

# Handbook of Microbial Biofertilizers

M. K. Rai, PhD  
Editor



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## CONTENTS

|  |              |
|--|--------------|
| <b>About the Editor</b>  | <b>xv</b>    |
| <b>Contributors</b>  | <b>xvii</b>  |
| <b>Foreword</b><br><i>Ian Robert Hall</i>  | <b>xxi</b>   |
| <b>Preface</b>   | <b>xxiii</b> |
| <b>Acknowledgments</b>   | <b>xxv</b>   |
| <b>Chapter 1. Potential and Possible Uses of Bacterial<br/>and Fungal Biofertilizers</b><br><i>Francesco Gentili</i><br><i>Ari Jumpponen</i> | <b>1</b>     |
| Introduction   | 1            |
| N <sub>2</sub> -Fixing Bacteria As Microbial Biofertilizers  | 3            |
| Symbiotic N <sub>2</sub> -Fixing Bacteria  | 3            |
| Cyanobacteria  | 6            |
| N <sub>2</sub> -Fixing Associated Bacteria   | 7            |
| Free-Living N <sub>2</sub> -Fixing Bacteria  | 8            |
| Plant-Growth-Promoting Rhizobacteria   | 8            |
| Phosphorus-Solubilizing Bacteria   | 9            |
| Plant Hormone Production by Bacteria   | 9            |
| Fungi and Their Potential As Biofertilizers  | 10           |
| Use of Arbuscular Mycorrhizal Fungi As Biofertilizers  | 11           |
| Use of Ectomycorrhizal Fungi As Biofertilizers   | 13           |
| Other Root-Colonizing Fungi  | 14           |
| Multiple Inoculations and Interactions Among Potential<br>Biofertilizers   | 15           |
| Future Perspectives and Conclusions  | 18           |

|  |           |
|--|-----------|
| <b>Chapter 2. Evaluation of the Functional Group of Microorganisms As Bioindicators on the Rhizosphere Microcosm</b> | <b>29</b> |
| <i>Ulisses Brigatto Albino</i>   |           |
| <i>Galdino Andrade</i>   |           |
| Introduction   | 29        |
| The Functional Groups  | 31        |
| Methods to Assess the Soil Functional Microorganism Groups   | 34        |
| Protocols  | 35        |
| Future Perspectives  | 46        |
| Conclusions  | 46        |
| <b>Chapter 3. Tripartite Relationship of <i>Rhizobium</i>, AMF, and Host in Growth Promotion</b>                     | <b>51</b> |
| <i>Nanjian Raman</i>   |           |
| <i>Thangaswamy Selvaraj</i>  |           |
| Introduction   | 51        |
| <i>Rhizobium</i> and AMF Interactions  | 52        |
| <i>Rhizobium</i> , AMF, and Biochemical Activities   | 58        |
| Enzyme Activities  | 60        |
| Growth Hormones  | 62        |
| Calcium and Phosphorus Requirements  | 63        |
| Heavy Metals   | 64        |
| Plant Uptake of Metals   | 68        |
| Plant-Growth-Promoting Rhizobacteria   | 69        |
| Coexistence of <i>Rhizobium</i> and AM Symbioses   | 69        |
| Conclusions and Future Perspectives  | 71        |
| <b>Chapter 4. Biological Fertilizers for Sustainable Rice Production</b>   | <b>89</b> |
| <i>S. Kannaiyan</i>  |           |
| <i>K. Kumar</i>  |           |
| Introduction   | 89        |
| Azolla Biofertilizer for Rice Crop   | 90        |
| Nitrogen-Fixing Blue Green Algae   | 92        |
| Acid-Tolerant Cyanobacteria  | 94        |
| N <sub>2</sub> -Fixing Stem-Nodulating <i>Sesbania</i> Biofertilizer for Rice  | 96        |
| <i>Sesbania</i> As Biofertilizer for Rice  | 100       |

|  |            |
|--|------------|
| Neem Cake and N Use Efficiency   | 100        |
| Urea Super Granules and N Use Efficiency   | 103        |
| Soil Enzyme Activity   | 104        |
| Induction of Nodule-Like Structures in Rice Roots<br>for N <sub>2</sub> Fixation                   | 104        |
| <i>Azospirillum</i> Biofertilizer for Rice   | 107        |
| Phosphobacterial Biofertilizer   | 107        |
| Future Perspectives  | 108        |
| Conclusions  | 109        |
| <br>   |            |
| <b>Chapter 5. Mycorrhiza Helper Bacteria: Their Ecological<br/>Impact in Mycorrhizal Symbiosis</b> | <b>117</b> |
| <i>Robin Duponnois</i>   |            |
| Introduction   | 117        |
| The Mycorrhizosphere Effect  | 118        |
| Evidence   | 123        |
| Biological Properties  | 125        |
| Mechanisms Involved in the MHB Effect  | 126        |
| Application  | 128        |
| Conclusions and Perspectives   | 129        |
| <br>   |            |
| <b>Chapter 6. Plant-Growth-Promoting Rhizobacteria<br/>As Biofertilizers and Biopesticides</b>     | <b>137</b> |
| <i>Manas Ranjan Banerjee</i><br><i>Laila Yesmin</i><br><i>Joseph Kevin Vessey</i>                  |            |
| Introduction   | 137        |
| Biofertilizers   | 139        |
| Endophytic and Rhizospheric Relationships Between PGPR<br>and Their Hosts                          | 141        |
| Mode of Action of PGPR As Biofertilizers   | 143        |
| Biopesticides  | 154        |
| Mode of Action of PGPR As Biopesticides  | 157        |
| Viability and Shelf Life of PGPR   | 164        |
| Benefits of PGPR   | 165        |
| Limitations of PGPR  | 167        |
| Future Perspectives  | 168        |
| Conclusions  | 170        |

|   |            |
|---|------------|
| <b>Chapter 7. Sustainable Agriculture<br/>and the Rhizobia/Legumes Symbiosis</b>        | <b>183</b> |
| <i>João Ruy Jardim Freire</i><br><i>Enilson Luiz Saccol de Sá</i>                       |            |
| Introduction  | 183        |
| Diversity of Rhizobia   | 184        |
| The Selection of Rhizobia Strains   | 188        |
| Desirable Characteristics   | 189        |
| Production and Quality of the Inoculants  | 190        |
| Techniques of Inoculation   | 190        |
| Soil Limiting Factors   | 191        |
| Some Management Practices   | 193        |
| Future Perspectives   | 195        |
| Conclusions   | 196        |
| <b>Chapter 8. Wild-Legume Rhizobia: Biodiversity<br/>and Potential As Biofertilizer</b> | <b>203</b> |
| <i>Hamdi Hussein Zahran</i>   |            |
| Introduction  | 203        |
| Taxonomy and Biodiversity of Wild-Legume Rhizobia                                       | 204        |
| Symbiotic Nitrogen-Fixing Systems Established<br>with Wild Rhizobia                     | 206        |
| Behavior of Wild-Legume Rhizobia Under Extreme<br>Conditions                            | 209        |
| Future Perspectives   | 212        |
| Conclusions   | 214        |
| <b>Chapter 9. Potential of Arbuscular Mycorrhizae<br/>in Organic Farming Systems</b>    | <b>223</b> |
| <i>Tanu</i><br><i>Anil Prakash</i><br><i>Alok Adholeya</i>                              |            |
| Introduction  | 223        |
| Mycorrhizae in Organic Farming  | 224        |
| Factors Affecting Mycorrhizal Inoculum Potential  | 226        |
| Selection of AM Fungi   | 230        |
| Integration of AM Fungi and Composts for Disease Control                                | 230        |
| Organic Manure and AMF Inoculum Production  | 231        |
| Application of AM Fungi   | 231        |

