Plant-Bacteria Interactions

Strategies and Techniques to Promote Plant Growth

Edited by
Iqbal Ahmad, John Pichtel, and Shamsul Hayat

WILEY-VCH Verlag GmbH & Co. KGaA

Plant-Bacteria Interactions

Edited by
Iqbal Ahmad, John Pichtel and
Shamsul Hayat

Related Titles

Roberts, K. (ed.)

Handbook of Plant Science

2 Volume Set

2007
ISBN: 978-0-470-05723-0

Kahl, G., Meksem, K. (eds.)

The Handbook of Plant Functional Genomics

Concepts and Protocols

2008
ISBN: 978-3-527-31885-8

Meksem, K., Kahl, G. (eds.)

The Handbook of Plant Genome Mapping

Genetic and Physical Mapping

2005
ISBN: 978-3-527-31116-3

Cullis, C. A.

Plant Genomics and Proteomics

2004
ISBN: 978-0-471-37314-8

Kayser, O., Quax, W. J. (eds.)

Medicinal Plant Biotechnology

From Basic Research to Industrial Applications

2007
ISBN: 978-3-527-31443-0

Dolezel, J., Greilhuber, J., Suda, J. (eds.)

Flow Cytometry with Plant Cells

Analysis of Genes, Chromosomes and Genomes

2007
ISBN: 978-3-527-31487-4

Plant-Bacteria Interactions

Strategies and Techniques to Promote Plant Growth

Edited by
Iqbal Ahmad, John Pichtel, and Shamsul Hayat

WILEY-VCH Verlag GmbH & Co. KGaA

The Editors

Dr. Iqbal Ahmad
Aligarh Muslim University
Department of Agricultural Microbiology
Aligarh 202002
India

Prof. Dr. John Pichtel
Ball State University
Department of Natural Resources and
Environmental Management
WQ 103
Muncie, IN 47306
USA

Dr. Shamsul Hayat
Aligarh Muslim University
Department of Botany
Aligarh 202002
India

Library of Congress Card No.: applied for

British Library Cataloguing-in-Publication Data
A catalogue record for this book is available from the British Library.

Bibliographic information published by the Deutsche Nationalbibliothek
Die Deutsche Nationalbibliothek lists this publication in the Deutsche Nationalbibliografie; detailed bibliographic data are available on the Internet at <http://dnb.d-nb.de>.

Composition Thomson Digital, Noida, India
Printing Betz-Druck GmbH, Darmstadt
Bookbinding Litges & Dopf GmbH, Heppenheim
Cover Design Adam Design, Weinheim

Printed in the Federal Republic of Germany
Printed on acid-free paper

ISBN: 978-3-527-31901-5

Contents

List of Contributors

Farah Ahmad
Department of Agricultural Microbiology
Faculty of Agricultural Sciences
Aligarh Muslim University
Aligarh 202002
India

Iqbal Ahmad
Department of Agricultural Microbiology
Faculty of Agricultural Sciences
Aligarh Muslim University
Aligarh 202002
India

Javier Andrés
Laboratorio de Interacción Microorganismo–Planta
Facultad de Ciencias Exactas Físico-Químicas y Naturales
Universidad Nacional de Río Cuarto
Campus Universitario Ruta 36
Km 601 (5800) Río Cuarto
Córdoba
Argentina

José Antonio Lucas García
Dpto. CC. Ambientales y Recursos Naturales
Facultad de Farmacia
Universidad San Pablo CEU
Urb. Montepríncipe
Ctra. Boadilla del Monte Km 5.3
28668 Boadilla del Monte
Madrid
Spain

Farrukh Aqil
Department of Agricultural Microbiology
Faculty of Agricultural Sciences
Aligarh Muslim University
Aligarh 202002
India

Germán Avanzini
Laboratorio de Interacción Microorganismo–Planta
Facultad de Ciencias Exactas Físico-Químicas y Naturales
Universidad Nacional de Río Cuarto
Campus Universitario Ruta 36
Km 601 (5800) Río Cuarto
Córdoba
Argentina

