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PRODUCTION OF SIDEROPHORES AND EVALUATION OF ITS SUITABILITY FOR APPLICATION ON THE CORRECTION OF IRON DEFICIENCIES IN AGRONOMIC CONDITIONS

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Abstract

Iron (Fe) is an essential element for the proper development of life. For example, plants require Fe for chlorophyll synthesis. However, due to the lack of Fe, plants grown in alkaline calcareous soils are very susceptible to a symptomatic array named iron-deficiency induced chlorosis (IDIC). The inappropriate chlorophyll production results in the reduction of crop yields worldwide with major implications in many agricultural regions worldwide.

Organic chelating agents, namely aminopolycarboxylic acids (APCAs), are adequate for IDIC amending. However, the low biodegradability of such compounds raises several environmental concerns: therefore, the replacement of APCA by new, more environment-friendly alternatives is needed.

It is known that plant-growth promoting rhizobacteria (PGPR) are capable of producing siderophores: low weight molecules (between 500 to 1500 dalton) with great affinity and selectivity to bind and complex Fe(III). In this study, we evaluated the capacity of several different PGPR strains for their biotechnological production of siderophores. We also evaluated the resulting siderophore-rich filtrates for their Fe(III)-complexation capacity, for their interactions with soil (and soil components) and finally their potential for amending IDIC in chlorotic soybean plants grown in calcareous soil.

Firstly, a review of the literature on PGPR was conducted to list the genera, that, in our understanding, were the most suitable to be applied in agronomic conditions for IDIC amendment. Siderophore production and type of

siderophore produced were considered. From this review, *Azotobacter*, *Bacillus*, *Pantoea*, and *Rhizobium* genera were selected.

Secondly, the performance of *Azotobacter vinelandii* Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) 2289; *Bacillus megaterium* American Type Culture Collection (ATCC) 19213; *Bacillus subtilis* DSM 10; *Pantoea allii* DSM 25133; and *Rhyzobium radiobacter* DSM 30205 was evaluated, regarding siderophore production kinetics, level of siderophore production (determined by chrome azurol S, CAS method), type siderophore produced (using Arnow and Csaky's tests) and iron-chelating capacity at pH 9.0. All bacteria were in stationary phase at 24 h, except *A. vinelandii* (at 72 h) and produced the maximum siderophore amount (80 - 140 $\mu mol.L^{-1}$) between 24 - 48 h, with the exception of *A. vinelandii* (at 72 h). The highest iron-chelating capacity, at pH 9.0, was obtained by *B. megaterium* followed by *B. subtilis*, and *A. vinelandii.* Therefore, these three bacterial strains were considered the most promising ones for siderophore production and chlorosis correction under alkaline conditions.

The three selected bacterial filtrates were subjected to tests on Fe chelate stability, in solution and in wet soil conditions. Results have shown that *A. vinelandii* iron fertilizer was more stable and interacted less with calcareous soils and its components than *B. subtilis* and *B. megaterium*. In fact, in face of the inadequate Fe(III) levels found for *B. megaterium,* only *A. vinelandii* and *B. subtilis* were tested in pot experiments. Freeze-dried preparations of *A. vinelandii* and *B. subtilis* filtrate were prepared to be used in plant pot experiments. Plants treated with *A. vinelandii* fertilizer responded more significantly and comparable to the positive control, ethylenediaminedi(o-

hydroxyphenylacetic) acid (*o,o*-EDDHA), than those treated with *B. subtilis* one, when evaluated by their growth (dry mass) and chlorophyll development (SPAD index). On average, iron content was also higher on *A. vinelandii* treated plants than in *B. subtilis* treated ones. These results suggest that the freeze-dried product prepared from *A. vinelandii* culture can be a viable alternative for mending IDIC of soybean plants grown in calcareous soils.

Finally, to elucidate the biodegradability of the siderophores that might be present in *A. vinelandii* and *B. subtilis* filtrates, a comprehensive analysis of the literature and metabolic databases was undertaken. Complementary, biodegradation estimation models, were also ran. For all siderophores a complete degradation (or partial in case of vibrioferrin and azotobactin) pathway was found. The metabolites resulting from the predicted degradation pathways are relevant biological molecules that may be further processed by microorganism. This provides an advantage to those to perform siderophore degradation. Biodegradation estimation models place the biodegradation of the most relevant siderophores on the “days” time-frame. This time frame is suitable for the purpose, as it grants siderophores have enough time to exert their positive influence on plants, without the risks associated with their persistency.

From the initially selected lot of bacterial strains, only *A. vinelandii* has shown great potential. Its filtrate composition has shown good siderophore production, good Fe complexation capability and easiness of production. Furthermore, its Fe solution has shown the lowest interaction with soils and soil components and was able to maintain adequate Fe levels in solution. When tested on chlorotic plants grown in calcareous soil, an overall improvement in comparison to the negative control was observed. The freeze-dried formulation

was also successful in improving the logistical handling of the filtrate as well as shelf life storage, without compromising the original properties.

Resumo

O ferro (Fe) é um elemento essencial para o desenvolvimento adequado da vida. Por exemplo, as plantas requerem Fe para a síntese de clorofila. No entanto, devido à falta de Fe, plantas cultivadas em solos calcários alcalinos são muito suscetíveis a um quadro sintomático chamado clorose induzida por deficiência de ferro (CIDF). Da produção inadequada de clorofila resulta a redução do rendimento das culturas a nível mundial, com importantes implicações em muitas regiões agrícolas em todo o mundo.

Agentes quelantes orgânicos, em particular os ácidos aminopolicarboxílicos (AAPC), são adequados para a alteração da CIDF. No entanto, a baixa biodegradabilidade destes compostos gera algumas preocupações ambientais: logo, a substituição dos AAPC por novas alternativas mais amigas do ambiente é necessária.

Sabe-se que as rizobactérias promotoras do crescimento vegetal (RPCV) são capazes de produzir sideróforos: moléculas de baixo peso molecular (entre 500 e 1500 dalton) com grande afinidade e seletividade para se ligar e complexar Fe(III). Neste estudo, avaliamos a capacidade de diversas linhagens de RPCV para a produção biotecnológica de sideróforos. Também avaliamos os filtrados bacterianos ricos em sideróforos pela sua capacidade de complexação de Fe(III), pelas suas interações com o solo (e componentes do solo) e, finalmente, pelo seu potencial para corrigir a CIDF em soja clorótica cultivada em solo calcário.

Inicialmente, foi realizada uma revisão da literatura sobre RPCV para selecionar os gêneros, que, no nosso entendimento, seriam os mais adequados para serem aplicados em condições agronômicas para a correção da CIDF. A produção de sideróforos e o tipo de sideróforos produzidos foram considerados. A partir desta revisão, os gêneros *Azotobacter*, *Bacillus*, *Pantoea* e *Rhizobium* foram selecionados.

Em segundo lugar, o desempenho de *Azotobacter vinelandii* Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) 2289; *Bacillus megaterium* American Type Culture Collection (ATCC) 19213; *Bacillus subtilis* DSM 10; *Pantoea allii* DSM 25133; e *Rhizobium radiobacter* DSM 30205, quanto à cinética de produção de sideróforos, nível de produção de sideróforos (determinado pelo método chrome azurol S, CAS), tipo sideróforo produzido (testes de Arnow e Csaky) e quelante de ferro a pH 9,0, foi avaliado. Todas as bactérias estavam em fase estacionária às 24 h, exceto *A. vinelandii* (às 72 h) e produziram a quantidade máxima de sideróforo (80 - 140 µmol.L-1) entre as 24 - 48 h, com exceção da *A. vinelandii* (em 72 h). A maior capacidade quelante de ferro, a pH 9,0, foi obtida pela *B. megaterium* seguida da *B. subtilis* e da *A. vinelandii*. Portanto, estas três bactérias são as bactérias mais promissoras para a produção de sideróforos e correção da clorose em condições alcalinas.

Os três filtrados bacterianos selecionados foram submetidos a testes de estabilidade do quelato de Fe em solução e em condições de solo húmido. Os resultados mostraram que o fertilizante de ferro da *A. vinelandii* foi mais o estável e interagiu menos com os solos calcários e seus componentes do que o da *B. subtilis* e da *B. megaterium*. De facto, os resultados de Fe(III) solúvel da *B. megaterium* foram inadequados, e apenas a *A. vinelandii* e a *B. subtilis*

foram testados em testes em vasos. Composições liofilizadas dos filtrados de *A. vinelandii* e *B. subtilis* foram preparadas para serem utilizadas nas experiências das plantas em vasos. Plantas tratadas com o fertilizante da *A. vinelandii* responderam de uma forma mais significativa e comparável ao controlo positivo, o ethylenediaminedi(o-hydroxyphenylacetic) acid (*o,o*-EDDHA), do que aquelas tratadas com *B. subtilis*, quando avaliado o seu crescimento (massa seca) e desenvolvimento de clorofila (índice SPAD). Em média, o teor de ferro também foi maior nas plantas tratadas com *A. vinelandii* do que nas tratadas com *B. subtilis*. Estes resultados sugerem que o produto liofilizado preparado a partir da cultura de *A. vinelandii* pode ser uma alternativa viável para a correção de CIDF de plantas de soja cultivadas em solos calcários.

Por fim, a fim de elucidar a biodegradabilidade dos sideróforos que podem estar presentes nos filtrados de *A. vinelandii* e *B. subtilis*, foi realizada uma análise abrangente da literatura e das bases de dados metabólicas. Modelos complementares de estimação da biodegradação também foram usados. Para todos os sideróforos foi encontrada uma forma de completa degradação (ou parcial no caso da vibrioferrina e azotobactina). Os metabolitos resultantes da degradação prevista são moléculas biologicamente relevantes que podem ser posteriormente processadas pelos microrganismos. Isto confere uma vantagem a aqueles que realizem a degradação dos sideróforos. Os modelos de biodegradação estimam que a biodegradação dos sideróforos mais relevantes ocorra numa janela de tempo de “dias”. Este arco de tempo é adequado para o propósito desejado, pois garante tempo suficiente para os sideróforos

exercerem a sua influência positiva nas plantas, sem os riscos associados com a sua persistência.

Do conjunto inicialmente selecionado de estirpes bacterianas, apenas a *A. vinelandii* mostrou grande potencial. Demonstrou uma boa produção de sideróforos, o seu filtrado uma boa capacidade de complexação de Fe e facilidade de produção. Além disso, a sua solução de Fe demonstrou a menor interação com solos e componentes do solo e foi capaz de manter níveis adequados de Fe em solução. Quando testado em plantas cloróticas cultivadas em solo calcário, foi observada uma melhoria global em comparação com o controlo negativo. A formulação liofilizada também foi bem sucedida na melhoria do manuseamento logístico do filtrado, bem como no aumento de tempo de vida útil de armazenamento, sem comprometer as propriedades originais.

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List of abbreviations and symbols

A: Absorbance at 630 nm

AAS-FA: Atomic Absorption Spectroscopy with Flame Atomization

ACC: AminoCyclopropane-1-Carboxylate

ACCD: AminoCyclopropane-1-Carboxylate Deaminase

APCA: AminoPolyCarboxylic Acid

A_{ref}: Absorbance of reference solution at 630 nm

ATCC: American Type Culture Collection

BM: Burk´s Medium

CAS: Chrome Azurol S

DAPI: 4,6-DiAmidino-2-PhenylIndole

DHBA: DiHydroxyBenzoic Acid

DPH: N,N-dihydroxy-N,N′-DiisoProylHexanediamide

DSMZ: Deutsche Sammlung von Mikroorganismen und Zellkulturen

DTPA: DiethyleneTriaminePentaacetic Acid

EDDCHA: EthyleneDiamineDi(5-Carboxy-2-Hydroxyphenylacetic) Acid

EDDHA: EthyleneDiamineDi(o-Hydroxyphenylacetic) Acid

EDDHMA: EthyleneDiamineDi(*o*-Hydroxy-*p*-Methylphenylacetic) Acid

EDDHSA: EthyleneDiamineDi(2-Hydroxy-5-Sulfophenylacetic) Acid

EDDS: EthyleneDiamineDiSuccinate

EDTA: EthyleneDiamineTetraacetic Acid

EPI: Estimation Programs Interface

Fe: Iron

[Fe]: Concentration of Fe

HBED: *N,N′*-bis(2-hydroxybenzyl)ethylenediaminediacetic acid

HEDTA: N-(Hydroxyethyl)EthyleneDiamineTriacetic Acid

HJB: *N,N′*-bis(2-hydroxy-5-methylphenyl)ethylediaminediacetic acid

IAA: Indole-3-Acetic Acid

IARI: Indian Agricultural Research Institute

IDIC: Iron Deficiency-Induced Chlorosis

KEGG: Kyoto Encyclopedia of Genes and Genomes

MM: Minimal Medium

MW: Molecular Weight

NCIM: National Collection of Industrial Microorganisms

[L]: Concentration of a complexing ligand

OD_{600}: Optic Density measured at 600 nm

PBS: Phosphate-Buffered Saline

PGPB: Plant-Growth Promoting Bacteria

PGPR: Plant-Growth Promoting Rhizobacteria

pH: Logarithmic concentration of H^+

TRBA: Technical Rules for Biological Agents

[Sid]: Concentration of Siderophore in filtrate

Chapter 1

Introduction

1. Understanding the issue

Iron (Fe) is an essential element for the proper development of life. For example, plants require Fe for numerous metabolic pathways (Barker and Stratton, 2015); one example is chlorophyll synthesis (Hansen et al., 2006). However, albeit present in appreciable quantities, Fe is not readily bioavailable in soils. Most Fe is found in the ferric form [Fe(III)], in a range of different minerals, which, under neutral pH are very insoluble. This results in concentrations of soluble Fe ranging as low as 10^{-10} $mol.L^{-1}$ (Colombo et al., 2014; Kaplan and Ward, 2013). Although acidification of the rhizosphere may help in certain circumstances to improve Fe uptake by plants, buffered calcareous soils render this method impractical (Lucena, 2000; Shenker and Chen, 2005). For this reason, plants grown in alkaline calcareous soils are very susceptible to a symptomatic array named chlorosis, sometimes referred as iron deficiency-induced chlorosis (IDIC), or even lime-induced chlorosis. This symptomatic array is characterized by the development of new yellow leaves, consequence of inappropriate chlorophyll production (Lucena, 2000). This inappropriate chlorophyll production results in crop yields losses worldwide with major implications in many agricultural regions (Hansen et al., 2006).

The challenge to overcome IDIC in agriculture and to increase crop yield is of great importance and interest. Organic chelating agents, namely aminopolycarboxylic acids (APCAs), which have high Fe complex stability, are adequate for soil application and IDIC amending. However, the low biodegradability of such compounds raises several environmental concerns (Bucheli-Witschel and Egli, 2001). Adverse effects on the wastewater treatment procedures in the treatment plants, toxic effects on aquatic and mammalian

organism, contribution to eutrophication of water bodies and mobilization of heavy metals are the main concerns regarding APCA use (Bucheli-Witschel and Egli, 2001). Therefore, the replacement of APCA for new, more environmentally friendly alternatives is needed.

2. Our proposal

If we look for environmentally friendly solutions, our best inspiration should be nature. In order to survive, natural systems developed ways to deal with the limited Fe supply. Such is the case of plant-growth promoting rhizobacteria (PGPR). PGPR, as named by Kloepper and Schorth (1978), are bacterial strains that live and interact in plants rhizosphere and promote their host plant growth (Bhattacharyya and Jha, 2012; Chauhan et al., 2015). The way how PGPR exert their positive contribution to plant growth is diverse. They may consist in: (i) mechanisms of supply of nutrients to the plant, either by nitrogen fixation, siderophore production, or organic matter and phosphate solubilization; (ii) the production of growth inducting phytohormones; (iii) removal of phytotoxic substances; (iv) competition with pathogenic organism; and (v) stimulation of mycorrhizae growth (Bhattacharyya and Jha, 2012; Jyoti Das et al., 2013).

Of relevance to our issue is the production of siderophores. Siderophores are low weight molecules (between 500 to 1500 dalton) with great affinity and selectivity to bind and complex Fe(III) (Hider and Kong, 2010). Being of natural source, they are expected to be more biodegradable than current APCA in use, while at the same time, being capable to complex and maintain Fe soluble and bioavailable. However, most siderophores have high structural complexity

(Hider and Kong, 2010). Due to this complexity, chemical synthesis would require several low yields steps. For this reason, chemical synthesis is not economically viable for agricultural purposes. Alternatively, siderophores can be produced by means of biotechnological processes. These would, in theory, reduce the cost of production, expand the range of options (as different PGPR produce different siderophores), and remove the insertion of bacteria into the environment and from human contact in the field. To further reduce costs and maximize yield, no isolation and purification steps were planned, and the direct bacterial broth were used.

3. Objectives

To achieve our proposed goals, several objectives were stablished. Firstly, a thorough analysis of the literature was conducted for the selection of the most suitable bacterial species. This deliberation had as basis the bacteria capability to produced siderophores, the type of siderophore produced by the bacteria, the risk group of the bacteria (only the lowest risk bacteria were selected), and the growth conditions of the bacteria.

Secondly, once bacteria were chosen, bacteria were monitored for their growth and siderophore production, and the bacterial media filtrates were tested for their capacity to complex Fe in alkaline conditions. Siderophore typology was also determined and compared to that described in literature. Given that the direct application of the bacterial media filtrate is not reasonable, due to logistical and product conservation issues, lyophilization of the bacterial media filtrate was optimized and verified to not impact its Fe complexation properties.

The formulation was to be tested for its suitability for agronomic conditions, that is, capacity to retain Fe in solution without being displaced or retained by soils constituents.

Thirdly, the capability of the formulation to mend IDIC in calcareous soils was assessed, using chlorotic soybean plants as biological model. And finally, to assess, by means of computational simulation and metabolic pathways analysis, the expected biodegradability of the main siderophores described in literature for the studied bacteria.

4. Thesis outline

The aforementioned work is organized in the present document divided by chapters. In the following (second) chapter, a comprehensive review of the state of the art is presented to the reader. In the third chapter, data regarding selected bacteria monitorized is presented and discussed, as well as results for siderophore quantitative and qualitative determination. The complexation capacity of each bacterium media is also presented and discussed. In the fourth chapter, data for the optimization of lyophilization process, soil interactions and biological assays is presented and discussed. The fifth chapter encompasses the discussion of the reviewed metabolic pathways that may be responsible for the biodegradation of the main siderophores present in media, as well as the results and discussion of computational data. Finally, in the sixth and last chapter, the main conclusions are drawn and discussed, and the future outcomes of the present work are discussed.

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Chapter 2

The state of art*

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1. Introduction

World population is ever increasing and currently there are around 7.7 billion people on planet Earth with current estimations placing a worldwide population of 10 billion by 2055 (Worldometers, 2018). This ever increasing number puts Earth and its limited resources on stress. Arable fertile land is one of such resources. As the population grows, more land is needed to supply enough food for all people. An alternative to progressively avoid damaging larger areas of wild land, is to achieve increasing means of production.

A worldwide agricultural problem, with major implications in crop production, is plant iron (Fe) deficiency. Fe bioavailability is particularly low in calcareous soils (Hansen et al., 2006). Since it is estimated that calcareous soils cover about 30 % of world´s cultivated soils, plant Fe deficiency arises as a major agricultural worldwide problem (Barker and Stratton, 2015; Hansen et al., 2006). The most common practice to overcome this problem is the application of synthetic Fe chelates (Nadal et al., 2012), mostly aminopolycarboxylate acids (APCAs), such as ethylenediaminetetraacetic acid (EDTA) or ethylenediaminedi(o-hydroxyphenylacetic) acid (EDDHA). Among APCAs, EDDHA/Fe(III) chelate is the most effective Fe fertilizer in neutral and alkaline soils (Alcañiz et al., 2017). In general, these compounds are strong chelating agents but usually they are not biodegradable (Bucheli-Witschel and Egli, 2001). Consequently, their accumulation in the environment is becoming a matter of great concern (Bucheli-Witschel and Egli, 2001).