|   |            |
|---|------------|
| Future Perspectives   | 232        |
| Conclusions   | 233        |
| <b>Chapter 10. Role of Mycorrhizae in Forestry</b>  | <b>241</b> |
| <i>Hanna Dahm</i>   |            |
| Introduction  | 241        |
| Ecological Significance of Mycorrhizae  | 241        |
| Types of Mycorrhizae on Forest Trees  | 243        |
| Occurrence of Mycorrhizal Fungi in Soils of Native Forests  | 248        |
| Soil Type and Humus Properties  | 249        |
| Ecological Specificity  | 250        |
| Competition Among Ectomycorrhizal Fungi in Forest Soil  | 250        |
| Favorable Effects of Mycorrhizae on Plant Growth  | 252        |
| Factors Affecting Ectomycorrhizal Reduction   | 258        |
| Receptiveness of Forest Soils to Ectomycorrhizal Association  | 261        |
| Practical Aspects of Mycorrhizae: Perspectives  | 262        |
| Conclusions   | 265        |
| <b>Chapter 11. Physiological and Molecular Aspects of Osmotic Stress Alleviation in Arbuscular Mycorrhizal Plants</b> | <b>271</b> |
| <i>Juan Manuel Ruiz-Lozano</i>  |            |
| Introduction  | 271        |
| Uptake and Transfer of Water Through the Fungal Hyphae to the Host Plant  | 274        |
| Amelioration of Plant Gas Exchange  | 276        |
| Changes in the Water Retention Properties of the Soil   | 277        |
| Stimulation of Assimilative Activities Essential for Plant Growth   | 278        |
| Osmotic Adjustment  | 278        |
| Protection Against the Oxidative Damage Generated by Drought  | 280        |
| Future Perspectives and Conclusions   | 283        |
| <b>Chapter 12. Arbuscular Mycorrhizal Inoculation in Nursery Practice</b>   | <b>289</b> |
| <i>Miguel Marin</i>   |            |
| Introduction  | 289        |

|   |            |
|---|------------|
| Types of Mycorrhizae  | 290        |
| Mycorrhizae Effects on Plant Physiology   | 292        |
| Interaction of Arbuscular Mycorrhizal Fungi with Soil Microbiota  | 295        |
| Mycorrhizae As Substitute for Fertilizers   | 296        |
| Mycorrhizal Symbiosis in Horticultural Systems  | 298        |
| Endomycorrhizal Technology in Different Propagation Systems   | 300        |
| Management of Arbuscular Mycorrhiza in Nursery Plant Production   | 302        |
| Future Perspectives   | 309        |
| Conclusions   | 310        |
| <b>Chapter 13. Interaction Between Arbuscular Mycorrhizal Fungi and Root Pathogens</b>  | <b>325</b> |
| <i>Leonor Costa Maia</i>  |            |
| <i>Norma Suely Sobral da Silveira</i>   |            |
| <i>Uided Maaze Tiburcio Cavalcante</i>  |            |
| Introduction  | 325        |
| Root Colonization   | 326        |
| Changes Produced by Mycorrhization  | 326        |
| Interaction: Arbuscular Mycorrhizal Fungi and Plant Pathogenic Fungi  | 329        |
| Arbuscular Mycorrhizal Fungi × Bacteria   | 334        |
| Arbuscular Mycorrhizal Fungi × Nematodes  | 336        |
| Future Perspectives   | 340        |
| Conclusions   | 341        |
| <b>Chapter 14. Production of Seedlings Inoculated with Arbuscular Mycorrhizal Fungi and Their Performance After Outplanting</b> | <b>353</b> |
| <i>Orivaldo José Saggin-Júnior</i>  |            |
| <i>Eliane Maria Ribeiro da Silva</i>  |            |
| Introduction  | 353        |
| Prerequisites for the Inoculation of Seedlings with AMF   | 355        |
| Production of Inoculants for Seedlings  | 362        |
| Production of Seedlings Inoculated with AMF   | 368        |
| Future Perspectives of Mycorrhizal Seedling Production  | 382        |
| Conclusions   | 383        |

|  |            |
|--|------------|
| <b>Chapter 15. Status of Endomycorrhizal (AMF)<br/>Biofertilizer in the Global Market</b>          | <b>395</b> |
| <i>Polachi Raja</i>  |            |
| Introduction   | 395        |
| World Fertilizer Consumption   | 396        |
| Alternative Farming Systems  | 397        |
| Sustainable Agriculture Systems  | 398        |
| Effective Microorganisms   | 400        |
| Evolution of AMF   | 401        |
| Techniques of AMF Inoculum Propagation   | 404        |
| AMF Benefits of Symbiosis  | 406        |
| Impact of AMF in Agriculture   | 406        |
| Availability and Cost of Mycorrhizal Inoculum  | 407        |
| Producers and Formulators of AMF Inoculum  | 407        |
| Future Perspectives  | 407        |
| Conclusions  | 412        |
| <b>Chapter 16. Role of Cyanobacteria As Biofertilizers:<br/>Potentials and Limitations</b>         | <b>417</b> |
| <i>Pilar Irisarri</i>  |            |
| Introduction   | 417        |
| Current Status of Cyanobacterial Use As Biofertilizers   | 418        |
| Availability of Fixed Nitrogen to Rice Plants  | 419        |
| Cultivation of Cyanobacteria for Biofertilizer   | 420        |
| Inoculation and Crop Yield   | 421        |
| Reasons for the Failure of Inoculation   | 422        |
| Benefits of Cyanobacteria Beyond Nitrogen Input<br>to the Agroecosystem                            | 424        |
| Is Inoculation with Cyanobacteria Worthwhile?  | 425        |
| Integrated Soil Fertility Management   | 426        |
| Future Perspectives  | 427        |
| Conclusions  | 428        |
| <b>Chapter 17. Cyanobacterial Biofertilizers for Rice:<br/>Present Status and Future Prospects</b> | <b>433</b> |
| <i>Siba Prasad Adhikary<br/>Bagmi Pattanaik</i>  |            |
| Introduction   | 433        |
| Cyanobacteria (Blue-Green Algae)   | 434        |

|  |            |
|--|------------|
| Factors Governing Distribution of Cyanobacteria<br>During the Crop Growth Cycle  | 440        |
| Seasonal Variation of Cyanobacterial Growth<br>in Paddy Fields   | 441        |
| Phosphorus   | 443        |
| Cyanobacteria Biofertilizer for Increased Crop Yield<br>and Maintenance of Soil Quality  | 445        |
| Algalization Technology  | 446        |
| Future Perspectives  | 449        |
| Conclusions  | 450        |
| <b>Chapter 18. A Comparative Study on Nitrogen-Fixing<br/>Cyanobacteria in South American and European<br/>Rice Fields</b>                                     | <b>459</b> |
| <i>Carlos Hugo Prospero</i>  |            |
| Introduction   | 459        |
| Measuring Nitrogen Fixation in the Field   | 462        |
| Determination of Nitrogenase Activity in Vivo  | 463        |
| Samples Collection and Identification  | 463        |
| The Diversity of Cyanobacteria   | 464        |
| Future Perspectives  | 471        |
| Appendix: Identification Key for Most Common<br>Cyanobacteria in Rice Fields of Argentina and Spain  | 472        |
| <b>Chapter 19. <i>Piriformospora indica</i> As a New<br/>and Emerging Mycofertilizer and Biotizer:<br/>Potentials and Prospects in Sustainable Agriculture</b> | <b>477</b> |
| <i>Sarika Shende</i> <i>Mahendra Rai</i><br><i>Kanchan Bhagwat</i> <i>Ajit Varma</i><br><i>Prasad Wadegaonkar</i>  |            |
| Introduction   | 477        |
| The Morphology and Cultural Characteristics  | 478        |
| Mycelial Penetration in Host   | 478        |
| Ultrastructure of Hyphal Wall  | 478        |
| Molecular Systematics  | 479        |
| Root Colonization Potential in Diverse Hosts   | 480        |
| In Vitro Rapid Proliferation of Roots of <i>Withania somnifera</i>   | 480        |
| Rhizoctonia-Like Growth Promotion of Orchids   | 480        |
| Enhancement of Secondary Metabolites   | 481        |

|  |            |
|--|------------|
| Mechanisms Involved During Colonization  | 482        |
| Mycorrhiza-Like Endophytes   | 483        |
| Protoplast Fusion for Better Understanding of Interaction                      | 483        |
| Strategies for Mass Production of Inocula                                      | 485        |
| Immobilization of Spores and Hyphae: A Step Forward<br>for Inoculum Production | 485        |
| Application in Horticulture  | 486        |
| Potential for Control of Phytopathogenic Rootborne<br>Microbes                 | 488        |
| Conclusions and Future Directions  | 490        |
| <b>Chapter 20. Matsutake: A Natural Biofertilizer?</b>                         | <b>497</b> |
| <i>Wang Yun</i>  |            |
| <i>Ian Robert Hall</i>   |            |
| Introduction   | 497        |
| Matsutake in the Commercial World  | 499        |
| Taxonomic History of Matsutake   | 502        |
| Ecology  | 502        |
| Morphology of Matsutake Infections   | 508        |
| Cultivation of <i>Tricholoma matsutake</i>                                     | 519        |
| Relationship Between Matsutake and Its Host Plants                             | 526        |
| Future Challenges  | 531        |
| Conclusions  | 532        |
| <b>Index</b>   | <b>543</b> |

