

Review

Deciphering the Symbiotic Plant Microbiome: Translating the Most Recent Discoveries on Rhizobia for the Improvement of Agricultural Practices in Metal-Contaminated and High Saline Lands

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Abstract: Rhizosphere and plant-associated microorganisms have been intensely studied for their beneficial effects on plant growth and health. These mainly include nitrogen-fixing bacteria (NFB) and plant-growth promoting rhizobacteria (PGPR). This beneficial fraction is involved in major functions such as plant nutrition and plant resistance to biotic and abiotic stresses, which include water deficiency and heavy-metal contamination. Consequently, crop yield emerges as the net result of the interactions between the plant genome and its associated microbiome. Here, we provide a review covering recent studies on PGP rhizobia as effective inoculants for agricultural practices in harsh soil, and we propose models for inoculant combinations and genomic manipulation strategies to improve crop yield.

Keywords: soil bioremediation; high-salinity soil; plant beneficial microbes; rhizobia; microbial inoculants; plant-growth promoting rhizobacteria (PGPR)

1. Introduction

The last ten years have witnessed a number of discoveries and an increased awareness of the importance of the microbiome for the health and the growth of host macroorganisms [1,2]. Plants and their related microbiota can be considered holobionts, complex systems ruled by interdependent and composite interactions [3–5]. Indeed, plants are colonized by an astounding number of microorganisms that can reach numbers much greater than those of plant cells. This is especially relevant for the rhizosphere, the thin layer of soil surrounding and influenced by plant roots, where it is possible to observe a staggering diversity of microorganisms; a single gram of rhizospheric soil hosts tens of thousands of distinct microbial species [6,7]. Plants influence the composition of their rhizosphere microbiota through the production of root exudates [8], which differ in space and time [9], contributing to the positive selection of plant-growth promoting (PGP) and beneficial bacteria [10]. Indeed, it has been suggested that plants may have evolved the beneficial trait of secreting specific compounds to recruit protective microorganisms in response to pathogen attacks [11,12]. Plant-based bacterial selection relies on the microorganism already present in the soils where they are grown. Therefore, crop productivity could be increased by modifying root microbiota with microbial inoculants, which may be composed of a single strain or a consortia of different PGP rhizobacteria (PGPR) [13,14].

Within the beneficial plant microbiota, rhizobia constitute one of the most studied fraction [15]. The “rhizobium” definition is based on the ability to induce the formation of root/stem nodules in leguminous plants [16]. However, rhizobia are found not only on legumes, but are also found in association with several plant species [17]. Within legume nodules, rhizobia differentiate into bacteroids and synthesize a protein complex called nitrogenase that converts atmospheric dinitrogen to ammonia (biological nitrogen fixation, BNF) [18]. The produced ammonia is then transferred to the host plant to sustain its biosynthetic pathways [16]. The establishment of the symbiotic interaction between the nitrogen fixing rhizobia and leguminous plants is highly regulated and begins with mutual recognition between the plant and the rhizobia present in the rhizosphere. To date, rhizobia have been identified in two bacterial classes, the *Alphaproteobacteria* and the *Betaproteobacteria*. In the *Alphaproteobacteria*, rhizobial strains are present in the genera *Sinorhizobium* (syn. *Ensifer*), *Rhizobium*, *Mesorhizobium*, *Bradyrhizobium*, *Azorhizobium*, *Methylobacterium*, *Devosia*, *Ochrobactrum*, *Aminobacter*, *Microvirga*, *Shinella*, and *Phyllobacterium*. In the *Betaproteobacteria*, rhizobia are present within strains of the genera *Paraburkholderia*, *Cupriavidus*, and *Trinickia* [19–21]. While the beta-rhizobia are mainly found in association with tropical legumes, the alpha-rhizobia appear to be more widespread and nodulate tropical to temperate legumes including pasture, tree, and grain legumes. The alpha-rhizobia have received more research attention than the beta-rhizobia; among the alpha-rhizobia, *Sinorhizobium* (syn. *Ensifer*) is likely the most studied genus, followed by the genera *Rhizobium* and *Bradyrhizobium* [22,23].

In this review, we highlight the most recent studies on PGP rhizobia isolated from, and adapted to, drought-affected and metal-contaminated soils and their possible use as effective inoculants for legumes grown in harsh agricultural soils. We cover the identification of the genetic determinants of their tolerance, as well as the mechanisms that allow rhizobia to survive and to improve host plant growth in harsh soils. Models of inoculant combinations and genomic manipulation strategies for the improvement of crop yield are discussed.

2. The Need for Rhizobial Inoculants

The demand for plant proteins for human nutrition has increased tremendously over the last fifteen years. This can be related to: (i) Demographic growth and urbanization, (ii) the limited land areas that can be used for the production of food crops while farming systems are switching to specialized but unsustainable cereal production (for market competitiveness), and (iii) decreases in animal protein production due to shortage of irrigation and/or rainfall water especially in arid areas. The demand for plant proteins can be met in part through the cultivation of protein-rich leguminous crops. Additionally, legumes can help improve soil fertility through symbiotic nitrogen fixation, and they can help protect ground water from toxicity resulting from excessive application of N-fertilizers [24].

