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WHEAT CULTIVAR INFLUENCE OVER NITROGEN-FIXING BACTERIAL COMMUNITIES IN AN ANDISOL AND A NOVEL PGPB TRACKING METHOD FOR *AZOSPIRILLUM* SPP. BASED ON CRISPR LOCI-TARGETED PCR

DOCTORAL THESIS IN FULFILLMENT OF THE REQUERIMENTS FOR THE DEGREE DOCTOR OF SCIENCES IN NATURAL RESOURCES

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"Wheat cultivar influence over Nitrogen-fixing bacterial communities in an Andisol and a novel PGPB tracking method for *Azospirillum* spp. based on CRISPR loci-targeted PCR"

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Summary and thesis outline

The applications of plant growth-promoting bacteria have been assessed since their first report in 1980. Such bacterial group presents diverse traits that promote the plant development in a direct or indirect manner. However, despite successful plant responses, their establishment is conditioned to a large number of externalities such as plant type, cultivar, plant-induced selection of rhizosphere taxa, nutrient availability, competition with indigenous microbial communities, water availability, among others. In that regard, PGPB products are part of a market that has grown exponentially since the 2000. However, after commercial applications farmers have not yet replaced chemical fertilization with these bioinputs, being most of the time combined with chemical fertilizers. In addition, most of these products are developed in base of a characteristic bacterial strain, and exported to other latitudes without proper colonization assessment. In Chile, the former is heavily misregulated. A considerable number of such preparations can be acquired and applied, however the effect of foraneous bacterial strains into Chilean soils has not pesquised by the governmental agencies in charge. Likewise, once bacteria are released into the environment, their colonization, persistence or ecological effect has been scarcely explored. The former is conditioned by a lack of methodologies developed to such endeavour. Either methods are laborious and expensive, or simple but not sensitive. This paradigm has directly conditioned the farmers to avoid the usage of this new fertilization type. On this thesis, we made a state-of-the-art revision in regards to PGPB tracking. We also explored the effect of different wheat cultivars on total bacterial and N_2-fixing bacterial communities in rhizosphere and root endosphere by culture and culture-independent approaches to assess the cultivar effect on free-living PGPB present in a potential target agroecosystem. Finally,

we used the CRISPR loci structure (repetitive conserved motives interspaced by non-conservative DNA) of a selected *Azospirillum* sp. as molecular marker for its tracking in rhizosphere and root endosphere on wheat plants. For this purpose, specific primers sets were designed, different endpoint PCR assays were standardized and performed to *Azospirillum* sp. B510 under different conditions to assess the tracking potential that CRISPR loci repeats presents. As a result, we successfully detected the presence of the *Azospirillum* strain in rhizosphere of wheat seedlings two weeks after inoculation. To our understanding, this is the first time that CRISPR loci has been described as a molecular marker for tracking of individual PGPB strains either in colonies, purified DNA, and inoculation studies.

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CHAPTER I

General Introduction

1.1 General Introduction

On the current climate change scenario, food security is considered among the top priorities around the globe (Fujimori et al. 2019). The former is directly related desertification and of culturable soils, where more food needs to be produced in smaller spaces (Moreno-Jimenez et al. 2019). In this sense, the traditional agricultural practices usually require large amounts of water, fertilizers and agrochemicals in order to sustain yields and economic profits. As a result, nutrients such as phosphorus (P), potassium (K) and nitrogen (N) are applied with surplus and due to externalities only a small amount is uptaken by plants, whereas the excess can be released to the atmosphere (in the case of N), or translocated water bodies (rivers, lakes and the oceans) inducing eutrophication (for N and P) (Svanbäck et al. 2019). To overcome this, new plant varieties as well as modernized techniques and farming methodologies must be developed to make food production more sustainable (Shan-e-Ali Zaidi et al, 2019).

The use of plant growth-promoting bacteria (or PGPB) has been proposed as potential alternative to chemical fertilzation since 1981 (Kloepper and Schroth 1981). Such bacterial group is present in almost every plant-colonized biome. Among the most common genera, it is possible to find representatives of *Actinobacteria, Azotobacter, Azospirillum*, *Bacillus*, *Burkholderia*, *Enterobacter*, *Microbacterium, Pseudomonas*, *Rhizobium*, *Serratia*, and others (Backer et al. 2018). Different direct or indirect growth-promotion traits can be exerted by PGPB representatives such as biological N_2-fixation (BNF), solubilization of organic P forms, phytohormones production (e.g.: indole-acetic acid, abscicic acid), biocontrol of pathogens, siderophores secretion, endophyte colonization, abiotic stress alleviation, antifreeze activity, and others (de Souza et al. 2015). Experiments conducted

with PGPB have proven to improve plant performance of several commercial crops, ranging from cereals and grasses, to fruits and vegetables (Pereg et al. 2016). However, as yield is a critical factor for farmers to make decisions, farm applications of such bacteria are combined with different rates of chemical fertilization. Thus, the ecological implicances of that can induce unbalanced yields that do not enable farmers to do a complete transition to PGPB application.

Among the commercial PGPB products, the *Azospirillum* genus is one the most studied, formulated, and applied. This derives from the cosmopolitan presence of different strains in more than 130 plants, their ability to fix N_2 as free-living bacteria, the presence of a signalling flagellum, and for presenting almost every other trait stated above (Pereg et al. 2015). Combinations of *Azospirillum* spp. and N fertilizers shows different responses when combined with chemical fertilization. Wheat biomass, grain yield and protein content has been increased on such combinations (Saubidet et al. 2002; El-Sirafy et al. 2006). However, negative correlations have also been observed between N doses and *Azospirillum* CFU g^{-1} rhizosphere, where treatments with over 40 kg N ha^{-1} affected its establishment (Alamri and Mostafa 2009). Higher amounts (150 – 200 kg N ha^{-1}) has overcome *Azospirillum* growth promotion effect in wheat, being most of the effect attributed to the chemical fertilizer (Namvar and Khandan 2013). The general consensus around this trend is commonly targeted towards competition. Chemical fertilization induces changes on soil bacterial communities and therefore, could affect PGPB establishment and efficiency. Others have considered that the introduction of PGPB is more effective in relatively N poor soils, due to the slow indigenous microflora activity, and low competition rates, hence, increasing colonization chances (Strigul and Kravchenko 2006). On the plant side, traits associated

with N acquisition regulate rhizosphere competition and therefore, its composition (Blagodatskaya et al. 2014). This can be explained by a reduced production of N_2-fixation related enzymes. The suppression of two bacterial N-acquisition enzymes in grass plants rhizosphere under common field N dosage has been reported (Zhu et al 2015). These uncertainties have kept the scientific community on hold in regards to a proper strategy on how free-living N_2-fixing PGPB must be applied at field scale.

Crop types, soil history and cultivars influence also represents a feasible part of the successful PGPB applications. This has been evident over the rhizosphere bacterial communities in potato (Inceoglu et al. 2012), cotton (Wei et al. 2019), and wheat (Mahoney et al. 2017). By default, management variations can modify the effectiveness of the PGPB strains on the target plants. In regards to *Azospirillum*, experiments conducted with *Azospirillum* sp. Sp7 revealed that plant responses will vary under the same growth protocol in four different barley cultivars (Lade et al. 2018), as well as in six wheat cultivars (Dehghani et al. 2020). Therefore, the compatibility between the target cultivar and the prospect PGPB strains must be assessed beforehand. Until now, few experiments have been performed on Chilean ash volcanic soils regarding the use of PGPB, assessing the target plants that will be inoculated.

Since the beginning of PGPB research, understanding their behavior and which plant niches are colonized once released to the environment has been subject of consideration (Bonaldi et al., 2015; De Weger et al., 1991). Most of the commercial PGPB products are formulated from already well characterized strains and in this sense, PGPB strains isolated from biomes or plants different than the target one could not perform as expected. The latter, has driven the discussion to consider the use of local PGPB strains to

assure compatibility between biome – target plants – and PGPB. Therefore, to successfully overcome the cultivar compatibility, N-availability influence and the external factors mentioned above, the tracking and monitoring of the PGPB is crucial for improve its performance under all specific and complex field conditions. Among the early proposals to assess this, Rattray et al. (1995) mentioned that non-invasive methodologies should be applied.

More recently, different research groups have developed modern methodologies that could assess the PGPB colonization. Efforts have been put in develop transparent soil-like matrixes, (Downie et al. 2012; 2014), gnotobiotic microfluidic devices (Massalha et al. 2017) or magnetic resonance analysis to plant roots (van Dusschloten et al. 2016). Others have tracked PGPB with Quartz Crystal Microbalance devices (QCMs) (Agrawal et al. 2012). Next generation sequencing (NGS) have also been proposed, but not recomended for PGPB tracking this far, once price, time, data analysis and sample preparation are evaluated. Nonetheless, farmers will always require the application of methods that relies on simplicity, easy application and inexpensiveness. The lack of methods that serves these criteria has conditioned both the scientific community and farmers to not being able of PGPB tracking. To overcome this, a combination of culture-dependent and independent methods can provide a better insight.

On a large proportion of bacteria and archaea genomes it has been described the presence of Clustered Regularly Interspaced Palindromic Repeats (or CRISPRs). The CRISPRs loci has been described as short repetitive sequences (~20 - 38 bp) interspaced by unique extra-genomic elements obtained from either virus or plasmids of similar size. The latter, is associated to a sort of primitive immune system against DNA infections

(Barrangou and Dudley 2016). The CRISPRs have been previously analyzed and used for strain typing of animal *Mycobacterium tuberculosis* (Shariat and Dudley, 2014), *Salmonella enterica* (Bachmann et al. 2014) and plant *Erwinia amylovora* pathogens (McGhee and Sundin 2012). Examples of the same approach are not existent to PGPB strains. Similar to CRISPRs, Sequence Characterized Amplified Regions (or SCARs) and Repetitive elements PCR (REP-PCR) have been successfully used for PGPB tracking purposes up until recently (de Melo Pereira et al. 2012; Reddypriya et al. 2019). Similarly, CRISPRs genetic structure can serve for this same purpose.

In Chile, the situation described in this chapter needs to be considered for future relevance. Food production represents a large amount of the Chilean gross domestic product (GDP), whereas climate change has extended the desertification from northern to southern Chilean soils endangering a market of around eighteen billion dollars (Resource Trade Earth, Chatham House, 2018). Most of the nation food production is concentrated between central to southern Chile, being the largest N consumer area of the country (ODEPA 2018). The desertification will bring serious consecuences to Chilean economic activity, as well as its food safety (CONAF 2016). To ameliorate those effects, novel agricultural local strategies must be studied, tested and implemented. Among those, the already mentioned PGPB usage is considered. However, a large number of biofertilizers in Chile are mainly imported and their application is being not properly monitored by government authorities. Examples of both are the laxative authorization lists of Agricultural and Livestock Service (SAG 2018), as well as the regulations established by congress (BCN 2018). To overcome such regulation issues, proper knowledge of the plant influence

over microbial communities behavior on local agroecosystems is required, as well as methods that allow the final users to properly track the PGPB once introduced to fields.

1.2 Hypotheses

Considering the lack of studies regarding total and N_2-fixing bacterial communities in Chilean andisols, the potential influence of those local cultivars on bacterial communities that will serve as new environment for inoculants, the similarities between the CRISPRs and SCAR and REP markers, and the requirements for novel tracking methods developed for implementation with unlaborious requisites we hypothesize that:

- ***Different cultivars modify the total and N_2-fixing bacterial communities on wheat plants grown in an andisol in rhizosphere and endosphere niches.***
- ***The CRISPRs loci can serve as template for tracking of a model N_2-fixing Azospirillum sp. strain in rhizosphere and endosphere of wheat plants.***

1.3 General objective:

- To develop a model PGPB *Azospirillum* spp. tracking method based on PCR with CRISPR loci repeat sites as template.

1.4 Specific objectives:

- To explore and identify differences exerted by different cultivars in total and N_2-fixing bacterial communities in wheat plants grown in andisol.
- To design and standardize a new CRISPR-PCR assay for the tracking of a model *Azospirillum* spp. strain.
- To validate a new CRISPR-PCR tracking assay for an *Azospirillum* spp. model strain in wheat plants rhizosphere and endosphere.

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CHAPTER II

Current opinion and perspectives on the methods for tracking and monitoring plant growth–promoting bacteria

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Current opinion and perspectives on the methods for tracking and monitoring plant growth–promoting bacteria

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Abstract

Since the 1980s, plant growth–promoting bacteria (PGPB) have been studied as a sustainable alternative to the use of chemical fertilizers to increase crop yields, and effective PGPB have been isolated from diverse plant (*e.g.*, endosphere and phyllosphere) and soil (*e.g.*, rhizosphere) compartments. Despite the promising plant growth promotion results commonly observed under laboratory and greenhouse conditions, the successful application of PGPB under field conditions has been limited, partly by the lack of knowledge of the ecological/environmental factors affecting the colonization, prevalence and activity of beneficial bacteria on crops. It is generally accepted that the effectiveness of PGPB depends on their ability to colonize a niche and compete with the indigenous plant microbiome under agronomic conditions. However, most studies do not include tracking or monitoring of PGPB in the environment after their application, and the beneficial effects on plants are measured by determining biomass– and physiology–related parameters without confirming bacterial colonization. To date, methods based on reporter genes, immunological reactions and nucleic acids have been applied to track or monitor PGPB in seeds, soils or *in planta* after inoculation. In this review, we describe, compare and discuss the methods used for tracking and monitoring PGPB, including challenges and perspectives on some novel methods.

Keywords: biofertilizer; bioproducts; inoculants; monitoring; plant growth–promoting bacteria; tracking.

2.1. Introduction

Plants are closely associated with an enormous variety of bacterial taxa that play a crucial role in their growth, tolerance and disease prevention. Thus, numerous beneficial bacterial strains, defined as plant growth–promoting bacteria (PGPB) by Kloepper and Schroth (1981), have been isolated from the rhizospheres (soil influenced by plant roots), endospheres (inner tissues of plants) and phyllospheres (aerial surfaces of plants, including leaves) of a great variety of plant species (Brimecombe et al., 2007). The promising results of PGPB at the laboratory scale have stimulated their biotechnological use as bioactive components in diverse bioproduct formulations (biofertilizers or inoculants) that have been commercialized to improve the health and yield of crops and pastures. However, the success of PGPB technology has been challenged by the lack of consistent responses under agronomic conditions, owing to different soils, crops and climatic conditions (Bashan et al., 2014). It is widely accepted that the effectiveness and prevalence of PGPB under field conditions depend on their versatility and adaptation to environmental changes as well as their ability to colonize and compete with other members of the indigenous plant microbiome (*e.g.*, bacteria, fungi, archaea, protozoa, etc.) (Thijs et al., 2016). At the field scale, the PGPB must also overcome or adapt to diverse factors such as plant characteristics, climate, soil composition and soil management, among others (Fageria and Stone 2006; Rattray et al., 1995). Therefore, the tracking and monitoring of the PGPB used for plant, soil or seed inoculation is crucial for determining the factors influencing their efficient behavior under changing and complex field conditions.

The tracking of PGPB released in the environment has been studied for at least 25 years (Ahmad et al., 2011; Brandt and Kluepfel 1991; Gamalero et al., 2003; Glick, 2015;

Kloepper and Beauchamp 1992; Podile and Kishore 2006; Stahl and Kane 1992), but despite the extensive research, especially during the last ten years, less than 25% of published articles have considered the use of tracking methods, according to indexed articles in the Thompson-Reuters Web of Science database (www.webofknowledge.com) (Fig. 1). The low number of studies using tracking methods is mainly due to the limited availability of an effective, cheap and rapid method to localize, count and evaluate the activity of inoculated PGPB. For this reason, the effect of such microorganisms on plants has generally been evaluated by measuring plant biomass (length, dry weight, stem diameter, leaf number, etc.) and plant physiological parameters (chlorophyll content, superoxide dismutase [SOD], thiobarbituric acid reactive substances [TBARS], osmolyte content, etc.) without an unequivocal confirmation of the capacity of bacteria to colonize the soil or plant. In this context, Ivnitski et al. (1999) suggested monitoring inoculated bacteria in the environment by detecting at least one cell and discriminating between viable and non–viable cells, using minimal amounts of reagents with an automatized method to identify the inoculated strain among native species. In addition, Rattray et al. (1995) suggested the need to develop non–destructive methods for the *in planta* study of PGPB. Finally, Stahl and Kane (1992) pointed out that the tracking and monitoring of bacteria are overlapping concepts, so their differences must be clearly established during their application. Tracking refers to phenotypic or genotypic detection, quantification and localization of inoculated PGPB strains, whereas monitoring includes tracking as well as determining the physiological activity of the inoculated PGPB strains, such as N–fixation, P–solubilization, and phytohormone production, among others. In general, the methods for tracking and monitoring PGPB are usually grouped into three main categories: i) reporter genes-based methods, ii) immunological methods, and iii) nucleic acid-based methods.

However, independent of the procedure, the selection of an appropriate method should consider its relative environmental safety, reproducibility, sensitivity, costs, and time consumption, among other aspects.

The main focus of this review is to discuss the state of the art of current methods for tracking and monitoring PGPB, pointing out the novel strategies developed for this purpose, their advantages, disadvantages and their particular perspectives.

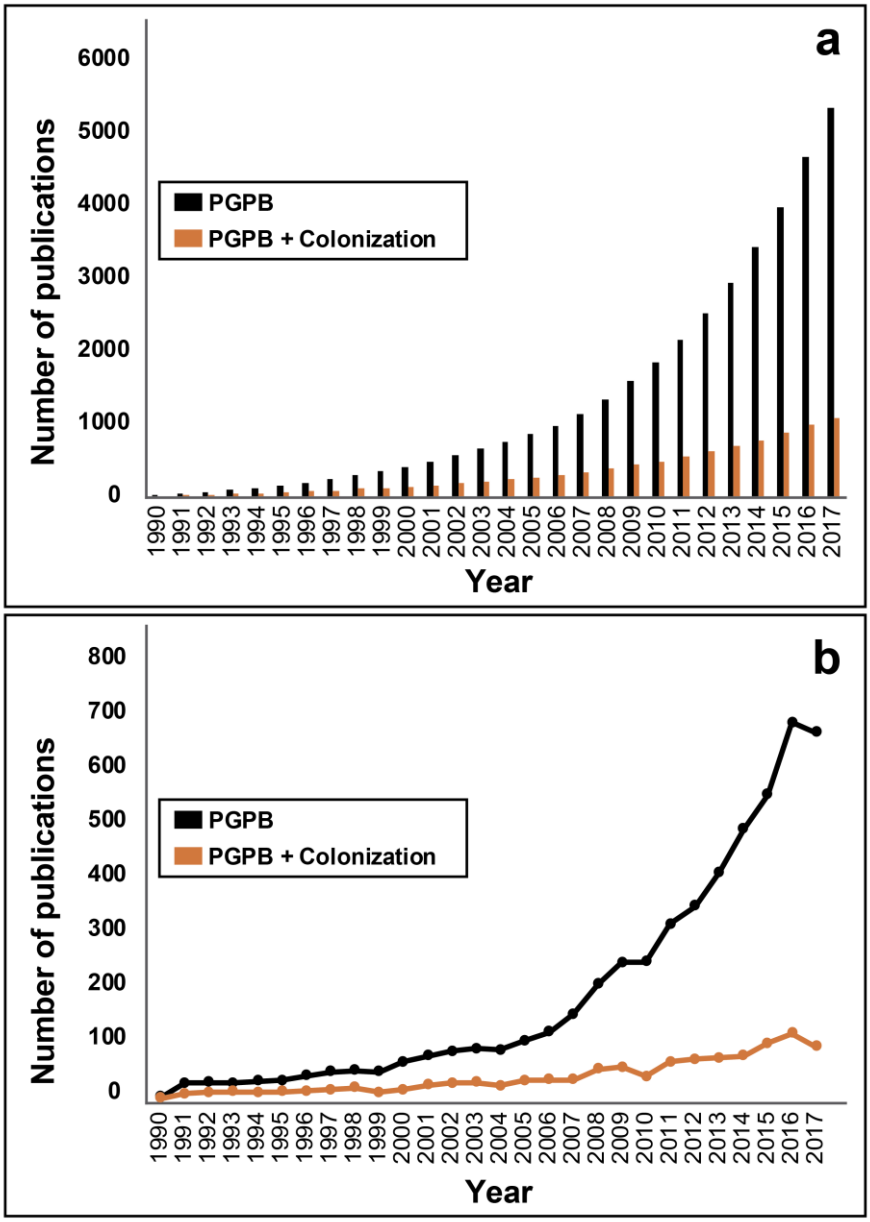

Figure 1. Accumulative (a) and non–accumulative (b) number of studies on plant growth–promoting bacteria including those with monitoring methods between 1990-2017. Data were extracted from the Thompson-Reuters Web of Knowledge (https://www.webofknowledge.com) using the following keywords: "*Monitoring, Tracking, Colonization, Plant growth-promoting bacteria, Plant growth-promoting rhizobacteria, PGPB* and *PGPR*". Analysis also included studies that provided information about inoculum persistence as evaluated in soil or in soil-plant systems using different techniques (such as reporter genes, immuno-associated techniques or nucleic acid-based methodologies). All searches were performed under default basic search parameters (SCI-EXPANDED 1975-Present).

2.2. Factors affecting plant growth–promoting bacteria colonization

Agro–ecosystems are complex environments with a high diversity of indigenous microorganisms, where the activity, abundance and composition of bacterial populations are modulated by diverse biotic and abiotic factors, such as plant characteristics (genotype, development stages, defense mechanisms, etc.), seed properties (hydrophobicity, the presence of chemical compounds, etc.), climate factors (temperature, UV irradiation, precipitation, dehydration, etc.), soil management (fertilization, rotation, tillage, etc.), soil properties (pH, organic matter, nutrients, texture, etc.), presence of grazers (nematodes, protozoa, etc.) and other members of microbial communities (archaea, fungi and viruses) (Fig. 2a). However, the specific factors affecting the colonization and survival of inoculated PGPB are poorly understood (Kragelund et al., 1997). According to Burns et al. (2015), the main factors influencing PGPB colonization are (in order of relevance) plant species, root exudates, soil chemical properties and spatial location. With respect to plant species, Singh et al. (2009) suggested that plant genotype can significantly affect colonization, so the interactions, including effect type (beneficial, neutral or harmful), between the inoculated microorganism and the target cultivar should be known for the plant to be efficiently

colonized by PGPB. During different phenological stages, plants can also select and modify the composition of associated or symbiotic bacterial communities, as occurs with the *Rhizobium* genus and the nodulation process (Depret and Laguerre 2008).

In the rhizosphere, which is the most-studied soil compartment with a higher abundance and activity of organisms compared to the bulk soil, the presence, prevalence and activity of bacteria is mainly attributed to their versatility in their use of rhizodepositions as nutrients and their ability to compete for niches with other members of microbial communities (Fig. 2b) (Brimecombe et al., 2007). It is well accepted that the presence of organic compounds in the rhizodepositions (including root exudates) affect the composition and activity of bacterial communities inhabiting the rhizosphere soil (Dennis et al., 2010). For example, Strigul and Kravchenko (2006) suggested that the survival and colonization of PGPB depend on their ability to consume organic acids, sugars, amino acids, cell debris, and other compounds in rhizodepositions. Thus, when PGPB cannot directly use some of these compounds as nutrients, their metabolic versatility, including co–metabolism, can be crucial for their survival and rhizosphere colonization. However, the competition between PGPB and indigenous rhizosphere bacteria is also very important for the success of bacterial inoculation (Canellas et al., 2015). For example, the colonization of the PGPB *Pseudomonas putida* W619 was lower in unsterile than sterile soils in hybrid poplar rhizospheres (Weyens et al., 2012). The success of the bacterial colonization can also depend on the presence of specific microorganisms in the rhizosphere. The presence of *Piroformospora indica*, a symbiotic fungus, in the rhizosphere increased the endophytic colonization rates of *Pseudomonas striata* in 4 of 6 maize cultivars (Singh et al., 2009).

Soil pH may be another relevant factor affecting the colonization, abundance and prevalence of PGPB in the rhizosphere, even if PGPB are commonly isolated from different

soils to which they are applied. Indeed, variations of more than 1.7 units below or above the optimum pH reduced the growth of bacterial communities in vineyard soils by 50% (Fernández–Calviño and Bååth 2010), whereas the colonization of the PGPB *P. fluorescens* strain CS85 on roots of cotton plants was unaffected between pH 5.2 and 8.5 (Wang et al., 2004). However, it is noteworthy that studies of PGPB under different soil pH values are mostly focused on evaluating the plant growth promotion effects than the effect of pH on PGPB colonization.

Soil management, such as fertilization and tillage, can also affect the activity, biomass and composition of bacterial communities and the colonization capacity of PGPB (Chen et al., 2016; Luo et al., 2017; Yang et al., 2017). Thus, beneficial *Pantoea agglomerans* and *Azotobacter chroococcum* strains showed higher rhizosphere colonization when phosphorus (P) and nitrogen (N) were added to soils (Narula et al., 2007). Vande Broek et al. (1993) observed a preference for lateral root colonization by *Azospirillum brasilense* strain Sp245 in the rhizosphere of wheat plants under N fertilization. Recently, a negative correlation was obtained between the colonization capacity of *Azospirillum* sp. and N-addition rates (Alamri and Mostafa 2009; Ramirez et al., 2012) that is probably explained by the Strigul and Kravchenko (2006) model, by which plant colonization by PGPB is more probable in nutrient–poor than nutrient–rich soils. In relation to tillage, which causes rapid changes in the composition of microbial communities (Bissett et al., 2013), PGPB colonization could be hampered once the microbial community stabilizes. In addition, the type of soil management combined with the inoculation method (*e.g.*, seed coating, liquid application in furrow or seeds, spray drying, etc.) may be relevant to the PGPB colonization capacity (Afzal et al., 2012). Sirohi et al. (2015) reported differences in bacterial survival of approximately 17% between a carrier matrix–based inoculation and

direct application on wheat plants. Similarly, Barra et al. (2016) observed differences in seedling emergence rates (67.5~92.5% versus 42.5~90%) when direct and seed coating with lyophilized bacteria inoculation methods were compared, but both inoculation treatments increased plant biomass compared with uninoculated controls.

