CATÓLICA
ESCOLA SUPERIOR DE BIOTECNOLOGIA
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UTILIZATION OF PLANT GROWTH-PROMOTING BACTERIA TO AMELIORATE IRON NUTRITION IN LEGUMES

Thesis submitted to *Universidade Católica Portuguesa* to attain the degree of PhD in Biotechnology, with specialization in Environmental Science and Engineering

Mariana Roriz Lemos Costa

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Mariana Roriz Lemos Costa

Under the supervision of Professor Marta Wilton Pereira Leite de Vasconcelos

Under the co-supervision of Professor Susana Maria Pinto de Carvalho and Professor Paula Maria Lima Castro

March 2021

To all who made me believe this could be possible.

RESUMO

A deficiência de ferro (Fe) é uma carência nutricional que leva a graves perdas de produtividade e valor nutricional das culturas, particularmente em solos calcários.

Existe uma necessidade urgente de encontrar práticas agrícolas mais sustentáveis para a produção de culturas com maior rendimento e valor nutricional. A biofortificação permite aumentar a concentração de micronutrientes nas culturas e contribui para cumprir estas necessidades.

A utilização de bioinoculantes (BIs) com bactérias promotoras de crescimento de plantas (BPCP) tem sido sugerida como uma abordagem de biofortificação e prevenção da deficiência de Fe. Até agora, existem poucos estudos sobre o papel das BPCP em soja crescida em condições alcalinas. O objetivo principal deste estudo foi testar o potencial de BPCP no crescimento das plantas e na absorção do Fe, elucidando os mecanismos subjacentes à absorção e acumulação do Fe. A soja foi escolhida pois é bastante afetada pela deficiência de Fe, e foram identificados vários fatores subjacentes relacionados com a sua homeostasia.

Inicialmente, fez-se uma revisão crítica da literatura tendo em conta a importância do Fe e da sua carência, o papel da soja face às políticas globais, e o potencial das BPCP como abordagem sustentável para melhorar a nutrição de Fe e combater a sua deficiência.

O primeiro estudo experimental teve como objetivo avaliar a capacidade de 24 estirpes de BPCP de uma coleção do CBQF em melhorar os processos de absorção do Fe em soja crescida em solo calcário durante 21 dias. *Sphingobium fuliginis* ZR 1-6 e *Pseudomonas jessenii* ZR 3-8 foram selecionadas com base na sua capacidade *in vitro* de produzir ácido indol-3-acético (AIA), ácido 1-carboxílico-1-aminociclopropano (ACC) oxidase, sideróforos e ácidos orgânicos, tolerar pH elevado, e reduzir Fe^{3+}. Os isolados bacterianos foram inoculados isoladamente e em mistura, e foram avaliados vários parâmetros morfológicos, fisiológicos e moleculares. *S. fuliginis* melhorou a atividade da redutase férrica (FC-R) (111 %), a expressão de FRO2 (646 %), e a concentração de Fe na raiz (62 %); a inoculação combinada promoveu a acumulação de Fe nos trifoliados (144 %) e aumentou a expressão de IRT1 (239 %) e FER4 (5036 %). No geral, a inoculação com *S. fuliginis* sozinho ou em combinação com *P. jessenni* revelaram-se os melhores tratamentos.

Num segundo estudo, as BPCP foram isoladas dos tecidos e da rizosfera de soja cultivada num solo português; foram isoladas 76 estirpes das raízes (53 %), rizosfera (29 %), e parte aérea (18 %), e foram identificados 29 géneros bacterianos. Foram selecionadas duas estirpes – *B. licheniformis* P2.3 e *B. aerius* S2.14 – para experiências *in vivo* em plantas crescidas até à maturidade. Os parâmetros fotossintéticos, teor de clorofila, peso fresco total e concentrações de Fe não foram afetados significativamente pela inoculação. No entanto, a inoculação com *B. licheniformis* aumentou o número de vagens (33 %), diminuiu a atividade da FC-R (45 %) e aumentou a expressão de genes relacionados com o Fe; a inoculação com *B. aerius* diminui o comprimento da raiz (20 %), a atividade da FC-R (55 %) e a expressão de FRO2, e aumentou a expressão dos restantes genes. Além disso, a inoculação com as bactérias melhorou a acumulação de Mn, Zn e Ca nos tecidos da

soja. Neste estudo, *B. licheniformis* apresentou potencial para ser incorporado em formulações para o melhoramento da soja crescida em solo calcário.

A formulação de BIs contempla vários requisitos e sua implementação representa ainda um desafio. No entanto, estes são uma tendência promissora para o cumprimento das políticas globais futuras, apresentado várias vantagens para práticas agrícolas mais “verdes”, analisadas na última parte desta tese.

No geral, os resultados apresentados contribuem para compreender melhor os mecanismos pelos quais as BPCP melhoram a absorção do Fe e o crescimento das plantas em condições alcalinas, e demonstram o seu potencial como bioinoculantes numa perspetiva sustentável.

Palavras-chave: biofertilizante, bioinoculante, *Glycine max*, deficiência de ferro, bactérias promotoras de crescimento de plantas

ABSTRACT

Iron (Fe) deficiency is an important micronutrient disorder that leads to severe yield losses and low nutritional crop value, particularly in calcareous soils.

There is an urgent need to find sustainable and greener agricultural practices to achieve higher crop yields with higher nutritional value. Biofortification allows the increase of micronutrient concentrations in edible crop tissues and contributes to achieving such demands.

The utilization of bioinoculants (BIs) with plant growth-promoting bacteria (PGPB) has been suggested as a promising approach for biofortification and prevention of Fe deficiency. So far, little work has been done on the role of PGPB in soybean (*Glycine max*) grown under alkaline conditions. The main purpose of this study was to test the potential of PGPB on plant growth and Fe uptake, unveiling mechanisms underlying Fe uptake and accumulation. Soybean was selected as a model species since it is severely affected by Fe deficiency and several underlying factors related to Fe homeostasis are identified.

Firstly, an in-depth and critical literature review was conducted concerning the global importance of Fe and the Fe deficiency, the importance of soybean in the fulfillment of global policies, and the potential of PGPB as a sustainable approach to improve Fe nutrition and cope with Fe deficiency.

Concerning the experimental work, the first study of this thesis aimed to evaluate the ability of 24 PGPB strains from a CBQF collection to enhance Fe uptake-related processes in soybean grown for 21 days in calcareous soil. *Sphingobium fuliginis* ZR 1-6 and *Pseudomonas jessenii* ZR 3-8 were selected based on their *in vitro* ability to produce indole-3-acetic acid (IAA), 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase, siderophores, and organic acids, to tolerate high pH, and to reduce Fe^{3+}. Bacterial isolates were inoculated singly and as a mixture, and a series of morphological, physiological, and molecular parameters were evaluated. *S. fuliginis* improved ferric chelate reductase (FC-R) activity (111 %), FRO2 expression (646 %), and root Fe (62 %); combined inoculation fostered Fe accumulation in trifoliates (144 %) and increased IRT1 (239 %) and FER4 expression (5036 %). Overall, *S. fuliginis* alone or in combination with *P. jessenni* were the best treatments.

In a second study, PGPB were isolated from root tissues and rhizosphere of soybean grown in a Portuguese soil; 76 bacterial strains were isolated from roots (53 %), rhizosphere (29 %), and shoots (18 %), and 29 genera were identified. Two bacterial strains – *B. licheniformis* P2.3 and *B. aerius* S2.14 – were selected for *in vivo* experiments, and inoculated plants were grown to maturity. Photosynthetic parameters, chlorophyll content, total fresh weight, and Fe concentrations were not significantly affected by inoculation. Nevertheless, inoculation with *B. licheniformis* increased pod number (33 %), decreased FC-R activity (45 %), and increased expression of Fe-related genes; inoculation with *B. aerius* decreased root length (20 %), FC-R activity (55 %), and FRO2 expression, and increased expression of the remaining genes. Furthermore, inoculation with bacterial isolates

improved the accumulation of Mn, Zn, and Ca in soybean tissues. In this study, *B. licheniformis* showed potential to be incorporated in formulations for improving soybean grown in calcareous soil.

The formulation of BIs contemplate a series of requirements and their effective implementation is still challenging. However, they are a promising trend to the accomplishment of future global politics and present a series of advantages to greener agriculture practices that are critically reviewed in the last part of this thesis.

In general, the results presented in this thesis contribute to better understand the mechanisms by which PGPB improve Fe uptake and plant growth, under alkaline conditions, and their potential as bioinoculants in a sustainable perspective.

Keywords: biofertilizer, bioinoculant, *Glycine max*, iron deficiency, plant growth-promoting bacteria

ACKNOWLEDGMENTS

I would like to thank Universidade Católica Portuguesa, Escola Superior de Biotecnologia, and CBQF for welcoming me as a PhD student, providing me all the conditions to carry out my work during these years.

To the European Union's Horizon 2020 research and innovation program through TRUE project (grant agreement No. 727973), and FCT within the scope of UIDB/50016/2020, UIDB/05748/2020 and UIDP/05748/2020, and PhD scholarship SFRH/BD/109432/2015 for supporting my work.

To my supervisor, Prof. Dr. Marta Vasconcelos, for once again trust my capacities, providing all the conditions for the development of this work. For your kindness, dedication, guidance and advice, that I truly esteem. Through this journey, you helped me to improve my skills and grow as a researcher, always being there to encourage and help overcome the difficulties. Thank you very much.

To my co-supervisors for having accepted to be part of the orientation of my work. To Prof. Dr. Susana Carvalho, for all the support, valuable sharing of scientific knowledge, and detailed appreciation of my work, which I consider very important, and for providing the ideal conditions for the growth of my plants. To Prof. Dr. Paula Castro, for the invaluable help, encouragement, and critical appreciation during these years, and for introducing me to people who were essential in the course of my work.

To Dr. Sofia Pereira, for your availability, help, advice, and valuable sharing of scientific knowledge that helped me to overcome all the difficulties. Thank you very much.

To the group of Prof. Dr. Paula Castro, in particular to Helena, Miguel, and Ana Paulo for the assistance and availability during these years. To Dr. Cristina Monteiro for supplying the expanded clay. To the "Chemistry Lab" for always being available to help and borrow reagents.

To Prof. Dr. António Ferreira and Rita, for providing the NIR equipment, and for sharing your scientific knowledge.

To the CBQF team, in particular to the technical staff, for always being ready to help and for contributing with reagents and equipment, helping to overcome the difficulties that were arising.

To the "Vairão team", Maling and Miguel, for always being available and ready to help.

To Eng. Rosa Moreira for providing the soybean plants, essential for my work. To Eng. João Vasconcelos, and Eng. Ricardo Carvalho from Quinta da Senhora da Ribeira for so kindly providing alkaline soils for my experiments. To Eng. Elisabete Pimenta from Horpozim for being available to help me find an agricultural soil with the ideal characteristics for my work. To Eng. Paulino Lourenço for the availability to provide soybean plants, and for the interest in my work.

To my dear PlanTech team, to those who are still part and to the former ones, Martinha, Patrícia, Elsa, Ana, Inês, Sofia, Vítor, Teresa, Zé, for the mutual help spirit and constant advice and for helping time to pass in a more fun and enjoyable way. These were very good and unforgettable times.

To Carla, my “oldest” lab mate, for our long conversations, for your advice in the best possible way, for being a good friend, and for always being there for me. You have always been a precious help. I am very proud of the excellent scientist you have become; you are an example to follow.

To Joana and Manel, for your help during my experiments, for always supporting me, advising, listening, and making me laugh; you are good friends who stay for life. You made this path lighter and more enjoyable, always sharing our achievements and difficulties.

To all my friends for life, who are special and unique, and in one way or another always contribute to brighten my days. To Anabela, for your unconditional support, for knowing well what a PhD involves, for our long conversations, for advising me, for encouraging me, for making me believe in my capacities; you know that you were a valuable help. To Joana and Guida, for patiently listening to my outbursts, for always encouraging me to believe in me, and for always being there to make me smile. Thank you very much, you are my compasses. To Ju, for always being interested in my work, and for giving unique and jovial support as only you know.

To my parents and brother, for being my pillar of support throughout my life and even more in this important time, for listening to my outbursts, for your patience and for helping me to move forward, always giving me good advice.

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LIST OF ABBREVIATIONS

ACC	1-aminocyclopropane-1-carboxylic acid
AMF	Arbuscular mycorrhizal fungi
BI	Bioinoculant
bHLH	Basic helix-loop-helix
Blast	Basic local alignment search tool
BPDS	Bathophenanthroline disulfonic acid
CAP	Common agriculture policy
CAS	Chrome azurol S
CBQF	Centre for Biotechnology and Fine Chemistry
cDNA	Complementary deoxyribonucleic acid
CFU	Colony-forming unit
CRISPR	Clustered regularly interspaced short palindromic repeats
DW	Dry weight
EDTA	Ethylenediaminetetraacetic acid
EC	European Commission
EU	European Union
F6'H1	Feruloyl-CoA 6'-hydroxylase
FBD	Fluid bed dryer
FC-R	Ferric-chelate reductase
FER	Ferritin
FIT	Iron deficiency-induced transcription factor
FRO	Ferric reductase oxidase
FW	Fresh weight
IAA	Indole-3-acetic acid
ICP-OES	Inductively coupled plasma optical emission spectrometer
IRGA	Infrared gas analyzer
IRT	Iron-regulated transporter
ISR	Immune system regulation
MES	2-(N-morpholino)ethanesulfonic acid
NAS	Nicotianamine synthase
NCBI	National Center for Biotechnology Information
OD	Optical density
PGPB	Plant growth-promoting bacteria
PGPR	Plant growth-promoting rhizobacteria
RAPD	Random amplification of polymorphic DNA
rRNA	Ribosomal ribonucleic acid

RT-qPCR	Real time-quantitative polymerase chain reaction
SDS	Sodium dodecyl sulphate
SEM	Standard error of mean
SPAD	Soil plant analysis development
TALEN	Transcription activator-like effector nuclease
TSB	Tryptic soy broth
UN	United Nations
WHO	World Health Organization
ZIP	Zinc finger nuclease

CHAPTER 1

GENERAL INTRODUCTION

Parts of this chapter were published in the two following articles:

Roriz, M., Barros, M., Castro, P.M.L., Carvalho, S.M.P., Vasconcelos, M.W. (2020) Improving iron nutrition in plant foods: the role of legumes and soil microbes, In: N. Benkeblia (ed)., *Vitamins and Minerals Biofortification of Edible Plants* (pp. 103-122). John Wiley & Sons Inc. https://doi.org/10.1002/9781119511144.ch6

Roriz, M., Carvalho, S.M.P., Castro, P.M.L., Vasconcelos, M.W. (2020) Legume biofortification and the role of plant growth-promoting bacteria in a sustainable agricultural era. *Agronomy, 10*(3), 435. https://doi.org/10.3390/agronomy10030435

1.1. Introduction

Iron (Fe) is an essential micronutrient for almost all living organisms due to its redox properties (Connorton et al., 2017). In plants, it is required for photosynthesis, respiration, chlorophyll synthesis, and nitrogen (N) fixation (Marschner, 2012). It is also the fourth most abundant element on Earth and despite its high geological abundance, Fe presents itself in a non-biological form which is not readily available for uptake by plants (Guerinot, 1994; Eng et al., 1998). This low availability is intensified in calcareous soils with pH ranging from 7.5 to 8.5, comprising 30 % of the world's cultivated areas, severely affecting plant productivity (del Campillo & Torrent, 1992; Miethke & Marahiel, 2007; Kobayashi et al., 2016). Other factors such as accumulation of bicarbonate, soluble salts and nitrate, poor aeration, low temperature, and high relative humidity, also contribute to the reduced solubility of Fe in the soil (Lucena, 2000; Schenkeveld et al., 2008; Bloom et al., 2011; Roriz et al., 2014; Vasconcelos & Grusak, 2014). Although plants use two distinct uptake strategies to take up Fe, Fe deficiency problems continue to appear in several crops, with serious implications for plant growth and yield.

Plants are the major source of Fe for humans in several parts of the world (Gibson et al., 2010) and legume grains, such as lentil, pea, common bean, chickpea, soybean, or pigeon pea are very important sources of nutrients in the human diet. In developing countries, where food availability and diet diversification are poor, Fe deficiency anemia problems are highly prevalent. Fe-deficiency anemia is a well-known health problem affecting two billion people, in particular children, pregnant and non-pregnant women (Stevens et al., 2013; Vasconcelos et al., 2017).

Soybean (*Glycine max* L.) is an economically important agricultural crop that is severely affected by Fe deficiency. Symptoms are characterized by stunted growth, and yellowing and interveinal chlorosis of the early trifoliate leaves (Jeong & Connolly, 2009). The outcomes are often diminished yields and crop quality (Vasconcelos & Grusak, 2014). Research efforts have identified cultivars with different susceptibilities to Fe restriction (Vasconcelos & Grusak, 2014) and have contributed to identify several of the underlying factors that trigger Fe uptake and accumulation responses (Santos et al., 2019).

In Portugal, the production of legumes with increased yield and nutritional value is essential, since its inhabitants are avid legume consumers, mainly of common bean (*Phaseolus vulgaris*), chickpea (*Cicer arietinum*), garden pea (*Pisum sativum*), and fava bean (*Vicia faba*), and a large volume of the legumes are imported (Lusa, 2013). Stimulating legume production in Portugal is important for the national economy, and it has also been encouraged in the last years in a European context due to their contribution to the sustainable development of agricultural and food systems (Lusa, 2013). Legume production requires extensive agricultural areas, mainly available in the south of Portugal, where calcareous, Fe deficiency-prone soils are predominant.

The traditional agronomic strategies available to overcome Fe deficiency include soil amendment, seed treatment and foliar application of Fe fertilizers, higher seeding rate, intercropping, and selection of cultivars with increased tolerance to Fe deficiency (Goos & Johnson, 2000; Helms et al.,

2010; Schenkeveld et al., 2010; Vasconcelos & Grusak, 2014; Dai et al., 2019). However, these conventional approaches present some drawbacks such as high costs and environmental risks. Therefore, finding new alternative sustainable strategies to prevent or ameliorate Fe deficiency symptoms is of utmost importance. Furthermore, these demands help in the production of highly nutritious foods in a sustainable manner as in the last years modern agriculture focused mainly on achieving high agricultural yields, leaving aside the nutritional needs (Shaikh & Saraf, 2017).

Scientists are making a great effort to help reducing micronutrient malnutrition, also called “hidden hunger”. This problem is one of the most relevant global societal challenges (Carvalho & Vasconcelos, 2013) and a common strategy to overcome it is through biofortification (i.e. increasing the concentration of bioavailable micronutrients in edible crop tissues). This can be implemented through plant breeding or agronomic practices (Allen et al., 2006; Carvalho & Vasconcelos, 2013; Finkelstein et al., 2017). Biofortification indeed serves both poor and rich populations by battling malnutrition and "hidden hunger", as the population is growing (~10 billion by 2050) (Ahirwar et al., 2019). Some examples of biofortification projects include Fe biofortification of rice, sweet potato, cassava, and legumes according to the World Health Organization (WHO) (Allen et al., 2006). Fe biofortification methods include either conventional breeding or genetic engineering (Vasconcelos et al., 2017). Nowadays, the use of conventional breeding is complemented by more modern techniques such as molecular breeding, genetic transformation, and in the future gene editing approaches. However, the success of these strategies is mainly dependent upon the molecular knowledge of Fe metabolism including root uptake, transport, remobilization, storage, and improvement in its bioavailability (Vasconcelos et al., 2017).

In this context, legume grains are good candidates for biofortification to assure a high nutritional value since they represent a benefic and cost-efficient dietary source of Fe (Suttle, 2010). Moreover, staple foods are the base diet in poverty scenarios (Allen et al., 2006).

The utilization of microorganisms that naturally colonize plant roots and promote the uptake of mineral nutrients more effectively is a promising sustainable strategy to be used in nutrient management programs. Soil microorganisms have a strong role in nutrient remobilization in the soil, enhancing root uptake of the most important macronutrients (N, phosphorus (P), and potassium (K)) and micronutrients (copper (Cu), Fe, manganese (Mn), and zinc (Zn)) involved in the growth and development of plants (Paul & Lade, 2014; Rashida et al., 2016). Plant growth stimulation and crop yield can be improved using plant-associated microorganisms such as plant growth-promoting bacteria (PGPB), which are naturally present in soils and plant tissues (Pii et al., 2015). Many studies have shown that the application of PGPB improve Fe uptake by legumes grown under Fe-deficient conditions (Zhou et al., 2016, 2018; İpek et al., 2017; Nagata, 2017; Aras et al., 2018; Arıkan et al., 2018; Patel et al., 2018; Rahimi et al., 2020; Roriz et al., 2021). Therefore, the utilization of PGPB as a Fe biofortification strategy and a way to avoid Fe deficiency appears as a good alternative or complement to other biofortification approaches.

1.2. Iron metabolism in plants

Either limited or excessive amounts of Fe have a negative impact on the plants and a proper regulation system is essential to maintain homeostasis. This can be kept by the action of five different processes: high-affinity uptake systems (Fe^{2+} and Fe^{3+}-chelates), transport and distribution (Fe^{3+}-citrate in the xylem and Fe^{2+}-nicotianamine in phloem), use of co-factors (Fe-sulfur clusters, heme, mono-Fe, and di-Fe), storage mechanisms (accumulation in seeds) and most importantly, thigh regulation of all the processes stated above, including remobilization (Connorton et al., 2017).

The oxidized form of Fe present in the soil (Fe^{3+}), reduces bioavailability to the plants particularly in calcareous soils, representing a negative impact to crop yield and total plant Fe content. Consequently, human health may be affected by the reduced nutritional quality of the consumed products. In a few words, Fe deficiency problems affect not only soils and crops but also potentially human health (Welch & Graham, 2004; Allen et al., 2006).

1.2.1. Iron uptake and absorption mechanisms

Fe uptake strategies at the root level affect the success of Fe nutrition in crops (Vasconcelos et al., 2017). Trace metals are frequently present in the environment in either low amounts or in configurations that are inaccessible to organisms. The efficacy of the transport systems is certainly of major importance for the uptake and accumulation of Fe in plants (Guerinot, 1994; Eng et al., 1998).

The acquisition of Fe from the soil can be separated into two main categories. The first is a reduction-based strategy (Strategy I) in which the rhizosphere is acidified by the secretion of protons, and Fe^{3+} is reduced to Fe^{2+} through the activity of the ferric reductase oxidase 2 (FRO2). Genes encoding the FRO enzyme include eight members involved in metal acquisition from the soil but also in the intracellular distribution of Fe (Jain et al., 2014). Alongside FRO, other compounds have been proposed to have a key role in the reduction step, such as phenolics, organic acids, sugar, and flavins (López-Millán et al., 2000; Rodríguez-Celma et al., 2011). Fe-deficient conditions trigger the synthesis and release of phenolic compounds (e.g. coumarins) under the control of some particular genes, including feruloyl-CoA 6'-hydroxylase (F6'H1) (Schmidt et al., 2014). In *Arabidopsis*, coumarins bind and possibly reduce Fe^{3+} being important for the Fe uptake process under Fe-deficient conditions (Fourcroy et al., 2014; Schmid et al., 2014; Schmidt et al., 2014; Tsai et al., 2018). The second is a chelation-based strategy (Strategy II) involving the production of phytosiderophores (e.g. mugineic acid) that bind to Fe^{3+} enabling its uptake by the root cells (Gamalero & Glick, 2011; Bulgarelli et al., 2013). Dicotyledonous, like soybean, and non-grasses mainly use a reduction-based strategy whereas most grass species rely on strategy II for an effective Fe uptake by the roots. Rice is a unique grass species able to use both Fe uptake strategies (Vasconcelos et al., 2017). The Fe uptake system also depends on the activity of specific transcription factors, such as Fer-like Fe deficiency-induced transcription factor (FIT) and basic helix-

loop-helix (bHLH) proteins. There are at least 16 bHLH proteins that have been associated with the regulation of Fe nutrition (Gao et al., 2020). bHLH38 physically interacts with FIT to induce expression of the Fe-uptake machinery in Strategy I plants (Walker & Connolly, 2008), inducing transcription activation of FRO2 and IRT1 genes (Yuan et al., 2008).