In the past three decades, eco-sustainable agronomic practices have been employed in an attempt to replace chemical fertilizers and pesticide-based agriculture [25,26]. Therefore, the exploitation of beneficial microorganisms as biofertilizer has become of primary importance [27]. In particular, rhizobial bioformulations could partially or completely substitute mineral nitrogen fertilizers [28,29]. Rhizobium-legume symbioses provide more than half of the world’s biologically fixed nitrogen [30], and it was reported that rhizobial nitrogen fixation introduces 40–48 million tonnes of nitrogen into agricultural systems each year [31]. The impact of BNF on the global agricultural economy was estimated to be worth the equivalent of USD160–180 billion [32]. Rhizobium inoculants are already widely used in agriculture, providing one of the most cost-effective ways to boost legume performances [33,34]. However, with a few exceptions, the last fifteen years has seen only small enhancements in the production of traditionally grown grain legumes such as fava bean, chickpea, lentils, or common beans [35]. Generally, yield instability is the main constraint for increasing plant productivity. Thus, special attention must be given to the factors that reduce soil quality and decrease plant yield.

Recent works have highlighted that microbial species associated with plants (rhizobial and non-rhizobial strains, including mycorrhizal fungi) can positively influence plant tolerance to water

deficiency [36]. This is due to their PGP features such as indoleacetic acid, siderophore production, phosphate and zinc solubilization [37], and the synthesis of 1-aminocyclopropane-1-carboxylate (ACC) deaminase [38], which are more evident and easily identified in stressful conditions [39]. Nevertheless, legumes are strongly affected by water deficit. In particular, BNF appears to be more sensitive to water deficit than other physiological functions such as photosynthesis or nutrient uptake [40]. Sometimes, this results in impaired nodule development [41] or the accumulation of small, generally organic, osmolytes called compatible solutes [42].

Aside from water deficiency and soil nutrient depletion, heavy-metal contamination due to anthropic activities (agricultural and industrial practices) or the weathering of metal-enriched rocks have recently increased exponentially, becoming a worldwide problem for crop productivity [43–45]. Generally, plant-associated microbes can contribute to a plant's ability to perform phytoextraction (accumulation of toxic compounds in the plant tissues) and phytostabilization (adsorption through the roots and conversion into harmless compounds). In legumes, phytostabilization is the key process when considering the phytoremediation of contaminated soils [46,47], and their associated rhizobia can promote chemical transformation and the chelation of heavy-metal compounds [48] (Figure 1). Therefore, plant growth and agricultural yield is related not only to the plant genotype and the soil condition, but, especially for legumes, their associated microbiota also play important roles [49,50]. As such, the selection of rhizobial strains resistant to water deficiency and capable of alleviating metal phytotoxicity could be a crucial strategy to improve the yield of legumes growth in arid or in metal-contaminated soils.

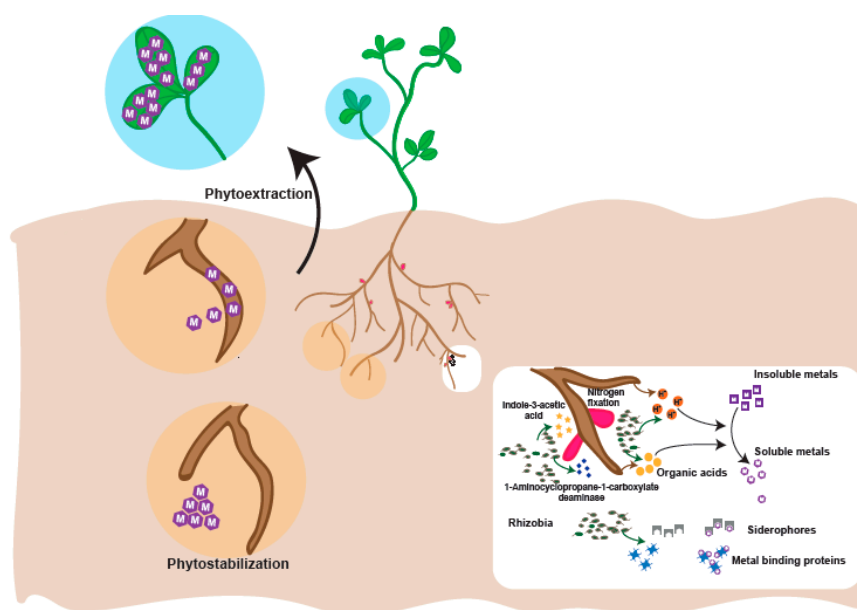


Figure 1. The mechanisms involved in bioremediation of heavy-metal contaminated soil and the contribution of PGP rhizobia.

3. Plant Growth Promoting Rhizobia in Saline and Harsh Soil

Salinity due to water deficiency is one of the largest environmental constraints for plant growth and productivity in stricken regions [51,52]. It was estimated that almost 40% of the world's lands can potentially become arid or semi-arid [52], most of which are located in the tropics and in the Mediterranean area [53,54]. The persistent increase of anthropic activities (such as poor agricultural practices) decreases soil water availability, alters the soil microbiota, and reduces the nutritive value of soils [55]. The progressive salinization of soil may cause several stresses to the plants that decrease their growth. This negatively affects crop productivity, hindering the agricultural economy of developing countries [56].