## Preface

The excessive use of chemical fertilizers and pesticides has generated several environmental problems including the greenhouse effect, ozone layer depletion, and acidification of water. These problems can be tackled by use of biofertilizers and biopesticides, which are natural, beneficial, and ecologically and user-friendly. The biofertilizers provide nutrients to the plants, control soilborne diseases, and maintain soil structure. Microbial biofertilizers play a pivotal role in sustainable agriculture. Arbuscular mycorrhizal fungi (AMF) are important microbes of soil that form symbiotic associations with most of the terrestrial plants on the earth. These fungi are chiefly responsible for phosphorus (P) uptake. Early inoculation at the seedling stage has been proven beneficial. Even in tissue culture industries, early mycorrhization of micropropagated plantlets overcome “transplant shock” due to their sophisticated nature. However, AMF cannot be cultured axenically, which poses a problem in rapid and mass multiplication in vitro conditions.

Another group of microbes is nitrogen-fixing bacteria, which are potent microbial inoculants now universally used for growth promotion of plants. The use of *Rhizobium* cultures in legume crops is increasing day by day to sustain agricultural productivity. These inoculants are commonly available in the market. An additional advantage of these microbes is that their axenic culture can be prepared in the laboratory and stored for mass inoculation. Phosphate-solubilizing bacteria are also immensely important as they have been reported to increase uptake of P by converting insoluble forms to soluble ones. *Azotobacter* and *Azospirillum* are two other efficient bacteria. The response of these organisms in increasing crop yield has been commonly experienced. In addition to these microbes, blue-green algae also contribute to a great extent in nitrogen economy of sustainable agriculture. The tripartite relationship of legume-rhizobium-mycorrhizae is the most efficient combination for growth promotion and for gaining higher yield of crops, vegetables, and trees.

Blue-green algae are the great suppliers of nitrogen. Their importance for bumper production of crops cannot be overstated and has been realized

the world over by farmers. Their multiplication is easy and they now can be obtained in packets in the open market.

As a matter of fact, application of microbial biofertilizers is a natural and effective way of increasing and maintaining the mineral economy of nature. However, they cannot be treated as substitute for chemical fertilizers. Their use reduces the use of chemical fertilizers, which is the only alternative for sustainable agriculture.

There has been great demand for a book on microbial fertilizers, wherein different issues concerning microbial fertilizers can be taken up. This book will certainly provide useful information dealing with a diverse group of microbes, beneficial effects, and the bottlenecks in their implementation.

Biotechnologists, microbiologists, botanists, growers, industrialists dealing with production of microbial fertilizers, environmentalists, and, above all, the users should find this book immensely useful.

## Chapter 1

# Potential and Possible Uses of Bacterial and Fungal Biofertilizers

Francesco Gentili  
Ari Jumpponen

### *INTRODUCTION*

During the past four decades we have witnessed the doubling of the human population and a concurrent doubling of food production (Vance, 2001). Plant nutrition has played a key role in this dramatic increase in demand for and supply of food. Increases in crop production have been made possible through the use of commercial man-made fertilizers. The use of nitrogen (N) fertilizer has increased almost ninefold and phosphorus (P) more than fourfold (Vance, 2001). The tremendous increase of N and P fertilization, in addition to the introduction of highly productive and intensive agricultural systems, has allowed these developments to occur at relatively low costs (Schultz et al., 1995; Vance, 2001). The increasing use of fertilizers and highly productive systems have also created environmental problems such as deterioration of soil quality, surface water, and groundwater, as well as air pollution, reduced biodiversity, and suppressed ecosystem function (Schultz et al., 1995; Socolow, 1999; Vance, 2001).

Environmental pollution resulting from greater nutrient availability can be either direct or indirect. Directly, misuse and excessive or poorly managed use of fertilizers can result in leaching, volatilization, acidification, and denitrification. Indirectly, the production (use of fossil fuel in Haber-Bosch process) and transport (combustion of fossil fuel) of fertilizers result in airborne CO<sub>2</sub> and N pollution, which will be eventually deposited into terrestrial ecosystems. A more comprehensive and complete view of the N cycle and impacts of N deposition at the global level can be found in Socolow (1999).

Community waste and sewage sludge provide an inexpensive and attractive alternative. One problem with the use of these sources of plant nutrition is their high content of heavy metals, which may have adverse effects on

crop growth, crop consumers, or microorganisms in soil or rhizosphere (Giller et al., 1998; Graham and Vance, 2000). Consequently, these sources of inexpensive plant nutrients may not be suitable for fertilization of crops intended for human consumption if the heavy metal content is not drastically reduced.

The most limiting nutrients for plant growth are N and P (Schachtman et al., 1998). Although soil may contain vast amounts of either nutrient, most is not readily available for plant use. Most of N is tied into the soil organic matter. Even after fertilization, plants have to compete with soil microbes for easily available soluble N. Problems with P are different. In acidic soils, even when added in substantial quantities as fertilizer, P precipitates with iron or aluminum, whereas in alkaline soils P precipitates as calcium phosphates (Hinsinger, 2001). Accordingly, P limitation may be a difficult problem to overcome through the addition of P-containing fertilizers. We refer the reader to Frossard et al. (1995) for a more comprehensive description of the reactions controlling P availability in soil. The extensive fertilization required to overcome N and P limitations may lead to (sub-)surface runoff, and the soluble nutrients can easily end up in surface-water bodies or groundwater. For example, the loss of P from agricultural systems is one of the main causes of eutrophication and hypoxia in lakes and estuaries in the developed world (Vance, 2001).

In conclusion, the recent great increases in crop yields and food production in developed countries have been achieved by intensive agricultural practices. These increases, however, have not come without tremendous environmental costs (Vance, 2001). In developing countries the problems are different. The lack of fertilizers and adequate agricultural practices do not allow intensive crop production, and a vast segment of the population remains undernourished. Clearly, there is an urgent need for sustainable agricultural practices on a global level. In the developed world a reduction of energy and environmental costs is necessary. In developing countries, efficient and sustainable practices are needed to allow cost-efficient production of adequate nutrition for the growing populations. To overcome the ecological problems resulting from the loss of plant nutrients and to increase crop yields in the absence of resources for obtaining costly fertilizers, microscopic organisms that allow more efficient nutrient use or increase nutrient availability can provide sustainable solutions for present and future agricultural practices. In this chapter we aim to provide a brief overview of potential use of various biological agents with shown or suggested crop-yield-improving properties. Further, we highlight a few well-studied systems and use those as examples for possible choices for practical large-scale applications.

### ***N<sub>2</sub>-FIXING BACTERIA AS MICROBIAL BIOFERTILIZERS***

Atmospheric N<sub>2</sub> composes approximately 80 percent of the air we breathe. Although abundant and ubiquitous in the air, N is the most limiting nutrient to plant growth because the atmospheric N is not available for plant uptake. Some bacteria are capable of N<sub>2</sub> fixation from the atmospheric N pool. These bacteria form various associations with plants:

1. Many free-living N<sub>2</sub>-fixing bacteria occur in soil.
2. Some have adapted to form symbioses; others have intimate endophytic associations with plants.
3. Others live in close association in the plant root zone (rhizosphere) without forming intimate endophytic symbioses.