1.2.2. Translocation and accumulation of iron

The entrance of Fe into the plant is only the first step towards reaching the grains, as Fe requires further transportation to reach edible plant parts (Connorton et al., 2017). After Fe^{3+} is reduced, Fe^{2+} is transported to the root epidermal cells by the iron-regulated transporter 1 (IRT1) (Guerinot, 2000). Fe, Mn, and Zn are all subject to IRT1-mediated transportation. However, in Fe deficiency scenarios, the overexpression of IRT1 results in an increase of Mn and Zn uptake. IRT1 is predominantly localized on the outward-facing membrane of the epidermal cells which suggests that it is the first entry to the symplastic pathway. When traveling through the apoplastic route and reaching the endodermis, nutrients face a barrier called the Casparian strip, which consists of a layer of waterproof lignin. When facing this structure, all Fe is forced to deviate from the apoplast to the symplast (Barberon, 2017). Fe is then loaded into the xylem for transport to the shoot in the Fe^{3+}-citrate form. In the leaves Fe is needed for photosynthesis, re-entering the symplast where FRO proteins reduce it to Fe^{2+}. Two main mechanisms are responsible for Fe storage: sequestration into ferritin, predominantly located in the plastids, or into vacuoles usually present in the aleurone layer. Fe stored in seeds is crucial for plant germination before seedling root development (Connorton et al., 2017). Ferritin proteins also protect cells from oxidative stress (Ravet et al., 2009) and are involved in the control of the root system architecture (Reyt et al., 2015). There are four ferritin genes (FER1, FER2, FER3, and FER4), with the latter two being expressed in the leaves (Briat et al., 2010).

1.3. Iron biofortification strategies

According to WHO, biofortification is “the process by which the nutritional quality of food crops is improved through agronomic practices, conventional plant breeding, or modern biotechnology” (World Health Organization, 2019). This process allows micronutrient enrichment of plant foods and can also target a reduction in the amount of antinutrients, that can negatively affect the bioavailability of nutrients in the human gut. The presence of some food substances such as phytates, tannins, polyphenols, oxalates, and trypsins, the oxidation state, and even the food matrix are all factors that contribute to the solubility and therefore the bioavailability of minerals (Etcheverry et al., 2012). As agricultural goods are the primary source of nutrients for humans, if agricultural systems fail to provide the recommended quantity of vitamins and minerals in plant foods, healthy lives cannot be supported (Welch & Graham, 2004). Ideally, the diet should be diverse and provide all the necessary macro- and micronutrients to sustain the proper growth and development of an organism.

Over the past decades, diet supplementation and food fortification have helped to alleviate this

problem (Tan et al., 2017). However, biofortification can more sustainably address the development of nutrient-dense staple crops that can be grown and distributed using existing agricultural practices, when comparing the drawbacks associated with other nutritional supplementation techniques (Díaz-Gómez et al., 2017).

There are several strategies to achieve Fe biofortification of crops (Figure 1.1). The agricultural strategies include conventional and molecular plant breeding; metabolic engineering techniques include genetic modification and fertilization techniques (Van Der Straeten et al., 2017). Conventional breeding focuses on the different hereditary values by improving multiple vegetative and reproductive traits, reflecting how responsive each character is for a genetic upgrade (Jacob et al., 2016). On the contrary, genetic modification allows the modification of the DNA in an organism's genome, enhancing or modifying the characteristics of an individual organism.

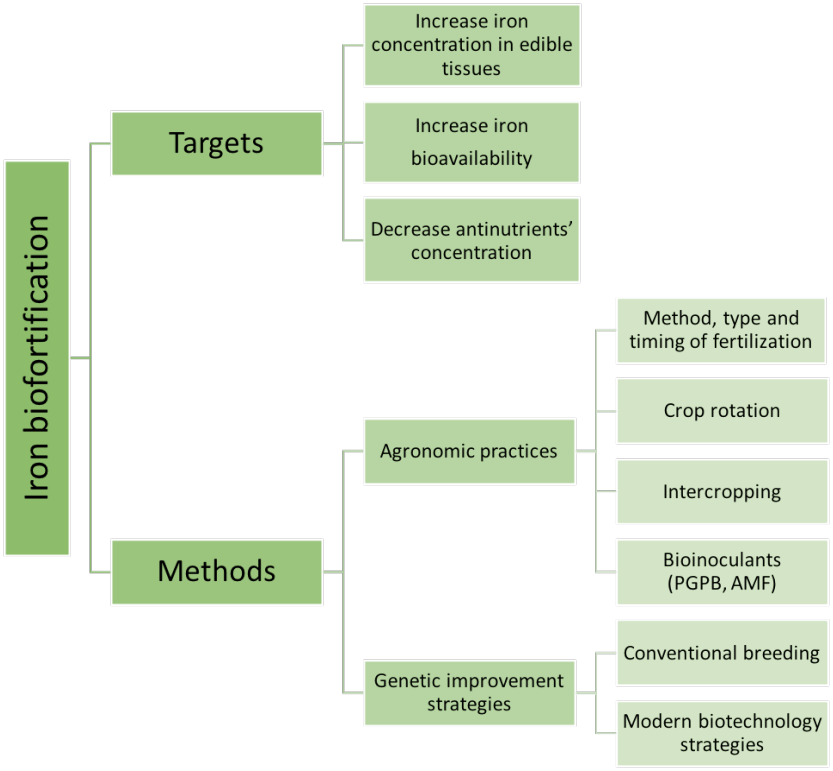

Figure 1.1. Possible Fe biofortification targets and strategies for plant-food products.

Nineteen years ago, the first breeding efforts were made to accomplish the recommended Fe content of 14 μg/g DW in polished rice grains (Trijatmiko et al., 2016). However, this goal was only achieved later on using genetic engineering strategies, revealing that conventional breeding *per se* was not effective at achieving nutritionally relevant values (Boonyaves et al., 2016; Trijatmiko et al., 2016). Besides focusing on the Fe homeostasis processes (uptake, transport, remobilization, and storage) to improve Fe nutrition, Fe must be bioavailable (Carvalho & Vasconcelos, 2013). Vasconcelos et al. (2003) transformed rice with ferritin expressed under the control of an endosperm-specific promoter. Also, the transformation of rice with genes related to phytosiderophore synthesis

or encoding Fe transporters resulted in grains with higher Fe content (Boonyaves et al., 2016). Several studies followed this line of research, and a few years ago, rice lines expressing both ferritin and NAS2 genes were able to produce grains with Fe levels reaching 30 % of the estimated average requirement of the HarvestPlus program (Trijatmiko et al., 2016), and in a trial conducted by Finkelstein et al. (2017) a significant increase of serum ferritin concentration and total body Fe content was demonstrated in at-risk populations that consumed Fe-biofortified rice, beans, and pearl millet, being higher in individuals who were Fe deficient from the beginning and that consumed a greater amount of biofortified food.

The development of new expertise in oligo-directed mutagenesis, RNA-directed DNA methylation, reverse breeding, sequence-specific nuclease technology, and genome editing have significantly advanced in Fe biofortification programs (Vasconcelos et al., 2017). The utilization of conventional breeding strategies for biofortification purposes is limited to the diversity in the gene pool and fertility of the species (Tan et al., 2017). Genome editing technologies usually use zinc (Zn) finger nucleases (ZIPs), meganucleases, transcription activator-like effector nucleases (TALENs), and clustered regularly interspaced short palindromic repeats – CRISPR-associated proteins (CRISPR-Cas) that allow more rapid progress in crop genetic improvement (Jacob et al., 2016; Schaart et al., 2016; Vasconcelos et al., 2017). Regardless of being a valuable tool, its utilization remains controversial due to public and political concerns for environmental and human safety (Frewer et al., 2013; Hefferon, 2015; Tan et al., 2017) and before it can be used the necessary allelic variation in Fe metabolism genes must first be unraveled.

1.4. Legume importance in future agriculture

Legumes belong to the Leguminosae family (Fabaceae). Soybean, pea, bean, lentil, chickpea, and peanut are some examples of edible seed members (Mann & Truswell, 2012). They meet effectively most of the recommended dietary guidelines hence they are low in fat and high in carbohydrates, dietary fiber, vitamins, proteins, and minerals (Erbersdobler et al., 2017). Fresh food consumption has scaled-up in recent years because plant protein from legumes, vegetables, and fruits has been associated with many health recommendations to the detriment of animal protein (Fabbri & Crosby, 2016).

Soybeans, lupins, beans, and peas contain 40 % to 60 % relative levels of Fe per 100 g concerning the daily reference values provided in the EU food information for consumers regulation (Erbersdobler et al., 2017), and legumes are also an important source of nutritional Fe (Balk & Schaedler, 2014). Many studies have been conducted to assess high Fe-accumulating traits in different legumes (Blair et al., 2013; Tan et al., 2017). For example, Blair et al. (2013) found that there are genes located on linkage groups B04 and B11 of common bean related with seed coat and cotyledonary Fe, important for Fe biofortification strategies.

Cooking and processing is an easy alternative to reducing most antinutrients and toxins present in legumes (Fabbri & Crosby, 2016). In the specific case of Fe, its absorption can be improved by

heat processing (Fabbri & Crosby, 2016). Moreover, ascorbic acid is capable of overcoming the negative effect on Fe absorption of all inhibitors comprising phytate and polyphenols (Abbaspour et al., 2014).

The Fabaceae family also plays an important role in ecosystem sustainability as legumes are ecologically known for successfully fixating N through nodulation (Denton et al., 2017). It is well known that the nutritional status of plant N affects the root-shoot translocation of nutrients. Legumes tend to develop symbiosis with N-fixing bacteria, where both combined generate a higher demand for Fe (Brear et al., 2013). Fe is used for the synthesis of leghemoglobin in the host as well as nitrogenase and cytochromes of the electron transport chain in bacteroids (Brear et al., 2013). Indeed, the ability of legumes to obtain fixed N from the bacteroid offers a growth advantage as soil N frequently limits plant growth (Graham, 2003).

The utilization of legume cover crops, cultivated in the fallow period of other crops, has a positive impact on soil stability (Dapaah & Vyn, 1998), control of weeds (Fisk et al., 2001; Sarrantonio & Gallandt, 2003; O'Reilly et al., 2011), nutrient cycling (Tonitto et al., 2006), pest management (Lundgren & Fergen, 2011), and soil N content (Vyn et al., 2000; Burket et al., 1997; O'Reilly et al., 2012), and thus on crop productivity. Although there are costs behind the establishment of such systems (Snapp et al., 2005), benefits are much higher than losses.

Plant-derived proteins such as those from legumes are a desirable option, sustaining a growing world population (Erbersdobler et al., 2017). The world is facing a time of important agricultural losses in terms of productivity and nutritional crop value due to the well-known climate change phenomenon. There has been a growing demand for sustainable agricultural practices to achieve food security in this panorama, and the so-called "climate-smart agriculture" which aims at using agricultural approaches that are "resistant" to the climate changes effects and are environment-friendly has emerged (Campbell et al., 2014). In 2015, the United Nations (UN) outlined 17 goals in a "Sustainable Development Goals Project", with the second one consisting of "end hunger, achieve food security and improve nutrition and promote sustainable agriculture" (United Nations, 2020). This concern is not only affecting human health and the environment but has also a great impact on the economy, representing the most important socio-economic challenge of the century (Daly et al., 2017). Amongst the strategies proposed by the UN to improve food nutrition and fight malnutrition are the increase in dietary diversity, food supplementation, food fortification, and biofortification (explored in section 1.3) (Rehman et al., 2019). In this context, the UN have highlighted the importance of legume biofortification programs, making them a good target to fight against hidden hunger. Also, in light of the European Green Deal, the incentive to the cultivation of legumes contributes to reduce the dependency on N fertilizers that compromise the environment (European Commission, 2020a)

1.5. Role of plant growth-promoting bacteria on crop improvement

Relationships between plants and microorganisms occur, generally, in the rhizosphere and exert beneficial effects on plant nutrition and growth, providing plant resistance and/or protection to cope

with biotic and abiotic stresses (Zamioudis & Pieterse, 2012). Bacteria are the most predominant microorganisms in the soil (95 %) and the greater concentration is found in the rhizosphere (Sattiraju et al., 2019).

There is a special type of bacteria, the PGPB, which includes either free-living bacteria or those that establish symbiotic relationships with plants in the rhizosphere or via endophytic colonization (Compant et al., 2011; Glick, 2020), that are very promising to be used as bioinoculants (BIs) in plant-based biofortification programs for nutritional improvement of food crops and to cope with Fe deficiency. Plant growth promotion can be achieved by a series of direct mechanisms including: N fixation (Islam et al., 2013), nutrient solubilization (Rodríguez et al., 2007; Delvasto et al., 2009), production of several phytohormones (auxins, cytokinins, gibberellins, ethylene, and abscisic acid) (Glick et al., 1995; Vacheron et al., 2013; Maheshwari et al., 2015), and production of siderophores and organic acids (Sayyed et al., 2005; Ahemad & Kibret, 2014; Pereira & Castro, 2014a). Indirectly, PGPB can enhance plant growth through: biocontrol activity through Fe chelation, induced resistance, production of antibiotics, extracellular enzymes and cyanide, and competition for niches in the rhizosphere (Beneduzi et al., 2012; Elshahat et al., 2016). This promotion involves a series of bacterial components that act in very specific ways. Molecules produced by PGPB are involved in several and overlapping mechanisms, influencing plant growth and nutrition, and resistance simultaneously. This thematic has been reviewed by Premachandra et al. (2016) and Rosier et al. (2018). Figure 1.2 illustrates the main mechanisms underlying plant growth promotion by bacteria, regarding important molecules involved in the different mechanisms.

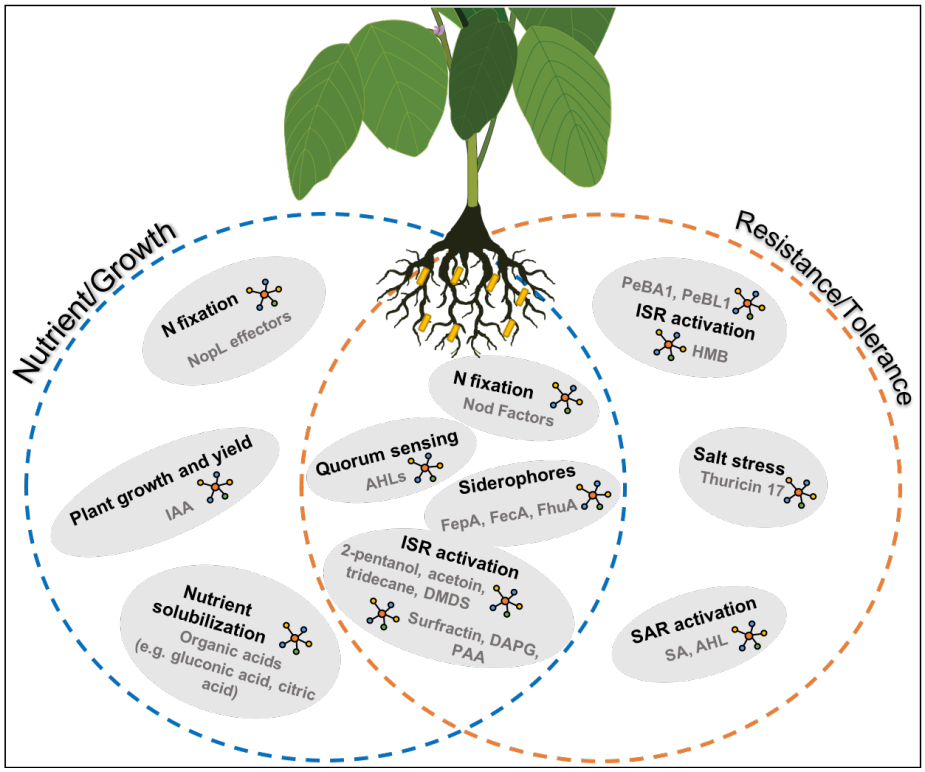

Figure 1.2. Illustration of the main mechanisms underlying plant growth promotion by bacteria, regarding important molecules involved in the different mechanisms.

The utilization of PGPB in the improvement of nutrient content in plants is an example of a biofortification strategy that seems to be very promising. Regarding legume biofortification with PGPB, some studies are showing their potential, most of them in chickpea, mungbean, and soybean (Table 1.1). Co-inoculation with strains *Rhizobium galegae* bv. *orientalis* HAMBI 540 and *Pseudomonas trivialis* 3Re27 increased nodule numbers and N content of fodder galega plants (Egamberdieva et al., 2010). Two similar studies in lentil and pea proved that co-inoculation with strains *Rhizobium leguminosarum*-PR1 and *Pseudomonas* sp. NARs1 or *Pseudomonas* sp. PGERs17 improved nodulation, leghaemoglobin, Fe, and chlorophyll content, and N and P uptake (Mishra et al., 2011, 2012). A study in common bean showed that co-inoculation with strains *Pseudomonas* sp. LG and *Rhizobium phaseoli* potentiated plant growth and content of N and P (Stajkovic et al., 2011). Also, inoculation with two *Bacillus aryabhattai* strains improved Zn uptake of soybean and wheat in Zn deficient soils (Ramesh et al., 2014a). Gopalakrishnan et al. (2016) tested seven PGPB in a study with chickpea and pigeon pea and found *Enterobacter ludwigii* to be the strain better-promoting root and shoot development, nodule formation, crop productivity, and soil nutritional factors, followed by *Brevibacterium antiquum* and *Acinetobacter tandoii*. In that study, *E. ludwigii* and *A. tandoii* significantly increased Fe, Zn, Cu, Mn, and calcium (Ca) uptake in chickpea and pigeon pea, respectively. Co-inoculation with strains *Bradyrhizobium japonicum* SAY3-7 and *Streptomyces griseoflavus* P4 improved N, P, K, Ca, and magnesium (Mg) uptake in soybean plants (Htwe et al., 2018). A recent study showed that seed coating with Zn solution in combination with a Zn solubilizer PGPB, *Enterobacter* sp., improved plant and grain yield and bioavailable Zn, rather than Zn application alone, in chickpea (Ullah et al., 2019). Co-inoculation of mungbean with *Bacillus aryabhattai* and *B. subtilis* proved to be effective in the improvement of plant growth and nutritional composition; N, P, and K concentration was significantly increased in shoots (Ahmad et al., 2019). Also, inoculation of two varieties of chickpea with five different PGPB (*Frauteria aurantia*, *Pseudomonas* sp., *P. citronellis*, *Serratia* sp., and *S. marcescens*) increased macro- and micronutrient concentration in plants (Dogra et al., 2019).

1.5.1. Plant growth-promoting bacteria and iron deficiency

Since the first description of the role of PGPB in the ‘80s (Kloepper, 1980), the continuous knowledge on the complex interaction between plants and microbes has awakened the interest in the utilization of soil microbes in improving Fe uptake processes by plants. A series of studies reported the importance of these microorganisms in the uptake of micro- and macronutrients (Krishnakumar et al. 2013; Wang et al. 2014; Wu et al. 2015; Berruti et al. 2016), suggesting that these agents may be used in biofertilization efforts aiming at increasing the Fe uptake and accumulation of plant foods and thus preventing Fe deficiency responses.

Table 1.1. Summary of studies showing the potential of some bacterial genera for increased nutritional content in legumes.

Bacterial genera	Crops	Contribution to biofortification	References
Rhizobium galegae bv. *orientalis* HAMBI 540 + *Pseudomonas trivialis* 3Re27	Fodder galega	Increase N content	(Egamberdieva et al., 2010)
Rhizobium leguminosarum-PR1 + *Pseudomonas* sp. NARs1/ *Pseudomonas* sp. PGERs17	Lentil, Pea	Increase Fe, N, and P uptake	(Mishra et al., 2011, 2012)
Pseudomonas sp. LG + *Rhizobium phaseoli*	Common bean	Increase P and N uptake	(Stajkovic et al., 2011)
Bacillus aryabhattai, Enterobacter sp.	Chickpea, Soybean, Wheat	Increase Zn uptake	(Ramesh et al., 2014a; Ullah et al., 2019)
Acinetobacter tandoii, Enterobacter ludwigii	Chickpea, Pigeonpea	Increase Fe, Zn, Cu, Mn, and Ca uptake	(Gopalakrishnan et al., 2016)
Bradyrhizobium japonicum SAY3-7 and *Streptomyces griseoflavus* P4	Soybean	Increase N, P, K, Ca, and Mg uptake	(Htwe et al., 2018)
Bacillus aryabhattai + *B. subtilis*	Mungbean	Increase N, P, and K uptake	(Ahmad et al., 2019)
Frauteria aurantia, Pseudomonas sp., *P. citronellis, Serratia* sp., *S. marcescens*	Chickpea	Increased macro- and micronutrient uptake	(Dogra et al., 2019)

PGPB can modulate the uptake and accumulation of several minerals (e.g. Fe, Zn, Mn, Mg, Ca, P, and K) in two different ways: increasing nutrient availability, and/or improving plant access to nutrients (Vessey, 2003). Several mechanisms are found to be associated with improved Fe uptake by bacteria (Table 1.2) which can be direct or indirect: i) rhizosphere acidification through the synthesis of organic acids which improves Fe solubility; ii) chelation and mobilization of Fe through the activation of ferric-chelate reductase (FC-R), and release of phenolic compounds, chelating agents, and siderophores; iii) induction of Fe-deficiency signaling pathways through the increased expression of FRO2 and IRT1 genes; and iv) improvement of photosynthetic capacity through the increase of chlorophyll content (Rajkumar et al., 2010; Bahadur et al., 2016; Zhou et al., 2016, 2018; Delaporte-Quintana et al., 2020; Kong et al., 2020). PGPB can synthesize low molecular weight compounds called siderophores, producing them in Fe-deficient conditions, and to date, more than

500 different siderophores have been reported (Boukhalfa, 2003). These can be divided into four groups: hydroxamate siderophores (the most common ones), catecholates, carboxylates, and pyoverdines. Species of *Pseudomonas fluorescens*, *P. aeruginosa*, *Escherichia coli*, and *Rhizobium meliloti* produce siderophores such as ferribactin, pyoverdine, enterochelin, and rhizobactin, respectively (Maurer & Keller-Scheirlin, 1968; Smith & Neilands, 1984; Schalk & Guillon, 2013). Bacterial siderophores are also associated with increased Fe uptake in Strategy I plants (including soybean) through the sharing of ferric ions between microbial siderophores and plant FC-R, promoting Fe reduction and transport into the plant, and indirectly by sequestering Fe required for the growth of phytopathogens (Crowley et al., 1988; Lucena et al., 2006; Bacaicoa et al., 2011; Meena et al., 2017). The synthesis of indole-3-acetic acid (IAA) by PGPB is often associated with the development of plant lateral roots and root hairs, potentiating a higher absorption surface area for Fe uptake, and increased porosity of the plant cell wall, enhancing root exudation (Jin et al., 2008; Chen et al., 2010; Glick, 2012; Wu et al., 2012; Zhou et al., 2016), while the enzyme 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase decreases the ethylene levels in plants, reducing its negative impact on plant physiological processes (Khan et al., 2009), having extreme importance when plants are exposed to stressful conditions (Grichko et al., 2000), such as alkaline conditions. Furthermore, inoculation with ACC deaminase-containing bacteria improves stem diameter and length, and root elongation of pea seedlings, suggesting that its activity influence such plant traits (Shaharoona et al., 2006), contributing also to improved mineral uptake.

Table 1.2. Strategies for increased Fe uptake and accumulation by PGPB.

Strategy	Description	Reference
Rhizosphere acidification	Increased Fe solubilization through the production of organic acids	(Kong et al., 2020)
Chelation and mobilization of Fe	Activation of FC-R, and release of phenolic compounds, chelating agents, and siderophores	(Rajkumar et al., 2010; Bahadur et al., 2016; Zhou et al., 2016, 2018; Delaporte-Quintana et al., 2020; Kong et al., 2020)
Induction of Fe-deficiency signaling pathways	Activation of FRO2 and IRT1 genes	(Zhang et al., 2009; Zhou et al., 2016, 2018)
Improved photosynthetic capacity	Increased chlorophyll content	(Zhou et al., 2016, 2018; Kong et al., 2020)
Production of IAA	Increased Fe solubilization through the development of plant lateral roots and root hairs	(Hardoim et al., 2008; Zhou et al., 2016, 2018)

It has been demonstrated that plants can select the “best” microorganisms when Fe-deficient conditions appear, namely those capable of producing auxins and siderophores. Also, when there is a high content of phenolic root exudates, produced by Fe-deficient plants, higher auxin-producing microorganisms appear in the rhizosphere.

Some studies have shown that the application of PGPB improve Fe uptake and accumulation by legumes grown under Fe-deficient conditions. A field study in chickpea grown in calcareous soil showed that inoculation with 19 *Acinetobacter* species increased overall seed nutrient content, with increases of 10-38 % in Fe concentration (Sathya et al., 2016). The inoculation with *Pseudomonas illinoisensis* and *Bacillus* sp. improved Fe nutrition of peanut grown in calcareous soil in pot and field experiments (Liu et al., 2017). Similarly, Patel et al. (2018) showed that the Fe content of mungbean grown in sterilized soil could be improved by 3.4-fold if inoculated with *Pantoea dispersa* MPJ9 strain. Plants of *Astragalus sinicus* grown in an artificial calcareous soil treated with *Burkholderia cepacia* JFW16 strain also showed improved Fe assimilation in a pot experiment (Zhou et al., 2018).

Bacterial composition in plants and soils vary according to the host species and genotype, season, and environment, having an important impact on plant fitness, growth, and productivity. The examples given above illustrate that PGPB are very promising as they can serve as natural BIs representing a solution for agro-environmental problems and nutritional deficiencies.

