Soil Biology

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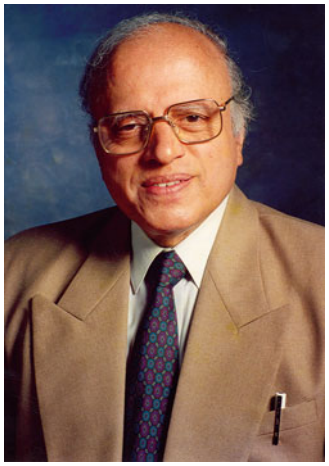
Foreword



It gives me an immense pleasure to write few glimpses on book "*Rhizobium* Biology & Biotechnology" written by series editor Prof. Dr. Ajit Varma.

Rhizobia have been widely used in agricultural systems for enhancing the ability of legumes to fix atmospheric nitrogen. Nitrogen was known to be an essential nutrient for plant growth and development. Intensive farming practices that accomplish high yields need chemical fertilizers, which are not only cost effective but also may create environmental problems. Nitrogen is essential in plant cells for synthesis of enzymes, proteins, chlorophyll, DNA and RNA, thus essential for plant growth and production of food and feed. The term rhizobia generally refer to members of the genus *Rhizobium* but in true sense it includes all bacteria that are capable of nodulation and fixing the nitrogen in association with leguminous crop. Rhizobia are soil-inhabiting heterogenous group of diverse bacteria with the

potential to stimulate nodule formation with the roots of both leguminous and non-leguminous plants. These bacteria enter in to the root tissues via root hairs or directly via wounded tissues during specific interactions with the host and induce nodule formation on roots and/or shoots. The rhizobia fix atmospheric nitrogen into ammonia through effective nodules and supports plant growth. In return the rhizobia obtain nutrition and protection by the host in symbiotic manner. This symbiotic relationship for nitrogen-fixation has been extensively studied in agriculture for improving soil health and crop yields.

The Rhizobia have diverse and heterogeneous group divided into alpha and beta-proteobacteria nevertheless they are united by their ability to form nodules on leguminous and non-leguminous plants. Due to nitrogen fixing ability and potential to replace nitrogen fertilizers, rhizobia are among the most intensively studied groups of microorganisms simultaneously, testing of nodulation by different bacteria led to the establishment of cross-inoculation groups. With the advent of modern biotechnological tools and techniques such as rDNA sequencing, 16S diversity, DNA–rRNA hybridizations, rRNA catalogues, more diversity of rhizobia could be exposed. The rhizobia isolated from leguminous plants of arid region largely belong to *Bradyrhizobium*, *Mesorhizobium*, *Rhizobium* and *Sinorhizobium* genera on the basis of morpho-physiological and molecular characterization. The specific stress tolerant traits of these bacteria can be exploited to mitigate climate resilience in context of global warming.

In the present volume Editors compiled researches to emphasize the role of *Rhizobium* in agriculture and its biotechnology with following objectives:

- Occurrence and distribution of *Rhizobium*
- Phenotypic and molecular characteristics of *Rhizobium*
- Impact of *Rhizobium* on other microbial communities in rhizosphere
- N_2-fixation ability of *Rhizobium*
- *Rhizobium* and abiotic/biotic stress
- *Rhizobium*-mediated restoration of an ecosystem
- In silico analysis of rhizobia pool
- Biotechnological perspectives of *Rhizobium*

Finally, I congratulate Prof. Dr. Ajit Varma and his team for their brilliant efforts in compilation of such fruitful volume which is a worthwhile compendium to explore *Rhizobium* technology and its dissemination to sustainable agriculture.

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Preface

Agriculture has a long history of research targeted at understanding how to improve the effectiveness of root symbionts viz., rhizobia. Plant-mediated mineralization for nutrient acquisition in agro-ecosystem would reduce the potential for nutrient losses because of tight coupling between net mineralization of N and P and plant uptake in the rhizosphere. Micro-organisms and their products in the rhizosphere react to the many metabolites that are released by plant roots in a variety of positive, negative, and neutral ways. Such interactions can influence plant growth and development, change nutrient dynamics, and alter plant's susceptibility to disease and abiotic stresses. Overall the general rhizosphere effect could help the plant by maintaining the recycling of nutrients through the production of hormones that help provide resistance to microbial diseases and to aid tolerance to toxic compounds. This benefit can either persist or lost in well fertilized agricultural soils where nutrients are readily available to plants and symbionts that reduce growth. Legumes are simultaneously one of the largest families of crop plants occupying nearly all terrestrial biomes. The unusual flower structure, podded fruits and ability of the 88.0% species to form root nodules with compatible rhizobacteria define the legumes. The wide use of legumes as food crops, forages and green manures is mainly associated with their ability to establish symbiotic associations with stem and root nodulating nitrogen (N_2) fixing bacteria, which are collectively referred as rhizobia. Rhizobia are of particular interest due to their symbiotic association with members of Leguminosae, which is the second largest family of flowering plants. Recent information indicates that about 3000 bacterial taxa are capable of nodulating 400 taxa, while information is lacking for more than 40% of the genera. A promising approach has been employed to understand how natural selection regulates changes in mutualistic interactions. A descriptive knowledge of basic evolutionary processes can be employed to develop agricultural management practices that favour the most effective symbionts. Mutually beneficial interactions between plant and associated rhizospheric microorganisms are ubiquitous which is important for ecosystem functioning. Symbiotic nitrogen fixation by bacteria e.g., *Rhizobium, Bradyrhizobium, Mesorhizobium, Sinorhizobium*, and *Azorhizobium*