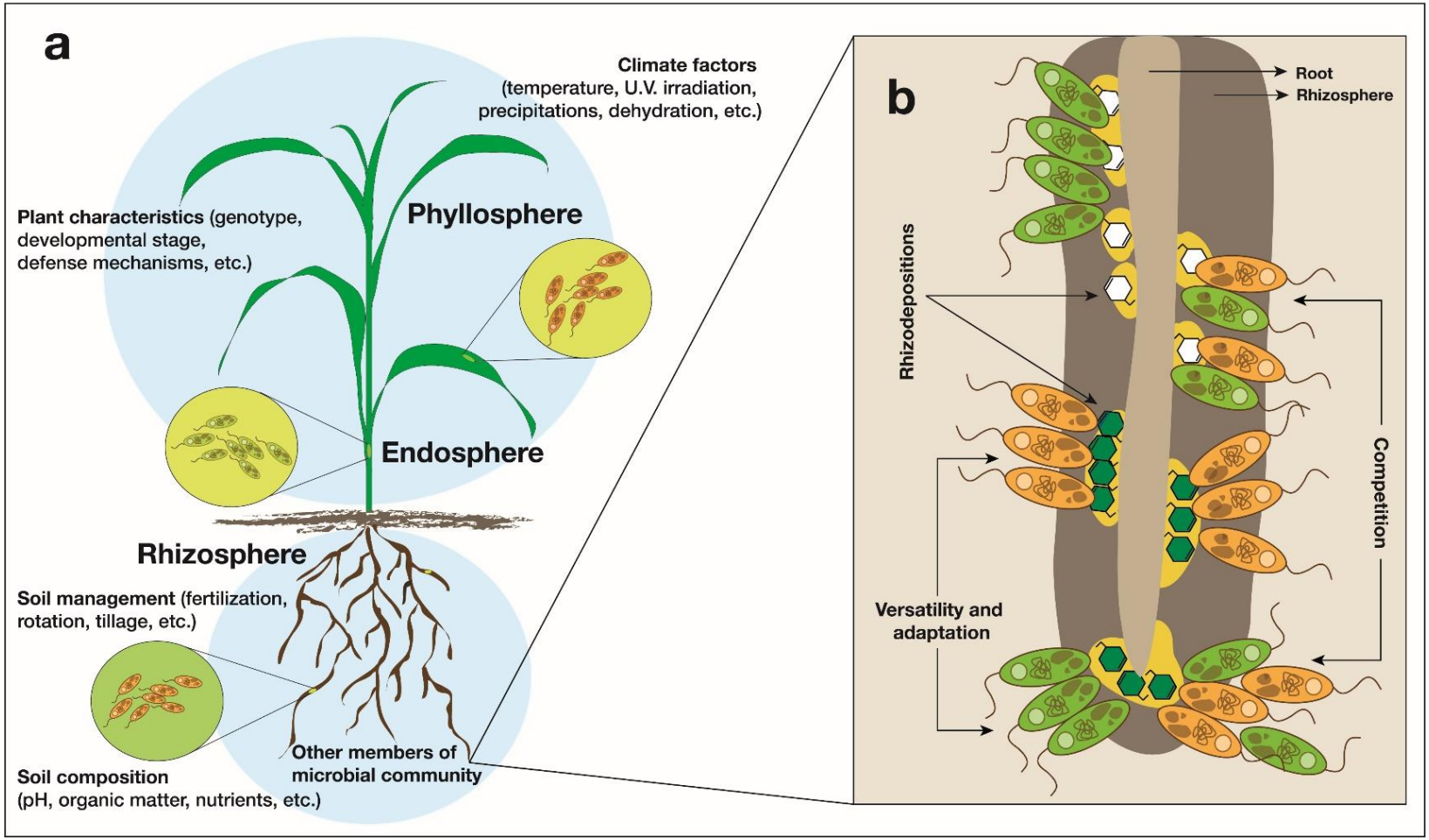

Figure 2. (**a**) Factors affecting the colonization of plant growth–promoting bacteria in the environment. (**b**) Niche–specific bacterial interactions in the plant rhizosphere.

2.3. Methods for tracking and monitoring plant growth–promoting bacteria

Inoculated bacteria in the environment have been tracked in studies of bioremediation (Elväng et al., 2001), plant growth promotion (Glick, 2015) and human safety (Ivnitski et al., 1999). Since early PGPB inoculation studies, authors have stated that knowledge of bacterial behavior *in situ* is highly required (Bonaldi et al., 2015; De Weger et al., 1991; Ramos et al., 2002). The estimated abundance of inoculated PGPB in roots and their activity on plants as well as the inoculation schedule are important parameters to be considered to maximize crop yields (Zhao et al., 2011). Rattray et al. (1995) suggested the use of non–extractable methods for PGPB tracking. Until now, PGPB tracking methods have included the use of reporter gene-based methods, immunological methods and nucleic acid-based methods (Solanki and Garg 2014). The principle and detection limits as well as the advantages and disadvantages of these methods are summarized in Table 1 and Fig. 3.

2.3.1. Reporter gene-based methods

The reporter gene (or simply reporter) is a gene attached to a regulatory sequence of a target gene that can be used to detect the presence and/or expression of the target. Commonly, the reporter genes are visually identified by color, luminescence or fluorescence, but diverse issues must be evaluated before they can be used for PGPB tracking. Gamalero et al. (2003) suggested that reporter genes should be specific, stable and previously tested on target bacteria in the environment, and the same authors consider that stability is essential to assure marker persistence and to prevent horizontal gene transfer from the inoculated PGPB to indigenous bacteria. In addition, reporter gene insertion should not affect plant growth–promoting (PGP) traits (Viazovaia et al., 2006), and the

release of genetically modified (including reporter gene–tagged) bacteria into the environment should avoid any biosafety risks (Glick 2015). As an example, De Weger et al. (1991) reported that approximately 80% of *lux*–tagged *Pseudomonas fluorescens* WCS374 lost the inserted reporter plasmid in wheat rhizosphere. Thus, methods based on reporter genes should only be used under controlled experimental controlled conditions (*e.g.*, laboratory or greenhouse experiments) and not for tracking in the field.

Despite this, the use of some reporter genes in PGPB are discussed below.

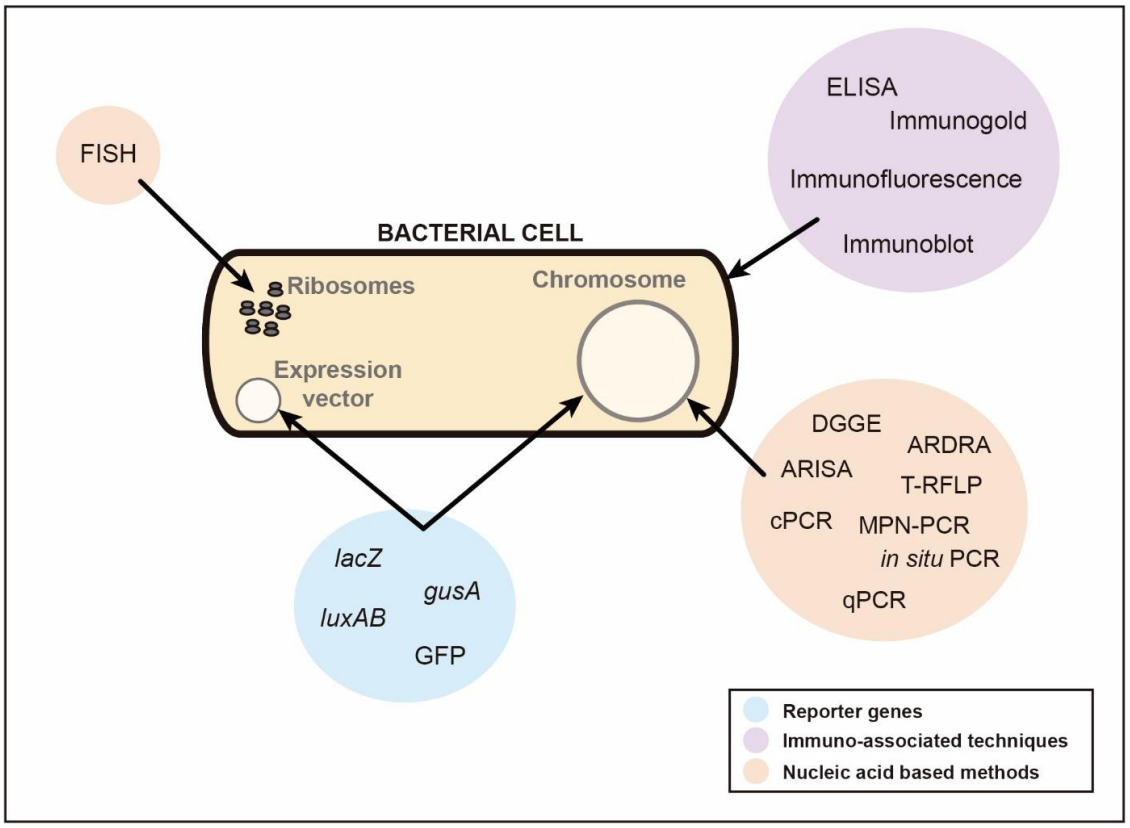

Figure 3. Methods commonly used for tracking and monitoring plant growth–promoting bacteria (also see Table 1).

2.3.1.1. β–galactosidase (lacZ)

The operon *lacZ* (derived from *Escherichia coli*) codifies a β–D–galactosidase (β–gal) that

cleaves lactose in presence of X–gal (5–bromo–4–chloro–3–indolyl–β–D–galactopyronaside), and the products of this reaction are galactose and 5,5'–dibromo–4,4'–dichloro–indigo, which is an insoluble blue compound. Thus, *lacZ*–tagged bacterial cells are visualized as blue colonies on agar plates when they are grown in media containing X–gal as a substrate and IPTG (Isopropyl β–D–1–thiogalactopyranoside) as a β–gal inducer (Sambrook and Russell, 2001).

Diverse studies have used *lacZ* as a reporter for PGPB tracking in the rhizosphere. Mourya and Jauhri (2002) monitored the colonization of P–solubilizing *Pseudomonas striata* str. P–27 in soybean rhizosphere and observed a decrease from 10^8 to 10^4 CFU g^{-1} soil after four months of inoculation. An increase of approximately 40% in the colonization of *Pseudomonas* sp. P–36 after 60 days was observed under different P-fertilization rates and manure fertilization (Sunita et al., 2010). In contrast, Fischer et al. (2000) reported a decrease in *A. brasilense* Cd from 1.25 to 0.77×10^6 CFU g^{-1} in the rhizoplane of wheat plants after two weeks under conditions of saline stress. The tracking of *Azotobacter chroococcum* Mac27 gave an estimated abundance of 10^5 CFU g^{-1} soil under field conditions after 60 days, and a higher colonization of *P. fluorescens* Psd was observed when inoculated with a talc–based carrier formulation (Sirohi et al., 2015).

Burn et al. (1987) observed a positive correlation between the expression of nodulation genes (*nodA,B,C*) and the response of the *lacZ* gene in *Rhizobium leguminosarum* 8401 exposed to root exudates of peas. Similarly, Göttfert et al. (1988) combined *nodA* and *lacZ* genes and observed a positive correlation between the production of a specific isoflavone by soybean and the production of *nod* factors by *Bradyrhizobium japonicum* I110. The expression of N–fixing genes in *B. japonicum* I110, monitored using *lacZ* as a reporter under different N concentrations, revealed a 2:1 expression ratio between

nifH and *nifD* genes (Yun et al., 1986). The use of *lacZ* reporter revealed that the alkaline phosphomonoesterase (commonly known as alkaline phosphatase) (*phoD*) gene was expressed by phosphate starvation in the soil bacterium *Bacillus subtilis* JH646M5 (Eder et al., 1996).

Endophytic bacterial populations have also been monitored by *lacZ* gene reporter, but the results can be affected by artifacts due to the indigenous β–gal activity of plant roots. However, Arsene et al. (1994) revealed no β–gal activity in roots by microscopy using 2% glutaraldehyde for tissue fixation. This modified method allowed the abundances of *A. brasilense* Sp7 and *A. brasilense* 7067 to be monitored in wheat plants, obtaining colonization values of 7×10^7 and 3×10^7 CFU g^{-1} root after 10 days, respectively. However, despite glutaraldehyde treatment, three endophytic *lacZ*–tagged bacteria (*Azospirillum. lipoferum*, *B. japonicum* and *Rhizobium melilotii*) were undetected by microscopy due to the root β–gal activity (Akao et al., 1999).

It is noteworthy that most of the above studies did not consider the occurrence of false positives produced by indigenous and endogenous β–gal activity of bacteria in the soil, rhizosphere or endosphere (Gamalero et al., 2003). Therefore, the use of *lacZ* gene as a reporter should only be recommended for tracking and monitoring PGPB under controlled gnotobiotic conditions.

2.3.1.2. β–glucoranidase (gusA)

Similar to *lacZ* and β–D–galactosidase, β–D–glucoranidase (β–glu), which is codified by *gusA* from *E. coli*, forms a blue insoluble compound in the presence of 5–bromo–4–chloro–3–indolyl–β–D–glucopyronaside (X–glu) in an induced or constitutive way (Vande Broek et al., 1993; Wilson et al., 1995). The major advantage in comparison

with *lacZ* is its low background activity in either plant tissue or indigenous soil bacteria (Akao et al., 1999; Compant et al., 2005; Ramos et al., 2002).

In the last 10 years, several studies have used *gusA* as a gene reporter to track PGPB strains inoculated on plants. Villegas and Paterno (2008) reported the incremental increase in the abundance of *gusA*-tagged *P. fluorescens* LV7 and *P. fluorescens* LV10 with values from 10^6 to 10^8 CFU g^{-1} fresh weight of roots between 14 and 30 days after inoculation. However, Huang et al. (2017) discussed the incorrect results obtained by evaluating the plant response to inoculation with PGPB considering fresh instead of dry weight data. Sathyapriya et al. (2012) reported a similar increase (from 10^5 to 10^8 CFU g^{-1} fresh weight of roots after 7 days) in *P. aeruginosa* UPMP3 in oil palm roots. Using the *gusA* gene as a reporter, the colonization of tomato roots by *A. brasilense* Cd, *Herbaspirillum seropedicae* Z67, *Burkholderia ambifara* PHP7 and *Gluconacetobacter diazotrophicus* Pal5 revealed populations of 10^7, 10^5, 10^7 and 10^8 CFU g^{-1} dry weight of roots, respectively, after 30 days of inoculation (Botta et al., 2013). Recently, Batista et al. (2015) monitored the colonization, nodulation and growth promotion of *gusA*–tagged strains of *R. leguminosarum* bv. *trifolii*, U204 (a commercial strain) and *R. leguminosarum* bv. *trifolii* 317 (a native strain isolated from red clover plants) in red clover (*Trifolium pretense* L.). After six months, growth promotion (2–fold) and nodulation rates were higher with inoculation with the native strain (317) than with the commercial strain (U204). Marek–Kozaczuk et al. (2014) determined the colonization rates of twelve *Ensifer melilotii* strains in alfalfa through *gusA* tagging. Three strains efficiently competed against indigenous rhizobia species, but indigenous rhizobia were dominant over the inoculated PGPB strains. Bernabeu et al. (2015) also used light microscopy to determine the presence of *gusA*–tagged *Burkholderia. tropica* ATCC BAA–569 in gnotobiotic and non–gnotobiotic

tomato plants. Under non–gnotobiotic conditions, the plate counts revealed 10^7 and 10^4 CFU g^{-1} FW for the rhizosphere and endosphere, respectively.

Similar to that published for the *lacZ* reporter, most studies focused on the use of *gusA* as a reporter did not consider the occurrence of false positives produced by the β–glu activity of indigenous bacteria and plant roots (Akao et al., 1999).

2.3.1.3. Bacterial luciferase (lux)

Bioluminescence is another strategy for tracking bacteria, and the related methods are based on the use of the *Vibrio fischeri* luciferase gene (*lux*), which requires the presence of oxygen and a fatty aldehyde to produce bioluminescence (Prosser et al., 1996). Bacteria are usually *lux*–tagged through chromosomal transformation with *luxA* and *luxB* genes using transposon delivery vectors (Gamalero et al., 2003), and the detection and semi–quantification of *lux*–tagged bacteria in the rhizosphere could be achieved through luminometry. This reporter gene is considered to be 1000–fold more sensitive than *lacZ* (De Weger et al., 1991; De Weger et al., 1997; Kragelund et al., 1997). In addition, bioluminescence methods are less time consuming and laborious than the previously mentioned plate-counting methods, so bioluminescence is suggested as an adequate method for monitoring inoculated bacteria in root colonization studies (Beauchamp et al., 1993).

First, Shaw and Kado (1986) used this method by monitoring the colonization of lux–tagged phytopathogen bacteria belonging to the genera *Xanthomonas*, *Agrobacterium*, *Rhizobium*, *Erwinia* and *Pseudomonas* in nine plants species (cauliflower, potato, carrot, pearl, soybean, rice, kiwi, blackberry and alfalfa). De Weger et al. (1991) compared the induced and constitutive expression of *lux* in *P. fluorescens* 5RL and *P. putida* LT2–139 inoculated on soybean roots, and the induced expression was 10^5 CFU cm^{-1} root, whereas

constitutive expression appeared to be metabolism dependent, requiring the addition of *n*–decyl aldehyde to detect 10^5 CFU cm^{-1} root, as observed by Malboobi et al. (2009). This substrate dependency was attributed to the inability of rhizosphere bacteria to produce aldehyde, which is necessary in the bioluminescence reaction. In contrast, Kozdrój (1996) pointed out that this limitation could be overcome by introducing the complete *lux* operon (*lux* A, B, C, D, E genes) in the target strain instead of just *luxAB*. Indeed, the detection of *lux*–tagged *E. cloacae* (pQF70/44) at single cell level in wheat roots was obtained by the insertion of more than one *luxAB* copy, thus increasing light emission (Rattray et al., 1995). Further experiments revealed that *in situ* luminometric *lux* quantification required separation of roots to enable the observation of colonization patterns in wheat (De Weger et al., 1997). In the same study, the tracking of *lux*–tagged *P. fluorescens* 5RL was not affected by nutrient–deficient conditions, and the same was also observed by Kragelund et al. (1997) in both gnotobiotic and natural barley rhizospheres inoculated with *lux*–tagged *P. fluorescens* DF57.

In terms of persistence, no differences were found in PGPB colonization of two contrasting soils (silt loam soil with a pH of 5.2 and sandy loam soil with a pH of 8.5), with values of 10^7 and 10^5 CFU of *lux*–tagged *P. fluorescens* KT2440 g^{-1} soil in bean and maize rhizospheres, respectively, 16 weeks after the inoculation (Molina et al., 2000). Malboobi et al. (2009) observed values of 10^7 CFU g^{-1} soil of each strain at distances of 2 and 5 cm from the roots when three *lux*–tagged P–solubilizing PGPB (*P. putida* P7, *Microbacterium laevaniformans* P13, and *Pantoea agglomerans* P5) were inoculated in potato rhizosphere under greenhouse conditions.

Studies have also described the difficulty in detecting *lux*–tagged PGPB in the endosphere due to the intense background generated by the tagged bacteria colonizing

epiphytic or rhizosphere areas (Rattray et al., 1995). Therefore, the combination of *luxAB* with *gusA* genes appear to be an attractive strategy to improve the tracking of rhizosphere or/and endorhiza-colonizing PGPB. Wang et al. (2004) studied the colonization, persistence and distribution of PGPB *P. fluorescens* CS85 tagged with both genes separately on native cotton roots, and their results showed the same colonization pattern and similar CFU number (10^6 CFU g^{-1} soil) during the 35-day assay.

Although the *lux* gene has advantages in PGPB tracking when compared with other reporter genes (*e.g.*, inexpensive substrate requirements and absence of exogenous or indigenous background), it has disadvantages such as metabolism dependency and challenges related to the physical manipulation of the samples.

2.3.1.4. Green fluorescent protein (GFP)

Chalfie (1995) first described green fluorescent protein (GFP) as a reporter gene. Isolated from the jellyfish *Aequorea victoria*, this protein emits fluorescent light when it is irradiated with nearly blue to UV light (Fig. 3d). Detection and quantification of GFP–tagged strains are performed by epifluorescence microscopy (Leff and Leff 1996), confocal laser scanning microscopy (CLSM) (Fan et al., 2011; Götz et al., 2006; Krzyzanowska et al., 2012), flow cytometry (Elväng et al. 2001), and with agar plates under UV exposition (Errampalli et al., 1999). The main advantages of using GFP are summarized as follows: i) no substrate is required, ii) the expression occurs inside the cell, iii) there is no background from indigenous bacteria, and iv) *in situ* detection is possible so studies may be carried out at the single cell level (Table 1). These characteristics make this method more efficient than *lacZ*, *gusA*, and *lux* gene reporter methods.

In PGPB research, the inoculation of muskmelon seeds with GFP–tagged *B. subtilis*

Y–IVI (10^9 CFU mL^{-1}) caused a 2–fold increase in plant growth, showing a colonization prevalence of 10^8 CFU g^{-1} in the rhizosphere and 10^5 CFU g^{-1} in the endorhiza over 30 days (Zhao et al., 2011). Similar results were obtained with GFP–tagged *Streptomyces* sp. ZEA17L and GFP–tagged *A. brasilense* FP2 in lettuce and wheat roots (Bonaldi et al., 2015; Ramos et al., 2002). Using CLSM, Annapurna et al. (2013) observed the colonization of GFP–tagged *Paenibacillus polymyxa* strain HKA–15 (10^5 CFU g^{-1}) in nodules formed by wild–type *B. japonicum* DS–1 after 30 days of incubation. However, when GFP–tagged *P. putida* strain PRD16 and *Enterobacter cowanii* strain PRD116 were quantified in tomato rhizosphere, the results showed 10^4 CFU g^{-1} of *P. putida* strain PRD16 in the rhizosphere by plate counting, but no fluorescent cells were observed under CLSM at 15 days after inoculation (Götz et al., 2006). In contrast, *E. cowanii* strain PRD116 was observed at all sampling times (2, 14 and 30 days).

As mentioned above, combining methods is an attractive strategy to track PGPB. Indeed, *B. tropica* MTo–293 colonization (10^5 CFU g^{-1} of roots) was tracked in tomato roots by combining *gusA* and GFP genes (Bernabeu et al., 2015), but intracellular pH, soil particle interference, anaerobic conditions, expression variability among species, and plasmid instability are factors that must be considered when GFP–tagged PGPB are used in colonization experiments (Gamalero et al., 2003; Ahmad et al., 2011; Weyens et al., 2011).

2.3.2. Immunological methods

Culture–independent immunology-associated methods have also been applied to track and monitor PGPB. These methods are based on the use of antibodies, which are conjugated with enzymes, isotopes, nanoparticles or fluorochromes to detect and/or quantify antigen–antibody interactions. According to Schloter et al. (1996), the use of these methods

in complex habitats, such as rhizospheres, should: i) localize the antigenic determinant on the cell surface, ii) not give a cross reaction, iii) allow the stabilization of the antigenic determinant *in situ*, and iv) have a high affinity to the antigen.

Table 1. Characteristics of methods used to monitor plant growth–promoting bacteria in literature (also see Fig. 3).

Methods	Detection	Advantages	Disadvantages	Detection limits	References
Reporter genes					
β–galactosidase (*lacZ*)	Blue color colonies on agar plates	–*In situ* detection	–Plant and indigenous bacteria with β-D-galactosidase activity –Culture dependent quantification –Substrate dependent	10^3 - 10^5 CFU g^{-1}	Mourya and Jauhri (2002); Solanki and Garg (2014)
β–glucoranidase (*gusA*)	Blue color colonies on agar plates	–*In situ* detection –Semiquantitative –Endophytic detection –Low indigenous β-D-glucoranidase activity	–Culture dependent quantification –Substrate dependent	10^3 – 10^5 CFU g^{-1}	Compant et al. (2005)
Bacterial luciferase (*lux*)	Light emission on agar plates or solution	–*In situ* detection –Semiquantitative –Single cell detection –No endogenous and indigenous activity –Inexpensive substrate	–Culture dependent quantification –Substrate dependent –Metabolism dependent –Luminometer required	10^3 – 10^4 CFU cm^{-1}	De Weger et al. (1997); Kragelund et al. (1997)
Green fluorescent protein (GFP)	Fluorescence microscopy	–*In situ* detection –Endophytic detection –Single cell detection –Constitutive expression –Substrate independent	–Oxygen dependent –pH interference –Variable expression –Plant tissue autofluorescence –Confocal or epifluorescence microscope required	10^3 CFU g^{-1}	Gamalero et al. (2003)
Immuno-associated techniques					
Enzyme–linked immunosorbent assay (ELISA)	Enzymatic reaction	–Easy-to-use –Inexpensive –Semiquantitative	–Non-*in situ* detection –Sample trituration required	10^3 CFU ml^{-1}	Quadt-Hallmann and Kloepper (1996)
Immunofluorescence	Fluorescence microscopy	–*In situ* detection –Semiquantitative	–Confocal or epifluorescence microscope required	Single-cell detection	Hansen et al. (1997)
Immunogold	Fluorescence microscopy	–*In situ* detection Semiquantitative	–Not enough surface area covered for quantification –Transmission electron microscope required	Single-cell detection	Gyaneshwar et al. (2002); Gamalero et al. (2003)
Immunoblot	Probe hybridization	–Qualitative	–Time consuming –Laborious –Expensive	NA	Krishnen et al. (2011)

Table 1. Characteristics of methods used to monitor plant growth–promoting bacteria in literature (also see Fig. 3).