1.6. Scope and thesis outline

The increase in world population, together with climate changes and increased hidden hunger, bring an urgent need for finding sustainable and eco-friendly agricultural approaches for improved crop yield and its nutritional value. With abiotic stresses intensifying, such as Fe deficiency, the existing methodologies for enhancing the concentration of bioavailable micronutrients, including Fe, in edible crop tissues (i.e. biofortification), including some agronomic strategies, conventional plant breeding, and genetic engineering, are not sufficient. In the last years, the use of PGPB has been suggested as a promising approach for the biofortification and prevention of nutritional deficiencies of important crops, including legumes.

The main purposes of this study were to further unveil the mechanisms underlying Fe uptake and accumulation in soybean by PGPB. PGPB were retrieved from two sources: i) from one existing collection at CBQF and ii) through microbial isolation from soybean plants growing in the field. PGPB were inoculated in soybean plants grown under alkaline conditions and plant morphological, physiological, and molecular parameters were evaluated to test their potential to enhance crop nutrition and stimulate soybean production. The specific objectives of this thesis were: (1) to study the *in vitro* traits of PGPB strains available within a CBQF collection to tackle Fe-deficiency; (2) to investigate the bacterial diversity of PGPB associated with soybean and to assess their *in vitro* traits to tackle Fe-deficiency; (3) to evaluate the role of PGPB in plant growth-promotion and Fe deficiency prevention/alleviation through evaluation of plant morphological and physiological parameters in *in vivo* trials; (4) to assess the expression of genes related to Fe nutrition as a result of bacterial

colonization; and (5) to review the role of BIs in the search for more sustainable agricultural practices exploiting relevant aspects of its formulation.

This thesis comprises five chapters. In the current chapter (**Chapter 1**), a brief review of the state of the art is presented concerning the importance of Fe in human and plant nutrition and focusing on the Fe deficiency problem, which severely affects crop yield and production in calcareous soils. Also, the role of legume crops, with particular focus on soybean, susceptible to Fe deficiency, in the search for more nutritional and sustainable food as suggested by the UN, and in a perspective to boost the national economy, is addressed. Finally, the potential of using PGPB as a sustainable agronomical approach to improve Fe nutrition and to cope with Fe deficiency is described. In **Chapter 2**, the first experimental part of this thesis, a collection of PGPB available at CBQF were selected based on their potential to promote plant growth and tested for their potential to improve Fe uptake. Two promising strains were tested *in vivo* for their potential to modulate Fe uptake and accumulation in soybean plants grown under alkaline conditions (Fe-deficient conditions). Isolation and characterization of native PGPB from soybean shoots, roots, and rhizosphere is described in **Chapter 3**. After the selection of two isolates with distinct traits related to plant growth-promotion and Fe uptake potential, an inoculation experiment was performed to evaluate their potential as BIs for improved soybean growth. **Chapter 4** presents a review of the current aspects and considerations in the formulation of BIs with PBPG. In the last chapter (**Chapter 5**), the main findings are summarized, highlighting major conclusions and presenting suggestions for further research.

CHAPTER 2

IRON METABOLISM IN SOYBEAN GROWN IN CALCAREOUS SOIL IS INFLUENCED BY PLANT GROWTH-PROMOTING RHIZOBACTERIA – A FUNCTIONAL ANALYSIS

This chapter was presented as poster communications and published articles:

Roriz, M., Pereira, S.I.A., Castro, P.M.L., Carvalho, S.M.P., Vasconcelos, M.W. (2021) Iron metabolism in soybean grown in calcareous soil is influenced by plant growth-promoting rhizobacteria – a functional analysis. *Rhizosphere* 17, 100274. https://doi.org/10.1016/j.rhisph.2020.100274

Roriz, M., Castro, P.M.L., Carvalho, S.M.P., Vasconcelos, M.W. (2018) Aplicação de bactérias promotoras de crescimento para controlo da clorose por deficiência de ferro em soja. *Agrotec* 26: 45. http://www.agronegocios.eu/noticias/aplicacao-de-bacterias-promotoras-de-crescimento-para-controlo-da-clorose-por-deficiencia-de-ferro-em-soja/

Roriz, M., Pereira, S.I.A., Castro, P.M.L., Carvalho, S.M.P., Vasconcelos, M.W. (2018) Phenotypic evaluation of the IDC profile of soybean plants after inoculation with plant growth-promoting bacteria. *Ciência 2018*. Lisbon, Portugal: 2-4 July 2018.

Roriz, M., Pereira, S.I.A., Castro, P.M.L., Carvalho, S.M.P., Vasconcelos, M.W. (2018) Phenotypic evaluation of the IDC profile of soybean plants after inoculation with plant growth-promoting bacteria. *COSTFA1306 Meeting - Plant phenotyping for future climate changes*, Leuven, Belgium: 20-21 March 2018.

Abstract

Iron deficiency results in severe yield losses, particularly in calcareous soils. Recent evidences suggest that bioinoculants with plant growth-promoting rhizobacteria (PGPR) may be an efficient strategy for enhancing iron (Fe) nutrition in legumes. This work aimed at evaluating the capacity of PGPR strains to enhance Fe uptake-related processes in soybean grown in calcareous soil. From the studied 24 PGPR, *Sphingobium fuliginis* ZR 1-6 and *Pseudomonas jessenni* ZR 3-8 strains were selected for the inoculation experiment based on their *in vitro* ability to produce indole-3-acetic acid, 1-aminocyclopropane-1-carboxylic acid deaminase, siderophores, and organic acids, to tolerate high pH, and to reduce Fe^{3+}. The effect of bacterial inoculation on improving Fe uptake was tested using each isolate alone or combined and through the evaluation of several morphological, physiological, and molecular parameters. Inoculation with *S. fuliginis* showed beneficial effects particularly at the root level by the improvement of ferric chelate activity (111 %) and FRO2 expression (646 %), resulting in increased Fe root content (62 %). Inoculation with *P. jessenii* increased Zn and Mn concentrations in the trifoliates (463 % and 51 %, respectively), decreased Zn concentration in the roots (88 %), and increased the expression of FER4 in the trifoliates (5260 %). Combined inoculation of both strains fostered Fe accumulation in the trifoliates and increased the expression of IRT1 and FER4 genes, indicating an improved capacity of Fe translocation to the shoots. These results suggest that inoculation with selected PGPR strains could be effective in improving Fe uptake and accumulation in soybean grown under Fe-deficient conditions.

2.1. Introduction

Iron (Fe) is an essential micronutrient required for several biological processes as an enzyme cofactor. In plants, it is required for photosynthesis, respiration, chlorophyll synthesis, and nitrogen (N) fixation (Marschner, 2012). Ferric Fe, Fe^{3+}, the predominant form present in the soil, is not easily absorbed by plants, especially in calcareous soils with pH ranging from 7.5 to 8.5 (del Campillo & Torrent, 1992; Miethke & Marahiel, 2007). Other factors such as accumulation of bicarbonate, soluble salts and nitrate, poor aeration, low temperature, and high relative humidity, also contribute to the reduced solubility of Fe in the soil (Lucena, 2000; Schenkeveld et al., 2008; Bloom et al., 2011; Roriz et al., 2014; Vasconcelos & Grusak, 2014). These conditions compromise the production of crops growing in calcareous soils around the world, comprising 30 % of the world's cultivated areas (Marschner, 2012; Li et al., 2016; Kobayashi et al., 2016). Soybean (*Glycine max* L.) is an economically important agricultural crop that is severely affected by Fe deficiency. Symptoms are characterized by stunted growth and yellowing and interveinal chlorosis of the early trifoliate leaves (Jeong & Connolly, 2009). The outcomes are often diminished yields and crop quality (Vasconcelos & Grusak, 2014). Research efforts have identified cultivars with different susceptibilities to Fe

restriction (Vasconcelos & Grusak, 2014) and have contributed to identify several of the underlying factors that trigger Fe uptake and accumulation responses (Santos et al., 2019).

To cope with Fe deficiency, plants have evolved two classic strategies to uptake Fe from the rhizosphere. Strategy I, also referred to as 'reduction strategy', is used by all dicotyledonous like soybean and non-gramineous. It is characterized by the release of protons which results in the acidification of the rhizosphere, followed by the reduction of Fe^{3+} to Fe^{2+} through the activity of the ferric reductase oxidase 2 (FRO2). Genes encoding the FRO enzyme include eight members involved in metal acquisition from the soil but also in the intracellular distribution of Fe (Jain et al., 2014). Alongside with FRO, other compounds have been proposed to have a key role in the reduction step, such as phenolics, organic acids, sugar, and flavins (López-Millán et al., 2000; Rodríguez-Celma et al., 2011). Fe-deficient conditions trigger the synthesis and release of phenolic compounds (e.g. coumarins) under the control of some particular genes, including feruloyl-CoA 6'-hydroxylase (F6'H1) (Schmidt et al., 2014). In *Arabidopsis*, coumarins bind and possibly reduce Fe^{3+} being important for the Fe uptake process under Fe-deficient conditions (Fourcroy et al., 2014; Schmid et al., 2014; Schmidt et al., 2014; Tsai et al., 2018). After Fe^{3+} is reduced, Fe^{2+} is transported to the root epidermal cells by the iron-regulated transporter 1 (IRT1) (Guerinot, 2000). Most grass species rely on strategy II for an effective Fe uptake by the roots. Strategy II consists of a chelation-based strategy involving the production of phytosiderophores that bind to Fe^{3+} enabling its uptake by the root cells (Gamalero & Glick, 2011; Bulgarelli et al., 2013). Rice is an unique grass species able to use both Fe uptake strategies (Ricachenevsky & Sperotto, 2014; Pereira et al., 2014; Vasconcelos et al., 2017; Wairich et al., 2019).

Ferritin proteins are involved in Fe storage and it was also found that these proteins protect cells from oxidative stress (Ravet et al., 2009) and are involved in the control of the root system architecture (Reyt et al., 2015). There are four ferritin genes (FER1, FER2, FER3, and FER4), with the latter two being expressed in the leaves (Briat et al., 2010).

The Fe uptake system depends on the activity of specific transcription factors, such as Fer-like iron deficiency-induced transcription factor (FIT) and basic helix-loop-helix (bHLH) proteins. There are at least 16 bHLH proteins that have been associated with the regulation of Fe nutrition (Gao et al., 2020). bHLH38 physically interacts with FIT to induce expression of the Fe-uptake machinery in Strategy I plants (Walker & Connolly, 2008), inducing transcription activation of FRO2 and IRT1 genes (Yuan et al., 2008).

The traditional agronomic strategies available to overcome Fe deficiency include soil amendment, seed treatment and foliar application of Fe fertilizers, higher seeding rate, intercropping, and selection of cultivars with increased tolerance to Fe deficiency (Goos & Johnson, 2000; Helms et al., 2010; Schenkeveld et al., 2010; Vasconcelos & Grusak, 2014; Dai et al., 2019). However, these conventional approaches are not cost-effective. Although some progress has been made in developing cultivars with enhanced tolerance to Fe deficiency, this effort has been hampered due to large environmental effects that interfere with the effectiveness of efficient cultivars (so-called

genotype × environment interaction) (Naeve & Rehm, 2006). Therefore, finding new alternative sustainable strategies to prevent or ameliorate Fe deficiency symptoms is of utmost importance.

The harnessing of microorganisms that naturally colonize plants and help them to uptake mineral nutrients more effectively is a promising strategy to be used in nutrient management programs. Many studies have shown that the application of plant growth-promoting rhizobacteria (PGPR) improve Fe uptake by legumes grown under Fe-deficient conditions. A field study in chickpea grown in calcareous soil showed that inoculation with 19 *Acinetobacter* species increased overall seed nutrient content, with increases of 10-38 % in Fe concentration (Sathya et al., 2016). The inoculation of *Pseudomonas illinoisensis* and *Bacillus* sp. improved Fe nutrition of peanut grown in calcareous soil in pot and field experiments (Liu et al., 2017). Similarly, Patel et al. (2018) showed that the Fe content of mungbean grown in sterilized soil could be improved by 3.4-fold if inoculated with the *Pantoea dispersa* strain. Plants of *Astragalus sinicus* grown in an artificial calcareous soil treated with *Burkholderia cepacia* JFW16 also showed improved Fe assimilation in a pot experiment (Zhou et al., 2018).

The improved Fe uptake promoted by PGPR can be related to several mechanisms: improvement of the root system through the production of the plant hormone indole-3-acetic acid (IAA), activation of Fe-deficiency signaling pathways through the increased expression of FRO2 and IRT1 genes, improvement of the photosynthetic capacity through the increase of chlorophyll content, activation of immune systemic responses through increased expression of defense-related genes (e.g. PR1, PR2, PDF1.2, and MYB72), rhizosphere acidification which improve nutrient solubility through the synthesis organic acids, and Fe chelation and mobilization through the release of phenolic compounds, chelating agents and siderophores (Rajkumar et al., 2010; Glick, 2014; Bahadur et al., 2016; Zhou et al., 2016, 2018; He et al., 2020; Delaporte-Quintana et al., 2020; Kong et al., 2020). Bacterial siderophores are associated with increased Fe uptake in Strategy I plants (including soybean) also through the sharing of ferric ions between microbial siderophores and plant ferric-chelate reductase (FC-R), promoting Fe reduction and transport into the plant, and indirectly by sequestering Fe required for the growth of phytopathogens (Crowley et al., 1988; Lucena et al., 2006; Bacaicoa et al., 2011; Meena et al., 2017).

Besides the limited number of studies on legume species, to the best of our knowledge, little work has been done so far on soybean (a legume species particularly susceptible to Fe deficiency), and the mechanisms by which PGPR improve Fe uptake are still not elucidated.

In the present study, we aimed at evaluating the capacity of PGPR strains to enhance Fe uptake and accumulation in soybean grown in calcareous soil through physiological and molecular approaches. To that end, we evaluated the potential of two PGPR (with known abilities related to Fe nutrition, such as siderophore production, capacity to grow at high pH levels, organic acid synthesis, and ability to reduce Fe^{3+}) inoculated independently or combined in preventing Fe deficiency in soybean plants grown in Fe-deficient conditions in a growth chamber experiment. We hypothesized that PGPR inoculation could improve the nutritional status and the physiological and molecular soybean response to Fe deficiency, and as such the plant responses in terms of fresh weight (FW),

chlorophyll content (soil plant analysis development (SPAD)), mineral content, FC-R activity, and Fe-related gene expression were evaluated.

2.2. Materials and Methods

2.2.1. Characterization and selection of PGPR

Twenty-four bacterial strains were selected from a collection of PGPR strains available at CBQF (Centre for Biotechnology and Fine Chemistry), based on their production of IAA and siderophores, and activity of 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase. Isolates *Arthrobacter nicotinovorans* EAPAA, *Rhodococcus* sp. EC35, *Bacillus subtilis* EAMR, *Pseudomonas putida* EAPC8, *Lysinibacillus fusiformes* EAR, *Pseudomonas* sp. EC8, *Bacillus weihenstephanensis* EC19, *Arthrobacter rombi* EC32, *Bacillus* sp. EC39, *Pseudomonas reactans* EDP 28, and *Pseudomonas fluorescens* S3X were isolated from a metal(loid) contaminated soil in Northern Portugal – Esteiro de Estarreja (S. Pereira & Castro, 2014a; S. Pereira et al., 2015), while the bacterial endophytes *Sphingobium fuliginis* ZR 1-6, *Pantoea allii* ZR 2-1, *Agrobacterium tumefaciens* ZR 2-9, *Chryseobacterium soldanellicola* ZR 2-12, *Enterobacter ludwigii* ZR 2-16, *Pseudomonas chlororaphis* ZR 2-17, *Stenotrophomonas maltophilia* ZR 2-18, *Ochrobactrum haematophilum* ZR 3-5, *Sphingobium fuliginis* ZR 3-6, *Pseudomonas jessenii* ZR 3-8, *Sphingomonas paucimobilis* ZS 1-5, and *P. allii* ZS 3-6 were isolated from tissues of *Zea mays* plants grown in an adjacent agricultural soil in the same location (Esteiro de Estarreja) (Pereira & Castro, 2014b).

All bacterial strains were first tested for their ability to grow in media with high pH. Strains were grown overnight in tryptic soy broth (TSB) medium with pH adjusted to 9.0 and 10.0 with bicarbonate-carbonate buffer, at 30 °C and 100 rpm on a rotary shaker. Bacterial growth in TSB medium (pH 7.0) was used as control. Optical density (OD) at 600 nm was recorded and the percent (%) of growth inhibition was determined. Seventy % was defined as the threshold below which we were able to choose the best isolates within the purpose of our study. The experiments were repeated three times, and OD determinations were made in triplicate.

After testing for pH tolerance, a set of 13 bacterial strains were selected and tested for their capacity to produce organic acids using a qualitative analysis. Strains were grown in MM9 agar medium at 30 °C. After 24 hours, plates were flooded with methyl red dye (1 % w/v) solution (Visser et al., 1997). The change of the colonies' color to pink was indicative of organic acid production. Three replicates were made for each bacterial strain. Their ability to reduce Fe^{3+} was also evaluated according to Coursolle et al. (2010) with some modifications. Briefly, bacterial strains were grown in specific media (SBM) and Fe reduction was evaluated by measuring absorbance at 562 nm immediately after the addition of ferrozine reagent. Standard curves (0.5-3.0 mM) were made using ferrous sulfate and Fe^{3+} reduction was evaluated as the amount of produced Fe^{2+}. The experiments were repeated three times, and absorbance determinations were made in triplicate. The two most

promising PGPR strains were selected for the growth chamber experiment.

2.2.2. Growth chamber experimental design

The growth chamber experiment consisted of a factorial design with four treatments: saline solution (Control), inoculation with *S. fuliginis* ZR 1-6, inoculation with *P. jessenii* ZR 3-8, and inoculation with a mixture of *S. fuliginis* and *P. jessenii* (Mix). Each treatment was replicated six times. Seeds of *G. max* cultivar Williams 82 were disinfected using 70 % (v/v) ethanol for 5 min, followed by a solution of 1.2 % (v/v) sodium hypochlorite, and 0.02 % (w/v) sodium dodecyl sulfate for 15 min. Seeds were then rinsed five times in sterile water. Germination was performed in filter paper for seven days in the dark, at 25 °C. Thereafter, one seedling per hole (125 cm^3) was transplanted to seedling trays containing a mixture (1:1) of vermiculite and calcareous agricultural soil (pH 7.8; Fe concentration: 5 mg/kg) collected in the South of Portugal. The physicochemical properties of this mixture are presented in Supplementary Table S2.1.

For inoculation, bacterial strains were grown overnight in TSB medium at 30 °C. Cells in the exponential phase were harvested by centrifugation at 5000 rpm for 10 min, washed twice with sterile saline solution (0.85 % NaCl), and centrifuged again. Bacterial inoculum was prepared by resuspending pellet cells in sterile saline solution to get an inoculum density of ca. 10^8 CFU/mL. Bacterial suspensions (3 mL/hole) were sprayed into the soil mixture surface at the time of seedlings transplantation.

The seedling trays were placed in a growth chamber (Aralab Fitoclima 10000EHF) with a 16h day photoperiod. Temperatures were set to 25 °C during the light period and 20 °C during the dark period, whereas relative humidity was maintained at 75 % throughout day and night. Plants were watered daily with 5 mL of a nutrient solution composed by 1.2 mM KNO_3, 0.8 mM $Ca(NO_3)_2$, 0.3 mM $MgSO_4.7H_2O$, 0.2 mM $NH_4H_2PO_4$, 25 mM $CaCl_2$, 25 mM H_3BO_3, 0.5 mM $MnSO_4$, 2 mM $ZnSO_4.H_2O$, 0.5 mM $CuSO_4.H_2O$, 0.5 mM MoO_3, and 0.1 mM $NiSO_4$, with pH adjusted to 10.0 with sodium carbonate and calcium oxide. This nutrient solution allowed maintaining the alkaline conditions provided by the calcareous soil through adjustment of pH to 10.0.

2.2.3. Total fresh weight and SPAD

After 21 days of growth, plants of each treatment were separated into trifoliates and roots and the FW was determined. Half of the samples were stored at -80 °C to be used in the molecular analysis and the other half was further dried at 60 °C for the mineral analysis.

Leaf chlorosis was assessed using a SPAD portable chlorophyll meter (Konica Minolta SPAD-502Plus; Minolta, Osaka, Japan). Readings were made in the youngest trifoliate leaf of six independent plants.

2.2.4. FC-R activity measurements

Root FC-R was quantified as described by Vasconcelos et al. (2006). The measurements were carried out in six plants through spectrophotometric determination of Fe^{2+} chelated to bathophenanthroline disulfonic acid (BPDS). Roots of each plant were submerged in assay solution containing: 1.5 mM KNO_3, 1 mM $Ca(NO_3)_2$, 3.75 mM $NH_4H_2PO_4$, 0.25 mM $MgSO_4$, 25 μM $CaCl_2$, 25 μM H_3BO_3, 2 μM $MnSO_4$, 2 μM $ZnSO_4$, 0.5 μM $CuSO_4$, 0.5 μM H_2MoO_4, 0.1 μM $NiSO_4$, 100 μM Fe (III)-EDTA (ethylenediaminetetraacetic acid), and 100 μM BPDS. All nutrients were buffered with 1 mM 2-(N-morpholino)ethanesulfonic acid (MES), pH 5.5. The assay was conducted in near darkness conditions at 20 °C and was concluded after 45 min by removal of the roots from the assay solution. Also, during this time, plants were covered with a black plastic bag to avoid any residual light exposure to the assay solution. Absorbance readings were obtained at 535 nm, and an aliquot of the solution that had no roots during the assay was used as blank. Rates of reduction were determined using the molar extinction coefficient of 22.14 mM/cm.

2.2.5. Microwave-assisted digestion and ICP analysis

About 200 mg per biological replicate of the dried plant tissues (trifoliates and roots; n=3) were mixed with 5 mL of 65 % nitric acid (HNO_3) and 2 mL of 30 % hydrogen peroxide (H_2O_2) in a Teflon reaction vessel and heated in a SpeedwaveTM MWS-3+ (Berghof, Germany) microwave system. Digestion procedure was conducted in five steps: 1 – 130 °C/10 min, 2 – 160 °C/15 min, 3 – 170 °C/12 min, 4 – 100 °C /7 min, 5 – 100 °C /3 min. The resulting clear solutions of the digestion procedure were then brought to 50 mL with ultrapure water for further analysis. Nutrient content (Fe, zinc (Zn), manganese (Mn), magnesium (Mg), calcium (Ca), phosphorus (P), and potassium (K)) was analyzed using the inductively coupled plasma optical emission spectrometer (ICP-OES) Optima 7000 DV (PerkinElmer, USA). All ICP analyses per biological replicate were done in triplicate.

2.2.6. RNA extraction and cDNA synthesis

Three biological replicates from each treatment were individually pulverized thoroughly in liquid nitrogen with a mortar and pestle until a fine powder was obtained, and total RNA was extracted using the Qiagen RNeasy Mini Kit (USA, #74904), according to the manufacturer's instructions. RNA quality and quantity were checked by UV-spectrophotometry, using a nanophotometer (Implen, Isaza, Portugal). Single-stranded cDNA was then synthesized using the First Strand cDNA Synthesis Kit (Thermo Scientific, #K1612) in a thermal cycler (VWR, Doppio, Belgium), according to the manufacturer’s instructions.

2.2.7. Primer design and RT-qPCR

The transcript levels of the FRO2, F6'H1, IRT1, bHLH38, and FER4 genes were analyzed, using 18S rRNA and ubiquitin as reference genes. Sequences for ubiquitin, F6'H1, IRT1, bHLH38, and FER4 in *G. max* were queried in the NCBI (National Center for Biotechnology Information) database and the sequences with the highest homology were selected. In all cases, primers were designed using the Primer-Blast tool from NCBI. Primers for the 18S and FRO2 were retrieved from available published literature (Santos et al., 2016). The primer pairs used for the quantitative polymerase chain reaction (qPCR) analysis are listed in Supplementary Table S2.2. FER4 expression is specific to the leaves and thus its expression was only evaluated in the trifoliates. The real time (RT)-qPCR analyses were performed in a CFX96 TouchTM Deep Well Real-Time PCR Detection System (Bio-Rad Laboratories Inc., CA, USA) using iQTM SYBR Green Supermix (Bio-Rad Laboratories, CA, USA). Primer efficiency was determined for all primers by qPCR analysis of a standard curve, constructed by serial dilutions of the synthesized cDNA from one test sample. The amplification protocol was set to cycle as follows: 95 °C denaturation for 10 min; 45 cycles of 95 °C for 15 s, followed by 54–56 °C (depending on the primer used) for 30 s; followed by melt curve stages to check that only single products were amplified. The stability of the reference genes was evaluated using the ΔCT method and the CFX ManagerTM software, version 3.1 (Bio-Rad Laboratories Inc., CA, USA). All expression data were normalized against the geometric mean of the expression of the two stable reference genes, using the ΔCT method incorporating individual amplification efficiencies. Three individual biological replicates were analyzed, and two technical repetitions of each biological replicate were performed. Non-template controls were included in each plate to discard the presence of primer-dimers and/or primer contamination.