The amount of N fixed by these different systems is considerable, although variation resulting from environmental conditions or different plant-microbe combinations is vast. The close proximity of these microorganisms to their host plants allows efficient plant use of fixed N and minimizes volatilization, leaching, and denitrification. In the following section, we discuss the different types of microorganisms capable of allowing plant access to the atmospheric N pool and consider their use as biofertilizers.

### ***SYMBIOTIC N<sub>2</sub>-FIXING BACTERIA***

#### **Rhizobia**

The best known and most exploited symbiotic N<sub>2</sub>-fixing bacteria are those belonging to the family Rhizobiaceae (*Rhizobia*) and include the following genera: *Rhizobium*, *Bradyrhizobium*, *Sinorhizobium*, *Azorhizobium*, *Mesorhizobium*, and *Allorhizobium* (Vance, 1998; Graham and Vance, 2000). More comprehensive information on rhizobial symbiosis can be found in Spaink et al. (1998). These bacteria infect legumes and have a global distribution ranging from high latitudes in Europe and North America to the equator, to tropics in Australia and South America. In equatorial and tropical areas, legumes are particularly important; they are utilized in silvo-pastoral and agroforestry systems (Dommergues and Subba Rao, 2000). The N<sub>2</sub>-fixing capability of rhizobia varies greatly (up to 450 Kg N·ha<sup>-1</sup>) among host plant species and bacterial strains (Stamford et al., 1997; see Table 9 in Unkovich et al., 1997; Unkovich and Pate, 2000). Therefore, selection of the best strains must take rhizobia-host compatibility into account for selection of biofertilizers. Additional key characteristics are essential

for rhizobial strains that are considered for practical applications as bio-fertilizers. They must have a high  $N_2$ -fixation rate and be able to compete with the indigenous rhizobia to maximize infection of the target crops (Stephens and Rask, 2000). From a practical perspective, the inoculum must be easily produced and have a high survivorship in field applications once inoculated on target seeds (Date, 2000).

Legume inoculation is an old practice that has been carried out for more than a century in agricultural systems in the United States and the United Kingdom (Brockwell and Bottomley, 1995; Catroux et al., 2001). Inoculation is particularly important when local and resident soil rhizobial populations are either absent or known to be very low (Catroux et al., 2001). For example, acidic soils generally contain no or low population densities of the alfalfa rhizobial symbiont *Sinorhizobium meliloti*, whereas basic soils contain a low inoculum potential of *Bradyrhizobium* sp., a rhizobial symbiont of *Lupinus* spp. (Amager, 1980, quoted by Catroux et al., 2001). Under such conditions inoculation with compatible rhizobia is likely to prove highly advantageous.

Prior to initiating a large-scale inoculation program with rhizobia, it is essential to evaluate the need for inoculation and perform a cost-benefit analysis. If rhizobial population densities are high, the inoculation is likely to prove unnecessary and investment in inoculation practice might be wasted. Catroux et al. (2001) suggested that when rhizobial population density is lower than 100 rhizobia per gram of soil, inoculation is likely to be beneficial for crop productivity. In such low population densities inoculation would prove cost efficient regardless of the  $N_2$ -fixation efficiency of the indigenous rhizobia. Unfortunately, fast, simple, and inexpensive methods for rhizobial population quantification are not readily available. Field experiments are time-consuming and can take several months. Microbiological assays are more expedient and take a few weeks but require substantial expertise (Brockwell and Bottomley, 1995). We refer the reader to Brockwell et al. (1988) for a description of assays estimating the  $N_2$ -fixing capacity of resident soil rhizobia.

The rhizobial inoculum can be produced and applied in numerous ways. Inoculum can be prepared as powder, liquid, and granular formulations. Granular formulations are convenient as they allow control of placement and application rate (Stephens and Rask, 2000). An additional important feature of an inoculum is the selection of the carrier (e.g., peat, perlite, mineral soil, charcoal). Whichever the carrier, its sterilization is necessary to maximize inoculum survival and subsequent infection rate (Brockwell and Bottomley, 1995; Stephens and Rask, 2000; Catroux et al., 2001). We refer the reader to Bashan (1998), Stephens and Rask (2000), and Lupwayi et al.

(2000) who provide extensive recent reviews on inoculation practices and choices on carriers and formulations.

It is important to control the quality of the inoculum. Unfortunately, the inoculum quality is often questionable and possibly up to 90 percent of all available inocula have no practical effect on the productivity of legumes (Brockwell and Bottomley, 1995). There are several possible reasons for the lacking crop yield response to inoculation. These range from poor control of inoculum production (low density of infective propagules or inadequate inoculum storage conditions) to incompatible inoculum-crop species combinations or edaphic conditions, which may preclude successful inoculation. Both federally and internationally controlled and standardized regulations are needed to simplify and clarify the rules on the quality and trade of commercially produced inocula. Academic institutions and agricultural experimental stations would provide an already existing network that could conveniently facilitate services to evaluate available inocula and provide much-needed quality control. Currently, purchase of inoculum in many cases is investment in a commodity of poor or questionable value. More information on inoculum quality control is available in recent reviews by Bashan (1998), Stephens and Rask (2000), and Lupwayi et al. (2000).

### **Frankia**

*Frankia* is the genus of N<sub>2</sub>-fixing actinomycetes that are capable of infecting and nodulating a group of eight families of mainly woody plants (Benson and Silvester, 1993; Huss-Danell, 1997; Wall, 2000). These so-called actinorhizal plants are used in land reclamation, for timber and fuel wood production, in mixed plantations, for windbreaks, as well as for shelterbelts along deserts and coastlines (Schwencke and Carù, 2001). Actinorhizal *Hippophaë rhamnoides* is cultivated for its berries. *Frankia* N<sub>2</sub> fixation has been estimated to be similar to rhizobial symbioses (Torrey, 1978; Dawson, 1986; Dommergues, 1995). Despite the potential importance of *Frankia* symbiosis, only limited information is available for inoculation practices and their use. *Frankia* inoculation can be advantageous in arid environments, disturbed sites, and areas where native actinorhizal plants are absent (Schwencke and Carù, 2001). Key factors for strain selection are similar to those described for *Rhizobium* sp. In addition to the symbiotic properties of the strains, characteristics such as age of the inoculum culture, cellular concentration, and the method of preservation may greatly affect inoculum infectivity. Since a universal strain adapted to all different environments and host genotypes does not exist, the best plant-*Frankia*

combination should be selected and customized for each target area and for each target species (Schwencke and Carù, 2001).

Inoculation and nodulation before seedling transplanting improves plant survival and performance (Prat, 1992). Consequently, inoculation in a nursery is likely to be most efficient (Sprent and Parsons, 2000). *Frankia* inoculum can be lyophilized, frozen in glycerol, or cultured on complex medium (Fontaine et al., 1986; Sougoufara et al., 1989). *Frankia* spores have also been successfully used for *Casuarina cunninghamiana* inoculation (Lalonde and Calvert, 1979; Burleigh and Torrey, 1990). Similar to rhizobial inocula, several different formulations and carriers can be utilized. This adds flexibility for selection of the most simple and applicable method of inoculation for each program.

We emphasize the need for developing techniques for nursery inoculation, which would allow the use of nodulated actinorhizal plants in land reclamation and revegetation applications. Studies from natural systems indicate clearly that the actinorhizal plants may facilitate and expedite early successional plant community development in nonvegetated terrain (Schwencke and Carù, 2001). More information on *Frankia* and actinorhizal plants can be found in Schwintzer and Tjepkema (1990).