spp., that collectively known as rhizobia or by *Frankia* spp., is the major N input to many natural and agricultural ecosystem in the root nodules of legumes or actinorhizal plants respectively. From an agricultural point of view, the most significant interactions are those of the Fabaceae-*Rhizobium* spp. *Bradyrhizobium* spp. root nodule symbioses. Recent work on root nodule bacteria has demonstrated that this interaction is not restricted to *Rhizobium/Bradyrhizobium* but includes N_2-fixing strains of *Ralstonia, Burkholderia*, and *Methylobacterium* that have been recovered from the nodules of several tropical Fabaceae.

Beside of nitrogen fixation, rhizobia have also been reported for plant growth promotion in legumes and non-legumes. Their associations modify the physiology and biochemistry of crop plants which enhanced plant growth under normal as well as stress conditions. Rhizobia can affect the plant growth in two different ways: directly or indirectly. The direct growth promotion of the plant is regulated by producing plant hormones; regulating endogenous ethylene level; enhanced total available nutrient contents and releasing other useful compounds like: exopolysaccharides (EPS), lumichrome, riboflavin etc. During rhizobial infection, ethylene produced into infected roots and several times caused inhibition of nodulation in various legumes. There are several rhizobial strains including *R. japonicum*, *R. leguminosarum*, *R. hedysari*, *R. gallicum*, *B. japonicum*, *B. elkanii*, *M. loti* and *S. meliloti* having an enzyme ACC deaminase which could reduce this stress by decreasing the level of ethylene in the host plant. The EPS producing rhizobial strains can relieve the effect of water deficit stress by altering soil properties. Various *Rhizobium* spp. are also studied for plant growth promotion via producing multiple phytohormones such as Auxins, abscisic acid, cytokinins, gibberellins, ethylene, and nitric oxide. Rhizobia can benefit plant growth indirectly by several mechanisms such as antibiosis, parasitism, competition for nutrients, and induction of systemic resistance (ISR). *Rhizobium* spp., namely, *R. leguminosarum*, *S. meliloti*, and *B. japonicum* have been found to show parasitism against fungal pathogens belonging to genera *Macrophomina*, *Rhizoctonia*, and *Fusarium*. Several studies have demonstrated that *Rhizobium* spp. enhanced defense mechanisms of plant via ISR in non-leguminous crops.

In the present volume editors compiled researches to elaborate the role of *Rhizobium* in agriculture with emphasis on biotechnological perspectives.

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Chapter 1
Impact of Rhizobial Inoculants on Rhizospheric Microbial Communities

Richa Sharma, Virendra S. Bisaria, and Shilpi Sharma

1.1 Introduction

Microorganisms under the order Rhizobiales (which includes genera like *Rhizobium, Mesorhizobium, Bradyrhizobium, Azorhizobium, Ensifer, Sinorhizobium* etc.) are legume-nodulating, gram-negative bacteria, belonging to α-proteobacteria, which enrich the soil by fixing atmospheric N_2 and, therefore, hold immense ecological significance. Not all organisms under this order are symbionts; some are methanotrophs while others are even pathogenic. Their application as bioinoculants in agriculture has been followed since decades. They reduce the requirement of chemical nitrogenous fertilizers as well as improve productivity of legumes in fields. Rhizobial inoculation, apart from leading to enhanced nodulation and nitrogen fixation, triggers the production of siderophores, phytohormones, and HCN (Trabelsi et al. 2011, 2012). It also helps in phosphate solubilization along with P and N uptake (Zahir et al. 2011; Flores-Félix et al. 2013; Yadav and Verma 2014). Flores-Félix et al. (2013) reported a strain of *Rhizobium leguminosarum* that produced siderophores and indole acetic acid, and solubilized phosphate. The strain was able to colonize two horticultural crops, *Lactuca sativa* L. (lettuce) and *Daucus carota* L. (carrot). The strain promoted the growth of both plant species as well as increased the uptake of N and P in the edible parts of both the plants, thus showing that it can be used as a biofertilizer for non-legumes as well. However, the issue of their establishment in the nodule due to competition with indigenous strains is critical (Triplett and Sadowsky 1992; Toro 1996; Trabelsi et al. 2011). An effective inoculant, therefore, must be highly competitive also (Mrabet et al. 2005; Mnasri et al. 2007a, b, c; Trabelsi et al. 2011). Mnasri et al. (2007a, b, c) have shown that *Rhizobium gallicum* strain 8a3 was highly