2.2.8. Statistical analysis

Data were subjected to analysis of variance (one-way ANOVA) for the effect of the bacterial inoculation using the GraphPad Prism version 7.0 (San Diego, CA, USA). Significant differences between treatments were determined using the Tukey's test ($P < 0.05$).

2.3. Results

2.3.1. Evaluation of Fe nutrition-related traits in selected PGPR

The ability of bacterial strains to grow at high pH values (9.0 and 10.0) is presented in Table 2.1. Growth inhibition was higher at pH 10.0 as compared to 9.0, ranging from 59 to 99 % increase. Thirteen PGPR strains showed growth inhibition lower than 70 % at pH 9.0 and were further tested for organic acid production and Fe^{3+} reduction. The strains *S. fuliginis* ZR 1-6 and *P. jessenii* ZR 3-

8 were selected for the growth chamber assay since they showed good production of organic acids and the highest ability to reduce Fe^{3+}.

Table 2.1. Percent of growth inhibition at pH 9.0 and 10.0, organic acid production, and concentration of reduced Fe^{2+} of bacterial strains. Data are mean ± SEM (n = 9).

Strain	% growth inhibition (pH 9.0)	% growth inhibition (pH 10.0)	Organic acid production[x]	Reduced Fe^{2+} (mM)[x]
Bacillus weihenstephanensis EC19	-24 ± 4	93 ± 0	[b]	0.545 ± 0.091
Lysinibacillus fusiformes EAR	-7 ± 1	66 ± 1	[b]	0.318 ± 0.000
Pantoea allii ZR 2-1	18 ± 1	91 ± 4	[a]	0.409 ± 0.045
Pseudomonas reactans EDP28	26 ± 1	92 ± 0	[b]	0.455 ± 0.045
Bacillus sp. EC39	32 ± 0	59 ± 1	[b]	0.591 ± 0.045
P. allii ZS 3-6	34 ± 0	97 ± 1	[a]	0.500 ± 0.045
Pseudomonas putida EAPC 8	39 ± 2	96 ± 1	[a]	0.364 ± 0.045
Bacillus subtilis EAMR	42 ± 5	92 ± 2	[b]	0.409 ± 0.045
Pseudomonas sp. EC8	44 ± 3	91 ± 1	[b]	0.955 ± 0.091
Sphingobium fuliginis ZR 1-6	48 ± 3	67 ± 2	[a]	0.682 ± 0.045
Pseudomonas jessenii ZR 3-8	50 ± 0	80 ± 0	[a]	1.045 ± 0.045
Rhodococcus sp. EC35	55 ± 1	94 ± 1	[a]	0.409 ± 0.045
Stenotrophomonas maltophilia ZR 2-18	67 ± 1	89 ± 1	[a]	0.500 ± 0.000
Ochrobactrum haematophilum ZR 3-5	72 ± 0	95 ± 1	n.d	n.d
Arthrobacter rombi EC32	73 ± 1	89 ± 1	n.d	n.d
Pseudomonas fluorescens S3X	79 ± 0	96 ± 1	n.d	n.d
S. fuliginis ZR 3-6	80 ± 4	89 ± 4	n.d	n.d
Pseudomonas chlororaphis ZR 2-17	80 ± 1	95 ± 0	n.d	n.d
Chryseobacterium soldanellicola ZR 2-12	81 ± 1	93 ± 1	n.d	n.d
Arthrobacter nicotinovorans EAPAA	83 ± 0	97 ± 1	n.d	n.d
Agrobacterium tumefaciens ZR 2-9	84 ± 2	94 ± 0	n.d	n.d
Enterobacter ludwigii ZR 2-16	87 ± 1	99 ± 1	n.d	n.d
Sphingomonas paucimobilis ZS 1-5	98 ± 1	98 ± 0	n.d	n. d

[a] organic acid production.

[b] no organic acid production.

[x] only determined when % growth inhibition at pH 9.0 was lower than 70 %; n.d, not determined.

2.3.2. Growth chamber assay

2.3.2.1. Fresh weight and SPAD of soybean plants

FW and chlorophyll content (SPAD readings) of plants grown for 21 days in the growth chamber are shown in Figure 2.1. No significant differences were observed between treatments.

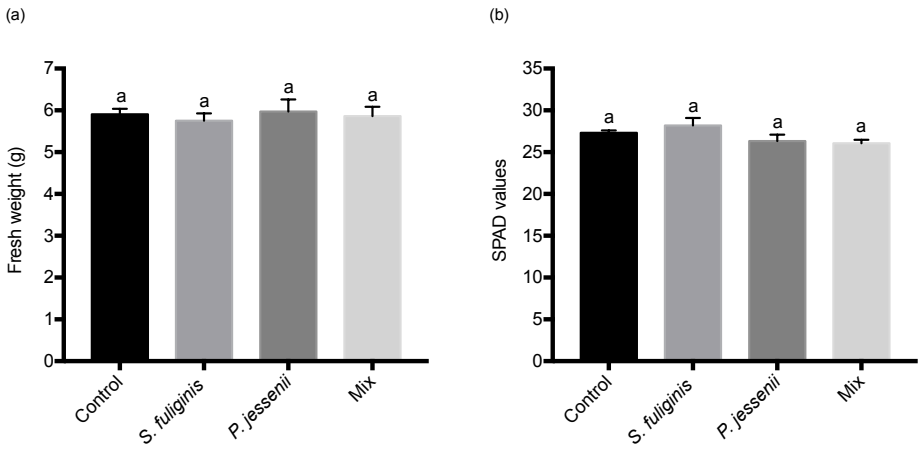

Figure 2.1. Total fresh weight (a) and SPAD values (b) of *G. max* plants, grown under Fe-deficient conditions, inoculated with saline solution (Control), *S. fuliginis*, *P. jessenii*, and a mixture of *S. fuliginis* and *P. jessenii* (Mix). Data are mean ± SEM of six biological replicates. Different letters indicate significant differences ($P < 0.05$) between treatments.

2.3.2.2. Mineral quantification and root FC-R activity

In general, for all treatments, plants accumulated far more nutrients per gram of dry tissue in the roots than in the trifoliates, and this difference was particularly evident in terms of Fe concentration. Moreover, a significant increase in the Fe concentration (144 %) in the trifoliates was observed in plants inoculated with a mixture of both strains (Mix), in comparison to non-inoculated plants. Further, a significant increase in the concentration of Fe (62 %) in the roots of plants inoculated with *S. fuliginis* and of Mn (97 %) in the roots of plants treated with the mixture of strains was observed, compared to Control. Zn concentration significantly decreased in the roots of plants inoculated with *P. jessenii* (88 %), and with both strains (76 %), compared to Control. Mg concentration decreased significantly (34 %) in the roots of plants inoculated with *P. jessenii* as compared to plant inoculated with *S. fuliginis*. No significant differences in the concentration of Ca, P, and K were observed in the inoculated plants when compared to the control plants (*data not shown*) (Table 2.2).

Table 2.2. Mineral concentration on trifoliates and roots (μg/g dry weight) of *G. max* plants, grown under Fe-deficient conditions, inoculated with saline solution (Control), *S. fuliginis*, *P. jessenii*, and a mixture of *S. fuliginis* and *P. jessenii* (Mix). Data are mean ± SEM of three biological replicates. Statistical analysis was performed independently for each mineral and plant tissue. Different letters indicate significant differences ($P < 0.05$) within the same plant tissue between treatments.

Plant tissue	Treatment	Mineral concentration (μg/g)			
		Fe	Zn	Mn	Mg
Trifoliates	Control	24.1 ± 5.7^{b}	0.7 ± 0.6^{b}	65.3 ± 5.3^{a}	5312.0 ± 212.4^{a}
	S. fuliginis	19.5 ± 7.0^{b}	2.7 ± 0.6^{a}	70.4 ± 1.8^{a}	5306.6 ± 191.7^{a}
	P. jessenii	29.9 ± 1.3^{b}	4.2 ± 0.9^{a}	98.4 ± 6.0^{a}	5570.0 ± 187.5^{a}
	Mix	58.8 ± 5.1^{a}	2.4 ± 0.3^{a}	90.2 ± 11.1^{a}	5777.3 ± 364.3^{a}
Roots	Control	1880.3 ± 56.8^{B}	13.4 ± 0.9^{A}	82.4 ± 8.3^{B}	17548.3 ± 433.8^{AB}
	S. fuliginis	3039.6 ± 330.7^{A}	8.8 ± 0.7^{A}	88.1 ± 1.4^{B}	22541.9 ± 2029.8^{A}
	P. jessenii	1930.6 ± 92.5^{B}	1.6 ± 1.4^{B}	114.3 ± 8.8^{AB}	14972.7 ± 852.1^{B}
	Mix	2372.3 ± 223.8^{AB}	3.2 ± 0.6^{B}	162.3 ± 20.8^{A}	17576.3 ± 1147.0^{AB}

The activity of FC-R in the roots of *G. max* plants is shown in Figure 2.2. A significant effect was only observed in soybean plants inoculated with *S. fuliginis*, which resulted in an increase of 111 % in the FC-R activity compared with the non-inoculated plants. Nonetheless, plants inoculated with *P. jessenii* also showed a trend to increase this enzyme activity (56 % increase), but this was not significantly different from the control. Thus, inoculation with the individual bacterial strains outperformed the dual inoculation regarding FC-R activity improvement.

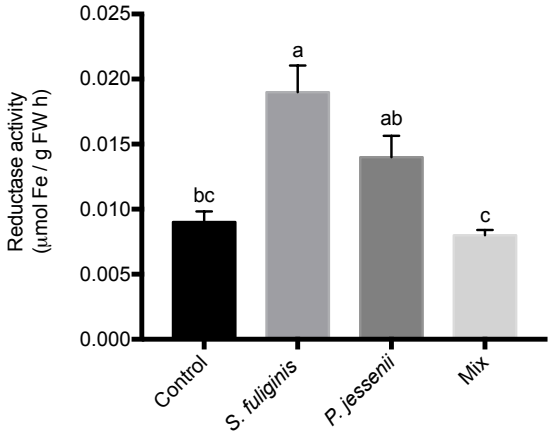

Figure 2.2. Root reductase activity of *G. max* plants grown under Fe-deficient conditions inoculated with saline solution (Control), *S. fuliginis*, *P. jessenii*, and a mixture of *S. fuliginis* and *P. jessenii* (Mix). Data are mean ± SEM of six biological replicates. Different letters indicate significant differences ($P < 0.05$) between treatments.

2.3.2.3. Expression of Fe-related genes

The expression of five genes associated with Fe deficiency response – FRO2, F6'H1, IRT1, bHLH38, and FER4 – were studied using qPCR. The first four were analyzed in the roots and the latter in the trifoliates.

In general, the expression of genes was higher in the inoculated plants, compared to non-inoculated ones (Control) (Figure 2.3). Plants inoculated with *S. fuliginis* showed the highest increase (646 %) in the expression of the gene FRO2 in roots when compared to control plants. F6'H1 expression in the roots showed a highly significant increase in plants inoculated with *S. fuliginis* (2674 %) and Mix (2510 %). Plants inoculated with the mixture of both strains showed a significant increase (239 %) in the expression of IRT1 compared to Control. The expression of bHLH38 significantly increased (391 %) in the roots of plants inoculated with Mix inocula. Finally, the expression of FER4 in the trifoliates had a significant increase in plants inoculated with *P. jessenii* (5260 %) and Mix (5036 %).

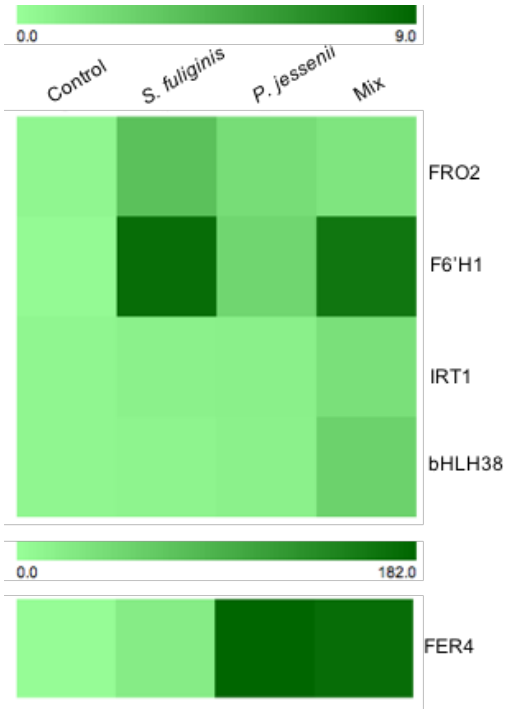

Figure 2.3. Heat map of the relative expression patterns of FRO2, F6'H1, IRT1, bHLH38, and FER4 genes in *G. max* plants, grown under Fe-deficient conditions, inoculated with saline solution (Control), *S. fuliginis*, *P. jessenii*, and a mixture of *S. fuliginis* and *P. jessenii* (Mix). FRO2, F6'H1, IRT1, and bHLH38 expression was measured in the roots; FER4 expression was measured in the trifoliates. Data are means of three independent biological replicates. Corresponding values are presented in Supplementary Table S2.3.

2.4. Discussion

Although inoculation with PGPR did not affect SPAD and FW of soybean plants, this study clearly demonstrated that bacterial inoculants (*S. fuliginis* ZR 1-6 and *P. jessenii* ZR 3-8) were able to modulate mineral uptake and accumulation in soybean. Inoculation with *S. fuliginis* improved FC-R activity and FRO2 expression, resulting in increased Fe root content; inoculation with *P. jessenii* increased Zn and Mn concentrations, and expression of FER4 in the trifoliates; combined inoculation of both strains fostered Fe accumulation in the trifoliates and increased the expression of IRT1 and FER4 genes.

Based on previous studies it was expected that non-inoculated *G. max* plants grown in calcareous soil would show Fe chlorosis as the cultivar used in this study (Williams 82) is susceptible to Fe shortage (Santos et al., 2013, 2016). Nonetheless, in those trials plants were grown under hydroponic conditions, where nutrient deficiencies symptoms are more pronounced due to the lack of cation-exchange capacity in such production system, which does not happen when plants are grown in soil or substrate. Another possible explanation for the lack of leaf chlorosis is the short duration of the experimental period (21 days), which may not have been enough to cause visual Fe deficiency symptoms under the current experimental conditions. However, when looking at the physiological and molecular parameters, evidence supports our hypothesis that PGPR positively contributed to the plant response to cope with Fe deficiency, improving plant Fe accumulation.

Concerning Fe concentration, which is the main focus of this study, higher Fe accumulation occurred in the roots than in the shoots. The same was reported by other authors with plants inoculated with PGPR growing under Fe-deficient conditions in a liquid medium or artificial calcareous soil (Nagata, 2017; Zhou et al., 2018). This could be related to the fact that the Fe measured in the roots includes both the apoplasmic and the cellular Fe, whereas the Fe measured in the trifoliates is only cellular (Bienfait et al., 1987). In our study, non-inoculated plants accumulated less Fe in the trifoliates and in the roots than the inoculated plants, indicating that the PGPR strains favored Fe accumulation by the host plant. Similar results were also reported in the studies of Nagata (2017) and Zhou et al. (2018) where PGPR were used to alleviate Fe deficiency in tomato and Chinese milk vetch plants, respectively. The content of Zn significantly decreased in the roots of plants inoculated with *P. jessenii* and Mix, but a trend to increase in the trifoliates was evident, although not significant. Low Zn concentrations were also found in the roots of soybean plants grown under Fe-deficient conditions in hydroponics (Roriz et al., 2014). The trend increase in the content of Zn in trifoliates can be due to its fast translocation to the shoots (Page & Feller, 2005) not necessarily corresponding to a lower Zn uptake by the roots. Fe deficiency promotes the uptake of Zn and Mn, being well-known that both elements share ZIP-like transporters, such as IRT1, with Fe, among other common molecular markers (Connolly & Guerinot, 2002; Ma et al., 2017). The increased Mn concentration in the roots of plants inoculated with Mix is consistent with the Fe-deficient conditions provided by the Fe-deficient growth environment.

Regarding root FC-R activity, *G. max* plants inoculated with *S. fuliginis* showed the highest increase (111 %), indicating that this strain may be more efficient in the activation of FC-R. Increased FC-R activity was also verified in barley, tomato, apple, and peach rootstocks, and camphor tree inoculated with PGPR and grown under Fe-deficient conditions (Scagliola et al., 2016; Nagata, 2017; Aras et al., 2018; Arıkan et al., 2018; Kong et al., 2020). The activity of the FC-R FRO2 is essential for the reduction of Fe^{3+} to Fe^{2+} in Strategy I plants, like soybean (Marschner, 2012). Several explanations can be put forward. A probable one is that the presence of the PGPR efficiently promoted the acidification of the rhizosphere through the release of organic acids, which was the case of the strains used in this study, decreasing the pH of the medium, and activating FC-R in the roots. However, some authors reported that the accumulation of organic acids and the increase in FC-R activity are not always related (Bienfait et al., 1989; Schmidt, 1999). Another explanation could be the good ability of these strains to produce IAA promoting lateral root formation which increases the plant's capacity to take up Fe and the surface area for FC-R activity (Kong et al., 2020).

Plants have evolved a set of molecular regulatory mechanisms to adapt to Fe deficiency. Several Fe homeostasis-regulated genes play essential roles in Fe uptake (Santi & Schmidt, 2009). In our study, the expression of Fe-related selected genes increased, in general, in all inoculated plants, indicating that PGPR inoculation induced molecular responses to Fe deficiency. As previously mentioned, FRO2 is important in the reduction of Fe^{3+} to Fe^{2+} and in the intracellular distribution of Fe. Under Fe-deficient conditions, usually, its expression increases in the roots (Robinson et al., 1999; Connolly et al., 2003). Our results showed that inoculation with PGPR further promoted an increase in the expression of FRO2 genes at the root level indicating that Fe uptake machinery was effectively activated in these plants to cope with Fe deficiency. These findings are corroborated by the highest Fe content verified in the roots of inoculated plants. In our study, a significant increase in FRO2 expression was verified in plants treated with *S. fuliginis* and these results are in line with the higher FC-R activity verified in plants inoculated with this strain. Increased FRO2 expression values after inoculation with PGPR in plants grown under Fe-deficient conditions has also been shown by Zhou et al. (2016, 2018) and Rahimi et al. (2020). Root F6'H1 expression increased in inoculated plants. F6'H1 is also involved in the Fe uptake process, and it is thought to be involved in coumarin biosynthesis (Mai et al., 2016). Fe-mobilizing coumarins are secreted by roots under Fe deficiency and mobilize Fe by reduction and/or chelation of Fe^{3+} (Tsai & Schmidt, 2017b). F6'H1 is under control of the transcription factor FIT, a key regulator of Fe acquisition that also controls the expression of FRO2 and IRT1 (Tsai & Schmidt, 2017a), also increased in the present study. Fe^{2+} is then transported to the plant by the IRT1 protein. The higher expression of this gene in the roots of inoculated plants indicates a more effective transport and translocation of Fe^{2+} from the roots to the shoots. Similar results were found in previous studies where several PGPR strains such as *B. subtilis* GB03 and *P. polymyxa* BFKC01 activated the transcription of FIT, IRT1, and FRO2 (Zhang et al., 2009; Zhou et al., 2016). bHLH38 is another transcription factor, which together with FIT is responsible for the regulation of Fe uptake (Wang et al., 2007; Walker & Connolly, 2008); bHLH38 confer transcription activation of FRO2 and IRT1 genes (Yuan et al., 2008). bHLH38 expression

significantly increased in plants dually inoculated. Similar results were found in shoots and roots of *Arabidopsis* grown under Fe-deficient conditions (Yuan et al., 2008). Expression of ferritin genes, such as FER4, is related to Fe storage in leaves, being important for general Fe homeostasis (Ravet et al., 2009). In our study, expression of FER4 increased in the trifoliates of inoculated plants suggesting a plant response to the high Fe concentration in the shoots. This allows plants to regulate Fe and maintain homeostasis, by storing or releasing the required Fe as needed, thus avoiding toxic effects (Ting-Bo et al., 2006). Higher rates of ferritin expression, particularly in the shoots, have been detected before in plants grown under high Fe concentrations (Vasconcelos et al., 2014; Santos et al., 2016). However, in our study, under Fe-deficient conditions, this increased expression can be justified by an effective enhancement of Fe uptake and storage in the shoots caused by PGPR inoculation.

Improved Fe nutrition promoted by inoculation with *S. fuliginis* and *P. jessenii* strains can be due to the enhancement of the biochemical and molecular mechanisms managing Fe availability and motility in the root surface area, either through the production of phytohormones (IAA and ACC deaminase) (Bacaicoa et al., 2011; Kong et al., 2020), siderophores (Crowley, 2006), and organic acids (Abadía et al., 2002), improvement of FC-R activity (Marschner, 2012), and/or regulation of key Fe-related genes (Santi & Schmidt, 2009). To date, no other studies have used PGPR to mitigate Fe deficiency in soybean grown under Fe-deficient conditions. Our results reveal that the utilization of *S. fuliginis* alone or in combination with *P. jessenii* are bioinoculants with the potential to improve Fe nutrition in soybean plants.

2.5. Conclusions

Soybean plants grown under Fe deficiency showed increased Fe concentrations in plant tissues, root FC-R activity, and expression of Fe-related genes after inoculation with *S. fuliginis* and *P. jessenii* strains, which suggest that plant Fe uptake and accumulation were successfully modulated. *S. fuliginis* provided more marked effects in the Fe deficiency responses at the root level. This strain significantly increased FC-R activity and FRO2 expression in the roots, thus allowing Fe^{3+} reduction and absorption, which could be seen by the significant increase in the root Fe content. However, in general, the combination of both PGPR strains (Mix) was more effective, particularly regarding Fe translocation to the shoots. Plants treated with the mixed inocula showed a significant increase in the Fe content in trifoliates and in the expression of IRT1, which is thought to be associated with the translocation of Fe to the shoots. Furthermore, plants inoculated with the mixture of strains also showed a marked increase in the expression of FER4. Our results show the potential of the selected PGPR strains as bioinoculants to improve Fe nutrition in *G. max* plants grown in calcareous soils, constituting a sustainable biological alternative to the currently available strategies. Nevertheless, additional studies would be important to test the robustness of their efficacy under field conditions where different soil compositions, environmental conditions, and other biological factors are present.

Supplementary material

Table S2.1. Physiochemical properties of the substrate (1:1 mixture vermiculite + agricultural soil).

Parameter	
pH	7.8
Organic content (%)	2.46
Texture	Thin
Slime (g/kg)	251
Clay (g/kg)	376
Coarse sand (g/kg)	196
Thin sand (g/kg)	177
Texture class	Clay loam
Total $CaCO_3$ (g/kg)	160.5
Extractable K[a] (mg K_2O/kg)	290
Extractable P[a] (mg P_2O_5/kg)	90
Extractable Cu[b] (mg/kg)	0.78
Extractable Zn[b] (mg/kg)	0.6
Extractable Fe[b] (mg/kg)	5.0
Extractable Mn[b] (mg/kg)	7.5
Total N (g/kg)	1.34
E.C (dS/m)	0.236

[a] analysis done by Égner-Riehm method.

[b] analysis done by Lindsay and Norvell – DPTA method.

Table S2.2. Primer sequences used for the quantification of transcripts via PCR.

Primer	Forward (5'-3')	Reverse (5'-3')
18S	TTAGGCCATGGAGGTTTGAG	GAGTTGATGACACGCGCTTA
Ubiquitin	GATTTATTTCATTGGCAGGC	AGGATCATCAGGATTTGGGT
FRO2	CAGAACATGGAAGGGTCAAC	AGCAAGAACTCCCACACTTG
F6'H1	TGCACTCCAAGTAATGAGCA	CAAAGGGCCAATAACATCTG
IRT1	ACAACAATGGCCACTTCACT	GCCAATTATGCTTGAGGCTA
bHLH38	GTGTTCTCAACCAAGGGATG	GGATGGAGAAGTGGACCTTT
FER4	GAACAAACGTGGTGGAAAAG	AACTGCACGTCACCATTCTT

Table S2.3. Relative expression values of FRO2, F6'H1, IRT1, bHLH38, and FER4 genes in *G. max* plants, grown under Fe-deficient conditions, inoculated with saline solution (Control), *S. fuliginis*, *P. jessenii*, and a mixture of *S. fuliginis* and *P. jessenii* (Mix). FRO2, F6'H1, IRT1, and bHLH38 expression was measured in the roots; FER4 expression was measured in the trifoliates. Data are means of three independent biological replicates.