Desiccated soils can lead to alterations in plant homeostasis due to a reduction in the osmotic potential and inappropriate ionic distribution [56]. The alteration in nutrient supply and the resulting nutritional imbalance induces a loss of turgor pressure and the growth of smaller leaves [56]. Furthermore, increasing Na⁺ and Cl⁻ concentrations commonly leads to the formation of burn-like lesions that alter leaf transpiration [57]. Continued growth interruption results in less efficient photosynthesis, respiratory variations, premature senescence, and the loss of cellular integrity leading to plant death [58,59]. Salt tolerance among legume species can fluctuate and is dependent on the chemical features of the soil, the climatic conditions, and the growth stage of the plant [53,60,61]. Tree legumes such as *Prosopis* [53,62] and *Acacia* spp. [53,63] are highly tolerant to salinity, as are the grain legumes *Glycine max* [51] and *Vicia faba* [61,64]. On the contrary, *Cicer arietinum*, *Phaseolus vulgaris*, and *Pisum sativum* are known to be extremely sensitive to salt stress [64–67]. BNF can also be strongly affected by a lack of water, influencing the mutual symbiotic interaction between the host plants and their associated rhizobia [53]. Generally, salt and osmotic stresses lead to a decrease in rhizobium nodulation, affecting the early stages of the symbiotic process [68–71]. This commonly includes poor root colonization by the bacteria and reduced curling and deformation of roots hairs [53,72,73]. Furthermore, a decrease in nitrogenase activity under drought/salinity stress is commonly attributed to a reduction in nodule respiration [67,74–76], which reduces the synthesis of cytosolic proteins such as leghemoglobin [67,76,77].

It was reported that host legumes are less tolerant to salt than their associated rhizobia [71,73,78]. For example, *Rhizobium leguminosarum* bv. *trifolii* TA-1 can tolerate up to 350 mM of NaCl in vitro [78]. The highest levels of salt tolerance seem to be associated with rhizobia isolated from woody legumes (e.g., *Prosopis*, *Acacia*, and *Leucaena*), which are tolerant of up to 850 mM of NaCl [73,79,80]. For example, Sakrouhi and collaborators [81] isolated 20 symbiotic N₂-fixing bacteria from *Acacia tortilis* and *Acacia gummifera* that were able to grow in high salt media (400 mM). The intracellular accumulation of low-molecular-weight organic solutes, osmo-protectants, is an osmotic adaptation mechanism used by a large variety of bacterial species [53]. These compounds are acquired through de novo synthesis or uptake from the environment, and they can be accumulated to high intracellular concentrations without interfering with cellular processes [56], such as in the *S. meliloti* 102F34 strain [82–84]. The accumulation of poly-hydroxybutyrate (PHB) has also been reported as a protective measure to help rhizobia survive in high saline environments [85]. Following a decrease in osmolarity, the osmo-protectants are released by bacteria into the surrounding environment and actively recovered by plants, which are unable to synthesize them de novo [84]. The successful uptake of these compounds by the plants improve their growth under osmotic stress [56]. Additionally, intracellular trehalose accumulation by *R. leguminosuarum* seems to be involved in metabolic osmoregulation of the host plants [86,87]. The intracellular accumulation of glycine betaine was identified as one of the most frequent osmotic stress responses of rhizobia [88,89]. Several lines of evidence suggest that this process plays a role in maintaining bacteroid nitrogenase activity in *Medicago sativa* nodules [53,90–92].

Nowadays, the massive use of fertilizers to offset the effect of soil nutrient loss (also due to salinization) on crop productivity seems to be the preferred solution. This choice has progressively contributed to the deterioration of the soils that were already compromised by intense agricultural practices. An alternative is to make use of bacterial inoculants adapted to these harsh conditions. Studying the bacterial communities associated with plants growing in saline soils, and the underlying mechanism of their effectiveness, can be a good starting point for the use of microbial inoculant in agricultural practices to reduce saline stress [56,93]. To begin addressing this, the genomes of several rhizobia that nodulate plants in harsh environments were sequenced to identify stress-adaption genes. Examples include: *Rhizobium* sp. LCM 4573 (a salt-tolerant rhizobium from Senegalese soil [94]), *R. leucaenae* (a stress-tolerant species nodulating plants in tropical acid soils [95]), *S. meliloti* AK21 (from the Aral Sea Region that experiences saline and drought conditions [96]) and *Ensifer aridi* (isolated from arid soils of diverse deserts [97]). Transcriptomic and proteomic studies have been instrumental in highlighting how rhizobia respond to environmental stresses (a detailed list is provided

elsewhere [98]). Studies with *Mesorhizobium loti* suggested heat shock results in a global downregulation of protein expression possibly to conserve energy [99], while salt stress leads to over-expression of ABC transporters and genes associated with nucleotide transport and metabolism [100]. Similarly, characterization of the acid stress response in *S. meliloti* suggested that this stress results in an elevated metabolic respiration rate [101]. However, to date only a few studies have examined the responses of differentiated bacteroids to environmental stress [102,103], although several studies have examined the responses of the plant partner [104]. Additional studies characterizing the response of bacteroids to environmental stresses would be beneficial to complement the free-living datasets.