Nucleic acid-based					
Fluorescent in situ hybridization (FISH)	–Probe hybridization required –Fluorescence under microscopy	–*In situ* detection –Semiquantitative	–Confocal or epifluorescence microscope required –Low resolution in small cells	10^2 CFU mm^{-2}	Watt et al. (2006)
Community analysis techniques (DGGE, T-RFLP, ARISA, ARDRA, among others)	DNA Fingerprinting	– Inexpensive	–Qualitative –Laborious	NA	Podile and Kishore (2006)
MPN-PCR	Primer hybridization	–Quantitative	–Laborious –External standard-dependent –Contamination during soil DNA extraction –Not discrimination between live or dead cells	10^2 CFU g^{-1}	Gamalero et al. (2003)
Competitive PCR (C-PCR)	Primer hybridization	–Quantitative	–Contamination during soil DNA extraction –Competitor internal standard-dependent –Competitor amplification efficiency-dependent –Not discrimination between live or dead cells	10^2 CFU g^{-1}	Zentilin and Giacca (2007)
Quantitative PCR (qPCR)	Primer hybridization	–Quantitative –High number of target sequences –Unaffected by cell size	–Soil DNA extraction contamination –Not discrimination between live or dead cells	10^2 CFU g^{-1}	Jansson et al. (1996)

NA: Not applicable. DGGE: denaturant gradient gel electrophoresis, T-RFLP: terminal restriction fragment length polymorphism, ARISA: automatic ribosomal interspace spacer analysis, ARDRA: amplified ribosomal DNA restriction analysis.

2.3.2.1. Enzyme–linked immunosorbent assay (ELISA)

Among immunoassays, one of the most commonly used for PGPB tracking is ELISA, which measures antigen–antibody interactions in different ways, such as by chemoluminescence, fluorescence or color production, and it depends on the enzyme or substrate being used (Schloter et al., 1992; Schloter et al., 1997). Using chemoluminiscence– and fluorescence–based ELISA in inoculated wheat plants in rhizotron cultivation systems, Schloter et al. (1992) detected 10^2 and 10^4 CFU of *A. brasilense* ml^{-1}, respectively, in rhizosphere soil extracts after 6 weeks. Then, a specific monoclonal antibody was developed for *A. brasilense* Sp245 with a tracking sensitivity of 10^2 CFU ml^{-1} (Schloter and Hartmann 1996). The use of a combination of monoclonal antibodies for three *A. brasilense* strains (Sp7, Sp245 and Wa5) discriminated the three inoculated strains and their colonization in rhizosphere and endorhiza of wheat plants at 14 weeks (Schloter and Hartmann 1998). Using the same method, Schloter et al. (1997) tracked the colonization of *R. leguminosarum* bv. *trifolii* R39 in maize, clover, pea and lupin with a sensitivity of 10^4 CFU ml^{-1} sample. The use of polyclonal and monoclonal antibodies determined the colonization of the endophytic *Enterobacter asburiae* JM22 in several plants (cotton, bean and cucumber) under gnotobiotic conditions (Quadt–Hallmann and Kloepper 1996). The sensitivity of ELISA reached 10^3 CFU ml^{-1}, detecting the inoculated bacteria in roots, stems and cotyledons with values of 10^5, 10^4 and 10^3 CFU per g^{-1} of sample, respectively. Monoclonal antibodies have also been used to track PGPB strains belonging to the genus *Pseudomonas*. Garcia et al. (2003) inoculated and tracked *P. fluorescens* Aur6 in pepper roots by the ELISA method reported by Schloter and Hartmann (1996), and the abundance of Aur6 strain in rhizosphere was approximately 10^7 CFU g^{-1} soil at 15, 30 and 15 days after inoculation.

Exopolysaccaride (EPS)-targeted polyclonal antibodies have also been developed (Yegorenkova et al., 2010). In wheat rhizosphere, the colonization of *P. polymyxa* 1465 tracked by indirect ELISA yielded 9.3×10^5, 7.6×10^6, and 2×10^7 CFU ml^{-1} at 15 min, 3 h and 24 h after inoculation, respectively.

ELISA variants, such as double antibody sandwich (DAS)–ELISA, have also been successfully used to track *P. agglomerans* D5/23 and *A. chroococcum* Mac 27 in wheat roots (Narula et al., 2007). The use of DAS–ELISA determined both the colonization of the root tip (3×10^7 CFU g^{-1} rhizosphere soils) and basal root (6×10^7 CFU g^{-1} rhizosphere soils) sections and resulted in numbers of inoculated strains 80% higher than those detected by plate counts. In contrast, plate counts yielded higher numbers when Rose et al. (2011) tracked *B. subtilis* B9, *Bacillus amyloliquefaciens* E19, and *P. fluorescens* 1N in a peat carrier biofertilizer. However, DAS–ELISA does not allow the inoculated PGPB to be detected *in situ* because of soil particle interference (Quadt–Hallmann and Kloepper 1996).

2.3.2.2. Immunofluorescence, immunogold staining and immunoblot

To overcome some of the ELISA disadvantages in tracking PGPB (*e.g.*, sample trituration), it is possible to use immunofluorescence methodology. Similar to ELISA, immunofluorescence can be applied as a direct or indirect method, with the latter being more reliable due to the use of two antibodies (Odell and Cook 2013). This method consists of the following steps: i) extracting rhizosphere or plant samples, ii) applying a fixation protocol, and iii) using fluorochrome–tagged antibodies to observe the fluorescent antigen–antibody reaction under microscopy. Antibodies can also be tagged with colloidal gold particles, and immunogold staining (IGS) of the antigen–antibody interaction is an alternative technique (Levanony et al., 1989; Grilli–Caiola et al., 2004). The main

advantage of both methods is the possibility of tracking the presence of bacteria at single cell levels *in situ* (Hansen et al., 1997), but during sample preparation the fixation affects some membrane component in cells that could potentially interfere with antigen binding. In addition, this method requires expensive equipment for epifluorescence or confocal microscopy for immunofluorescence and transmission electron microscopy (TEM) for IGS. Finally, all immunological techniques involving the use of monoclonal antibodies to target specific strains are time consuming and expensive.

In relation to *in situ* studies, the presence of indigenous fluorescent bacteria and root autofluorescence may interfere with immunofluorescence assays (Schloter et al., 1992), so combining methods may solve limitations associated with the use of a single technique. Indeed, Schloter et al. (1993) used *A. brasilense* Sp7 monoclonal antibodies tagged with FITC and TRITC, and to overcome the problems related to autofluorescence, it was suggested that TRITC–tagged antibodies and DAPI (4',6–diamino–2–fenilindol) be used as a counterstain for estimating the colonization of *A. brasilense* Sp7 and Wa3 in wheat plants. Using immunofluorescence and chemoluminiscence ELISA, Aßmus et al. (1997) discovered competition when both strains (*A. brasilense* Sp7 and Wa3) were co–inoculated. In contrast, Hansen et al. (1997) did not observe competition between *P. fluorescens* DF57 and Ag1 during their early colonization of barley roots, as revealed by immunofluorescence and CLSM. Quadt–Hallmann et al. (1997) used the IGS technique in combination with ELISA and immunofluorescence to determine specific colonization of *E. asburiae* JM22 in cotton. Previously, Levanony and Bashan (1989) considered IGS as an antigen localization tool for *A. brasilense* Cd rather than a potential tracking method. Contrarily, Gyaneshwar et al. (2001, 2002) used IGS to detect *Serratia marcescens* IRBG500 and *H. seropedicae* Z67 in rice tissues. Additionally, both immunofluorescence and IGS were used to describe the

colonization patterns of *A. brasilense* Cd in tomato roots (Grilli–Caiola et al., 2004). Nevertheless, the high resolution of TEM constitutes a disadvantage for tracking PGPB by IGS because only a very small part of the root sample can be observed. Therefore, IGS must be combined with tracking methods (Gamalero et al., 2003).

Krishnen et al. (2011) used immunoblot (also known as western blot) to track three PGPB (*P. fluorescens* 1N, *A. brasilense* Sp245 and *R. Leguminosarum* bv. *trifolii* R4) in soil cropped to rice and reduced the costs by approximately 80% by substituting the nylon membrane with Whatman paper. However, the use of immunoblot to track PGPB is more laborious than immunofluorescence, and it does not allow *in situ* localization. It is probably for these reasons that no further immunoblot assays have been reported for tracking PGPB.

Overall, immunoassays used in PGPB tracking are advantageous because: i) there is no need to transform bacteria before inoculation; ii) they allow *in situ* tracking; iii) they have a high diversity of target molecules; and iv) they possess a high specificity depending on the antibody used (monoclonal or polyclonal). Nevertheless, antibody production is difficult and requires immunization of an animal (commonly goats, rabbits or rats) or a mammalian cell. In addition, due to the specific characteristics of some PGPB, detection of a particular strain requires very specific antibodies, whose development involves time and elevated costs (at least greater than inserting a reporter gene).

2.3.3. Nucleic acid–based methods

PGPB can be tracked using DNA– or rRNA–based methods. This approach focuses on the detection of fingerprint patterns or specific sequences, and in contrast to other methods, it does not require of antisera production or bacterial transformation (Podile and Kishore 2006). Nucleic acid–based approaches can be grouped into PCR–independent and

PCR–dependent methods.

2.3.3.1. PCR–independent methods

The PCR–independent methods are mainly based on DNA cleavage or hybridization with probes that allow the detection of target strains (Gamalero et al., 2003). Among them, the most used and efficient for PGPB tracking is fluorescence *in situ* hybridization (FISH). Metabolically active cells contain approximately 50,000–70,000 ribosomes (Podile and Kishore 2006) that are targeted by fluorescently–tagged, 15–30-bp oligonucleotide probes (Moter and Göbel 2000) designed to bind 16S or 23S ribosomal RNA (rRNA) sequences. The technique consists of five steps: i) sample fixation, ii) sample preparation (pre–treatment), iii) probe hybridization, iv) washing (removal of unbound probes), and v) visualization (Moter and Göbel 2000). The FISH method can detect target PGPB strains *in situ* using epifluorescence microscopy (Compant and Mathieu 2013) or CLSM (Rothballer et al., 2003; Wu et al., 2008). Rothballer et al. (2003) detected *A. brasilense* Sp245 and Sp7 in different wheat varieties, and high colonization rates occurred in older basal roots but decreased toward the root tip. In addition, differences in tissue colonization and strains were determined. *A. brasilense* Sp245 was detected in the apoplast, whereas strain Sp7 was only detected in the rhizoplane. Colonization of diazotrophic bacteria (*G. diazotrophicus* PAL5, *H. seropedicae* HRC54, *Herbaspirillum rubisubalbicans* HCC103, *Azospirillum amazonense* CBAmC, and *B. tropica* Ppe8) was tracked by FISH in sugarcane rhizosphere by Oliveira et al. (2009), who reported that all strains were traceable 12 h after inoculation as also observed by Rothballer et al. (2003). Wu et al. (2008) designed probes for tracking *P. putida* TOM20 in wheat rhizosphere, and Watt et al. (2006) used FISH to quantify native bacteria, *Pseudomonas* sp. and filamentous bacteria in wheat roots. However, few studies

have reported the quantification of PGPB using FISH, and most FISH studies have been complemented with culture-dependent quantification methods, such as colony-forming units (CFU) or most probable number (MPN) counting.

In addition to the advantages mentioned above, the FISH method also has disadvantages such as the interference of root and indigenous bacteria by autofluorescence (Briones et al., 2002). Nevertheless, this disadvantage can be overcome by using fluorochromes that emit signals in near far–red spectra such as Cy5 and Cy5.5 (Watt et al., 2006) or Alexa647 (Wu et al., 2008). Other limiting factors are cell size and bad washing procedures that reduce signal quality. Cell products, such as EPS, have also blocked probe insertion in cells (Watt et al., 2006). Olivera et al. (2009) reported that a designed probe did not hybridize the EPS–producer *G. diazotrophicus* BR11281, whereas other bacteria in the sample were properly detected. Both the signal quality and sensitivity of FISH can be improved to overcome some of these problems. Catalyzed reported deposition (CARD)–FISH has been developed to increase signal detection in marine bacteria (Pernthaler et al., 2002). Probes tagged with horseradish peroxidase (HRP) were hybridized and then treated with fluorescent–tagged tyramide; they emitted the fluorescent signal upon cleavage of the tyramide (Amann and Fuchs 2008), which increased FISH sensitivity up to 40–fold (Hoshino et al., 2008). The CARD–FISH probes effectively detected indigenous bacterial classes on rice rhizoplane (Schmidt and Eickhorst, 2014), but this technique increases FISH complexity due to the enzymatic treatment required to achieve cell penetration of large–sized HRP–tagged probes. Contrarily, double–tagged probes (DOPE)–FISH increased signal intensity by avoiding the enzymatic sample treatment (Stoecker et al., 2010). Positive results of DOPE–FISH were observed in tracking *Saccharothrix algeriensis* NRRL B–24137 in grapevine rhizosphere (Compant and Mathieu

2013) and cucurbit fruits when using this technique to determine bacterial diversity (Glassner et al., 2015).

2.3.3.2. PCR–dependent methods

Generally, bacteria inoculated in the rhizosphere soil have been tracked with community analysis methods, such as denaturing gradient gel electrophoresis (DGGE), terminal restriction fragment length polymorphism (T–RFLP), automatic ribosomal interspace spacer analysis (ARISA), and amplified ribosomal DNA restriction analysis (ARDRA) as well as PCR variants, such as most probable number PCR (MPN–PCR), competitive (C–PCR), or quantitative (qPCR) Gamalero et al., 2003).

One of the most used methods in microbial community analysis is DGGE, which is based on the separation of double-strand DNA fragments from a target gene during their migration on denaturant gradient acrylamide gels, but the complete separation of the two strands is avoided by coupling a rich GC-content DNA fragment (named GC–clamp) during PCR amplification (Sheffield et al., 1989). The separation of the different denatured dsDNA fragments depends on their %GC content (Strathdee & Free, 2013). Changes in community composition by the inoculated PGPB occurred in rape plants as shown by DGGE (Chen et al., 2013). After their inoculation, both *Burkholderia* sp. J62 and *P. thivervalensis* Y139 were observed in rhizosphere and endorhiza at the end of a 60 days study. Bands were separated by DGGE and then excised with solubilization of the relative DNA, the final sequencing of which allowed the detection of inoculated PGPB but not its relative quantification (Menezes-Blackburn et al., 2016; Podile and Kishore 2006).

Similar to DGGE, ARISA and ARDRA produce fingerprint patterns that are obtained by enzymatic restriction of PCR products of the 16S rRNA gene from complex

DNA samples (such as soil) (Rincon-Florez et al., 2013). Despite being sensitive to bacterial diversity, the ARDRA technique is biased in that different bacterial genomes can produce more than one detection peak (Nocker et al., 2007). Similar disadvantages are observed with ARDRA, where more than one band could be attributed to the same microbial group (Kirk et al., 2004). Thus, these techniques are more suitable to assess the effect of inoculation on host bacterial communities rather than for tracking specific strains.

In the case of the MPN–PCR, once the PCR product is detected, quantification is achieved through serial dilutions of PCR products until no bands are observed in electrophoresis gels, and the results are compared with a PCR–positive external standard of known concentration. However, Gamalero et al. (2003) suggested that this technique, which relies on probabilistic evaluation and dilutions, may yield biased results, mainly due to the potential differences in reaction specificity between the samples and the standard because the samples contain mixtures of different DNA. In contrast, C–PCR requires the use of two DNA templates (*e.g.*, sample and internal standard) containing the same primer recognition sites, and the reactions are performed in a series of tubes containing different dilutions of the internal standard. Since the amount of the competitor is known, target DNA can be quantified. Zentilin and Giacca (2007) have suggested that both C–PCR and qPCR are accurate quantification methods, but C–PCR is more laborious and requires the construction of a competitor in at least three different reactions and with high chances of cross contamination. In contrast, qPCR is an easy–to–use technique that is preferred despite the low costs of C–PCR. Additionally, no recent PGPB tracking studies have been reported using either MPN– or C–PCR.

To track and quantify *P. polymyxa* strains, Timmusk et al. (2009) designed 16 rDNA-targeted qPCR primers, and using commercial soil DNA extraction kits, they

estimated the abundance of native *P. polymyxa* populations in wild barley rhizosphere. Fernandes et al. (2014) designed qPCR primers for *G. diazotrophicus*, and primer efficiency was tested in sugarcane rhizosphere, revealing 6.5×10^4 CFU g^{-1} FW. Furthermore, Couillerot et al. (2010a) assessed the applicability of BOX, ERIC and RAPD fragments in the design of qPCR strain–specific primers for *A. brasilense* UAP–154 and CFN–535. In maize rhizosphere, designed primers were sensitive at 4.0×10^3 and 4×10^4 CFU g^{-1} soil for UAP–154 and CFN–535 strains, respectively, using rhizosphere DNA as a template, and primers designed for a strain–specific RFLP marker in *A. lipoferum* CRT1 also reached 4×10^4 CFU g^{-1} soil. Another *A. brasilense* strain (FP2) was recently tracked in wheat rhizosphere; Stets et al. (2015) used the whole genome sequence of the FP2 strain and fragmented it into 500 bp, avoiding fragment overlap. BLASTn fragments with no hits were used to design qPCR primers, and in rhizosphere, primers revealed 10^7 CFU g^{-1} FW root, 13 days after strain inoculation. A similar method was applied to monitor *H. seropedicae* SmR1 with specific qPCR primers designed by Pereira et al. (2014), who reported an increase in copy number from 10^7 to 10^9 g^{-1} FW 10 days after the inoculation in maize roots. Considering its applicability, efficiency and sensitivity, and strain–specificity, qPCR has been described as the best method for PGPB tracking (Couillerot et al., 2010b).

Another PCR variant used for tracking is *in situ* PCR, which has the sensitivity of PCR with the spatial resolution of FISH. *In situ* PCR reactions are performed on glass slides, which are hybridized with fluorescent probes and visualized under a microscope. However, it is an invasive method, and bacterial cells could be lost during the temperature denaturation treatment, thus affecting the abundance counts. Ruppel et al. (2006) detected *Enterobacter radicincitans* DSM 16656, after its inoculation in cauliflower, competing with native bacterial populations around roots using the *in situ* PCR technique. A minimum of

2.7×10^4 and a maximum of 10^9 *E. radicincitans* cells g^{-1} root was detected, and 10~16% of the rhizobacterial community was represented by the inoculant strain 14 days after inoculation.

Nevertheless, the main disadvantage of PCR–dependent methods for tracking PGPB in rhizosphere is that this technique does not distinguish living from dead cells (Holmberg et al., 2009). Additionally, factors such as poor primer specificity and DNA extraction contamination with soil particles might negatively affect PCR.

Table 2. Novel strategies for tracking plant growth–promoting bacteria.

Method	Detection	Advantages	Disadvantages	Detection limit	References
Transparent soil	–GFP tagged cells or FISH probe required –Fluorescence microscopy	–*In situ* detection –Single cell detection	–Plant tissue autofluorescence –Confocal or epifluorescence microscope required	NR	Downie et al. (2012); Downie et al. (2014)
MRI	-Cellular proliferation spots detection	-*In situ* detection	-Pot dependence -Heavy equipment required -Nanoparticle stability dependency	ND	van Dusschoten et al. (2016); Pflugfelder et al. (2017)
TRIS	–GFP tagged cells or FISH probe required –Fluorescence microscopy	–*In situ* detection –Single cell detection	-Not applicable to soil or substrate -Plant culture media dependency -Confocal or epifluorescence microscope required -Plant tissue autofluorescence	NR	Massalha et al. (2017)
QCM Sensor	–Piezoelectric frequency changes	–Precise quantification –Strain specific –Automatable	–Expensive –Reusable –Plates can only be used 10 times	23 CFU mL^{-1} (in PBS)	Shen et al. (2011); Agrawal et al. (2012)
Next generation sequencing (NGS)	-Sequence data analysis	-High resolution -Sensitivity	-Sample destruction -Time consuming analysis -Expensive equipment required (sequencer)	ND	Qichao et al. (2014); Albanese & Donati (2017)
CRISPR–PCR	–Electrophoresis –Loci sequencing	–Inexpensive and fast –Sensitive –Strain-specific –Sensitivity can be increased if coupled to qPCR	–Requires genome characterization –Plate count or PCR–assay dependent	ND	Bachmann et al. (2014); Barrangou and Dudley (2016)

MRI: Magnetic resonance imaging; TRIS: Tracking root interaction system; QCM: quartz crystal microbalances; CRISPR: clustered regularly interspaced short palindromic repeats; qPCR: quantitative polymerase chain reaction; NR: not reported; ND: not done.

2.4. Novel strategies for tracking PGPB

A couple of novel methodologies have been developed for evaluating PGPB colonization in plant–root systems (Table 2). Downie et al. (2012) developed a nafion–based transparent substrate for *in situ* root imaging and reported that growth rates of alfalfa, barley, maize, tobacco, lettuce and *Arabidopsis thaliana* were similar in this substrate to those occurring in soil, although root morphology was altered. Their methodology requires flooding the pots with a refraction inhibitor (RI) to diminish light dispersion through transparent soil particles under a microscope. Thus, *E. coli* O157:H7 was GFP–tagged, and its colonization and attachment to the rhizoplane was observed in lettuce seedlings using CLSM. In a similar experiment, Downie et al. (2014) inoculated GFP–tagged *P. fluorescens* SBW25 on lettuce roots, and using calcofluor, they discriminated the root autofluorescence from the inoculated bacterium. Differences were observed between the formation of bacterial aggregates on controls without plants, whereas large bacterial aggregates were observed on root tips in the presence of plants. However, no CFU counts were reported in either of the articles. In addition, although this transparent polymer has physical similarities with soil, it is chemically completely different (Roose et al., 2016).

Complementary to this imaging approach, magnetic resonance imaging (MRI) can be applied to track the development of bacteria in mice (Hoerr et al., 2013). After labeling *Staphylococcus aureus* with coated iron oxide nanoparticles, the authors tracked the development of the inoculum and observed *in vivo* colonization in different organs using MRI. Regarding plants, two recent studies (van Dusschoten et al., 2016; Pflugfelder et al., 2017) have revealed that it is possible to image barley and maize roots either in substrate or soil using MRI. However, this technique should be verified in pot experiments, and the

selected nanoparticle stability in soil should be evaluated (Tourinho et al., 2012). Nonetheless, studies on bacterial tracking by this technique have not been conducted so far.

The development of specific equipment has contributed to the study of bacterial colonization in plants. Massalha et al. (2017) designed and implemented a microfluidic chamber named TRIS (tracking root interactions system), which, coupled to CLSM and dark-field microscopy, allowed the colonization progress of GFP-labeled *B. subtilis* NCIB 3610 to be tracked in *A. thaliana* roots. The authors were also able to observe real-time competition of *B. subtilis* with GFP-labeled *E. coli* OKN-3 after co-inoculation. However, this technique can only be applied under laboratory conditions due to its design and the requirement of synthetic plant culture media for both plant development and microscopical observations.

Another proposed strategy combines the use of antibodies and piezoelectric quartz microbalances (QCM). The mass of the quartz crystals increases under specific nanoparticle–tagged DNA probes or antigen–antibody reactions. These variations can be measured with a piezoelectric crystal. As the mass of the crystal increases, baseline oscillation proportionally decreases, and this signal can be used to detect the presence of the target strain. This method has a measurement threshold of 23 CFU *E. coli* O157:H7 ml^{-1} on phosphate saline buffer (PBS) (Shen et al., 2011). By testing several antibody concentrations, two cadmium–resistant PGPB *Pseudomonas* strains (Z9 and S2) were detected by coupling anti–Z9 and anti–S2 on a QCM, but they were not quantified as CFU data (Agrawal et al., 2012). This method is faster, more sensitive and more reliable than the ELISA assays. Authors suggested that this immunosensor could be used in future PGPB tracking studies and coupled with automatization processes. In a similar manner, a recently developed QCM device based in DNA probes successfully reached a detection limit of a

minimum 22 copies μL^{-1} PCR product of *Ehrilichia canis* 16s rRNA gene (Bunroddith et al. 2017). Novel molecular chromosome markers could also be applied for PGPB tracking. Indeed, next generation sequencing (NGS) has increased the genotyping resolution of bacteria. By using a technique described as ribosomal multilocus strain typing (rMLST), Jolley et al. (2012) reported that the *rps* loci was more suitable than 16S rRNA for genotyping due to its ubiquitous presence in all bacteria, its coding capacity, and its capability of predicting taxonomy at the strain level. However, this technique was applied to cultured isolates, so future studies should be performed to verify its applicability to a metagenomic sample to evaluate if it can track bacterial inoculant. Similarly, Qichao et al. (2014) designed a pipeline to identify strains in raw metagenomic datasets. Their methodology was based on the identification of genome specific-markers (GSMs) obtained from > 5000 genome sequences available in public databases, and it was validated on synthetic metagenomes and further tested on diabetes raw metagenomes, which accurately detected 45 strains. Albanese & Donati (2017) also reported single nucleotide variant (SNV) strain profiles from isolated bacterial genomic data, and these profiles were further compared with metagenomic datasets to search for the presence of co-occurrence and discrimination at the strain level. It is important to highlight that both metagenomic NGS tracking methodologies require further analysis and annotation of genomic data. Additionally, despite the capabilities, resolution and decreased costs of the NGS approaches, they are poorly sensitive when applied to complex bacterial communities (Vierheilig et al., 2015).

