C and N	202
13.2.2	Hexanol Extraction Method for Microbial P	205
13.3	Protocol for Total Microbial Activity Determination	207
13.3.1	Equipment	207

13.3.2 Reagents	207
13.3.3 Protocol for Extraction	208
13.4 Protocol for Soil Dehydrogenase Enzyme Analysis	208
13.4.1 Equipment	209
13.4.2 Reagents	209
13.4.3 Protocol for Extraction	209
13.5 Conclusions	209
References	210
14 Immuno-Technology for the Localization of Acid Phosphatase Using Native Gel Bands in <i>Piriformospora indica</i> and Other Soil Microorganisms	213
<i>R. Malla, U. Pokharel, R. Prasad, R. Oelmüller, and A. Varma</i>	
14.1 Introduction	213
14.1.1 Taxonomic Status	213
14.1.2 Phosphatases	214
14.2 Immunotechnology for the Detection and Localization of Acid Phosphatase in <i>P. indica</i>	216
14.2.1 Extraction of Protein and Enzyme Assay	216
14.2.2 Purification of Protein by Column Chromatography ..	217
14.2.3 Purification of Protein by Ion Exchange Chromatography	217
14.2.4 Native Polyacrylamide Gel Electrophoresis	219
14.2.5 Detection of Enzyme in Native PAGE	220
14.2.6 Isolation of Acid Phosphatase for Raising Antibody ..	222
14.2.7 Production of Antibodies using Acid Phosphatase in Native Gel	222
14.2.8 Antiserum Preparation	224
14.2.9 Purification of Immunoglobulin from Serum	225
14.2.10 Western Blot	226
14.2.11 Immuno-Fluorescence	228
14.2.12 Localization of ACPase by Immunogold Technique ..	228
14.3 Troubleshooting	232
14.4 Conclusions	232
References	232
15 Use of Short Oligonucleotide Primers in Random Amplified Polymorphic DNA Techniques for Species Identification	237
<i>R. Malla and A. Varma</i>	
15.1 Introduction	237
15.2 Polymorphism between <i>Piriformospora indica</i> and <i>Sebacina vermicifera</i> , Members of the Order Sebacinales	239
15.3 General Protocol for RAPD Technique to Show Polymorphism	241
15.3.1 Experimental Procedures	242

15.4	Troubleshooting	244
15.5	Conclusions	244
	References	245
16	Co-Cultivation with Sebacinales	247
	<i>A.C. Kharkwal, R. Prasad, H. Kharkwal, A. Das, K. Bhatnagar, I. Sherameti, R. Oelmüller, and A. Varma</i>	
16.1	Introduction	247
16.2	Sebacinaceae – Novel Fungi	248
16.3	Host Spectrum	249
16.4	Functions of the Sebacinaceae	251
16.5	Eco-Functional Identity	252
16.6	Axenic Co-Cultivation of Sebacinaceae	254
16.6.1	Procedure	254
16.6.2	Protocol	256
16.7	Media Compositions	256
16.8	Seed Surface Sterilization and Germination	261
16.8.1	Protocol for Seed Surface Sterilization	262
16.8.2	Inoculum Placement in the Pots	262
16.8.3	Results	262
16.9	Comparative Study on Plant Growth with Treated Endosymbionts	264
16.10	In Vivo Co-Cultivation of Sebacinales	264
16.11	Conclusions	266
	References	267
17	Quantitative Histochemistry: a Forgotten Tool with New Applications	271
	<i>R. Hampp and S. Haag</i>	
17.1	Introduction	271
17.2	Sample Preparation and Handling	272
17.3	Microphotometry	274
17.4	Biochemical Analysis: Real Time Microassays	276
17.5	Spatial Resolution of Basic Steps of Fungal Trehalose Metabolism in Symbiosis	277
	References	279
18	Ion Cyclotron Resonance Fourier Transform Mass Spectrometry for Non-Targeted Metabolomics of Molecular Interactions in the Rhizosphere	281
	<i>P. Schmitt-Kopplin, N. Hertkorn, M. Frommberger, M. Lucio, M. Englmann, A. Fekete, and I. Gebefugi</i>	
18.1	Introduction	281
18.2	The Chemical Biology Approach	282