The agricultural significance of uncovering the genetic and metabolic basis of stress resistance in rhizobia and other PGP bacteria has been emphasized from at least the early 1990s [105]. In fact, numerous studies have demonstrated that genetically modifying rhizobia can increase or decrease their symbiotic abilities in stressful environments [106]. The grain yield of common bean plants grown in drought conditions was significantly higher when inoculated with a *Rhizobium etli* strain overexpressing *otsA*, encoding a trehalose-6-phosphate [107]. Similar results were obtained for maize plants inoculated with a non-rhizobium diazotroph *Azospirillum brasilense* strain overexpressing a trehalose biosynthesis gene [108]. In contrast, soybean plants inoculated with a *Bradyrhizobium japonicum putA* mutant that is unable to catabolize proline produced fewer seeds than plants inoculated with the wild-type parental strain when grown in moderate drought conditions [109]. *S. meliloti* strains overexpressing *betS* displayed improved nitrogen fixation phenotypes during salt stress [110], while salt-sensitive *Rhizobium tropici* mutants were poor symbionts even in the absence of stress [111]. Finally, ACC-deaminases encoded by some rhizobia can reduce the overproduction of the plant gas hormone ethylene during abiotic stresses [38,112], reducing the deleterious effect of ethylene and thus improving plant growth [113].

The inoculation of plants with microbial communities has also been shown to improve plant tolerance to environmental stresses. The co-inoculation of soybean (*Glycine max*) with *Chryseobacterium balustinum* Aur9 and *Ensifer (Sinorhizobium) fredii* SMH12 led to increased symbiotic performance under saline conditions (25 mM NaCl) [114]. In the same study, co-inoculation of common bean (*Phaseolus vulgaris* L.) with *R. tropici* CIAT899 and *C. balustinum* Aur9 enhanced bean growth in both saline (25 mM NaCl) and control conditions compared to single strain inoculation [114]. Moreover, co-inoculation of *Rhizobium phaseoli* M6 and M9, *Pseudomonas syringae* Mk1, *Pseudomonas fluorescens* Mk20, and *Pseudomonas fluorescens* biotype G Mk25 strains decreased the effects of salinity stress in bean, enhancing its nodulation process in vitro and in fields conditions [115,116].

4. Plant Growth Promoting Rhizobia in Heavy Metal Contaminated Soil

Anthropogenic activities, such as the use of fertilizers and pesticides in agricultural soils, the production of sewage sludge waste, and industrial and mining activities, are responsible for the accumulation of toxic heavy metals in the food chain [117]. Low concentrations of metals such as zinc (Zn), copper (Cu), iron (Fe), nickel (Ni), manganese (Mn), molybdenum (Mo) and cobalt (Co) are necessary for the metabolism of all organisms [118]. However, high concentrations of these metals, as well as the long term persistence in the soil of elements such as cadmium (Cd), lead (Pb), and arsenic (As), negatively affect the composition of microbial communities [119], the dynamics of the rhizosphere niche [120], and the growth, the biomass, and the photosynthesis of plants [121]. Plant species used for the remediation of heavy metal polluted sites represent an environment-friendly, aesthetically appealing, and cost-effective solution. Legumes may be ideal species for bioremediation as surveys of plant species surviving in long-term metal-contaminated environments have shown legumes to account for a dominant portion of these populations [122]. The metal tolerant plant species used for bioremediation have developed several mechanisms that allow them to thrive in these contaminated environments and to accumulate high concentrations of specific metals in the aboveground tissue. Among these, both enzymatic and non-enzymatic molecular mechanisms have been described. Heavy-metal stressed plants may protect themselves from reactive oxygen species through the production of antioxidant

enzymes or scavenger compounds [123]. Recently, PGP bacteria, including rhizobia, have been shown to reduce the toxicity of plant exposure to heavy metals [124,125]. Heavy-metal contaminated soil remediation can be performed with different strategies [126]. Plants able to decrease the mobility and/or the bioavailability of metals can be used in both phytostabilization and phytoimmobilization to prevent their leaching into ground water or their entry into the food chain. Mechanisms involved in this process include adsorption by roots, and the precipitation and complexation of the metals in the root zone [127]. Phytovolatilization involves the conversion of a metal (i.e., Hg as the mercuric ion) into the volatile form and its release into the atmosphere through the stomata [128]. However, the most important phytoremediation approach for removal of metals and metalloids from contaminated soils, water, and sediments is phytoextraction [129–131]. The main contribution of rhizobia towards phytostabilization and phytoimmobilization is plant growth enhancement [132]. Bacteria that nodulate their hosts may increase metal accumulation in root nodules, while those that remain in the rhizosphere would reduce metal toxicity locally by precipitation, chelation, immobilization, and biosorption. The nodule itself has an important role in metal-resistance: Once the symbiosis is established, nodules could serve as storage areas that provide plants an extra place to stock metals and reduce the risk of direct exposure [133].