### CYANOBACTERIA

Cyanobacteria are ecologically important. For example, an aquatic cyanobacterium, *Trichodesmium*, contributes approximately 36 percent of global N<sub>2</sub> fixation (Gallon, 2001). Cyanobacterial N<sub>2</sub> fixation has been essential in the cultivation of rice. Until the end of the 1970s *Azolla-Anabaena* symbiosis was the major N source for the 6.5 × 10<sup>6</sup> ha of rice cultivation in China. Presently, population pressure and increased labor costs have decreased the reliance on cyanobacterial symbioses in Chinese rice cultivation (Graham and Vance, 2000). In Uruguay and many parts of Asia, the cyanobacteria (mainly *Nostoc* and *Anabaena*) still have a vital importance for rice-field fertility (Kundu and Ladha, 1995; Irisarri et al., 2001). Cyanobacteria and their N<sub>2</sub> fixation find further application in the remediation of arid soils. In the Sahelian soil in Niger, N<sub>2</sub>-fixing cyanobacteria are present in the soil surface crust. These cyanobacteria have been shown to increase soil N content (Malam Issa et al., 2001), therefore bearing a great promise for reclamation of extreme, arid environments.

Despite the traditional importance of the N<sub>2</sub>-fixing cyanobacteria in rice cultivation and their possible reclamation uses in arid environments (Malam Issa et al., 2001) or ecosystems prone to frequent and/or seasonal flooding (Bashan et al., 1998), the production and application of cyanobacteria is

still fairly poorly developed (Hashem, 2001). However, cyanobacteria should be seriously considered as a biofertilizer supporting sustainable agricultural practices in various environments (Hashem, 2001).

### ***N<sub>2</sub>-FIXING ASSOCIATED BACTERIA***

In addition to symbiotic bacteria infecting plant roots, numerous taxa of less intimately associated N<sub>2</sub>-fixing bacteria can be considered for crop yield improvement. Examples of such bacteria include *Acetobacter diazotrophicus* and *Herbaspirillum* spp. associated with sugarcane, sorghum, and maize (Triplett, 1996; James et al., 1997; Boddey et al., 2000), *Azoarcus* spp. associated with kallar grass (*Leptochloa fusca*) (Malik et al., 1997), and *Alcaligenes*, *Azospirillum*, *Bacillus*, *Enterobacter*, *Herbaspirillum*, *Klebsiella*, *Pseudomonas*, and *Rhizobium* associated with rice and maize (James, 2000). The genus *Azospirillum* colonizes a great variety of annual and perennial plants, many of which have never been reported to be colonized by N<sub>2</sub>-fixing bacteria. Accordingly, *Azospirillum* possesses a great potential as a general root colonizer, whose use is not limited by host specificity (Bashan and Holguin, 1997). Indeed, several studies indicate that *Azospirillum* can increase the growth of various crops. These include sunflower, carrot, oak, sugarbeet, tomato, eggplant, pepper, and cotton in addition to wheat and rice (Bashan et al., 1989; Bashan and Holguin, 1997). In two decades of field experiments, general consensus is that in 60 to 70 percent of the cases *Azospirillum* application results in a significant crop yield increase (Okon and Labandera-Gonzalez, 1994). The yield increases can be substantial, up to 30 percent, but generally range from 5 to 30 percent. These yield increases by *Azospirillum* are possibly a result of the production of growth-promoting substances rather than N<sub>2</sub> fixation (Okon, 1985).

The main problem that limits the use of *Azospirillum* on a large scale is the great uncertainty and unpredictability of the results. Regardless of these uncertainties *Azospirillum* bears great promise as a growth-promoting N<sub>2</sub>-fixing biofertilizer. Its growth-promoting properties are fairly well documented, and its commercial production as well as field application are simple. Inoculum can be produced and applied as in peat formulation, production of which is inexpensive. The peat formulation can also be directly utilized in field research and agricultural applications. However, alternative carriers warrant and require further research to allow selection of a reliable and effective means for inoculum production and field application (Vande Broek et al., 2000).

To emphasize the potential significance of N<sub>2</sub> fixation by associated bacteria, we use *Acetobacter diazotrophicus* and sugarcane as an additional ex-

ample. As with actinorhizal or rhizobial symbioses, the quantity of fixed N is substantial and therefore associated bacteria are attractive candidates for biofertilizers. *Acetobacter diazotrophicus* can fix up to 70 percent of the sugarcane N requirement (Boddey et al., 1995). This translates to an annual 150 kg N·ha<sup>-1</sup> that is fixed from the atmosphere and made available to the crop plant (Boddey et al., 1995). These estimates and their generality require further work as it appears that the amount of N<sub>2</sub> fixed depends on the plant genotype and environmental or edaphic conditions.

### **FREE-LIVING N<sub>2</sub>-FIXING BACTERIA**

Many free-living bacteria also fix atmospheric N<sub>2</sub>. Examples of such free-living bacteria include *Azotobacter*, *Beijerinckia*, and *Clostridium*. Furthermore, if environmental conditions allow, nodulating bacterial symbionts (e.g., *Frankia*) of plant roots can also fix N<sub>2</sub> when not in a symbiotic association (free-living culture) (Benson and Silvester, 1993) with their plant host. More interestingly, it has been found that *Frankia* can occur and possibly fixes atmospheric N<sub>2</sub> in the rhizosphere of nonhost plants. *Frankia* has been recorded in the rhizosphere of *Betula pendula* (Smolander and Sarsa, 1990) and in soil where actinorhizal plants were not present (Benson and Silvester, 1993). These observations strongly suggest *Frankia* N<sub>2</sub> fixation in the rhizosphere of nonactinorhizal plants. These results open exciting possibilities for utilization of *Frankia* in biofertilizer applications but should be confirmed and further evaluated.

Estimation of the N<sub>2</sub> fixation by free-living bacteria is difficult. In an alfalfa (*Medicago sativa*) stand, the contribution of free-living N<sub>2</sub>-fixing bacteria was estimated to range from 3 to 10 kg N·ha<sup>-1</sup> (Roper et al., 1995). In a greenhouse experiment using different types of bacterial inoculation methods (leaf spray, seed soaking, side dressing), *Beijerinckia mobilis* and *Clostridium* spp. stimulated growth in cucumber and barley plants (Polyanskaya et al., 2002). The mechanism of the growth stimulation, however, remained unclear. Although the N<sub>2</sub> fixation by the inoculated bacteria may have played a significant role, other mechanisms cannot be excluded. It is possible that the detected growth response was also due to the bacterial synthesis of secondary growth-promoting compounds, such as plant growth hormones (Polyanskaya et al., 2002).

### **PLANT-GROWTH-PROMOTING RHIZOBACTERIA**

Various bacteria can promote plant growth (Bashan, 1998). Collectively such bacteria are called plant-growth-promoting rhizobacteria (PGPR).

PGPR is a generic acronym that indicates bacteria, in some often unknown way, can stimulate plant growth. These bacteria vary in their mechanism of plant growth promotion but generally influence growth via P solubilization, nutrient uptake enhancement, or plant growth hormone production (Bashan et al., 1990; Okon and Labandera-Gonzalez, 1994; De Freitas et al., 1997; Goldstein et al., 1999; Richardson, 2001). Bertrand et al. (2000) showed that a rhizobacterium belonging to the genus *Achromobacter* could enhance root hair number and length in oilseed rape (*Brassica napus*). *Achromobacter* increased NO<sub>3</sub> and K uptake and, consequently, shoot and root dry weights by 22 to 33 percent and 6 to 21 percent respectively (Bertrand et al., 2000).

### **PHOSPHORUS-SOLUBILIZING BACTERIA**

We first discussed the importance of the bacterial N<sub>2</sub> fixation because N is generally the most limiting nutrient for plant growth. Phosphorus is the second most limiting plant nutrient after N (Schachtman et al., 1998). Total P content in soil is usually high, but most of this soil P pool is not in forms available for plant uptake. Bacteria that can mobilize P from unavailable soil pools and increase P availability to plants are of great importance. Most predominant phosphorus-solubilizing bacteria (PSB) belong to the genera *Bacillus* and *Pseudomonas* (Richardson, 2001).