Treatment	Gene				
	FRO2	**F6'H1**	**IRT1**	**bHLH38**	**FER4**
S. fuliginis	3.66	8.60	0.85	0.66	23.04
P. jessenii	2.01	2.43	0.89	0.85	181.69
Mix	1.52	8.09	1.83	2.70	174.10
Control	0.49	0.31	0.55	0.55	3.39

CHAPTER 3

PLANT GROWTH-PROMOTING BACTERIA ISOLATED FROM SOYBEAN: CHARACTERIZATION AND IMPACT ON MODULATING GROWTH UNDER ALKALINE CONDITIONS

This chapter was submitted for publication as:

Roriz, M., Pereira, S.I.A., Castro, P.M.L., Carvalho, S.M.P., Vasconcelos, M.W. (2021) Plant growth-promoting bacteria isolated from soybean: characterization and impact on modulating growth under alkaline conditions. *Applied and Soil Ecology*.

Abstract

Knowledge of crop-specific diversity and functional traits of plant growth-promoting bacteria (PGPB) potentiates their applicability as bioinoculants. Conventional strategies to manage iron (Fe) deficiency constraints, found in calcareous soils, still present drawbacks. Here, we isolated and characterized PGPB from soybean, and evaluated their impact on plant growth under alkaline conditions. Seventy-six bacterial strains were isolated from roots (53 %), rhizosphere (29 %), and shoots (18 %). Twenty-nine genera were identified, with *Bacillus* and *Microbacterium* being the most predominant. Based on the distinct PGP traits, two bacterial strains were selected to assess their potential to improve plant growth under alkaline conditions: *B. licheniformis* P2.3 showed ability to reduce Fe^{3+} and to produce IAA and organic acids, weak siderophore production and ACC deaminase activity; *B. aerius* S2.14 showed strong production of siderophores, moderate IAA production and ACC deaminase activity, and low ability to reduce Fe^{3+}. *In vivo* tests showed that soybean photosynthetic parameters, chlorophyll content, total fresh weight, and Fe concentrations were not significantly affected by inoculation with PGPB. However, inoculation with *B. licheniformis* increased pod number (33 %), decreased FC-R activity (45 %), and increased the expression of Fe-related genes (FRO2, IRT1, F6'H1, bHLH38, and FER4); inoculation with *B. aerius* decreased root length (20 %), FC-R activity (55 %), and FRO2 expression, and increased the expression of the remaining genes. Although Fe concentrations were not significantly affected, inoculation with PGPB impacted significantly the accumulation of other essential minerals (Mn, Zn, and Ca). *B. licheniformis* showed potential to be incorporated in formulations for improving soybean growth under alkaline conditions.

3.1. Introduction

Plants co-exist in association with a diversity of bacteria that modulate their growth and development. Plant growth-promoting bacteria (PGPB) are often found in the plant rhizoplane attached to the root surface, in the rhizosphere, and inside plant tissues as endophytic bacteria colonizing different organs such as roots, stem, leaves, flowers, fruits, and seeds (Compant et al. 2011; Glick 2020).
Legumes grown in calcareous soils, under alkaline conditions (pH 7.5 to 8.5), usually suffer from iron (Fe) deficiency, exhibiting symptoms such as yellowing and interveinal chlorosis of the young leaves, and stunted growth (Jeong & Connolly, 2009), severely affecting plants' yield and quality (Marschner, 2012; Vasconcelos & Grusak, 2014; Li et al., 2016; Kobayashi et al., 2016). Moreover, the reduced chlorophyll content of young leaves leads to a decrease in the photosynthetic rate, stomatal conductance, and transpiration rate (Jin et al., 2017).

Distinct mechanisms are used by plants to efficiently uptake Fe from the soils. Non-gramineous plants (e.g. soybean), known as Strategy I plants, use a reduction-based strategy, in which the release of protons results in the acidification of the rhizosphere and activation of the ferric chelate

reductase (FC-R) FRO2 which reduces Fe^{3+} to Fe^{2+}. Soluble Fe is then imported into the root epidermal cells by the iron-regulated transporter 1 (IRT1) which is induced under Fe-limited conditions (Guerinot, 2000; Vert et al., 2002). It was found that several other compounds play important roles in the reduction step conducted by FRO2, such as organic acids, flavins, phenolics, and sugars (López-Millán et al., 2000; Rodríguez-Celma et al., 2011). These compounds are regulated by specific genes, such as feruloyl-CoA 6'-hydroxylase (F6'H1) which controls the synthesis and release of phenolic compounds (e.g. coumarins) (Schmidt et al., 2014). After F6'H1 activation, coumarins bind and reduce Fe^{3+}, improving Fe uptake under limiting conditions (Fourcroy et al., 2014; Schmid et al., 2014; Schmidt et al., 2014; Tsai et al., 2018). The basic helix-loop-helix protein 38 (bHLH38) is also involved in the regulation of Fe uptake in Strategy I plants (Walker & Connolly, 2008; Gao et al., 2020). Fe storage is under the control of ferritin proteins, which also protect cells from oxidative stress (Ravet et al., 2009) and play a role in the root system architecture (Reyt et al., 2015). There are four ferritin genes (FER1, FER2, FER3, and FER4), with the latter two being expressed in the leaves (Briat et al., 2010).

The conventional strategies used to manage the constraints associated with Fe deficiency present some drawbacks such as high costs and environmental risks (Naeve & Rehm, 2006; Šrámek & Dubský, 2009). The search for more sustainable and ecological approaches is therefore essential.

The knowledge of the multiplicity of plant-associated bacteria is of extreme importance as it allows the understanding of their ecological role and potential for biotechnological application in agriculture, such as the management of crop performance grown under alkaline conditions. Previous studies have shown that *Acinetobacter, Bacillus, Enterobacter, Microbacterium, Paenibacillus, Pseudomonas,* and *Stenotrophomonas* are amongst the predominant genera with plant growth-promoting potential associated with soybean plants (Bagalkar, 2013; Ramesh et al., 2014b; Sugiyama et al., 2014; de Almeida Lopes et al., 2016; Jain et al., 2016; Nhu & Diep, 2017; Arfaoui et al., 2018; Ma et al., 2019; Temesgen et al., 2019).

PGPB can modulate the uptake and accumulation of several minerals (e.g. Fe, zinc (Zn), manganese (Mn), magnesium (Mg), calcium (Ca), phosphorus (P), and potassium (K)) in two different ways: increasing nutrient availability, and/or improving plant access to nutrients (Vessey, 2003). Several mechanisms are found to be associated with improved Fe uptake by bacteria: i) indirect rhizosphere acidification through the synthesis of organic acids which improves Fe solubility; ii) chelation and mobilization of Fe through the release of phenolic compounds, chelating agents, and siderophores; iii) induction of Fe-deficiency signaling pathways through the increased expression of FRO2 and IRT1 genes; and iv) improvement of photosynthetic capacity through the indirect increase of chlorophyll content (Rajkumar et al., 2010; Bahadur et al., 2016; Zhou et al., 2016, 2018; Delaporte-Quintana et al., 2020; Kong et al., 2020). Bacterial siderophores are also associated with increased Fe uptake in Strategy I plants (including soybean) through the sharing of ferric ions between microbial siderophores and plant ferric chelate reductase (FC-R), promoting Fe reduction and transport into the plant, and indirectly by sequestering Fe required for the growth of phytopathogens (Crowley et al., 1988; Lucena et al., 2006; Bacaicoa et al., 2011; Meena et al., 2017).

The synthesis of indole-3-acetic acid (IAA) by PGPB is often associated with the development of plant lateral roots and root hairs (Patten & Glick, 2002), contributing to increased nutrient absorption, while the enzyme 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase decreases the ethylene levels in plants, reducing its negative impact on plant physiological processes (Khan et al., 2009), having extreme importance when plants are exposed to stressful conditions (Grichko et al., 2000), such as alkaline conditions. Furthermore, inoculation with ACC deaminase-containing bacteria improves stem diameter and length, and root elongation of pea seedlings, suggesting that its activity influence such plant traits (Shaharoona et al., 2006), contributing also to improved mineral uptake.

The role of PGPB on Fe uptake and accumulation has been demonstrated in recent works with legumes (Sathya et al., 2016; Liu et al., 2017; Zhou et al., 2018; Patel et al., 2018). Although the diversity of rhizo- and endophytic bacteria on soybean has been studied, most works focus on rhizobia isolated from root nodules, and to the best of our knowledge, no work explored their potential to improve crop performance under alkaline conditions.

In previous work, we have shown that two endophytic bacteria isolated from maize with known plant growth-promoting traits and Fe-uptake abilities were able to improve Fe nutrition in 21 days old soybean plants grown in calcareous soil (Roriz et al., 2021). In the present study, we aimed to isolate and characterize PGPB from soybean tissues and rhizosphere to assess whether they could trigger a more pronounced effect in soybean performance grown under alkaline conditions than non-soybean-associated PGPB and further investigate whether that would be reflected in the final yield of soybean plants.

3.2. Materials and Methods

3.2.1. Isolation of PGPB

Four healthy soybean plants cv. PI 635039 and their rhizospheric soil were sampled at random from an agricultural soil in Retorta (Vila do Conde, Portugal; 41349612, -8.719009). For the isolation of bacterial endophytes from plant tissues, primarily, plant surface was sterilized according to Luo et al. 2011 with some modifications: a first wash with tap water was done followed by three rinses with deionized water. After separation into shoots and roots, plant tissues were first dipped for 2 min in 75 % (v/v) ethanol and then 2 min in 25 % (v/v) commercial bleach; final washes with deionized sterile water were made to remove the sterilization agents. To achieve the success of surface disinfection process, 100 µL of water from the final rinse was spread onto Trypticase Soy Agar (TSA; Liofilchem, Italy) medium and incubated for 3 days at 30 °C. No bacterial growth was found. One gram of each plant tissue (shoot and root) was macerated with 9 mL of sterile phosphate-buffered saline solution (PBS, g/L: Na_2HPO_4, 1.44; KH_2PO_4, 0.24; KCl, 0.20; NaCl, 8.00; pH 7.4) with a mortar and pestle under sterile conditions. Serial dilutions were prepared in 0.85 % (w/v) saline solution; 100 µL of each dilution were plated, in duplicate, on TSA medium supplemented with 10 mg/L of fungicidin (Amresco, USA) after autoclaving, and incubated at 30 ºC for 7 days. For the isolation of

rhizobacteria from the rhizosphere, 1 g of fresh soil was suspended in 0.85 % (w/v) saline solution with 2 drops of Tween 20 (Sigma-Aldrich, USA) and vortexed for 10 min. Serial dilutions were prepared in 0.85 % (w/v) saline solution and 100 µL of each dilution were plated, in duplicate, on TSA medium and incubated at 30 ºC for up to 10 days. Bacterial isolates from plant tissues and soil were daily monitored and selected based on their morphology and color. They were further purified by subculturing on TSA medium.

3.2.2. Identification of bacterial isolates

Genomic DNA was obtained by the heat-shock extraction method (Calheiros et al., 2010). Random Amplification of Polymorphic DNA (RAPD) analysis was used as a primary method to group the isolates. Amplification reactions (25 µL) were performed as follows: 12.5 µL of NZYTaq II 2x Green Master Mix (Nzytech, Lisbon, Portugal), 1 µL of primer M13 (5′GAGGGTGGCGGT TCT-3′) (MWG-Biotech), and 2.5 µL of sample DNA. A negative control was included for each PCR reaction. Amplification was performed in a thermocycler DOPPIO (VWR, USA) programmed for an initial denaturation step at 94 °C for 5 min, followed by 45 cycles of 1 min at 94 °C, 2 min at 34 °C and 2 min at 72 °C, and a final extension step at 72 °C for 10 min. PCR products were analyzed on a 1.5 % agarose gel stained with GelRed (Biotium, USA) for 135 min at 80 V. RAPD patterns were compared using Bionumerics software (Applied Maths, St- Martens-Laten, Belgium) and clustered according to their similarities. Band matching position tolerance was set at 1 %. Seventy-three different profiles were recognized after RAPD analysis.

Partial sequence of the 16S ribosomal RNA (rRNA) gene was carried out using the universal primers 27F (5′-GAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-ACCTTGTTACGACTT-3′) (MWG-Biotech). PCR amplifications were performed in a 25 µL-reaction mixture containing: 12.5 µL of NZYTaq II 2x Green Master Mix (Nzytech, Lisbon, Portugal), 0.25 µL of each primer, and 8 µL of DNA template. The cycling procedure was performed using an initial denaturation step at 94 °C for 5 min, followed by 30 cycles of 94 °C for 1 min, 52 °C for 1 min, 72 °C for 1 min, and a final extension at 72 °C for 10 min. PCR products were sent for purification and sequencing to Macrogen Inc. (Seoul, Republic of Korea). Sequence editing and inspection were performed using BioEdit program 7.0. The 16S rRNA sequences of the bacterial strains with quality were deposited in GenBank database under the accession numbers MW414477-MW414500, MW544871, and MW544872.

The selection of the isolates for further testing excluded those considered human or plant pathogens.

3.2.3. Screening for Fe nutrition-related traits

Bacterial strains were tested for their ability to grow in Tryptic Soy Broth (TSB; Liofilchem, Italy) medium with pH adjusted to 9.0, to reduce Fe^{3+}, and to produce organic acids as described before (Roriz et al., 2021). For each analysis, experiments were repeated three times for each bacterial

strain and OD determinations were made in triplicate. A growth inhibition percentage lower than 70 % was defined as an exclusion criterion for the greenhouse experiment.

3.2.4. Screening for plant growth-promoting traits

Production of siderophores by bacterial strains was evaluated using a chrome azurol S (CAS) agar medium as described by Schwyn & Neilands (1987). Orange halos around the colonies on CAS agar were indicative of siderophore excretion. Three replicates were made for each bacterial strain.

The amount of IAA produced by bacterial isolates was determined according to the method of Gordon and Weber (1951). Briefly, an aliquot of 500 µL of the supernatant obtained from bacterial cultures grown in the presence of L-tryptophan (1 %) was mixed with 350 µL of Salper reagent. The absorbance of pink color developed after 30 min incubation in dark was read at 530 nm. The IAA concentration was determined using a calibration curve (0-80 µg/mL) of pure IAA as a standard. The experiments were repeated three times, and IAA determinations were made in triplicate.

The ACC deaminase activity of cell-free extracts was determined by estimating the amount of α-ketobutyrate generated by the enzymatic hydrolysis of ACC (Saleh & Glick, 2001) according to the procedure of Honma and Shimomura (1978). The experiments were repeated three times, and determinations were made in triplicate.

3.2.5. Greenhouse experimental design

The endophyte *Bacillus licheniformis* P2.3 and the rhizobacteria *Bacillus aerius* S2.14 were selected for the pot greenhouse assay. The choice of these strains relied on distinct characteristics related to their ability to reduce Fe^{3+}, to produce siderophores, organic acids, IAA, and ACC deaminase activity. The greenhouse pot experiment consisted of a factorial design with three treatments: i) saline solution (Control); ii) inoculation with *B. licheniformis* P2.3; and iii) inoculation with *B. aerius* S2.14. Each treatment was replicated 12 times. Seeds of *Glycine max* cultivar Williams 82 were disinfected using 70 % (v/v) ethanol for 5 min, followed by a solution of 1.2 % (v/v) sodium hypochlorite, and 0.02 % (w/v) sodium dodecyl sulphate (SDS) for 15 min. Seeds were then rinsed five times in sterile water. Germination was performed in filter paper for seven days in the dark, at 25 °C. One seedling was transferred to pots (3 L) containing 150 g of a lower bed of light expanded clay aggregate (LECA®) and 2 kg of an upper bed of sieved (<2 mm) calcareous agricultural soil (pH 8.2) collected in the South of Portugal. The soil was not autoclaved to better mimic real-life conditions to which plants would be exposed in a future agricultural setting. The physicochemical properties of the soil are presented in Table 3.1.

Table 3.1. Physicochemical properties of substrate samples collected before inoculation (day 1) and 128 days after inoculation with saline solution (Control), *B. licheniformis,* and *B. aerius*.

Parameter	Substrate sample			
	Day 1	Day 128		
		Control	*B. licheniformis*	*B. aerius*
pH	8.2	8.3	8.1	8.1
Organic content (%)	0.86	1.24	2.45	1.81
Texture	Medium	Medium	Coarse	Coarse
Slime (g/kg)	104	94	92	94
Clay (g/kg)	216	206	191	190
Coarse sand (g/kg)	488	522	537	549
Thin sand (g/kg)	193	178	181	167
Texture class	Sandy clay loam	Sandy clay loam	Sandy loam	Sandy loam
Total $CaCO_3$ (g/kg)	225.3	203.8	191.5	226.7
Extractable K (mg K_2O/kg)†	107	93	90	110
Extractable P (mg P_2O_5/kg)†	13	6	6	5
Extractable B (mg/kg)‡	0.09	0.14	0.11	0.18
Extractable Cu (mg/kg)§	0.75	0.62	0.58	0.57
Extractable Zn (mg/kg)§	0.18	0.39	0.34	0.33
Extractable Fe (mg/kg)§	8.24	6.96	5.96	5.39
Extractable Mn (mg/kg)§	7.11	3.51	3.16	3.94
E.C (dS/m)	0.227	0.259	0.227	0.350
Total N (g/kg)	0.58	0.82	1.35	1.09

†analysis done by Égner-Riehm method.

‡analysis done by hot-water extraction.

§analysis done by Lindsay and Norvell – DPTA method.

For the inoculation, bacterial strains were grown overnight in TSB medium at 30 °C. Cells in the exponential phase were harvested by centrifugation at 5000 rpm for 10 min, washed twice with sterile saline solution (0.85 % NaCl), and centrifuged again. Bacterial inoculum was prepared by

resuspending pellet in sterile saline solution to get an inoculum density of ca. 10^8 CFU/mL. Bacterial suspensions (50 mL/pot) were poured into the substrate surface at the time of seedling transplantation. Soybean plants grown in a commercial substrate were used as border plants and pots with treated plants were randomly distributed and watered daily with tap water through an automatic irrigation system.

Plants were collected in two phases: 24 days after inoculation (V3 growth stage), half of the plants were collected and analyzed for photosynthetic parameters (using an InfraRed Gas Analyzer (IRGA)), chlorophyll content (using a Soil Plant Analysis Development (SPAD) chlorophyll meter), total fresh weight (FW), plant height, root length, FC-R activity, mineral quantification, and gene expression; 128 days after inoculation (full maturity), the remaining plants were collected and analyzed for dry weight (DW), no. of pods, and seeds per pod.

3.2.6. IRGA analysis

The photosynthetic rate (μmol $CO_2/m^2/s$), stomatal conductance (mol $H_2O/m^2/s$), and transpiration rate ($mol/m^2/s$) were measured in the youngest trifoliate leaf of six independent plants 24 days after inoculation, using an IRGA LI-6400 portable photosynthesis system (LI-COR Inc., Lincoln, NE, USA).

3.2.7. SPAD and plant biometrical parameters

Twenty-four days after inoculation, for six independent plants, leaf chlorosis was assessed using a SPAD chlorophyll meter (Konica Minolta SPAD-502Plus; Minolta, Osaka, Japan) in the youngest trifoliate leaf. Plant height and root length were measured, and FW was determined after separation into trifoliates and roots. At the end of the experiment (128 days), the no. of pods and seeds per pod were determined for the other six plants of each treatment, as well as DW after drying plant tissues at 60°C.

3.2.8. FC-R activity measurements

Twenty-four days after inoculation, root Fe reductase was quantified in six independent plants as described by Roriz et al. (2021).

3.2.9. Microwave-assisted digestion and ICP analysis

Dried plant tissues (trifoliates and roots; n =3) were digested through microwave-assisted digestion and the concentration of Fe, Zn, Mn, Mg, Ca, P, and K was analyzed as described before (Roriz et al., 2021). All analyses per biological replicate were done in triplicate.

3.2.10. rRNA extraction and cDNA synthesis

rRNA extraction and cDNA synthesis were performed in three biological replicates from each treatment according to Roriz et al. (2021).

3.2.11. Primer design and RT-qPCR

The transcript levels of FRO2, IRT1, F6'H1, bHLH38, and FER4 genes were analyzed, using 18S rRNA and ubiquitin as reference genes, as described before (Roriz et al., 2021). FER4 expression is specific to the leaves and thus its expression was only evaluated in trifoliates. Three biological replicates were analyzed, and two technical repetitions of each biological replicate were performed. The primer pairs used for the qPCR analysis are listed in Supplementary Table S3.1.

3.2.12. Physicochemical soil properties

The physicochemical properties of the soil (pH, organic content, texture, concentration of slime, clay, coarse sand, and thin sand, texture class, total $CaCO_2$, extractable K, P, B, Cu, Zn, Fe, and Mn, electrical conductivity, and total nitrogen (N)) were evaluated in samples collected at the beginning, before inoculation with bacteria (day 1), and at the end of the greenhouse experiment (day 128). For this purpose, for each treatment, we analyzed a 3-sample pool of the rhizospheric region.

3.2.13. Statistical analysis

Data were subjected to analysis of variance (one-way ANOVA) for the effect of bacterial inoculation using the GraphPad Prism version 7.0 (San Diego, CA, USA). Significant differences between treatments were determined using Tukey's test ($P < 0.05$).

3.3. Results

3.3.1. PGPB isolated from soybean plants

Overall, based on the distinct colony characteristics, a total of 76 bacterial strains were isolated from roots (53 %), rhizosphere (29 %), and shoots (18 %) of soybean plants. After RAPD analysis, 73 different profiles were recognized. Based on the 16S rRNA gene sequencing, 51 isolates were identified (Table 3.2). However, a large number of the sequences obtained (53 %) showed poor quality, and so bacterial identification associated with those sequences should be interpreted carefully.

Table 3.2. List of NCBI accession number, closest blast match, and similarity of bacterial isolates from the shoots, roots, and rhizosphere of soybean plants.

Origin	Strain	NCBI accession no.	Closest blast match (accession number)	Similarity (%)
Shoots	P1.2		*Microbacterium sediminicola* YM10-847 (NR_145621.1)	75
	P1.3		*Brachybacterium paraconglomeratum* JCM 17781 (NR_113401.1)	75
	P1.5		*Kocuria dechangensis* NEAU-ST5-33 (NR_137239.1)	96
	P2.2#	MW414477	*Staphylococcus epidermidis* 3039 (MT613456.1)	100
	P2.3	MW544872	*Bacillus licheniformis* IND706 (MT642946.1)	100
	P3.1		*Luteimonas terrae* THG-MD21 (NR_149233.1)	92
	P3.3	MW414478	*Pseudoclavibacter terrae* w11 (MK696245.1)	100
	P3.4#		*Achromobacter pulmonis* R-16442 (NR_117644.1)	87
	P3.5#	MW414479	*Staphylococcus xylosus* LS160 (MT409908.1)	100
	P3.6#	MW414480	*Bacillus oleronius* M1/25 (KX826966.1)	100
	P4.1	MW414481	*Methylorubrum extorquens* B44 (MN629084.1)	100
Roots	R1.1#		*Rheinheimera mesophile* IITR-13 (NR_137339.1)	83
	R1.2		*Stenotrophomonas chelatiphaga* LPM-5 (NR_116366.1)	97
	R1.4	MW414482	*Pseudomonas flavescens* SY17 (MH259981.1)	100
	R1.5#	MW414483	*Bosea thiooxidans* NRB220 (MK543115.1)	100
	R1.9	MW414484	*Agrococcus* sp. KB2M3ps (HG313894.1)	100
	R1.10		*Flavobacterium johnsoniae* UW101 (NR_074455.1)	82
	R1.12#		*Sphingomonas faucium* E62-3 (NR_151915.1)	90
	R1.13#		*Stenotrophomonas pavanii* LMG 25348 (NR_118008.1)	94
	R1.15#		*Dyadobacter jiangsuensis* L-1 (NR_134721.1)	98
	R2.1		*Microbacterium hydrothermale* 0704C9-2 (NR_134084.1)	96

Table 3.2. (cont.) List of NCBI accession number, closest blast match, and similarity of bacterial isolates from the shoots, roots, and rhizosphere of soybean plants.