Jorge Barriuso
Dpto. CC. Ambientales y Recursos Naturales
Facultad de Farmacia
Universidad San Pablo CEU
Urb. Montepríncipe
Ctra. Boadilla del Monte Km 5.3
28668 Boadilla del Monte
Madrid
Spain

Evelin Carlier
Laboratorio de Interacción Microorganismo–Planta
Facultad de Ciencias Exactas Físico-Químicas y Naturales
Universidad Nacional de Río Cuarto
Campus Universitario Ruta 36
Km 601 (5800) Río Cuarto
Córdoba
Argentina

Alex T. Chow
College of Environmental Science and Engineering
South China University of Technology
Guangzhou
China

Frank B. Dazzo
Department of Microbiology and Molecular Genetics
Michigan State University
East Lansing, MI 48824
USA

Dilfuza Egamberdiyeva
Department of Biotechnology
Faculty of Biology
National University of Uzbekistan
100174 Tashkent
Uzbekistan

Ana García-Villaraco Velasco
Dpto. CC. Ambientales y Recursos Naturales
Facultad de Farmacia
Universidad San Pablo CEU
Urb. Montepríncipe
Ctra. Boadilla del Monte Km 5.3
28668 Boadilla del Monte
Madrid
Spain

Stephen R. Giddens
Department of Plant Sciences
University of Oxford
South Parks Road
Oxford OX1 3RB
UK

Reeta Goel
Department of Microbiology
G.B. Pant University of Agriculture & Technology
Pantnagar, Distt. U.S. Nagar
Uttarakhand 263145
India

J. González-López
Institute of Water Research
University of Granada
Calle Ramón y Cajal 4
180071 Granada
Spain

F.J. Gutiérrez Mañero
Dpto. CC. Ambientales y Recursos Naturales
Facultad de Farmacia
Universidad San Pablo CEU
Urb. Montepríncipe
Ctra. Boadilla del Monte Km 5.3
28668 Boadilla del Monte
Madrid
Spain

S. Hayat
Department of Botany
Aligarh Muslim University
Aligarh 202002
India

Khandakar R. Islam
Crop, Soil and Water Resources
The Ohio State University South Centers
1864 Shyville Road
Piketon, OH 45661
USA

Robert W. Jackson
School of Biological Sciences
University of Reading
Whiteknights
Reading RG6 6AJ
UK

Hemant K. Jaiswal
Department of Genetics and Plant Breeding
Institute of Agricultural Sciences
Banaras Hindu University
Varanasi 221005
India

Alexander A. Kamnev
Institute of Biochemistry and Physiology of Plants and Microorganisms
Russian Academy of Sciences
410049 Saratov
Russia

Abdul G. Khan
14, Clarissa Place
Ambarvale NSW 2560
Australia

Mahejibin Khan
Department of Biotechnology
Kumaun University
Nainital
India

M.S. Khan
Department of Agricultural Microbiology
Faculty of Agricultural Sciences
Aligarh Muslim University
Aligarh 202002
India

Sarita Kumari
Department of Microbiology
G.B. Pant University of Agriculture & Technology
Pantnagar, Distt. U.S. Nagar
Uttarakhand-263145
India

M.V. Martínez-Toledo
Institute of Water Research
University of Granada
Calle Ramón y Cajal 4
180071 Granada
Spain

Ravi P.N. Mishra
Department of Genetics and Plant Breeding
Institute of Agricultural Sciences
Banaras Hindu University
Varanasi 221005
India

Christina D. Moon
AgResearch Ltd
Grasslands Research Centre
Private Bag 11008
Palmerston North
New Zealand

Javed Musarrat
Department of Agricultural Microbiology
Faculty of Agricultural Sciences
Aligarh Muslim University
Aligarh 202002
India

Carolina Pasluosta
Laboratorio de Interacción Microorganismo–Planta
Facultad de Ciencias Exactas Físico-Químicas y Naturales
Universidad Nacional de Río Cuarto
Campus Universitario Ruta 36
Km 601 (5800) Río Cuarto
Córdoba
Argentina