From a simpler perspective, clustered regularly interspaced palindromic repeats (CRISPR) might serve as a molecular marker. CRISPRs have a particular loci structure (non–coding unique sequences of 21–48 bp interspaced by conserved sequences of constant

length) (Sorek et al., 2008) and are present in the genome of 40% of Bacteria and 90% of Archaea (Horvath and Barrangou 2010), including known PGPB (Table 3). Recently, the CRISPRs loci has been used for high–resolution phylogenetic studies and for tracking a broad spectrum of specific pathogenic bacteria (Barrangou & Dudley 2016). As an example, Bachmann et al. (2014) were able to discriminate between two *Salmonella enterica* (subsp. *Enterica serovar* Virchow SVQ1 and SL491) by comparing the genomes of the two strains with 27 previously published genomes of *Salmonella* spp. Fabre et al. (2012) found a correlation between CRISPR loci polymorphisms and serotypes on 744 strains and suggested that the CRISPR–based methodology was faster and more sensitive than most of the previously applied methods. Similarly, Delannoy et al. (2012) were able to discriminate seven *E. coli* strains from a panel of 958 sequenced genomes through CRISPR loci–targeted PCR with a sensitivity range of 95.7–100%. CRISPR typing of plant–related bacteria have been applied to plant pathogenic *Erwinia amylovora* (McGhee and Sundin 2012) but not to plant-soil systems. The screening of CRISPR loci in whole genomes of soil–plant associated bacteria deposited in CRISPR databases (CRISPI, http://crispi.genouest.org/; CRISPRfinder, http://crispr.i2bc.paris-saclay.fr/) revealed the presence of CRISPR loci in 20 of 27 genera (Fig. 4). In addition, the genera *Azospirillum*, *Azotobacter*, *Nitrobacter*, *Nitrosomonas*, *Xanthomonas*, *Sarcina*, and *Frankia* show CRISPR loci in all deposited genomes in CRISPR databases. Therefore, studies based on the use of CRISPR loci structure (instead of the enzymatic machinery associated to them) as molecular markers for typing or monitoring PGPB should be carried out with the aim of developing sensitive methods (Table 3).

Table 3. CRISPR repetitive sequences in representative plant growth–promoting bacteria.

Organism	Repetitive sequence (5'-3')[a]	Accession no.[b]
Azospirillum lipoferum 4B	GTCTTCCCGAGCAGAAATGCTTGGGCCTCATTGAAGC	FQ311868
Paenibacillus polymyxa CR1	GTCGCACTCTGTATGGAGTGCGTGGATTGAAAT	CP006941
Pseudomonas sp. B10	GGAGCGAGCCTGCTCGCGAAGGCG	LT707063
Serratia plymuthica AS9	AAGCCTTGCAGGAAGCGGCGCTGCGTCAGGCGTTTGC CGA	CP002773

[a] Sequence obtained from CRISPRfinder database (http://crispr.i2bc.paris-saclay.fr/Server/) and confirmed by PILERCR tool (https://www.drive5.com/pilercr/)
[b] Accesion number of whole genome in NCBI Genome database.

It needs to be addressed that the development of this technique requires an extensive analysis of the PGPB genomes available in databases other that those that are CRISPR related due to the absence of this loci in deeply characterized members of the PGPB group (*e.g.*, *Azospirillum* sp. Az39).

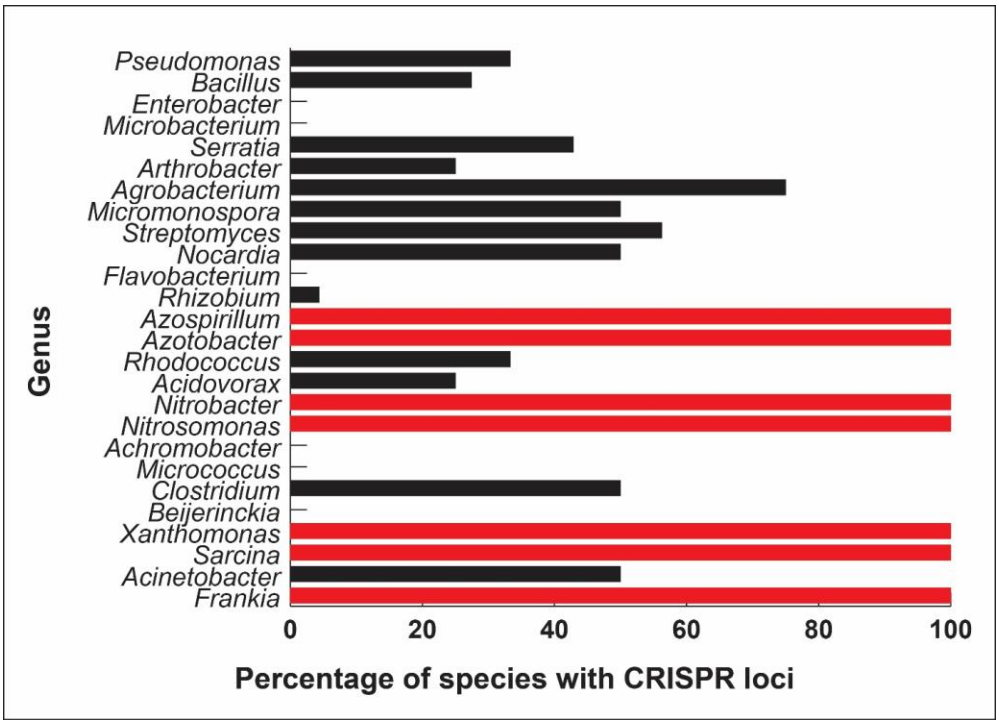

Figure 4. Percentage of species with CRISPR loci in genomes of soil–associated bacterial genera available in databases. Red bars represent species with CRISPR loci in all genomes available in databases. Genome sequences were obtained from the databases CRISPI (http://crispi.genouest.org/) and CRISPRfinder (http://crispr.i2bc.paris-saclay.fr/) and then processed by PILER–CR (https://www.drive5.com/piler/).

2.5. Future perspectives

The market of products based on PGPB strains around the world has grown rapidly over the last decade, although, several issues arise from their massive use (*e.g.*, host specificity, environment specific conditions, required co-habitants, and others). Important is to elucidate the specific dynamics of selected PGPB with their hosts (Timmusk et al. 2017), especially considering. the development of a large number biofertilizer products formulated with indigenous PGPB strains (Cassan & Diaz-Zorita, 2016), which (as described) are difficult to track once released to nature.

Besides the various advantages that novel methodologies might provide to PGPR tracking in terms of sensitivity and specificity (Table 2), there is still little information of how efficient these methodologies would be under a real plant-soil scenario. The imagen-based techniques, such as the transparent soil matrix and MRI or a microfluidic device could not be suitable outside laboratory-controlled conditions, as both methods present pot-dependency and neither of them allows total quantification. The use of labelled bacteria (either fluorescent or nanoparticle-tagged) also has drawbacks, due to their inability to be released to environment but, for inoculant preparation stages, these novel methods could provide important information on how target host plants interact with potential PGPB strains.

For PGPB tracking in field, there are large differences between the use of a QCM device, NGS, or our proposed CRISPR loci PCR assay. The use of a QCM device appears to be promissory, as the technique has demonstrated to be highly sensitive and can used with DNA probes, as demonstrated by Bunroddith et al. (2017). This last advance improves its applicability, as DNA probes are less expensive, and less laborious to develop than a

monoclonal antibody, being only required a proper genomic knowledge of the strain to track. Oppositely to a targeted device such as QCM, the use of a high throughput technique as NGS requires more effort for the detection of a single strain, since the approach points to discriminate one strain from a large metagenomic dataset, therefore, databases needs to be improved in order to increase the resolution of the technique. It also needs to be considered the presence of membrane-damaged or dead cells in environmental samples, whose DNA (if not inactivated) could generate false positive data (Nocker et al. 2007; Wagner et al. 2008). If the last is taken into account, NGS results have high specificity. Although the post-sequencing analysis required to confirm the presence of a PGPB needs to be performed by a bioinformatic analyst using large scale computing servers, which are not available for every laboratory. Complementarily, on some scenarios the sequencing performance will rely on sample type, as different soil- or plant samples could contain inhibitors, affecting the PCR performance prior to sequencing (Sagova-Mareckova et al. 2007).

Regarding our proposal, the specificity of CRISPR repeat sequences from representative PGPR of Table 2 only presented association with the same species under BLAST analysis. It needs to be considered that this approach is not related to the CRISPR-Cas enzymatic machinery currently used for DNA engineering. Our proposal is based in CRISPR repeat sequences that form genetic clusters (at genus scale) according to their composition (Kunin et al. 2007). In this sense, these short repeat sequences are specific enough to develop primer sets for the PGPB detection in different soil-plant compartments, either under a culturable approach, such as colony PCR, or by total DNA PCR from different plant niches in a standard-equipped microbiology laboratory. Additionally, as the proposed method depends on specific (and conserved genus) DNA fragments, probes could

be designed for it to be coupled on QCM devices for *in situ* detection or fluorescent microscopy for more specific laboratory analysis.

Finally, it is necessary to mention that the most of studies of tracking are focused in the detection and prevalence of specific inoculated PGPB strains, but their functionality *in situ* still represents a big challenge to be boarded in the application of PGPB at field level. Studies on the presence or expression of known PGP genetic functions as molecular markers, such as N-fixation (*e.g.*, *nif*H gene), phosphate-solubilization (*e.g.*, pyrroloquinoline quinone [*pqq*E] genes), indole acetic acid production (*e.g.*, *ipd*C genes), antibiotic production (*e.g.*, 2,4-diacetylphloroglucinol [DAPG] gene), chitinase activity (*chi*A gene), 1-aminocyclopropane-1-carboxylate deaminase (ACCD) activity (*e.g.*, *acd*S gene) among others, should be considered and complemented with current tracking studies of PGPB *in situ*. However, these genes have showed a low reliability when PGPB screening have been done in diverse ecosystems, revealing a poor representability (and low coverage on current databases) in relation to the wide diversity of bacteria in the environment (Jorquera et al. 2014).

2.6. Concluding remarks

Despite plant growth–promoting bacteria (PGPB) have been extensively applied in agriculture, studies on the prevalence, colonization and activity of PGPB to evaluating the PGPB effectiveness under field conditions are scarce. The low number of studies that include monitoring of PGPB is due to the lack of proper methods to determine their colonization or efficiency *in situ* and our poor knowledge of the mechanisms underlying plant–microbiome interactions in the environment. Currently, the methods for monitoring

of inoculated PGPB are time-consuming, laborious, expensive and strain–unspecific; and their adoption as tool to evaluate the effectiveness of PGPB under field conditions is questionable or unpracticable. Thus, the development and establishment of rapid, easy and inexpensive methods for evaluating the colonization and monitoring of PGPB strains at field scales is highly needed. A proper application of PGPB in agriculture requires a better understanding the behavior of PGPB *in situ* and their influence on or interaction with plant–microbiome. In this sense, the advances in PGPB omics (such as metagenomics, metatranscriptomics and proteomics) can allow developing novel methods based on their genomes (such as CRISPR loci) and plant–microbiome response as result of PGPB inoculation. With this knowledge, it should be possible determine the influence of PGPB on plant and its microbiome, and adopt better sustainable management strategies reducing our dependency to chemical inputs in agriculture.

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CHAPTER III

Putative Nitrogen-fixing Bacteria Associated with the Rhizosphere and Root Endosphere of Wheat Plants Grown in an Andisol from Southern Chile

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Putative Nitrogen-fixing Bacteria Associated with the Rhizosphere and Root endosphere of Wheat Plants Grown in an Andisol from Southern Chile

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Abstract

Acidic ash derived volcanic soils (Andisols) support 50% of cereal production in Chile. Nitrogen (N) is essential for cereal crops and commonly added as urea with consequent environmental concerns due to leaching. Despite the relevance of N to plant growth, few studies have focused on understanding the application, management and ecological role of N_2-fixing bacterial populations as tool for improve the N nutrition of cereal crops in Chile. It is known that N_2-fixing bacteria commonly inhabits diverse plant compartments (*e.g.*, rhizosphere and root endosphere) where they can supply N for plant growth. Here, we used culture-independent and dependent approaches to characterize and compare the putative N_2-fixing bacteria associated with the rhizosphere and root endosphere of wheat plants grown in an Andisol from southern Chile. Our results showed significantly greater bacterial loads in the rhizosphere than the root endosphere. Quantitative PCR results indicated that the copy number of the 16S rRNA gene ranged from 10^{12}~10^{13} and 10^{7}~10^{8} g^{-1} sample in rhizosphere and root endosphere, respectively. The *nif*H gene copy number ranged from 10^{5}~10^{6} and 10^{5} g^{-1} sample in rhizosphere and root endosphere, respectively. The total culturable bacteria number ranged from 10^{9}~10^{10} and 10^{7}~10^{8} CFU g^{-1} sample in rhizosphere and 10^{4}~10^{5} and 10^{4} CFU g^{-1} sample in root endosphere using LB and NM-1 media, respectively. Indirect counts of putative N_2-fixing bacteria were 10^{3} and 10^{2}~10^{3} CFU g^{-1} sample in rhizosphere and root endosphere using NFb medium, respectively. Sequencing of 16S rRNA genes from randomly selected putative N_2-fixing bacteria revealed the presence of members of Proteobacteria (*Bosea* and *Roseomonas*), Actinobacteria (*Georgenia*, *Mycobacterium*, *Microbacterium*, *Leifsonia* and *Arthrobacter*), Bacteroidetes (*Chitinophaga*) and Firmicutes (*Bacillus* and *Psychrobacillus*) taxa.

Differences in 16S rRNA and putative *nif*H-containing bacterial communities between rhizosphere and root endosphere were shown by denaturing gradient gel electrophoresis (DGGE). This study shows a compartmentalization between rhizosphere and root endosphere for both the abundance and diversity of total (16S rRNA) and putative N_2-fixing bacterial communities on wheat plants grown in Chilean Andisols. This information can be relevant for the design and application of agronomic strategies to enhance sustainable N-utilization in cereal crops in Chile.

Keywords: Andisol; root endosphere; N_2-fixing bacteria; rhizosphere; wheat

3.1. Introduction

Agricultural production in southern Chile is established in acidic ash derived volcanic soils (Andisols), which support around 50% of cereal production in Chile (Laval & Garcia, 2018). In these soils, nitrogen (N) fertilization (as urea and other chemicals) is a common practice to improve agricultural production. The application of N is essential for crop yields and its availability is crucial during plant vegetative development and seed development (Ohyama et al. 2014), but also contributes to Andisol acidification and contamination of water bodies by N leaching (Demanet et al. 2010).

Currently, it is widely accepted that the plant rhizomicrobiome contributes in a direct or indirect way to the growth and fitness of plants, providing phytohormones, solubilizing nutrients, fixing nitrogen (N_2), establishing biocontrol of phytopathogens, and chelating metallic ions (De La Peña & Loyola-Vargas 2014). Biological N_2 fixation by bacteria is the most ecologically and agronomically relevant benefit obtained by plants from their interaction with bacteria, Atmospheric N_2 is reduced to ammonia (NH_3) by the bacterial nitrogenase enzyme complex making it accessible for plant uptake. Thus, the recruitment of N_2-fixing bacteria under symbiotic or non-symbiotic relationships (*e.g.* nodulation of legume plants by *Rhizobium* spp. or interaction with free-living associative N_2 fixers) helps the host plant to obtain N directly from atmosphere and fulfill its nutritional requirements (de Brujin, 2015). Studies have also show that some genera of free-living bacteria (*e.g.*, *Azospirillum* and *Azotobacter,* and others) can colonize diverse plant niches such as the rhizosphere (soil influenced by plant roots) and endosphere (inner tissues of plants), contributing to the N needs of non-leguminous plants (Bhattacharyya & Jha 2012).

The inoculation or bioaugmentation of plants with N_2-fixing bacteria is an attractive alternative to traditional N-fertilization practices and results in decreased fertilization costs and an environmentally friendly alternative to use of agrochemicals. In pastures grown in Chilean Andisols, studies have demonstrated that N_2 fertilization induces changes in total rhizobacterial populations, including potential plant growth-promoting rhizobacteria and populations harboring the *nif*H gene (Jorquera et al. 2014a; Martinez et al. 2011). Symbiotic N_2-fixing bacteria (e.g. *Bradyrhizobium*) have been isolated from nodules of yellow lupin (*Lupinus luteus*) grown in Chilean Andisols (Campos et al. 2014). Partial sequencing of 16S rRNA genes, the application of denaturing gradient gel electrophoresis (DGGE), and 454-Roche pyrosequencing revealed a great diversity of bacterial group present in pasture and cereal rhizospheres of plants grown in Chilean Andisols, including also some N_2-fixing bacteria such as the bradyrhizobia (Jorquera et al. 2014b; Lagos et al. 2014).

However, despite the relevance of N nutrition in cereal production in Andisols in southern Chile, few studies have been done to explore the association of N_2-fixing bacteria with cereals grown in Chilean acid volcanic soils. Several studies have demonstrated that the abundance, diversity, and activity of bacterial populations associated with plants may play a central role in its productivity (Turner et al. 2013; Berg et al. 2014). Therefore, information on N_2-fixing bacterial populations in cereal crops can be relevant for bioprospecting of native bacterial strains as inoculants as well as the develop of management strategies to improve the N nutrition of plants and decreasing our dependency to chemical N fertilization.

In this study, we used culture-independent and dependent approaches to characterize and compare putative N_2-fixing bacterial populations associated with the rhizosphere and root endosphere of wheat plants grown in an Andisol from southern Chile.

3.2. Material and Methods

3.2.1 Sampling

Wheat plants and their adhered rhizosphere soil were placed into sterile flasks (in triplicates) and immediately transported on ice to the Applied Microbial Ecology Laboratory (EMAlab) of Universidad de La Frontera, Temuco, Chile. The samples were taken from four wheat cultivars (*Triticum aestivum* cv. Feña, Patras, Joker, and Rocky, labelled as F, P, J and R, respectively) grown in an Andisol located in the La Araucanía region (38°32'47.5"S, 72°27'43.6"W) of Chile under yearly rotation with rapeseed (*Brassica napus*) and oat (*Avena sativa*) since 2012. Prior to sampling, the soil was fertilized with 140 kg of urea ha^{-1} and treated with Bacara (Bayer Crop Science, Inc.), and a commercial mixture of the pre-emergence herbicides flufenacet, flurtamone, and diflufenican, to a final concentration of 1 L ha^{-1}. Rhizosphere, roots and shoots were separated separately prior to further activities.

The chemical properties of rhizosphere samples was determined from composited samples as follows: the soil pH was measured in 1:2.5 soil:deionized water suspensions, extractable P (P_{Olsen}) was extracted using Na-bicarbonate (0.5 M) and analyzed using the molybdate-blue method (Murphy and Riley, 1962), exchangeable cations (K, Ca, Mg, and Na) were extracted with CH_3COONH_4 (1 M) at pH 7.0 and analyzed by flame atomic adsorption spectrophotometry (FAAS) (Warncke and Brown, 1998), and exchangeable

aluminum was extracted with KCl (1 M) and analyzed by FAAS (Bertsch and Bloom, 1996).

3.2.2 Counts of putative N_2-fixing bacteria

Total and N_2-fixing bacteria populations in rhizosphere and root endosphere samples were estimated by quantitative PCR (qPCR) using the 16S rRNA and *nif*H as target genes. Total DNA was extracted from rhizosphere samples (0.25 g) by using DNeasy PowerSoil Kits (Qiagen, Inc.), according to manufacturer instructions. For endosphere samples (0.15 g), root samples were initially vigorously washed with sterile distilled water (SDW) and rhizoplane surfaces were disinfected by three continuous washes in 80% ethanol for 5 min, followed by a 20 min immersion in 4% sodium hypochlorite and three rinses with SDW as described by Duran et al. (2014). The roots were macerated on sterile ceramic mortars and homogenized in 1.5 mL of sterile 0.85% saline solution. To verify sterility of rhizoplane samples, SDW from the third rinse was collected and processed as a sample for sterility control purposes. Root endosphere total DNA was extracted from 0.25 mL of surface sterile root homogenate using Quick-DNA™ Plant/Seed Miniprep Kit (Zymo Research Corp.), according to manufacturer instructions. The DNA concentrations were adjusted by dilution to 20 ng ul^{-1} and quality (260:280 ratio) was confirmed at ~1.8.

The quantitation of *nif*H genes was done by using the primer set nifH-g1-forB (5'-GGT TGT GAC CCG AAA GCT GA-3') and nifH-g1-rev (5'-GCG TAC ATG GCC ATC ATC TC-3') (Bürgmann et al. 2003). The following PCR conditions were used: 95°C for 11 s followed by cycles of 95°C for 15s, 60°C for 30 s, and a final extension step at 75°C for 8 s and at 72°C for 10 s (Bürgmann et al. 2003). The quantitation of 16S rRNA genes was done by using the mitochondria- and chloroplast excluding primer set 799f (5'-AAC

MGG ATT AGA TAC CCK G-3') and 1115r (5'-AGG GTT GCG CTC GTT G-3') (Shade et al. 2013) with the following program: cycles of 94°C for 1 min, annealing at 53°C for 1 min and extension at 72°C during 1 min; with a final extension step at 72°C during 10 min. (Beckers et al. 2016). Both qPCR assays were done using PowerUp™ SYBR® Green Master Mix (Thermo Fisher Scientific Inc.). The quantitation of gene copies was estimated by using standard curves prepared with synthetic ~1500 bp dsDNA ultramers (Integrated DNA Technologies, Inc.) of the *nif*H gene from *Azospirillum brasilense* Sp7 (NCBI accession no. X51500) and the 16S rRNA gene from *Azospirillum picis* (NCBI accession no. AM922283), respectively.

3.2.3. Counts of culturable and putative N_2-fixing bacteria

Rhizosphere soil (2 g) was suspended in 50 mL of SDW. Previously macerated root samples (1 mL) from section 2.2 above were resuspended in 25 mL of SDW. Both sample types were homogenized by sonication for 30 s at 120 kHz. Suspensions were serially diluted in 0.85% saline used in plate counting methods described as follow. Counts of total culturable bacteria were done on Luria Bertani agar medium (LB) (10 g l^{-1} Triptone; 5 g l^{-1}; Yeast Extract; 5 g l^{-1} NaCl; Sambrook and Russell, 2001), and on NM-1 oligotrophic medium (0.5 g l^{-1} D-glucose, 0.5 g l^{-1} polypeptone, 0.5 g l^{-1} Na- glutamate, 0.5 g l^{-1} yeast extract, 0.44 g l^{-1} KH_2PO_4, 0.1 g l^{-1} $(NH_4)2SO_4$, 0.1 gl^{-1} $MgSO_4 \cdot 7H_2O$, 15 g l^{-1} agar, and 1 ml vitamin solution containing 1 g l^{-1} nicotinamide, 1 g l^{-1} thiamine hydrochloride, 0.05 g l^{-1} biotin, 0.5 g l^{-1} 4-aminobenzoic acid, 0.01 g l^{-1} vitamin B12, 0.5 g l^{-1} D- pantothenic acid hemicalcium salt, 0.5 g l^{-1} pyridoxamine dihydrochloride, 0.5 g l^{-1} folic acid; Nakamura et al. 1995). Both culture media were amended with 10 µg ml^{-1} cycloheximide prevent fungal growth. Autoclaved agar was added before plating at final concentration of

1.5% for both media, as recommended by Tanaka et al. (2014), to prevent hydrogen peroxide formation, resulting in bacterial growth inhibition with the concomitant underestimation of total culturable bacteria counts (Supplementary Fig. 1). Aliquots (50 µl) of appropriated dilutions of the rhizosphere and root endosphere suspensions were separately plated onto petri dishes containing LB and NM-1 media, and incubated for 4 days at 28°C. Colonies grown on agar plates were automatically counted by using CLIQS Colony Counter software (TotalLab Inc., UK).

In parallel, indirect counts of putative N_2-fixing bacteria were carried-out by MPN analyses as recommend by Baldani et al. (2014), using semisolid (0.5% agar) NFb medium tubes (5 g L^{-1} malic acid, 0.5 g L^{-1} K_2HPO4, 0.1 g L^{-1} NaCl, 0.02 g L^{-1} $CaCl_2 \times 2H_2O$, 2 mL of micronutrient solution [0.4 g L^{-1} $CuSO_4 \times 5H_2O$, 0.12 g L^{-1} $ZnSO_4$, 1.4 g L^{-1} H_3BO_3, 1 g $Na_2MoO_4 \times 2H_2O$, 1.5 g and $MnSO_4 \times H_2O$], 2 mL 0.5% bromothymol blue in 0.2N KOH, 4 mL of 1.64% Fe[III] EDTA, and 1 mL of vitamin solution containing 100 mg L^{-1} biotin and 200 mg L^{-1} pyridoxamine dihydrochloride; Hartmann et al. 2006). Tubes containing 5 mL of NFb medium were inoculated with 100 µL of sample and incubated for 4 days at 37°C. The presence of white layer (or pellicle), an indicator of N_2-fixing bacterial growth, was checked, and aseptically removed by using sterile cork borers. After MPN analyses, the isolation of putative N_2-fixing bacteria was performed by resuspension of the pellicle on 1 mL 0.85% NaCl, and 100 µL reinoculated into fresh tubes of NFb medium. Tubes were incubated at 37°C for 4 days. After second incubation, the pellicle was newly aseptically removed, resuspended on 1 mL 0.85% NaCl, serially diluted and plated onto LB and Congo red malic-acid (CRMA; 0.5 g L^{-1} K_2HPO_4; 0.2 g L^{-1} $MgSO_4 \times 7H_2O$; 0.1 g L^{-1} NaCl, 0.5 g^{-1} yeast extract, 0.015 g L^{-1} $FeCl_3 \times H_2O$; 5 g L^{-1} DL-malic acid, 4.8 g L^{-1} KOH, and 15 mL^{-1} of 1:400 Congo red; Rodriguez-Caceres 1982) agar media. Colonies (75) were randomly

selected from CRMA agar plates according to their morphology, purified by streaking on NFb agar plates, and stored in 1 mL sterile LB-Glycerol (7:3) at -80°C.