Therefore, the search for a better, alternative, environmental-friendly and cheap means for the increase of food production or to solve specific agricultural

problems (such as Fe-fertilizers, composed by suitable Fe chelators) are of utter importance and became a great challenge. Such alternatives would replace non-green or unsustainable means, such as chemical fertilization. The use of natural compounds, produced for instance by plant-growth promoting bacteria (PGPB), can be regarded as an alternative proposal for a more sustainable and environmental-friendly development of agricultural.

The objective of this chapter is to perform a comprehensive review of the potential application of bacteria, in sustainable agriculture, crossing, at the same time, relevant information regarding their safety. Thus, the most promising PGPB genera, for agronomic practices, are presented, in respect to their siderophore production capacity. In addition, examples of studies using these bacteria in the promotion of plant growth and biosafety aspects linked with their use in an agronomic context are critically reviewed.

2. Agriculture challenges

2.1. Chlorosis in plants: an increasing issue

2.1.1. Iron function in plants

It is generally recognized that Fe is an essential nutrient for plant development at the same time as the macronutrients (Barker and Stratton, 2015). Although not present in chlorophyll, Fe is necessary for their synthesis and for the functioning of the photosynthetic apparatus (Barker and Stratton, 2015; Broadley et al., 2012). In addition, non-heme Fe-S proteins (such as ferredoxin) and heme proteins, such as several enzymes (catalases and

peroxidases) and cytochromes (which participate in the electron transport in photosynthesis, in chloroplasts, and in oxidative phosphorylation, in mitochondria) require the presence of Fe to fulfil their functions in plant metabolism (Broadley et al., 2012).

2.1.2. Iron deficiency in plants: chlorosis

The lack of Fe in plants leads to a symptomatic array named iron-deficiency induced chlorosis (IDIC), which is characterized by the appearance of yellow young leaves as a consequence of the inefficient chlorophyll production (Lucena, 2000). Plants under Fe deficiency present chloroplasts with marked changes in their ultrastructure, being smaller under extreme Fe deficiency (Broadley et al., 2012).

IDIC is a problem in agriculture as it can significantly reduce crop yields. Chlorotic plants will develop less, produce less biomass and yield less flowers and fruits, or even lead to complete crop failure (Guerinot and Yi, 1994). Some crops known to be susceptible to IDIC are peach, kiwifruit, citrus and pear (Lucena, 2003), but some other valuable crops have also been described to suffer from IDIC, such as rice, soybean, cotton, peanut (Hansen et al., 2006), tomato (Chaney et al., 2008) and pea (Kabir et al., 2016). Furthermore, Fe shortage leads to loss of nutrient value on harvested crops. For example, on developing countries, the major Fe source are vegetables (Barton and Abadia, 2006). Furthermore, Fe deficiency may hinder the production of proteins (Broadley et al., 2012), reducing the nutritional value of food. Therefore, insufficient Fe in crops may have detrimental effects on animal and human

health. This further reinforces the need to solve the issue of Fe nutrition of plants, especially in susceptible crops.

The low bioavailability of Fe is a result of its chemical nature and its low solubility and dissolution kinetics. Although generally present in high quantities in soil, in well drained soils most Fe is found as crystalline Fe (hydro)oxides, such as, goethite and hematite (Colombo et al., 2014). These minerals have different solubilities. Yet, under aerated conditions and pH values above 7, these species are highly insoluble, and the free Fe concentration can be as low as 10^{-10} mol.L^{-1} (García-Marco et al., 2006), whereas the required optimal concentration for plant development is about 10^{-7} to 10^{-8} mol.L^{-1} (Lindsay and Schwab, 1982). This is further aggravated in Fe-minerals presenting a low surface area, especially in well crystalized minerals. This limits the absorption of Fe by plants, especially in alkaline calcareous soils. In plants, the effects of bicarbonate on Fe transport may also limit the distribution and availability of Fe on leaves (Mengel, 1994). The presence of nitrates can also interfere in the pH and redox balance. Nitrate uptake system of plants is normally mediated by a proton cotransport system, which can increase local rhizosphere pH. Also, for strategy I plants, root reductases are speculated to reduce nitrate to nitrite in favour of Fe(III) to Fe(II), compromising Fe uptake by the root (Lucena, 2000). Finally, heavy metals in soil can also disrupt the Fe uptake system on roots (Bashmakova et al., 2015). The main factors that influence the Fe availability and uptake by plants are summarized in Table 1.

Table 1. Factors affecting Fe availability and uptake to plants.

Causes of low Fe availability	Causes for low plant uptake
High pH, due to carbonates in soil (pH>7 is critical)	Bicarbonate buffering
Types of minerals	Presence of nitrates affects pH and redox state
Low Fe mineral surface	Presence and competition of heavy metals
Absence or inefficiency of Fe carriers	

2.1.3. The use of iron fertilizers

2.1.3.1. Drawbacks of the use of inorganic and organic (synthetic) compounds

The challenge to overcome IDIC in agriculture and to increase crop yield has been of great importance and interest. The first response to the problem was the use of inorganic Fe fertilizers, such as $FeSO_4$. However, the supply of Fe in this manner is inconsequent because Fe readily precipitates due to the buffering capacity of carbonate/bicarbonates present in some soils (Lucena, 2003). Thus, organic chelating agents are more adequate for this purpose. While some organic acids, such as malic, citric and oxalic acids (Figure 1), are suitable for foliar application or hydroponics (a method of growing plants using mineral nutrient aqueous solution instead of soil) where chlorosis is not severe, they are ineffective in providing Fe to plants in alkaline calcareous soils (Lucena, 2003). Another approach consists in the use of synthetic organic chelating agents, namely APCAs, which have high Fe complex stability and are more adequate for soil application (Bucheli-Witschel and Egli, 2001). APCAs

are compounds containing one or more nitrogen atoms to which carboxyl groups are linked (Figure 1). Examples of synthetic APCAs are: diethylenetriaminepentaacetic acid (DTPA), EDDHA, ethylenediaminedi(*o*-hydroxy-*p*-methylphenylacetic) acid (EDDHMA), ethylenediaminedi(2-hydroxy-5-sulfophenylacetic) acid (EDDHSA), ethylenediaminedi(5-carboxy-2-hydroxyphenylacetic) acid (EDDCHA), EDTA and N-(hydroxyethyl)ethylenediaminetriacetic acid (HEDTA) (Figure 1). The use of these compounds is regulated in the EU (European Union, 2003).

However, several issues arise from the use of such synthetic chelates in the agriculture. Firstly, the synthesis of EDDHA, which is one of the most commonly used synthetic chelating agent, originates a mixture of regioisomers, *o,o*-EDDHA, *o,p*-EDDHA, and *p,p*-EDDHA, with the two last isomers having lower complexing capability and therefore reduced value and efficiency as Fe fertilizer (Hernández-Apaolaza et al., 2006). Similar issues related to impurities in EDDHMA, EDDHSA and EDDCHA production were also found (Alvarez-Fernández et al., 2002). EDTA, although widely used in agriculture, has shown lower performances compared to EDDHA due to its lower chelate stability constants (Ferreira et al., 2019; López-Rayo et al., 2019). New APCA agents, such as *N,N′*-bis(2-hydroxy-5-methylphenyl)ethylediamine-*N,N′*-diacetic acid (HJB) (López-Rayo et al., 2009) or *N,N′*-bis(2-hydroxybenzyl)ethylenediamine-*N,N′*-diacetic acid (HBED) (Nadal et al., 2012) have been developed to cope with the issue of racemic mixtures of EDDHA and related chelating agents, since their synthesis yields purer compounds than the previous ones.

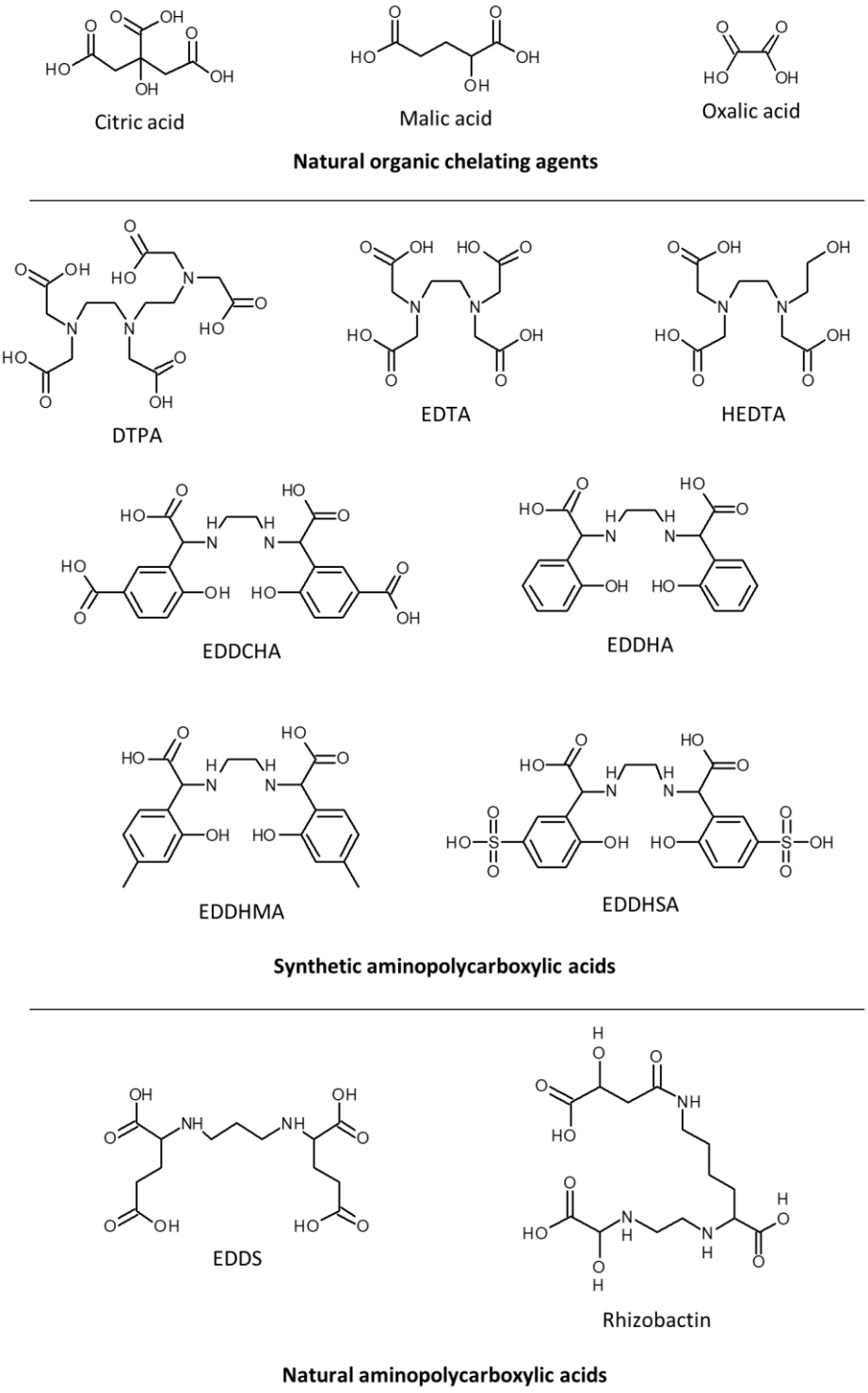


Figure 1. Examples of natural and synthetic chelating agents used to correct Fe chlorosis.

Another important problem associated with the extensive use of APCAs is related to their environmental impact. In fact, synthetic APCAs are not

biodegradable or poorly biodegradable (Bucheli-Witschel and Egli, 2001). For example, EDTA has been described as poorly biodegradable (Oviedo and Rodríguez, 2003), and its persistence and translocation in soil has been described (Bloem et al., 2017). As consequence of the limited biodegradability of APCAs, questions regarding its possible toxic effects on aquatic organisms and in mammalians, as result of the mobilization of heavy metals, have been raised (Bucheli-Witschel and Egli, 2001).

2.1.3.2. Use of APCAs of biological origin as alternative

It is important to underline that a chelating agent must have some degree of persistence in soil in order to achieve its goal: efficient supply of Fe for correction of chlorosis in plants. However, regarding to the biodegradability of the chelating agent, a balance should be achieved in order to avoid either an inefficient supply of Fe (in the case of a fast biodegradability) or a possible toxic effect due to the leaching of heavy metals (if not biodegradable).

In this context, new alternatives to synthetic APCAs should be sought, in an attempt to find even better candidates for being used as biodegradable Fe fertilizers in agricultural practices. As possible candidates can be the APCAs of biological origin (natural occurring APCAs), such as ethylenediaminedisuccinate (EDDS), produced by *Amycolatopsis orientalis* or rhizobactin, produced by *Rhizobium meliloti* (Figure 1) (Bucheli-Witschel and Egli, 2001). The latter compound is classified as siderophore (please see siderophore definition in section 3.3.2.1), which present a better biodegradability comparing with the most synthetic APCAs (Fazary et al., 2016).

3. Plant-growth promoting rhizobacteria

3.1. Definition

Plant growth promoting bacteria (PGPB), consists in an large group of microorganisms that can be found in the rhizosphere (and thus also known as plant growth promoting rhizobacteria, PGPR), on the root surface or associated to it; these bacteria live and interact with plants and improve plant growth (Kloepper and Schroth, 1978). Bacteria are an indispensable part of this ecosystem and their presence is beneficial for plant growth (Bhattacharyya and Jha, 2012; Chauhan et al., 2015). Plants often stimulate colonization of bacteria on the rhizosphere by exuding organic compounds that can be used as energy sources for bacteria; therefore, the bacterial population may be as much as one thousand times more dense in the rhizosphere than in the bulk soil (Glick, 2014).

In more recent years, the PGPR classification was refined to include only species that satisfy two of the following three conditions: aggressive colonization, plant growth stimulation and biocontrol of pathogenic organisms (Bhattacharyya and Jha, 2012). PGPR can also be classified as: 1) extracellular-PGPR, which corresponds to bacteria mostly found on the rhizosphere or rhizoplane (surface of plant root that is in contact with soil) and more rarely on the spaces between root cortex and 2) intracellular-PGPR, which is related to bacteria found inside specialized nodules in roots (Jorquera et al., 2010).

3.2. Mechanisms of action

The PGPB can exert their positive input on plant growth by different mechanisms, which can be classified by different ways. One possibility consists on the classification of the bacteria according to their function: biofertilizer, biostimulator or biocontrol (Figure 2). Biofertelizers are a mixture of living microorganisms that when applied to seeds, plants or soil, promote the increase of nutrient supply, such as NH_4^+, SO_4^{2-} or PO_4^{3-}. A biostimulator is a microorganism with the capability to produce phytohormones, such as auxins (plant growth regulators with morphogen-like characteristics, like indole acetic acid, IAA) and cytokinins (substance that promote cell division). A biocontrol microorganism is that which promotes plant growth by controlling pathogenic populations, for instance by producing antibiotics, hydrogen cyanide (HCN) or enzymes with the ability to hydrolyze the fungal cell wall (Bhattacharyya and Jha, 2012).

The mechanisms of action of PGPB can also be classified as being direct or indirect. Direct mechanisms consist on the supply of nutrients to the plant (either by nitrogen fixation or phosphate solubilization) increasing Fe bioavailability (through siderophore production), as well as by production of growth inducting phytohormones, like IAA (Figure 2). Indirect mechanisms include antibiotic production, removal of phytotoxic substances, competition with pathogenic organism and stimulation of mycorrhizae (symbiotic association between fungus and plant roots) growth (Bhattacharyya and Jha, 2012).

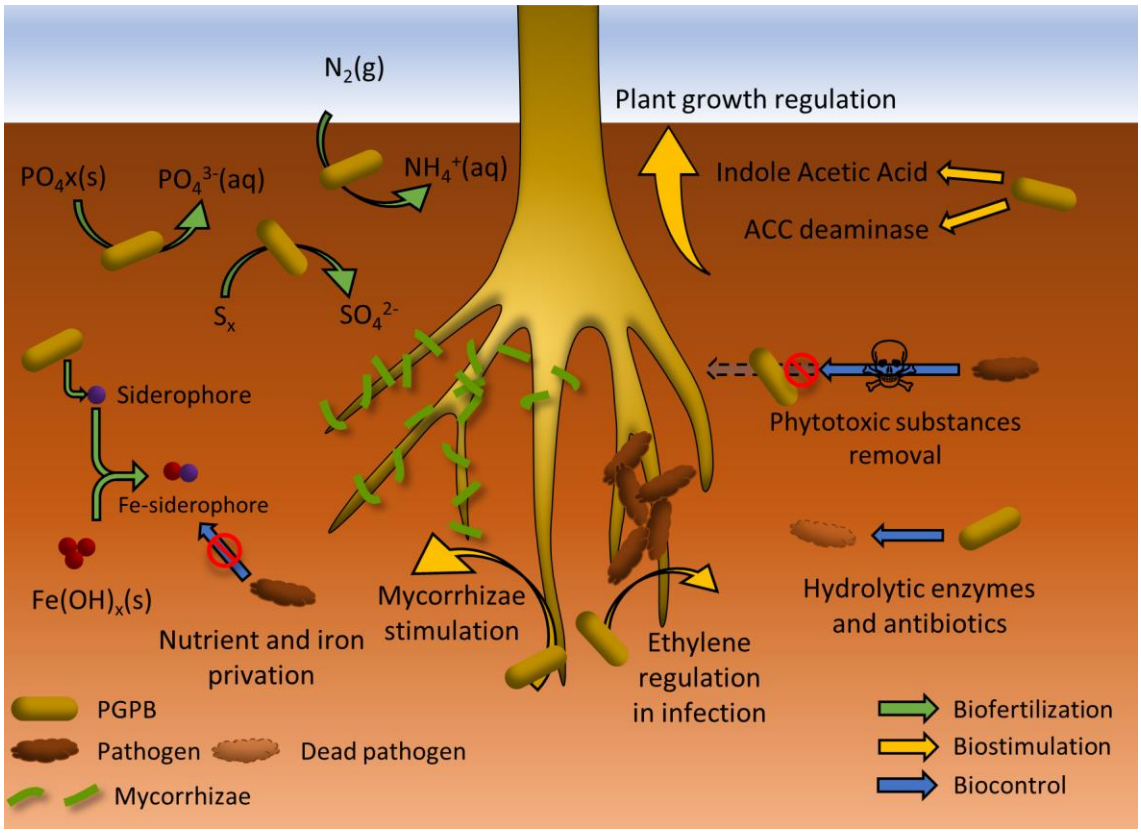


Figure 2. Some mechanisms of action of PGPB in the rhizosphere. Biofertilization mechanisms (green arrows) are those which act at the nutrition level. Biostimulation mechanisms (yellow arrows) act on the plant's regulatory system, by action of phytohormones and signalling components. Biocontrol mechanisms (blue arrows) rely on the control of pathogenic microbial populations by direct and indirect means of action, such as privation of siderophores (nutrient privation).

3.3. Benefits of PGPB

3.3.1. Macronutrients

Plants need an array of elements/nutrients in higher quantity than others, namely carbon, which can be obtained from the atmosphere. Plants also obtain other important macronutrients from the soil such as nitrogen, phosphorus, or sulphur (Figure 2). Regarding soil macronutrients, PGPB should, by definition, be capable of supplying host plants with additional nutrients or facilitate the acquisition of existing ones. For example, the most common case is the

nitrogen fixating bacteria, known to be symbiotic with plants in fixing and supplying nitrogen to plants. Genera, such as *Azotobacter* (Kraepiel et al., 2009), *Azospirillum* (Steenhoudt and Vanderleyden, 2000), *Azorhizobium* and *Rhizobium* (Cocking, 2003), among others, are known to help plant growth by nitrogen fixation (conversion of atmospheric nitrogen to ammonium) (Figure 2).

