

Plant-Bacteria Interactions

Strategies and Techniques to Promote Plant Growth

Edited by

Iqbal Ahmad, John Pichtel, and Shamsul Hayat



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Contents

List of Contributors XIII

1	Ecology, Genetic Diversity and Screening Strategies of Plant Growth Promoting Rhizobacteria (PGPR)	1
	<i>Jorge Barriuso, Beatriz Ramos Solano, José A. Lucas, Agustín Probanza Lobo, Ana García-Villaraco, and F.J. Gutiérrez Mañero</i>	
1.1	Introduction	1
1.1.1	Rhizosphere Microbial Ecology	1
1.1.2	Plant Growth Promoting Rhizobacteria (PGPR)	3
1.2	Rhizosphere Microbial Structure	4
1.2.1	Methods to Study the Microbial Structure in the Rhizosphere	4
1.2.2	Ecology and Biodiversity of PGPR Living in the Rhizosphere	5
1.2.2.1	Diazotrophic PGPR	6
1.2.2.2	<i>Bacillus</i>	6
1.2.2.3	<i>Pseudomonas</i>	6
1.2.2.4	Rhizobia	6
1.3	Microbial Activity and Functional Diversity in the Rhizosphere	7
1.3.1	Methods to Study Activity and Functional Diversity in the Rhizosphere	7
1.3.2	Activity and Effect of PGPR in the Rhizosphere	8
1.4	Screening Strategies of PGPR	9
1.5	Conclusions	13
1.6	Prospects	13
	References	13
2	Physicochemical Approaches to Studying Plant Growth Promoting Rhizobacteria	19
	<i>Alexander A. Kamnev</i>	
2.1	Introduction	19
2.2	Application of Vibrational Spectroscopy to Studying Whole Bacterial Cells	20

2.2.1	Methodological Background	20
2.2.2	Vibrational Spectroscopic Studies of <i>A. brasilense</i> Cells	20
2.2.2.1	Effects of Heavy Metal Stress on <i>A. brasilense</i> Metabolism	20
2.2.2.2	Differences in Heavy Metal Induced Metabolic Responses in Epiphytic and Endophytic <i>A. brasilense</i> Strains	21
2.3	Application of Nuclear γ -Resonance Spectroscopy to Studying Whole Bacterial Cells	25
2.3.1	Methodological Background	25
2.3.2	Emission Mössbauer Spectroscopic Studies of Cobalt(II) Binding and Transformations in <i>A. brasilense</i> Cells	26
2.4	Structural Studies of Glutamine Synthetase (GS) from <i>A. brasilense</i>	29
2.4.1	General Characterization of the Enzyme	29
2.4.2	Circular Dichroism Spectroscopic Studies of the Enzyme Secondary Structure	30
2.4.2.1	Methodology of Circular Dichroism (CD) Spectroscopic Analysis of Protein Secondary Structure	30
2.4.2.2	The Effect of Divalent Cations on the Secondary Structure of GS from <i>A. brasilense</i>	31
2.4.3	Emission Mössbauer Spectroscopic Analysis of the Structural Organization of the Cation-Binding Sites in the Enzyme Active Centers	32
2.4.3.1	Methodological Outlines and Prerequisites	32
2.4.3.2	Experimental Studies of <i>A. brasilense</i> GS	33
2.4.3.3	Conclusions and Outlook	35
2.5	General Conclusions and Future Directions of Research	36
	References	37
3	Physiological and Molecular Mechanisms of Plant Growth Promoting Rhizobacteria (PGPR)	41
	<i>Beatriz Ramos Solano, Jorge Barriuso Maicas, and F.J. Gutiérrez Mañero</i>	
3.1	Introduction	41
3.2	PGPR Grouped According to Action Mechanisms	41
3.2.1	PGPR Using Indirect Mechanisms	42
3.2.1.1	Free Nitrogen-Fixing PGPR	42
3.2.1.2	Siderophore-Producing PGPR	44
3.2.1.3	Phosphate-Solubilizing PGPR	45
3.2.2	PGPR Using Direct Mechanisms	45
3.2.2.1	PGPR that Modify Plant Growth Regulator Levels	46
3.2.2.2	PGPR that Induce Systemic Resistance	50
3.3	Conclusions	51
3.4	Future Prospects	51
	References	52

4 **A Review on the Taxonomy and Possible Screening Traits of Plant Growth Promoting Rhizobacteria** 55