3.2.4. Characterization of putative N_2-fixing bacteria

Total DNA from selected rhizosphere and root endosphere isolates was extracted by using the Proteinase K-CTAB (cetyl-trimethylammonium bromide) method as described by Wilson (1997). To prevent analysis of clones, all isolates were firstly genotyping by using rep-PCR DNA fingerprinting and ERIC primers as described by Versalovic et al. (1991). The 16S rRNA genes were amplified from 20 and 18 genetically-different rhizosphere and root endosphere isolates, respectively, by using PCR and the universal bacterial primer set 27f (5'-AGA GTT TGA TCC TGG CTC AG-3') and 1492r (5'-TAC GGY TAC CTT GTT ACG ACT T-3') (Lane 1991) and by using the PCR conditions suggested by Jorquera et al. (2012). In parallel, *nif*H genes was also amplified by using the primer set PolF (5'-TGC GAY CCS AAR GCB GAC TC-3') and PolR (5'-ATS GCC ATC ATY TCR CCG GA-3') and PCR condition suggested by Jorquera et al. (2014a).

Synthetic 16S rRNA and *nif*H genes from *Azospirillum picis* and *Azospirillum brasilense* Sp7 were used as positive controls, respectively.

In addition, a variety of universal *nif*H primer combinations were also tested, including PolFI and PolR; PolF and AQER; nifH-g1-forA and nifH-g1-rev; nifH-g1-forB and nifH-g1-rev; MehtaF and MehtaR (Supplementary Table 1). Despite the large number of primers used, however, sequence analyses indicated that the amplicons were unspecific and not related to *nif*H.

The PCR products were sequenced by Macrogen Inc. (Seoul, South Korea), trimmed, cleaned up, and compared with those deposited in GenBank database using

BLASTn tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi). To the presence of *nif*H, nucleotide sequences were translated into amino acids and compared with those present in GenBank by using BLASTx. Sequences (1 from rhizosphere and 7 from the root endosphere) showing positive alignment with the *nif*H enzyme were used to build a neighbor-joining tree using representative sequences of *nif*H reported in literature, including *Azoarcus*, *Azospirillum*, *Azotobacter*, *Bosea*, *Bradyrhizobium*, *Bacillus*, *Burkholderia*, *Chitinophaga*, *Herbaspirillum*, *Mesorhizobium Microbacterium*, *Pontibacter*, *Rhizobium*, and *Roseomonas*. The alignment of amino acids sequences was done by CLUSTAL W (Larkin et al. 2007) and neighbor-joining trees were built using Geneious version R11 (Bootstrap = 1000) (Kearse et al., 2012).

Sequences obtained in this study were deposited in GenBank under accession numbers MG835569 to MG835606 for 16S rRNA gene, and MH175481 - MH175487 as well as MH175490 for *nif*H gene sequences.

3.2.5. DGGE Fingerprinting of total and N_2-fixing bacterial communities

Fingerprinting of total and N_2-fixing bacterial communities in rhizosphere and root endosphere samples was done by using denaturing gradient gel electrophoresis (DGGE) of 16S rRNA and *nif*H as target genes, respectively. For total bacterial communities, 16S rRNA genes were first amplified by using primer set 933f (5'-GCA CAA GCG GTG GAG CAT GTG G-3') and 1492r. Then, ~600 bp bands were confirmed on electrophoresis, and used as template for a nested PCR with primer set 933f-gc and 1387r (5'-GCC CGG GAA CGT ATT CAC CG-3'). The GC-clamp (5'-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG-3') was attached to the 5'-end of primer 933f, and PCR reactions were carried as described by Jorquera et al. (2012). In parallel, the amplification

of *nif*H was done by nested PCR using in the first PCR round the PolF (5'-TGC GAY CCS AAR GCB GAC TC-3') and PolR (5'-ATS GCC ATC ATY TCR CCG GA-3') primer set. After specific ~400 bp bands were confirmed on electrophoresis, a second PCR reaction was carried with the PolFI (5'-TGC GAI CCS AAI GCI GAC TC-3') and AQER-GC30 (5'- GAC GAT GTA GAT YTC CTG GGG-3') primer set. The GC-clamp (5'-CGC CCG CCG CGC CCC GCG CCC GGC CCG CCC GAC GAT GTA GAT YTC CTG-3') was attached to the 5'-end of primer AQER and the PCR conditions were carried out according to described by Jorquera et al. (2014a).

The DGGE analysis was performed using a DCode system (Bio-Rad Laboratories, Inc.). PCR products (20 µL) were loaded onto 6 % (w/v) polyacrylamide gel with a 50–75 % denaturing gradient (7 M urea and 40 % formamide) and electrophoresis was run for 16 h at 80 V. The gel was stained with SYBR Gold (Molecular Probes, Invitrogen Co.) for 30 min and photographed on a UV transilluminator. Image analysis and clustering of DGGE banding profiles were done under CLIQS 1D Pro software (TotalLab Ltd). Based on the matrix obtained from CLIQS 1D Pro analysis, the distances between the bacterial communities from rhizosphere and root endosphere 16S rRNA and *nif*H genes were calculated by similarity profile analysis (SIMPROF test) with Bray-Curtis similarity index with a 5% significance level and <0.15 stress values with Primer-E v6 (Primer-E Ltd.; http://www.primer-e.com/) (Clarke et al. 1993 and 2004). A graphical representation of this results was generated through non-metric multidimensional scaling (nMDS) plots, developed with the same software. The similarities of rhizosphere and root endosphere 16S rRNA and *nif*H communities were compared at 40 and 60%.

3.2.6. Statistical analysis

The culture-dependent (dilutions, plating, and isolation) as well as -independent (DNA extractions, PCR, DGGE, and qPCR) procedures were performed in triplicates and analyzed by one-way ANOVA. Comparisons were carried out for each pair with Tukey HSD test using IBM SPSS Statistics 24 (IBM Corporation). Values are given as means ± standard deviation on means. Differences were considered to be significant when the *P* value was ≤ 0.05.

3.3. Results

3.3.1. Rhizosphere soil properties

No large differences in soil chemical properties were observed between the rhizosphere soils of different wheat cultivars, showing typical characteristics of Chilean Andisols used in agriculture. The properties of rhizosphere soils (Table 1) were as follows: pH ranged from 6.29 to 6.39, organic matter ranged from 15 to 16%, and the ranges for the macronutrients N, P and K were 52.7~63.1, 29~37 and 138~159 mg kg^{-1}, respectively. The cation exchange capacity ranged from 16.08 to 18.01 $cmol_{(+)}$ kg^{-1}, with an Al saturation between 0.06 and 0.25%.

Table 1. Chemical properties of rhizosphere soils used in this study.

Property	Wheat cultivars			
	Feña (F)	**Patras (P)**	**Joker (J)**	**Rocky (R)**
N (mg kg^{-1})	60	52.7	63.1	55.3
P (mg kg^{-1})	35	29	37	31
K (mg kg^{-1})	156	141	138	159
pH (H_2O)	6.35	6.31	6.39	6.29
Organic matter (%)	16	15	16	16
K ($cmol_{(+)}$ kg^{-1})	0.4	0.74	0.25	0.43
Na ($cmol_{(+)}$ kg^{-1})	0.03	0.07	0.02	0.03
Ca ($cmol_{(+)}$ kg^{-1})	15.87	14.35	16.23	14.07
Mg ($cmol_{(+)}$ kg^{-1})	1.46	1.49	1.5	1.51
Al ($cmol_{(+)}$ kg^{-1})	0.01	0.03	0.01	0.04
CEC ($cmol_{(+)}$ kg^{-1})	17.77	16.68	18.01	16.08
Σ Bases ($cmol_{(+)}$ kg^{-1})	17.76	16.65	18	16.04
Al saturation (%)[a]	0.06	0.18	0.06	0.25

[a] Calculated as Al/cation exchange capacity [Σ (K, Ca, Mg, Na, and Al)] × 100]

3.3.2. Counts of total- and N-fixing bacteria

The qPCR analyses indicated that there were larges differences in total bacteria loads between rhizosphere and root endosphere samples (Fig. 1). Significantly greater ($P \leq 0.05$) counts of total bacteria were observed in the rhizosphere (1.8×10^{12}~9.2×10^{13} copies of 16S rRNA genes g^{-1} sample) compared with the root endosphere (2.2×10^{7}~3.6×10^{8} copies of 16S rRNA genes g^{-1} sample) samples. Similarly, significantly greater ($P \leq 0.05$) counts of total N_2-fixing bacteria were also observed in the rhizosphere (3.3×10^{5}~8.1×10^{6} copies of *nif*H gene g^{-1} sample) compared with root endosphere samples (1.7~6.5×10^{5} copies of *nif*H gene g^{-1} sample). However, the differences between rhizosphere and root endosphere samples were lower when counts of the *nif*H gene are compared with 16S rRNA genes.

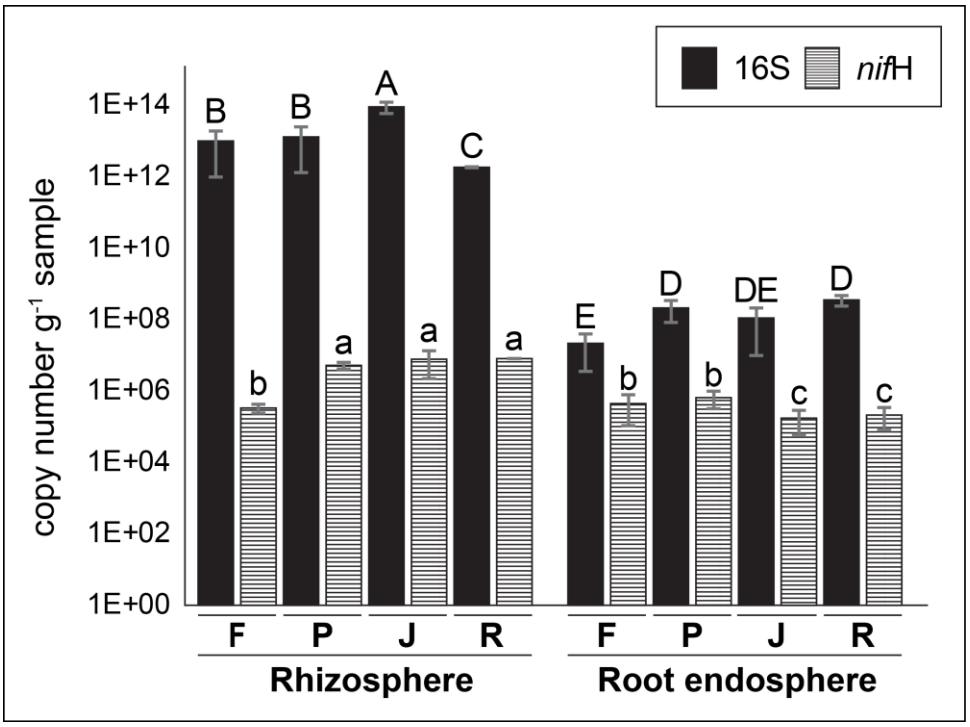

Figure 1. Counts (gene copy number per g of sample) of total and N_2-fixing bacteria in rhizosphere and root endosphere samples of wheat plants by quantitative PCR (qPCR) using specific primer set for 16S rRNA and *nif*H genes. Samples labelled as F, P, J and R correspond to Feña, Patras, Joker and Rocky wheat cultivars, respectively. Error bars represent standard deviation and different lower letters denote statistical difference ($P \leq 0.05$, Tukey HSD test) ($n=3$).

3.3.3. Counts of culturable and putative N_2-fixing bacteria

The use of culture media also showed larges differences in total culturable bacterial counts between rhizosphere and root endosphere samples (Fig. 2a). Significantly greater ($P \leq 0.05$) counts of total cultural bacteria were observed in the rhizosphere (1.1×10^{9}~1.9×10^{10} and 9.5×10^{7}~1.6×10^{8} CFU g^{-1} sample with LB and NM1 media, respectively) compared with root endosphere samples (1.9×10^{4}~3.4×10^{5} and 1.2~5.1×10^{4} CFU g^{-1} sample) on the same media. Smaller distances (less than one order) ($P \leq 0.05$) were observed between on putative N_2-fixing culturable bacteria in CRMA medium for rhizosphere samples (2~8.2×10^{3} CFU g^{-1}) compared with those from the root endosphere (8.5×10^{2}~1.9×10^{3} CFU g^{-1}) (Fig. 2b).

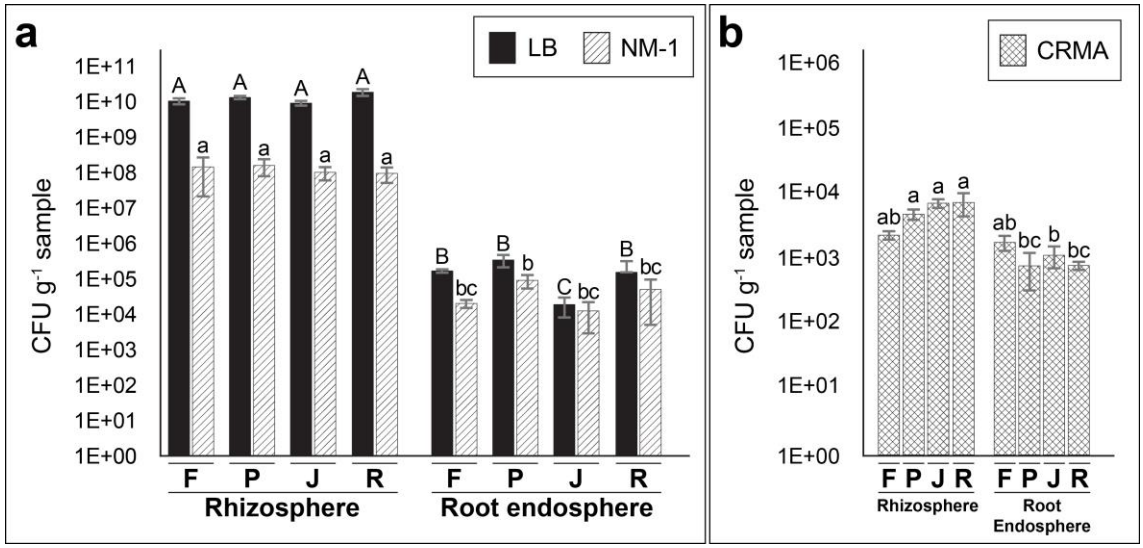

Figure 2. Counts (CFU per g of sample) of total culturable (a) in rhizosphere and root endosphere samples of wheat plants using general (LB and NM–1) and putative N_2-fixing bacteria (b) with selective (Congo red malic–acid; CRMA) media. Samples labelled as F, P, J and R correspond to Feña, Patras, Joker and Rocky wheat cultivars, respectively. Error bars represent standard deviation and different lower letters denote statistical difference ($P \leq 0.05$, Tukey HSD test) (n=3).

3.3.4. Characterization of putative N_2-fixing bacteria

Thirty-eight of 77 strains examined with ERIC-PCR analysis (49.3%) were recognized as non-redundant putative N_2-fixing bacterial isolates, whose taxonomic assignments are shown in Table 2. Based on partial sequencing of 16S rRNA genes, most of isolates from the rhizosphere samples had an affiliation with the genus *Bacillus* (15 of 20 isolates), followed by members of genera *Microbacterium* (3 isolates), *Chitinophaga* (1) and *Arthrobacter* (1) genera. In contrast, only 4 of 18 isolates from the root endosphere samples were characterized as member of the genus *Bacillus* based on sequencing of 16S rRNA genes. Other isolates were characterized as being members of the genera *Roseomonas* (3), *Mycobacterium* (3), *Georgenia* (2 isolates), *Bosea* (2), *Microbacterium* (1), *Psychrobacillus* (1), *Chitinophaga* (1) and *Leifsonia* (1).

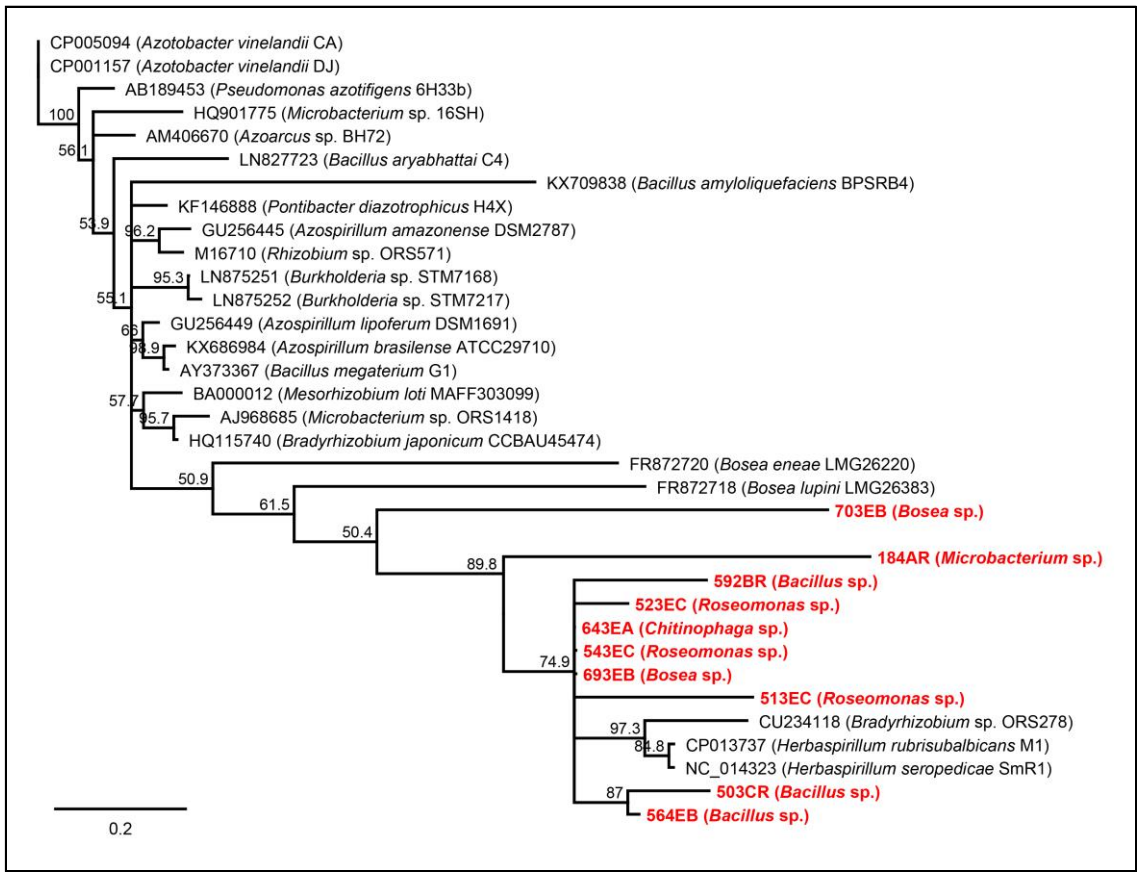

Figure 3. Neighbor-joining tree showing the phylogenetic affiliation between predicted amino acid sequences from *nif*H gene obtained from rhizosphere and root endosphere isolates in this study (red) and representative *nif*H-coded enzyme amino acid sequences from known representative plant-soil bacteria deposited in NCBI GenBank database (black). Scale represents substitution sites (Bootstrap = 1,000). In parenthesis is shown the accession number of representative sequences in GenBank or the taxonomic affiliation based on 16S rRNA gene sequencing of isolates.

Table 2. Taxonomic identity of putative N_2-fixing bacteria isolates obtained by sequencing of 16S rRNA genes.

Isolate	Taxonomy group[a]	Closest relatives or cloned sequences (accession no.)[b]	Identity	Accession no.
Rhizosphere				
62CR	*Actinobacteria; Micrococcales; Microbacteriaceae; Microbacterium*	*Microbacterium proteolyticum* from *Halimione portulacoides* roots (NR_132869)	99%	MG835569
72CR	*Firmicutes; Bacilli; Bacillales; Bacillaceae; Bacillus*	*Bacillus megaterium* strain p102_H06 from maíze rhizosphere (JQ832067)	99%	MG835570
102BR	*Firmicutes; Bacilli; Bacillales; Bacillaceae; Bacillus*	Nitrogen-fixing *Bacillus megaterium* strain ADU08 from date palm soil (KX694270)	99%	MG835571
112BR	*Firmicutes; Bacilli; Bacillales; Bacillaceae; Bacillus*	*Bacillus megaterium* strain ARD47 from acid soil (KX023249)	100%	MG835572
154AR	*Firmicutes; Bacilli; Bacillales; Bacillaceae; Bacillus*	*Bacillus* sp. strain SZ177 from straw decomposition (KU986708)	99%	MG835573
173CR	*Firmicutes; Bacilli; Bacillales; Bacillaceae; Bacillus*	*Bacillus megaterium* strain PSC1 from sugarcane rhizosphere (KU196781)	99%	MG835574
184AR	*Actinobacteria; Micrococcales;7 Microbacteriaceae; Microbacterium*	Diazotrophic *Microbacterium* sp. S2SP302 from sugarcane rhizosphere (KT183549)	100%	MG835575
184AR-1	*Firmicutes; Bacilli; Bacillales; Bacillaceae; Bacillus*	*Bacillus megaterium* strain yangyueK8 from *Juglans regia* rhizosphere (KU977121)	100%	MG835576
214AR	*Firmicutes; Bacilli; Bacillales; Bacillaceae; Bacillus*	*Bacillus megaterium* strain yangyueN10 from *Juglans regia* rhizosphere (KU977110)	100%	MG835577
214AR-1	*Firmicutes; Bacilli; Bacillales; Bacillaceae; Bacillus*	*Bacillus megaterium* strain yangyueN10 from *Juglans regia* rhizosphere (KU977110)	100%	MG835578
222BR	*Firmicutes; Bacilli; Bacillales; Bacillaceae; Bacillus*	*Bacillus* sp. MR35 from rice rhizosphere (LT629146)	99%	MG835579
623EA	*Bacteroidetes; Chitinophagia; Chitinophagales; Chitinophagaceae; Chitinophaga*	*Chitinophaga arvensicola* strain MRP-16 from *Dioscorea batatas* rhizoplane (AB908086)	99%	MG835588
243AR	*Actinobacteria; Micrococcales; Micrococcaceae; Arthrobacter*	*Arthrobacter* sp. strain JQ-1 from phtalate contaminated soil (KX055564)	100%	MG835580
Isolate	**Taxonomy group[a]**	**Closest relatives or cloned sequences (accession no.)[b]**	**Identity**	**Accession no.**
322CR	*Firmicutes; Bacilli; Bacillales; Bacillaceae; Bacillus*	*Bacillus megaterium* strain ARD47 from acid soil (KX023249)	100%	MG835583
342CR	*Firmicutes; Bacilli; Bacillales; Bacillaceae; Bacillus*	*Bacillus huizhouensis* strain WJB150 from rice rhizosphere 1(KU877672)	100%	MG835584
354AR	*Actinobacteria; Micrococcales; Microbacteriaceae; Microbacterium*	*Microbacterium* sp. SIB_Cu_R3 from *Betula pendula* L. rhizosphere (KX036571)	100%	MG835585

354AR-1	*Firmicutes; Bacilli; Bacillales; Bacillaceae; Bacillus*	*Bacillus megaterium* strain Hd from arsenic contaminated soil (KY098770)	100%	MG835586
372EC	*Firmicutes; Bacilli; Bacillales; Bacillaceae; Bacillus*	Nitrogen-fixing *Bacillus megaterium* strain ADU08 from date palm soil (KX694270)	99%	MG835587
503CR	*Firmicutes; Bacilli; Bacillales; Bacillaceae; Bacillus*	*Bacillus aryabhattai* strain He from arsenic contanimated soil (KY098771)	100%	MG835581
503CR-1	*Firmicutes; Bacilli; Bacillales; Bacillaceae; Bacillus*	*Bacillus amyloliquefaciens* strain Y14 from peanut rhizosphere (CP017953)	99%	MG835582

Root endosphere

Isolate	**Taxonomy group[a]**	**Closest relatives or cloned sequences (accession no.)[b]**	**Identity**	**Accession no.**
223EC	*Actinobacteria; Corynebacteriales; Mycobacteriaceae; Mycobacterium*	*Mycobacterium* sp. Site1-11a from manure-fertilized grassland (JF304596)	99%	MG835593
382EC	*Actinobacteria; Micrococcales; Microbacteriaceae; Microbacterium*	*Microbacterium* sp. SIB_Cu_R3 from *Betula pendula* L. rhizosphere (KX036571)	100%	MG835590
424EC	*Actinobacteria; Micrococcales; Bogoriellaceae; Georgenia*	*Georgenia soli* strain MC-14-2 from *Populus euphratica* endosphere (KF848489)	99%	MG835591
444EC	*Firmicutes; Bacilli; Bacillales; Bacillaceae; Psychrobacillus*	*Psychrobacillus psychrodurans* strain EC1 from corn roots (KP334982)	99%	MG835592
491EC	*Actinobacteria; Corynebacteriales; Mycobacteriaceae; Mycobacterium*	*Mycobacterium* sp. strain OTB74 from Sandy soil (KN022865)	99%	MG835594
513EC	*Proteobacteria; Alphaproteobacteria; Rhodospirillales; Acetobacteraceae; Roseomonas*	*Roseomonas* sp. A2 from tomato rhizosphere (KP789481)	99%	MG835595
523EC	*Proteobacteria; Alphaproteobacteria; Rhodospirillales; Acetobacteraceae; Roseomonas*	*Roseomonas* sp. Esch5-313 from poplar endosphere (AM489616)	99%	MG835596
543EC	*Proteobacteria; Alphaproteobacteria; Rhodospirillales; Acetobacteraceae; Roseomonas*	*Roseomonas* sp. strain THG-N2.22 from soil (KX456186)	98%	MG835598
564EB	*Firmicutes; Bacilli; Bacillales; Bacillaceae; Bacillus*	*Bacillus amyloliquefaciens* strain Y14 from peanut rhizosphere (CP017953)	100%	MG835599
584EA-1	*Firmicutes; Bacilli; Bacillales; Bacillaceae; Bacillus*	*Bacillus aryabhattai* strain He from arsenic contanimated soil (KY098771)	100%	MG835600
592BR	*Firmicutes; Bacilli; Bacillales; Bacillaceae; Bacillus*	*Bacillus aryabhattai* strain He from arsenic contanimated soil (KY098771)	100%	MG835601
643EA	*Bacteroidetes; Chitinophagia; Chitinophagales; Chitinophagaceae; Chitinophaga*	*Chitinophaga* sp. W-9 from tomato rhizosphere (KX082640)	99%	MG835602
693EB	*Proteobacteria; Alphaproteobacteria; Rhizobiales; Bradyrhizobiaceae; Bosea*	*Bosea* sp. strain SR 5-12 from *Artemisa prínceps* endorhiza (KM253172)	100%	MG835603
703EB	*Proteobacteria; Alphaproteobacteria;*	*Bosea* sp. strain J1 from soil (KP125320)	100%	MG835604

	Rhizobiales; Bradyrhizobiaceae; Bosea			
274EB	*Actinobacteria; Micrococcales; Microbacteriaceae; Leifsonia*	*Leifsonia* sp. URHA0017 from grassland rhizosphere (LN876290)	99%	MG835589
223EC	*Actinobacteria; Corynebacteriales; Mycobacteriaceae; Mycobacterium*	*Mycobacterium* sp. Site1-11a from manure-fertilized grassland (JF304596)	99%	MG835593
714EA	*Firmicutes; Bacilli; Bacillales; Bacillaceae; Bacillus*	*Bacillus megaterium* strain Hd (KY098770)	100%	MG835605
734EA	*Actinobacteria; Micrococcales; Bogoriellaceae; Georgenia*	*Georgenia soli* strain CC-NMPT-T3 from iron ore-contaminated soil (NR_116959)	98%	MG835606

[a] The phylogenetic assignment is based on sequence analysis by BLASTn of GenBank database from NCBI (http://www.ncbi.nlm.nih.gov). It is given the phylum as well as the lowest predictable phylogenetic rank.