Origin	Strain	NCBI accession no.	Closest blast match (accession number)	Similarity (%)
	R2.2	MW414485	*Aliihoeflea aestuarii* 2WW (HF565048.1)	100
	R2.5#		*Microbacter margulisiae* ADRI (NR_126217.3)	81
	R2.12#	MW414486	*Brachybacterium faecium* Nb6MB-3 (KP296218.1)	100
	R2.14	MW414487	*Paenibacillus massiliensis* EB373 (MH127819.1)	100
	R3.2#		*Flexithrix dorotheae* NBRC 15987 (NR_113831.1)	75
	R3.3		*Microbacterium maritypicum* DSM 12512 (NR_114986.1)	86
	R3.4#		*Bacteroides fragilis* NCTC 9343 (NR_074784.1)	100
	R3.7		*Cellulomonas fimi* ATCC 484 (NR_074509.1)	85
	R3.11#	MW414488	*Sphingobacterium chuzhouense* DH-5 (NR_153692.1)	96
	R4.2#		*Microbacterium jejuense* THG-C31 (NR_134085.1)	87
	R4.3	MW414489	*Stenotrophomonas rhizophila* strain KR2-13 (MN753976.1)	96
	R4.4#	MW414490	*Luteimonas aestuarii* L22 (KT719210.1)	97
	R4.7#		*Vitreoscilla stercoraria* Gottingen 1488-6 (NR_025894.1)	80
	R4.8		*Pseudomonas punonensis* LMT03 (NR_109583.1)	89
	R4.12	MW414491	*Bacillus toyonensis* WS2-2 (MT605503.1)	100
	R4.13#		*Staphylococcus warneri* AW 25 (NR_025922.1)	91
Rhizosphere	S1.1	MW414492	*Kocuria rhizophila* DP1TSA86 (MH972188.1)	100
	S1.3		*Sporosarcina contaminans* CCUG 53915 (NR_116955.1)	84
	S1.6#	MW414493	*Bacillus licheniformis* IND706 (MT642946.1)	100
	S1.7#	MW414494	*Bacillus cereus* MD152 (MT642947.1)	100

Table 3.2. (cont.) List of NCBI accession number, closest blast match, and similarity of bacterial isolates from the shoots, roots, and rhizosphere of soybean plants.

Origin	Strain	NCBI accession no.	Closest blast match (accession number)	Similarity (%)
	S2.2	MW414495	*Ochrobactrum thiophenivorans* 0312MAR12L7 (LN774753.1)	100
	S2.3		*Agrococcus jejuensis* SSW1-48 (NR_042551.1)	88
	S2.4	MW414496	*Bacillus* sp. BAB-4377 (KM388720.1)	100
	S2.6	MW414497	*Paenibacillus camelliae* b11s-2 (NR_116303.1)	99
	S2.8[#]		*Arthrobacter humicola* KV-653 (NR_041546.1)	91
	S2.10		*Paenibacillus lactis* MB 1871 (NR_025739.1)	80
	S2.14	MW544871	*Bacillus aerius* OsEnb_PLM_L10.1 (MN889250.1)	99
	S3.4	MW414498	*Microbacterium testaceum* BAC2029 (HM355620.1)	100
	S4.1	MW414499	*Bacillus zhangzhouensis* cqsV18 (MN826587.1)	100
	S4.2[#]	MW414500	*Fictibacillus enclensis* Ng120-7 (MK519114.1)	100

[#]isolates excluded from the screening tests.

Bacterial endophytes isolated from shoots were affiliated to *Achromobacter*, *Bacillus*, *Brachybacterium*, *Kocuria*, *Luteimonas*, *Methylorubrum*, *Microbacterium*, *Pseudoclavibacter*, and *Staphylococcus* genera, while the endophytic strains isolated from the roots belonged to the genera *Agrococcus*, *Aliihoeflea*, *Bacillus*, *Bacteroides*, *Bosea*, *Brachybacterium*, *Cellulomonas*, *Dyadobacter*, *Flavobacterium*, *Flexithrix*, *Luteimonas*, *Microbacter*, *Microbacterium*, *Paenibacillus*, *Pseudomonas*, *Rheinheimera*, *Sphingomonas*, *Sphingobacterium*, *Staphylococcus*, *Stenotrophomonas*, and *Vitreoscilla*. Rhizobacterial strains were affiliated to genera *Agrococcus, Arthrobacter, Bacillus, Fictibacillus, Kocuria, Microbacterium, Ochrobactrum, Paenibacillus, Sphingomonas, Sporosarcina*, and *Stenotrophomonas*.

Strains *Staphylococcus epidermidis* P2.2, *Achromobacter pulmonis* P3.4, *Staphylococcus xylosus* P.3.5*, Bacillus oleronius* P3.6, *Rheinheimera mesophile* R1.1*, Bosea thiooxidans* R1.5*, Sphingomonas faucium* R1.12*, Stenotrophomonas pavanii* R1.13*, Dyadobacter jiangsuensis* R1.15*, Microbacter margulisiae* R2.5*, Brachybacterium faecium* R2.12*, Flexithrix dorotheae* R3.2*, Bacteroides fragilis* R3.4*, Sphingobacterium chuzhouense* R3.11*, Microbacterium jejuense* R4.2*, Luteimonas aestuarii* R4.4*, Vitreoscilla stercoraria* R4.7*, Staphylococcus warneri* R4.13, *Bacillus licheniformis* S1.6*, Bacillus cereus* S1.7*, Arthrobacter humicola* S2.8*,* and *Fictibacillus enclensis* S4.2 were excluded from the subsequent assays as they are considered human or plant pathogens.

3.3.2. Fe nutrition and plant growth-related traits in selected PGPB

The ability of bacterial strains to grow at high pH values, to reduce Fe^{3+}, to produce organic acids, siderophores, and IAA, and the ACC deaminase activity are presented in Table 3.3. Ninety percent of PGPB strains showed growth inhibition at pH 9.0 lower than 70 % (the value above which we defined as an exclusion criterion for the greenhouse experiment). The ability to reduce Fe^{3+} was evaluated by the produced amount of Fe^{2+}. Concentrations of Fe^{2+} ranged from 0.017 to 0.055 mM and 76 % of the isolates produced an amount of reduced Fe^{2+} above 0.030 mM. The mean value of reduced Fe^{2+} was significantly higher in root endophytes (0.038 mM) if compared with rhizospheric strains (0.029 mM). All tested strains produced siderophores, with 66 % showing intermediate production, while 14 % showed strong production. Production of organic acids was only observed in 10 % of the bacterial isolates. IAA production was detected in all bacterial isolates in the presence of 1 % of tryptophan after 48 h of incubation. The levels of IAA produced by PGPB ranged from 0.849 to 16.568 mg/L. Moreover, the mean value was significantly higher in rhizobacteria (9.325 mg/L) compared with root (4.756 mg/L) and shoot (2.111 mg/L) endophytes. ACC deaminase activity was detected in all bacterial strains with values ranging from 1.206 to 12.467 nmol α-ketobutyrate/g/h.

Table 3.3. Percentage of growth inhibition at pH 9.0, concentration of reduced Fe^{2+}, and production of siderophores, organic acids, indole-3-acetic acid (IAA), and 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase in 29 studied bacterial strains. Data are means ± SEM (n = 9).

Isolate	% inhibition (pH 9.0)	Reduced Fe^{2+} (mM)	Siderophores	Organic acids	IAA (mg/L)	ACC (nmol/g/h)
P1.2	40 ± 3	0.047 ± 0.001	++	-	1.089 ± 0.145	1.206 ± 0.119
P1.3	-39 ± 1	0.038 ± 0.001	++	-	0.849 ± 0.194	1.481 ± 0.662
P1.5	-812 ± 2	0.036 ± 0.001	++	-	2.185 ± 0.339	2.610 ± 0.447
P2.3	-105 ± 2	0.035 ± 0.001	+	+	2.082 ± 0.194	5.469 ± 0.297
P3.1	14 ± 4	0.026 ± 0.001	++	-	4.171 ± 0.145	7.591 ± 0.264
P3.3	45 ± 5	0.033 ± 0.001	++	-	2.151 ± 0.022	12.467 ± 0.488
P4.1	96 ± 1	0.035 ± 0.001	++	+	2.253 ± 0.436	1.455 ± 0.651
R1.2	-27 ± 4	0.038 ± 0.001	+	-	2.014 ± 0.053	11.118 ± 0.547
R1.4	38 ± 6	0.040 ± 0.000	+	-	2.082 ± 0.291	4.273 ± 0.417
R1.9	60 ± 0	0.040 ± 0.001	++	-	2.562 ± 0.291	3.269 ± 0.266
R1.10	97 ± 1	0.029 ± 0.001	+++	-	3.966 ± 0.145	7.263 ± 0.119
R2.1	58 ± 3	0.031 ± 0.001	++	-	2.767 ± 0.090	7.989 ± 0.876
R2.2	3 ± 4	0.038 ± 0.000	++	-	3.452 ± 0.291	4.161 ± 0.101
R2.14	85 ± 3	0.029 ± 0.000	++	-	2.836 ± 0.097	2.317 ± 0.088
R3.3	31 ± 1	0.030 ± 0.001	++	-	2.288 ± 0.102	1.552 ± 0.101
R3.7	-231 ± 3	0.038 ± 0.001	++	-	2.288 ± 0.029	3.266 ± 0.117
R4.3	32 ± 5	0.055 ± 0.001	+++	-	13.144 ± 0.048	8.213 ± 0.118
R4.8	22 ± 6	0.041 ± 0.001	++	+	11.979 ± 0.533	10.070 ± 0.269
R4.12	37 ± 5	0.048 ± 0.001	++	-	7.699 ± 0.097	3.840 ± 0.118

Table 3.3. (cont.) Percentage of growth inhibition at pH 9.0, concentration of reduced Fe^{2+}, and production of siderophores, organic acids, indole-3-acetic acid (IAA), and 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase in 29 studied bacterial strains. Data are means ± SEM (n = 9).

Isolate	% inhibition (pH 9.0)	Reduced Fe^{2+} (mM)	Siderophores	Organic acids	IAA (mg/L)	ACC (nmol/g/h)
S1.1	-327 ± 4	0.036 ± 0.001	+	-	2.493 ± 0.048	4.103 ± 0.235
S1.3	37 ± 5	0.031 ± 0.001	++	-	9.377 ± 0.048	8.890 ± 0.288
S2.2	16 ± 3	0.033 ± 0.001	++	-	12.527 ± 0.145	8.917 ± 0.128
S2.3	30 ± 4	0.030 ± 0.001	++	-	8.521 ± 0.387	7.275 ± 0.288
S2.4	-73 ± 0	0.023 ± 0.000	++	-	7.288 ± 0.581	2.746 ± 0.134
S2.6	24 ± 3	0.025 ± 0.001	+	-	9.103 ± 0.048	3.865 ± 0.138
S2.10	17 ± 6	0.037 ± 0.001	+++	-	13.760 ± 0.726	4.235 ± 0.130
S2.14	-48 ± 6	0.017 ± 0.001	+++	-	9.514 ± 0.242	8.750 ± 0.108
S3.4	25 ± 4	0.032 ± 0.001	++	-	16.568 ± 0.145	5.050 ± 0.147
S4.1	6 ± 2	0.025 ± 0.001	+	-	4.103 ± 0.145	9.069 ± 0.153

-, negative.

+, positive/weak.

++, intermediate.

+++, strong.

Two promising strains, *B. licheniformis* P2.3 and *B. aerius* S2.14, were selected for pot experiments based on their distinct characteristics. *B. licheniformis* is an endophyte isolated from the shoots that showed increased ability to reduce Fe^{3+} and to produce IAA, tested positive for the production of organic acids, but showed weak ability to produce siderophores and ACC deaminase; the strain *B. aerius* was isolated from the rhizosphere, showed strong production of siderophores, moderate IAA synthesis and ACC deaminase activity, low ability to reduce Fe^{3+}, and tested negative for the production of organic acids.

3.3.3. Influence of inoculation with PGPB on Fe nutrition in soybean

Inoculation with bacteria did not significantly affect photosynthetic and transpiration rate, and stomatal conductance of soybean plants (Figure 3.1). Moreover, after 24 days of inoculation, no significant differences were observed between treatments for SPAD and FW measurements (Table 3.4). Plants inoculated with *B. licheniformis* showed higher height (+23 %) than plants inoculated with *B. aerius*, despite no significant differences were observed in relation to control. Nevertheless, plants inoculated with *B. aerius* showed a significant decrease (25 %) in root length compared with control plants. After 128 days of inoculation, no significant differences were observed between treatments for DW and seeds per pod (Table 3.5). However, plants inoculated with the strain *B. licheniformis* showed an increase of 33 % in the no. of pods compared with control plants, and of 37 % compared with inoculation with *B. aerius*.

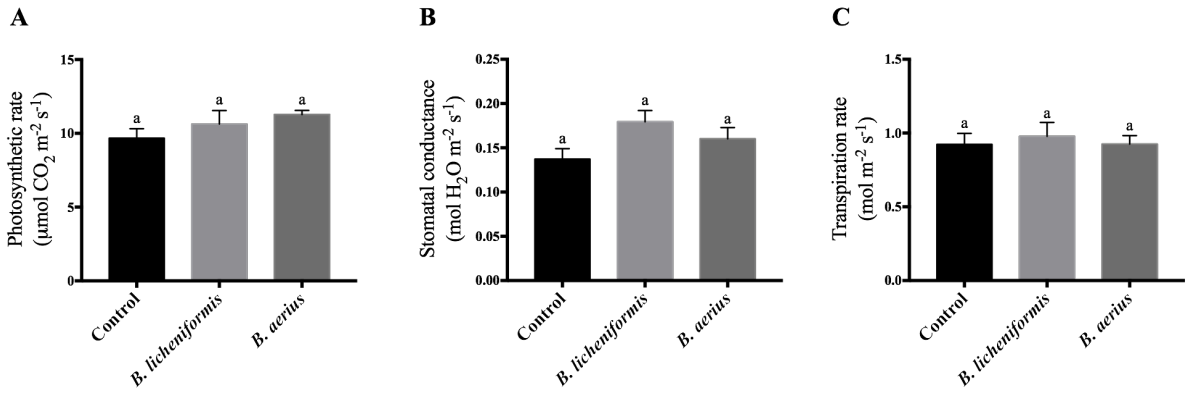

Figure 3.1. Photosynthetic rate (A), stomatal conductance (B), and transpiration rate (C) of soybean plants 24 days after inoculation with saline solution (Control), *B. licheniformis*, and *B. aerius*. Data are mean ± SEM of three biological replicates. Different letters indicate significant differences ($P < 0.05$) between treatments.

Table 3.4. SPAD values and morphological parameters of soybean plants 24 days after inoculation with saline solution (Control), *B. licheniformis*, and *B. aerius*. Data are mean ± SEM of six biological replicates. Different letters indicate significant differences ($P < 0.05$) between treatments. Statistical analysis was performed independently for each analysis.

Treatment	SPAD	Fresh weight (g)	Plant height (cm)	Root length (cm)
Control	33.12 ± 2.18[a]	5.22 ± 0.82[a]	40.08 ± 1.15[ab]	26.98 ± 0.92[a]
B. licheniformis	29.48 ± 2.44[a]	5.81 ± 0.67[a]	43.77 ± 1.77[a]	30.33 ± 1.66[a]
B. aerius	32.67 ± 1.61[a]	5.56 ± 0.59[a]	35.67 ± 1.18[b]	21.60 ± 1.21[b]

Table 3.5. Dry weight, no. of pods and seeds per pod of soybean plants 128 days after inoculation with saline solution (Control), *B. licheniformis*, and *B. aerius*. Data are mean ± SEM of six biological replicates. Different letters indicate significant differences ($P < 0.05$) between treatments. Statistical analysis was performed independently for each analysis.

Treatment	Dry weight (g)	No. of pods	No. of seeds per pod
Control	2.39 ± 0.23[a]	3.6 ± 0.2[b]	1.6 ± 0.1[a]
B. licheniformis	3.07 ± 0.38[a]	4.8 ± 0.4[a]	1.6 ± 0.2[a]
B. aerius	2.75 ± 0.26[a]	3.5 ± 0.2[b]	1.8 ± 0.1[a]

The concentration of micro- (Fe, Mn, Zn) and macronutrients (Ca, K, Mg, P) in the trifoliates and roots of *G. max* plants is presented in Table 3.6. No significant differences were observed in the concentration of Fe in the trifoliates and roots of inoculated plants compared with control plants, while Mn concentration significantly decreased in the trifoliates of plants inoculated with *B. licheniformis* (32 %) and *B. aerius* (29 %), compared with non-inoculated plants. Root Zn concentration in plants inoculated with *B. licheniformis* was increased by 62 % compared with control plants. A significant increase (20 %) was also verified for Ca concentration in trifoliates inoculated with *B. licheniformis* compared with non-inoculated plants. No significant differences in the concentration of K, Mg, and P were observed between treatments.

The activity of the Fe reductase in the roots of *G. max* plants is shown in Figure 3.2. A significant decrease in FC-R activity was verified in plants inoculated with *B. licheniformis* (45 %) and with *B. aerius* (55 %) compared with non-inoculated plants.

Table 3.6. Mineral concentration on trifoliates and roots of *G. max* plants, grown under alkaline conditions, 24 days after inoculation with saline solution (Control), *B. licheniformis*, and *B. aerius*. Data are mean ± SEM of three biological replicates. Statistical analysis was performed independently for each mineral and plant tissue. Different letters indicate significant differences ($P < 0.05$) within the same plant tissue between treatments.

Plant tissue	Treatment	Mineral concentration (μg/g)						
		Fe	Mn	Zn	Ca	K	Mg	P
Trifoliates	Control	108.5 ± 15.3^{a}	115.6 ± 11.1^{a}	13.0 ± 0.5^{a}	18834.0 ± 453.3^{b}	7123.5 ± 879.1^{a}	5151.7 ± 97.7^{a}	1275.7 ± 117.2^{a}
	B. licheniformis	89.7 ± 7.9^{a}	78.4 ± 4.6^{b}	13.4 ± 1.0^{a}	22541.6 ± 232.3^{a}	6480.7 ± 332.3^{a}	5052.7 ± 388.8^{a}	1219.9 ± 102.9^{a}
	B. aerius	102.8 ± 1.2^{a}	82.2 ± 3.1^{b}	12.2 ± 0.6^{a}	20652.3 ± 950.0^{ab}	7453.5 ± 463.2^{a}	5323.0 ± 523.0^{a}	1219.1 ± 38.1^{a}
Roots	Control	4127.5 ± 160.4^{A}	98.4 ± 9.6^{A}	23.3 ± 0.2^{B}	7822.8 ± 471.2^{A}	7155.5 ± 880.8^{A}	7108.5 ± 692.4^{A}	749.0 ± 92.6^{A}
	B. licheniformis	4604.5 ± 485.9^{A}	139.6 ± 15.8^{A}	37.8 ± 2.9^{A}	9947.9 ± 1076.0^{A}	6651.0 ± 961.0^{A}	7474.8 ± 773.8^{A}	792.7 ± 57.1^{A}
	B. aerius	3173.8 ± 260.9^{A}	99.2 ± 11.4^{A}	28.8 ± 1.6^{B}	6917.7 ± 410.7^{A}	6710.8 ± 998.7^{A}	6787.2 ± 373.0^{A}	872.4 ± 86.6^{A}

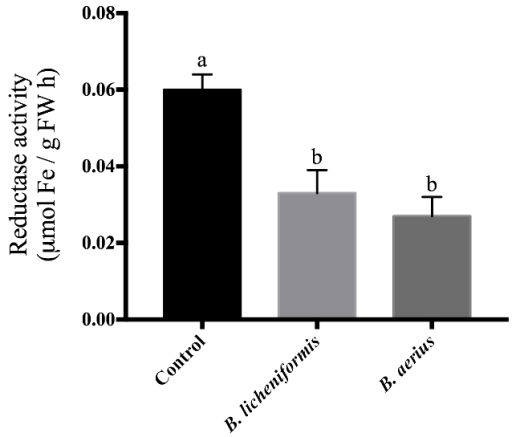

Figure 3.2. Root reductase activity of *G. max* plants grown under alkaline conditions inoculated with saline solution (Control), *B. licheniformis*, and *B. aerius*. Data are mean ± SEM of six biological replicates. Different letters indicate significant differences ($P < 0.05$) between treatments.

The expression of five known genes associated with Fe nutrition was studied using qPCR: FRO2, IRT1, F6'H1, and bHLH38 in the roots, and FER4 in the trifoliates. Gene expression increased in all inoculated plants compared with non-inoculated plants (Figure 3.3). FRO2 expression increased by 38 % in the roots of plants inoculated with *B. licheniformis* and decreased by 20 % with inoculation with *B. aerius* compared with non-inoculated plants. Expression of IRT1, F6'H1, and bHLH38 genes increased in the roots of plants inoculated with *B. licheniformis* by 135 %, 167 %, and 59 % respectively, while in the roots of plants inoculated with *B. aerius* the expression increased 16 %, 183 %, and 16 % respectively compared with control plants. In the trifoliates, a similar trend was observed in the expression of FER4 in inoculation treatments (increases up to 252 %).

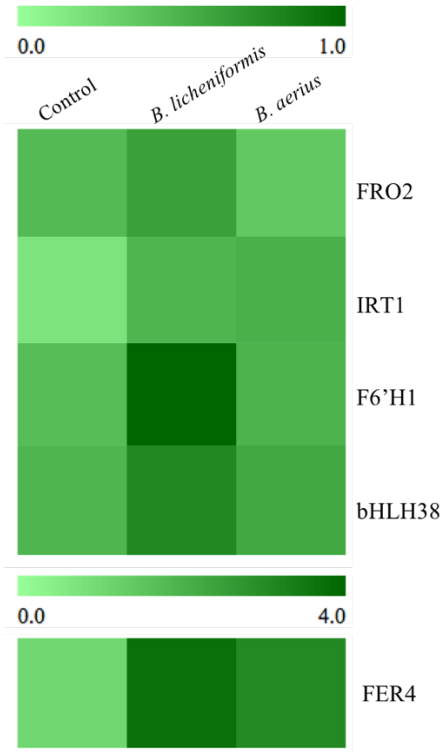

Figure 3.3. Heat map of the expression patterns of FRO2, IRT1, F6'H1, bHLH38, and FER4 genes in *G. max* plants, grown under alkaline conditions, inoculated with saline solution (Control), *B. licheniformis*, and *B. aerius*. FRO2, IRT1, F6'H1, and bHLH38 expression was measured in the roots; FER4 expression was measured in the trifoliates. In dark green: increased gene expression; in light green: lower gene expression. Data are means of three independent biological replicates. Corresponding values are presented in Supplementary Table S3.2.

3.3.4. Soil composition

The physicochemical characterization of soil samples was performed at the beginning, before inoculation with bacteria (day 1), and at the end of the experiment (day 128) (Table 3.1). In general, OM content tended to be higher at the end of the experiment, especially in soil inoculated with the

strain *B. licheniformis.* Regarding the nutrient composition, the concentration of boron (B), Zn, and N tended to be higher at the end of the experiment, while an opposite trend was observed for P, cooper (Cu), Fe, and Mn concentrations.

3.4. Discussion

In this study, we successfully isolated and characterized soybean-associated PGPB and tested their potential to improve soybean performance under alkaline conditions. Bacterial inoculants impacted differently growth parameters, mineral balance, FC-R activity, and gene expression of plants grown under alkaline conditions, even if none of the typical phenotypic traits of Fe deficiency were observed. The inoculation with *B. licheniformis* P2.3 (isolated from soybean shoots) increased the no. of pods (33 %) of soybean plants at maturity, decreased FC-R activity (45 %), and increased the expression of Fe-nutrition related genes (FRO2, IRT1, F6'H1, bHLH38, and FER4), whereas the inoculation with *B. aerius* S2.14 (isolated from soybean rhizosphere) decreased root length (20 %), FC-R activity (55 %) and FRO2 expression, despite having increased the expression of the remaining genes.

The diversity of bacterial genera varied with the isolation source. The number of bacterial isolates recovered from soybean roots was higher than those obtained from rhizospheric soil, followed by the shoots, which may reflect the intimate contact of radicular system with the rhizosphere during plant growth and development, facilitating the entry of bacteria into the root tissues (Kobayashi & Palumbo, 2000). The presence of bacterial isolates internally of tissues reflects their ability to colonize plant organs, a relevant trait for their applicability as inoculants to improve plant growth. Seventy-six bacteria were first isolated from shoots and roots of soybean plants, and rhizospheric soil and 29 different genera were identified, indicating a high variety of culturable bacteria associated with soybean plants. Interestingly, it is known that legumes own a notable diversity of associated bacteria due to their long history of cultivation and selection under various agroclimatic and geographic conditions (Hung et al., 2007). *Bacillus* and *Microbacterium* were the most common genera followed by *Paenibacillus* and *Stenotrophomonas*. These four genera have already been reported in several studies associated with soybean plants with enhanced plant growth abilities (de Almeida Lopes et al., 2016; Jain et al., 2016; Nhu & Diep, 2017; Arfaoui et al., 2018; Temesgen et al., 2019). Usually, the culturable bacterial communities vary significantly among studies. This may happen due to differences in the isolation and identification protocols, and also because of the different environmental growth conditions and host development stage (Rosenblueth & Martínez-Romero, 2006; Liu et al., 2013). Many other factors may also contribute to these differences: plant tissue (Mocali et al., 2003), plant species, cultivar, and soil type (Dalmastri, 1999; Kinkel et al., 2000; Fromin et al., 2001), and the plant´s ability to select specific benefic microorganisms (Hardoim et al., 2008).