M. Rodríguez-Díaz, B. Rodelas, C. Pozo, M.V. Martínez-Toledo, and J. González-López

- 4.1 Introduction 55
- 4.2 Taxonomy of PGPR 56
- 4.3 Symbiotic Plant Growth Promoting Bacteria 63
 - 4.3.1 LNB 63
 - 4.3.1.1 Alphaproteobacteria 63
 - 4.3.1.2 Betaproteobacteria 67
 - 4.3.2 Bacteria Capable of Fixing Dinitrogen in Symbiosis with Plants Other Than Legumes 67
 - 4.3.2.1 Actinobacteria 68
 - 4.3.2.2 Cyanobacteria 68
 - 4.3.2.3 *Gluconacetobacter* 69
- 4.4 Asymbiotic Plant Growth Promoting Bacteria 69
 - 4.4.1 Alphaproteobacteria: Genera *Acetobacter*, *Swaminathania* and *Azospirillum* 69
 - 4.4.1.1 *Acetobacter* and *Swaminathania* 69
 - 4.4.1.2 *Azospirillum* 70
 - 4.4.2 Gammaproteobacteria 70
 - 4.4.2.1 Enterobacteria 70
 - 4.4.2.2 *Citrobacter* 70
 - 4.4.2.3 *Enterobacter* 70
 - 4.4.2.4 *Erwinia* 71
 - 4.4.2.5 The *Klebsiella* Complex 71
 - 4.4.2.6 *Kluyvera* 71
 - 4.4.2.7 *Pantoea* 72
 - 4.4.2.8 *Serratia* 72
 - 4.4.2.9 *Pseudomonas* 72
 - 4.4.2.10 *Azotobacter* (*Azomonas*, *Beijerinckia* and *Derxia*) 72
 - 4.4.3 Firmicutes. Genera *Bacillus* and *Paenibacillus* 73
 - 4.4.3.1 *Bacillus* 73
 - 4.4.3.2 *Paenibacillus* 73
- 4.5 Screening Methods of PGPR 74
 - 4.5.1 Culture-Dependent Screening Methods 74
 - 4.5.2 Culture-Independent Screening Methods 75
- 4.6 Conclusions and Remarks 75
- References 76

5 **Diversity and Potential of Nonsymbiotic Diazotrophic Bacteria in Promoting Plant Growth** 81

Farah Ahmad, Iqbal Ahmad, Farrukh Aqil, M.S. Khan, and S. Hayat

- 5.1 Introduction 81
- 5.2 Rhizosphere and Bacterial Diversity 82

5.2.1	Diazotrophic Bacteria	84
5.2.1.1	Symbiotic Diazotrophic Bacteria	85
5.2.1.2	Asymbiotic Diazotrophic Bacteria	86
5.3	Asymbiotic Nitrogen Fixation and Its Significance to Plant Growth	89
5.4	Plant Growth Promoting Mechanisms of Diazotrophic PGPR	90
5.5	Interaction of Diazotrophic PGPR with Other Microorganisms	93
5.5.1	Interaction of Diazotrophic PGPR with Rhizobia	93
5.5.2	Interaction of Diazotrophic PGPR with Arbuscular Mycorrhizae	96
5.6	Other Dimensions of Plant Growth Promoting Activities	97
5.6.1	ACC Deaminase Activity	97
5.6.2	Induced Systemic Resistance (ISR)	98
5.6.3	Improved Stress Tolerance	98
5.6.4	Quorum Sensing	99
5.7	Critical Gaps in PGPR Research and Future Directions	100
	References	102
6	Molecular Mechanisms Underpinning Colonization of a Plant by Plant Growth Promoting Rhizobacteria	111
	<i>Christina D. Moon, Stephen R. Giddens, Xue-Xian Zhang, and Robert W. Jackson</i>	
6.1	Introduction	111
6.2	Identification of Plant-Induced Genes of SBW25 Using IVET	113
6.3	Regulatory Networks Controlling Plant-Induced Genes	119
6.4	Spatial and Temporal Patterns of Plant-Induced Gene Expression	123
6.5	Concluding Remarks and Future Perspectives	126
	References	126
7	Quorum Sensing in Bacteria: Potential in Plant Health Protection	129
	<i>Iqbal Ahmad, Farrukh Aqil, Farah Ahmad, Maryam Zahin, and Javed Musarrat</i>	
7.1	Introduction	129
7.2	Acyl-HSL-Based Regulatory System: The Lux System	130
7.3	QS and Bacterial Traits Underregulation	132
7.4	QS in Certain Phytopathogenic Bacteria	137
7.4.1	<i>E. carotovora</i>	137
7.4.2	<i>R. solanacearum</i>	138
7.4.3	<i>Xanthomonas campestris</i>	138
7.4.4	Other Bacteria	139
7.5	Quorum-Sensing Signal Molecules in Gram-Negative Bacteria	139
7.5.1	Bioassays for the Detection of Signal Molecules	141
7.5.2	Chemical Characterization of Signal Molecules	142
7.6	Interfering Quorum Sensing: A Novel Mechanism for Plant Health Protection	144