Accelerating the phytoremediation of metalliferous soils by increasing mobilization and phytoextraction of heavy metals though the metabolic activity of rhizobia is a well-known practice [37,131,134–137]. Currently, the most studied metal resistance mechanisms in microorganisms include metal exclusion, protein binding-mediated extra- and intra-cellular sequestration, enzymatic detoxification, active transport of the metal, passive tolerance, and reduction in metal sensitivity of the cellular targets [138,139]. Bacteria can also contribute to phytoremediation through the production of extracellular polymeric substances. For example, studies of the interaction between metals and extracellular polymeric substances demonstrated that biosorption can reduce heavy metal contamination of wastewater systems [140,141]. Unlike salt-tolerant bacteria, there have been numerous studies on the use of bacteria isolated from metal contaminated soil as inoculants to promote plant growth in contaminated environments [37,48]. Although not all rhizobia are intrinsically tolerant to metals, metal-tolerant strains of taxonomically diverse rhizobia have been isolated from various plants in heavy metal contaminated environments [37,142]. Metal resistance determinants provide protection for rhizobia to survive and maintain effective nodulation of legumes, allowing them to play a role in promoting plant growth. In addition, the existence of a symbiotic relationship may provide protection for the survival of rhizobia in soils with elevated metal concentrations [143,144].

Arsenic toxicity, and the oxidative damage that it produces in cells through the overproduction of reactive oxygen species, affects DNA, proteins, and lipids. This provokes chlorosis, necrosis, delays in flowering, and a reduction in yield [145]. PGP rhizobia may play a beneficial role in protecting plants from arsenic contamination. This can be accomplished by stimulating the antioxidant enzymatic activities in plants, and stabilizing heavy metals and metalloids thereby reducing their accumulation in aerial organs [146–148]. For this reason, the use of PGP rhizobia in heavy metal and metalloid contaminated soils should not only promote the growth of the plant but should also immobilize and decrease the concentration of these elements in plant organs to reduce human exposure to toxic concentrations [149,150].

The presence of heavy metals can also influence the results of inoculant treatment of crops. For example, inoculation of soybean plants with two different strains of *Bradyrhizobium*, *B. diazoefficiens* USDA110 and *Bradyrhizobium* sp. Per 3.61, was studied in the Córdoba province of Argentina where arsenic contamination of groundwater is a consistent environmental problem [151]. The results demonstrated that only *B. diazoefficiens* USDA110 could nodulate soybean at the highest tested As(V) concentrations, while *Bradyrhizobium* sp. Per 3.61 was the better symbiont in the presence of low As(V) concentrations as it limited the translocation of the metal to the legume aerial compartments [151]. Numerous studies have also examined the effect of plant inoculation with pairs of PGP bacteria [152–155]. For instance, the co-inoculation of soybean with *B. japonicum* E109 and *Azospirillum brasilense* Az39

influenced plant growth and arsenic phytostabilization in arsenic contaminated conditions [156]. Furthermore, it was observed that the indole acetic acid (IAA) produced by *A. brasilense* Az39 had a protective effect on *B. japonicum* E109 when exposed to arsenic [156]. *B. japonicum* strains can use IAA as a carbon source, which seems to serve as a signal to coordinate bacterial behaviour to enhance protection under adverse conditions [157]. The presence of high levels of lead in soil is toxic for plants, resulting in chlorosis, blackening of roots, and reduced growth [158]. A study using *Brassica juncea* showed that the inoculation of autochthonous PGP rhizobial strains can alleviate the harmful effects of lead exposure. *Sinorhizobium* sp. Pb002 was isolated from the rhizosphere of *B. juncea* grown in Pb-contaminated soil [159]. In a microcosm experiment, the presence of strain Pb002 stimulated biomass formation by *B. juncea* and increased plant survival and lead uptake [159]. Nickel and zinc are essential elements for plant growth; however, excessive amounts of these nutrients can be toxic [160,161]. This toxicity can be alleviated, at least in part, through rhizobium inoculation. For example, inoculation of green gram plants with *Bradyrhizobium* sp. (vigna) RM8 or *Rhizobium* sp. RP5 increased both seed yield and grain protein in the presence of excessive nickel or zinc [162,163]. The presence of Cd in soil can impair plant growth due to a reduction in chlorophyll content and photosynthesis [164]. Moreover, Cd alters the cell redox potential and increases the amount of reactive oxygen species in the cell, which in turn negatively impacts cell membranes and biomolecules [164,165]. *Bradyrhizobium* sp. YI-6, isolated from *G. max* nodules grown in Chinese Cd-contaminated soil, displayed an ability to increase mineral nutrient (Fe) uptake while reducing Cd accumulation [166].