[b] Based on partial sequencing of 16S rRNA gene and comparison with those present in GenBank by using BLASTn algorithm.

BLASTx analyses (Table 3) predicted eight amino acid sequences (1 from the rhizosphere and 7 from the root endosphere) that coded the nitrogenase-characteristic P-loop NTPase conserved superfamily domain. The neighbor-joining tree analysis also revealed that our partial predicted nitrogenase-like enzymes showed higher dissimilarities compared with those representatives of nitrogenase enzymes taken from GenBank (Fig. 3), except for sequences from *Chitinophaga* sp. 643EA and *Roseomonas* sp. 523EC.

Table 3. Characterization of predicted nitrogenase enzymes obtained in this study.

Isolate	Closest relative (accession no.)[a]	Identity	Accession no.
Rhizosphere			
503CR-1	Dinitrogenase reductase from uncultured bacterium of saline-alkaline soil (AEO13485)	79%	MH175490
Root endosphere			
513EC	Nitrogenase iron protein from uncultured bacterium of Sorghum rhizosphere (ABW87180)	48%	MH175481
523EC	Nitrogenase reductase from endosymbiont *Burkholderia* sp. (AF194084)	100%	MH175482
543EC	Dinitrogenase reductase from uncultured bacterium of saline-alkaline soil (AEO13447)	94%	MH175483
564EB	Dinitrogenase reductase from *Azospirillum zeae* of wheat rhizosphere (CBL85086)	85%	MH175484
592BR	Dinitrogenase reductase from uncultured bacterium of *Spartina alterniflora* biomass (AAK91227)	68%	MH175485
643EA	Nitrogenase reductase from *Azospirillum doebereinerae* of *Miscanthus* plant roots (ACO35353)	99%	MH175486
693EB	Nitrogenase reductase from *Azospirillum thiophilum* of sulfide spring (ACO35352)	92%	MH175487

[a] Assignment based on the closest sequence according to BLASTx analysis.

3.3.5. Fingerprinting of total and N_2-fixing bacterial communities

Fingerprint analysis of bacterial communities by DGGE revealed significant differences ($P \leq 0.05$) between the total bacterial communities found in rhizosphere and root endosphere samples from the wheat cultivars examined (Fig. 4a). Two clusters were clearly observed at the 40% similarity level. However, a specific bacterial community for each of the wheat

cultivars was not observed, even at higher similarity percentages (60%). Similarly, significant differences ($P \leq 0.05$) between rhizosphere and root endosphere samples of wheat cultivars with respect to *nif*H-harboring bacterial populations. However, a specific *nif*H-harboring bacterial community for each cultivar was not observed at higher similarity (60%), except for the root endosphere samples from wheat cv 'Feña' (Fig. 4b).

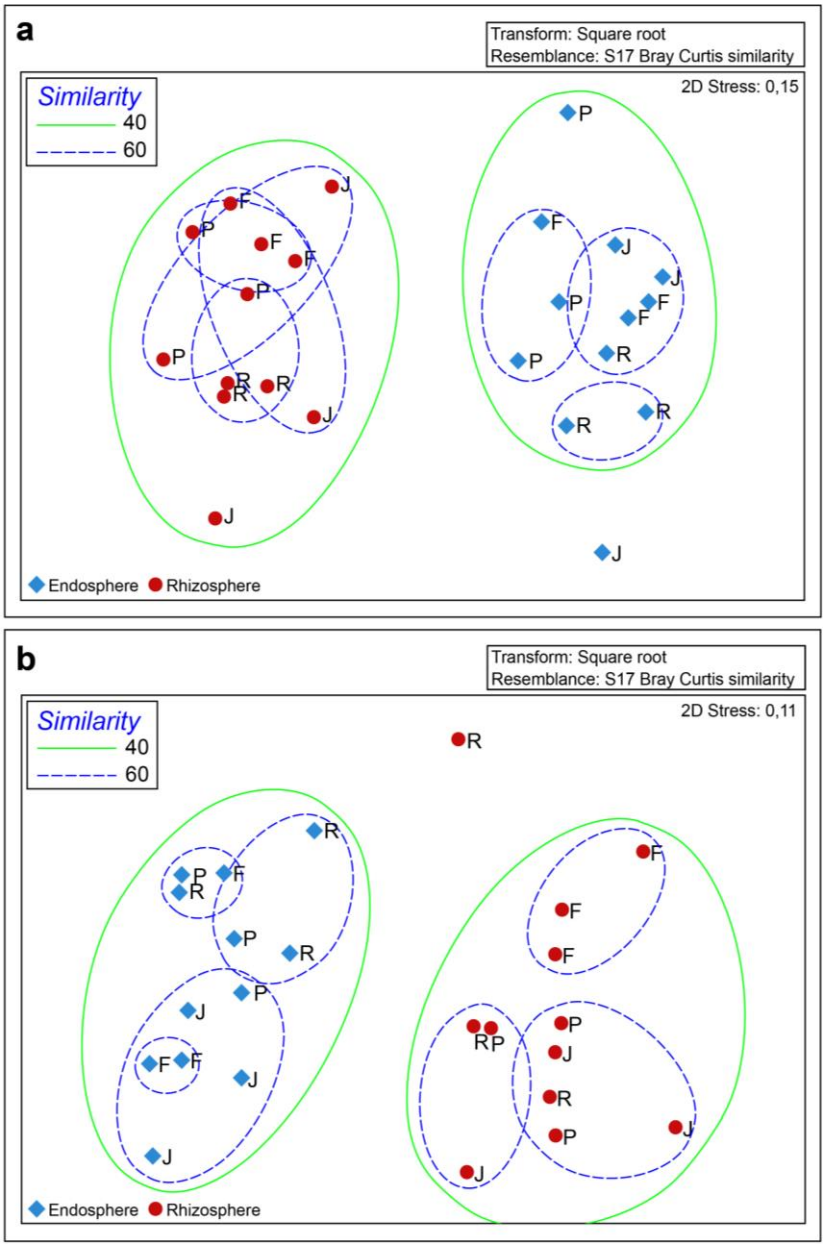

Figure 4. Non-metric multidimensional scaling (nMDS) analysis of DGGE fingerprinting analysis generated by Primer v6 software (http://www.primer-e.com) with the Bray-Curtis

similarity index, 5 % significance level, and < 0.1 stress values. Red and blue shapes represent rhizosphere (a) and root endosphere (b), respectively. Samples labelled as F, P, J and R correspond to Feña, Patras, Joker and Rocky wheat cultivars, respectively.'

4. Discussion

Nitrogen is an essential nutrient for plant growth and N_2-fixing bacteria play an important role in plant nutrition. Studies focused to N_2-fixing bacteria in Chilean agroecosystems are scarce, particularly with respect to cereal cropping systems. By using culture-dependent methods, N_2-fixing bacteria have previously been isolated from alfalfa and lupin plants grown in Andisols from southern Chile (Campos et al. 2014; Langer et al. 2008). Culture-independent methods based on partial sequencing of 16S rRNA genes have also revealed the occurrence of *Sinorhizobium* strains on wheat (Jorquera et al. 2014b) and *Azospirillum* in ryegrass (Lagos et al. 2014) rhizospheres. In addition, and to our knowledge, no studies on endophytic N_2-fixing bacteria in Chilean agroecosystems have not been done so far.

In this study, qPCR revealed the occurrence of *nif*H-harboring bacterial population in all samples of wheat cultivars analyzed, with a significantly higher abundance of total and N_2-fixing bacteria in the rhizosphere, compared with root endosphere samples. Our results showed a large number of copies, 10^{12}~10^{13} of 16S rRNA genes g^{-1} are present in rhizosphere soils, which is two orders of magnitude greater than those reported by Sanguin et al. (2009) in rhizosphere soils from wheat. Lower abundances (~10^8 gene copies g^{-1}) were also reported in wheat rhizospheres by Reardon et al. (2014). The difference between our results and other wheat studies in literature could be attributed to the variability of 16s rRNA copy number of present in environmental bacteria, which could contain as much as

15 copies per cell (Kembel et al. 2012; Větrovský and Baldrian, 2013). Similarly, 16S rRNA copies in cotton plants has been reported around the values that we report here (10^{12} copies g^{-1} rhizosphere; Zhang et al. 2016).

In relation to the abundance of *nif*H genes, the values obtained in this study were close to those reported by Reardon et al. (2014). We found ~10^6 copies of *nif*H genes g^{-1} of rhizosphere soils in wheat plants. In contrast, Bouffaud et al. (2016) reported greater *nif*H gene abundances (~10^9 gene copies g^{-1}) in rhizosphere soils of wheat plants.

Reported counts of 16S rRNA genes, by qPCR, in inner tissues or root endosphere of plants are scarce because of the potential for biased results due to the presence of ribosomes in chloroplasts and mitochondria (Shade et al. 2013). The use of the chloroplast- and mitochondria-excluding primer set 799f and 1115r (Shade et al. 2013) has been reported to produce reliable qPCR results on root endosphere samples (Beckers et al. 2016). Our results showed ~ 10^{7}-10^{8} copies of 16S rRNA genes g^{-1} root, which are similar to those obtained by Ruppel et al. (2006) in the rice endosphere. Higher loads of endophytic bacteria (10^{10}~10^{13} copies of 16S rRNA genes g^{-1} root) were reported in a rice-maize rotation (Breidenbach et al. 2017). In relation to *nif*H genes, studies have reported abundances of ~10^8 copies of *nif*H genes g^{-1} root in wheat and rapeseed plants (Bouffaud et al. 2016; Puri et al. 2016). Both studies reported three orders higher *nif*H gene numbers, compared to those obtained in this study (~10^5 genes copies g^{-1} root).

Similar to qPCR results, bacterial numbers obtained by the plate-counting studies done here suggested the occurrence of culturable N_2-fixing bacteria in all samples of wheat cultivars analyzed, with a significant higher abundance in rhizosphere compared with root endosphere samples. Our results showed counts of total culturable bacteria of 10^9~10^{10} CFU g^{-1} and 10^7~10^8 CFU g^{-1} on LB and NM-1 media, respectively. In this context,

Jorquera et al. (2014b) reported counts of 10^7 CFU g^{-1} rhizosphere on cereals (wheat and oats) and pastures (ryegrass) by using NM-1 medium, similar to the results described by Jia et al. (2015) in wheat rhizosphere on meat-peptone agar (10^8 CFU g^{-1} rhizosphere). In addition, the counts of culturable bacteria in root endosphere samples we examined (10^4-10^5 CFU g^{-1} root) were similar to those observed on wheat roots by Ruppel (1989) and Robinson et al. (2016) with counts of ~10^4 CFU g^{-1} root in wheat.

Our counts of putative N_2-fixing bacteria in the rhizosphere (10^3 CFU g^{-1}) of wheat were lower than those reported in the wheat (10^4 CFU g^{-1}), chickpea and sugarcane (10^4-10^5 CFU g^{-1}) rhizospheres examined by Neemisha et al. (2014) and Ahmad et al. (2006). This may, in part, due to different culture conditions used in each study. However, our counts of endospheric N_2-fixing bacteria (10^3 CFU g^{-1} root) are similar to those obtained by Ruppel et al (1989) (10^4 CFU g^{-1} root) in wheat and Patel & Archana (2017) in several *Poaceae* plant tissues (10^3 – 10^5 CFU g^{-1} root). Most studies on N_2-fixing bacterial communities in the wheat root endosphere have examined colonization niches and physiological effect of different diazotrophic endophytes (Liu et al. 2017a; Liu et al. 2017b), instead of determining how abundant are the N_2-fixing root endosphere communities. That said, however, it is well known that culture medium type greatly affects the reported numbers of bacteria obtained via plate-counting. Media bias is always an issue in examining microbiota in environmental niches. In this context it has been described that the use of diluted of culture media improves CFU number determination and enhance isolation of N_2-fixing bacteria (Janssen et al. 2002; Hashimoto et al. 2009). Despite this limitation, however, our analyses do allow relative comparisons of total and N_2-fixing microbes in the plant compartments we examined.

Sequencing of 16S rRNA genes of rhizosphere isolates revealed the occurrence of members of genera *Bacillus, Microbacterium, Chitinophaga* and *Arthrobacter.* Most of isolates were characterized as belonging to the genus *Bacillus,* which is a common inhabitant in the rhizosphere soil of plants grown in Andisols from southern Chile (Jorquera et al. 2011; Martinez et al. 2011). In this context, diazotrophic *Bacillus* sp. strains have been shown to be associated with N_2-fixation in wheats (Neemisha et al. 2014), as well as sugarcane (Madhaiyan et al. 2011). It is noteworthy that most of isolates characterized as *Bacillus* (10 of 15) were phylogenetically close to *Bacillus megaterium,* a well-known N_2-fixing and phosphate-solubilizing bacterium commonly studied as plant growth-promoting bacteria (Ding et al. 2005; Elkoca et al. 2008). The remaining five rhizosphere isolates we examined were characterized as *Microbacterium, Chitinophaga,* and *Arthrobacter* sp. strains, these microorganisms were previously proposed to be associated with N_2-fixation in the rhizospheres of other plant species (Mirza et al. 2012; Beneduzi et al. 2013; Moyes et al. 2016).

Our results also showed the occurrence of members of the genera *Bacillus, Georgenia*, *Mycobacterium*, *Bosea*, *Microbacterium*, *Psychrobacillus*, *Roseomonas*, *Chitinophaga* and *Leifsonia* genera in the root endosphere of wheat. Bacteria belonging to the phylum Actinobacteria, such as members of the genera *Georgenia, Mycobacterium,* and *Leifsonia*, have been described as common inhabitants of the root endosphere of plants, as well as have many diazotrophs (Martenson et al. 2009; Han et al. 2011; Liaqat et al. 2016). It is noteworthy, that the isolation of Proteobacteria belonging to the genera *Bosea* and *Roseomonas* have not been previously reported either soils or *in planta* in Chile. Isolates characterized as *Bosea* have been isolated from lupin root nodules by De Meyer and Williams (2012). In this context, *Bosea* spp. appear to be related to bacteria within the

genus *Rhizobium*, well known for forming N_2-fixation symbioses with legumes worldwide, as well as with plants grown in acidic soils in Chile (Langer et al. 2008).

In addition, despite that our analysis with BLASTx suggested the presence of the nitrogenase enzyme in genomes of our isolates; the neighbor-joining tree analysis did not show a high similarity when our predicted nitrogenase-like enzymes were compared with representative nitrogenases taken from GenBank. This result also might explain the low specificity of universal primer sets found in the literature and used in this study (Supplementary Table 1), which could not adequately cover the nitrogenases harbored by native bacteria living in Chilean Andisols. Accordingly, and as discussed by Gaby et al. (2012), while several universal primers have been designed and empirically tested for nitrogenase, some of them can generate false positive reactions; and therefore, primers must be used with caution and validated with genomic DNA from phylogenetically diverse N_2-fixing strains from different environments.

Likewise, members of the genus *Roseomonas* have been recognized as a PGPB found in a wide variety of environments, including the in rhizospheres of rice (Ramaprasad et al. 2015) and Chinese cabbage (Kim and Ka, 2014), and in contaminated soils (Chen et al 2014). In this study, we noted that there were large differences in microbiota present in rhizosphere and root endosphere samples. Similarly, Robinson et al. (2015) found differences in endophytic bacteria between roots and leaves, which were attributed to tissue type, phenological stage of plants, and soil nutrient availability. Bouffaud et al. (2016) also proposed that plants recruit its own N_2-fixing endophytic microbiome. These differences in the composition and structure of bacterial communities between rhizosphere and root endosphere were also confirmed by DGGE. Our results suggest a compartmentalization between rhizosphere and root endosphere for both studied communities (16s rRNA and

*nif*H). Such separation has been described as being common in plants (Mahaffee and Kloepper, 1997) and we propose that these differences might also be influenced by a combination of different factors, including soil composition (pH, organic matter, and nutrients), soil management (fertilization, rotation, and tillage) and plant (genotype, phonological stages, and defense mechanisms) and the presence of other microbial communities (fungi, nematode, and protozoa).

In Chilean Andisols, as wells as other agroecosystems, our knowledge on N_2-fixing bacterial populations associated with plants is very limited. In this sense, based on the relevance of plant microbiome upon fitness and production of crops, an exhaustive study on the abundance, diversity and activity of N_2-fixing bacterial populations could be essential to the develop of novel fertilizers and management agronomic strategies to improve the efficiency of N fertilization in the field with the consequent low cost for the farmers and environmental benefits.

3.5. Acknowledgements

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CHAPTER IV

PCR of Clustered repetitive interspaced palindromic short repeats (CRISPR-PCR) as tool for Azospirillum sp. B510 tracking

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Combined use of PCR and clustered repetitive interspaced palindromic short repeats (CRISPR-PCR) as tool for tracking of *Azospirillum* sp. B510

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Abstract

Azospirillum spp. are widely used as microbial inoculants to improve the nutrition and growth of crops in agriculture worldwide. However, the colonization and persistence of *Azospirillum* strains after inoculation is scarcely tracked and their effect on crops is indirectly measured such as biomass increase, nitrogen content, productivity, etc. Despite that molecular methods have been proposed for tracking of specific inoculated strains in agriculture, they are expensive, laborious and strain–unspecific at field level; therefore, *Azospirillum* strains are generally estimated by plate–counting methods, which is also laborious, time–consuming and strain–unspecific. On the other hand, studies have revealed the occurrence of clustered regularly interspaced palindromic short repeats (CRISPR) loci in the genome of several bacterial taxa, including *Azospirillum* genus, which play a pivotal role in the defense system against extragenomic DNA elements. CRISPR loci are specific for *Azospirillum* strains and here we propose the use of its structure as a potential molecular marker for tracking of inoculated strains. Specific PCR primer sets for CRISPR loci repeats of *Azospirillum* sp. B510 were designed and evaluated by conventional endpoint PCR. The specificity of CRISPR-PCR for *Azospirillum* sp. B510 strain was standardized and demonstrated against other two collection strains, seven native *Azospirillum* strain isolates, as well as thirty–eight native strains from wheat plants. In addition, colony CRISPR-PCR was validated in a wheat seedlings assay mounted in sterile and unsterile substrates (1:1:2 soil:perlite:pea) with and without inoculation of *Azospirillum* sp. B510 strain. The presence of *Azospirillum* sp. B510 strain in plant compartments (rhizosphere and root endosphere)

was detected at two weeks after inoculation in both sterile and unsterile substrates. To our knowledge, this is the first approach using CRISPR-PCR as a molecular tool for *Azospirillum* tracking in inoculated plants. However, further studies are still required for its validation as an inexpensive, less laborious and strain–unspecific molecular tool at field level in agriculture.

Keywords: *Azospirillum*, CRISPR, tracking, colony PCR, soil inoculants.

4.1. Introduction

Biofertilizer products based on microbial inoculants are currently commercialized worldwide. Predictions suggest that by 2022 the revenues obtained from biofertilizers could reach between USD 1.65 billion in USA, more than 100% increase since 2016 (787.8 million) (Grand View Research, 2016). In this context, members of *Azospirillum* genus are one of the most studied and used microbial inoculants in agriculture because to their growth–promoting functions, mainly atmospheric nitrogen (N) fixation and phytohormones production (*e.g.*, auxins). *Azospirillum* spp. have also shown the ability to colonize inner plant tissues (*e.g.*, root and shoot endosphere), solubilize organic phosphorus (P), phytopathogens biocontrol, reduction of abiotic stress in plants by ACC (1–aminocyclopropane–1–carboxylate) deaminase activity, among others (Hartmann & Baldani, 2006). Thus, *Azospirillum* spp. have been applied in more than 133 plant species, including 35 families of cereals, legumes, vegetables, grasses, weeds and wild plants, and resulting in root and shoot elongation, grain yield increase, nutrition improvement, higher photosynthetic rate, and higher protein content under different inoculation strategies (Pereg et al. 2015). These growth–promoting traits have raised a huge commercial interest, particularly in South America, where 1 and 6 million ha^{-1} were sowed in 2016 with soybean, corn, wheat and winter grasses in Argentina and Brazil, respectively (Coniglio et al. 2019).

An efficient colonization of crops and pastures by inoculated *Azospirillum* spp. is pivotal to increase the yields in agroecosystems; therefore, specific colonizing mechanisms are desired in *Azospirillum* strains used as inoculants. In this context, intrinsic factors such as exo and lipopolysaccharides production and flagellar motility by can be determinant in

terms of competence and colonization (Rossi et al. 2016 and Greer-Phillips et al. 2004). Similarly, comparative genomics have showed that despite that core genome in *Azospirillum* genus present large similarities, unique genetic determinants can also be related to niche–specific adaptation, affecting their plant–inoculum compatibility (Wisniewski-Dyé et al. 2012). Complementarily, extrinsic factors can also be relevant as determinants in the efficient colonization of *Azospirillum* spp. In this sense, agricultural practices exert an effect on the colonization of inoculated bacterial strains. As example, different N sources (KNO_3, NH_4Cl, $CO[NH_2]_2$, and NH_4NO_3) can influence the endosphere colonization of *Azospirillum* sp. B510 in rice (Naher et al. 2018). Plant genotypes also exert selection in their associated microbiome as well as history and management of soils affects the rhizosphere community structures and their functions involved in N cycling (Schmidt et al. 2019), where *Azospirillum* spp. applications are evaluated. In addition, how (*e.g.*, seed coating, foliar spraying, etc.), how much (10^n cells per mL^{-1} or g^{-1} or cm^2), and when (once, months, seasonally, etc.) are they inoculated must also be considered in terms of efficient colonization of inoculants (Bashan et al. 2014; Puente et al. 2019). Despite of the importance of colonization and prevalence in the use of microbial inoculants in agriculture, ~80% of the published scientific articles do not considered them, and their effects are indirectly measured as biomass gained, productivity increased, nutrient content improved, etc. (Rilling et al. 2019).

Once inoculated, the tracking of *Azospirillum* spp. and other microbial inoculants has been mainly analyzed under laboratory conditions, where a variety of culture–dependent methods based on reporter genes (*e.g.*, *lac*Z, *gus*A and *lux*) and culture–independent merthods based on chromosome gene markers (*e.g.*, fluorescent *in situ* hybridization and qPCR) have been used; however, most of them present biases in terms

sensitivity and/or specificity when are used in complex matrixes such as the soil-plant continuum (Rilling et al. 2019). Under fields conditions, the current approximation for the tracking of inoculated *Azospirillum* spp. still relies in culture–dependent methods, mainly plate-counting on differential or selective media agar, which after laborious work and timeconsuming procedure, do not give data on strains under unculturability as well as specific counts at strain level. Therefore, a specific and sensitive method are required for the tracking *Azospirillum* spp. and other microbial inoculants in agriculture.

During last decade, studies have revealed that a large proportion of prokaryotes (bacteria and archaea) present clustered regularly interspaced short palindromic repeats (CRISPRs) in their chromosome. The CRISPRs loci have a particular structure, where extragenomic DNA (28-60 bp) is incorporated after being cleaved. As more extragenomic elements are incorporated, the loci increase its size. New fragments are interspaced by repetitive sequences (28-60 bp) providing a fingerprinting specific for each strain, including members of *Azospirillum* genus (Rilling et al. 2019). Based on this, we hypothesized that CRISPR loci structure can be used as strain–specific chromosome marker for *Azospirillum*, which can be revealed by endpoint PCR, an inexpensive and less laborious technique with the potential to be used for tracking of strains at commercial level. In this study, we developed a PCR–CRISPR approach based on combined use of a designed primer set for CRISPR loci and standardized endpoint PCR reaction for strain–specific detection of *Azospirillum* sp. B510 (Fig. 1). In addition, the use of PCR–CRISPR approach as tracking tool was validated in two plant niches (rhizosphere and endosphere) through a inoculation wheat seedlings assay.