Field experiments highlight the potential importance of PSB. Sundara et al. (2002) applied rock phosphate with a PSB (*Bacillus megaterium* var. *phosphaticum*) in lignite-based culture medium in a field experiment. They found that without P application PSB amendment could increase sugarcane yield by 12.6 percent. PSB and P fertilizer together reduced the P requirement by 25 percent. Furthermore, 50 percent of the costly superphosphate could be replaced with inexpensive rock phosphate. PSB also improved the sugar yield and juice quality (Sundara et al., 2002). In conclusion, PSB may be of greatest value in allowing use of cheaper P sources (e.g., rock phosphate instead of superphosphate).

### **PLANT HORMONE PRODUCTION BY BACTERIA**

In addition to improving plant nutrition by fixing atmospheric N or solubilizing plant-unavailable pools of P, bacteria can influence plant growth by production of plant hormones. *Bacillus pumilus* and *B. licheniformis*, isolated from the rhizosphere of alder (*Alnus glutinosa*), can produce high amounts of physiologically active gibberellins (Gutierrez-Mañero

et al., 2001). *Paenibacillus polymyxa* (recently transferred from genus *Bacillus*) is able to produce cytokinins (Timmusk et al., 1999). According to Timmusk et al. (1999), *P. polymyxa* may possess a great variety of properties that are of interest in the development of biofertilizers. The reported properties include N<sub>2</sub> fixation, P solubilization, and production of antibiotics, chitinase, and other hydrolytic enzymes, as well as enhancement of soil porosity. Similarly, taxa in the genus *Azospirillum* can fix atmospheric N<sub>2</sub>, enhance plant mineral uptake, and produce growth-promoting plant hormones (Bashan et al., 1990; Bashan and Holguin, 1997). *Azospirillum* and *P. polymyxa*, along with numerous other bacteria, are examples of bacteria, which may warrant further study due to their highly diverse, potentially beneficial effects on plant growth.

### ***FUNGI AND THEIR POTENTIAL AS BIOFERTILIZERS***

#### ***Mycorrhizal Fungi***

##### *Benefits of Mycorrhizal Symbiosis to Plant Growth*

It is not our intention to provide an extensive review on the benefits of the mycorrhizal symbioses; we will briefly summarize the available literature with special emphasis on the crop yield improvement and potential applications that may prove useful in designing biofertilizers. For more in-depth review, we refer the reader to extensive published volumes on the importance and benefits of mycorrhizal symbiosis to plant growth and performance (Harley and Smith, 1983; Bolan, 1991; Newsham et al., 1995; Smith and Read, 1997).

Mycorrhizal fungi form mutualistic symbioses with a vast majority of land plants (Smith and Read, 1997) Possibly more than 80 percent of all land plants form mycorrhizal symbioses. The extent of mycorrhizal symbioses emphasizes the ancient evolutionary history and potential importance of fungal symbioses for plant production and physiology. The association between plants and their root-colonizing mycorrhizal fungi is a functional symbiosis in which the mycorrhizal fungus is obligately or facultatively dependent on host photosynthates and energy. The plant-acquired carbon is traded for various mycorrhizal benefits to the host plant. The fungal mycelium that extends from the root surfaces into the soil matrix captures nutrients from soil solution. The minuscule diameter of the fungal hyphae increases the surface area that the plants are able to utilize for their nutrient acquisition. Resulting from the more efficient nutrient uptake, plant growth is generally improved when mycorrhizal fungi colonize the root systems.

The increasing yield can easily be demonstrated in laboratory and greenhouse experiments, although host-fungus species combinations and environmental factors may cause variation in the host response to mycorrhizal inoculation (Johnson et al., 1997). Additional benefits from the mycorrhizal symbiosis include increased tolerance of heavy metal contamination or drought, as well as lesser susceptibility to root pathogens or herbivory. Mycorrhizal fungi may also improve soil quality by having a direct influence on soil aggregation (Rillig et al., 2002) and therefore aeration and water dynamics. An interesting potential application for mycorrhizal fungi is their ability to allow plant access to nutrient sources generally unavailable to the host plants. For example, crop plants may be able to use insoluble sources of P when inoculated with mycorrhizal fungi but not in the absence of inoculation (Smith and Read, 1997).

In summary, the various potential benefits of mycorrhizal symbiosis on plant performance and crop yield suggest that they have substantial applications in agriculture (Menge, 1983) and in land reclamation or vegetation restoration (Sylvia, 1990). We do, however, concur with the conclusions of Ryan and Graham (2002) that mycorrhizal inoculation practices are often not compatible with industrial-scale agriculture, but they may find applications in alternative agricultural and horticultural practices.

#### ***USE OF ARBUSCULAR MYCORRHIZAL FUNGI AS BIOFERTILIZERS***

The unculturability and obligate biotrophy of arbuscular mycorrhizal (AM) fungi have precluded the development of large-scale inoculation programs (Wood and Cummings, 1992). The only feasible means for production of infective propagules is growing the inoculum in symbiosis with living host plants or in root organ cultures, in other words, never in the absence of living host tissue. Although such production systems provide an advantage by allowing a continuous monitoring of the infective capability of the inoculum, their major drawbacks include extensive production costs, slow turnover time, and difficulty excluding secondary root colonizers such as root pathogens.

AM inoculum can be applied as spores, fragments of roots colonized by AM fungi, or a combination of the two and incorporated soil mycelium. AM spores and hyphae can be isolated from the soil substrate and mixed with carrier substrate. Commonly used carriers include pumice or clay, sand, perlite, vermiculite, soilrite, and soil or glass pellets (Mallesha et al., 1992; Redecker et al., 1995; Gaur and Adholeya, 2000). AM taxa and strains may vary in their ability to colonize host plants depending on the

source of inoculum (Biermann and Linderman, 1983; Klironomos and Hart, 2002). Spores may be the most reliable source of inoculum across various AM taxa, whereas fragments of colonized roots are effective for some taxa but not others (Biermann and Linderman, 1983; Klironomos and Hart, 2002). The entire substrate can also be used and homogenized into a crude soil carrier that includes plant roots and fungal spores as well as the soil mycelium. Alternative methods, including soil-free aeroponic (Sylvia and Jarstfer, 1992; Jarstfer and Sylvia, 1995), nutrient film (Elmes et al., 1983; Elmes and Mosse, 1984), and root organ culture (Mugnier and Mosse, 1987) systems, have been tried experimentally, but the extensive costs of these methods seem preclusive. Furthermore, large-scale production of inoculum using these alternative methods has been poorly developed.

Given the large costs and difficulty of producing inoculum, the need for AM inoculation should be carefully evaluated. The determining factors including expected crop response to AM inoculation, availability of soilborne inoculum, and alternative practices such as cropping system management for AM inoculum maintenance may need to be considered. Recent evidence also suggests that, contrary to general assumptions, AM fungi may have limited host specificity (Eom et al., 2000; Helgason et al., 2002). The possibility of host specificity patterns among the AM fungi underlines the importance of strain and taxon selection for each inoculation application. Although AM inoculum is commercially available, large-scale inoculation has not become practical or achievable in everyday agricultural practices. Accordingly, the AM inoculation has been limited to production of high value nursery stocks. In such nursery applications the inoculation is often highly advantageous, resulting in improved crop growth, more expedient development, and homogeneous end product. In the absence of practical applications for production of AM inoculum for agricultural practices, the importance of management for maintenance of soilborne fungi must be emphasized. The potential and relative benefits of different inoculation and land management practices have been reviewed and discussed elsewhere (Wood and Cummings, 1992; Dodd and Thomson, 1994). We summarize only a few general recommendations. Continuous plant cover by intercropping or sequential cropping systems may allow maintenance of adequate inoculum levels in soil (Tisdall and Adem, 1990). Similarly, minimizing disturbance is likely to support indigenous inoculum (Jasper et al., 1992; Miller and Jastrow, 1992). Extensive fertilization may also need to be avoided as P, in particular, often inhibits AM colonization (Allison and Goldberg, 2002). The last point underlines the importance of management practices for greater soil AM inoculum in agricultural systems, which may not allow economically viable application of commercial fertilizers. In intensive agricultural systems relying heavily on N and P fertilization, the

management for AM inoculum is unlikely to prove cost-effective (Ryan et al., 2002).