Researchers have identified potential metalloid stress-adaptation genes in rhizobia and they have investigated their transcriptional responses. Putative nickel adaptation genes were identified using association mapping with 47 symbiotic *Mesorhizobium* strains isolated from either nickel-enriched serpentine soils or nearby non-serpentine soils [167]. The identified genes included several transporters, an opine dehydrogenase, and an exopolysaccharide export protein, among others [166]. Additionally, investigation of the transcriptional response of *S. meliloti* strain CCNWSX0020 upon exposure to copper or zinc stress allowed the identification of several upregulated genes, including four genes (*yedYZ*, *fixH*-like, *cusA*-like, and *cueO*) whose mutation impaired either early or late symbiotic processes [168]. As for rhizobia colonizing saline and arid environments, genetically modified strains can have different symbiotic abilities. For example, a *M. amorphae* Δ *copA* deletion mutant displayed impaired symbiotic capabilities in copper contaminated soils, whereas overexpression of a flavodoxin gene in *S. meliloti* led to a more efficient symbiosis under cadmium stress [169].

Together, the studies discussed in this review (and summarized in Table 1) highlight how the rhizobial genotype can strongly influence symbiotic effectiveness and the plant response in harsh environments. Fully elucidating the genetic and molecular bases of these phenotypes would lay a strong foundation to aid the development of improved bio-inoculants, either through genetic engineering or the rational selection of optimal wild isolates.

Table 1. Studies of plant growth improvement mediated by rhizobium-inoculants on harsh soil.

Strain	Isolation Conditions		Crop	Effect/Action Mechanism	Reference
	Site	Metal Contamination			
<i>Bradyrhizobium diazoefficiens</i> USDA110	Ref. strain	As	Soybean	Limits metalloid translocation and accumulation in edible parts of the legume	[151]
<i>Bradyrhizobium</i> sp. Per 3.61	Nodules of soybeans	As			
<i>A. brasilense</i> Az39		As	Soybean	Enhances growth of the plant and phytostabilization of As when co-inoculated	[156]
<i>B. japonicum</i> E109		As			
<i>Sinorhizobium</i> sp. Pb002	Rhizosphere of <i>Brassica juncea</i>	Cd	<i>Brassica juncea</i>	Increases plant survival and lead uptake	[159]

Table 1. Cont.

Strain	Isolation Conditions		Crop	Effect/Action Mechanism	Reference
	Site	Metal Contamination			
<i>Bradyrhizobium</i> sp. (<i>vigna</i>) RM8 <i>Rhizobium</i> sp. RP5	Nodules of greengram	Ni, Zn	Greengram	Increases the number of nodules on the plant, as well as IAA and siderophore production	[163]
<i>Bradyrhizobium</i> sp.	Nodules of Glycine max	Cd	Glycine max	Increases mineral nutrient uptake (Fe) and reduces cadmium accumulation in the plant	[166]
<i>Rhizobium leguminosarum</i> (LR-30), <i>Mesorhizobium ciceri</i> (CR.30 and CR-39) and <i>Rhizobium phaseoli</i> (MR-2)	<i>Lens culinaris</i> L., <i>Vigna radiata</i> L., <i>Cicer arietinum</i> L.		Wheat (Triticum aestivum)	Improves plant growth, biomass, and drought stress through production of catalase, IAA, and exopolysaccharides under PEG-6000 simulated drought conditions	[170]
Strain	Isolation conditions		Crop	Effect/Mechanism of action	Reference
<i>Azospirillum</i> Sp245	Surface-sterilized wheat roots of (Brazil) [171]		Lattuce (<i>L.sativa</i> L.)	Promotes aerial biomass, higher ascorbic acid content accompanied by a lower oxidation rate, better overall visual quality due to higher chlorophyll content, hue, Chroma, L and lower browning intensity	[172]
<i>Sinorhizobium medicae</i> WSM419	Nodules of <i>Medicago murex</i>		<i>Medicago truncatula</i>	Delays stress-induced leaf senescence and abscission and nutrient acquisition during drought stress	[173]
<i>Sinorhizobium meliloti</i> A2 strains	Commercial strain, Eastern Canada [174]		<i>Medicago sativa</i> cv Apica, <i>Medicago sativa</i> cv Halo	Increases shoot/root ratio, shoot water content, and the concentrations of starch and pinitol in nodules	[175]
<i>Sinorhizobium meliloti</i> Rm1521	Ottawa vicinity [176]				
<i>Rhizobium etli</i> CE3 overexpressing trehalose-6-phosphate synthase gene	CFN42 derivate [177], original isolate from <i>P. vulgaris</i> nodule		<i>P. vulgaris</i> var. Negro Jamapa	Enhances drought tolerance due to upregulation of genes involved in stress tolerance, carbon and nitrogen metabolism by trehalose	[107]
Co-inoculation			Crop	Effect/Mechanism of action	Reference
Rhizobia	PGPRs				
<i>Rhizobium phaseoli</i> M6; M9, <i>phaseoli</i> M6 and M9 and PGPR	<i>Pseudomonas syringae</i> , Mk1; <i>Pseudomonas fluorescens</i> Mk20; <i>Pseudomonas fluorescens</i> biot. G Mk25		<i>Vigna radiata</i> L.	Decreases damaging effect of salinity stress on mung bean growth	[116]