7.7	Conclusion	147
	References	148
8	<i>Pseudomonas aurantiaca</i> SR1: Plant Growth Promoting Traits, Secondary Metabolites and Crop Inoculation Response	155
	<i>Marisa Rovera, Evelin Carlier, Carolina Pasluosta, Germán Avanzini, Javier Andrés, and Susana Rosas</i>	
8.1	Plant Growth Promoting Rhizobacteria: General Considerations	155
8.2	Secondary Metabolites Produced by <i>Pseudomonas</i>	156
8.3	Coinoculation Greenhouse Assays in Alfalfa (<i>Medicago sativa</i> L.)	157
8.4	Field Experiments with <i>P. aurantiaca</i> SR1 in Wheat (<i>Triticum aestivum</i> L.)	158
8.5	Conclusions	161
	References	161
9	Rice–Rhizobia Association: Evolution of an Alternate Niche of Beneficial Plant–Bacteria Association	165
	<i>Ravi P.N. Mishra, Ramesh K. Singh, Hemant K. Jaiswal, Manoj K. Singh, Youssef G. Yanni, and Frank B. Dazzo</i>	
9.1	Introduction	165
9.2	Landmark Discovery of the Natural Rhizobia–Rice Association	166
9.3	Confirmation of Natural Endophytic Association of Rhizobia with Rice	168
9.4	Association of Rhizobia with Other Cereals Like Wheat, Sorghum, Maize and Canola	170
9.5	Mechanism of Interaction of Rhizobia with Rice Plants	171
9.5.1	Mode of Entry and Site of Endophytic Colonization in Rice	171
9.5.2	Systemic Movement of Rhizobial Endophytes from Rice Root to Leaf Tip	176
9.5.3	Genetic Predisposition of Rice–Rhizobia Association	176
9.6	Importance of Endophytic Rhizobia–Rice Association in Agroecosystems	177
9.6.1	Plant Growth Promotion by <i>Rhizobium</i> Endophytes	177
9.6.2	Extensions of Rhizobial Endophyte Effects	180
9.6.2.1	Use of Rhizobial Endophytes from Rice with Certain Maize Genotypes	180
9.6.2.2	Rhizobia–Rice Associations in Different Rice Varieties	180
9.7	Mechanisms of Plant Growth Promotion by Endophytic Rhizobia	182
9.7.1	Stimulation of Root Growth and Nutrient Uptake Efficiency	182
9.7.2	Secretion of Plant Growth Regulators	185
9.7.3	Solubilization of Precipitated Phosphate Complexes by Rhizobial Endophytes	185
9.7.4	Endophytic Nitrogen Fixation	186
9.7.5	Production of Fe-Chelating Siderophores	187
9.7.6	Induction of Systemic Disease Resistance	188

9.8	Summary and Conclusion	188
	References	190
10	Principles, Applications and Future Aspects of Cold-Adapted PGPR	195
	<i>Mahejibin Khan and Reeta Goel</i>	
10.1	Introduction	195
10.2	Cold Adaptation of PGPR Strains	196
10.2.1	Cytoplasmic Membrane Adaptation	197
10.2.2	Carbon Metabolism and Electron Flow	198
10.2.3	Expression of Antifreeze Proteins	199
10.3	Mechanism of Plant Growth Promotion at Low Temperature	201
10.3.1	Phytostimulation	201
10.3.2	Frost Injury Protection	202
10.4	Challenges in Selection and Characterization of PGPR	202
10.5	Challenges in Field Application of PGPRs	202
10.6	Applications of PGPRs	203
10.6.1	Applications of PGPR in Agriculture	203
10.6.2	Application of PGPR in Forestry	204
10.6.3	Environmental Remediation and Heavy Metal Detoxification	207
10.7	Prospects	208
	References	209
11	Rhamnolipid-Producing PGPR and Their Role in Damping-Off Disease Suppression	213
	<i>Alok Sharma</i>	
11.1	Introduction	213
11.2	Biocontrol	214
11.2.1	Antibiotic-Mediated Suppression	214
11.2.2	HCN Production	216
11.2.3	Induced Systemic Resistance	216
11.3	Damping-Off	217
11.3.1	Causal Organisms	217
11.3.2	Control	218
11.4	Rhamnolipids	219
11.4.1	Biosynthesis of Rhamnolipids	222
11.4.2	Genetics of Rhamnolipid Synthesis	222
11.4.3	Regulation	223
11.4.4	Rhamnolipid-Mediated Biocontrol	224
11.4.5	Other Agricultural Applications	226
11.5	Quorum Sensing in the Rhizosphere	226
11.5.1	The Dominant System (<i>las</i>)	226
11.5.2	The <i>rhl</i> System	226
11.6	Conclusions and Future Directions	228
	References	228

12	Practical Applications of Rhizospheric Bacteria in Biodegradation of Polymers from Plastic Wastes	235
	<i>Ravindra Soni, Sarita Kumari, Mohd G.H. Zaidi, Yogesh S. Shouche, and Reeta Goel</i>	
12.1	Introduction	235
12.2	Materials and Methods	236
12.2.1	Chemicals and Media	236
12.2.2	LDPE-g-PMMA	236
12.2.3	LDPE-g-PMH	236
12.2.4	Isolation of Bacteria	236
12.2.5	Screening of Bacterial Isolates to Grow in the Presence of Polymer	237
12.2.6	Optimization of Growth Conditions	237
12.2.7	Biodegradation Studies	237
12.3	Results and Discussion	237
12.3.1	Growth in the Presence of Polymer	238
12.3.2	Biodegradation Studies	238
12.3.2.1	<i>B. cereus</i>	238
12.3.2.2	<i>Bacillus</i> sp.	238
12.3.2.3	<i>B. pumilus</i>	239
12.3.2.4	Bacterial Consortium and LDPE	240
12.3.2.5	FTIR Spectroscopy	241
12.4	Conclusions	242
	References	243
13	Microbial Dynamics in the Mycorrhizosphere with Special Reference to Arbuscular Mycorrhizae	245
	<i>Abdul G. Khan</i>	
13.1	The Soil and the Rhizosphere	245
13.2	Rhizosphere and Microorganisms	245
13.2.1	Glomalian Fungi	245
13.2.2	Arbuscular Mycorrhiza–Rhizobacteria Interactions	247
13.2.3	Plant Growth Promoting Rhizobacteria	249
13.2.4	Co-occurrence of AMF and PGPR/MHB	250
13.3	Conclusion	252
	References	252
14	Salt-Tolerant Rhizobacteria: Plant Growth Promoting Traits and Physiological Characterization Within Ecologically Stressed Environments	257
	<i>Dilfuza Egamberdiyeva and Khandakar R. Islam</i>	
14.1	Introduction	257
14.2	Diversity of Salt-Tolerant Rhizobacteria	259
14.3	Colonization and Survival of Salt-Tolerant Rhizobacteria	261
14.4	Salt and Temperature Tolerance	263