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competitive with respect to its nodule occupancy as compared to the indigenous strain of *Sinorhizobium meliloti*, a known symbiont of *Medicago*. In fact, the strain had an antibiotic activity against the indigenous strain. Since the inoculants are released into the field in numbers much higher than their actual numbers in the soil, and their nutritional requirement might overlap with the resident microbial community, it would lead to at least transient perturbation of the soil equilibrium because of their competition for the localization of niche. Hence, their effect must be assessed with respect to the potential risk they might possess on the resident microfloral diversity. Diversity is defined as the number of species present and their relative abundance (Felske and Osborn 2005). If their application leads to loss of crucial native species, it will affect the subsequent crop and therefore would be tagged as undesirable (Trabelsi et al. 2011). This disturbance can be buffered by ecosystem elasticity, resistance, and resilience, which are in turn consequences of diversity and plant–soil–biota interaction (Holling 1973; Grimm and Wissel 1997; Kennedy 1999; Reinhart et al. 2003). Due to bacterial redundancy, the negative impact on certain resident microbial species may not drastically change the ecosystem's functioning (Kennedy 1999; Nannipieri et al. 2003). When rhizobial inoculation affects the composition of soil microbiome, it in turn affects the synthesis and liberation of the enzymes in the rhizosphere (Antoun and Prévost 2005; Sun et al. 2009; Saharan and Nehra 2011). Rhizobial inoculants have also proved to have pathogen-suppressive capability in both legumes and non-leguminous plants, thereby controlling several plant diseases (Antoun and Prévost 2005; Huang and Erickson 2007; Shaban and El-Bramawy 2011).

1.1.1 Scope of the Chapter

The prime focus of the chapter is on the indirect effects caused by rhizobial inoculants on resident rhizospheric microbial communities both positively (by enhancing the nutritional status of the soil, and by exerting antibiosis against plant pathogens) and negatively (by having detrimental consequence on the resident microflora), which are well documented. On the basis of available literature, we conclude with an interpretative summary so far and the future perspective.

1.2 Individual Inoculation Versus Combined Inoculation of Rhizobial Strains

Rhizobial inoculants have been used individually as well as in combination with other bioinoculants (sometimes with other rhizobial strains) for the betterment of plant growth. Such combinations have yielded variable results in terms of their effect on plant growth parameters and also on the resident microflora. Yadav and Verma (2014) reported that the direct effect of rhizobial inoculation on plant growth parameters was

greatly affected by its co-inoculation with other PGPR. Co-inoculation of *R. leguminosarum* with *Pseudomonas aeruginosa* resulted in enhancement of shoot dry weight per plant, grain yield, and uptake of N and P by chickpea as compared to no inoculation and inoculation with *R. leguminosarum* alone. Zahir et al. (2011) have also determined the positive effect of co-inoculation of *Pseudomonas* sp. and *Serratia* sp. with *R. leguminosarum* on the growth and yield of lentil. On the contrary, in certain cases there seems to be no effect of co-inoculation (*Ensifer meliloti* and *R. gallicum*) when compared with mono-inoculation in terms of plant growth parameters (nodule number, shoot dry weight, and grain yield) as shown by Trabelsi et al. (2011). The effect was, however, visible when assessed with respect to the total bacterial community. Co-inoculation significantly increased the total bacterial community when compared with mono-inoculation and control samples. Effect of these two strains on the species richness of the rhizosphere of potato cropping revealed that co-inoculation appeared less effective than mono-inoculation (Trabelsi et al. 2012).

Sharma et al. (2017) compared several treatments in a field experiment and showed that shoot weight at vegetative and flowering stage, and grain yield at harvest stage, were significantly increased when *Bradyrhizobium* sp. was co-inoculated with *Azotobacter chroococcum*, *Bacillus megaterium*, and *Pseudomonas fluorescens*. The effect was also assessed on major groups of resident microflora. It was found that fungal population and nitrogen fixers significantly decreased at harvest stage, whereas phosphate solubilizers were significantly reduced at flowering stage in the mono-inoculation with *Bradyrhizobium* when compared with the co-inoculation. On the other hand, it was observed that *Actinomycetes* population significantly increased at vegetative stage in the mono-inoculation as opposed to co-inoculation. Rhizobium–legume symbiosis being a complicated partnership involves key enzyme cellulase for the primary root infection. Diez-Mendes et al. (2015) showed that co-inoculation of the native strain of *R. leguminosarum* with cellulase-overproducing strain *Rhizobium cellulosilyticum* exhibited higher grain production in *Phaseolus vulgaris*. Also, the grains had increased N content compared to mono-inoculation with the native rhizobial strain and uninoculated plants. This suggested co-inoculation to have significantly enhanced the N fixation efficiency of the native strain.

1.3 Soil Nutritional Status

Apart from fixing nitrogen symbiotically, rhizobium inoculation has a positive effect on the crop by several mechanisms involving production of plant hormones, increasing the phosphorous and iron supply, etc (Antoun and Prévost 2005; Saharan and Nehra 2011). Although iron is abundant in the soil, its acquisition is difficult due to its low solubility. Iron and phosphorous concentration in the nodules has been found to have linear correlation with the efficiency of nitrogen fixation (Rotaru and Sinclair 2009). Siczek and Lipiec (2016) observed an increase in enzymatic activities (dehydrogenases, urease, protease, and acid phosphomonoesterase) in the rhizosphere of *Vicia faba* throughout the growing season in soil inoculated with *R. leguminosarum* as compared

to uninoculated soil. Increment in the enzymatic activities of urease and dehydrogenase was much higher compared to the activities of protease and acid phosphomonoesterase. This was explained by the fact that inoculation with rhizobium has significant prospective for N cycling and ATP production by the oxidation of organic matter in the soil. On the contrary, Zhang et al. (2010) showed that *Rhizobium* inoculation did not exert a significant effect on available K, N, organic matter, and pH of the rhizospheric soil. In yet another study, rhizobial inoculation was reported to enhance inorganic nitrogen content in the soil, together with exerting an adverse effect on the microbial biomass (Herrmann et al. 2012). Sun et al. (2009) showed that in alfalfa rhizosphere, there was an enhancement in the activity of urease (15.65%) and invertase (19.34%) in intercropping-rhizobial inoculation as compared to monoculture. However, there was no pronounced effect of treatment on acid phosphatase activity.