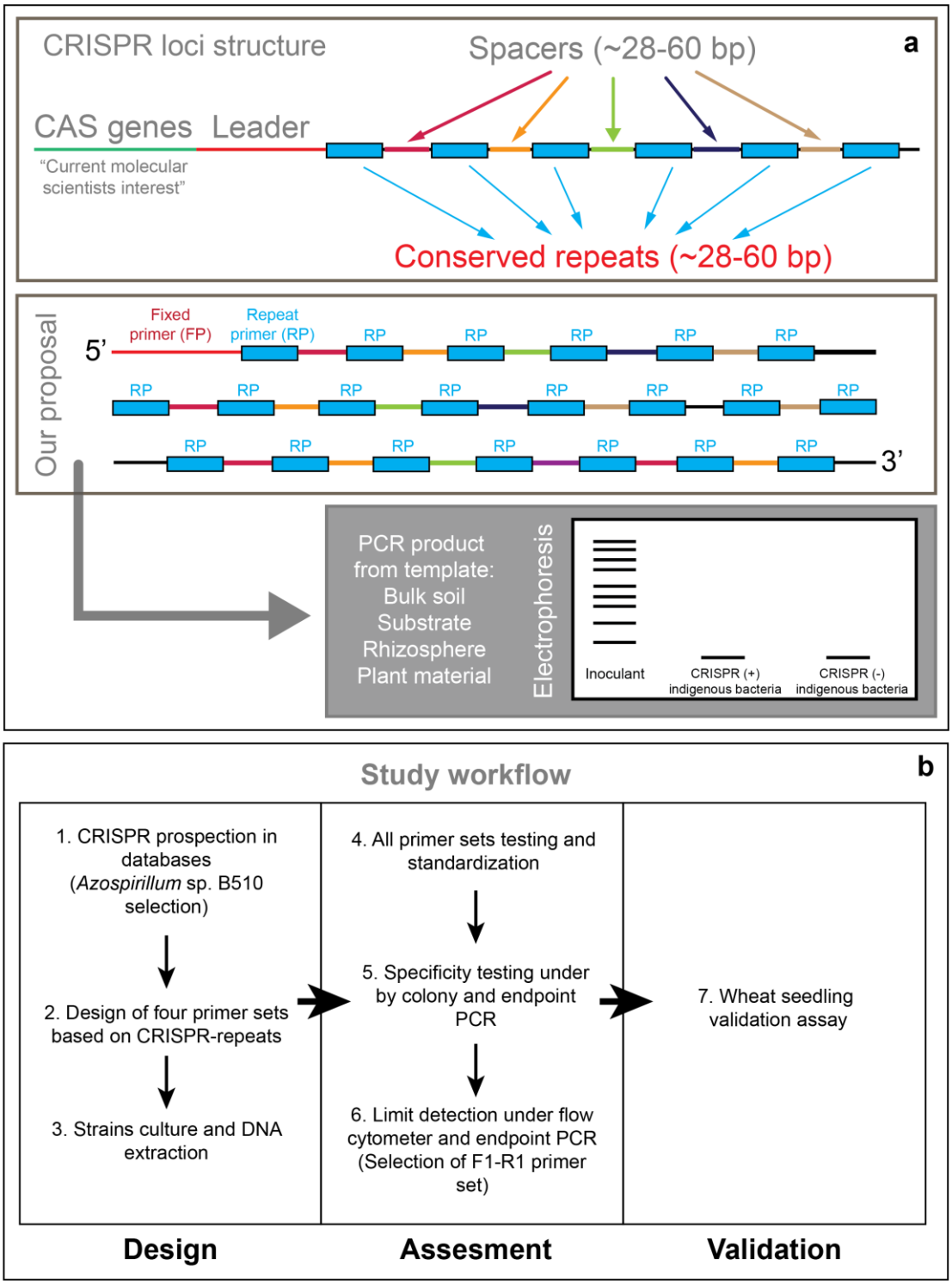

Figure 1. A) Proposed strategy for the strain-specific tracking of *Azospirillum* spp. based on combined use of PCR and CRISPR loci structure. B) Methodology workflow applied on this study.

4.2. Methodology

4.2.1. Bacterial strains used in this study

The search of CRISPR loci was performed in *Azospirillum* genomes available by using the following databases: CRISPRdb (https://crispr.i2bc.paris-saclay.fr/), CRISPI (https://genoweb1.genouest.org/Serveur-GPO/outils/repeatsAnalysis/CRISPR/index.php), and NCBI genome (https://www.ncbi.nlm.nih.gov/genome) databases. Thirteen representatives (among sixteen) of *Azospirillum* presented either CRISPR or putative CRISPR loci, where *Azospirillum* sp. B510 showed two longer CRISPR loci with lengths of 2494 bp and 5243 bp and repeat fragment sizes of 37 bp and 37 bp, respectively. Thus, *Azospirillum* sp. B510 (Elbeltagy et al. 2001) was selected target bacterium in this study and purchased from Japan Collection of Microorganisms (http://www.jcm.riken.jp). *Azospirillum* sp. B4 (Elbetagy et al. 2000) and *Azospirillum* sp. B506 (Elbetagy et al. 2001), both harboring CRISPR loci, were also acquired from the Japanese collection and used as negative controls of *Azospirillum* strains in this study. Native *Azospirillum* strains isolated from wheat rhizosphere (Acuña et al. 2020) were also chosen and used as negative controls of *Azospirillum* strains. In addition, negative controls of *Azospirillum* strains were complemented with twenty rhizosphere strains and eighteen endosphere strains of different taxa isolated from wheat plants. All strains used in this study are listed in Table 1.

Table 1. Bacterial strains used in this study.

Strain	Plant niche source	Country	16S rRNA gene accession no.[a]	Reference
Target bacterium				
Azospirillum sp. B510	Oryza sativa stems	Japan	AB049111	Elbetagy et al. (2001)
Negative Azospirillum strains				
Azospirillum sp. B506	Oryza sativa stems	Japan	AB049110	Elbetagy et al. (2001)
Azospirillum sp. B4	Oryza sativa stems	Japan	AB027690	Elbetagy et al. (2000)
Azospirillum sp. I53	Wheat rhizosphere	Chile	MK216930	
Azospirillum sp. I84	Wheat rhizosphere	Chile	MK216960	
Azospirillum sp. I118	Wheat rhizosphere	Chile	MK216988	
Azospirillum sp. I146	Wheat rhizosphere	Chile	MK217013	Acuña et al. (2020)
Azospirillum sp. I148	Wheat rhizosphere	Chile	MK217015	
Azospirillum sp. I171	Wheat rhizosphere	Chile	MK217036	
Azospirillum sp. I51	Wheat rhizosphere	Chile	MK216928	
Negative strains				
Microbacterium sp. 62CR	Wheat rhizosphere	Chile	MG835569	
Bacillus sp. 72CR	Wheat rhizosphere	Chile	MG835570	
Bacillus sp. 102CR	Wheat rhizosphere	Chile	MG835571	
Bacillus sp. 112BR	Wheat rhizosphere	Chile	MG835572	
Bacillus sp. 154AR	Wheat rhizosphere	Chile	MG835573	
Bacillus sp. 173CR	Wheat rhizosphere	Chile	MG835574	
Microbacterium sp. 184AR	Wheat rhizosphere	Chile	MG835575	
Bacillus sp. 184AR-1	Wheat rhizosphere	Chile	MG835576	
Bacillus sp. 214AR	Wheat rhizosphere	Chile	MG835577	
Bacillus sp. 214AR-1	Wheat rhizosphere	Chile	MG835578	Rilling et al. (2018)
Bacillus sp. 222BR	Wheat rhizosphere	Chile	MG835579	
Chitinophaga sp. 623EA	Wheat rhizosphere	Chile	MG835588	
Arthrobacter sp. 243AR	Wheat rhizosphere	Chile	MG835580	
Bacillus sp. 322CR	Wheat rhizosphere	Chile	MG835583	
Bacillus sp. 342CR	Wheat rhizosphere	Chile	MG835584	
Microbacterium sp. 354AR	Wheat rhizosphere	Chile	MG835585	
Bacillus sp. 354AR-1	Wheat rhizosphere	Chile	MG835586	
Bacillus sp. 372EC	Wheat rhizosphere	Chile	MG835587	
Bacillus sp. 503CR	Wheat rhizosphere	Chile	MG835581	
Bacillus sp. 503CR-1	Wheat rhizosphere	Chile	MG835582	

Mycobacterium sp. 222EC	Wheat root endosphere	Chile	MG835593
Microbacterium sp. 382EC	Wheat root endosphere	Chile	MG835590
Georgenia sp. 424EC	Wheat root endosphere	Chile	MG835591
Psychrobacillus sp. 444EC	Wheat root endosphere	Chile	MG835592
Mycobacterium sp. 491EC	Wheat root endosphere	Chile	MG835594
Roseomonas sp. 513EC	Wheat root endosphere	Chile	MG835595
Roseomonas sp. 523EC	Wheat root endosphere	Chile	MG835596
Roseomonas sp. 543EC	Wheat root endosphere	Chile	MG835598
Bacillus sp. 564EB	Wheat root endosphere	Chile	MG835599
Bacillus sp. 584EA-1	Wheat root endosphere	Chile	MG835600
Bacillus sp. 592BR	Wheat root endosphere	Chile	MG835601
Chitinophaga sp. 643EA	Wheat root endosphere	Chile	MG835602
Bosea sp. 693EB	Wheat root endosphere	Chile	MG835603
Bosea sp. 703EB	Wheat root endosphere	Chile	MG835604
Leifsonia sp. 274EB	Wheat root endosphere	Chile	MG835589
Mycobacterium sp. 222EC	Wheat root endosphere	Chile	MG835593
Bacillus sp. 714EA	Wheat root endosphere	Chile	MG835605
Georgenia sp. 734EA	Wheat root endosphere	Chile	MG835606

[a]Accession number according to NCBI nucleotide database (http://ncbi.nlm.nih.gov/nucleotide).

[b]Data not published.

4.2.2. Primer design

The CRISPR loci NC_013854_8 of *Azospirillum* sp. B510 was selected for primer design. The repeat sequence (5'–GCT TCA ATG AGG CCC AAG CAT TTC TGC CTG GGA AGA C–3') was used as template for the design of three forward and three reverse primers (Table 2) by using Primer3 (Untergasser et al. 2012).

With the designed six primer oligos, four combinations were set up by combination of 'fixed' and 'repeat' primers. Fixed primers were targeted to non-repetitive zones of the loci as annealing zone, whereas repeat primers were designed to target the strain-specific repeats of the CRISPR assembly. Thus, considering fixed primers as criteria the forward 'fixed' primer sets were F1–R1 and F2–R1 and reverse 'fixed' primer sets were R1F–R1R and R1F–R2R.

Table 2. Primer oligos designed for this study.

Primer name	Sequence (5'-3')	Melting temperature (°C)	Primer hybridation strategy
Forward			
F1	CGAACGCTTTCTCAAACCAC	60.81	Fixed
F2	CCTTTTCTCTCACACACGCTAC	59.05	Fixed
R1F	ATGAGGCCCAAGCATTTCTG	62.43	Repeat
Reverse			
R1	AGAAATGCTTGGGCCTCATT	60.96	Fixed
R1R	CCACTTCGACAGCTTTCTCC	59.99	Fixed
R2R	AGGCCCAAGCATTTCTGC	61.30	Repeat

4.2.3 DNA extraction and PCR reaction

Prior to DNA extraction, the purity of each strain was checked by striking onto plates of R2A agar (0.5 g L^{-1} yeast extract, 0.5 g L^{-1} peptone, 0.5 g L^{-1} casaminoacids, 0,5 g L^{-1} glucose, 0.5 g L^{-1} starch, 0.3 g L^{-1} sodium pyruvate, 0.3 g L^{-1} K_2HPO_4, 0.05 g L^{-1}

$MgSO_4 \times 7H_2O$, and 15 g L^{-1} bacteriological agar; pH 7.42) and incubated at 30°C for 3 days. Once confirmed, strains were again cultured and 1 mL was of culture was used for DNA extraction according to cetyl trimethylammonium bromide (CTAB) - Proteinase K protocol (Wilson, 1997). First PCR reactions were performed with 15 ng μL^{-1} DNA. Each tube contained 1x PCR Buffer, 1.5 mM $MgCl_2$, 1 mM dNTPs, 0.5 mM of repeat targeted primer and 0.5 mM of fixed primer, 0.5 U ul^{-1} Promega GoTaq™ DNA polymerase (Promega Inc.). The PCR conditions were the following: 95°C for 7 min, followed by 35 cycles of denaturation at 94°C for 30 min, annealing from 60°C for 30 min, extension at 72°C for 30 min and final extension of 72°C x 7 min. PCR products were then revealed on 1% TBE gels stained with GelRed® 1× Nucleic Acid Gel Stain.

4.2.4. CRISPR-PCR reaction standardization

Once primer sets amplification was confirmed in 4.2.3, fifteen ng μL^{-1} of purified DNA from *Azospirillum* sp. B510 were used as template for PCR reaction standardization. The standardized PCR parameters were: i) DNA Polymerases, ii) primers and dNTPs concentrations, iii) annealing temperatures, and iv) use of DMSO as PCR additive to enhance denaturation. Three commercial brands of DNA polymerases were tested: GoTaq™ Flexi (Promega Inc., USA), Invitrogen™ Taq DNA Polymerase (ThermoFisher Scientific Inc., USA) and KAPA Taq PCR Kit (KAPA Biosystems, Switzerland). The primers (IDT Inc., USA) and dNTPs (Promega Inc., USA) concentrations were tested from 0.1 to 1 uM and from 0.1 to 1 mM, respectively. CRISPR repeats–targeted primer sets were set at 2x the concentration of the fixed primer. The PCR reactions were set as follow: 95°C for 10 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing from 60 to 65°C for 1 min, extension at 72°C for 1 min and final extension of 72°C for 15 min.

Dimethyl sulfoxide (DMSO) was added from 1 to 10%. In addition, the PCR amplicons were visualized in different electrophoresis gels using 1.5% of agarose gels prepared with 1% TAE (Tris–acetate–EDTA), TBE (Tris–Borax–EDTA) and/or SB (Sodium borate) buffers stained with GelRed® Nucleic Acid Gel Stain 1x (Biotium Inc., USA).

4.2.5. Specificity of CRISPR-PCR

The specificity of CRISPR-PCR approach using designed F1 and R1 set was assessed on all strains by colony PCR and endpoint PCR. For colony PCR each strain was incubated in nutrient broth (NB) agar at 28°C for 24 h. Each colony was scraped and used as template for standardized PCR (without DMSO). Endpoint PCR was performed with 15 ng μL^{-1} DNA extracted in section 2.3. Each tube contained 1x PCR buffer, 1.5 mM $MgCl_2$, 1 mM dNTPs, 0.1 mM of repeat targeted primer and 0.05 mM of fixed primer, 0.25 U ul^{-1} DNA polymerase and 10% DMSO. The PCR conditions were the following: 95°C for 10 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 1 min, extension at 72°C for 1 min and final extension of 72°C x 15 min. PCR products were then revealed on 1% TBE gels stained with GelRed® 1x Nucleic Acid Gel Stain.

4.2.6. Detection limit of CRISPR-PCR

Azospirillum sp. B510 was overnight cultured (28°C at 100 rpm) and cells were washed three times and resuspended in sterile saline solution (0.8% NaCl). Cultures were homogenized and cell number in 1 mL was determined by flow cytometry in a Facs Canto II (BD Life Sciences, USA) in triplicates. In parallel, 1 mL aliquot was separated for DNA extraction with DNeasy UltraClean® Microbial Kit (QIAGEN GbmH) in triplicates. After quantitation, samples were diluted to 10, 5, 2.5, 1.25 and 0.625 ng μL^{-1} DNA and

conventional endpoint PCR was performed to each with primers set F1-R1, F2-R1, R1F-R1R and R2F-R1R (Table 1). Each PCR reaction was performed according to 2.6 and verified on 1.5% Agarose-TBE gels. The highest sensitivity was observed using F1–R1 primer set, which was chosen and used in further experiments.

4.2.7. Validation of CRISPR-PCR assay

A wheat seedlings assay was carried out to validate the use of PCR–CRISPR as specific detection tool for *Azospirillum* sp. B510 in complex matrixes. Wheat seeds (*Triticum aestivum* L. var. Otto) were disinfected for 5 min in NaClO 5%, followed by EtOH 70% for 20 min, rinsed three times with sterile distilled water Rilling et al. (2018) and set to germinate for 48 h at room temperature in dark as described by. In parallel, a 1:1:2 of soil:perlite:peat substrate was prepared in sterile and non-sterile conditions. After germination, seeds were sown into substrate and kept in growth chamber for two weeks under the following treatments: a) sterile substrate (SS), b) sterile substrate inoculated with *Azospirillum* sp. B510 (SSI), c) sterile substrate with wheat seedlings (SSW), d) sterile substrate inoculated with *Azospirillum* sp. B510 with wheat seedlings (SSWI), e) unsterile substrate (US), f) unsterile substrate inoculated with *Azospirillum* sp. B510 (USI), g) unsterile substrate with wheat seedlings (USW), and h) unsterile substrate inoculated with *Azospirillum* sp. B510 with wheat seedlings (USWI).

Once finished, adhered rhizosphere substrate was shaken gently and DNA was extracted from 0.25 g^{-1} of sample with QIAGEN DNeasy PowerSoil Kit (QIAGEN GbmH) following manufacturer instructions. For treatments without plants, 0.25 g of subtrate was used as sample. Endosphere DNA was also extracted from 0.15 g^{-1} sample of root tissue with Zymo Research Plant and Seed DNA Miniprep Kit (Zymo Research Inc.). PCR was

performed to DNA samples with primer set F1–R1 (Table 2) using the 5 ng μL^{-1} *Azospirillum* sp. B510 pure DNA as positive control. Each PCR reaction was performed according to section 2.6 and verified on 1.5% Agarose-TBE gels as described above. In addition, the PCR products were also loaded and revealed in a 5200 Fragment Analyzer (Advanced Analytical, Inc., USA) with DNF915 kit (from 5 to 1000 bp) to confirm the information obtained under conventional electrophoresis procedures.

3. Results

4.3.1. CRISPR-PCR Standardization

The results of CRISPR-PCR standardization for *Azospirillum* sp. B510 using designed F1 and R1 primer set are shown in Fig. 2. On all three DNA polymerase enzymes assayed, Promega GoTaq™ Flexi was the most effective for the CRISPR loci fingerprint obtention (Figure 2a). Comparatively, Invitrogen Taq produced the desired fingerprint, although less defined bands when compared with the Promega GoTaq™. The KapaTaq™ enzyme PCR results revealed only one band in all PCR assays, being unreliable for further usage.

In terms of primers and dNTPs concentration, a low concentration of primers was required to obtain a proper fingerprint of the targeted CRISPR loci for all primer sets. In this case, 0.1 µM and 0.05 µM of the repeat-targeted and fixed primers were set as optimal (Figure 2b). On the other hand, dNTPs concentration was required to be as high as 1 mM (Figure 2c).

Among the other parameters standardized for this PCR assay, optimal annealing temperature was 60°C, which was the average of all primers in Table 2 and provided better amplification. The increasing DMSO concentrations improved the efficiency of the reactions (Figure 2d). The use of 10% DMSO improved band definition, diminishing the

unspecific amplification observed in the other PCR standardization proceedings (Figure 2a, b and c).

In relation to SB and TBE buffer (Figure 2e and f, respectively) for electrophoresis, both buffers are suitable to reveal the desired fingerprint. Although, TBE buffer has proven to produce better defined bands, whereas SB buffer allowed to run electrophoresis in half the time, as the buffer for both gel casting and electrophoresis run does not generate heat.

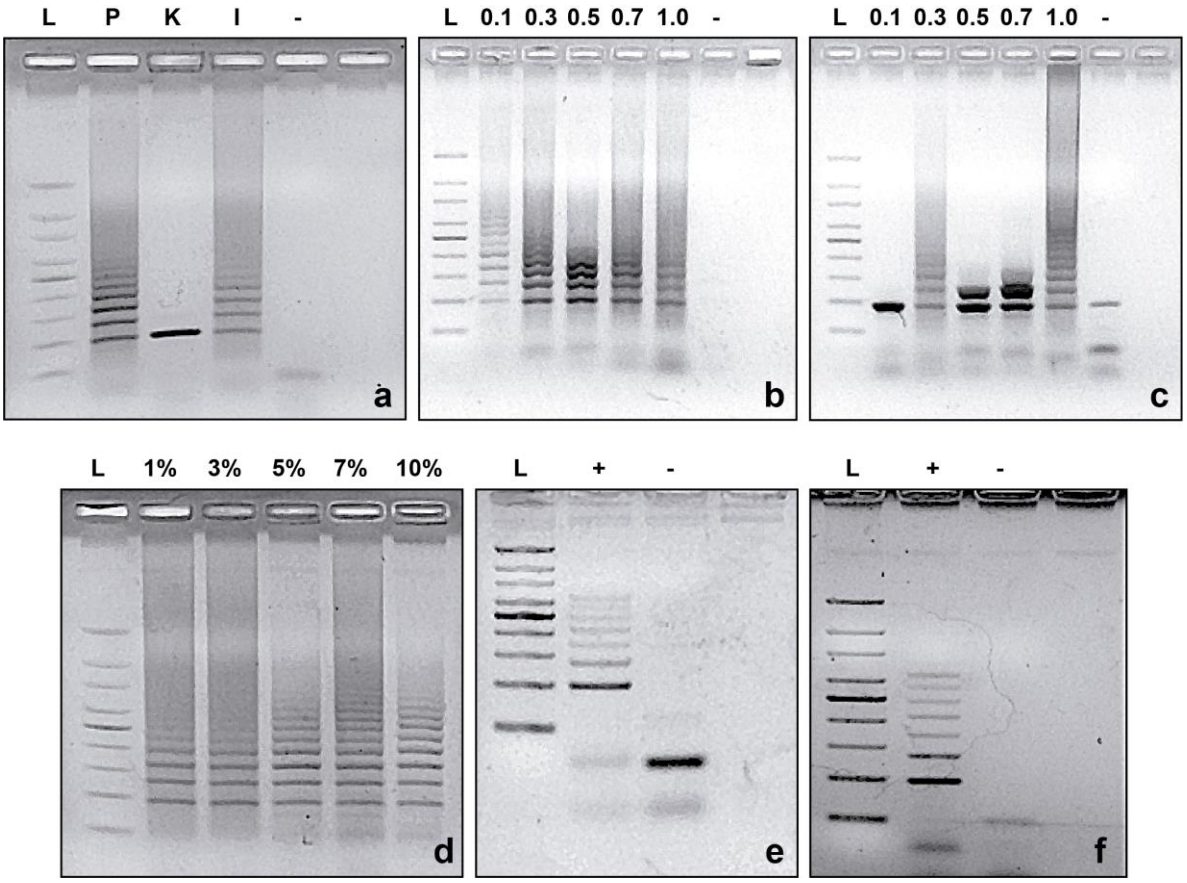

Figure 2. Standardization of PCR–CRISPR approach using designed F1 and R1 primer set. **(a)** Polymerases (P: GoTaq™ Flexi from Promega Inc.; K: KAPA Taq PCR Kit from KAPA Biosystems; I: Invitrogen™ Taq DNA Polymerase from ThermoFisher Scientific Inc.). **(b)** Primer concentrations (µM). **(c)** dNTPs concentrations (mM); **(d)** Dimethyl

sulfoxide (DMSO) percentages. Electrophoresis on **(e)** Tris–Borax–EDTA (TBE) and **(f)** sodium borate (SB) agarose gels. L = Ladder; –: Negative control; +: positive control (pure DNA from *Azospirillum* sp. B510).

4.3.2. Specificity of CRISPR–PCR

Both colony and endpoint PCR assays performed to all strains present in Table 1 revealed amplification only for *Azospirillum* sp. B510. Biological control *Azospirillum* B4 and B506 isolated from the same source as *Azospirillum* sp. B510 revealed no bands. On the seven native *Azospirillum* isolates from Region de La Araucania no amplification was observed. The same occurred on the PCR reactions conducted on the other thirty-eight strains from different taxa tested (Table 1). On colony PCR gels, bands were discernible only for our model strain, although amplification was heavily unspecific. The other forty-seven strains assayed showed no amplification. All other wells presented a large band was observed under 100 bp, characteristic of unutilized primer residue.

4.3.3. Detection limit

With regards to the detection limit, after 24 h of incubation the cell number was determined as 7.49 x 10^6 cells ml^{-1} culture under flow cytometer. On further DNA extraction of the same samples 15.1 ng μl^{-1} DNA was recovered. With that in consideration, all the four DNA concentrations tested (10, 5, 2.5 and 1.25 ng μl^{-1}) revealed band amplification with all the designed primer sets (Figure 3). Although, at 2.5 and 1.25 ng μl^{-1} DNA bands were almost undiscernible. The lowest concentration 0.625 µg ul^{-1} DNA revealed no amplification. With that in consideration, the detection limit for this PCR assay under our consideration must was set at 5 ng ul^{-1} DNA, the equivalent of 2.5 x 10^6 cells

considering the prior cytometer measurement values.

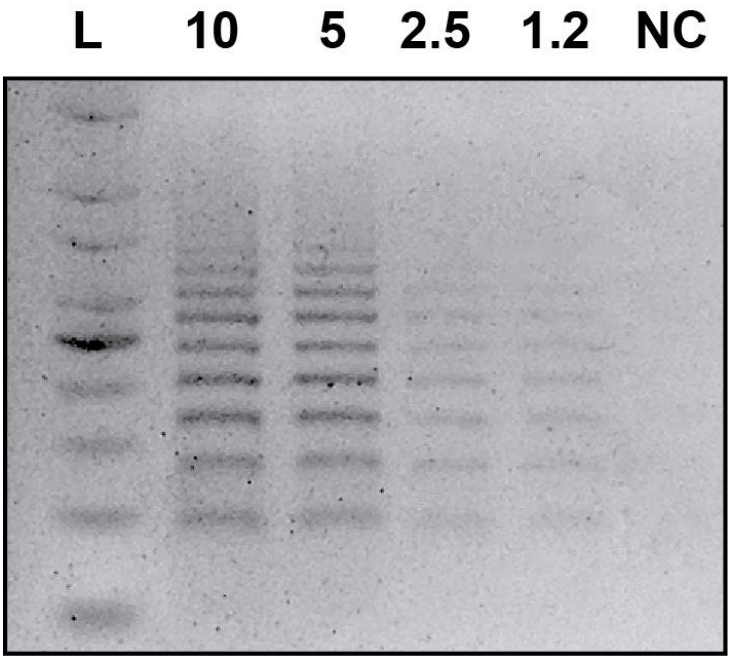

Figure 3. Detection limit of CRISPR repeats targeted PCR based on different *Azospirillum* sp. B510 DNA concentrations (10, 5, 2.5 and 1.25 ng ng μl^{-1}). L (1000 bp ladder); NC (Negative control).