14.5	Physiological Characterization of Rhizobacteria	264
14.6	Plant Growth Stimulation in Arid Soils	268
14.7	Biomechanisms to Enhance Plant Growth	273
14.8	Conclusions	275
14.9	Future Directions	276
	References	276
15	The Use of Rhizospheric Bacteria to Enhance Metal Ion Uptake by Water Hyacinth, <i>Eichhornia crassipe</i> (Mart)	283
	<i>Lai M. So, Alex T. Chow, Kin H. Wong, and Po K. Wong</i>	
15.1	Introduction	283
15.2	Overview of Metal Ion Pollution	284
15.3	Treatment of Metal Ions in Wastewater	285
15.3.1	Conventional Methods	285
15.3.2	Microbial Methods	285
15.3.3	Phytoremediation	286
15.3.3.1	An Overview of Phytoremediation	286
15.3.3.2	Using Water Hyacinth for Wastewater Treatment	287
15.4	Biology of Water Hyacinth	290
15.4.1	Scientific Classification	290
15.4.2	Morphology	291
15.4.3	Ecology	292
15.4.4	Environmental Impact	293
15.4.5	Management of Water Hyacinth	293
15.5	Microbial Enhancement of Metal Ion Removal Capacity of Water Hyacinth	294
15.5.1	Biology of the Rhizosphere	294
15.5.2	Mechanisms of Metal Ion Removal by Plant Roots	295
15.5.3	Effects of Rhizospheric Bacteria on Metal Uptake and Plant Growth	296
15.6	Summary	298
	References	299
	Index	305

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1

Ecology, Genetic Diversity and Screening Strategies of Plant Growth Promoting Rhizobacteria (PGPR)

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1.1

Introduction

1.1.1

Rhizosphere Microbial Ecology

The German agronomist Hiltner first defined the rhizosphere, in 1904, as the ‘effect’ of the roots of legumes on the surrounding soil, in terms of higher microbial activity because of the organic matter released by the roots.

Until the end of the twentieth century, this ‘effect’ was not considered to be an ecosystem. It is interesting to make some brief observations about the size, in terms of energy and extension, of this ecosystem to determine its impact on how the biosphere functions. First, in extension, the rhizosphere is the largest ecosystem on earth. Second, the energy flux in this system is enormous. Some authors estimate that plants release between 20 and 50% of their photosynthates through their roots [1,2]. Thus, rhizosphere’s impact on how the biosphere functions is fundamental.

A large number of macroscopic organisms and microorganisms such as bacteria, fungi, protozoa and algae coexist in the rhizosphere. Bacteria are the most abundant among them. Plants select those bacteria contributing most to their fitness by releasing organic compounds through exudates [3], creating a very selective environment where diversity is low [4,5]. A complex web of interactions takes place among them, and this may affect plant growth, directly or indirectly. Since bacteria are the most abundant microorganisms in the rhizosphere, it is highly probable that they influence the plant’s physiology to a greater extent, especially considering their competitiveness in root colonization [6].

Bacterial diversity can be defined in terms of taxonomic, genetic and functional diversity [7]. In the rhizosphere, the metabolic versatility of a bacterial population (functional diversity) is based on its genetic variability and on possible interactions with other prokaryotic and eukaryotic organisms such as plants.

However, a question still to be answered regarding microbial communities in the rhizosphere is the relationship between the ecological function of communities and soil biodiversity. In spite of the lack of information about the importance of the diversity and the richness of species related to their ecological function [8,9], soil organisms have been classified several times in functional groups [10].

This lack of knowledge about bacterial diversity is partly owing to the high number of species present, as well as to the fact that most bacteria are viable but not culturable.

The biological diversity of soil microorganisms has been expressed using a variety of indexes [11,12] and mathematical models [13], but there is no accepted general model to describe the relationship among abundance, species' richness and dominance. It is, therefore, reasonable that the components of diversity are studied separately to quantify them [14].

Bacterial diversity studies are more complex at taxonomic, functional and genetic levels than are similar studies on eukaryotic organisms owing to the minute working scale and the large number of different bacterial species present in the environment. Torsvik and coworkers [15] identified more than 7000 species in an organic forest soil.

The variations in populations through space and time and their specialization in ecological niches are two important factors in the rhizosphere that must be considered in studying how species' richness influences the functioning of the system. The functioning of soil microbial communities is based on the fact that there is appropriate species diversity for the resources to be used efficiently and that this can be maintained under changing conditions [14].