5. Development of Rhizobial Inoculants

The use of rhizobial bioinoculants began in the USA at the end of 19th century, where soil containing naturally-occurring rhizobia was mixed with seeds. Since then, rhizobium inoculation has become a common practice to improve crop production [178]. Since the first marketed rhizobium biofertilizer “Nitragin”, which was developed by Nobbe and Hiltner in 1896, rhizobial bioformulations have improved dramatically. From the second half of the 19th century, liquid inoculants formulation [179] moved initially to freeze-dried inoculant lyophilization [180], and then to gel-based products such as polyacrylamide (PER) [181], alginate (AER), or xanthan (XER) [27,182]. Over the last 30 years, a huge number of formulations have been patented and commercialized; examples include vermiculite-based Gold Coat™ *Rhizobium* inoculant [183], liquid seed applied soybean inoculant Cell-Tech® [184], liquid in-furrow inoculant LIFT, and air-dried clay powder for alfalfa Nitragin® Gold [27,184]. In 1997, the first marketing of a genetically engineered *S. meliloti* strain RMBPC-2 was approved [185].

Eventually, additives and cell protectant-based liquid inoculant formulations were developed that increased cell survival through the use of compounds such as the polymer polyvinyl pyrrolidone (PVP), carboxymethyl cellulose (CMC) [186], gum Arabic [142], sodium alginate [182], and glycerol [187]. The choice of inoculant carriers that can promote the long-lasting maintenance and protection of viable microbial cells is a global issue [24,188]. Peat is currently the most common organic carrier material for bioformulation production [189], especially in North and South America, Europe, and Australia [190],

although other materials (such as coal, bagasse, coir, dust, and perlite) are also used [191]. Supported by successful in vitro experiments, Albareda and collaborators suggested the use of broth culture media as a rhizobial carrier for soybean cultivation [191]. It was demonstrated that after 3 months of storage, liquid cultures were able to maintain more than 10⁹ cfu/mL of *Sinorhizobium fredii* SMH12 and *Bradyrhizobium japonicum* USDA110 [191]. Therefore, aqueous-, oil-, or polymer-based liquid formulations have increased enormously in recent years (for details see [190,192–194]). Additionally, the inclusion of microbial or plant secondary metabolites, such as flavonoids and phytohormones, has become a common practice in bioformulation preparation to improve the efficiency of the inoculants [25,195–197].

6. Inoculant Combinations and Phenomic Strategies for Improving Crop Yield

Screening for rhizobia with high nitrogen fixation rates is performed in many laboratories; however, the use of effectiveness as the sole criterion for rhizobia selection may not always be the most relevant criterion for field applications [75]. Indeed, in the soil the rhizobia have to overcome many different adverse conditions (pH, desiccation, nutrient deficiencies, salinity/alkalinity, extreme temperatures, toxicities) [75,198] and they have to outcompete other rhizobial strains [199,200]. It is thought that there is generally an alignment between the fitness of rhizobia and the fitness of their host plants [201]. However, rhizobia are not vertically transmitted but are instead soil bacteria that colonize plant roots. Therefore, ineffective or less effective rhizobia can become abundant and outcompete more effective strains. Moreover, a single plant can be infected by multiple strains with different nitrogen fixation efficiency [202,203]. Recent data suggested that legumes cannot discriminate between effective and ineffective strains prior to infection [204]. Instead, legumes limit the loss of resources by sanctioning individual nodules containing ineffective strains [205]. However ineffective strains may escape from plant sanctions by co-infecting nodules together with effective strains [202].

One way to overcome these competition issues is to select or create highly competitive strains [3]. Rhizobial symbiosis genes (*nod*, *nif*, and *fix*) are generally located on chromosomal mobile elements or on symbiosis plasmids [206]. Taking advantage of these features, it is possible to create hybrid strains without the insertion of exogenous DNA. For example, a hybrid strain of *S. meliloti* was recently created by moving the pSymA megaplasmid (accounting for nearly 20% of total genome content) from a donor *S. meliloti* strain to an acceptor strain [207]. Interesting, the resulting cis-hybrid strain seemed to exhibit a cultivar-specific improvement in symbiotic properties, compared to the parental strains, in controlled laboratory conditions [207]. Similarly, the transfer of symbiotic plasmids between different *R. leguminosarum* strains improved various measures of symbiotic efficiency in laboratory settings [208–211]. Therefore, genome-wide replicon-based remodeling of bacterial strains, potentially supported with a metabolic modelling framework [212], could be a powerful tool in precision agriculture by creating highly efficient strains depending of the farm/soil features [213]. This “Natural Genome Assisted Breeding” approach, based on the transfer of replicons among different strains, will also prevent the introduction of non-natural genes into the environment (Figure 2).