The isolated PGPB showed distinct traits related to plant growth promotion and Fe uptake according to their origin. The higher mean value of reduced Fe^{2+} registered for root endophytes compared with that found for rhizobacteria can be related to the protection conferred by the plant against environmental stresses, nutrient competition, and phytopathogens (Sturz et al., 2000), which

may enhance their capacity to produce siderophores, and organic acids, as well as the ability to reduce Fe^{3+}. An opposite trend was observed for IAA, since rhizobacteria, in general, produced more IAA than bacterial endophytes. Indeed, the existence of high nutrient concentrations and root exudates in the rhizosphere contributes to the improved growth and metabolism of rhizobacteria, improving their ability to produce IAA (Etesami et al., 2015). Furthermore, as bacterial IAA production is often associated with the development of plant lateral roots and root hairs (Patten & Glick, 2002), it would be expected a higher production of this phytohormone by rhizospheric bacterial strains.

In this study, based on the morphological parameters (SPAD, FW, plant height, and root length) of non-inoculated plants and according to previous studies (Roriz et al., 2014; Santos et al., 2015, 2016), we can assume that soybean plants showed no Fe deficiency symptoms. The same was verified in a previous work conducted by Roriz et al. (2021). It would be expected that non-inoculated plants showed visual Fe deficiency symptoms as Williams 82 cultivar usually shows intermediate susceptibility to Fe shortage (Santos et al., 2013, 2016). In the present study, the absence of symptoms can be explained by the fact of soybean plants were grown in a substrate instead of a hydroponic system where the lack of cation-exchange capacity induces Fe deficiency symptoms. Moreover, the chosen cultivar and the growth time may also contribute to the observed differences. Furthermore, it would be expected that inoculation with PGPB improved general plant growth parameters if Fe-deficient conditions were present (Khalid et al., 2015; Zhou et al., 2016; Nagata, 2017; Patel et al., 2018).

IRGA analysis, although promising, did not provide significant differences regarding the effect of PGPB inoculation on photosynthetic parameters. We would expect that inoculation with bacteria, by improving nutrient uptake, could enhance chlorophyll synthesis and increase the photosynthetic parameters; furthermore, it has been shown that bacteria can modify the leaf structure which in turn affects gas exchange, improving also these photosynthetic parameters (Paradiso et al., 2017).

Regarding the plant mineral analysis, it was observed that inoculation influenced the general nutritional level of soybean plants grown under alkaline conditions. Fe concentration, our main focus, was in general higher in the roots than in the shoots. Similar results were described by Nagata (2017) and Zhou et al. (2018) in plants grown under Fe-deficient conditions in a liquid medium or artificial calcareous soil after inoculation with PGPB. Several studies showed that PGPB improve legume growth by enhancing Fe uptake under alkaline conditions (Zhou et al., 2016, 2018; İpek et al., 2017; Nagata, 2017; Aras et al., 2018; Arıkan et al., 2018; Patel et al., 2018; Rahimi et al., 2020; Roriz et al., 2021). However, no significant differences were observed in the Fe concentration of inoculated plants, although a trend to the increase in Fe concentration was observed in the roots of plants inoculated with *B. licheniformis*. It is possible that the Fe-nutrition-related mechanisms promoted by bacteria were insufficient to enhance its accumulation in the plant tissues; maybe the constant high soil pH, even after inoculation with bacteria, hindered Fe solubilization. Nevertheless, inoculation with PGPB significantly impacted the accumulation of other important minerals (Mn, Zn, and Ca) showing the potential of these inoculants to modulate mineral uptake, which is also important for plant nutrition. Under Fe-deficient conditions, Mn concentration usually increases. This happens

since Mn shares with Fe ZIP-like transporters, such as IRT1 (Connolly & Guerinot, 2002). In our study, as growth conditions did not point to Fe deficiency, this increase was not found. Furthermore, its concentration decreased in the trifoliates of inoculated plants, which could indicate that Mn was not efficiently translocated to the shoots. The concentration of Zn significantly increased in the roots of plants inoculated with *B. licheniformis*. This increase can be justified by the fact that Mn, Zn, and Fe share the same transporters, and Fe-deficient conditions usually increase Zn uptake (Connolly & Guerinot, 2002; Ma et al., 2017), although these conditions have not been seen in our study.

Fe reductase activity is usually induced under Fe-deficient conditions (Vert et al., 2003; Kong et al., 2013; Wang et al., 2013; Zha et al., 2014), and that has been shown in studies where inoculation with PGPB improved FC-R activity under Fe-limiting conditions (Zhou et al., 2016, 2018; Scagliola et al., 2016; İpek et al., 2017; Nagata, 2017; Aras et al., 2018; Arıkan et al., 2018; Kong et al., 2020; Rahimi et al., 2020; Roriz et al., 2021). Our results showed that the inoculation of *G. max* plants with PGPB resulted in a decreased FC-R activity compared with non-inoculated plants. These findings could indicate that Fe uptake was effectively promoted by bacteria through, for example, IAA and ACC deaminase synthesis, the release of siderophores, and/or production of organic acids, and there was no need to increase FC-R activity under these conditions; thus, FC-R values decreased in plants inoculated with PGPB compared with the non-inoculated ones, as a shutdown mechanism due to the presence of bacteria. This can be corroborated by the increased Fe concentration found in the roots compared with the shoots, which may indicate that Fe absorption was effective.

Fe uptake is under the control of several important genes (Santi & Schmidt, 2009). Inoculation with PGPB resulted in a general increase in the expression of Fe-related genes. FRO2 expression increased in the roots of plants treated with *B. licheniformis* but decreased with *B. aerius*. FRO2 expression is usually activated so that Fe^{3+} is reduced to Fe^{2+} (Robinson et al., 1999; Connolly et al., 2003). An increase in FRO2 expression after inoculation with PGPB in plants grown under Fe-deficient conditions has been shown by Zhou et al. (2016, 2018), Rahimi et al. (2020), and Roriz et al. (2021). However, as mentioned before, our findings don't reflect the presence of Fe-deficient growth conditions. Nevertheless, the increased expression of IRT1 in inoculated plants suggests that Fe^{2+} was effectively collected from the rhizosphere and absorbed by the roots, showing the potential of bacterial isolates on Fe uptake. Similar to FRO2, IRT1 expression was found increased in previous studies with plants inoculated with *B. subtilis* GB03 and *P. polymyxa* BFKC01 (Zhang et al., 2009; Zhou et al., 2016). The expression of F6´H1 increased in inoculated plants. F6´H1 plays a role in the Fe uptake process and in the coumarin biosynthesis (Mai et al., 2016) being related to FRO2 and IRT1 expression (Tsai & Schmidt, 2017a). Under Fe deficiency, the roots produce Fe-mobilizing coumarins that mobilize Fe by reduction and/or chelation of Fe^{3+} (Tsai & Schmidt, 2017b). However, again, as Fe-deficient growth conditions have not been proven, this hypothesis can't be corroborated. Similarly, inoculation of soybean plants with PGPB led to an increase in the expression of bHLH38 gene which can indicate that Fe uptake was successfully activated. FIT together with several bHLH proteins, including bHLH38, regulates Fe uptake, and activates FRO2 and IRT1 expression (Wang et al., 2007; Yuan et al., 2008). Yuan et al. (2008) also found an increased bHLH38 expression in

the shoots and roots of *Arabidopsis* triggered by the Fe-deficient conditions. The increased expression of FER4 in the trifoliates of treated plants can be related to an effective enhancement of Fe uptake and storage in the shoots caused by inoculation with PGPB. Ferritins, including FER4, are involved in the maintenance of Fe homeostasis and are responsible for Fe storage in the leaves (Ting-Bo et al., 2006; Ravet et al., 2009). These findings can support our theory that PGPB effectively promoted Fe uptake through the activation of other mechanisms than FC-R activation.

Analysis of the composition of the soil showed a trend to the increase in OM concentration after inoculation with PGPB, as expected since the residues produced by plant growth and the activity of bacteria can contribute to this increase (Kallenbach et al., 2016; Rao et al., 2019). This can be supported by the increased no. of pods verified after inoculation with *B. licheniformis* indicative of improved plant growth and further increased OM. Regarding some of the analyzed minerals, we could say that the trend to the increase in their concentration after inoculation with *B. licheniformis* (for root P, Fe, and Mn) and with *B. aerius* (for root P, and K in the trifoliates), can be related with the ability of bacteria to solubilize these nutrients and make them available to the plant.

A previous study of our group has shown that two maize endophytic bacteria with known abilities related to plant growth promotion and Fe uptake were able to improve Fe nutrition in 21 days old soybean plants grown in calcareous soil (Roriz et al., 2021). Inoculation with *Sphingobium fuliginis* ZR 1-6 alone or in combination with *Pseudomonas jessenni* ZR 3-8 strain improved FC-R activity and general nutrient concentration (including Fe), contrary to what was seen in the present study. Although we expected that the selected native soybean isolates, being better adapted to soybean plants, would show a better performance in the improvement of plant growth and Fe uptake, when comparing with previous results this cannot be assumed (particularly for *B. aerius* strain).

We can conclude that, in general, the potential to improve crop performance was more pronounced with inoculation with *B. licheniformis* P2.3. This strain improved plant yield through the increase in the no. of pods, which did not happen with inoculation with *B. aerius*, bringing some light on the potential of this strain at the plant yield level. Several PGP traits can be related to the growth enhancement of soybean plants by *B. licheniformis*, which can be related to the Fe uptake process or not. This strain showed better results in terms of the ability to reduce Fe^{3+} and to produce organic acids, which could mean that within the conditions of our work, these characteristics overlap, for example, the production of siderophores and phytohormones. The fact that *B. licheniformis* is an endophytic bacteria from soybean shoots also contributes to its potential to promote plant growth since endophytes are naturally protected within the plant tissues from environmental stresses, competition for nutrients, and plant pathogens (Sturz et al., 2000). Plant growth promotion under alkaline conditions by PGPB can be the result of a combination of different traits during their life cycle, a process that involves the activation of several mechanisms. Furthermore, other important factors related to mineral balance, FC-R activity regulation, and/or regulation of key Fe-related genes may contribute to the improvement of Fe uptake. The potential shown by *B. licheniformis* P. 2.3 in this study makes this strain an interesting isolate to be used in bioinoculants formulations for

improved soybean growth, regardless of growth conditions; a future similar study with a non-calcareous soil would be interesting.

3.5. Conclusions

G. max plants growing in a selected agricultural Portuguese soil harbor several culturable bacterial strains in their tissues and in the rhizosphere. A large fraction of the isolates showed improved capacities to grow at high pH, to reduce Fe^{3+}, to produce siderophores, organic acids, IAA, and ACC deaminase, making them potential candidates for exploitation as future bioinoculants. Inoculation with *B. licheniformis* P2.3 improved plant yield (no. of pods), mineral nutrition (Zn and Ca), increased the expression of Fe-related genes, despite having decreased FC-R activity and Mn concentration in the trifoliates; inoculation with *B. aerius* S2.14 decreased root length, FC-R activity, Mn concentration in the trifoliates, and FRO2 expression, despite having increased the expression of other important Fe-related genes. *B. licheniformis* was more effective at improving crop performance under alkaline conditions, but with potential to improve soybean growth regardless of growth conditions.

Supplementary Material

Table S3.1. Sequences, accession number, and melting temperature (Tm) of primers used for the quantification of the transcripts via PCR.

Primer	Forward (5'-3')	Reverse (5'-3')	Accession number	Tm
18S	TTAGGCCATGGAGGTTTGAG	GAGTTGATGACACGCGCTTA	X75080.1	58.4
Ubiquitin	GATTTATTTCATTGGCAGGC	AGGATCATCAGGATTTGGGT	AF461687.1	54.3
FRO2	AGAACATGGAAGGGTCAACA	AGCAAGAACTCCCACACTTG	XM_003528793.4	56.4
IRT1	ACAACAATGGCCACTTCACT	GCCAATTATGCTTGAGGCTA	NM_001287456.1	56.4
F6'H1	TGCACTCCAAGTAATGAGCA	CAAAGGGCCAATAACATCTG	XM_003530041.2	56.4
bHLH38	GTGTTCTCAACCAAGGGATG	GGATGGAGAAGTGGACCTTT	NM_001367104.1	58.4
FER4	GAACAAACGTGGTGGAAAAG	AACTGCACGTCACCATTCTT	NM_001250120.1	56.4

Table S3.2. Relative expression values of FRO2, IRT1, F6'H1, bHLH38, and FER4 genes in *G. max* plants, grown under alkaline conditions, inoculated with saline solution (Control), *B. licheniformis*, and *B. aerius*. FRO2, IRT1, F6'H1, and bHLH38 expression was measured in the roots; FER4 expression was measured in the trifoliates. Data are means of three independent biological replicates.

Treatment	Gene				
	FRO2	IRT1	F6'H1	bHLH38	FER4
Control	0.45	0.18	0.43	0.49	1.06
B. licheniformis	0.62	0.48	1.01	0.78	3.73
B. aerius	0.36	0.51	0.50	0.57	3.08

CHAPTER 4

CURRENT ASPECTS AND CONSIDERATIONS IN THE FORMULATION OF BIOINOCULANTS WITH PLANT GROWTH-PROMOTING BACTERIA

Abstract

There is currently a huge need for more sustainable agricultural approaches to counteract the effects of the intensive agricultural practices of the past century that have been contributed to soil degradation and detrition of natural resources. The inclusion of microbial-based formulations that contain living or latent cells of microorganisms – bioinoculants (BIs) – has great potential in the fulfillment of the global policies launched by the United Nations for sustainable development, which focus on the environment and climate change towards a greener and sustainable agriculture. These formulations are a natural source of nutrient recycling, providing solutions for pest and pathogens management, and alleviation of the negative impact of abiotic stresses. Furthermore, they represent an environmental-friendly approach to avoid the negative impact of chemical fertilizers. The most common microorganisms present in BIs include nitrogen-fixing- and phosphate-solubilizing bacteria, different species of plant growth-promoting bacteria, and arbuscular mycorrhizal fungi. These must possess several functional traits to better survive in the plant environment, such as high rhizosphere competence, plant growth-promotion ability, and adaptation to several environments. The choice of formulation and type of application (seed, soil, or leaves) relies on their composing microorganisms and the purpose of the application. There are several BIs formulations, including soil carrier-based, liquid, and more recently those based on bioencapsulation, biofilms, nanostructures, and fluid bed driers technologies. BIs global implementation is still very difficult since the quality control guidelines are ambiguous between countries, which makes their utilization limited. This review focus on: (i) the importance of BIs in the new direction of European global policies (ii) main microorganisms used in BIs formulations and desired traits; (iii) different BIs formulations; (iv) possible application and delivery methods; and (v) the importance of quality control procedures.

4.1. Introduction

The soil and its biodiversity is perhaps the most important system to agriculture and food production. The excessive use of chemical fertilizers and pesticides has been deteriorating soil and the environment, reflecting on both human and animal health. Efforts have been made in the study of alternative biological means to efficiently utilize agricultural resources and enhance crop productivity. The European Commission (EC) has launched a series of missions to raise awareness of the urgency in the adoption of greener global policies for sustainable agriculture development. The presence of several soil microbes and mammals is a powerful tool to better manage soil potential and to cope with these important future challenges. The utilization of “microbial-based formulations that contain living or latent cells of microorganisms that when applied to the soil, seeds, or plant surfaces, improve the uptake of nutrients by the plant” – bioinoculants (BIs) – deserves particular interest in this context (du Jardin, 2015). These are the key to green agriculture and include plant dead tissues and organic matter, different algae species, and several microorganisms (Sahoo et al.,

2013). Among the countless advantages of BIs usage, we can highlight the safety, cost-effectiveness, being environmentally friendly, and being a renewable source of plant nutrients that improve soil fertility (Ramasamy et al., 2020).

There is numerous evidence regarding the potential of inoculation with microorganisms in the improvement of crop growth and nutrition. The inclusion of BIs in agriculture was estimated to potentially increase up to 10–40 % in the crop yield (Schütz et al., 2018). These microbes play vital roles to plants by participating in nitrogen (N) fixation, contributing to nutrient enrichment, recycling, and solubilization, secreting plant hormones (auxins, cytokinins, biotins, and vitamins) and exopolysaccharides, participating in organic matter decomposition, producing siderophores, acting as biocontrol agents by the production of antibiotics, and protecting plants from biotic and abiotic stresses (Sahoo et al., 2013; Kumar & Gopal, 2015; Naik et al., 2019).

Even though about 150 microbe-based registered products are available for use in agriculture, BIs account for only 5 % of the global fertilizer market (Verma et al., 2019). N fixers-based BIs are the most commonly found (78 %) (e.g. Azonik, Bio-N), followed by phosphorus solubilizers-based (15 %) (e.g. Get-Phos) (Mishra & Das, 2014). There are also BI composed of potassium-solubilizers (e.g. BioPotash, Green Earth Reap K), sulfur-solubilizers (e.g. Siron), zinc-solubilizers (e.g. MicroZ-109), and silica-solubilizers (e.g. BioSilica) microbes (Thomas & Singh, 2019). These are composed of strains of *Azospirillum, Azotobacter, Bacillus, Rhizobium,* and *Thiobacillus* but also of blue-green algae, fungi, mycorrhizae, and vermicompost (Sahoo et al., 2013; García-Fraile et al., 2015; Thomas & Singh, 2019). The use of BIs, in general, is still very limited but with the recent impulse of the EC in the adoption of greener agricultural practices, an increase is expected.

In this review, current aspects and applications of BIs formulations, focusing on their importance in the current global policies of the EC, addressing the most commonly used microorganisms, their desired traits, the different strategies used in their formulation, their application and delivery methods, and the importance of quality control procedures, are discussed.

4.2. Importance of bioinoculants in future policy frameworks for sustainable agriculture

Several concerns are severely compromising the success of agricultural practices in terms of crop yield and productivity and food security. One of the main problems deals with the deterioration of soil, water, and biodiversity, as a result of the over-exploitation of natural resources, and excessive use of inorganic fertilizers and pesticides. It is estimated that 33 % of world soils are degraded, and erosion affects 25 % of European agricultural land (European Commission, 2020b). Moreover, farmers are still little open to the cultivation of “biodiversified” crops, including legumes, which present many advantages to agriculture, including participation in N fixation (reducing need of N fertilizers application), decrease of greenhouse gas emissions by up to 25 %, and general improvement of soil composition (Karkanis et al., 2018; Ma et al., 2018). In Europe, less than 2 % of the agricultural land

includes legume cultivation (Watson et al., 2017), and in Portugal this percentage is residual. The world population is expected to reach around 10.2 billion by 2050, particularly in developing countries (Ahirwar et al., 2019). Furthermore, climate change is increasing the severity of abiotic stresses, including abrupt temperature changes, drought, and salinity, which severely compromises soil quality and crop productivity (Onyekachi et al., 2019). These concerns affect not only the environment, but also human health, and an important socio-economic challenge of the century is arising (Daly et al., 2017).

The EC launched a series of missions that deal with the above-mentioned concerns. These are included in the Horizon Europe framework program that started in 2021 and point in the direction of the European Green Deal to make Europe the first climate-neutral continent by 2050, and the Sustainable Development Goals launched in 2015 by the United Nations as part of the 2030 Agenda for Sustainable Development. One of these goals includes "end hunger, achieve food security and improve nutrition, and promote sustainable agriculture" (United Nations, 2020). These are also connected with one of the central points of the Green Deal, called “the Farm to Fork Strategy” which aims to understand the value that the Europeans give to food sustainability, with the concern of reaching a healthy planet and a healthy society. Also, with the impact that the COVID-19 pandemic brought to the world, the need for more sustainable food systems that deal with several important social concerns is essential and highlighted the importance of the establishment of robust and resilient food systems (European Commission, 2020a). These demands focus on the essential role of the soil as an essential part of the food supply, whose ecosystems and biodiversity contribute to the water quality, cycling of nutrients, and climate balance (European Commission, 2020b). They highlight the urgent need to reduce fertilizers, pesticides, and even antimicrobials utilization, intensify organic farming, improve biodiversity, and focus on animal welfare (European Commission, 2020a). In this context, the definition of “climate-smart agriculture” appeared, referring to the adoption of climate change-“resistant” and environment-friendly agricultural approaches.

Several strategies are required to deal with these demands. The main way is to put aside the strategies adopted during the agricultural revolutions of the 20th century, that reduced the efficiency of agricultural systems by seriously affecting soil health, biodiversity, and crop susceptibility to pests/pathogens. Agriculture should focus on the so-called Sustainable Intensification, defined as “a process or system where yields are increased without adverse environmental impact and without the cultivation of more land” (The Royal Society, 2009). This concept has the advantage of being very open and does not refer to any specific agricultural approach, and can involve strategies of soil conservation or other conventional and modern agricultural activities (Garnett & Godfray, 2012; Smith, 2013). The purpose is to focus on new techniques to better use the available resources aiming to produce healthy foods and giving importance to all the constituents of the agro-ecosystems. Research and innovation activities should direct to the fields and markets to efficiently achieve these goals. A good example of a way to restore soil ecology and its enrichment is to stimulate the cultivation of plant diversity, through crop rotation or cover crop practices (Cruz, 2018).

In 2020, the EC also concentrated on the improvement of the Common Agriculture Policy (CAP)

to meet these new demands. The objective is to focus the European farming model on the environment and climate change towards a greener and sustainable agriculture based on nine objectives. A nutrient management tool that aims to reduce ammonia and nitrous oxide emissions and improve water quality was launched to enable farmers to easily achieve crops' nutritional requirements with low costs (Fertilizers Europe, 2019). Furthermore, one of the goals of the CAP post-2020 deals with the encouragement of the farmers into the use of more sustainable and environmentally friendly nutrient delivery methods, including BIs application (e.g. N fixers) (Fertilizers Europe, 2019).

The recovery of a functional soil biota by the inclusion of BIs in future agricultural practices can undoubtedly make a great contribution to the current directions of European policies. They act in the improvement of soil quality, nutrient recycling, plant health, pest and pathogens management, and alleviation of the negative impact of abiotic stresses. The microorganisms present in BIs formulations are a natural, environment-friendly pathway of plant nutrients.

4.3. Microorganisms used in bioionoculants formulations and desired traits

The first step in the formulation of a BI is the isolation and selection of appropriate microorganisms. Usually, BIs formulations include N-fixing bacteria, phosphate-solubilizing bacteria, different species of plant growth-promoting bacteria, and arbuscular mycorrhizal fungi (AMF) (Bhattacharyya et al., 2020). The most common strains used in BIs formulations for different crops are *Azospirillum* spp., *Bacillus* spp., *Dyadobacter* spp., *Frankia* spp., *Pseudomonas* spp., and *Rhizobium* spp. (Suyal et al., 2016; Aremu et al., 2017; Tahir et al., 2017). They can be found inside the plant or in the rhizosphere, contributing directly or indirectly to plant growth.

A promising microorganism must possess high rhizosphere competence, improve plant growth and development, enable an ease biomass production, act in several environments and conditions, and be environmentally friendly (Nakkeeran et al., 2005). Indeed, the most important characteristic of a BI formulation and which defines its effectiveness is the survival ability of the selected microorganism within the rhizosphere soil environment. It is well known that climate conditions have a direct effect on the growth and survival of microorganisms (Hebeisen et al., 1997). Environmental conditions such as pH, temperature, drought, mineral concentrations, soil type, moisture content, and presence of toxic compounds impact microbial performance (Bhattacharjee & Dey, 2014; Kaur et al., 2017). Other factors such as the presence of grazers and competition with other native microbes equally determine the success of the applied inoculant (Kaur et al., 2017). Several functional traits determine the rhizocompetence of each microbial strain and dictate its survival, such as the ability to produce siderophores and proteases, antibiosis potential, capacity to form biofilms, motility ability, and utilization of root exudates (Kaur et al., 2017).

Ideally, co-inoculation or multi-strain is preferred over single strain inoculation, which despite requiring different growth media with specific characteristics, potentiates the benefic effects associated with plant growth, as the different microorganisms possess different working mechanisms

and act synergistically increasing their efficiency (Chaudhary et al., 2020). However, competitiveness effects can arise when we mix different strains and the plant growth effect can be compromised (Rani & Kumar, 2019). Thus, microbe interactions should be carefully evaluated to understand their effectiveness.

After the selection of promising microbes, a large-scale production on a selected medium is carried out. This process must be cost-efficient, easily obtainable, and reunite all the properties for the proper growth of microbial strains in sufficient amounts (Glick, 2015). Scaling up of BIs microbes is achieved using liquid, semisolid, and solid fermentation techniques (Stamenković et al., 2018); the fed-batch technique is usually used (Mutturi et al., 2017).