1.4 Effect on the Resident Microbial Community

A concise representation of the impact of rhizobial inoculation on bacterial diversity, nutrient status, and enzymatic activity in the rhizosphere has been provided in Table 1.1. Competition occurs between inoculated species and the resident microbial community due to overlapped niche colonization and nutritional requirement (de Weger et al. 1995; Anyango et al. 1998). This results in either the inoculated microbe outcompeting the indigenous microbes or vice versa. If the crucial processes, such as nutrient cycling or plant pathogen protection, are inhibited as a result of application of bioinoculants, then the out-competition is considered as an ecological risk. Therefore, the release of bioinoculants in huge amount may either result in non-target effects, which in turn enhance plant growth, or lead to an ecological risk (Schwieger and Tebbe 2000, Gupta et al. 2012). Rhizobia–legume interaction is assumed to act as a driving force for maintaining the nitrogen balance in soil. This effect also depends on the rhizosphere and internal nitrogen turnover (Babić et al. 2008). It is therefore important to determine the effect of rhizobial inoculants on the resident microbial community.

Various techniques have been used to examine soil resident microflora. Cultivation-dependent methods such as enumeration on plates and CLPP are laborious and only $<1\%$ of the total microbial flora is cultivable. Though cultivation-independent methods, such as qPCR, fingerprinting [amplified ribosomal DNA restriction analysis (ARDRA), denaturing gradient gel electrophoresis (DGGE), single-strand conformation polymorphism (SSCP), temperature gradient gel electrophoresis (TGGE), terminal restriction fragment length polymorphism (T-RFLP), etc.], and next-generation sequencing (NGS) are able to provide a much clearer vision, a strong bias is introduced by DNA extraction, together with inherent limitations of PCR amplification.

Only limited reports have addressed the question of non-target effects of application of rhizobial inoculants. A look into the available reports reveals mixed results concerning the impact exerted by rhizobial amendments. Sun et al. (2009), using the

Table 1.1 Impact of rhizobial inoculants on rhizospheric properties

S. No.	Host plant(s)	Rhizobial inoculant(s)	Remarks	References
1.	*Vicia faba* L.	*R. leguminosarum* bv. *viciae*	– Enhanced enzyme (dehydrogenases, urease, protease, and acid phosphomonoesterase) activities in inoculated rhizosphere soil	Siczek and Lipiec (2016)
2.	*Vicia faba* L.	*R. leguminosarum* bv. *viciae*	– Rhizobium inoculation decreased the microbial biomass C in the rhizosphere by 26–30% – ARDRA results showed *Rhizobium* inoculation decreased bacterial diversity – *Rhizobium* inoculation negatively affected Planctomycetes and Actinobacteria but had a positive impact on member of α-proteobacteria	Zhang et al. (2010)
3.	*Phaseolus vulgaris*	*E. meliloti* 4H41, *R. gallicum* 8a3	– Inoculation with strain 4H41 induced an increase in soil ammonium concentration, but inoculation with 8a3 led to a decrease – Nitrate was below detectable limit for uninoculated as compared to the inoculated treatment at the end of the plant cycle	Trabelsi et al. (2011)
4.	*Phaseolus vulgaris* cv. Flamingo and Potatoes	*Rhizobium gallicum* 8a3, *Ensifer meliloti* 4H41	– T-RFLP analysis of 16S rRNA gene was used to evaluate and compare total bacterial communities in different treatments – Co-inoculation appeared to be less effective than simple inoculation with 8a3 or 4H41 – Composition of the bacterial communities was significantly affected by inoculation	Trabelsi et al. (2012)
5.	*Indigofera tinctoria*, *Pueraria mirifica,* and *Derris elliptica* Benth.	10 indigenous rhizobial strains	– Bacterial community structure of native rhizosphere and inoculated rhizospheres was different as revealed by DGGE – Sorensen's index showed the bacterial community structure of the rhizosphere inoculated with Rhizobium divergent from uninoculated control – Slight differences were observed upon rhizobial inoculation; stronger effect of plant type was noted	Nimnoi et al. (2010)

(continued)

Table 1.1 (continued)