In the rhizosphere, as in other well-formed ecosystems with an appropriate structure, changes in some of the components can affect entire or part of the system. The degree of impact will depend on features of the system such as its resistance or resilience. The state of this system changes depending on variables such as the age of the plant, root area, light availability, humidity, temperature and plant nutrition [16,17]. Under stressful conditions, the plant exerts a stronger control on release of root exudates [18,19]. From this viewpoint, it is reasonable to assume that the changes that occur in the plant will change the root exudation patterns and, thereby, the rhizosphere microbial communities. There have been many studies that relate the quality and quantity of the exudates with changes in the structure of rhizosphere microbial communities [20].

In 1980, Torsvik [21] published the first protocol for the extraction and isolation of microbial DNA from soil. Since then, there have been many studies directed at the development of new methods and molecular tools for the analysis of soil microbial communities. However, molecular genetics is not the only tool used in solving the difficulties in analyzing soil microbial communities. A multimethodological approach using conventional techniques such as bacterial isolation and physiological studies, together with molecular genetics, will be necessary to fully develop the study of microbial ecology [22,23].

The bacterial community can be studied using several approaches: first, a structural approach, attempting to study the entire soil bacterial community; second, the

relationships between populations and the processes that regulate the system; and finally, a functional approach.

Recent research has shown that, within a bacterial population, cells are not isolated from each other but communicate to coordinate certain activities. This communication is key to their survival since microbial success depends on the ability to perceive and respond rapidly to changes in the environment [24]. Bacteria have developed complex communication mechanisms to control the expression of certain functions in a cell density-dependent manner, a phenomenon termed as *quorum sensing* (QS).

Quorum sensing confers an enormous competitive advantage on bacteria, improving their chances to survive as they can explore more complex niches. This mechanism is also involved in the infection ability of some plant bacterial pathogens (such as *Xanthomonas campestris* and *Pseudomonas syringae*) [25].

Bacterial communication by quorum sensing is based on the production and release of signal molecules into the medium, termed autoinducers, concentration being proportional to cell density. When bacteria detect the signal molecule at a given concentration, the transcription of certain genes regulated by this mechanism is induced or repressed. There are many microbial processes regulated by quorum sensing, including DNA transference by conjugation, siderophore production, bioluminescence, biofilm formation and the ability of some bacteria to move, called swarming [26,27].

Recent studies have shown the importance of this type of regulation mechanism in putative beneficial bacterial traits for the plant, such as plant growth promotion, protection against pathogens or saline stress protection [28,29]. In addition, coevolution studies of plants and bacteria have determined that some plants release molecules, which mimic acyl homoserine lactones (AHLs) and even enzymes that are able to degrade the AHL molecule in root exudates. Somehow, plants have 'learned' the language of bacteria and use it for their own benefit. Some studies have discovered that this behavior leads to defense against plant bacterial pathogens, altering or blocking communication among bacteria, thus dramatically reducing their infection efficiency.

1.1.2

Plant Growth Promoting Rhizobacteria (PGPR)

Bacteria inhabiting the rhizosphere and beneficial to plants are termed PGPR [30]. Thus, the rhizosphere of wild plant species appears to be the best source from which to isolate plant growth promoting rhizobacteria [4,31].

A putative PGPR qualifies as PGPR when it is able to produce a positive effect on the plant upon inoculation, hence demonstrating good competitive skills over the existing rhizosphere communities. Generally, about 2–5% of rhizosphere bacteria are PGPR [32].

Some PGPR have been produced commercially as inoculants for agriculture, but it must be borne in mind that the inoculation of these bacteria in soil may affect the composition and structure of microbial communities, and these changes must be

studied since they have, at times, been related to the inefficiency of biofertilizers when applied to plant roots [33,34]. On the contrary, many studies [35] have tested the efficiency of PGPR in various conditions, observing that PGPR are efficient under determined conditions only [36]. Knowledge of the structure of rhizosphere microbial communities and their diversity, as related to other essential processes within the system such as complexity, natural selection, interpopulational relations (symbiosis, parasitism, mutualism or competence), succession or the effect of disturbances, is the key to a better understanding of the system and for the correct utilization of PGPR in biotechnology.

Taking all of the above into consideration, it appears that quorum sensing can be a very useful tool in agriculture, with the potential to prevent bacterial pathogen attack and improve PGPR performance. There already exist transgenic plants that have been engineered to produce high levels of AHLs or an enzyme capable of degrading AHLs and that have demonstrated considerable capacity in blocking pathogen infection or altering PGPR performance [24].

1.2

Rhizosphere Microbial Structure

1.2.1

Methods to Study the Microbial Structure in the Rhizosphere

As mentioned above, the bacterial community can be studied through two approaches: structural and functional. To understand the structural approach, we must know the groups of individuals, their species and abundance. Traditionally, this has been done by extracting microorganisms from the system, culturing them in the laboratory and performing many morphological, biochemical and genetic tests. Bacteria extraction methods require a dispersing agent to disintegrate the links among cells and need to be performed using either physical or chemical agents or a combination of both.

When handling bulk soil, rhizosphere soil and plant roots, dispersion methods need to be used owing to the intimate relationship between bacteria and the substrate. The efficiency of these methods is evaluated by comparing the microbial biomass of the original substrate before and after extraction. However, microbial biomass is difficult to calculate. There are several ways to approach these parameters including direct counting under a microscope (e.g. by using acridine orange dye) [37], microbial respiration (i.e. substrate induced respiration, SIR [38]), ATP level assay [39], counting viable cells with the most probable number (MPN) [40], using biomarkers such as lipids [41] and soil fumigation with chloroform [42].