Phosphorus is found in soil, as phosphates, but usually it is found in insoluble forms and, thus, not bioavailable for plants. Some bacteria have the ability of dissolving inorganic phosphates, therefore helping plants to cope their phosphorus needs (Figure 2). Such bacteria comprise genera, such as *Azotobacter* (Kannapiran and Ramkumar, 2011), *Burkholderia*, *Enterobacter*, *Serratia* (Mamta et al., 2010) and *Pantoea* (Sulbarán et al., 2009).

Reduced inorganic sulfur compounds can be oxidized to sulfate by some bacteria genera like *Aquaspirillum, Bacillus*, *Pseudomonas*, *Thiobacillus* or *Xanthobacter* (Friedrich et al., 2001). This sulfate can be further absorbed by plants and promote their growth (Figure 2).

3.3.2. Siderophores

3.3.2.1. Definition and function

Siderophores (from the greek *sideros* meaning iron and *phores* meaning bearer) are low weight molecules (between 500 to 1500 Da) that possess a great affinity and selectivity to bind and complex Fe(III) (Ahmed and Holmström, 2014a).

Siderophores are produced by microorganisms, such as bacteria, fungi or cyanobacteria, as well as by some gramineous plants, as part of a strategy to obtain Fe from the environment (Sah and Singh, 2015). As previously discussed

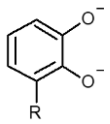
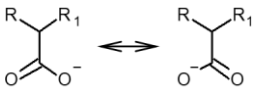
(Fe deficiency in plants: chlorosis; section 2.1.2), Fe(III) is very bio unavailable; in physiological conditions, the dissolved Fe levels can be as low as 10^{-10} mol.L^{-1} (Kraemer, 2004). Microorganisms need an optimal Fe concentration of about 10^{-5} to 10^{-7} mol.L^{-1} (Saha et al., 2016) while for plants, some estimate place optimal Fe concentration at 10^{-9} to 10^{-4} mol.L^{-1} (Guerinot and Yi, 1994) while other authors estimates a value of 10^{-7} to 10^{-8} mol.L^{-1} (Lindsay and Schwab, 1982) for proper plant development.

3.3.2.2. Composition and structure

More than five hundred siderophores have been reported; among them, more than two hundred seventy have been structurally characterized (Barry and Challis, 2009). Despite this great variety, some common moieties are found among siderophores. These compounds usually have negatively charged oxygen atoms with electron charge displaced (the higher the negative charge, the higher the affinity for Fe[III]). In order to favour the more thermodynamically stable high-spin Fe(III) complexes, the most common geometry is octahedral with a six donor atoms arrangement and minimal ligand repulsion (Hider and Kong, 2010).

The most common moieties are oxygen bidentate moieties, namely, catecholate, hydroxamate and carboxylate (Table 2). Others moieties exist, such as oxygen and nitrogen/oxygen-based, but are less frequent among microorganisms (Saha et al., 2016).

Table 2. Main moieties found in siderophores.

Functional moiety	Structure
Catecholate, Catechol	
Hydroxamate, Hydroxamic	
Carboxylate; If R_1= OH; α-Hydroxy-carboxylate	

The catecholate moiety (2,3-dihydroxybenzenyl) has a great affinity to Fe(III) due to its *ortho*-phenolates, with high charge density. This results in the usually high pKa found for siderophores containing catecholate moieties (between 9 and 13) (Hider and Kong, 2010) and also the high complex stability constants with Fe (ranging from 10^{40} to 10^{54}) (Dertz et al., 2006; Robert and Chenu, 1992). Catecholates are produced predominantly by bacteria, such as *Bacillus* spp. (Bharucha et al., 2013; May et al., 2001), *Azospirillum* spp. (Bachhawat and Ghosh, 1987), *Streptomyces* spp. (Fiedler, 2001; Matsuo et al., 2011), *Azotobacter* spp. (Cornish and Page, 1998) or *Escherichia* spp. (Fiedler, 2001). However, no catecholate production was found on both soil and maritime fungi strains (Baakza et al., 2004). The most commonly used method for identifying the catecholate group relies on the Arnow's assay (Arnow, 1937).

On its turn, the hydroxamate moiety (hydroxycarbamoyl) has two mesomeric forms, one of which has a carbonyl oxygen with high charge density.

This can be further enhanced by the presence of side chains through the delocalization and concentration of electron density on the oxygen, which further increases Fe(III) affinity (Hider and Kong, 2010). Siderophores of this kind have stability constants ranging from 10^{22} to 10^{37} (Ahmed and Holmström, 2014a; Saha et al., 2016). Siderophores of the hydroxamate type are most frequent in fungi (Renshaw et al., 2002). This is particularly true for species belonging to the *Ascomycota* phylum that produce only hydroxamates, in opposition to those of the *Zygomycota* phylum, that produce α-hydroxycarboxylate (Baakza et al., 2004). However, some bacteria, such as *Streptomyces* spp. and *Nocardia* spp., produce hydroxamate siderophores (Hider and Kong, 2010). Also, maritime strains from *Vibrio*, *Alteromonas*, *Alcaligenes*, *Pseudomonas*, and *Photobacterium* genera also produce hydroxamate siderophores predominately over catecholate siderophores (Trick, 1989). Different means for the identification of hydroxamate siderophores exist, such as the Csaky's test (Csáky, 1948).

The carboxylate moiety, like the previous groups, also allow for a high-density charge on its oxygen atoms. Their properties can be further enhanced by substitutions on its α-carbon, namely with hydroxyl (α-hydroxycarboxylate) or less frequently with amino (α-aminocarboxylate) (Hider and Kong, 2010; Kraemer, 2004). These substitutions are reflected by the high pKa that can be as high as 9, for the α-aminocarboxylate, or 14, for α-hydroxycarboxylate (Hider and Kong, 2010). Carboxylate complexes of 1:1 coordination have stability constants of about 10^{40} (Robert and Chenu, 1992). These are more commonly found in marine siderophore (Ito and Butler, 2005) although some soil microorganisms may also produce carboxylate siderophores (Drechsel et al.,

1991). Also, plant siderophores (phytosiderophores) are commonly α-hydroxycarboxilate (Harrington et al., 2015) or carboxylates conjugated with amine groups (Ahmed and Holmström, 2014a). The carboxylate type can be identified with the aid of Shenker's spectrophotometric method (Shenker et al., 1992).

In addition to these types, some microorganisms produce siderophores that do not fall into a single type, being a mixture of different types or even of peptide nature, such as siderophores from the pyoverdin family from some *Pseudomonadaceae* such as *Pseudomonas* spp., *Azotobacter* spp. or *Azospirillum* spp. (Bachhawat and Ghosh, 1987; Menhart et al., 1991; Wendenbaum et al., 1983).

Besides the type of donor groups, side chains and complexation energies, other factors are equally important to define the stability strength of the complex. Among them, the denticity (number of donor groups in a single ligand that bind to the central atom) of the chelating agent is of paramount importance, as higher denticity results in a higher stability of the chelate formed. Then, in nature, it is expectable that the most common siderophores are hexadentate (six donor atoms) and can be found in linear or cyclic fashions (Hider and Kong, 2010). The pH is also a determinant factor on the kind of siderophores found. Usually, hydroxamates are more commonly found in neutral to acid environments while catecholates are more commonly found in neutral to alkaline environments (Saha et al., 2013)

Several bacterial genera were described to produce siderophores, such as *Azotobacter* (Baars et al., 2016; McRose et al., 2017; Romero-Perdomo et al.,

2017), *Azospirillum* (Banik et al., 2016), *Bacillus* (Kesaulya et al., 2018; Pourbabaee et al., 2018), *Dickeya* (Sandy and Butler, 2011), *Klebsiella* (Bailey et al., 2018; Zhang et al., 2017), *Nocardia* (Hoshino et al., 2011), *Pantoea* (Burbank et al., 2015; Soutar and Stavrinides, 2018), *Paenibacillus* (Liu et al., 2017), *Pseudomonas* (Baune et al., 2017; Deori et al., 2018; Pourbabaee et al., 2018), *Serratia* (Coulthurst, 2014) and *Streptomyces* (Gáll et al., 2016; Goudjal et al., 2016; Schütze et al., 2014).

3.3.2.3. Stability and environmental fate

In the environment, the majority of siderophore producers are confined to plant rhizosphere (Chauhan et al., 2015). For this reason, the siderophore concentration is maximum in the rhizosphere zone, where concentrations as high as 0.1 $\mu mol.L^{-1}$ and 1 $mmol.L^{-1}$ for bacterial siderophore and plant phytosiderophore, respectively, were reported (Harrington et al., 2015). Conversely, siderophore concentration drops dramatically from rhizosphere to bulk soil, where siderophore concentrations can be as low as 10 $nmol.L^{-1}$ (Ahmed and Holmström, 2014b). However, these values may be underestimated due to sorption phenomena and inadequate extraction and detection protocols for siderophore quantification (Harrington et al., 2015). In fact, in soil, siderophores may adsorb to clay minerals, (oxyhydr)oxides and organic matter (Powell et al., 1982). For example, the desferrioxamine B–$Fe(III)^{+}$ complex has been shown to adsorb strongly with montmorillonite (Siebner-Freibach et al., 2006). However, this may be a positive aspect since the adsorbed Fe(III) complexes may be released over a greater period of time for plant usage (Ahmed and Holmström, 2014b). Similarly, it was demonstrated

that desferrioxamine B adsorbs to Fe -containing kaolinite and facilitates the dissolution of Fe present on the clay-mineral (Rosenberg and Maurice, 2003).

In the environment, other fate for siderophores is its abiotic degradation, which may take place through hydrolyses and/or oxidation processes. The siderophores hydroxamate moieties may suffer hydrolyses and generate hydroxylamine groups; this process is followed by the reduction of Fe(III) to Fe(II) (Simanova et al., 2010). Laboratory studies have shown that hydrolyse products from coprogen (tri-hydroxamate siderophore) were also effective Fe-carriers to cucumber and maize plants (Hördt et al., 2000). All these facts evidence a possible strategy for siderophore utilization where the presence of “sacrificial” moieties may help on the reduction, dissolution and transport of Fe to (micro)organisms (Harrington et al., 2015).

The exposure to sunlight has also influence on the mechanisms of siderophore degradation and mineral dissolution. The type of siderophore as well as the presence of chelated Fe, can induce different behaviours. For example, hydroxycarboxylates, when complexed with Fe are photo-reactive, while catecholates are only photo-reactive when not complexed; hydroxamates are not photo-reactive in both circumstances (Barbeau et al., 2003). The exposure to light also improves the rate of dissolution of some minerals by siderophores. Desferrioxamine B and aerobactin have been shown to dissolve lepidocrocite (an Fe oxide-hydroxide mineral) faster when under UV-light exposure (Borer et al., 2009). Some other non-siderophore compounds carboxylates-containing, such as oxalate and malonate (and their α-hydroxycarboxylate versions), have been shown to be photo-reactive when adsorbed into some Fe minerals, such as hematite (Fe_2O_3) or lepidocrocite, with

possible dissolution of the minerals (Borer and Hug, 2014). Therefore, on its many forms, siderophore degradation, may have a positive influence on the Fe uptake by organisms. This is due to the promotion of dissolution rates of mineral phases, Fe reduction and transport to cells, while part of a dynamic environment of varied strategies for Fe sequestration by organisms.

As for their biotic fate, siderophores interact inadvertently with (micro)organisms, being taken in by bacteria and plants (Figure 2). Some reports also describe the capacity of some bacteria to use siderophore as their source of carbon and nitrogen (Pierwola et al., 2004; Villa et al., 2014). As for plants capability to use siderophores for Fe intake, two strategies exists: The first one relies on the reduction of Fe(III) in the siderophore complex to Fe(II) and transport to the conductor system. For that matter, first the Fe(III)-siderophore complex has to be transported to the aploplast space to be reduced (Mengel, 1994). Once reduced, it may accumulate on the root, which can explain Fe accumulations found in plant roots (Kosegarten et al., 1999). The second is based on siderophores production by microorganisms to dissolve and scavenge Fe in soil minerals and then in the induction of a mass-exchange with their own phytosiderophores for the intake of Fe. This has the disadvantage that phytosiderophores have lower stability constants that those of bacterial siderophores (Ahmed and Holmström, 2014a), and therefore is reliant on the concentration, pH or redox condition for its success (Crowley, 2006).

3.3.3. Phytohormones and ACC deaminase

The plants´ growth is controlled by phytohormones, namely auxins and ethylene, which control and model several aspects on plant, such as cell

expansion and division, cell elongation and differentiation, and a variety of physiological responses (Jiang et al., 2017).

Among auxins, the most common is indole-3-acetic acid (IAA), which is known to be produced not only by plants but also by fungi and bacteria (Duca et al., 2014). Given its importance to the plant, there are several redundant tryptophan dependent metabolic pathways: the indole-3-pyruvic acid, indole-3-acetamide and indole-3-acetonitrile based pathways. These metabolic pathways have been previously described and reviewed by Duca et al. (2014). Besides plants, some bacteria genera are also capable of producing IAA, such as *Agrobacterium* (Rashid et al., 2012; Sitbon et al., 1992), *Azospirillum* (Perrig et al., 2007), *Azotobacter* (Ahmad et al., 2008; Farajzadeh et al., 2012; Torres-Rubio et al., 2000), *Bacillus* (Ahmad et al., 2008; Mehta et al., 2010), *Enterobacter* (Shailesh et al., 2011; Viruel et al., 2011), *Nocardia* (Khamna et al., 2009), *Pantoea* (Shailesh et al., 2011; Viruel et al., 2011), *Rhizobium* (Shailesh et al., 2011; Tan et al., 2014), *Serratia* (Mamta et al., 2010), and *Streptomyces* (Gopalakrishnan et al., 2014). The IAA produced is part of the signaling and communication system between the plant and the bacteria present in the rhizosphere (Spaepen et al., 2007).

The correlation between IAA concentration and plant growth is not linear. Plants have an optimal IAA concentration for their best development (Duca et al., 2014). Excess quantities of IAA may cause pathogenic effects on plants, such as the inhibition of root growth (Duca et al., 2014). Therefore, the use of IAA for plant stimulation has to be carefully regulated to avoid inhibitory effects from over dosage. In this context, plants possess neutralization mechanisms to

control IAA excess, like inactivation of IAA by conjugation with sugars, amino acids or peptides (Sitbon et al., 1992).

Other important plant growth regulator is ethylene, which controls the growth of root, leaves, flowers and fruits (Dubois et al., 2018) as well as the interaction with microorganisms on roots (Gamalero et al., 2008). Ethylene is derived from 1-aminocyclopropane-1-carboxylate (ACC) and is produced in plants as response to multiple stresses. Thus, different stresses such as, metals and chemicals, mechanical damage, water and temperature extremes, induce ethylene biosynthesis through the induction of ACC synthase and other hormones (Dubois et al., 2018). When in excess, ethylene may be toxic to the plant causing defoliation and other harmful cellular effects (Desbrosses et al., 2009).

ACC deaminase (ACCD) is an enzyme produced by some bacteria, such as *Azotobacter* (Dubey et al., 2012; Farajzadeh et al., 2012), *Bacillus* (Belimov et al., 2001) and *Pseudomonas* (Kamran et al., 2016; Sandhya et al., 2010) and is considered as a key trait for PGPB strains (Glick, 2014). ACCD is beneficial for plants as it decreased ACC levels by cleaving it into ammonia and α-ketobutyrate. This helps to regulate ethylene adverse effects by reducing its levels. Therefore, PGPB capable of producing ACCD are beneficial for plants grown in stress conditions such as drought (Sandhya et al., 2010), salt (Mayak et al., 2004) and heavy metals (Belimov et al., 2001) by regulating plant's ACC levels and, thus, of ethylene to non-toxic levels.

3.3.4. Pathogen antagonism

As an indirect method of plant growth promotion, antagonistic properties to plant pathogens are a valuable asset, as they can severely reduce or eliminate the incidence of plant diseases caused by fungi and bacteria. PGPB can achieve these effects by several mechanisms: production of hydrolytic enzymes; competition for nutrients; modulate ethylene levels caused by pathogenic infection; production of siderophores and antibiotics (Figure 2) (Beneduzi et al., 2012). Several genera have been described as having antagonistic behaviour to one or more plant pathogen, such as *Bacillus* (Heidarzadeh and Baghaee-Ravari, 2015), *Azospirillum* (Tortora et al., 2011), *Pseudomonas*, *Coniothyrium*, *Pythium* (Woo and Lorito, 2007), *Serratia*, *Burkholderia* and *Staphylococcus* (Opelt et al., 2007). The use of these microorganisms has shown good results on improving plant growth over time when colonization of rhizosphere is successful.

3.4. Microbiological safety

3.4.1. The need of using safe microorganisms

One of the main problems when working with microorganism is their safety. In fact, the spread of microorganisms is a cause of concern, particularly when they are pathogenic to humans and/or to other organisms, such as animals and plants. Such considerations should also be taken into account when it is intended to use microorganisms for agricultural practices.

The use of microorganisms for agronomic purposes can fall in one of the two categories: 1) production of a compound (for instance, a fertilizer), that can be used in agriculture, by microbial fermentation or 2) bio-inoculants: the

microorganisms (individual strains or microbial consortia) are added directly to the soil or as a seed coating when re-seeding (Owen et al., 2015). In the first scenario, the organisms are confined to the place of production. However, production conditions and appropriate safety measures should be considered in order to reduce risks. The process, which includes the microbial cultivation and downstream processing (microbial harvesting, recovering and product processing), equipment and facilities should be designed in order to avoid or minimize the release of biological agents into work place. In addition, proper treatment and disposal of biological wastes should be taken into account as well as the training of the operators and the implementation of collective and individual protection measures. Even when working with biological agents with lowest risk, i.e, no or low individual and community risk, unlikely to cause human disease (group 1 biological agents; please see below), the procedures of good occupational safety and hygiene should be considered (European Union, 2000). According to EU directive (2000/54/EC), activities in which workers are (or are potentially) exposed to biological agents as a consequence of their work, the replacement of biological agents that pose a risk to human health for other microorganisms which pose no risk is mandatory, whenever possible (European Union, 2000). In the second case (bio-inoculants) the biological agent goes beyond the controlled area of the place of production. In this kind of application, it is very likely that the microorganisms will be set loose on the environment and may disrupt the natural microbial communities. For these reasons, the use of non-pathogenic microorganisms (to humans, animals and plants) is not only advisable but also desirable and even mandatory.

3.4.2. Biosafety classification

Organisms can be classified according to four risk-to-humans groups, as described in the Directive 2000/54/EC (European Union, 2000). Those biological agents unlikely to cause human disease are classified in group 1. If capable of causing disease and be a hazard to the workers, but no risk of spreading to the community, is classified in group 2; if can cause severe human disease or can spread to the community, is classified in group 3. In both these cases (2 and 3) there is usually effective prophylaxis or treatment available. If the biological agent causes severe human disease and is a serious hazard to workers and may present a high risk of spreading to the community and there is usually no effective prophylaxis or treatment available, then it is classified as group 4.