S. No.	Host plant(s)	Rhizobial inoculant(s)	Remarks	References
6.	*Medicago sativa* L.	*Sinorhizobium meliloti* S26/O26	– Bacterial genes involved in nitrogen turnover were affected by inoculation as shown by qPCR. Effectiveness of inoculation was related to the abundance of *nif*H genes in the late flowering stage – Higher number of *amo*A copies was observed during flowering	Babić et al. (2008)
7.	*Medicago sativa* L. cv. Arago´n	*S. meliloti* strain M4	– RFLP and TGGE profiles suggested that inoculation with *S. meliloti* permitted certain γ-proteobacterial populations to be maintained longer in the rhizosphere without affecting others	van Dillewijn et al. (2002)
8.	*Medicago sativa* and *Chenopodium album*	*S. meliloti* L33	– PCR–single-strand conformation polymorphism (SSCP) profiles of a 16S rRNA gene region confirmed the bacterial diversity in the rhizosphere of *Medicago sativa* affected qualitatively and quantitatively – Members of γ-proteobacteria decreased while the number of members of α-proteobacteria increased	Schwieger and Tebbe (2000)
9.	*Acacia senegal*	Mixture of four *Ensifer* strains	– DGGE profiles showed a significant increase in total bacterial diversity due to seasonal changes as compared to rhizobial inoculation – No significant difference was revealed between inoculated and non-inoculated soil samples	Herrmann et al. (2012)
10.	*Cajanus cajan*	*Bradyrhizobium* sp.	– Rhizobial inoculation positively affected the population of *Actinomycetes* and *Pseudomonas* compared to the rest of the treatments including control and chemical fertilizers during the early stages of plant – Fungi, gram-negative enteric bacteria, *Azotobacter*, and nitrogen fixers were negatively affected by rhizobial inoculation as compared to control	Sharma et al. (2017)

technique of T-RFLP, showed an enhancement in the *Nitrosomonas* and an adverse effect on *Nitrospira* in the intercropping-rhizobial inoculation treatment. Both treatments tended to increase the diversity of *amoA*. Zhang et al. (2010) upon employing ARDRA and T-RFLP determined that *Rhizobium* inoculation led to a detrimental impact on microbial biomass C with a reduction by 26–30% as shown by Shannon diversity index (H_A'). Diversity of Planctomycetes and Actinobacteria were negatively affected, while those of α-proteobacteria increased with *Rhizobium* inoculation. Both intercropping and application of *Rhizobium* inoculant were reported to influence the less abundant phyla, without affecting the dominant phyla. On the other hand, studies conducted by Zhang et al. (2011) and Herrmann et al. (2012) showed almost no effect of these amendments on the resident microflora. Schwieger et al. (2000) reported that *Sinorhizobium meliloti* strain and its $RecA^-$ derivative did not have any effect on the residential microbial community in a lysimeter experiment. Using fingerprint techniques of ARDRA and SSCP, Schwieger and Tebbe (2000) reported that inoculation with a strain of *S. meliloti* affected the structure of rhizospheric community of *Medicago sativa* (target plant), whereas the same strain exhibited no effect on the rhizospheric diversity of *Chenopodium album* (non-target plant). As a consequence of the inoculation, the most dominant species *Acinetobacter calcoaceticus* and *Pseudomonas* were almost eradicated, as shown by ARDRA profiles, from the rhizosphere of *M. sativa*. Inoculation reduced the abundance of γ-proteobacteria, with simultaneous enhancement of α-proteobacterial members. Also, greater rhizospheric diversity was shown by the rhizospheric samples of *M. sativa* as compared to *C. album*. The shift can be explained as replacement of more general bacteria by amendment with rhizobia. Field release of genetically engineered rhizobial strains (containing genes encoding trifolitoxin) resulted in killing many of the α-proteobacterial members, without affecting other groups. This can be explained by trifolitoxin sensitivity of α-proteobacteria (Triplett et al. 1994; Robleto et al. 1998). van Dillewijn et al. (2002) on the contrary adopted the techniques of RFLP and TGGE to conclude that the inoculation with *S. meliloti* strains enhanced the γ-proteobacterial population in soil. Lower level of persistence of the inoculants led to a modest effect on the indigenous microbes. By using DGGE technique and analyzing the Sorensen's index of the rhizobial inoculated and non-inoculated soil, Nimnoi et al. (2010) concluded that the resident microbial community structure was different for both the soils. This implied that the inoculation had modified the rhizospheric community structure.

An extensive study by Babić et al. (2008) assessed the effect of two *Sinorhizobium* strains on genes involved in N cycle. qPCR revealed that in the late flowering stage of alfalfa there was an abundance of *nif*H and *amo*A genes; this in turn complements the effectiveness of rhizobial inocula. Despite high throughput and resolution, only limited reports are available employing such techniques to address the question of non-target effect of bioinoculant at gene and transcript levels.

It has been seen that the grain yield of legumes is greatly affected by the attack of several pathogens. Both underground and aerial parts are affected by these phytopathogens. There are a few studies which report the efficacy of rhizobial inoculants against these phytopathogens. Huang and Erickson (2007) and Shaban and El-Bramawy (2011) showed that treatment with *R. leguminosarum* resulted in significant decrement in

damping-off when compared with untreated control in faba bean and pea seeds, respectively. On the other hand, Reitz et al. (2000) showed that intact cells were not necessary to combat the disease, as lipopolysaccharides (concentrations as low as 0.1 mg ml^{-1}) produced by a strain of *Rhizobium etli* resulted in significant (up to 37%) reduction of *Globodera pallida* infection of potato roots by acting as an inducing agent of systemic resistance in potato roots.