After extracting bacteria, several simple methods can be applied to isolate and count soil bacteria, such as growing them in a nonselective medium to obtain the total viable count (TVC). The data obtained with this method are expressed as colony forming units (CFUs).

These studies, in which bacteria are grown on plates, are used to calculate the soil bacterial diversity, by observing the number and abundance of each species. Diversity indexes, such as the Shannon index (H), the Simpson index and the equitability index (J), have all been used to describe the structure of communities from a mathematical viewpoint [43].

The percentage of culturable microorganisms in soil is very low; however, some researchers estimate this at only 10% [44], while others suggest 1% [43] or even lower (between 0.2 and 0.8%) [45]. Because of the limitation of some methods, techniques in which it is not necessary to culture microorganisms on plates are required. One such technique is the phospholipid fatty acid analysis (PLFA) [33,34,46–48]. Phospholipids are integrated in the bacterial cell membranes [49]. Different groups of microorganisms possess different fatty acid patterns. It is not usually possible to detect specific strains or species, but changes in the concentration of specific fatty acids can be correlated to changes in specific groups of microorganisms.

Another approach to nonculturable diversity is through techniques of molecular genetics, which, in the past 20 years, has revealed new information about soil microbial communities [50]. Techniques include DNA and/or RNA hybridization [51], polymerase chain reaction (PCR), ribosomal RNA sequencing [52], G + C percentages [53] and DNA reassociation between bacteria in the community [53,54].

At present, the most notable techniques are temperature gradient gel electrophoresis (TGGE) and denaturing gradient gel electrophoresis (DGGE), both based on the direct extraction of DNA or RNA from soil; the amplification of this DNA (by means of PCR), followed by electrophoretic separation in a temperature gradient for the former or by using chemical denaturing substances for the latter. These techniques allow the separation of DNA fragments of exactly the same length but with different sequences, based on their melting properties [54–56]. Other techniques include restriction fragment length polymorphism (RFLP) [57,58], techniques related to the analysis and cutting of different restriction enzymes (amplified ribosomal DNA restriction analysis, ARDRA) [59] or cloning the rDNA 16S and then sequencing [5]. The use of microarrays [22] is also an emerging technique with a promising future, which permits the identification of specific genes [60].

Each of the methods described above possesses its own distinctive advantages and disadvantages. Generally, the more selective the method, the less able it is to detect global changes in communities and vice versa. Using these tools can provide an estimate of the microbial diversity in the soil.

1.2.2

Ecology and Biodiversity of PGPR Living in the Rhizosphere

In the last few years, the number of PGPR that have been identified has seen a great increase, mainly because the role of the rhizosphere as an ecosystem has gained importance in the functioning of the biosphere and also because mechanisms of action of PGPR have been deeply studied.

Currently, there are many bacterial genera that include PGPR among them, revealing a high diversity in this group. A discussion of some of the most abundant genera of PGPR follows to describe the genetic diversity and ecology of PGPR.

1.2.2.1 Diazotrophic PGPR

Free nitrogen-fixing bacteria were probably the first rhizobacteria used to promote plant growth. *Azospirillum* strains have been isolated and used ever since the 1970s when it was first used [61]. This genus has been studied widely, the study by Bashan *et al.* [62] being the most recent one reporting the latest advances in physiology, molecular characteristics and agricultural applications of this genus.

Other bacterial genera capable of nitrogen fixation that is probably responsible for growth promotion effect, are *Azoarcus* sp., *Burkholderia* sp., *Gluconacetobacter diazotrophicus*, *Herbaspirillum* sp., *Azotobacter* sp and *Paenibacillus (Bacillus) polymyxa* [63]. These strains have been isolated from a number of plant species such as rice, sugarcane, corn, sorghum, other cereals, pineapple and coffee bean.

Azoarcus has recently gained attention due to its great genetic and metabolic diversity. It has been split into three different genera (*Azovibrio*, *Azospira* and *Azonexus*) [64]. The most distinctive characteristic of these genera, which particularly differentiates them from other species, is their ability to grow in carboxylic acids or ethanol instead of sugars, with their optimum growth temperature ranging between 37 and 42 °C. *Azoarcus* is an endophyte of rice and is currently considered the model of nitrogen-fixing endophytes [65].

1.2.2.2 *Bacillus*

Ninety-five percent of Gram-positive soil bacilli belong to the genus *Bacillus*. The remaining 5% are confirmed to be *Arthrobacter* and *Frankia* [66]. Members of *Bacillus* species are able to form endospores and hence survive under adverse conditions; some species are diazotrophs such as *Bacillus subtilis* [67], whereas others have different PGPR capacities, as many reports on their growth promoting activity reveal [33,68,69].

1.2.2.3 *Pseudomonas*

Among Gram-negative soil bacteria, *Pseudomonas* is the most abundant genus in the rhizosphere, and the PGPR activity of some of these strains has been known for many years, resulting in a broad knowledge of the mechanisms involved [33,70,71].

The ecological diversity of this genus is enormous, since individual species have been isolated from a number of plant species in different soils throughout the world. *Pseudomonas* strains show high versatility in their metabolic capacity. Antibiotics, siderophores or hydrogen cyanide are among the metabolites generally released by these strains [72]. These metabolites strongly affect the environment, both because they inhibit growth of other deleterious microorganisms and because they increase nutrient availability for the plant.