Besides the classification of biological agents taking into account the possible risks to humans, remarks can also be made on possible pathogenicity on vertebrates, invertebrates and plants (Committee on Biological Agents, 2015). The technical rules for biological Agents (TRBA)-466 (Committee on Biological Agents, 2015) are a well-suited tool for assessing the appropriateness of a given bacterium regarding its safety used in agricultural practices. For further crosscheck on risk groups of a given bacterium, it is possible to consult the American Biological Safety Association site (https://my.absa.org/Riskgroups), which hosts most risk classifications for pathogenic species (although non-pathogenic species are not found there) or the Canadian Pathogen Safety Data Sheets (Public Health Agency of Canada, 2018).

Due to the reasons presented above, for agricultural purposes is desirable the use of microorganisms characterized as Risk 1; this requirement is absolutely necessary when biological agents are used directly applied in the soils.

4. Promising bacterial genera for agricultural practices

A thorough review of the literature was conducted for the bacteria genera with plant growth promotion (PGP) properties belonging mostly to risk 1 which also have no or low phytotoxicity. In the following subsections, alphabetically, several genera of bacteria were reviewed taking into consideration all the PGP attributes discussed above (namely, siderophore production, plant nutrient provision, biocontrol properties and auxin production) and biosafety level (risk groups evaluation).

4.1. *Azospirillum*

4.1.1. General genus description

The members of genus *Azospirillum* are free living, but usually found colonizing plant roots, normally gram-negative, typically rod or spiral-shaped bacteria. The species *Azs brasilense* and *Azs lipoferum* were first described (Lin et al., 2015). Subsequently, more species have been isolated and added to *Azospirillum* genus, such as *Azs. amazonense* (Cecagno et al., 2015) and *Azs. melinis* (Peng et al., 2006). *Azospirillum* genus is currently one of the most used PGPB as a result of its known properties for promoting the growth of several cereals, as it is the case of maize (Cassán and Diaz-Zorita, 2016).

4.1.2. Siderophore production

Azospirillum species are capable of producing siderophores. A catechol-type siderophore, named spirilobactin, was identified in Fe-depleted medium of *Azs. brasilense* (Bachhawat and Ghosh, 1987). A strain of *Azs. lipoferum*, was described to produce 2,3-dihydroxybenzoic acid (2,3-DHBA) and 3,5-DHBA conjugated with threonine and lysine (Shah et al., 1992), which are also responsible for siderophore-mediated Mo intake by *Azs. lipoferum* (Saxena et al., 1989). Other *Azospirillum* spp. have been described to produce siderophores (Banik et al., 2016; Sahoo et al., 2014).

4.1.3. Other PGP properties

Azospirillum spp. were reported to produce phytohormones, such as: IAA (Sahoo et al., 2014), kinetin (a type of cytokinin) (Tien et al., 1979), abscisic acid, zeatin (Perrig et al., 2007; Sahoo et al., 2014), ACCD, Gibberellic acid (GA) (Sahoo et al., 2014). *Azospirillum* spp. are also able to fix nitrogen (Bellone et al., 2011; Gadagi et al., 2004), solubilize phosphate (Rodriguez et al., 2004) as well producing HCN (Sahoo et al., 2014) and as of having antagonistic properties towards some plant pathogens, such as *Colletotrichum acutatum* (Tortora et al., 2011).

4.1.4. Applied studies

Field studies with *Azospirillum* species yield, in a general way, good results (Fukami et al., 2016). For this reason, this genus has received a lot of increasing attention. Banik et al. (2016) used new *Azospirillum* strains for improvement of rice production. Inoculation studies with *Azospirillum* spp. were conducted on wheat (Bashan et al., 1990; Noreen and Noreen, 2014), rice

(García de Salamone et al., 2012) and soybean (Bashan et al., 1990). More recently, the interest in *Azospirillum* strains has increased due to positive results for plant growth enhancing in soils under osmotic stress (Fasciglione et al., 2015; García et al., 2017; Gonzalez et al., 2015).

4.1.5. Microbiological safety

All *Azospirillum* spp. belong to risk group 1 (Committee on Biological Agents, 2015). In addition, no adverse effects are predicted to plants, invertebrates or other non-human vertebrates, making *Azospirillum* a presumably safe and eco-friendly genus to use (Committee on Biological Agents, 2015). In fact, some studies have shown that the inoculation of soybean with *Azospirillum brasilense* did not affect native rhizobia population while increasing grain yield (Bellone et al., 2011).

4.2. *Azotobacter*

4.2.1. General genus description

The *Azotobacter* genus is characterized by spherical or ovoid Gram-negative bacteria, usually motile, that can be found in soils, mostly in neutral or alkaline soils, aqueous medium and associated with plants (Voets and Dedeken, 1966). *Azotobacter* strains are also known to form resistant cysts during dormant phases or when exposed to some reagents (Diaz-Barrera and Soto, 2010). This genus is of great importance to the environment due to their great nitrogen fixation capacity, which is achieved as a consequence of the high metabolic rate of the three nitrogenases (McRose et al., 2017).

4.2.2. Siderophore production

Azotobacter vinelandii has the ability to produce several siderophores (Figure 3). Azotobactin, initially called yellow-green fluorescent peptide, was isolated by Bulen & LeComte (1962). Later, Corbin & Bulen isolated 2,3-DHBA and azotochelin, produced by *Azt. vinelandii* under Fe stress (Corbin and Bulen, 1969). In the meanwhile, the structure of azotobactin was characterized by Fukasawa *et al.* (1972), and Fekete *et al.* (1983) concluded that all these three compounds are produced in Fe depleted media and act as siderophores. Page & Tigerstrom (1988) isolated the mono-catecholate aminochelin and, finally, in 1995, protochelin was isolated and characterized. This siderophore is a tri-catecholate, which is believed to be resultant of the condensation of aminochelin and azotochelin (Cornish and Page, 1995). The medium conditions for all siderophore production by *Azt. vinelandii* have been determined. In medium containing Fe(III) concentrations higher than 7 $\mu mol.L^{-1}$, only 2,3-DHBA is produced; when Fe concentrations are below 7 $\mu mol.L^{-1}$, azotochelin and aminochelin are produced; if Fe concentration in medium is under 3 $\mu mol.L^{-1}$, azotobactin is produced; finally, if Fe is limited and Mo concentration is high (> 100 $\mu mol.L^{-1}$), protochelin is produced (Duhme et al., 1998). More recently, in a broad genetic study on *Azt. vinelandii*, vibrioferrin was hinted, opening the possibility that one more siderophore is produced by *Azt. vinelandii* (Baars et al., 2016). Vibrioferrin was later described to be regulated by vanadium and molybdenum levels of culture medium (McRose et al., 2017).

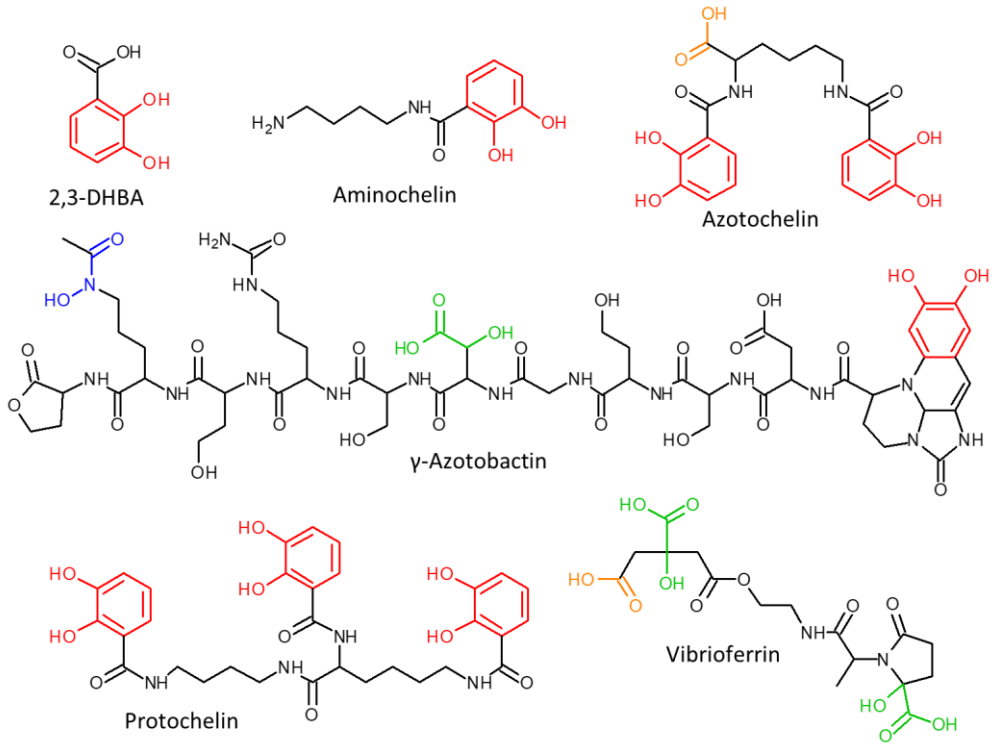


Figure 3. Siderophores produced by Azotobacter vinelandii. Red: catechol moieties; Orange: carboxylate moieties; Green: α-hydroxycarboxylate moieties; Blue: hydroxamate moieties. 2,3-DHBA: 2,3-dihydroxybenzoic acid.

In *Azt. chroococcum*, hydroxamate type siderophore production was described (Suneja et al., 1994), being dependent on the Fe levels of the medium (Page, 1987). It was found an inverse relationship between nitrogen fixation and the (uncharacterized) siderophore production in *Azt. chroococcum;* (Suneja et al., 1996). It was also found that ammonium citrate decreases the production of hydroxamate siderophores on *Azt. chroococcum* while boosting the production of catechol siderophores (Suneja et al., 1996).

Other *Azotobacter* species also produce siderophores (some of which were not yet characterized). For instance, *Azt. paspali* (reclassified as *Azorhizophilus paspali*) produces 3,4-dihydroxybenzoic acid (Collinson et al., 1987), *Azt. salinestris* produces hydroxamate type siderophores (Page and

Shivprasad, 1995) and *Azt. beijerinckii* produces an unidentified siderophore type (Kannapiran and Ramkumar, 2011).

4.2.3. Other PGP properties

Azotobacter spp have the capacity to fix nitrogen, solubilize phosphate, produce HCN and produce IAA (Ponmurugan et al., 2012) and ACCD (Sahoo et al., 2013). *Azotobacter* spp. were also reported as possible biocontrol agents against *Fusarium oxysporum* (Chauhan et al., 2012)*, Rhizoctonia solani* (Fatima et al., 2009) or *Macrophomina phaseolina* (Dubey et al., 2012).

4.2.4. Applied studies

Due to all their plant-growth promoting traits, *Azotobacter* spp. have been applied into plants to evaluate their capabilities to improve crop yields; examples are: cotton (Chauhan et al., 2012; Romero-Perdomo et al., 2017), sesame (Dubey et al., 2012), maize (Shirinbayan et al., 2019), wheat (Mahato and Kafle, 2018), rice (Sahoo et al., 2013), tomato (Chauhan et al., 2012), or maize (Shirinbayan et al., 2019). Growth improvement was measured through the increase in disease resistance (Chauhan et al., 2012; Dubey et al., 2012), plant biomass (Mahato and Kafle, 2018; Romero-Perdomo et al., 2017; Shirinbayan et al., 2019), nitrogen plant concentration (Shirinbayan et al., 2019), Fe concentration (Shirinbayan et al., 2019), grain yield (Dubey et al., 2012; Mahato and Kafle, 2018) and drought resistance (Shirinbayan et al., 2019).

4.2.5. Microbiological safety

All the seven species registered on TRBA 466 are classified as risk group 1 (Committee on Biological Agents, 2015). Therefore, *Azotobacter* is a safe

genus to be manipulated by humans. In addition, no indicated pathogeny to plants or other organisms was described (Committee on Biological Agents, 2015). Piromyou *et al* (2013) have studied the effects of some PGPB inoculants (including *Azotobacter sp.)* in the microbial diversity, and have concluded that the diversity indices were not different between the PGPB-inoculated and un-inoculated.

4.3. *Bacillus*

4.3.1. General genus description

Bacillus genus is constituted by rod shaped, Gram-positive bacteria, which can be either obligate aerobes or facultative aerobes, catalase positive and endospores producer (Turnbull, 1996). It is an omnipresent genus in nature, being found either in soil or water and even in extreme conditions, such as extremely alkaline (*Bcl. alcalophilus*) (Ntougias et al., 2006) or hot environments (*Bcl. thermophilus*) (Yang et al., 2013). Other known species from the genus are *Bcl. subtilis*, a model species broadly studied and described (Graumann, 2012), *Bcl. anthracis*, anthrax agent (Sheff, 2003), *Bcl. thuringiensis*, which is used as a natural insecticide (Sanchis and Bourguet, 2008) and *Bcl. megaterium*, which is amongst the biggest known bacteria; more recently, it became popular due to its recombinant protein production capacity (Bunk et al., 2010).

4.3.2. Siderophore production

It was described the ability of *Bcl. megaterium* to produce schizokinen, a di-hydroxamate, α-hydroxycarboxylate siderophore (Figure 4) (Byers et al., 1967). Later on, the ability of this bacterium to produce a deprotonated siderophore version (N- schizokinen) and respective amine versions (N-

schizokinen-A) (Hu and Boyer, 1995) was also described. In this particular species, siderophore production is strongly correlated with carbon source in medium as well as with shaking and aeration conditions (Santos et al., 2014).

For *Bcl. subtilis*, the production of 2,3-dihydoxylbenzoylglycine, also known as itoic acid (Figure 4), was described by Ito & Neilands (1958) in Fe depleted conditions. Subsequently, the trimeric ester, named bacillibactin (Figure 4) was described (May et al., 2001). The genes responsible for bacillibactin production have also been found in *Bcl. amyloliquefaciens* (Dunlap et al., 2013; Niazi et al., 2014). The production of bacillibactin was also reported in *Bcl. anthracis*, *Bcl. cereus* and *Bcl. thuringiensis* (Wilson et al., 2006). Petrobactin (Figure 4), a di-catecholate and α-hydroxycarboxylate siderophore, is synthetized by *Bcl. anthracis* (Koppisch et al., 2005) as well as by some strains of *Bcl. thuringiensis* (Koppisch et al., 2008), and probably by *Bcl. cereus* (Wilson et al., 2006).

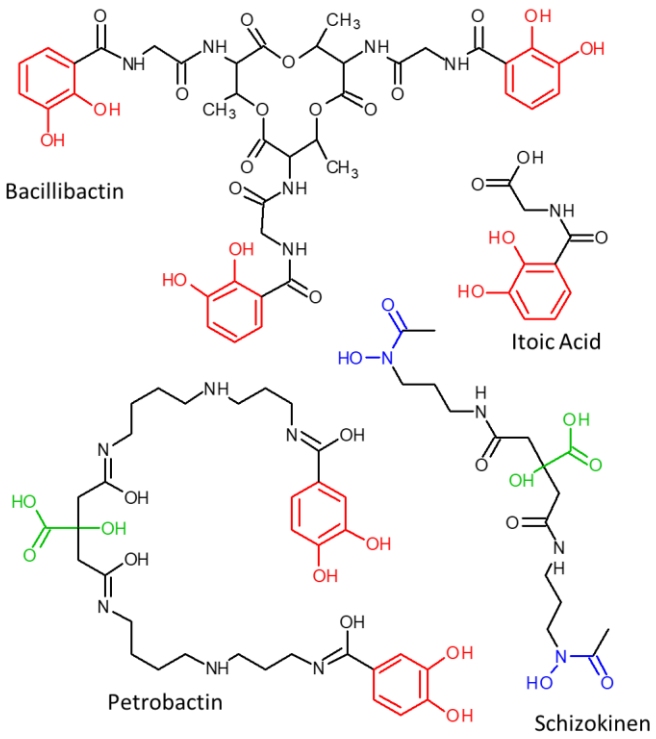


Figure 4. Example of siderophores produced by Bacillus genus. Red: catechol moieties; Green: α-hydroxycarboxylate moieties; Blue: hydroxamate moieties. Other PGP properties.

4.3.3. Other PGP properties

Concerning to plant growth promotion characteristics, several works report different traits in *Bacillus* spp. The production of ACCD, IAA (Kumar et al., 2014), GA (Lenin and Jayanthi, 2012), ammonia (Ahmad et al., 2008) and the capacity to solubilize inorganic phosphates (Pourbabaee et al., 2018) by *Bacillus* spp. has been reported. In addition the production of HCN (Pourbabaee et al., 2018) and biocontrol potential of *Bacillus* spp. against *M. phaseolina* (Kesaulya et al., 2018), *Ralstonia solanacearum (Huang et al., 2016), or Rht. solani* and *F. oxysporum (Kumar et al., 2014)* was described.

4.3.4. Applied studies

Some authors applied *Bacillus* spp. to plants in order to test their magnitude effects on the plant/crop yield. For example, *Bacillus* spp. were applied to banana (Kesaulya et al., 2018), sunflower (Pourbabaee et al., 2018), bean (Sabaté et al., 2017), tobacco (Wu et al., 2016), and tomato (Akram et al., 2015; Heidarzadeh and Baghaee-Ravari, 2015), with positive outcomes, such as: increase of biomass (Akram et al., 2015; Pourbabaee et al., 2018; Sabaté et al., 2017), Fe and chlorophyll concentration (Pourbabaee et al., 2018) and resistance to pathogens (Kesaulya et al., 2018; Sabaté et al., 2017; Wu et al., 2016).

4.3.5. Microbiological safety

According to the TRBA 466, only three *Bacillus* species are not classified in risk group 1: *Bcl. anthracis* (risk group 3), *Bcl. cereus* and *Bcl. weihenstephanensis* (risk group 2) (Committee on Biological Agents, 2015). The most commonly found species (*Bcl. subtilis*, *Bcl. megaterium*, *Bcl. amyloliquefaciens* and *Bcl. pumilus)*, belong to risk group 1. *Bcl. thuringiensis* is pathogenic to invertebrates and *Bcl. cereus* is a toxin producer but safe in technical conditions (Committee on Biological Agents, 2015).

Regarding the impact of *Bacillus* spp. inoculation on local rhizosphere populations, contradicting results have been reported. Felici et al. (2008) reported no changes on the rhizosphere communities after inoculation with *Bcl. subtilis*, whereas Probanza et al. (2002) have concluded that the introduction of two *Bacillus* (*Bcl. licheniformis* CECT 5106 and *Bcl. pumilus* CECT 5105) inoculants caused an alteration in the microbial rhizosphere composition.

However, some changes in the microbial rhizosphere composition can be positive; for example, the application of *Bcl*. *amyloliquefaciens* ZM9 affected positively the rhizosphere composition of tobacco plants by reducing pathogen populations (Wu et al., 2016).

4.4. *Pantoea*

4.4.1. General genus description

The *Pantoea* genus, which has *Pa. agglomerans* as type species, was recently separated from the *Enterobacter* genus (Walterson and Stavrinides, 2015). It is a motile Gram-negative rod shaped bacteria, forming yellow mucoid colonies (Walterson and Stavrinides, 2015). Given its versatility, ubiquity and genetic tractability, it is an ideal genus for the development of commercially relevant medical, agricultural and environmental bioproducts (Walterson and Stavrinides, 2015). Since the creation of the genus with *Pa. agglomerans*, new species have strengthen the ranks of *Pantoea* genus, such as *Pa. allii* (Brady et al., 2011), *Pa. vagans, Pa. eucalypti, Pa. deleyi* and *Pa. anthophila* (Brady et al., 2009).