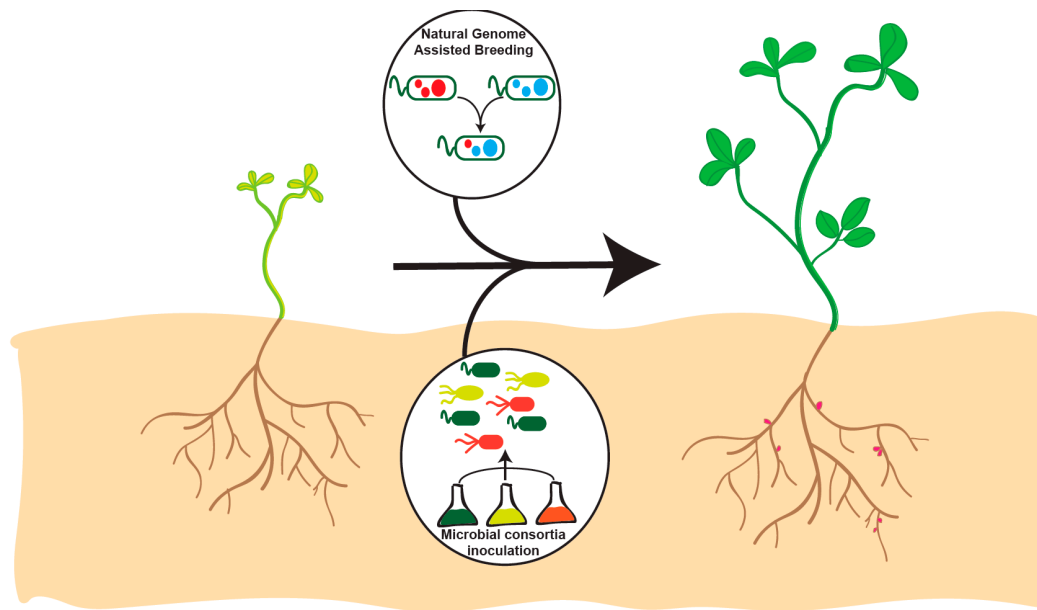


Figure 2. Different approach aimed to promote plant growth: Natural genome assisted breeding as a genomic manipulation strategy, and microbial consortia (multi-strains combination) for the improvement of plant-specific rhizobial inoculants in harsh environments.

One of the major issues related to the application of rhizobium inoculants is the inclusion of other additives (e.g., fungicides, nutrients [193], and fertilizers) that may reduce the viability or effectiveness of rhizobia. A recent trend to overcome this issue is to use microbial consortia instead of single strain biostimulants [214]. Consortia are formed by a combination of bacterial and/or fungal species to cover a broader spectrum of usage and soil conditions [214]. Microbes within a consortium are better able to handle biotic and abiotic stresses as they may work synergistically by exchanging nutrients and removing toxic compounds [215,216]. Using rhizobia in combination with other PGPR may improve their effect; for example, it has been reported that the combination of *Rhizobium* strains with *Bacillus* strains can improve root structure and increase nodule formation in bean, pigeon pea, and soybean (see [217] and references therein). Other well documented examples of mixed inoculants involving rhizobia and PGPR strains that led to improved symbiotic phenotype are: *Rhizobium* with *Bacillus subtilis* and *Bacillus megaterium*; *Rhizobium tropici* with *Chryseobacterium balustinum*, *Bacillus atrophaeus*, and *Burkholderia cepacia*; *Mesorhizobium* with *Pseudomonas*; *Mesorhizobium* in combination with *Azotobacter chroococcum*, *Pseudomonas aeruginosa*, and *Trichoderma harzianum*; and *S. meliloti* with a consortia of *Burkholderia* spp. (see [217] and references therein). Increased growth promotion could be due to a direct effect on nodulation, the production of phytohormones, or enhanced resistance to crop diseases [216,217]. Additionally, the synergistic interaction between arbuscular mycorrhiza and rhizobia for enhancing crop yield through improving nutrients uptake has also been heavily investigated [217]. Indeed, arbuscular mycorrhiza symbiosis can increase rhizobium nodulation of legumes under control [218] and saline conditions [219]. The consortium can also improve the uptake and transfer of nitrogen in a soybean/maize inter-cropping system [220].

Overall, the use of consortia composed of rhizobia and other PGPR combined with recent advances in rhizobium genomic manipulation could lead to increased inoculum efficiency in field conditions.

7. Concluding Remarks

In recent years, many studies were focused on the development and the optimization of technologies for the improvement of sustainable crop production, in particular in harsh (arid and/or metal-contaminated) environments. The studies were mainly spurred by an increasing requirement for plant proteins, which is due to the increasing worldwide human population and the need to reduce

the use of chemical fertilizers. The demand for increasing plant protein production and reducing the use of fertilizers can be accomplished, in part, through the cultivation of legumes; legumes are rich in protein and are able to improve soil fertility through BNF performed by their associated rhizobia. A huge number of studies conducted in the last thirty years have highlighted the ability of rhizobia to colonize particularly harsh soils, and to promote the growth of the leguminous plants to which they are associated. In this review, we summarized the most recent and detailed literature on plant growth promoting rhizobia isolated from (and thus, adapted to) arid and heavy metal contaminated soils, as well as their possible use as inoculants for legume-based agriculture in harsh soils. Despite the current knowledge on the topic, which ranges from genetic to molecular mechanisms, further research should be conducted on the feasibility of plant or soil specific rhizobia-based inoculations. Here, we proposed genetic manipulation strategies, which simulate natural evolution, and strain combination to optimize plant-specific rhizobial inoculants for the improvement of crop yield.

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