1.2.2.4 *Rhizobia*

Among the groups that inhabit the rhizosphere are rhizobia. Strains from this genus may behave as PGPR when they colonize roots from nonlegume plant

species in a nonspecific relationship. It is well known that a number of individual species may release plant growth regulators, siderophores and hydrogen cyanide or may increase phosphate availability, thereby improving plant nutrition [73]. An increase in rhizosphere populations has been reported after crop rotation with nonlegumes [74], with this abundance benefiting subsequent crops [75].

1.3

Microbial Activity and Functional Diversity in the Rhizosphere

1.3.1

Methods to Study Activity and Functional Diversity in the Rhizosphere

The classical approach to determining functional diversity is to use culturable bacteria grown on a plate and subject them to selected biochemical tests. Another method involves analyzing bacterial growth rate on a plate, which is considered as an indicator of the physiological state of the bacteria in the environment, the availability of nutrients and the adaptation strategy [76]. It is known that culturable bacteria are scarce in soil but are considered responsible for the most important chemical and biochemical processes. This is based on the fact that nonculturable bacteria are mostly 'dwarfs', measuring less than 0.4 μm in diameter and are considered as dying forms with almost no activity [77]. Bååth [37] studied the incorporation of radioactive precursors of DNA ($[\text{H}^3]$ -thymidine, to assess population growth), and proteins ($[\text{C}^{14}]$ -leucine, to assess population activity) in various fractions of soil filtrates. His research revealed that the culturable bacteria fraction (the larger size) is responsible for most of the growth and activity of the soil communities, whereas the fraction of cells less than 0.4 μm , considered nonculturable, had little importance in the metabolism and soil activity. Finally, using the PLFA technique, it has been demonstrated that there are no significant differences between the phospholipid fatty acids of bacteria in soil and bacteria culturable from this soil.

In contrast, other authors state that in rhizospheric communities, there are some difficulties in culturing groups of bacteria present in low densities that are metabolically very active; they can synthesize high amounts of proteins, use different substrates [78] and are believed to be important in fundamental processes in the soil. These bacteria are called keystone species, some of which include *Nitrosomonas* and *Nitrobacter*, playing a very important role in the nitrogen cycle [79].

At present, enzymatic activity measurement is one of the more widely used techniques to determine microbial diversity, in which it is possible to perform studies with a specific enzyme. An other approach is to use Biolog plates, which permit microbial communities to be characterized according to their physiological profile (community-level physiological profile, CLPP [47,80]) calculated from the different utilization patterns of many carbon and nitrogen sources, determined by a redox reaction that changes color after inoculation and incubation of the microbial communities [47,81].

New approaches such as the search for new catabolic, biosynthetic or antibiotic functions in soil samples [82] are required to identify new, potentially nonculturable genotypes. The cloning and sequencing of large DNA fragments (BAC library) will provide researchers with information about the metabolic diversity of nonculturable and culturable strains in the future and also provide important information on ecological laws and the operation of the soil ecosystem [22]. Undoubtedly, future studies on soil communities will involve microarray techniques [22] that will permit the study of differences in the structure of communities, identifying groups that are active or inactive during a specific treatment [60] leading to the identification of strains isolated from different environments and explaining differences or similarities in the operation of niches [83]. These techniques are complemented with transcriptomic techniques, based on the description of the activity of a gene by its expressed mRNA, and the proteomic approximation [22,82].

1.3.2

Activity and Effect of PGPR in the Rhizosphere

Some researchers approach the study of biochemical diversity in soil by identifying biochemical activities related to putative physiological PGPR traits in bacteria isolated from the rhizosphere (Table 1.1) [31].

Microbial activity in the rhizosphere indicates how metabolically active the microbial communities are. Using PGPR as inoculants in soil, besides altering the structure of the communities, will also influence microbial activity, and this could be related to the survival of the PGPR in the environment [34]. Some of the factors influencing the survival and activity of bacteria in the rhizosphere are physical (texture, temperature and humidity), while others are chemical, such as pH, nutrient

Table 1.1 Frequency of physiological PGPR traits in the mycorrhizosphere of *P. pinaster* and *P. pinea* and the associated mycosphere of *L. deliciosus* [31].

PGPR trait	<i>P. pinaster</i>		<i>P. pinea</i>	
	Mycorrhizosphere	Mycosphere	Mycorrhizosphere	Mycosphere
Aux (%)	14	0	50	42
Aux + PDYA (%)	0	0	0	2
Aux + CAS (%)	0	3	11	2
Aux + ACC (%)	0	0	7	0
Aux + CAS + PDYA (%)	0	3	0	0
PDYA (%)	47	35	11	32
PDYA + ACC (%)	3	0	0	0
CAS (%)	36	40	14	11
CAS + PDYA (%)	0	3	0	0
CAS + PDYA + ACC (%)	0	3	0	0
ACC (%)	0	13	7	11

Aux, auxin production; PDYA, phosphate solubilization; CAS, siderophore production; ACC, 1-aminocyclopropanecarboxylic acid degradation.

availability, organic matter content and, above all, interactions with other rhizosphere microorganisms. The interaction with the biotic factor is very important because PGPR must occupy a new niche, adhering to the plant roots, and the inoculum must compete for available nutrients released, essentially, by the root exudates, maintaining a minimum population able to exert its biological effect.