4.4.2. Siderophore production

The production of siderophores was described for several *Pantoea* species, such as *Pa. agglomeran*, *Pa. eucalyptii* (Viruel et al., 2011), *Pa. allii* (Pereira and Castro, 2014), *Pa. ananatis* (Kim et al., 2012; Loaces et al., 2011), as well as for other *Pantoea* spp. (Loaces et al., 2011). However, little is known about these siderophores. The strain *Pa. vagans* C9-1 is known to produce an enterobactin-like (catechol) siderophore and a desferrioxamine-like siderophore (Feistner and Ishimaru, 1996); the production of the latter compound is

dependent on plasmatic transcription; therefore, this characteristic is susceptible to be lost on some mutants (Smits et al., 2010). *Pa. stewartii* subsp. *stewartii* produces a siderophore under Fe-limiting conditions, which biosynthesis and export of proteins are encoded by the *iuc*ABCD-*iut*A operon; this operon is homologous to the aerobactin biosynthetic gene cluster found in a number of enteric pathogens (Burbank et al., 2015). It was also described that the strain *Pa. eucalypti* M91 is capable of producing pyoverdine-like and pyochelin-like siderophores under alkaline growth conditions (Campestre et al., 2016). More recently, in a phylogenetic and comparative genomic study, desferrioxamine-like, enterobactin-like, and aerobactin-like siderophore biosynthetic gene clusters were found in several *Panteoa* spp. strains (Soutar and Stavrinides, 2018). Judging from these findings, *Pantoea* strains are potentially capable of producing multiple and different types of siderophores.

4.4.3. Other PGP properties

The production of phytohormones, such as IAA, abscisic acid and GA (Feng et al., 2006) and cytokines (Omer et al., 2004) by *Pantoea* spp. was reported. The production of ACCD was also described for *Panteoa* spp. strains (Trifi et al., 2017). In addition, nutrient based promoting traits, such as phosphate solubilisation or nitrogen fixation (Kim et al., 2012) were described. The biocontrol potential of *Pantoea* spp. against *Pseudomonas syringae* (Xie et al., 2017) or *Erwinia amylovora* (Ait Bahadou et al., 2018) has also been recently reported.

4.4.4. Applied studies

The use of *Pantoea* spp isolates to be used for agronomic practices was tested in pot or field studies. Thus, the increased mass in plant bean (Gopalakrishnan et al., 2017), and in canola (Trifi et al., 2017), biocontrol action in mulberry seedlings (Xie et al., 2017), Fe intake in *Lotus japonicus* (Campestre et al., 2016), root development in olive trees (Montero-Calasanz et al., 2013), and increased crop yield and biomass in tomato, cucumber or pepper (Kim et al., 2012) was described.

4.4.5. Microbiological safety

Regarding safety aspects of working with *Pantoea* strains, from the twenty seven species listed on the TRBA-466, only four are not listed as risk group 1: *Pa. agglomerans*, *Pa. brenneri*, *Pa. eucrina* and *Pa. septica* (Committee on Biological Agents, 2015). Eleven species have been classified as plant pathogens strains (Committee on Biological Agents, 2015). Despite this, the use of *Pantoea* strains, namely *Pa. agglomerans*, at commercial level, as adjuncts to agricultural practice, has been reported (Glick, 2012). *Pa. allii*, *Pa. ananatis* and *Pa. vagans*, which also have plant-growth properties, as reported above, and are risk 1 species, can be possible species alternatives.

4.5. *Pseudomonas*

4.5.1. General genus description

The *Pseudomona*s genus is of medical and biotechnologically importance. It consists in aerobic, straight or slightly curved rods, Gram-negative bacteria. *Pseudomonas* strains are found in biofilms or in planktonic forms with one or more polar flagella. Due to their high metabolic versatility, these bacteria are

able of using most carbon sources (Özen and Ussery, 2012). The *Pseudomonas* genus is widely spread in the environment and some biotechnological important bacteria are inhabitants of a wide range of niches, such as soil and water environments, in addition to plant and animal associations (Özen and Ussery, 2012).

The *Pseudomonas* genus is one of the main PGPB genus that has received wide attention from the scientific community, being recognized for their biocontrol (Beneduzi et al., 2012) and agricultural potential (Bhattacharyya and Jha, 2012; Hayat et al., 2010).

4.5.2. Siderophore production

Several siderophores produced by the *Pseudomonas* genus have been identified. Liu & Shokrani (1978) coined the term pyochelin to designate the large quantity of siderophores produced by *Ps. aeruginosa* capable of harvesting Fe from transferrin. The structural characterization of pyochelin (A) (Figure 5) was later made by Cox and co-workers (Cox et al., 1981; Cox and Graham, 1979). Meyer & Abdallah (1978) isolated and characterized the yellow-green, fluorescent, water-soluble pigment, produced by *Ps. fluorescens,* suggesting that it was a siderophore. Kloepper et al. (1980) described the production of a siderophore in *Pseudomonas* strain B10. Later on, their colleagues characterized the structure of both pseudobactin (Figure 5) and pseudobactin A (different in the quinoline derivatives) produced by *Pseudomonas* strain B10 (Teintze et al., 1981; Teintze and Leong, 1981). Pyoverdines are a large family of siderophores composed by a chromophore and a peptide chain (Figure 5). The composition of this chain varies greatly from

one pyoverdine to another and between different fluorescent *Pseudomonas* species (Ringel and Brüser, 2018). For example, three different pyoverdines, named, pyoverdine, pyoverdine$_0$ and pyoverdine A (as well as ferribactin), were isolated from *Ps. fluorescens* (Philson and Llinas, 1982). The production of pyoverdines by *Ps. aeruginosa*, such as pyoverdine Pa, (Wendenbaum et al., 1983) and pyoverdine C, D and E (Briskot et al., 1986), as well as a blue pigment named pyocyanin (Cox, 1986), was also reported. Other *Pseudomonas*, such as *Ps. syringae,* also produce pyoverdine type siderophores (Torres et al., 1986). Other example is *Ps. chlororaphis* ATCC 9446 that produces pyoverdines and ferribactins similar to those produced by *Ps. fluorescens* ATCC 13525 (Hohlneicher et al., 1995). Others authors described the production of hydroxamate siderophores by *Ps. fluorescens* NCIM 5096 and *Ps. putida* NCIM 2847 (Sayyed et al., 2005). The production of pyoverdine siderophores by *Ps. aureofaciens* was also reported (Beiderbeck et al., 1999). In fact, given the wide production of siderophores by *Pseudomonas* spp., it has been proposed the use of siderophore typing as a form of identification of fluorescent and non-fluorescent *Pseudomonas* (Meyer et al., 2002, 1997). The importance, synthesis and variability of pyoverdines in *Pseudomonas* was also consequently reviewed (Visca et al., 2007). Some *Pseudomonas* have been described to produce non-fluorescent Fe chelating compounds, such as 7-hydroxytropolone, which is produced in large quantities by *Ps. donghuensis* (Jiang et al., 2016).

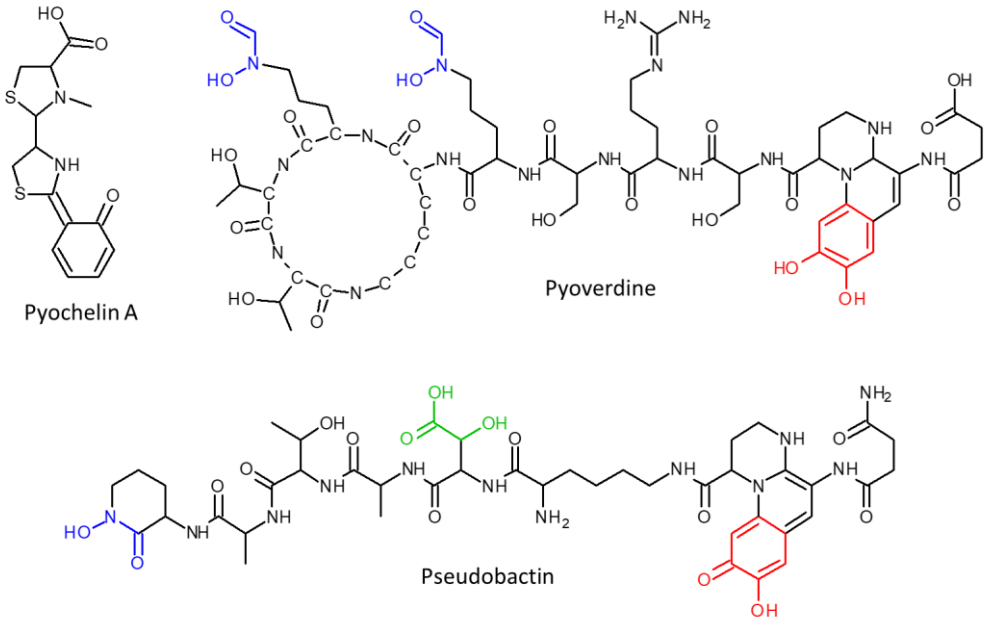


Figure 5. Some siderophores produced by Pseudomonas genus. Red: catechol moieties; Green: α-hydroxycarboxylate moieties; Blue: hydroxamate moieties

4.5.3. Other PGP properties

Other PGP characteristics have been extensively reported. The production of IAA and ACCD (Rashid et al., 2012) by *Pseudomonas* spp. has been reported. In addition, HCN (Subramanian and Satyan, 2014) and ammonia production (Rashid et al., 2012; Subramanian and Satyan, 2014) as well as phosphate solubilisation (Subramanian and Satyan, 2014) and nitrogen fixation (Pham et al., 2017) by *Pseudomonas* spp. was described. Antagonistic behaviour on several plant pathogens, such as *Erw. amylovora* (Ait Bahadou et al., 2018), *F. oxysporum* (Deori et al., 2018) or *Py. ultimum*, and *F. udum* (Sulochana et al., 2014) was also described.

4.5.4. Applied studies

Given the described properties of *Pseudomonas* spp., these bacteria have been tested for their potential as agronomic inoculants. Test studies have been conducted in crops such as sunflower (Pourbabaee et al., 2018), tomato (Nagata, 2017), chickpea (Gopalakrishnan et al., 2017; Sulochana et al., 2014), green gram (Kumar et al., 2015), wheat (Rasouli-Sadaghiani et al., 2014), rice (García de Salamone et al., 2012) or olive (Montero-Calasanz et al., 2013). Thus, it was reported the increase of Fe concentration (Nagata, 2017; Pourbabaee et al., 2018; Rasouli-Sadaghiani et al., 2014) (dry) plant mass (García de Salamone et al., 2012; Pourbabaee et al., 2018; Sulochana et al., 2014), resilience to drought (Pourbabaee et al., 2018), grain yield (García de Salamone et al., 2012; Gopalakrishnan et al., 2017), shoot length (Sulochana et al., 2014) or root length (Montero-Calasanz et al., 2013).

4.5.5. Microbiological safety

Regarding *Pseudomonas* spp. safety, a total of two hundred fifty five species (subspecies included) belonging to the group risk 1 were isolated (Committee on Biological Agents, 2015). However, some of the most mentioned species, as PGPB, are not risk 1, or, have other notes of pathogenicity in invertebrates/plants. For example, both *Ps. aeruginosa* and *Ps. putida* are risk 2 level, with known human and vertebrate pathogenic properties. However, there are some species that are not pathogenic and are suitable for safe application, with PGP traits. For instance, *Ps. fluorescens* (a risk group 1 with only a remark for invertebrates), when inoculated in plants roots was reported as innocuous for the rhizosphere (De La Fuente et al., 2002). Also, the inoculation of *Ps.*

fluorescens in rice seems to not impact significantly, culturable microbial communities in rhizosphere (García de Salamone et al., 2012).

4.6. *Rhizobium*

4.6.1. General genus description

The *Rhizobium* genus includes Gram-negative rod shape bacteria, usually mobile and able to use simple carbohydrates, predominantly aerobic chemoorganotrophs. Some species have shown to fix nitrogen while most of them do not fix it when they present as free living (Somasegaran and Hoben, 1994). Although not mandatory, they are also commonly found in the nodules of plant's roots; in this case, they are found as nitrogen fixators, forming symbiotic relations with the host plant (Lodwig et al., 2003). *Rhb. leguminosarum* is the representative species of this ever-growing genus (Parte, 2018), which has more than 90 species registered on microorganisms banks such as the Deutsche Sammlung von Mikroorganismen und Zellkulturen.

Being symbionts with plants, it is predictable that *Rhizobium* species have traits that help the plants growth, as part of its symbiotic relation. In fact, not only *Rhizobium* species fixate nitrogen when in symbiosis with plants but they also produce other compounds, such as siderophores, phytohormones and other growth regulators.

4.6.2. Siderophore production

Several siderophores have been described to be produced by *Rhizobium* spp. *Rhb. radiobacter* (then known as *Agrobacterium tumefaciens* and later as *Agrobacterium radiobacter*) was shown to produce a tri-catecholate siderophore named agrobactin (Figure 6) (Eng-Wilmot and Van der Helm, 1980; Ong et al.,

1979). Some strains of *Rhb. radiobacter* were also reported to produce hydroxamate type siderophores (Leong and Neilands, 1982). Smith et al. (1984) reported that *Rhb. meliloti* produces a non-catecholate and a non-hydroxamate siderophore named rhizobactin (Figure 1); later on, these authors have been working on its structure and described it as a di α-hydroxy-carboxylate siderophore (Smith et al., 1985). *Rhb. meliloti* 1021 strain (also known as *Sinorhizobium meliloti* or *Ensifer meliloti*) was found to produce a variant of rhizobactin, named rhizobactin 1021 (Persmark et al., 1993). *Rhb. leguminosarum IARI 102 produced* 2,3-DHBA conjugated with threonine (2,3-DHBA-Thr, Figure 6), under Fe starved conditions (Patel et al., 1988). On the other hand, the *Rhb. leguminosarum* IARI 917 strain was described to produce the schizokinen siderophore (Figure 4), (Storey et al., 2006). *Rhb. leguminosarum* ATCC 14479 was described to produce vicibactin (Figure 6), a try-hydroxamate, and two other Fe-binding siderophore-like compounds (Wright et al., 2013). It can then be concluded that *Rhizobium* genus has the ability to produce several siderophores, which can be tapped for possible uses in agricultural, medical and engineering purposes.

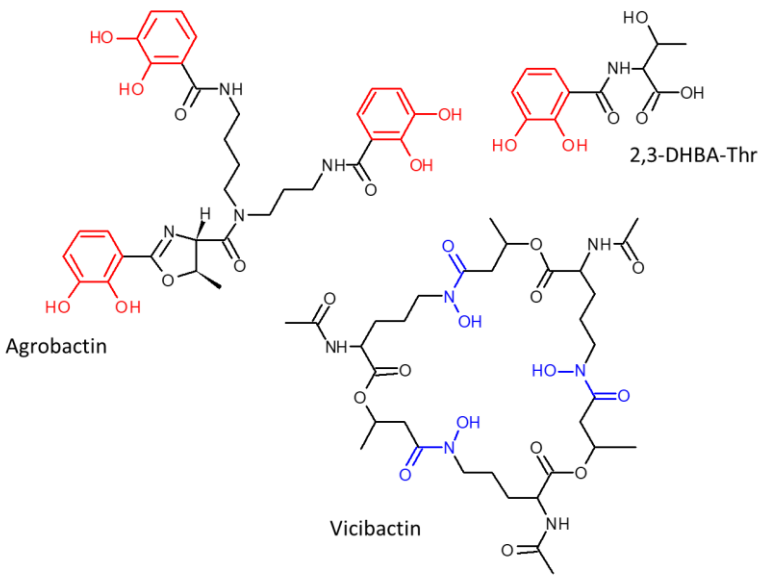


Figure 6. Different siderophores produced by Rhizobium genus. Red: catechol moieties; Orange: carboxylate moieties; Blue: hydroxamate moieties. 2,3-DHBA-Thr: 2,3-dihydroxybenzoic threonine.

4.6.3. Other PGP properties

The production of ACCD and IAA has been described in *Rhizobium* spp (Hernández et al., 2017). Has also the ability to solubilize phosphate (Kumar et al., 2015; Xing et al., 2016) and to fix atmospheric nitrogen when in root nodules (Datta et al., 2015). The antagonistic activity against different plant pathogens, such as *F. oxysporum* (Arfaoui et al., 2006), *Rht. solani*, *Sclerotium rolfsii* (Sai Prasad et al., 2014) or *M. phaseolina*, and *Asp. niger* (Sharma and Das, 2010), was also documented.

4.6.4. Applied studies

The development of crops such as bean (de Souza et al., 2016), green gram (Kumar et al., 2015), rice (Bhattacharjee et al., 2012), groundnut (Arora et al., 2001), crambe (de Aquino et al., 2018), lettuce or carrot (Flores-Félix et al.,

2013) have been tested with the inoculation of different *Rhizobium* spp. The inoculation has resulted in different positive outcomes, such as increase of shoot mass (Bhattacharjee et al., 2012; de Souza et al., 2016; Flores-Félix et al., 2013), nitrogen plant content (Baba et al., 2015; Bhattacharjee et al., 2012; Flores-Félix et al., 2013) and root development (de Aquino et al., 2018) or reduced effects of pathogenic agents (Arora et al., 2001; Siddiqui et al., 2007).

4.6.5. Microbiological safety

Rhizobium genus has about sixty-three species identified in the TRBA-466 list, belonging all of them to risk group 1 bacteria (Committee on Biological Agents, 2015). Some species, such as *Rhb. radiobacter*, have strains with plant phytopathogenicity. *Rhizobium* inoculation studies have shown that this genus does not have a negative impact on the native community; instead, it contributes to an increase of the bacterial community (Ambrosini et al., 2016). Therefore, considering the proven capabilities to promote plant growth without causing any harm to human health and rhizosphere communities, the bacteria belonging to *Rhizobium* genus can be seen as an alternative for agronomic application.

5. Concluding remarks

As the world's population continues to increase, as well as the quality of life in some developing countries, the demand for food of quality equally increases. The limited space available for farming means that the usable space must be worked with the best efficiency possible. On the other hand, agriculture presents some problems such as the development of chlorotic plants due to the

Fe limitation, which is particularly severe under calcareous soils, or the loss of crops due diseases caused by phytopathogenic microorganisms. Both cases contribute to the reduction of crops yield.

In this chapter, it was presented one of the possible solutions for the development of sustainable agriculture: the use of bacteria to improve the quality and quantity of harvest crops. More specifically, it was discussed the suitability of some bacteria genera (*Azotobacter*, *Azospirillum*, *Bacillus*, *Pantoea*, *Pseudomonas* and *Rhizobium*) as source of biological Fe-fertilizers (siderophores) and other compounds that promotes the plant growth and prevent the action of infectious agents that cause plant diseases. Additionally, the application of bacteria in different crops improvement was presented and the biosafety aspects associated with the use of microorganisms in agronomic conditions were also addressed. In Table 3, it was summarized the main characteristics of promising bacterial genera for agriculture practices. Within these bacterial genera, particular strains may be more specialized in a given trait, while others, presented a wide properties spectra. But all genera have in common the presence of strains belonging to group 1 of biological agents, which makes them safe to be used for agricultural practices.

Table 3. Main characteristics of promising bacterial genera for agricultural practices.

	Siderophore		Other agronomic characteristics		
Genus	**Name**	**Type**	**Biofertelization**	**Biostimulation**	**Biocontrol**
Azospirillum	Spirilobactin	Catechol	Nitrogen fixation	IAA	Produces HCN: Yes
	2,3 - DHB acid	Catechol	Phosphate solubilization	ACCD	Example pathogen:
	3,5- DHB-threonine	Catechol		GA	*Col.acutatum*
	3,5 - DHB-lysine	Catechol		Kinetin	
				Abscisic acid	
				Zeatin	
Azotobacter	2,3 - DHB acid	Catechol	Nitrogen fixation	IAA	Produces HCN: Yes
	Aminochelin	Catechol	Phosphate solubilization	ACCD	Example pathogen:
	Azotochelin	Catechol			*F. oxysporum*
	Protochelin	Catechol			*Rht. solani*
	Azotobactin	Mixed			*M. phaseolina*
	Vibrioferrin	Mixed			
	3,4 - DHB acid	Chatecol			
Bacillus	Schizokinen	Hydroxamate	Phosphate solubilization	IAA	Produces HCN: Yes
	N- schizokinen	Hydroxamate	Ammonia production	ACCD	Example pathogen:
	N-schizokinen-A	Hydroxamate		GA	*M. phaseolina*
	Itoic acid	Catechol			*Ral. solanacearum*
	Bacillobactin	Catechol			*Rht. solani*
	Petrobactin	Mixed			*F. oxysporum*

IAA = Indole-3-acetic acid production; ACCD = 1-aminocyclopropane-1-carboxylate deaminase; GA = Gibberellic acid; DHB = dihydroxybenzoic

Table 3 (Cont.) Main characteristics of promising bacterial genera for agricultural practices.