C. Pozo Clemente
Institute of Water Research
University of Granada
Calle Ramón y Cajal 4
180071 Granada
Spain

Agustín Probanza Lobo
Dpto. CC. Ambientales y Recursos Naturales
Facultad de Farmacia
Universidad San Pablo CEU
Urb. Montepríncipe
Ctra. Boadilla del Monte Km 5.3
28668 Boadilla del Monte
Madrid
Spain

Beatriz Ramos Solano
Dpto. CC. Ambientales y Recursos Naturales
Facultad de Farmacia
Universidad San Pablo CEU
Urb. Montepríncipe
Ctra. Boadilla del Monte Km 5.3
28668 Boadilla del Monte
Madrid
Spain

B. Rodelas González
Dpto. de Microbiología
Facultad de Farmacia
University of Granada
Calle Ramón y Cajal 4
180071 Granada
Spain

M. Rodríguez-Díaz
Institute of Water Research
University of Granada
Calle Ramón y Cajal 4
180071 Granada
Spain

Susana Rosas
Laboratorio de Interacción Microorganismo–Planta
Facultad de Ciencias Exactas Físico-Químicas y Naturales
Universidad Nacional de Río Cuarto
Campus Universitario Ruta 36
Km 601 (5800) Río Cuarto
Córdoba
Argentina

Marisa Rovera
Departamento de Microbiología e lmmunologia
Universidad Nacional de Río Cuarto
Ruta 36, Km 601
5800 Río Cuarto
Córdoba
Argentina

Alok Sharma
Department of Structural Biology
Helmholtz Centre for Infection Research
Inhoffenstraße 7
D-38124 Braunschweig
Germany

Yogesh S. Shouche
National Center for Cell Sciences
Pune University Campus
Gomeskhind
Pune 411007
India

Manoj K. Singh
Department of Genetics and Plant Breeding
Institute of Agricultural Sciences
Banaras Hindu University
Varanasi 221005
India

Ramesh K. Singh
Department of Genetics and Plant Breeding
Institute of Agricultural Sciences
Banaras Hindu University
Varanasi 221005
India

Lai M. So
Department of Biology
The Chinese University of Hong Kong
Shatin, NT
Hong Kong SAR
China

Ravindra Soni
Department of Microbiology
G.B. Pant University of Agriculture & Technology
Pantnagar, Distt. U.S. Nagar
Uttarakhand 263145
India

Kin H. Wong
Department of Biology
The Chinese University of Hong Kong
Shatin, NT
Hong Kong SAR
China

Po K. Wong
Department of Biology
The Chinese University of Hong Kong
Shatin, NT
Hong Kong SAR
China

Youssef G. Yanni
Sakha Agricultural Research Station
Dept. of Soil Microbiology
Kafr El-Sheikh 33717
Egypt

Maryam Zahin
Department of Agricultural Microbiology
Faculty of Agricultural Sciences
Aligarh Muslim University
Aligarh 202002
India

Mohd G.H. Zaidi
Department of Chemistry
G.B. Pant University of Agriculture & Technology
Pantnagar, Distt. U.S. Nagar
Uttarakhand 263145
India

Xue-Xian Zhang
School of Biological Sciences
University of Auckland
Private Bag 92019
Auckland
New Zealand

1
Ecology, Genetic Diversity and Screening Strategies of Plant Growth Promoting Rhizobacteria (PGPR)

Jorge Barriuso, Beatriz Ramos Solano, José A. Lucas, Agustín Probanza Lobo, Ana García-Villaraco, and Francisco J. Gutiérrez Mañero

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 dominancy. 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 ($[H^3]$-thymidine, to assess population growth), and proteins (L-$[C^{14}]$-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].

	P. pinaster		*P. pinea*	
PGPR trait	**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 ([H^3]) incorporation, radioactive DNA precursors to assess population growth and leucine (L-[C^{14}]) 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 Winogradsky 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