18.3	Complementary Analytical Approaches	283
18.3.1	Targeted Analysis	284
18.3.2	Metabolite Profiling	285
18.3.3	Non-Targeted Analysis	285
18.4	Resolving Structural Information from Molecular Complexity with ICR-FT/MS	286
18.4.1	Top-Down Approach: From ICR-FT/MS-Profiling Analysis to Structural Hypothesis	288
18.4.2	Complementary Analytical Tools	290
18.4.3	Bottom-Up Approach: From Hypothesis-Driven Experiments Upwards to ICR-FT/MS	290
18.5	Conclusion	292
	References	292
19	Application of Terminal-Restriction Fragment Length Polymorphism for Molecular Analysis of Soil Bacterial Communities	295
	<i>A. Mengoni, E. Giuntini, and M. Bazzicalupo</i>	
19.1	Introduction	295
19.2	A General Protocol for Taxonomic T-RFLP Profiling of Soil Bacterial Communities	297
19.2.1	Materials	297
19.2.2	Experimental Procedure	298
19.2.3	Troubleshooting	299
19.3	Standardization of T-RFLP Profiles	299
19.4	Other Applications of T-RFLP to Soil Bacterial Communities	301
19.5	Conclusions	302
	References	302
20	Molecular Symbiotic Analysis Between <i>Arabiopsis thaliana</i> and <i>Piriformospora indica</i>	307
	<i>B. Shahollari, K. Bhatnagar, I. Sherameti, A. Varma, and R. Oelmüller</i>	
20.1	Introduction	307
20.2	Beneficial Interaction Between Plants and Fungi: <i>Piriformospora indica</i> and <i>Arabidopsis thaliana</i> as a Model System	308
20.3	Co-Cultivation of <i>P. indica</i> and <i>Arabidopsis</i> under Standardized Growth Conditions	309
20.4	Map-Based Cloning of a Mutated Gene	312
20.5	Rapid DNA Extraction	313
20.6	Confirmation of a Mutated Phenotype of an EMS Mutant by the Analysis of an Independent T-DNA Insertion Line	313
20.7	Differential Display to Identify Genes which are Regulated in Response to <i>P. indica</i>	314
20.8	Activation Tagged Lines	315

20.9	Identification of Biochemical Pathways in <i>A. thaliana</i> which are Regulated by <i>P. indica</i>	317
	References	317
21	Biophysical Phenomics Reveals Functional Building Blocks of Plants Systems Biology: a Case Study for the Evaluation of the Impact of Mycorrhization with <i>Piriformospora indica</i>	319
	<i>R.J. Strasser, M. Tsimilli-Michael, D. Dangre, and M. Rai</i>	
21.1	Introduction	319
21.2	Biophysical Phenomics of the Fast Fluorescence Rise O-J-I-P	320
21.2.1	The Energy Cascade in the Photosynthetic Apparatus	320
21.2.2	Microstates – Functional Building Blocks of Photosynthesis	320
21.2.3	Measuring Fluorescence Transients with PEA, Handy-PEA and FIM- Fluorimeters	322
21.2.4	How Fluorescence Kinetics Provide an Insight to the Microstates – Functional Blocks of PSII	323
21.3	Case Study	332
21.3.1	Mycorrhization and the Advantages of <i>Piriformospora indica</i> , an Emerging Growth Booster	332
21.3.2	Phenomics of the O-J-I-P Fluorescence Transient for the Study of Cadmium Stress on Chick Peas (<i>Cicer arietinum</i> L. Chafa variety) With and Without Symbiosis With <i>Glomus mosseae</i> , <i>G. caledonium</i> and <i>Piriformospora indica</i>	333
21.3.3	Correlation of Physiological with Biophysical Parameters	337
21.4	Conclusions	338
	References	338
22	Analysis of the Plant Protective Potential of the Root Endophytic Fungus <i>Piriformospora indica</i> in Cereals	343
	<i>F. Waller, B. Achatz, and K.-H. Kogel</i>	
22.1	Introduction	343
22.2	Plant Responses and Resistance to Pathogens	344
22.2.1	Local Reactions	344
22.2.2	Systemic Reactions and Resistance in Cereals	344
22.2.3	Beneficial Microbial Endophytes Protecting Cereals from Pathogens	345
22.3	Interaction of <i>P. indica</i> with Cereals	345
22.3.1	<i>P. indica</i> Colonizes Root Cortical Cells in Barley	346
22.3.2	<i>P. indica</i> Enhances Biomass and Yield in Barley	346
22.4	Approaches to Study the Mechanism of <i>P. indica</i> -Induced Pathogen Resistance	347