1.5 Conclusion

Rhizobial inoculants cause changes in the diversity and abundance of resident rhizospheric microflora in plants. Plant growth promotion and their protection are not necessarily the direct effect of these agricultural amendments but may also result from induction or suppression of indigenous microbial community. These changes in turn lead to changes in beneficial soil processes like nitrogen cycling. There is a paucity of literature available to conclusively state whether rhizobial amendments have a beneficial or an inhibitory effect on resident microflora. However, the majority of results indicate at least a disturbance in the structure and function of rhizospheric community upon application of rhizobial inoculants. There is, therefore, a need to perform further assessment of the magnitude of such changes on soil functioning. The assessment of the non-target effects of rhizobial inoculants at genomic, transcriptomic, and proteomic levels will lead to a greater understanding of such changes on soil functioning. This, in turn, will help in making a more judicious choice about their application on leguminous crops.

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Chapter 2
Restoration of Degraded Pasture Soils on the Basis of EM Associations

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2.1 Introduction

Analysis of the current state of the global agribusiness has shown that in many countries of the world the priority is given to the development of pasture-based livestock farming (Ivanov 2010; Kotlyarova et al. 2013). Natural pasturable potential of agricultural development fully meets the rational management requirements in livestock farming (Dickhoefera et al. 2010; Abseitov 2012). However, the irrational use of pastures (overloading, overgrazing, violating seasonality of grazing) results in soil degradation and landscape desertification. Currently, most of the pasture ecosystems are seriously disturbed, a number of valuable forage grasses have disappeared or become rare, and soils have been severely depleted. The existing range of perennial pasture grasses does not meet environmental standards (Han et al. 2008; Angassa and Oba 2010). Particularly, pronounced manifestation of pasture vegetation degradation is observed around the wells, where the species composition is reduced, and uneaten grasses take the place of eaten ones (Kandalova 2009a, b). Degraded pastures are not resistant to erosion and desertification; the loss of humus is in the range from 25 to 30%, and not replenished. 60% of pasture lands are exposed to wind erosion; more than 50% of soils are saline in varying degrees. All these negative processes lead to the biodiversity depletion, reduced productivity of natural pasture ecosystems, and, as a consequence, deterioration of forage resources in grassland farming (Squires 2012). In this connection,

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the scientists from various countries are carrying out research on restoration of disturbed pastures and developing measures for rational land resource management (Han et al. 2008; Hou et al. 2008; Penkov 2009; Petrov 2012).

One of the most promising solutions to this problem lies in biological farming, in which maintaining the soil in a biologically active state that ensures its fertility is seen as a decisive factor. Biological farming is based on the use of effective microorganisms (EM), which represent the primary environmental soil forming factor and consists in the application of effective microorganism associations (EM associations) (Higa and James 1994; Condor et al. 2007). In this case, mineral fertilizers, pesticides, and other chemical protection agents should not be used (Gorski and Kleiber 2010; Jusoh et al. 2013; Reddy and Giller 2014). EM associations represent a multispecies and multifunctional composition or artificially created community. The composition of EM associations includes microorganisms belonging to different genera and species. In the main, EM associations comprise nitrogen-fixing, phosphate-mobilizing, cellulolytic, and silicate groups of microorganisms. When EM associations are introduced into soil, it is enriched with readily available nutrients and becomes more fertile, and microorganisms themselves supply the plants with necessary products of their vital activity (enzymes, vitamins, amino acids, etc.) (Tokeshi et al. 2007; Mayer et al. 2010).

Nitrogen-fixing microorganisms represent one of the important components of EM associations. They play the leading part in the fixation of atmospheric nitrogen and soil enrichment with plant-available nitrogen (Schott 2007; Orr et al. 2011; Emer et al. 2014). To normalize the microbial flora of the depleted and degraded soils and enhance soil fertility, the nitrogen-fixing microorganisms should be a mandatory component of EM associations.

To restore the degraded pastures, we are planning to use EM associations, composed of nitrogen-fixing, phosphate-mobilizing, cellulolytic, and silicate bacteria, with simultaneous sowing of perennial meadow grasses. In the future, these grasses will become the basis of the pasture grass stand. The composition of meadow grasses will include legumes such as wild alfalfa, sweet clover, and white clover. These plants are the major forage crops. Alfalfa and clover are the most valuable nutritious animal feed. Melilot, in addition, enriches soil with nutrient elements, promotes humus accumulation, and improves the soil properties. It is planned to introduce EM association by the pre-sowing treatment of seeds of meadow grasses with this association.

However, when studying soil microflora of degraded pastures, it was found that there are practically no nitrogen-fixing root nodule bacteria rhizobia in the soil. Therefore, the composition of EM associations should comprise the nodule bacteria—symbionts of these valuable forage crops for the successful development of these legumes.

The aim of this research was to isolate, identify, and study nitrogen-fixing root nodule bacteria of the genus *Rhizobium* and select the most promising among them for developing EM associations.