One aspect of AM inoculation that has only recently received attention is the need for diverse populations and communities of fungi in soil. Van der Heijden, Klironomos, et al. (1998) concluded that a greater number of inoculated fungi significantly increased plant community yields. The impact of increasing mycorrhizal diversity can be attributed to functional complementarity: different fungi may perform different tasks in their natural environment (van der Heijden, Boller, 1998). Although only a limited body of literature exists in support of host specificity patterns among AM fungi, recent evidence suggests that different AM fungi vary in their effects in respect to their ability to increase host yield and/or nutrient use (Helgason et al., 2002). The mechanisms for these effects remain rather unclear to date, but it is possible that there is a need to test each crop species, possibly even variety, prior to recommendations on broad-scale inoculation programs. This way the most efficient host variety and fungal strain combinations can be identified for different environmental conditions.

#### ***USE OF ECTOMYCORRHIZAL FUNGI AS BIOFERTILIZERS***

The importance of ectomycorrhizal (EM) symbiosis for tree growth and nutrient acquisition was already hypothesized by Frank (1885). Accordingly, there is a long history of technique development for incorporation of EM fungal inoculation into nursery or forestry plantation practices (White, 1941; Wilde, 1944; Mikola, 1969, 1970). General evidence suggests that tree plantations will fail unless endemic or inoculated EM fungi are available on site. Selection of the inoculated EM fungi has been largely based on tree crop enhancement and/or applicability for a large-scale inoculum practices (Smith and Read, 1997).

Although various alternatives have been used and surveyed, the most widespread inoculum programs have been developed for *Pisolithus tinctorius*. The general advantages for the use of *P. tinctorius* include its wide host range and extensive geographic distribution, as well as its occurrence on sites burdened by recent disturbance, drought, high temperatures, and/or chemical contaminants. *Pisolithus tinctorius* inoculum can be produced and applied as vegetative mycelium in a peat vermiculite carrier. The nutrient solution, which is necessary for the vegetative growth of *P. tinctorius* throughout the substrate, will also facilitate the competitive exclusion of other root-colonizing fungi (Smith and Read, 1997). Various alternative techniques and formulations for inoculation have been developed (Marx and Kenney, 1982; Marx et al., 1984, 1991). Although liquid or spore sus-

pension techniques would avoid the problems resulting from bulky solid inoculum production and storage, they often suffer from delayed EM establishment (basidiospore inoculation) or mycelial fragmentation and shredding (axenic liquid culture techniques).

Inoculation programs with EM fungi have had some success. However, as with AM or bacterial inoculum applications, there seems to be no single fungal species or strain that could be universally applied across different sites and host species. When compared to local strains and species in the northwestern United States, the *P. tinctorius* strain that had proven extremely favorable for seedling growth and establishment elsewhere seemed less beneficial (Perry et al., 1987). In many cases, the strains that easily colonize seedlings in the nurseries and are easy to manipulate have only limited positive effects on the performance of the planted seedlings (Perry et al., 1987; le Tacon et al., 1992; Jackson et al., 1995). The limited success of the fungi, which have been selected for the inoculation programs, may be simply due to the ubiquitous presence of endemic mycorrhizal fungi in reforested sites and the competitive exclusion of the nursery-inoculated fungi in the field. Accordingly, the inoculation with EM fungi may be most important on sites with poor reforestation history or on plantations, which will be established on previously nonforested sites.

In the research focusing on the development of the forest nursery inoculation programs, one issue that has received relatively little attention is the impact that imported and possibly invasive EM fungi have on the endemic fungi and their community composition. The inoculated fungi may persist in the root systems for extended periods of time and outcompete less invasive endemic strains and species (de la Bastide et al., 1994). Although no direct evidence for such competitive exclusion currently exists, introductions may homogenize local fungal populations and communities. For example, *Eucalyptus* species imported and planted in the United States often support extensive colonization by a false truffle, *Hydnangium carneum*. Similarly, *Amanita phalloides*, which was likely imported from Europe with cork oaks, is spreading in the native oak stands in California. The question that remains is whether these successful invaders have had any negative impacts on the local EM communities.

#### **OTHER ROOT-COLONIZING FUNGI**

Root systems of all higher and lower plants support fungal communities, which are composed of fungi occupying the rhizosphere or rhizoplane environment and fungi forming intimate symbiotic associations with the plant roots colonizing them inter- and intracellularly. It is generally acknowl-

edged that these root-associated fungal communities are diverse (Vandenkoornhuysen et al., 2002). However, the functions of these communities and of the individual species in these communities are poorly understood. Studies focusing on the fungi isolated from the root environment provide, not surprisingly, inconsistent results. Even studies focusing on a fairly limited number of anamorphic taxa or a limited number of fungal strains produce incongruent results (Jumpponen and Trappe, 1998a; Jumpponen, 2001). The bottom line is that these communities incorporate a vast diversity and contain species which may prove beneficial in biofertilizer applications. Based on the fairly limited number of existing studies, the benefits resulting from association with the root-colonizing miscellaneous fungi are similar to those reported for mycorrhizal symbioses. The root-associated fungi benefit plants by promoting plant growth and crop yield (Shivanna et al., 1994) and by reduction of root pathogen infection (Dewan and Sivasithamparan, 1989). Recent studies also indicate that these fungi may improve plant nutrient uptake (Jumpponen et al., 1998; Jumpponen and Trappe, 1998b) or allow plant access to otherwise unavailable nutrient sources (Barrow and Osuna, 2002). Several strains originating from crop roots have been shown to be able to promote plant growth and crop yields under field conditions, not only in controlled laboratory or greenhouse studies (Shivanna et al., 1994).

Very little effort has been made in the development of inoculum programs for these various potentially beneficial fungi. Although many of these fungi may be easily cultured and therefore also easily manipulated in inoculation applications, the problems of inoculation are similar to those described with root-associated bacteria and mycorrhizal fungi. The beneficial effects, growth promoting or otherwise, can be highly variable and depend on host genotype and selected fungal strain or species, and can change with plant development or environmental conditions. However, the ubiquitous presence and possible global distribution of many of these fungi suggest a great environmental tolerance. This, in addition to the suggested lacking host specificity (Jumpponen and Trappe, 1998a), makes these fungi a group of great potential interest.

#### ***MULTIPLE INOCULATIONS AND INTERACTIONS AMONG POTENTIAL BIOFERTILIZERS***

We refer to the practice of inoculation and introduction of more than one fungus and/or bacterium into the target crops as *multiple inoculation*.

The rhizosphere presents a challenging environment to manipulate in agricultural practices. Plant roots and their carbohydrates control the bacterial

and fungal populations and their dynamics in the soil matrix adjacent to the plant roots (Hadacek and Kraus, 2002). Such host-plant and root-microbe interactions may partly explain the species-specific responses to inoculation programs. The complexity of the rhizosphere environment is further emphasized by the various interactions among the different bacteria and fungi possibly competing for resources or facilitating presence and occurrence by modification of the rhizosphere environment. Although only poorly understood, various examples on inhibition of root pathogens by rhizosphere-associated bacteria and fungi presented elsewhere in this chapter are likely to be at least partly a result of competitive exclusion. Facilitation of other microscopic organisms in the rhizosphere environment is also possible. For example, bacteria have been reported to adhere superficially or intracellularly to fungal hyphae on the root surfaces and in soil or be generally associated with the rhizosphere of mycorrhizal plants (Bianciotto et al., 1996, 2000; Mogge et al., 2000; Poole et al., 2001; Minerdi et al., 2002). Although AM fungi are exclusively biotrophic, organic matter facilitates the growth of extramatrical mycelium—a response that has been suggested to be a result of bacterial activities in the organic matter (Green et al., 1999; Ravnskov et al., 1999). Some bacterial genera—for example *Paenibacillus* discussed earlier in this chapter—are able to stimulate mycorrhizal colonization and are often isolated from rhizosphere of mycorrhizal plants (Garbaye, 1994; Budi et al., 1999; Poole et al., 2001). These bacteria may also provide a crop yield or plant-growth-promoting effect. Ruiz-Lozano and Bonfante (2001) hypothesized that the bacterial association with the AM fungi may positively influence the host plant nutrient uptake and nutrient transport from the AM fungus to the plant.