Studies of characterization of the soil microbial community activity are conducted using various techniques, such as thymidine ($[^3\text{H}]$) incorporation, radioactive DNA precursors to assess population growth and leucine ($\text{L-}[^{14}\text{C}]$) radioactive protein precursor to assess the metabolic activity of the population [37,84–86]. Stable isotope probing (SIP), based on radioactive labeling of different substrates, is considered to have enormous potential [23]. A further approach to quantifying the activity in the rhizosphere is by means of SIR [38].

1.4

Screening Strategies of PGPR

The rhizosphere of wild populations of plants is proposed as one of the optimal sources in which to isolate PGPR. This is because of the high selective pressure a plant exerts in this zone. The plant selects, among others, beneficial bacteria [4,31]. In the screening of PGPR, the different soil types, plant species, seasons and the plant's physiological moment must be considered to ensure the successful isolation of putative beneficial rhizobacteria.

The first step in obtaining a PGPR is the isolation of rhizospheric bacteria. It is generally accepted that the rhizosphere is the soil volume close to the roots (soil at 1–3 mm from the root and the soil adhering to the root). To collect this soil fraction, the root is normally shaken vigorously and soil still adhering is collected as the rhizosphere. Depending on the type of study, the root containing the endophyte bacteria is included, as some have been described as PGPR. Other researchers refer to the rhizosphere as the soil adhering to the roots after they have been washed under running water.

Rhizobacteria extraction starts with the suspension of soil in water, phosphate buffer or saline solution. Some compounds such as pyrophosphate are effective for soil disgregation, but can alter cell membranes [87]. Sample dispersion is made with chemical dispersants such as chelants that exchange monovalent ions (Na^+) for polyvalent cations (Ca^{2+}) of clay particles, reducing the electrostatic attraction between the soil and the bacterial cells. Various researchers have used ionic exchange resins derived from iminodiacetic acid, for example, Dowex A1 [88] or Chelex-100 [89,90]. Other dispersants are Tris buffer or sodium hexametaphosphate [91]. Detergents are used because the microbial cells present in the treated sample adhere by extracellular polymers to the soil particles. MacDonald [88] demonstrated that using detergents (sodium deoxycholate at 0.1%) together with Dowex A1 increased the microbial extraction from soil to 84%. This method was modified later by Herron and Wellington [89], replacing Dowex with Chelex-100 and combining with polyethylene glycol (PEG 6000) to dissolve and separate the phases. Other chemical

solvents used in extraction protocols are Calgon at 0.2% for the extraction of bacteria from soil in studies of bacterial counts with acridine orange [40,85], citrate buffer used in studies of membrane phospholipids from soil microbes [92] and Winoogradsky solution [54] for microbial diversity studies using molecular techniques (ARDRA, DGGE or REP-PCR) or phenotypical tests (Biolog).

Chemical extraction methods may be combined with physical methods, and these can be divided into three categories: shaking, mixing (homogenizing or grinding) and ultrasonics. Shaking is probably the least efficient method but adequate for sensitive bacteria or bacteriophages [93]. Techniques based on homogenization could damage some groups of bacteria, such as Gram-negative bacteria, and extraction would be selective. A combined method of grinding and chemical dispersants would be more effective [94]. Ultrasonic treatments are the best among methods used to break the physical forces between soil particles. In clay soils, pretreatment of the sample is necessary [95]; however, most sensitive bacteria, such as Gram-negative ones, could be damaged. This effect can be avoided using less aggressive ultrasonic treatments [96].

After rhizobacterial isolation, a screening of the putative PGPR is performed using two different strategies:

- (a) Isolation, to select putative bacteria beneficial to the plant using specific culture media and specific isolation methods. For example, Founoune *et al.* [97] isolated *Pseudomonas fluorescens* from the Acacia rhizosphere as a species described as PGPR.
- (b) After isolation of the maximum number of bacteria to avoid the loss of bacterial variability, different tests are performed to reduce the various types of bacteria chosen, so that only the putative beneficial ones remain. The test is performed *in vitro* to check biochemical activities that correspond with potential PGPR traits. Genetic tests may also be performed to remove genetic redundancy, that is, select different genomes that may have different putative beneficial activities [31,98,99].

Among the biochemical tests used to find putative PGPR traits, the most common are the following: (i) test for plant growth regulator production (i.e. auxins, gibberellin and cytokinins); (ii) the ACC (1-aminocyclopropanecarboxylic acid) deaminase test; this enzyme degrades the ethylene precursor ACC, causing a substantial alteration in ethylene levels in the plant, improving root system growth [100]; (iii) phosphate solubilization test, phosphate solubilization may improve phosphorous availability to the plant [101,102]; (iv) siderophore production test, which may improve plant's iron uptake [103]; (v) test for nitrogen-fixing bacteria to improve the plant's nitrogen nutrition [63]; and (vi) test for bacteria capable of producing enzymes that can degrade pathogenic fungi cell walls (i.e. chitinase or β -1,3-glucanase) preventing plant diseases [98].

The most common genetic techniques are PCR-RAPD (randomly amplified polymorphic DNA, ERIC-PCR, BOX-PCR and REP-PCR). They all compare bacterial