22.4.1	<i>P. indica</i> Induces Disease Resistance Against Root Pathogens	347
22.4.2	<i>P. indica</i> Induces Systemic Disease Resistance	348
22.4.3	Assessment of the Antioxidant Capacity of <i>P. indica</i> -Infested Roots	350
22.4.4	Gene Expression Induced by <i>P. indica</i> in Barley Leaves	351
22.5	Conclusions	351
	References	352
23	Members of Sebacinales Confer Resistance Against Heavy Metal Stress in Plants	355
	<i>N. Hahn, A. Varma, R. Oelmüller, and I. Sherameti</i>	
23.1	Introduction	355
23.2	Scientific Background	355
23.3	Differential Display to Understand Cd ²⁺ Resistance Mediated by Endophytic Fungi	357
23.4	Studies on Protein Level	357
23.4.1	Two-Dimensional Gel Electrophoresis, Preparation of Proteins	359
23.4.2	Mass Spectrometry, Preparation of Samples by Tryptic Digestion	359
	References	360
24	Screening of Plant Growth-Promoting Rhizobacteria	363
	<i>C.S. Nautiyal and S.M. DasGupta</i>	
24.1	Introduction	363
24.2	Candidature for Being a Rhizobacteria	364
24.3	Screening Methods	365
24.3.1	Criteria for Screening	365
24.3.2	Selection of Screening Methods	365
24.3.3	Classic Methods	366
24.3.4	Modern Methods	368
24.3.5	Molecular Methods	370
24.4	Metagenomics	371
24.5	Tracking of GEMs	372
24.6	Conclusions	372
	References	373
25	Research Methods in Arbuscular Mycorrhizal Fungi	377
	<i>A. Gaur and A. Varma</i>	
25.1	Introduction	377
25.2	Assessment of AM Fungal Propagules in Soil	378
25.2.1	Soil Sampling	378

25.2.2	Spore Extraction	378
25.2.3	Quantification of Spore Numbers	379
25.2.4	Infectivity Assays	379
25.2.5	Identification of AM Fungi	380
25.2.6	Use of Fatty Acids for Identification of AM Fungi	381
25.3	Quantification of AM Fungal Root Colonization in Root	382
25.3.1	Clearing and Staining Roots	382
25.3.2	Modifications of Staining Procedure	383
25.3.3	Measurement of Root Colonization by AM Fungi	384
25.4	Extraction and Quantification of Extra-Radical Mycelium of AM Fungi in Soils	384
25.5	Assessment of Growth Response of Effective Isolates	385
25.6	Inoculum Production of AM Fungi	386
25.6.1	On-Farm Production of AM Fungi	386
25.6.2	Traditional Culture Methods	387
25.6.3	AM Fungal Culture Using Aeroponic and Hydroponic Culture	388
25.6.4	Monoaxenic Culture of AM Fungi	389
25.6.5	Storage of AM Fungal Inoculum	390
25.7	Conclusions	390
	References	391
26	Field Trials of Bioinoculants	397
	<i>I. Ortas and A. Varma</i>	
26.1	Introduction	397
26.2	Effect of Mycorrhizal Infection on Nutrient Uptake	398
26.3	Effect of Soil Fumigation and Mycorrhizal Inoculation on Plant Growth Under Field Conditions	399
26.4	Effect of Mycorrhizal Inoculation on Plant Growth and Nutrient Uptake under Non-Sterile Field Conditions	403
26.5	Soil and Crop Management System	408
26.6	Inoculation Techniques	409
26.7	Conclusion	411
	References	412
	Subject Index	415