	Siderophore		Other agronomic characteristics		
Genus	**Name**	**Type**	**Biofertelization**	**Biostimulation**	**Biocontrol**
Pantoea	Enterobactin-like	Catechol	Nitrogen fixation	IAA	Produces HCN: No
	Desferrioxamine-like	Hydroxamate	Phosphate solubilization	ACCD	Example pathogen:
	Pyoverdine-like	Mixed		GA	*Ps. syringae*
	Pyochelin-like	Mixed		cytokines	*Erw. amylovora*
				abscisic acid	
Pseudomonas	Pseudobactin(s)	Mixed	Nitrogen fixation	IAA	Produces HCN: Yes
	Pyoverdine(s)	Mixed	Phosphate solubilization	ACCD	Example pathogen:
	Pyocyanin	Other	Ammonia production		*Erw. amylovora*
	Ferribactin	Mixed			*F. oxysporum*
	Hydroxytropolone	Other			*F. udum*
					Py. ultimum
Rhizobium	Agrobactin	Catechol	Nitrogen fixation	IAA	Produces HCN: No
	Vicibactin	Hydroxamate	Phosphate solubilization	ACCD	Example pathogen:
	Schizokinen	Hydroxamate			*F. oxysporum*
	2,3 - DHB-threonine	Catechol			*Rht. solani*
	Rhizobactin	Catechol			*Scl. rolfsii*
	Rhizobactin 1021	Carboxylate			*M. phaseolina*

IAA = Indole-3-acetic acid production; ACCD = 1-aminocyclopropane-1-carboxylate deaminase; GA = Gibberellic acid; DHB = dihydroxybenzoic

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Chapter 3

Searching for suitable siderophore(s) bacteria producers for correcting iron-deficiency (chlorosis) under alkaline conditions[*]

* Ferreira, C.M.H., Vilas-Boas, Â., Sousa, C.A., Soares, H.M.V.M., Soares, E. V., 2019. Comparison of five bacterial strains producing siderophores with ability to chelate iron under alkaline conditions. AMB Express 9, 78. https://doi.org/10.1186/s13568-019-0796-3

1. Introduction

Iron is an essential nutrient for plant development together with macronutrients (Barker and Stratton, 2015). Although not present in chlorophyll, iron is necessary for its synthesis and for the normal functioning of the photosynthetic apparatus (Broadley et al., 2012). The lack of iron in plants leads to the develop of a symptomatic array, named iron deficiency induced chlorosis (IDIC). It is characterized by the development of yellow young leaves as a result of insufficient iron for the efficient chlorophyll production (Lucena, 2000). Chlorotic plants will under develop, produce less biomass, yield less flowers and fruits, or, ultimately, lead to complete crop failure (Guerinot and Yi, 1994). Some crops known to be susceptible to iron chlorosis are peach, kiwifruit, citrus and pear (Lucena, 2003), although some other valuable crops have also been described to suffer from chlorosis, such as rice (Yoshida et al., 1996), soybean (Froechlich and Fehr, 1981), tomato (Chaney et al., 2008) and pea (Kabir et al., 2016).

To surpass the problem of IDIC, organic chelating agents have been used, such as aminopolycarboxylic acids (APCA), to correct iron deficiency. Although, these compounds are strong chelating agents, usually they are not biodegradable. Thus, their persistence and consequent accumulation in aquatic systems is becoming a matter of great concern, as they can mobilize toxic metals (Bucheli-Witschel and Egli, 2001). Therefore, the search for environmental-friendly and suitable iron chelators, with specific properties to be used, as iron fertilizers, has become a great challenge. Among a great number of compounds considered, siderophores are an interesting object of study since

they are effective iron chelating compounds and have the advantage of being more biodegradable than synthetic APCAs (Fazary et al., 2016a, 2016b).

Plant-growth promoting rhizobacteria (PGPR) are bacterial strains that live and interact in plants rhizosphere and promote their host plant growth (Bhattacharyya and Jha, 2012; Chauhan et al., 2015). The plant-growth promoting mechanisms can be described as direct or indirect. Supply of nutrients to the plant, either by nitrogen fixation, siderophore production, or organic matter and phosphate solubilization, as well as the production of growth-inducing phytohormones, are example of direct mechanisms of action. Indirect mechanisms may include the removal of pathogenic substances, competition with pathogenic organism and stimulation of mycorrhizae growth (Bhattacharyya and Jha, 2012; Jyoti Das et al., 2013).

One important trait is the production of siderophores: low weight molecules (between 500 to 1500 dalton) with great affinity and selectivity to bind and complex Fe(III) (Hider and Kong, 2010). This particular trait has drawn the attention of many researchers in recent years. For instance, many studies have been conducted in search on new siderophore producing species. As one example, Grobelak & Hiller have conducted a screening of bacteria (*Bacillus* spp*.* and *Pseudomonas* spp.) isolated from plant's roots for catechol and hydroxamate producing bacteria (Grobelak and Hiller, 2017). The positive effects of siderophore producing bacteria have been studied as well, as for example in the works of Liu et al. (2017), Trifi et al. (2017) or by Sabaté et al. (2017).

Considering that iron deficiency is a yield-limiting factor with major implications for crop management, the production of siderophores compounds

is an important challenge. In recent works (Ferreira et al., 2019; Martins et al., 2018), it was shown that synthetic compounds containing catecholate and hydroxamate groups are potential iron chelators for iron nutrition in plants. However, due to the high structural complexity of siderophores, its production by chemical synthesis involves several steps with low yields, which limits the feasibility of their use for agriculture purposes. As an alternative, siderophores can be produced by biotechnological processes. The work present in this chapter focused in searching for bacteria that can be further used in the production of environment-friendly and suitable iron chelators. These compounds should be produced at low cost, in order to be used in agriculture as iron fertilizers. For this purpose, five risk 1 (Committee on Biological Agents, 2015) bacteria belonging from the previously reviewed genera, were evaluated in order to select those which seem to be the most promising siderophore producers, considering the following parameters: amount of siderophore produced, efficiency of iron-complexing capacity at pH 9.0, type of siderophore, speed of siderophore production and growth and handling of the strain (growth, nutritional requirements, culture medium composition, and easiness of biomass separation from the culture medium).

2. Materials and methods

2.1. Microorganisms, media and culture conditions

In this work, the following microorganisms were used: *Azotobacter vinelandii* Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) 2289; *Bacillus megaterium* American Type Culture Collection (ATCC) 19213; *B.*

subtilis DSM 10; *Pantoea allii* DSM 25133; and *Rhizobium radiobacter* DSM 30205. The original strains were obtained from DSMZ, Germany, or ATCC, U.S.A. All these bacteria belong to Risk 1 group, according to the Technical Rules for Biological Agents (TRBA)-466 (Committee on Biological Agents, 2015).

The microorganisms were maintained in minimal medium (MM) agar, except *A. vinelandii,* which was maintained in Burk´s medium (BM) agar, at 4 ºC. MM agar contained per litre: 10 g glucose, 1.47 g glutamic acid, 3.0 g potassium hydrogenophosphate (K_2HPO_4), 1.0 g potassium dihydrogenophosphate (KH_2PO_4), 0.5 g ammonium chloride (NH_4Cl), 0.1 g ammonium nitrate (NH_4NO_3), 0.1 g sodium sulphate (Na_2SO_4), 10 mg magnesium sulphate heptahydrate ($MgSO_4.7H_2O$), 1 mg magnesium sulphate tetrahydrate ($MnSO_4.4H_2O$), 0.5 mg calcium chloride ($CaCl_2$) and 20 g agar. BM agar was prepared as previously described (HiMedia Laboratories, 2015) replacing sucrose by glucose; the medium contained per litre: 10 g glucose, 0.8 g K_2HPO_4, 0.2 g KH_2PO_4, 0.20 g $MgSO_4.7H_2O$, 0.253 mg sodium molybdate, 0.13 g calcium sulphate and 20 g agar. The final pH of the media was set to 7.0 ± 0.1. For iron-replete media, 29 mg of iron(III) chloride ($FeCl_3$) was also added.

All reagents used were obtained from Merck, Panreac, Sigma-Aldrich or BD Difco. Inorganic chemicals were *pro-analysis* grade while organic chemicals were *Ph. Eur.* grade.

In order to avoid iron contamination (on iron-deficient-cultures), all glassware was soaked in 10 % nitric acid, overnight and, subsequently, washed with deionized water prior to use.

All pre-starter cultures, with exception of *A. vinelandii,* were prepared by inoculating the bacteria in 20 mL of iron-replete MM broth in 100 mL Erlenmeyer flasks. Cells were incubated at 30 ºC, for 8 h, in an orbital shaker at 150 rpm. Starter cultures were prepared by inoculating 40 mL of iron-replete MM broth, in 100 mL Erlenmeyer flasks, with an appropriate volume of the pre-starter cultures, and then incubated overnight to an OD_{600} ≈ 2.0, under the same conditions described for the pre-starter cultures. Cells, in exponential phase of growth, were harvested by centrifugation (3000 x *g*, 10 to 30 min), rinsed and suspended in iron-deficient media. Next, cultures were obtained by inoculating the cells in 400 mL of iron-deficient MM broth (initial OD_{600} ≈ 0.1), in 1 L Erlenmeyer flasks. Cells were incubated under the same conditions as described above. For *A. vinelandii*, there were some differences in the protocol: BM broth was used instead of MM broth and only a starter culture was grown, for 24 h, due to the slower growth rate of the bacterium.

2.2. Determination of siderophore production

Due to its easiness and ability for a high and sensitive detection of siderophores (Schwyn and Neilands, 1987), the chrome azurol S (CAS) method was used (Alexander and Zuberer, 1991) in the estimation of the bacterial siderophore production. Therefore, samples were taken and cells were pelleted by centrifugation (3000 x *g*, 10 to 30 min). The supernatant was carefully removed and, subsequently, filtered through a 0.45 µm pore size filter and immediately stored at −20 °C until siderophore determination.

CAS method consists in mixing 1.0 mL of filtrate, properly diluted with deionized water, with 1.0 mL of CAS assay solution, prepared as described by Alexander & Zuberer (Alexander and Zuberer, 1991). One reference solution

was also prepared by mixing 1.0 mL of CAS solution with 1.0 mL of deionized water. A zero-absorbance solution, was also prepared by mixing 1.0 mL of CAS solution with 1.0 mL of 100 µmol.L^{-1} desferrioxamine mesylate salt (desferal). The solutions were left to reach chemical equilibrium at room temperature in the dark for 24 h. Next, absorbance was read at 630 nm. A calibration curve was performed by plotting the ratio A/A_{ref} *versus* the concentration of desferal; where, A is the standard solution absorbance at 630 nm and A_{ref} stands for absorbance of the reference at 630 nm. Siderophore production is expressed as µmol.L^{-1} desferal equivalent. For each culture, samples were read in triplicate and repeated independently at least two times (that is, two independent cultures read in triplicate).

2.3. Siderophore qualification

The type of siderophore present in each bacterial medium was characterized using Arnow's and the Csaky's methods for catecholates and hydroxamates, respectively (Payne, 1994).

Arnow method is based on the reaction between catechol and nitrite-molybdate reagent, in acidic conditions, originating a yellow colour. The colour changes to an intense orange-red in alkaline conditions. For this purpose, 1.0 mL of culture filtrate was combined with 1.0 mL of HCl 0.5 mol.L^{-1}. Subsequently, 1.0 mL of nitrite-molybdate reagent was added and then 1.0 mL of NaOH 1.0 mol.L^{-1}. The assay was incubated at room temperature, for approximately 5 min, to allow full colour development. As blank, 1.0 ml of deionised water was used. Nitrite-molybdate reagent was prepared by dissolving 10 g of sodium nitrite and 10 g of sodium molybdate in 100 mL of deionized water. If catecholate-type siderophore is present, the solution

presents an orange-red colour. The colour intensity is dependent of the amount of catechol present (Arnow, 1937).

The Csaky's test detects hydroxamate-type siderophores and depends on oxidation to nitrite and formation of a coloured complex via diazonium coupling (Csáky, 1948). Firstly, 1.0 mL of culture filtrate was hydrolysed with 1.0 mL of 6 N H_2SO_4, at 130 ºC, for 30 min. The solution was then buffered with the aid of 3.0 mL of sodium acetate (350 $g.L^{-1}$) and 1.0 mL of sulfanilic acid [10 $g.L^{-1}$, 30 % acetic acid (v / v)] was added, followed by 0.5 mL of iodine solution (13 $g.L^{-1}$, in glacial acetic acid). The solution was allowed to settle for 3 - 5 min, after which the excess of iodine was neutralized by the addition of 1.0 mL of 20 $g.L^{-1}$ sodium arsenite. Finally, 1.0 mL of α-Naphthylamine solution [3 $g.L^{-1}$, in 30 % (v / v) acetic acid] was added and the colour was allowed to develop for 20 - 30 min. The presence of hydroxamates in solution was confirmed by the presence of a deep pink colour (Csáky, 1948).

Synthetic N,N-dihydroxy-N,N′-diisopropylhexanediamide (DPH), a hydroxamate, and Azotochelin, a catecholate, were used as controls (Martins et al., 2018). As blank, 1.0 ml of deionised water was used.

2.4. Complexation capacity assays

The complex capability of each culture medium containing siderophore was tested using a procedure adapted from Villen et al. (2007). Briefly, to a fixed volume of culture filtrate, $FeCl_3$ was added, the solution pH was set to 9.0 ± 0.1 and let to rest for 30 min. Then, pH was corrected again and let to settle for 3 h. The solution was subsequently centrifuged (3000 x *g*, 10 min) and filtered by a 0.45 µm pore size membrane. The amount of Fe in solution was

then determined (see “Iron determination” bellow). A graphical representation of the ratio $[Fe]_{complex}/[L]$ *versus* $[Fe]_{added}/[L]$ was plotted in order to represent the iron complexation capability of the siderophore in solution; where $[Fe]_{complex}$ is the concentration of Fe found in the filtrate, $[Fe]_{added}$ is the total Fe concentration added and [L] is the concentration of siderophore determined by CAS method.

2.5. Bacteria staining

Bacterial cultures were collected and cells were fixed with 3.5 % (w / v) formaldehyde for two hours. Cells were then harvested by centrifugation and re-suspended in 10 $mmol.L^{-1}$ phosphate-buffered saline (PBS) solution (pH 7.0) with 3.5 % (w / v) formaldehyde and stored at 4 ºC, until use.

Previously stored cells were pelleted by centrifugation and washed twice with PBS buffer (pH 7.0). Cells were re-suspended in PBS buffer with 3 $\mu mol.L^{-1}$ 4,6-diamidino-2-phenylindole (DAPI) and incubated for 15 min in darkness at room temperature. DAPI is a cell membrane permeant stain, which exhibits a strong blue fluorescence upon bonded to adenine-thymine rich regions in DNA (Haugland, 2005).

2.6. Epifluorescence Microscopy

Bacteria were observed using a Leica DLMB epifluorescence microscope coupled with an HBO 100 mercury lamp and appropriate filter setting (A, excitation filter BP 340-380, dichromatic mirror 400, and suppression LP 425) from Leica. The images were captured with a Leica DC 300 F camera using a 100× oil immersion N plan objective, using Leica IM 50-Image manager software processing.

2.7. Iron determination

Iron determinations in cell cultures and in complexation capacity assays were carried out by atomic absorption spectroscopy with flame atomization (AAS-FA) using a Perkin Elmer AAnalyst 400 spectrometer (Norwalk, CT, USA).

2.8. Reproducibility of the results

All experiments were repeated, independently, at least, two times. Data is presented as mean values ± standard deviations (SD). Growth-curves were performed in duplicate; for each time, growth was monitored in triplicate. Data reported for siderophore concentrations and iron complexation experiments are the mean ± SD of at least six determinations.

3. Results and discussion

3.1. Microbial growth and siderophore production

3.1.1. Microbial growth

In the present chapter, it was our objective to select promissory siderophore producing bacteria that can be used in the agriculture in the correction of IDIC, in calcareous soils. It was our aim to select microorganisms producers of efficient siderophores, such as catecholates and hydroxamates, since these compounds display the highest affinity to iron (Hider and Kong, 2010). A literature evaluation revealed the presence of more than 20 bacteria genera that produces these siderophore-types (Bhattacharyya and Jha, 2012; Hider and Kong, 2010). From the available pool, five risk 1, non-pathogenic,

bacteria (Figure 7) were selected to cover different genus and siderophores types.

All tested bacteria were grown in MM broth, except *A. vinelandii,* which was grown in BM broth, at 30 ºC, with agitation (150 rpm). The pre-cultures were prepared in iron-complete media. To limit iron carryover (from iron-complete media), which inhibit siderophore production, cells in exponential phase of growth were washed and re-inoculated in the same media but without iron. At the end of the growth, the iron concentration in culture media was below the detection limit (0.09 $mg.L^{-1}$) of the method used (section 2.7). With the exception of *A. vinelandii*, all bacteria grown in MM had a duplication time between 1.1 and 1.8 h (Table 4) and were in stationary phase of growth after 24 h of incubation. *A. vinelandii*, a bacterium with the ability to fix atmospheric nitrogen (Hamilton et al., 2011), grew more slowly in BM broth (a culture medium without nitrogen source). Under the cultural conditions used, *A. vinelandii* presented a duplication time of about 14.4 h (Table 4) and reached the stationary phase after 72 h (Figure 8).

Table 4. Duplication time and growth media for the bacteria studied.