2.2 Materials and Methods

The symbiotic bacteria or rhizobia that form nodules on the roots of legumes such as wild alfalfa (*Medicago sativa* L.), sweet clover yellow *Melilotus officinalis* (L.) Desr., and white clover (*Trifolium repens* L.) were used as objects of the study. These legumes compose the natural grass stand of environmentally undisturbed or natural pastures in Kazakhstan and are among the most nutritious forage plants for agricultural livestock.

2.2.1 Isolation of Rhizobia

Gathering of leguminous plants for isolation of the rhizobia was carried out on natural high-yielding pastures of Kazakhstan. Rhizobia were isolated from nodules on the roots of alfalfa, melilot, and white clover during the budding-flowering period of the host plant. Nodules were taken from the freshly gathered plant roots. The plant roots were washed thoroughly under running water to remove mud and soil particles. The largest healthy and pink nodules were separated with forceps from thoroughly washed root and placed in 70% ethanol for 30 s. They were then treated with 0.1% $HgCl_2$ for 2 min. Thereafter, the nodules were repeatedly washed with sterile water. The nodules were transferred into a Petri dish using sterile forceps and crushed with a scalpel under aseptic conditions. A small amount of nodule contents was transferred using an inoculating loop into 100 μL of sterile water, further on the surface of the agar medium in a Petri dish, and spread with a spatula. Another 2–3 Petri dishes were inoculated using the same spatula to get isolated colonies. The inoculated dishes were incubated at 25–27 °C. Fast-growing rhizobia grew in 3–4 days and slow-growing in 7–9 days. The appearance of colonies within 1–2 days is indicative of culture contamination (Vincent 1970; Carter and Gregorich 2007). The cultures were maintained on nutrient agar slants with regular subculturing.

2.2.2 Culture Conditions

The Ashby and Maze media with pea broth were used to isolate the symbiotic rhizobacteria. Nitrogen-free Ashby medium has the following composition (g/L): mannitol—20.0; K_2HPO_4—0.2; $MgS0_4$—02; NaCl—0.2; K_2SO_4—0.1; $CaCO_3$—5.0; agar—20.0; pH 7.1–7.3. The medium is sterilized at 0.5 atm and 120 °C (Zenova et al. 2002).

The Maze medium has the following composition (g/L): K_2HPO_4—1.0; $MgSO_4$—0.3; sucrose—10.0; broth made from 100 g of pea—up to 1 L, pH 6.8—7.0 (Emtsev 2005; Stiles 2013).

To prepare pea broth, 100 g of peas were poured with 1 L of tap water and boiled until the skin swells and cracks. The broth was filtered through cotton wool (gauze) and the volume adjusted with tap water to 1 L.

Nodule bacteria growing on these media form colorless or milky white mucous colonies. Rhizobia growing on pea agar slants were richly developed, forming transparent mucus, often flowing down massive colonies or streaks.

Cultivation of rhizobia was carried out in flasks using an incubator shaker at 28 ± 2 °C and 180 rpm and in Petri dishes placed in a thermostat at 28 ± 2 °C.

2.2.3 Selection of Active Strains of Nitrogen-Fixing Rhizobia

Primary selection of the nitrogen fixer's active strains was carried out in the nitrogen-free Ashby liquid medium. Based on the fact that the higher the accumulation of bacterial biomass, the more active the culture is in fixation of atmospheric molecular nitrogen. The bacterial biomass was measured nephelometrically with the PD-303 spectrophotometer ("Apel," Japan) in optical density units (RODU), calculated per absolute dry biomass (a.d.m.) using the calibration curve, and expressed in g/1000 mL.

2.2.4 Studies on Growth-Promoting Activity and Rhizobial Nodulation

To study the growth-promoting activity of rhizobia and their nodulation ability (ability to form nodules on the roots of plants), bacteria were cultivated in the liquid Ashby medium on shaking conditions at a rotary speed of 180 rpm and temperature of 28 °C for 3–5 days. Seeds of alfalfa, melilot, and clover before sowing were inoculated with bacterial suspension with a titer of 10^7–10^8 cell/mL for 2 h at a temperature of 23 °C. The seeds were then sown in the vegetation vessels. Vermiculite was used as a substrate for plant growth, and the liquid Knopp medium lacked a nitrogen source as a feed for seedlings. Before setting up the experiment, the substrate and Knopp solution were sterilized; sterile tap water was used for watering plants. Seeds of alfalfa, melilot, and clover that were not inoculated with rhizobia served as a control. All the experiments were performed in triplicates.

Model laboratory experiments on the effect of rhizobia on the pasture grasses were carried out in a climatic chamber (Constant Climate Chamber HPP-750, "Memmert", Germany). The parameters of the moisture, illumination, and temperature in the climate chamber were coincided with the spring vegetation period of the year.

Biometric parameters of the plants, such as stem length and root length, were measured after 30 days of cultivation. Nodule number and average weight were determined after 45 days of cultivation. By this time, the nodules were well developed, and it was easy to detect and count them.

2.2.5 *Identification of Nitrogen-Fixing Root Nodule Bacteria of the Genus Rhizobium*

A number of methods including classical microbiological, based on studying the cultural-morphological and biochemical characteristics and properties of microorganisms (Holt et al. 1997), and molecular genetic techniques were used to determine the taxonomic position of rhizobia.