Clearly, there is some evidence that bacteria may influence mycorrhizal fungi and fungal colonization of plant roots. Similarly, bacteria and their growth or activity are affected by fungi and their exudates in the rhizosphere (Christensen and Jakobsen, 1993; Olsson et al., 1996). Meyer and Linderman (1986) enumerated bacteria and surveyed their community structure in mycorrhizal and nonmycorrhizal plants. They observed no difference in the total numbers of culturable bacteria, but noted specific bacteria present more frequently in the mycorrhizal rhizospheres. Mansfeld-Giese et al. (2002), in contrast, reported large differences in the bacterial community structures between rhizosphere and nonrhizosphere soil, but found only few bacterial taxa whose occurrence differed between mycorrhizal and nonmycorrhizal plants. Most interestingly, they found *Paenibacillus* spp. almost exclusively associated with not only the rhizosphere of mycorrhizal plants but also the mycelium of the AM fungus *Glomus intraradices*. It remained unclear from that study whether the bacteria were living in the proximity of the mycelium, on the hyphal surface, or within the

fungal hyphae. Whichever the case, the possibility of intimate association between fungi and helper bacteria exists (Garbaye, 1994; Minerdi et al., 2002). Further examples suggest that fungal symbionts of plant roots may stimulate bacterial activity. Coinoculation with AM fungi and *Frankia* stimulated N<sub>2</sub> fixation and AM development in *Hippophaë tibetana* (Tian et al., 2002). Alfalfa (*Medicago sativa*) N and P acquisition was stimulated by inoculation with *Rhizobium*, AM fungi, and PSB (Toro et al., 1998). From the examples presented here, one general conclusion can be drawn: Fungi and bacteria have various inhibitory and facilitative interactions, which may be of use in biofertilizer applications once the compatible combinations of fungi and bacteria are identified.

Compatibility and interactions among microscopic organisms are poorly understood. It is possible that different species of fungi have specific bacterial associates. Andrade et al. (1997) reported that bacterial populations in the rhizosphere environment were different depending on which species or strains of AM fungi were used as inoculum. Similar results are likely also in the EM systems as various bacteria have been reported to be associated with structures of various EM fungi (Poole et al., 2001). In addition, Danell et al. (1993) reported the association of *Pseudomonas* and ectomycorrhizal fungus *Cantharellus cibarius*.

Similar interactions also occur among bacteria. For example, we briefly list a few experimental systems that present evidence for facilitative interactions among bacteria. *Pseudomonas fluorescens* increased nodulation and nitrogenase activity of *Bradyrhizobium japonicum* in a soybean culture system (Chebotar et al., 2001). Zhang et al. (1996) found that certain PGPR belonging to the genera *Serratia* and *Aeromonas* could increase soybean nodulation and N<sub>2</sub> fixation of *Bradyrhizobium japonicum* at suboptimal root zone temperature. Bashan and Holguin (1997) have reviewed several examples of coinoculation with *Azospirillum* and *Rhizobium*, *Azotobacter*, *Arthrobacter*, *Enterobacter*, or *Klebsiella*. Based on a study on the coinoculation of the N<sub>2</sub>-fixing *Phyllobacterium* sp. and the P-solubilizing *Bacillus licheniformis* in mangrove, Rojas et al. (2001) suggested that the interaction between different rhizosphere bacteria should be considered when evaluating the growth-promoting effects of these bacteria.

An interesting new application is the introduction of “artificial” associations. In addition to combining stimulatory bacteria and fungi, novel applications of microbes may allow extension of microbial symbioses beyond their usual host range. Koval’skaya et al. (2001) showed that treating rape (*Brassica napus* var. *napus*) with an abiotic agent (auxinlike growth-promoting substance) or the bacteria *Micrococcus* sp. and *Rhodococcus* sp. induced formation of paranodules. The paranodules were then colonized by

the N<sub>2</sub> fixing *Azotobacter nigrificans* and *Bacillus* sp. As a result of the paranodulation and N<sub>2</sub> fixing, the rape N and protein content increased.

These examples strongly suggest the possibility of a complex web of interactions between root-associated fungi, rhizosphere bacteria, and host plants. The numerous benefits provided by various bacteria and fungi to plant growth and crop yield may open new avenues for developing biologically active fertilizers. Combinations of growth- and yield-promoting bacteria and fungi as well as combinations of organisms facilitating establishment or plant-derived benefit from inoculation deserve further study. For example, production of inoculum, which would integrate the growth-promoting and pathogen-inhibiting effects of *Paenibacillus* and AM fungi, seems like a reasonable first step in order to generate a product that may prove superior in agricultural systems. An add-on benefit from such inoculum would also be the facilitation of AM establishment and colonization by *Paenibacillus*. EM fungi and their mycorrhizal helper bacteria (Garbaye, 1994) are likely to provide new combination inocula with benefits similar to the AM fungal-bacterial systems.

### ***FUTURE PERSPECTIVES AND CONCLUSIONS***

In order to increase our understanding of the role of various root-associated organisms in plant growth and health as well as make use of their potential beneficial features as biofertilizers in plant production, more information is urgently needed on the interactions among plants and rhizosphere microorganisms. We have briefly presented a few examples of bacteria and fungi, which may bear a great promise as biofertilizers. We acknowledge that we selected simplified examples. However, studies utilizing simplified laboratory experiments are essential in uncoupling different factors and identifying the best possible candidates for biofertilizers. Such experiments and their results, however, are often difficult to extrapolate to field conditions. Furthermore, the rhizosphere presents additional challenges as an environment to be manipulated. The rhizosphere is a highly dynamic system with a vast number of fungi and bacteria interacting simultaneously; the difficulty of excluding endemic bacteria and fungi may preclude clear conclusions from inoculation experiments in the field. We understand the difficulty of conducting such experiments on a scale that would be meaningful in everyday agricultural practice. However, unless the positive effects can be repeatably shown in practical applications, the commercial viability of inoculation programs will be uncertain.

We emphasized earlier the importance of the evaluation of soil inoculum potential of N<sub>2</sub> fixers, PGPR, and mycorrhizal fungi. The inocula made commercially available should conform to minimal quality standards. Simple and clear governmental and international regulations are essential to guarantee efficiency of the inocula. Local or regional strains should preferably be selected and used for the target crops. We underlined in this chapter the difficulty of obtaining universal “wonder” strains that perform equally across different host taxa and environments.

Use of multiple inoculations can aim to stimulate N<sub>2</sub> fixation, P uptake, and mineral nutrition in general, but they can also help in controlling plant pathogens. Such applications would be welcomed as they allow reduction in chemical fertilizers and pesticides that are expensive and environmentally unsound. An interesting issue, in addition to the infection potential, is the persistence of the biofertilizer after inoculation. If inoculum potential can be built into agricultural soils, the interval between biofertilizer applications could be increased and costs lowered.

Various fungi and bacteria provide a battery of extracellular enzymes, which may be utilized for improved crop yields and reduced costs for inorganic fertilizers. We emphasize the need for field trials with multiple organism inoculations. These inoculum combinations may be of greatest value if various organisms with different proven or suspected benefits to the crop plants can be integrated. The integration of different microbial capabilities into combined biofertilizers with numerous potential yield-promoting effects is desirable. This is likely to be best achieved by approaching the application and research of biofertilizers at a scale that is relevant to agricultural practices. Finding avenues and funding for collaboration between research facilities and biotechnology industry seems like a reasonable first step. Producing inocula at large scales is essential for achievement of the research and practical application goals stated previously. Connection between research and industry allows not only the inoculum production for field trials but also testing of the industrial scale inoculum production for direct marketing.

Finally, we emphasize the importance of establishing federal and international guidelines for inoculum production and trade. To protect the end user of inoculum and to promote safe choice of commercial inocula collaboration among research facilities, federal agencies, farmers, and the inoculum producers are of pivotal importance. Such innovative collaborative approaches will allow a head start for commercial and economically viable production of biofertilizer inocula for marketing directly to primary target consumers.

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