Bacteria	Duplication time (h)	Growth media
Azotobacter vinelandii	14.4	Burk's medium
Bacillus megaterium	1.1	Minimal medium
Bacillus subtilis	1.2	Minimal medium
Pantoea allii	1.7	Minimal medium
Rhizobium radiobacter	1.8	Minimal medium

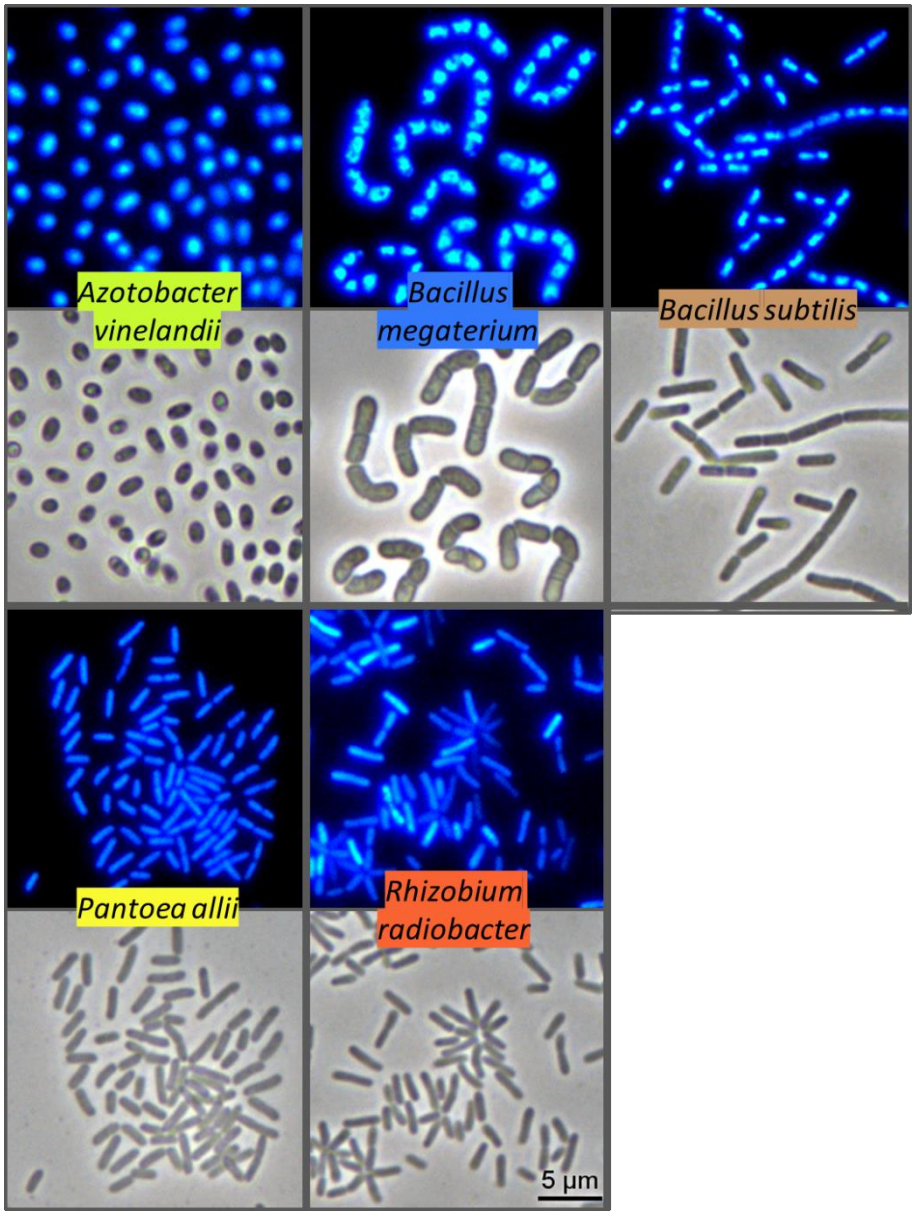

Figure 7. Microphotographs illustrative of the morphology of the bacteria studied, taken with DAPI fluorescent dye (upper image) and in phase contrast (lower image). All microphotographs were taken to cell cultures 6 h old, with exception of A. vinelandii, which was 48 h old.

The amount of biomass reached, in stationary phase, varied widely (Figure 8). After 24 h of growth, *B. megaterium* and *B. subtilis* reached a biomass between 1.7 and 1.5 $mg.mL^{-1}$, while *R. radiobacter* only reached approximately 1 $mg.mL^{-1}$. *A. vinelandii* had a maximum biomass less than 1 $mg.mL^{-1}$ while *P. allii* had a maximum biomass of 1.3 $mg.mL^{-1}$ (Figure 8).

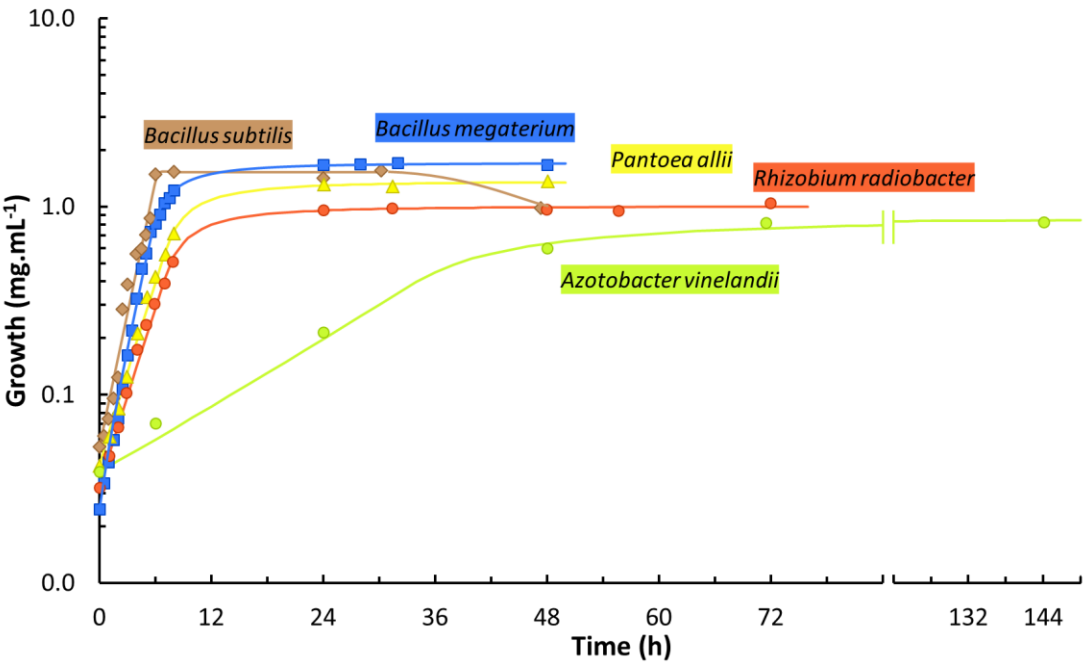

Figure 8. Typical growth of the microorganisms tested. The bacteria, in exponential phase of growth, were inoculated in MM broth (except A. vinelandii, which was inoculated in BM broth), iron-deficient and incubated at 30 °C with agitation (150 rpm). This is a typical example of an experiment performed at least two times. Each point represents the average of three determinations.

In the case of *B. subtilis,* after 30 h of growth, a decrease of biomass was observed (Figure 8), probably due to the formation of spores (Figure 9), with a subsequent change in solution hue (Figure 10). A similar behaviour was reported for other *Bacillus* species (Ait Kaki et al., 2013; Bharucha et al., 2013; Santos et al., 2014).

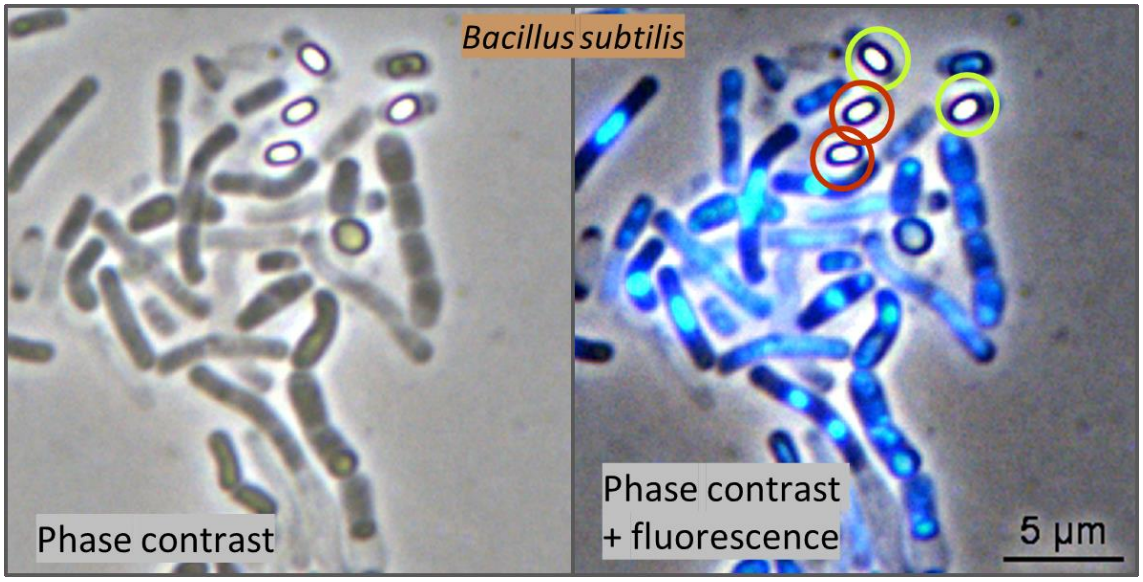

Figure 9. Microphotographs illustrative of the morphology of B. subtilis (lower), at 48 h of culture growth. Red circles: free spores; Green circles: Endospores.

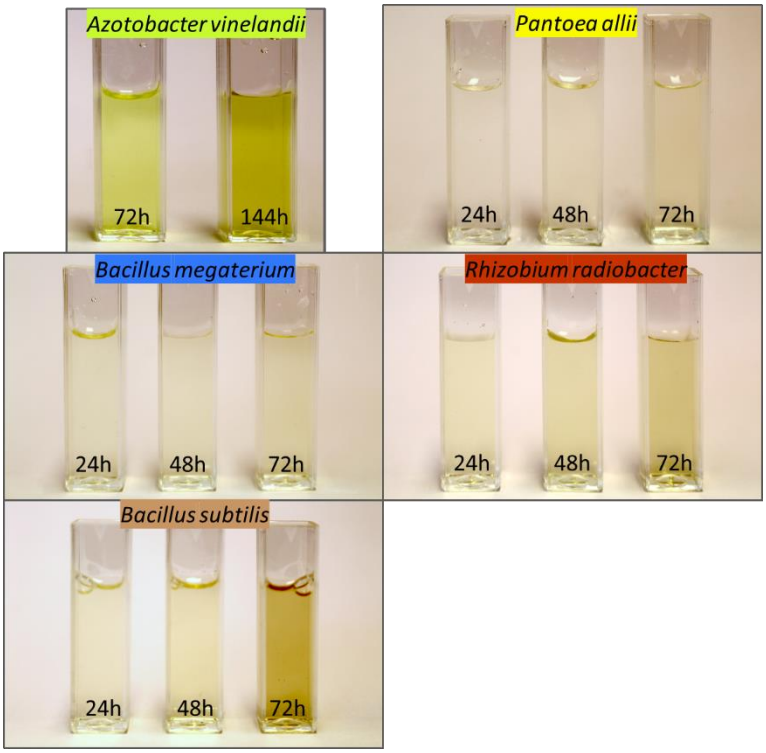

Figure 10. Representative photographs of microbial filtrate at different times of growth.

3.1.2. Siderophore production

Under the cultural conditions tested (culture medium composition, temperature and agitation), the siderophore concentration, found in the culture filtrates of *B. megaterium* reached the maximum level at 24 h (Figure 11), when the bacterium was in stationary phase (Figure 8). After this time, the level of siderophore remained stable (Figure 11). In contrast, using a different culture medium and different cultural conditions, it was described that *B. megaterium* continued to produce siderophore during the stationary phase (Santos et al., 2014).

In the case of *A. vinelandii*, the maximum siderophore production was observed when the strain was in stationary phase (72 h) (Figure 8 and Figure 11). The production of siderophores is not only visible by CAS tests, but also hinted by the appearance of a strong green fluorescent hue in culture, which may be linked to azotobactin production (Figure 10) (Bulen and LeComte, 1962; Fukasawa et al., 1972).

Siderophore concentration, from 24 to 48 h of growth, in *B. subtilis* filtrates was also relatively stable; only a small increase at 48 h was observed. The pattern of growth and the siderophore production of *B. subtilis* strain studied are in agreement with those described in the literature (Patel et al., 2009). However, under the cultural conditions used in the present work, the strain presented an higher level of siderophore compared to that described by Miethke et al. (2006) using a minimal culture medium. Also, a faster maximum concentration of siderophore production was achieved with our conditions than those used by Fazary et al. (2016a) to grow *Bacillus* spp*. ST13.*

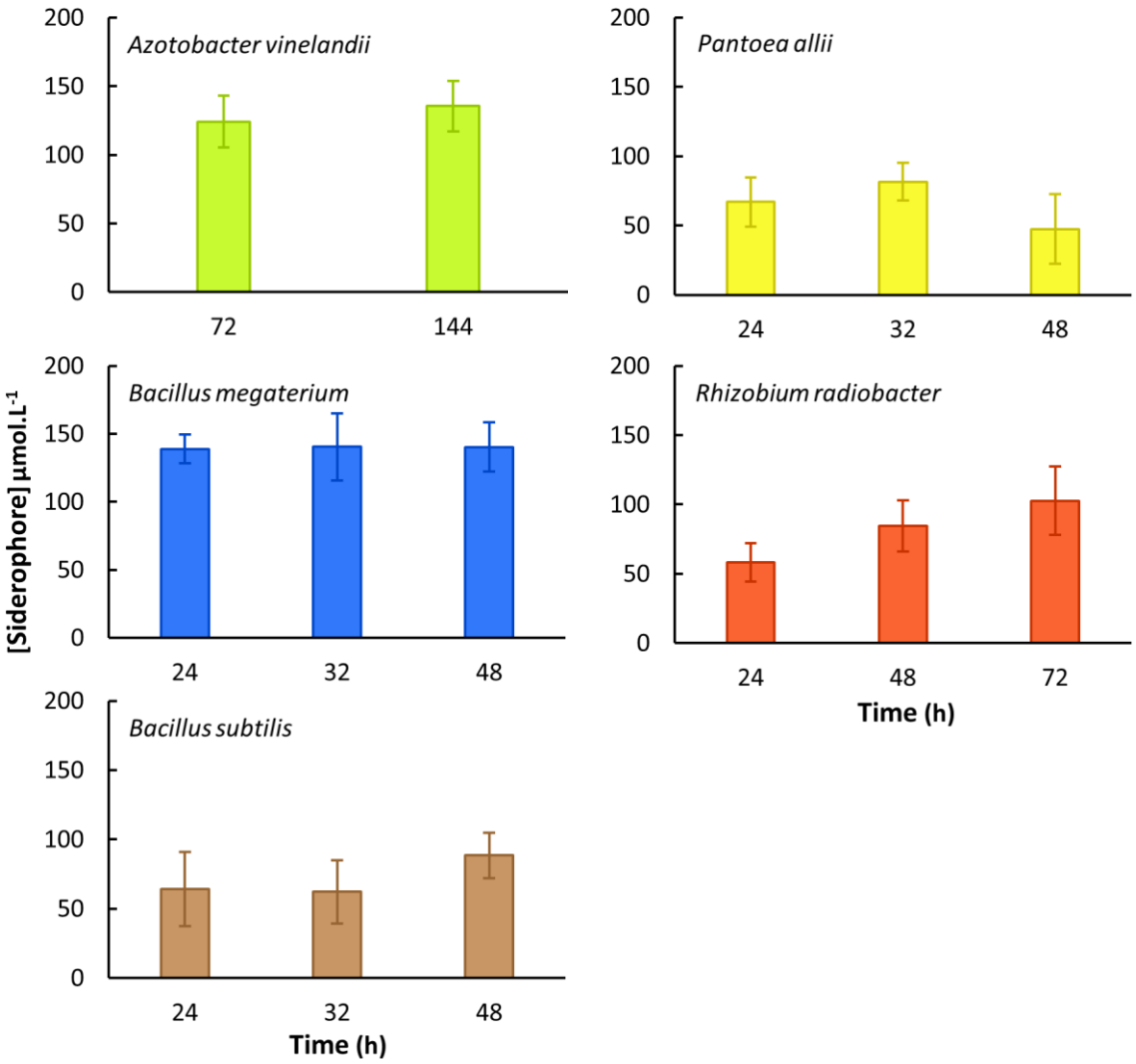

Figure 11. Siderophore production by the different microorganisms tested. Siderophore was quantified by CAS assay on culture filtrates and expressed as µmol.L-1 desferal equivalent. Each bar represents the mean (± standard deviation) of at least six determinations.

A similar pattern for *P. allii* was observed: however a small decrease of siderophore production was observed during the stationary phase of growth (24 – 48 h) (Figure 8 and Figure 11). To our knowledge, no studies of siderophore production, over time, have been carried out before with *Pantoea* spp.*;*

therefore, no comparison with the literature data is possible. This is very like due to the novelty factor of *Pantoea* spp*.* as PGPR (Chauhan et al., 2015).

A similar growth/siderophore production profile to that of *B. subtilis* was observed for *R. radiobacter* (Figure 8 and Figure 11). Interestingly, using a different *Rhizobium* sp. (*R. militoli*), the maximum siderophore production was described after 24 h of growth (Reigh, 1991). A *Rhizobium* strain, isolated from cowpea also had a similar growth/siderophore production behavior (Jadhav and Desai, 1992).

In a general way, the maximum level of siderophore in cultures filtrates was attained when the bacteria entered the stationary phase (Figure 8 and Figure 11). Unless for *A. vinelandii*, these results indicate that, for future siderophore production using these bacteria, it is not necessary to prolong the culture more than 48 h; for *A. vinelandii*, it is necessary to prepare a culture with 72 h.

3.2. Siderophore qualification

The type of siderophore present in each medium filtrate was tested using the Arnow's and Csaky's tests. Arnow's test allows the identification of the presence of catechol groups in siderophores, while Csaky's test is used to identify the presence of hydroxamic groups (Arnow, 1937; Csáky, 1948).

A. vinelandii had a positive result for both Arnow's and Csaky's test, which indicates the presence of both catechol and hydroxamate siderophores in the filtrate of the culture medium (Table 5, Figure 12). Several siderophores have been described for *A. vinelandii*, namely azotobactin, previously known as an yellow-green fluorescent peptide (Bulen and LeComte, 1962), 2,3-

dihydroxybenzoic acid, azotochelin (Corbin and Bulen, 1969), aminochelin (Page and Tigerstrom, 1988) and protochelin (Cornish and Page, 1995), which are, respectively, mono-, mono-, di-, mono- and tri-catecholates. Vibrioferrin, another siderophore described, has two hydroxy-carboxylate moieties which should not react in neither test. Finally, azotobactin has a complex structure in which a catechol and a hydroxamate moieties can be found (Duhme et al., 1998). The positive result in both tests (Table 5), can be attributed to the possible presence of azotobactin, aminochelin, azotochelin or/and protochelin. Given the low colour development compared to that of *B. subtilis* on Arnow's test (Figure 12), a low quantity of catecholate is expected. Considering the similar average molar quantities of siderophores on *B. subtilis* and *A. vinelandii* filtrates evaluated by CAS (~100 $\mu mol.L^{-1}$ *vs* ~120 $\mu mol.L^{-1}$) and the more intense color developed by *B. subtilis* compared to *A. vinelandii*, these facts suggest that the number of catechol sites per molecule may be lower in the case of *A. vinelandii* filtrates. Therefore, the presence of mono and di catecholates, such as azotobactin, aminochelin or/and azotochelin is very likely. Given the positive result for Csaky's test and the fluorescent hue (Figure 10), the presence of azotobactin is very plausible.

Table 5. Characterization of the siderophore-type produced by the bacteria: catechol- and hydroxamate type siderophores using Arnow´ and Csaky's tests, respectively

Bacteria	Arnow	Csaky
Azotobacter vinelandii	+	+
Bacillus megaterium	-	++
Bacillus subtilis	++	-
Pantoea allii	-	+
Rhizobium radiobacter	+	-

++ strong positive result; + positive result; – negative result.

The two *Bacillus* species studied produce different siderophore types. *B. megaterium* had a strong reaction to Csaky's and none to Arnow's, which indicate the presence of a hydroxamate-type siderophore in the supernatant of the culture. *B. subtilis* had a strong positive reaction with the Arnow's test and no reaction in Csaky's, which suggest the presence of catechol-type siderophore (Table 5, Figure 12).

These results are in agreement with those described in the literature. In the case of *B. megaterium*, only schizokinen and its derivatives were reported and characterized as siderophores produced under iron-limited conditions (Mullis et al., 1971). Schizokinen is a di-hydroxamate with a citrate backbone (Goldman et al., 1983; Hu and Boyer, 1995). On the other hand, the positive result in Arnow's test for *B. subtilis* is compatible with the literature, which describes the ability of this bacterium to produce bacillibactin (a tri-catecholate siderophore, which has a 1:1 coordination with iron) and, to a less extent, the more simple itoic acid, with a coordination of 3:1 (Dertz et al., 2006).