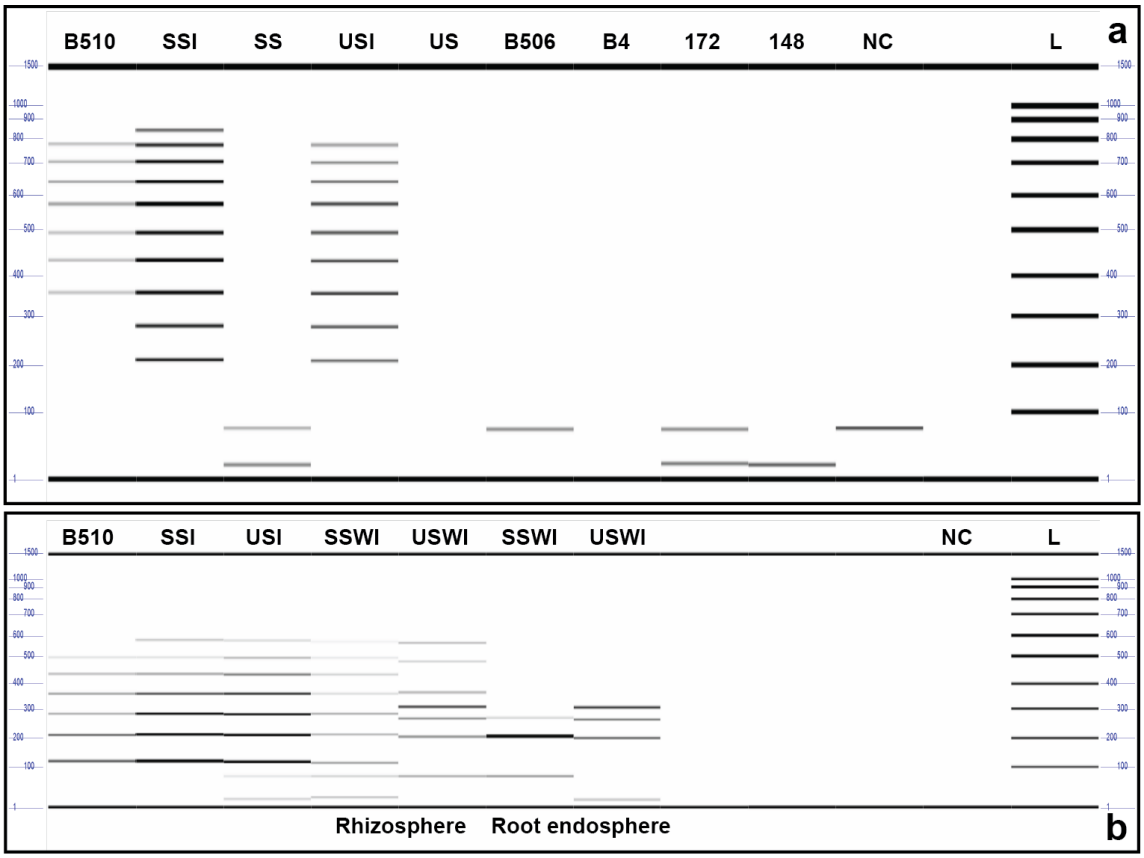

Figure 4. Fragment analysis of CRISPR-PCR validation assay. **a)** B510 (positive DNA control); SSI (sterile substrate inoculated; SS (sterile substrate); USI (unsterile substrate inoculated); US (unsterile substrate); B506, B4, 172, 148, NC (negative controls); L (1000 bp DNA ladder). **b)** B510 (positive DNA control); SSI (sterile substrate inoculated); USI (unsterile substrate inoculated); SSWI (sterile substrate + seedlings inoculated); USWI (sterile substrate + seedlings inoculated); NC (negative control); L (1000 bp ladder).

4.3.4. Method validation

On our method validation assay, revealed the strain-specific fingerprint banding expected (Figure 4a). Both treatments without plant or inoculation (SS and US) revealed no amplification. Oppositely, under inoculation (SSI and USI) presented the specific

Azospirillum sp. B510 fingerprint when compared with *Azospirillum* sp. B510 pure DNA (Figure 4a). Fragment analysis revealed the molecular weight of the fragments to be 358, 433, 494, 574, 640, 701 and 778 bp for *Azospirillum* sp. purified DNA, and 209, 280, 358, 433, 494, 574, 640, 701, 774 and 843 bp for the SSI treatment (Figure 5). Similarly, USI treatment presented slight molecular weight variations (1 – 2 bp) being the fragments measured as 207, 278, 355, 431, 494, 574, 640, 700 and 774 bp. Neither of the negative controls (B506, B4, 172, 148 and NC) revealed peaks on the synthetic or agarose gels.

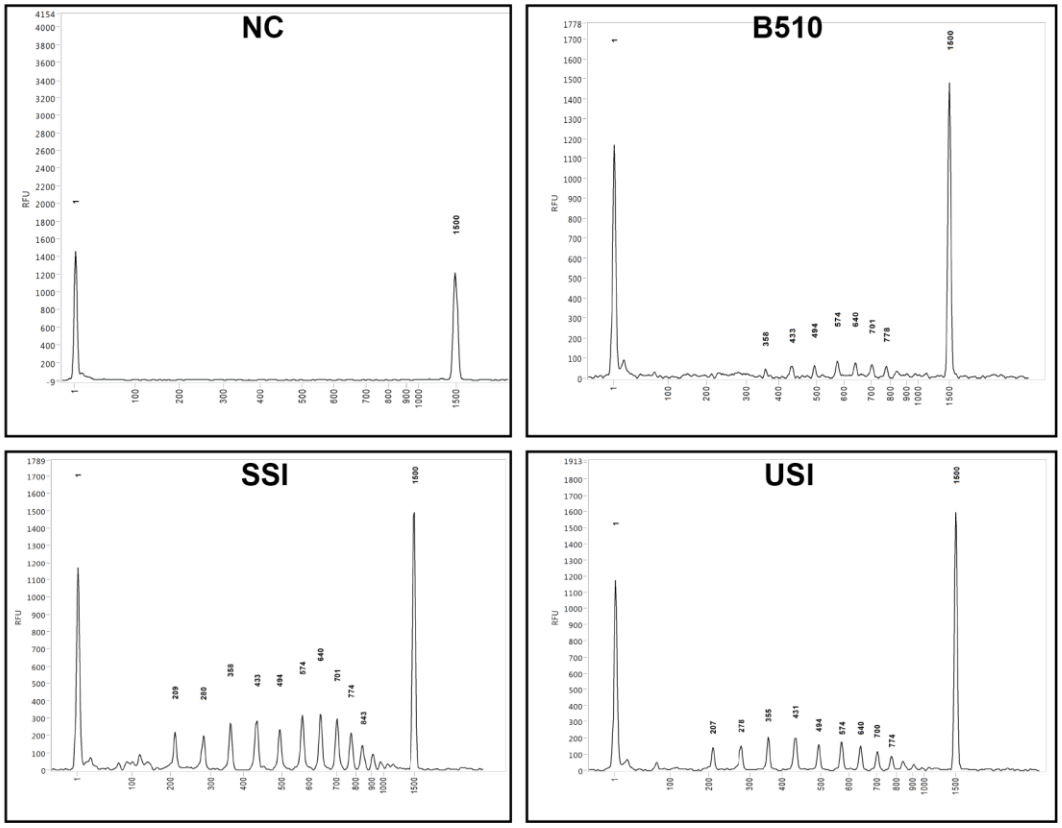

Figure 5. Electropherogram of fragment analysis for CRISPR-PCR validation assay observed in figure 4a. NC (negative control); B510 (positive DNA control); SSI (sterile substrate inoculated); USI (unsterile substrate inoculated).

On a second PCR assay, similar results to those obtained previously were observed (Figure 4b). Treatments revealed the same banding profile when compared with B510

strain, although bands were observed at smaller molecular weights (Figure 6). In this sense, for B510 control strain bands were defined at 119, 209, 282, 360, 434 and 496 bp. SSI treatment presented bands at 119, 211, 282, 360, 434, 496 and 577 bp, whereas USI treatment presented differences of about 2 bp in some fragments, revealing 77, 117, 209, 280, 360, 432, 494 and 575 bp. In the both SSWI and USWI treatments the specific *Azospirillum* sp. B510 fingerprint was observed. SSWI rhizosphere samples presented bands at 77, 113, 211, 282, 360, 432, 494 and 569 bp, although peaks detected at 494 and 569 bp are were not noticeable under horizontal gel electrophoresis (Figure 6). On other hand, USWI rhizosphere samples presented differences with SSWI treatment, presenting dissimilarities in all bands (203, 266, 306, 365, 481 and 563 bp). For root endosphere, both SSWI and USWI treatments yielded bands of similar size to those observed in control and other treatments. However, samples of the same treatments, the largest band observed was around 300 bp. Under our criteria those fragments do not reveal a clear fingerprint as observed in other samples.

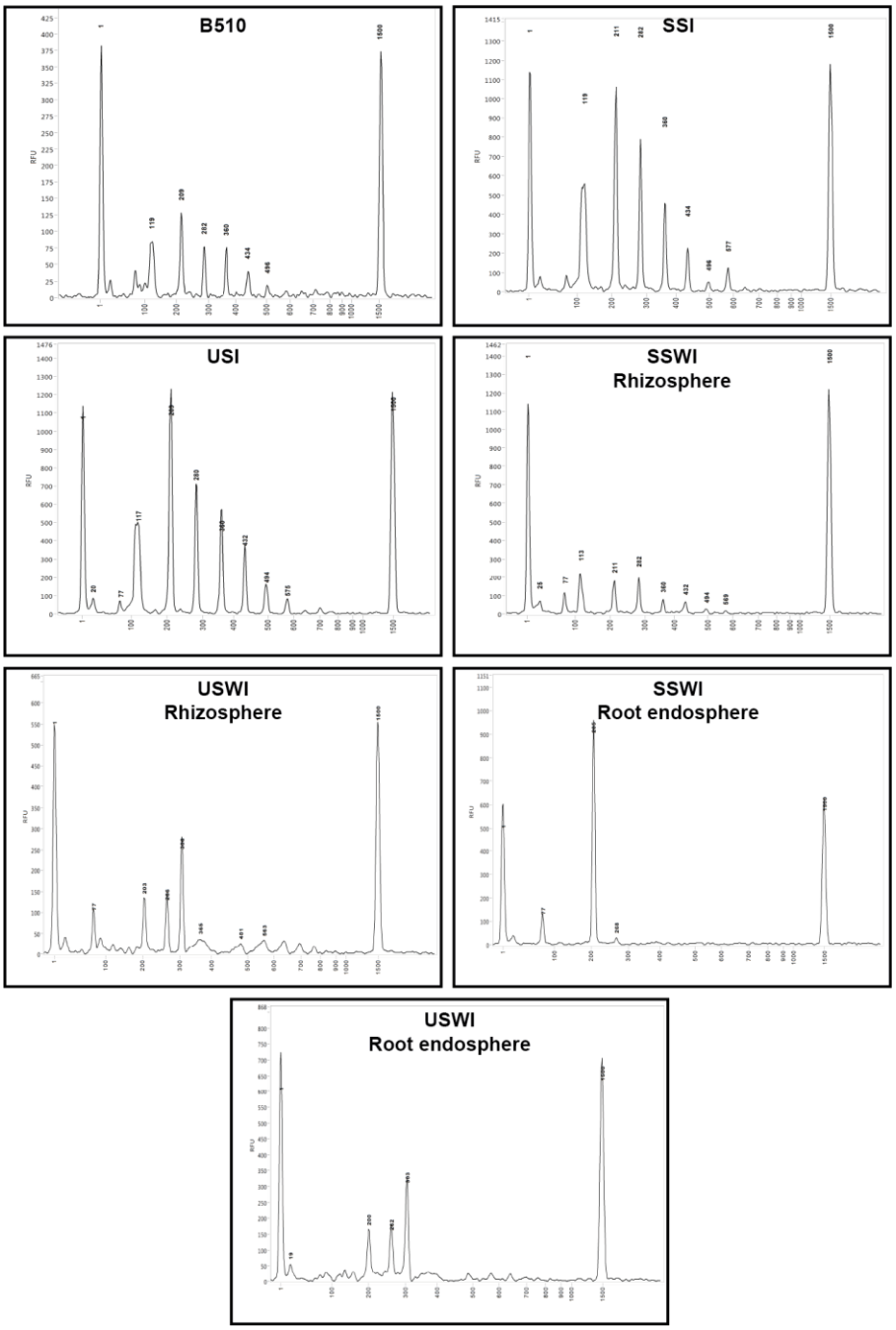

Figure 6. Electropherogram of fragment analysis for CRISPR-PCR validation assay observed in figure 4b. B510 (positive DNA control); SSI (sterile substrate inoculated); USI (unsterile substrate inoculated); SSWI (sterile substrate + seedlings inoculated); USWI (sterile substrate + seedlings inoculated); NC (negative control); L (1000 bp ladder).

4.4 Discussion

Tracking the fate of PGPB once released to the environment is a fundamental aspect of their technological usage. Assessing the colonization of a strain has been the bottleneck of PGPB studies since the first studies that tried to assess the effects of such bacterial group into the environment (Ahmad et al. 2011). The former needs to be addressed in order to reduce the significant breach between chemical fertilization and microbial inoculation.

In this study we focused in the development of a potential tracking method based on endpoint PCR for the tracking of an inoculated *Azospirillum* sp. B510. In the past, effort to achieve proper bacterial tracking have been implemented, a large number of them present considerable caveats (Rilling et al. 2019). In such sense and due to its simplicity, the current and future most effective approach for tracking *Azospirillum* and other PGPB strains once inoculated will still be based on PCR. This is mainly based on the easefulness for sampling and sample preparation, as well as broad range of laboratories that has access to the required equipment (Coleman and Tsongalis, 2006). For our study we assessed and developed four primer sets targeted to the strain-specific *Azospirillum* sp. B510 CRISPR loci repeat sequence 5'-GCTTCAATGAGGCCCAAGCATTTCTGCCTGGGAAGAC-3'. Despite all primer sets demonstrated to be specific, the F1-R1 set (5'-CGAACGCTTTCTCAAACCAC-3'; 5'-AGAAATGCTTGGGCCTCATT-3', respectively) demonstrated to produce the most reliable amplification at low concentrations of purified DNA. The specificity of such primers was tested *in silico* under NCBI BLASTn (http://blast.ncbi.nlm.nih.gov), where both the designed primers and the CRISPR-repeat sequence selected revealed no homology with other organisms or metagenomic datasets. We acknowledge that databases are in constant curation and do not contain all the DNA

sequences information present in nature, however our results validate our hypothesis at laboratory scale. These was addressed by a standardization and different PCR assays that revealed a strain-specific fingerprint not observed either in other nine *Azospirillum* strains as well as thirty-eight native strains previously isolated from Region de La Araucania wheat rhizosphere and root endosphere (Acuña et al. 2020; Rilling et al. 2019).

To determine the strain-specificity and PCR performance mentioned, several prior steps were considered. The identification of CRISPRs relied exclusively in the availability of completely and properly assembled genomes, which were analyzed. CRISPR-dedicated databases are in constant curation (Grissa et al. 2007), nonetheless as genome databases have a much faster growth and curation rate, external software such as PILER-CR (Edgar 2007) can be used to detect CRISPRs without the use of such specific databases. In addition to the former, the target CRISPR repeats usually presents palindromic sequences (Horvath and Barrangou 2010), being required to evaluate among several primer set combinations for the design to be successful. To assure proper amplification these strain-specific PCR assay, standardization was determinant. In such terms, these procedures must be performed for all PGPB strains that are subjected to our methodology.

Similar approaches to those proposed in this article have been reported. Reddypriya et al. (2019) designed and implemented a three primer set endpoint and possible qPCR assay for the quality control of a biofertilizer consortium based in *Azotobacter chroococcum*, *Bacillus megaterium* and *Azospirillum brasilense*. Authors describe this method as reliable for field applications, however, once those primer sequences are input into BLASTn, different soil bacteria taxa are revealed as homologous, which could lead to a false positive bias. Similar results were observed by Holmberg et al. (2009) and Couillerot et al. (2010) on a qPCR SCAR markers assay for *Pseudomonas brassicacearum*

MA250 and *A. lipoferum* CRT1, respectively. Despite the primer specificity for such SCAR genes, strain-specificity cannot be assured. To overcome such issues, we propose the combination of culture-dependent and culture-independent approach for the tracking of the target strains. The use of a culture-dependent approach will guarantee a proper evaluation of the viability of bacteria in sample. The former can be directed using selective culture media that targets the desired PGPB trait that the inoculum should be exerting into the rhizosphere. Complementarily, the culture of bacteria in rhizosphere can provide a qualitative perspective to the tracking procedure, which is not obtainable under an endpoint PCR. However, the use of a method that assesses colonization or persistence of a PGPB strain in field will not define that the strain is properly active in the sampled plant niche, hence. Although, the former can be used to improve both the knowledge related to plant-PGPB compatibility, to improve quality control and shelf life measurements, as well as to provide certainty to farmers in terms of PGPB establishment after a considerable period of time past inoculation.

During the validation experiments, the selected B510 strain was detected in a growth chamber assay under conventional PCR assays on sterile and unsterile substrate, as well as on rhizosphere of wheat seedlings sown in substrate under the same conditions (sterile and unsterile). Unsterile rhizosphere did presented differences of banding as observed in Figure 6, however we attribute those to the rhizosphere complexity. In regards to the root endosphere detection, our method was not sufficient and there are multiple factors that could be involved such as the number of cells that can enter the plant root tissues, as well as interferent plant DNA present in the sample. Those results were expected, as the DNA extraction kit used was designed for plant DNA, however methods for the extraction of endosphere bacteria are scarce (Simmons et al. 2018). The strain

specificity observed in the PCR reactions confirms that the designed primer set, as well as the target loci could be used for PGPB tracking in field if deeper studies are performed. These could represent a step forward when compared with the tracking methodologies applied to date. However, it must be taken to consideration that both technological and ecological CRISPR studies in soil are scarce, given its novelty and soil complexity, being present in literature only reports of *Erwinia amilovora* strain typing (McGhee and Sundin 2012) until this study.

Under our understanding, the advances in PGPB genomics and metagenomics can allow the development of novel methods based on new genomes identified strain-specific sequences (such as CRISPR loci) and plant–microbiome response as result of PGPB inoculation. Likewise, in this study we used such knowledge to develop an inexpensive and easy-to-apply method for *Azospirillum* sp. B510 tracking once inoculated. Nonetheless and considering the previously stated, the improvement of the method is required for its proper application in field samples.

4.5. Acknowledgements

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CHAPTER V

Concluding remarks and future directions

Concluding remarks and future directions

In the modern world, the technology involved food supply and production for the increasing population needs to reconsidered soon. On hand with the increment on food demands, the environmental impact that agriculture exerts is noteworthy. Thus, farmers are subjected to a permanent demand for increased yields, but without the environmental impact that agriculture traditionally has exerted onto different ecosystems. The former has pushed the market into the assessment of solutions that goes in hand with both principles. In such terms, despite the expectatives that the use of PGPB has generated, their use still is restricted as farmers require similar yields to those obtained under chemical fertilization. However, as the soil-plant continuum microbial communities are dynamic and dependant on a large number of external conditions (temperature, host plant, pH, water availability, competition, etc.), proper explanations on how to manipulate the rhizosphere of plants with the introduction of PGPB are scarce.

Currently, despite the yearly increasing number of techniques, most of the modern high-throughput methods developed for the tracking or monitoring of introduced bacteria on plant roots are laborious, expensive and do not fulfill the simplicity criteria that agricultural assessment methods require. Although, that problematic does not rely entirely on the methods but also the community complexity that environments such as the rhizosphere or the endosphere presents. On this thesis, first we explored the different approaches used for PGPB tracking since 1990. The different focuses observed reveal that despite PGPB induce a plant response, the current applied focus has proven to be unsufficient. This is given by the fact that bacteria in environment will interact in a strictly different manner to what is observed under laboratory conditions, where most of the

tracking experiments needs to be applied. In addition, the access to the laboratory available technologies is not guaranteed to farmers due to both costs and complexity. In such sense our initial proposal was the development of inexpensive and simple methodology that required widespread laboratory equipment, as well as a simple design that does not rely in the complexity of the method, but in the specificity of the target microorganism.

In a different focus, the target plants can present a different response to different inoculated bacteria. Thus, we explored the effect of four wheat cultivars over their total and N_2-fixing bacterial communities in the same plot. Few differences were found among the cultivars, with the exemption of one cultivar that presented different structure in terms of its bacterial communities. However, despite the final focus of this thesis was directed to the *Azospirillum* spp. presence in wheat roots (either rhizosphere or root endosphere), we did not isolate representatives of this genus. This can be explained by a methodological mishap during the experimental design or either sample nature. The culture media used in that study were utilized considering a broad range of N_2-fixing bacteria, and not a particular genus such as *Azospirillum*. The observed N content of the samples was high enough to exert influence on N_2-fixing communities, inducing a sample bias on the results. Nonetheless, the results homogenicity in terms of CFU g^{-1} sample, as well as the differences observed between plant niches are a clear example of plant selection of its microbiome under agronomic management conditions. In future studies, non-fertilized sown plots must be used as target for studies that are directed in this endeavour. This is clearly a key factor to be considered prior to do PGPB inoculation in field and must be assessed before farmers make the transition from chemical fertilization to an inoculation-based one.

Despite the prior mentioned statements, current commercial marketing practices have pushed users and producers of PGPB-based products to skip such assessments. Currently, PGPB biofertilizer products are sold as an all-in-one benefic solution for an entire plant species without considering the implicances of cultivar influence. The main reason to the former is the time consumption that those assays would precise, taking into account the enormous number of worldwide available commercial cultivars, as well as climatic, edaphic and management conditions.

To overcome the former issue, we developed, standardized and validated an inexpensive and easy-to-use method that is based on endpoint PCR of strain-specific CRISPR loci. Those loci are known to be present in a broad range of bacterial genera, and naturally serves as an adaptative immunity barrier against extragenomic DNA elements such as phages or plasmids. The selection of PGPB based on the presence of CRISPR loci can also increase the survival and establishment chances once inoculated, due to a natural defense strategy against the infection of soil phages. Our results revealed that it was achievable to determine the presence of DNA of our model *Azospirillum* sp. B510 strain in samples inoculated under sterile and unsterile substrate, as well as into wheat seedlings rhizosphere sown in those substrates. In terms of costs considering sampling, DNA extraction and PCR our approach costs around $10 USD per sample if our proposed the pipeline is followed (Figure 1).

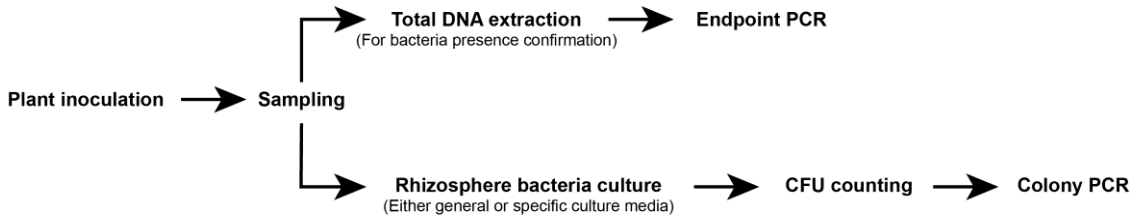

Figure 1. Proposed pipeline for CRISPR-PCR application in PGPB tracking.

On the other hand, the method developed in our study prior mentioned still present certain caveats. Among those, not all bacteria present CRISPR loci, and to determine the presence of a CRISPR loci the entire genome sequence of the target bacteria is required. This could pose a major setback for the proposed methodology, nonetheless, cost reductions on genome sequencing on the last ten years has been a trend. In addition to the former, it needs to be considered that in its current development state, our approach requires standardization for all PGPB strains, due to its strain-specificity (Figure 2). That must be further validated both under laboratory and field conditions. During this thesis, we only reached validation at laboratory scale under a qualitative approach, which explains the need of colony or endpoint PCR performed from a culture-dependent perspective in order to generate quantitative data. However, in future experiments the use of high-resolution qPCR tools such as TaqMan probes can be useful to determine the presence of the PGPB strain, as well as to obtain a quantitative approach of the colonization and hence, provide useful results to farmers.

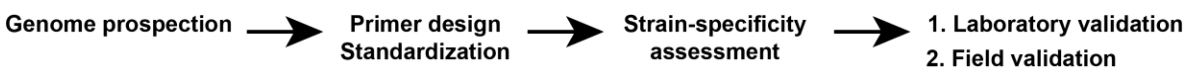

Figure 2. Experimental pipeline for CRISPR-PCR PGPB tracking design.

Finally, the strain-specificity observed on a complex environment such as rhizosphere, combined with the availability of endpoint PCR thermalcyclers in small microbiology laboratories makes it interesting for future experiments and PGPB products development. In such regard, we consider that methods for this endeavour in the future must be developed serving the simple mass applicability and inexpensiveness criteria. Once achieving those considerations, the PGPB products could increase both its value and performance. This is key for farmers that need to verify if the inoculated are being properly managed and delivered to target plants.