Three strains of nodule bacteria L23, D26, and K24 were identified by amplifying their 16S rRNA. Genomic DNA was isolated using a set PureLink® Genomic DNA Kits. The concentration of DNA in the samples was determined using a fluorometer Qubit (Invitrogen). Sequencing was performed using universal primers for 16S rRNA gene: 8f-5′–AGAGTTTGATCCTGGCTCAG-3 and 806R-5′-GGACTACCAGGGTATCTAAT-3. The reaction mixture for amplification consisted of 1 μL of primers, 2.5 μL dNTP, 2.5 μL buffer, 0.2 μL Polymerase, 2 μL DNA, and H_2O. PCR was carried out in a thermocycler Mastercycler pro S (Eppendorf). The reaction was started by incubating the mixture at 95 °C for 7 min and then followed by 30 cycles consisting of incubations: 95 °C—30 s, 55 °C—40 s, 72 °C—1 min. The final elongation was performed at 72 °C for 10 min.

The sequencing reactions were performed using BigDye Terminator v3.1 Cycle Sequencing Kit (Applide Biosystems) according to manufacturer's instructions (BigDye® Terminator v3.1 Cycle Sequencing Kit Protocol Applied Biosystems USA), followed by separation of the fragments on an automated Genetic Analyzer 3500 DNA Analyzer (Applide Biosystems). Sequencing results were processed in the program SeqAn (Applide Biosystems). The resulting nucleotide sequence of 16S rRNA gene was compared with the GenBank database (www.ncbi.nih.gov), using the BLAST program. Phylogenetic analysis was performed using the software MEGA6. The alignment of the nucleotide sequences was performed using ClustalW algorithm.

2.2.6 *Statistical Analysis*

The statistical significance of the results obtained was determined using the Student's *t*-test for the confidence level $p < 0.01$ (Tolchenov et al. 2009).

2.3 Results

Rhizobia have been isolated from root nodules of wild alfalfa (*M. sativa* L.), sweet clover yellow (*M. officinalis* (L.) Desr.), and white clover (*T. repens* L.). Plants were harvested on environmentally undisturbed pastures of Kazakhstan. Healthy and strong plants with a well-developed root system were selected to isolate rhizobia. Plant

monitoring was also carried out, and plants with a large number of nodules on the roots were selected. Isolation of rhizobia from nodules was performed according to the protocol (see Sect. 2.2.1). As a result of the work, 24 rhizobium isolates were obtained from legume nodules. Of these, nine isolates were obtained from alfalfa nodules, ten from melilot nodules, and five from clover nodules.

Identification of nodule bacteria was carried out by studying the cultural-morphological and biochemical characteristics. Table 2.1 shows the identification of rhizobium strains and legumes from which they were isolated.

Table 2.1 The nodulating Rhizobial species from some legumes of natural pastures

No.	Strains	Rhizobial species	Legume species	
			Common name	Latin name
1	L12	*Sinorhizobium medicae*	Wild alfalfa	*Medicago sativa* L.
2	L17	*Sinorhizobium meliloti*	Wild alfalfa	*Medicago sativa* L.
3	L19	*S. meliloti*	Wild alfalfa	*Medicago sativa* L.
4	L20	*S. spp.*	Wild alfalfa	*Medicago sativa* L.
5	L23	*S. meliloti*	Wild alfalfa	*Medicago sativa* L.
6	L24	*S. medicae*	Wild alfalfa	*Medicago sativa* L.
7	L28	*S. medica*	Wild alfalfa	*Medicago sativa* L.
8	L35	*S. meliloti*	Wild alfalfa	*Medicago sativa* L.
9	L36	*S. meliloti*	Wild alfalfa	*Medicago sativa* L.
10	D09	*S. meliloti*	Sweet clover yellow	*Melilotus officinalis* (L.) *Desr.*
11	D12	*S. meliloti*	Sweet clover yellow	*Melilotus officinalis* (L.) *Desr.*
12	D14	*S. meliloti*	Sweet clover yellow	*Melilotus officinalis* (L.) *Desr.*
13	D20	*S. medicae*	Sweet clover yellow	*Melilotus officinalis* (L.) *Desr.*
14	D24	*S. meliloti*	Sweet clover yellow	*Melilotus officinalis* (L.) *Desr.*
15	D26	*S. medicae*	Sweet clover yellow	*Melilotus officinalis* (L.) *Desr.*
16	D34	*S. meliloti*	Sweet clover yellow	*Melilotus officinalis* (L.) *Desr.*
17	D36	*S. medicae*	Sweet clover yellow	*Melilotus officinalis* (L.) *Desr.*
18	D38	*S. meliloti*	Sweet clover yellow	*Melilotus officinalis* (L.) *Desr.*
19	D39	*S. medicae*	Sweet clover yellow	*Melilotus officinalis* (L.) *Desr.*
20	K06	*Rhizobium leguminosarum*	Clover white	*Trifolium repens* L.
21	K14	*Rh. spp.*	Clover white	*Trifolium repens* L.
22	K16	*Rh. leguminosarum bv. trifolii*	Clover white	*Trifolium repens* L.
23	K22	*Rh. spp.*	Clover white	*Trifolium repens* L.
24	K24	*Rh. leguminosarum bv. trifolii*	Clover white	*Trifolium repens* L.