4.4. Bioinoculants formulations

4.4.1. Solid carrier-based

The formulation of a BI comprises the presence of a vehicle for the delivery of microorganisms that provide them protection and a supportive niche, called a carrier (Chaudhary et al., 2020). This methodology improves the effectiveness and water ration ability of the BI (Ritika & Utpal, 2014). A suitable carrier must compile several characteristics such as: be efficient, affordable, readily and plentily available, be cost-efficient, easy to process, free of lump-forming materials, be easy to sterilize by autoclaving or other processes, non-toxic to microbes and plants, possess a high moisture absorption capacity, and a high pH buffering capacity, have preferably a high organic matter content, be suitable for as many microbial strains as possible, be non-polluting and biodegradable, and have an adequate shelf life (Malusá et al., 2012; Sahu et al., 2018; Misra et al., 2020; Naik et al., 2020).

Materials used as carriers can have several sources: organic – clay, coal, compost, diatomaceous earth, manure, peat, rice, soybean meal, and wheat bran; or inert – bentonite, biochar, charcoal, kaolin, perlite, sawdust, silicates, talc, vermiculite, zeolite, and more recently polyacrylamide gels, and alginate beads (Sahu & Brahmaprakash, 2016; Rani & Kumar, 2019). The choice of the best material is dependent on the microorganisms and the purpose of the application. When choosing the suitable carrier material, we must be aware of the advantages and drawbacks of each material. For example, compost materials have the advantage of being biodegradable and non-polluting, potentiating the survival of soil microorganisms and improving plant growth and yield. It is therefore a low-cost, safe, and zero-waste material (Naik et al., 2020). Peat-based formulations are the most used worldwide, as they are easily available, with high water-holding capacity, and high organic matter content (Bhattacharyya et al., 2020). However, peat utilization does not represent a sustainable approach as issues such as changes in composition and quality, possibility of releasing toxic compounds during sterilization which can compromise the viability of the microorganisms, are associated with its utilization (Malusá et al., 2012). Biochar, like compost, is a good eco-friendly

alternative, which improves the viability of the BI and as it has a low water content its storage is facilitated (Chaudhary et al., 2020).

Although it is still difficult to find a carrier that combines all the desired characteristics, modern technologies are putting a step forward in this issue (Naik et al., 2020) and will be better discussed below (section 4.4.3). Still, the majority of the available solid carrier-based BIs bring some issues related to the reduced shelf life (6 months), susceptibility to UV and low stability at high temperatures, microbial densities which decrease with time, and proneness to contaminations (Rana & Ramesh, 2013).

4.4.2. Liquid formulations

The development of liquid BIs overcome some of the problems associated with solid carried-based BIs, being a novel and innovative approach. The liquid formulations involve the use of mineral or organic oils, oil-in-water suspensions, and several microbial metabolites such as amino acids, biosurfactants, flavonoids, humic acid, molasses, starch wastewater, sugars, etc. (Brar et al., 2012; Chaudhary et al., 2020). The additives present in liquid BIs (e.g., glycerol, polyvinylpyrrolidone, and sucrose) improve the survival of microorganisms by providing nutrients and conferring cellular protection under stress conditions. Furthermore, these compounds improve plant growth, playing an important role in N fixation, and confer protection against phytopathogens (Chaudhary et al., 2020; Naik et al., 2020). Strains belonging to genera *Bacillus, Mesorhizobium, Pseudomonas,* and *Rhizobium* are used in the production of metabolite-based BIs. Liquid formulations are composed of high cell concentrations which make a low amount of inoculum necessary. Usually, liquid BIs formulations are conceived as emulsions that are easily applied to soil and seed, commonly used in legume inoculation. These formulations allow the addition of cell protectants and nutrients which improve their efficiency (Bhattacharyya et al., 2020). Contrary to what happens with some solid carrier-based BIs, liquid BIs tolerate high temperatures and UV radiations, usually have a shelf life of 2 years, microbial density is stable over time, and are less prone to contaminations (Mahdi et al., 2010). Liquid formulations are easy to apply, through hand or power sprayers, or by incorporation in the irrigation systems (VanderGheynst et al., 2006). Nevertheless, the high costs associated with liquid BIs usage restrict their applicability.

4.4.3. New promising approaches

4.4.3.1. Bioencapsulation-based

The encapsulation process allows microbial cells to be covered with a protective shell or entrapped within a polymeric material through the creation of beads permeable to nutrients, gases, and metabolites (John et al., 2011; Sathvika et al., 2018). There are two types of encapsulation: microencapsulation, and macroencapsulation, depending on the size of the polymeric beads

(Nordstierna et al., 2010). This is a very promising approach for the development of carriers for BIs formulations in which the nutritive capsule created around microorganisms enables their gradual release in the environment, avoid the risk of contamination during transport and storage, and improve their physiological activity and viability particularly under stress conditions, which results in an increased product shelf life (Mortazavian et al., 2007; Schoebitz et al., 2013). Polymers such as agar, alginate, casein, chitosan, carrageenan, gelatin, gellan gum, glycerol, polyacrylamide, polyvinyl alcohol, skimmed milk, soy oil, starch, xanthan gum, and whey protein are usually used in the bioencapsulation process (Rathore et al., 2013; Chaudhary et al., 2020). Strains of *Azospirillum brasilense*, *Bacillus subtilis, Klebsiella oxytoca, Mesorhizobium spp*. and *Raoultella planticola* have already been tested in encapsulated BIs (Alvarez et al., 2010; Wu et al., 2011; Trejo et al., 2012; He et al., 2015; Tu et al., 2016). Alginate is the most popular polymer used in bioencapsulation programs; it is non-toxic, biodegradable, and with a gradual releasing capacity (Chaudhary et al., 2020). Dried capsules with encapsulated cells can be stored at room temperature for long periods (Chaudhary et al., 2020). Although very promising, the bioencapsulation-based BIs formulations strategy still involves high investments particularly associated with bead production (Chaudhary et al., 2020).

4.4.3.2. Biofilms-based

The utilization of biofilms containing microorganisms is very promising, as they are natural carriers where microbial cells are enclosed in their own produced matrix, being protected from hostile conditions (Parween et al., 2017a; Parween et al., 2017b). The biofilms composed of beneficial plant root microbes promote plant growth and improve the uptake of minerals in several crops (Müller et al., 2013; Weidner et al., 2017). BIs microorganisms face a heterogeneity of biotic and abiotic factors and have to compete for their survival with the indigenous soil organisms. The utilization of biofilms containing mixed cultures of beneficial microbes can help to overcome these issues as they are better protected and possess several distinct characteristics that make them better prepared to face the environment (Zakeel & Safeena, 2019). Production of hormones, siderophore, and hydrogen cyanide, nitrogenase activity, biocontrol properties, and solubilization and mineralization of nutrients are amongst the properties of developed microbial biofilms (Bandara et al., 2006; Herath et al., 2013; Triveni et al., 2013). Several crops (maize, rice, rubber, soybean, tea, and several vegetables) have been successfully fertilized with biofilm-based BIs under field or greenhouse conditions (Zakeel & Safeena, 2019). For example, nitrogen fixation was improved when a biofilm composed of fungi and *Rhizobium* spp. strains was inoculated in soybean, compared to traditional *Rhizobium* BIs inoculation (Parween et al., 2017a; Parween et al., 2017b).

4.4.3.3. Bionanotechnology-based

The incorporation of microbial cells in nanostructures (1-100 nm) made of organic or inorganic materials (chitosan, zeolite, and polymers) is another promising approach for the development of new carrier-based BIs (Veronica et al., 2015). There is also the possibility to incorporate nutrients in nanomaterials whose release can be managed according to the temperature, moisture, and pH of the environment (El-Ghamry et al., 2018) improving its solubility and bioavailability (Naderi & Danesh-Shahraki, 2013). The use of nanotechnologies in future agriculture is deserving particular interest as its utilization is related to improved bioinoculant viability, activity, and stability, increased plant production rates, yield, and nutrient use efficiency, biocontrol properties, and improved resource utilization efficiency (VanderGheynst et al., 2007; Sekhon, 2014; Sharma et al., 2016; Mącik et al., 2020). Furthermore, nano-bioinoculant production involves the utilization of a low quantity of fertilizer and within a shorter period at large-scale production, it is cost-efficient and an eco-safe perspective and renewable fertilization system (Duhan et al., 2017; Thirugnanasambandan, 2018).

The inclusion of *Bacillus subtilis, Paenibacillus elgii,* and *Pseudomonas fluorescens* within silver and gold nanoparticles has already shown promising results in the promotion of growth in several crops (Dikshit et al., 2013). The application of a nano-zinc chelate and a nano-bioinoculant improved the plant yield of *Zea mays* plants grown under water stress conditions (Farnia & Omidi, 2015). The yield of sugar beet increased by 22.9 % after the application of the bioorganic nano fertilizer “Nagro” (Jakienė et al., 2015). Rajendran et al. (2017) showed that the application of a nanostructured fertilizer with neem cake and PGPB improved the growth of *Vigna radiata* through stimulation of seed germination and nutrient solubilization.

4.4.3.4. Fluid bed dryer-based

In a fluid bed dryer (FBD) solid particles or granules are suspended against gravity in an upward flowing warm or hot air stream creating a fluidized condition (Brahmaprakash & Sahu, 2012). There are two ways by which microorganisms can be incorporated: they can be sprayed onto blowing carriers before drying or can be dispensed as dry mass and then coated with a protective shell in a fluidized bed (Mącik et al., 2020). In this type of formulations, the water content is highly reduced, which prevent contaminations; furthermore, microbial cell counts are constant over time, the drying temperature and addition of ingredients can be managed according to the needs, and room temperature is enough for the drying process (Sahu & Brahmaprakash, 2016). Although FBD is a low-cost drying approach, its applicability is limited when slurry-like or liquid original matrices are present (Berninger et al., 2018). The development of solid carriers composed of *Pseudomonas fluorescens*, vermiculite, and EB™ (clay and wood particles) is an example of a FBD formulation (Moenne-Loccoz et al., 1999).

In Table 4.1 a compilation of the composition, selected microorganisms, advantages, and limitations of the available BIs formulations is presented.

Table 4.1. Composition, selected microorganisms, advantages, and limitations of available bioinoculant formulations.

Bioinoculants formulations	Composition	Microorganisms	Advantages	Limitations	References
Solid carrier-based	• Organic: clay, coal, compost, diatomaceous earth, manure, peat, rice, soybean meal, and wheat bran • Inert: bentonite, biochar, charcoal, kaolin, perlite, sawdust, silicates, talc, vermiculite, zeolite, polyacrylamide gels, and alginate beads	Ectomycorrhizal and arbuscular mycorrhizal fungi	• Microbial protection • Improved effectiveness and water ration ability	• Reduced shelf life (6 months) • UV susceptibility • Release of toxic compounds	(Rana & Ramesh, 2013; Ritika & Utpal, 2014; Sahu & Brahmaprakash, 2016; Rani & Kumar, 2019; Chaudhary et al., 2020)
Liquid-based	Mineral or organic oils, landfill leachates, oil-in-water suspensions, amino acids, biosurfactants, flavonoids, humic acid, molasses, starch wastewater, and sugars	*Bacillus, Mesorhizobium, Pseudomonas,* and *Rhizobium* strains	• Improved microbial survival • Low amounts • High temperature and UV tolerance • Increased shelf-life • Less prone to contaminations • Easy application	High production costs	(Mahdi et al., 2010; Brar et al., 2012; Bhattacharyya et al., 2020; Chaudhary et al., 2020)

Table 4.1. (cont.) Composition, selected microorganisms, advantages, and limitations of available bioinoculant formulations.

Bioinoculants formulations	Composition	Microorganisms	Advantages	Limitations	References
Bioencapsulation-based	Agar, alginate, casein, chitosan, carrageenan, gelatin, gellan gum, glycerol, polyacrulamide, polyvinyl alcohol, skimmed milk, soy oil, starch, xanthan gum, and whey protein	*Azospirillum brasilense*, *Bacillus subtilis, Klebsiella oxytoca, Mesorhizobium spp*. and *Raoultella planticola*	• Microbial protection • Improved microbial viability • Gradual release • Increased shelf-life • Less prone to contaminations • Non-toxic and biodegradable materials	High production costs	(Mortazavian et al. 2007; Alvarez et al. 2010; Wu et al. 2011; Trejo et al. 2012; Rathore et al. 2013; Schoebitz et al. 2013; He et al. 2015; Tu et al. 2016; Chaudhary et al. 2020)
Biofilms-based	Plant root microbes	Fungi and *Rhizobium* spp.	• Microbial protection • Improved microbial survival and competition ability • Reduced fertilizer needs by 50 %	Technical issues during the production system	(Seneviratne et al., 2011; Parween et al., 2017a; Parween et al., 2017b; Zakeel & Safeena, 2019)

Table 4.1. (cont.) Composition, selected microorganisms, advantages, and limitations of available bioinoculant formulations.

Bioinoculants formulations	Composition	Microorganisms	Advantages	Limitations	References
Nanostructured-based	Organic or inorganic materials (chitosan, zeolite, and polymers)	*Bacillus subtilis,* *Paenibacillus elgii,* and *Pseudomonas fluorescens*	• Possibility to add ingredients • Improved solubility and bioavailability • Low amounts • Short application period • Eco-safety • Cost efficiency	Reduced soil adsorption and fixation	(Dikshit et al., 2013; Naderi & Danesh-Shahraki, 2013; Veronica et al., 2015; Duhan et al., 2017; El-Ghamry et al., 2018; Thirugnanasambandan, 2018;)
Fluid bed dryer-based	Solid particles or granules	*Pseudomonas fluorescens*	• Constant microbial counts • Manageable temperature and ingredients • RT in the drying process • Contamination-free • Cost efficiency	Limited application	(Brahmaprakash & Sahu, 2012; Sahu & Brahmaprakash, 2016; Berninger et al., 2018)

4.5. Application and delivery methods for bioinoculants

4.5.1. Seed application

BIs can be applied to the seeds by three distinct methodologies: dusting, slurry, or seed coating. Through seed dusting, dry seeds are used and mixed directly with the inoculant. However, this strategy is considered the least effective due to problems associated with microorganism's adherence. Through seed slurry, wetted seeds are used and mixed with the inoculant (Malusá et al., 2012). An adhesive material (e.g. carboxymethyl cellulose, gum arabic, jaggery solution, methyl ethyl cellulose, sucrose solutions, vegetable oils, and vermiculite) is used to coat seeds uniformly (Ghosh et al., 2015). Through seed coating, seeds are mixed with the slurry prepared from the inoculant and coated with inorganic inert materials such as calcium carbonate, charcoal, clay, dolomite, lime, rock phosphate, and talc (Malusá et al., 2012). After seed treatment, seeds are spread, dried in the dark, and sown (Kumar et al., 2017; Misra et al., 2020). Seed coating is usually suggested for pulses, oilseeds, and fodder (García-Fraile et al., 2015).

Although seed coating is the most used technique, its success is not always guaranteed as the survival of the inoculated strains is limited. Usually, the application of microorganisms in the soil is preferred as this technique brings the possibility to use larger concentrations of the microbial population (Chen et al., 2011).

4.5.2. Soil application

Direct inoculation to the soil enables the application of a larger population of microorganisms, which does not happen with seed application. Utilization of granules composed of peat, perlite, soil aggregates, or talc is usually preferred in this type of delivery method (Mącik et al., 2020). BIs are usually applied to the soil at the time of seed sowing or just before transplanting (Misra et al., 2020). Granules-based BIs can be applied under, above, or alongside the seeds; liquid BIs usually are sprayed on the seeds or incorporated in hydroponic systems.

Among the advantages of soil application are the inclusion of the inoculant in a protected environment, preventing the possible damage effect of pesticides and fungicides, and protecting the seed coat, in addition to allowing to handle the location and application rate of inoculant. However, soil inoculation requires higher amounts of BI and specific equipment which significantly increase their general associated costs, which make it less available in developing countries (Mącik et al., 2020).

Some examples of plant-growth promoting microorganisms, their application methods, including seed and soil treatment, and effect on different crops including cereals, legumes, and vegetables, have been reviewed by Verma et al. (2020).

4.6. Quality control of produced bioinoculants

Quality control is a key step in BI formulation and commercialization, which significantly affects BI efficacy (Herrmann & Lesueur, 2013; Yadav & Chandra, 2014). This process influences the farmer's perception and the success of BIs production and commercialization requiring that all production stages are under supervision (Sethi & Adhikary, 2012).

The main problems that the quality establishment patterns face deal with the presence of contaminants that reduce the counts of viable selected microorganisms, diminishing their efficiency. Several other factors affect the quality and efficacy of the BIs from their production to their delivery: (i) during the large-scale production, issues such as proper media composition and growth conditions, culture purity, and minimization of production costs; (ii) during their formulation, the choice of the proper carrier and the best sterilization procedure, alternatives to peat application, effective speed drying process, need of stickers and other additives, and contaminants presence; (iii) during their storage and transport, appropriate water content, competition issues due to the presence of contaminants, proper storage temperature and shelf-life; and (iv) when inoculated, the best concentration, the best application method, and competitive ability (Petrova & Petrov, 2021).

In countries where BIs are mostly used, such as China and India, the production standards and quality parameters are established. The quality parameters are usually based on the appearance, counts of microbial cells, the content of water and carbon, pH, carrier dimension (in case of solid BIs), contamination level, and shelf-life of BI. A minimum validity period of 6 months and organic content of at least 20 % is required. The amount of living cells is still the major parameter, varying between countries and defined for specific strains. In the European Union and the USA, these parameters are yet ambiguously stipulated (Mącik et al., 2020).

4.7. Conclusions

The inclusion of BIs to improve soil quality and plant growth is undoubtedly a growing trend that replaces the use of chemical fertilization and contributes to sustainable agriculture. In the current scenario of growing population and climate change, BIs utilization in future agriculture is a promising approach to increase food production and nutritional value in a sustainable manner. There are many types of BIs formulations, each of one with its limitations. The appearance of modern technologies brings new perspectives in the formulation of BIs and helps to overcome some of the limitations present in the solid and liquid formulations, mainly through the increase in the BI shelf-life and effectiveness. However, the high costs associated with such formulations still limit their usability in developed countries, as chemical fertilizers reveal more inexpensive. Although some countries already have well-established production standards and quality parameters, in Europe and the USA these are still ambiguous, compromising their usability. The appearance of a more universal quality control system, with the same regulations and standards, should be advantageous for the effective

implementation of BI. There is still a long way to go before BIs are widely used, and much research is still needed, but with the recent demands for the use of more sustainable agricultural strategies, their attention is being stimulated.

CHAPTER 5

GENERAL CONCLUSIONS AND FUTURE WORK

5.1. General conclusions

5.1.1. Inoculation with maize endophytes influenced iron metabolism in soybean grown in calcareous soil

The potential to enhance plant growth and Fe uptake was successfully evaluated in 24 PGPB strains available in a CBQF collection, which was obtained from isolates of a metal(loid) contaminated soil or tissues of maize plants. To this end, several *in vitro* analyses were performed including: (i) ability to produce IAA, ACC deaminase, siderophores, and organic acids; (ii) tolerance to high pH; and (iii) capacity to reduce Fe^{3+}. Two promising PGPR were tested for their ability to modulate Fe uptake-related processes in soybean grown in calcareous soil. Although inoculation with PGPR did not affect the chlorophyll content (SPAD values) and fresh weight, an increase in the shoot and root Fe concentrations, root ferric-chelate reductase activity, and expression of Fe-related genes (FRO2, F6'H1, IRT1, bHLH38, and FER4) was verified. These results suggest that bacterial inoculation successfully modulated Fe uptake and accumulation. The selected inoculants acted differently in the plant and two of the treatments proved to be more promising: (1) Inoculation with *S. fuliginis* ZR 1-6 showed more marked effects at the root level, inducing Fe reduction and uptake through the increase of ferric-chelate reductase activity and FRO2 expression, which was reflected in an increased Fe root concentration; (2) Mixed inoculation with the selected PGPR strains resulted in increased Fe concentration in the trifoliates, IRT1 expression in the roots, and FER4 in the trifoliates, suggesting its potential regarding Fe translocation to the shoots. These findings show the perspective of the utilization of *S. fuliginis* alone or in combination with *P. jessenii* as bioinoculants to improve Fe uptake and accumulation in *G. max* plants grown in calcareous soils, regardless of the existence of typical Fe deficiency symptoms.

5.1.2. Soybean grown in a Portuguese soil harbor several PGPB with the potential to modulate plant growth under alkaline conditions

Soybean plants grown in a Portuguese soil were collected and their associated PGPB were successfully isolated from shoots, roots, and rhizosphere. A total of 76 bacterial strains were isolated (53 % from roots, 29 % from rhizosphere, and 18 % from shoots) and 29 genera were identified. A large fraction of the isolates showed improved capacities to grow at high pH, to reduce Fe^{3+}, to produce siderophores, organic acids, IAA, and ACC deaminase, rendering them potential candidates for exploitation as future bioinoculants. After *in vitro* characterization, two PGPB with distinct plant growth-promoting traits were selected: (i) *B. licheniformis* P2.3 showed the ability to reduce Fe^{3+} and to produce IAA and organic acids, weak siderophore production, and ACC deaminase activity; (ii) *B. aerius* S2.14 showed strong production of siderophores, moderate IAA production and ACC deaminase activity, and low ability to reduce Fe^{3+}. Their impact on plant growth under alkaline conditions was also evaluated. Although inoculation with PGPB did not significantly affect

photosynthetic parameters, chlorophyll content, fresh weight, and Fe concentrations of soybean plants, other important parameters were influenced: inoculation with *B. licheniformis* increased pod number (improved yield), decreased ferric-chelate reductase activity, and increased the expression of Fe-related genes (FRO2, IRT1, F6'H1, bHLH38, and FER4); on the other hand, inoculation with *B. aerius* decreased root length, ferric-chelate reductase activity, and FRO2 expression, and increased the expression of the remaining genes. Although Fe concentrations were not significantly affected, inoculation with PGPB impacted significantly the accumulation of other essential minerals (Mn, Zn, and Ca) showing their potential in the modulation of mineral uptake, equally important for plant nutrition. Though Fe deficient conditions were not proven in this study, the results showed that *B. licheniformis* was effective at improving crop performance under alkaline conditions, and with the potential to improve soybean growth regardless of growth conditions.

5.1.3. Comparison of inoculation studies and concluding remarks

In this work, two different studies were conducted to find potential bioinoculant candidates for improved soybean growth under Fe-deficient conditions: (1) in Chapter 2, PGPR previously isolated from maize tissues and with potential to improve Fe nutrition were inoculated in soybean plants grown in a mixture of vermiculite and calcareous soil in a growth chamber for 14 days; (2) in Chapter 3, PGPB were isolated from soybean tissues and rhizosphere, tested for their potential to improve plant growth and Fe nutrition, and inoculated in soybean plants grown in calcareous soil in a greenhouse (plants were collected after 24 days, and at full maturity). The selection of native soybean isolates (Chapter 3), in spite of being more promising due to their improved adaptation to soybean plants, showed less potential to improve plant growth and Fe uptake than non-soybean associated PGPB (Chapter 2). Although in Chapter 2 some results point to the existence of Fe-deficient growth conditions, by the activation of physiological and molecular Fe deficiency-responses, the typical symptoms of Fe deficient plants were not observed even when using a calcareous soil (particularly in Chapter 3). Nevertheless, PGPB inoculants showed promising results as soybean-growth promoters, improving plant yield and nutritional value. This work shows the potential of selected PGPB strains as bioinoculants for improved soybean growth and nutritional value. Here we present a sustainable and ecologic alternative to prevent/ameliorate Fe deficiency constrains in calcareous soils and improved crop yield, that can be extrapolated to other important legume crops. This is an initial contribution to the stimulation of legume crop production with improved yield in Portuguese soils. In Portugal, there is still a large dependency on the import of legumes, including soybean, which is often associated with intensive cultivation practices. Furthermore, there are no approved environmentally friendly agrochemicals at national level for the production of soybean and other legumes, which reduces their competitiveness compared to others (e.g. maize). In a global context of population growth and climate change, we give here our contribution to the discovery of sustainable agronomic approaches for improved food nutritional value to help fulfill the second

“sustainable development goal" – “end hunger, achieve food security and improve nutrition and promote sustainable agriculture” – proposed by the UN through the Horizon 2020 program.

5.2. Future work

In this section, some suggestions of future research regarding the obtained results and their potential are given:

- Further explore the molecular mechanisms related to bacterial colonization, looking at other important genes such as the ones related to the onset of bacteria-induced systemic resistance and plant survival under Fe deficient conditions (e.g. MYB72, BGLU42);

- Perform a more extensive study of the microbial community associated with soybean plants, looking at the total plant microbiome of different edaphoclimatic conditions, and focusing on the important role of Rhizobia;

- Perform additional studies to test the robustness of the selected inoculants under field conditions where heterogeneity prevails regarding soil composition, environmental conditions, and other biological factors;

- Test the applicability of the selected inoculants to other important legume crops to combat Fe deficiency;

- Develop a sustainable commercial formulation for improved soybean growth that can be a complement or an alternative to the currently available strategies.

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