P. allii had a negative result on Arnow's test and positive on Csaky's test (Table 5, Figure 12), which suggests the absence of catechol-type and the presence of hydroxamate-type siderophores in the filtrate. For *P. allii*, little information regarding siderophore production and/or structure has been published. Although it was described that a strain similar to *P. allii* had great siderophore production capacity, no information regarding the type of siderophore was provided (Pereira and Castro, 2014). For other *Pantoea* species, a recent genome-wide survey has found three gene clusters homologous to those of enterobactin (catecholate-type siderophore), desferrioxamine (hydroxamate-type siderophore), and aerobactin (hydroxamate-type siderophore) (Soutar and Stavrinides, 2018). These findings are in agreement with the results found for *P. allii* as a hydroxamate-type siderophore producer (Csaky positive test).

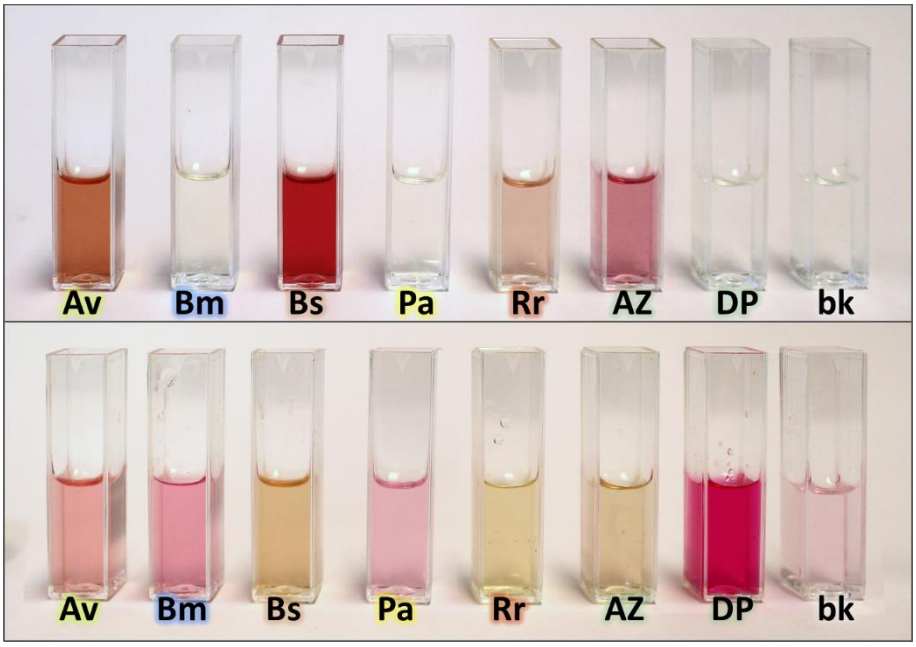

Figure 12. Arnow (top) and Csaky's (bottom) tests conducted in filtrates. Av: Azotobacter vinelandii; Bm: Bacillus megaterium; Bs: B. subtilis; Pa. Pantoea allii; Rr: Rhizobium radiobacter; DP: DPH, positive control for hidroxamates; AZ: Azotochelin, positive control for catecholates; Bk: blank.

R. radiobacter filtrate also presented a positive result for Arnow's test, which indicates that this microorganism produces a catechol-type siderophore. (Table 5, Figure 12). This observation is in agreement with the literature, which describes the ability of *R. radiobacter* to produce a siderophore so called agrobactin, a tri-catecholate with a structure simpler than that of *B. subtilis* (Ong et al., 1979).

3.3. Iron Complexation capacity

The problem of plant IDIC is particularly critical under alkaline conditions due to the low concentration of iron bioavailable (Colombo et al., 2014; Hansen et al., 2006). Bacterial filtrates can be further used in the preparation of siderophore concentrate intended to be used in the IDIC amendment in

calcareous soils. Even though all bacteria tested in this work evidenced siderophore production of similar order of magnitude (Figure 11) (when evaluated by the CAS assay and expressed as µmol.L^{-1} desferal equivalent), these results do not mean that these culture filtrates have ability to complex Fe at high pH conditions, which represent the real situation that the complexes have to endure when they are applied in alkaline soils. In order to assess the iron chelating potential of the culture filtrates, for the pH range typical of calcareous agronomic conditions (pH ~ 9.0), the bacterial culture filtrates were subjected to the procedure described in Materials and Methods, “Complexation capacity assays”.

3.3.1. EN15962 standard protocol modification

The EN15962 standard protocol (European Committee for Standardization, 2011) procedures and calculations were changed due to the fact that we do not have a chelator in a pure solid form, but as an “impure” solution instead. Therefore, it is not possible to add to a fixed amount of mass of ligand, increasing amounts of Fe, as described in the EN15962 standard protocol. As an alternative, instead of the total mass of chelate (eq.1), we used siderophore concentration, as read by CAS method, as a normalization factor.

The results given will then deviate from those typically presented in literature, such as those reported by Hernández-Apaolaza and her co-workers (Carrasco et al., 2012; Kovács et al., 2013; Martín-Ortiz et al., 2009).

When using the unmodified standard, a triangle-like shape is expected from the representation of the experimental points. Each experimental point represents a fixed mass of chelating agent to which a varying mass of Fe is

added. Thus, increasing quantities of Fe represent increasing total mass of the product:

$$m_{product} = m_{chelating\ agent} + m_{Fe} \quad \text{(eq.1)}$$

This total mass is used as normalizing factor and it is the common denominator on both axis of the graphical data representation (Figure 13). As maximum Fe complexation capability is archived, the iron complexed remains stable, and therefore the numerator in eq.2, *y* axis in Figure 13, remains constant, whereas the denominator increases (as a consequence of more iron being added), resulting in lower values of the calculated ratio.

$$m_{Fe\ complexed} / m_{product} \quad \text{(eq.2)}$$

For this reason, the maximum capability of a given chelator should be, in theory, represented by a vertex of the two intersecting lines of the experimental points, as seen in Figure 13.

On the other hand, by using siderophore concentration instead, we change the data representation to (eq.3, *x* axis in Figure 13) and (eq.4, *y* axis in Figure 13), where the common denominator remains constant.

$$[Fe]_{added} / [Siderophore] \quad \text{(eq.3)}$$

$$[Fe]_{complexed} / [Siderophore] \quad \text{(eq.4)}$$

As the dominator remains constant, once the maximum complexation capacity is reached, and no more Fe is solubilized, the ratio should remain constant, as represented by the grey line in Figure 13.

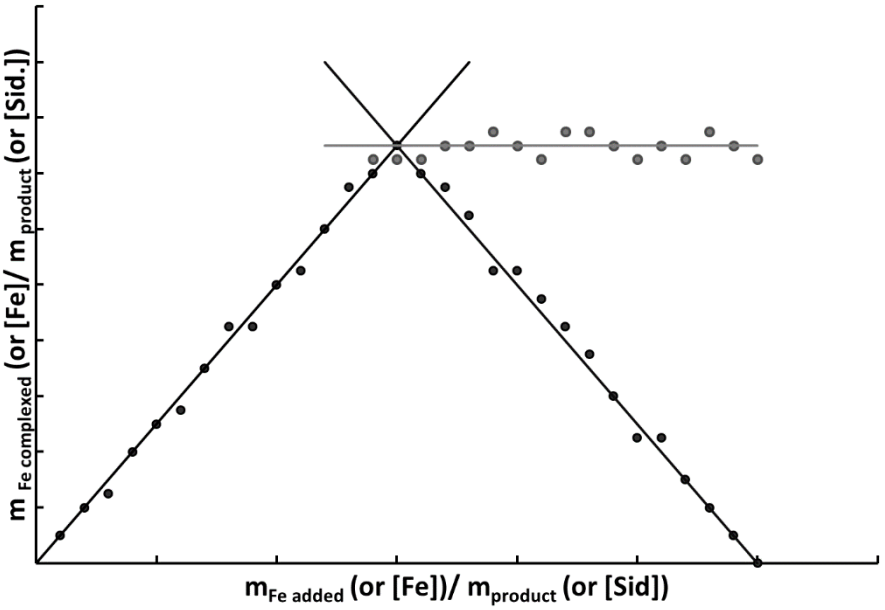

Figure 13. Typical (Black, A) and expected (Grey, B) lines for the complexation capacity assay. Typical lines adapted from Villen et al. (Villén et al., 2007)*. [Sid] = Siderophore concentration in filtrate.*

As an added advantage, by using the siderophore concentration as denominator, not only can we perform this modified assessment of the filtrate complex capacity, but also allows us to make comparisons between different filtrates of the same bacteria strain (which will undoubtedly have varing siderophore concentrations) but also compare different bacteria filtrates to each other. It also allows us to predict, based in siderophore concentrations readings in CAS, the amount of Fe that a given filtrate will be able to complex effectively in solution.

3.3.2. Experimental Results

The main results of the complexation capacity assays are shown in Figure 14. In regard to *B. megaterium* and *B. subtilis*, the initial slopes of the experimental data are close to 1 (dashed line) up to a $[Fe]_{added}/[Siderophore]$ ratio of about 2 and 2.5, respectively, which indicates that iron is being complexed with fairly good effectiveness up to that amount of iron added. For *A. vinelandii*, although some early deviations are seen, the only major shift from the slope = 1 is seen at $[Fe]_{added}/[Siderophore]$ ratio of about 1.5. At this point, an efficiency of about 75 % is observed with $[Fe]_{added}/[Siderophore]$ of 1.5 corresponding to a $[Fe]_{soluble}/[Siderophore]$ of 1. In both cases, early deviations may be related to inefficient complexation in the pH range used (pH = 9.0 ± 0.1); on the other hand, more severe deviations observed for higher $[Fe]_{added}/[Siderophore]$ ratios are more likely due to the complexation of weaker siderophores/chelating agents, where the added iron is not effectively complexed and promptly precipitates.

For each culture filtrate, based on the values of highest $[Fe]_{added}/[Siderophore]$, one can, based upon CAS values, estimate the amount of Fe soluble possible to obtain. For example, for one *A. vinelandii* filtrate with a CAS reading of 140 µmol.L^{-1}, one can expect to have 140 µmol.L^{-1} of dissolved iron upon addition of about 210 µmol.L^{-1} of Fe (1.5 x 140 µmol.L^{-1}). Likewise, a *B. subtilis* filtrate, with a CAS reading of 100 µmol.L^{-1}, is expected to be able to dissolve the entirety of 250 µmol.L^{-1} of added Fe (2.5 x 100 µmol.L^{-1}).

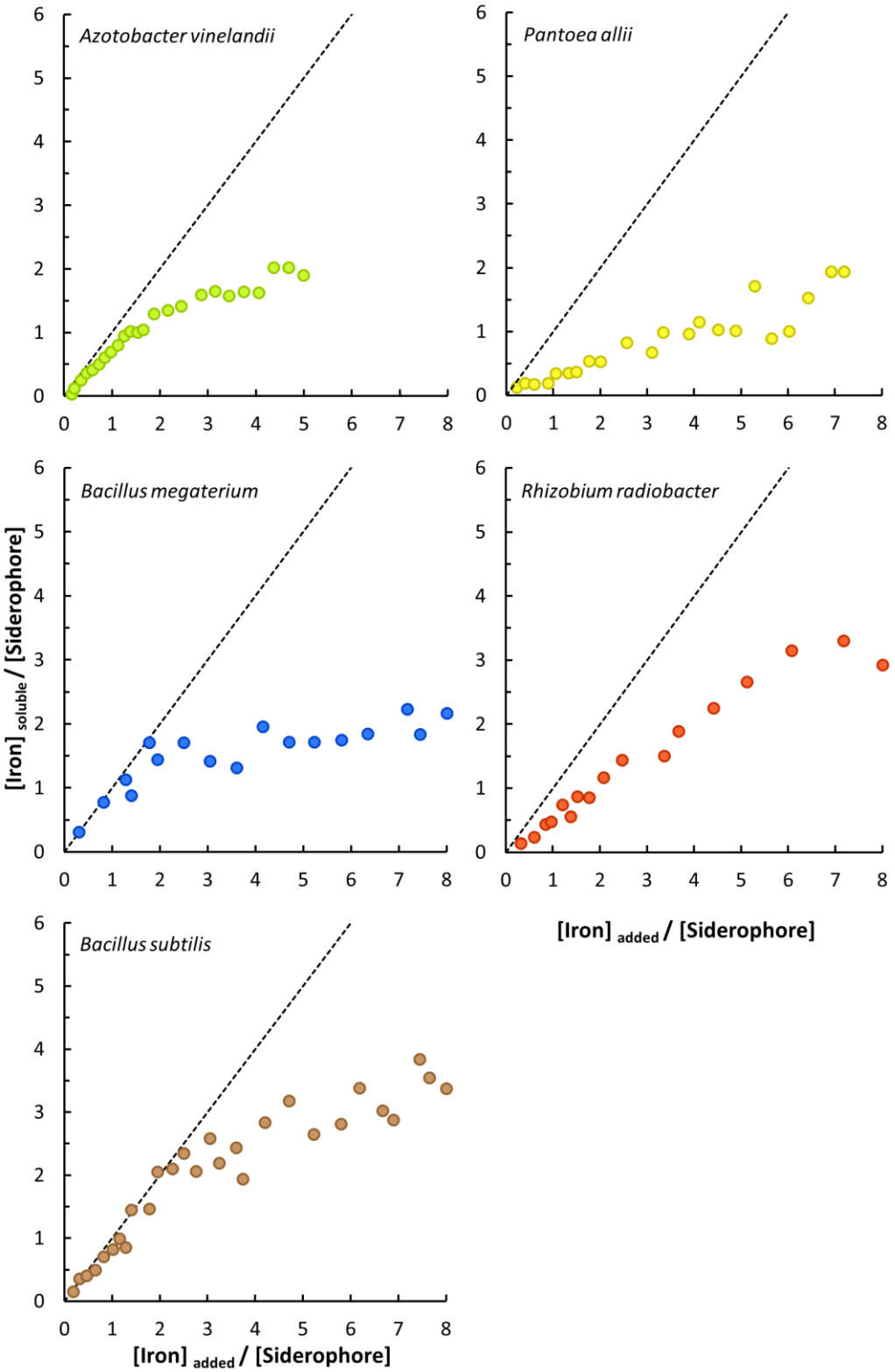

Figure 14. Average (n≥2) complexed iron versus added iron as function of total siderophore concentration as determined by CAS method. Dashed grey line represents a line with slope = 1.

On the other hand, *P. allii* and *R. radiobacter* evidenced lower slopes from early on, which is somehow contradictory to what would be expected. For example, literature describes that *R. radiobacter* produces agrobactin, which complexes iron in a 1:1 ratio, in an hexadentate conformation; thus, a slope close to 1 would be expected (Figure 14), as a result of the high complex stability and consequent effectiveness of complexation. The observed results may be due to the lower efficiency for chelating iron at this pH and/or due to the presence of other(s) siderophore(s) with a different stoichiometry (and stability). Due to the lack of information of the siderophores produced by *P. allii* and the results shown in Figure 14, it is possible that the siderophores produced are not of the 1:1 type, as it is the case of enterobactin, desferrioxamine, and aerobactin, which were described for others species of *Pantoea* (Soutar and Stavrinides, 2018). Nonetheless, in case of a 1:1 complexation, the complexed formed may not be very strong and, thus, not capable of complexing most iron added at pH 9.

3.4. Overall comparison of bacteria performance

In order to select the strain(s) producer(s) of siderophores with potential ability to be subsequently tested for correcting IDIC in plants, the bacteria's performance was compared, regarding the following characteristics: iron complexation capacity at pH 9.0; amount of siderophore produced; type; growth and handling of the strain: growth rate, nutritional requirements (culture media composition) and easiness of biomass separation from the culture medium (Table 6).

Table 6. Summary of the main properties of the bacteria studied.

Bacteria	Average complexation capacity at pH 9 (µmol/L)	Siderophore type [a]	Growth & Handling [b]
Azotobacter vinelandii	188	C+H	+++
Bacillus megaterium	280	H	+++
Bacillus subtilis	225	C	+
Pantoea allii	weak	H	+
Rhizobium radiobacter	weak	C	+

[a] Siderophore type: C = catecholate; H = hydroxamate.

[b] Growth & handling: it was considered the growth rate of the bacteria, culture media composition and easiness of the bacterial removal by centrifugation/filtration.

A. vinelandii, *B. megaterium* and *B. subtilis* revealed to be the bacteria with the best performance since the respective medium filtrate had the ability to complex iron efficiently at pH 9.0 with the highest iron complexation capacity (Table 6). This is an indispensable condition in order for the culture filtrates to be used in the preparation of a siderophore concentrate intended to for IDIC amendment in calcareous soils. Also, the culture media used for both bacterial growth are cheap since they are only composed by mineral salts and glucose. In the case of BM (used for *A. vinelandii*), the medium is selective due to the lack of nitrogen source, which reduces the risk of culture contamination. The slow growth of *A. vinelandii* is not a hindrance to the siderophore production since the maximum concentration is achieved at 72 h, which is a culture time in the same order of magnitude of the other bacteria studied. Probably, due to its size and clump formation (Figure 7, Figure 10), biomass separation of *A. vinelandii* and *B. megaterium* by centrifugation/filtration is easy to perform. However, in *B. megaterium* case, sporulation should be avoided as spores will

make filtration more difficult. To avoid this problem, the bacteria should not be left to grow more than 48 h (Figure 8). Biomass separation of *B. subtilis,* by centrifugation/filtration, presented some difficulties, which can be attributed to the relatively small size of the bacterium (Figure 7). It also suffers from sporulation issues as *B. megaterium.*

The siderophores produced by *P. allii* and *R. radiobacter,* presented inefficient iron-complexation capacity at pH 9.0 (Table 6), which prevents their use for agricultural purposes. *P. allii* suffers from the same handling issues reported for *B. subtilis*. Probably, due to their small size (Figure 7), these bacteria easily clog the pores, being hard to filter.

4. Conclusions

In the present chapter, the performance of five bacteria was studied in view of their potential use for IDIC amendment in calcareous soils. *A. vinelandii*, *B. megaterium and B. subtilis* were the most promising, taking into account their strong iron complexation capacity at pH 9.0 (this is an important parameter if correction of IDIC in plants grown in calcareous soils is the aim) and the siderophore type produced (catechol and hydroxamate). The three bacteria also presented the maximum siderophore production, evaluated by CAS assay, between 24 and 72 h, did not require expensive ingredients in culture media formulation and are easily removed (except *B. subtilis*) from the culture medium by centrifugation/filtration.

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Chapter 4

Evaluation of the efficacy of two freeze-dried iron fertilizers, of biological origin, for correcting iron chlorosis of soybean plants grown in calcareous soils[*†]

* Work submitted to an international peer-review journal.
† Work from this and previous chapter were used for the submission of a patent registration.

1. Introduction

Iron (Fe) deficiency-induced chlorosis (IDIC) is a yield limiting factor in a large range of crops world-wide (Hansen et al., 2006), especially severe in calcareous soils. It is estimated that calcareous soils cover about 30 % of world´s land surface (Hansen et al., 2006). Among field crops, soybean is one of the most studied species suffering Fe deficiency chlorosis. Despite extensive study, the incidence of Fe deficiency is increasing and it has been estimated to have expanded by 160% in the last three decades, potentially affecting nearly two million hectares (Hansen et al., 2006). Thus, alleviation of Fe-chlorosis in soybean crops remains a major agronomic problem.

Although commonly found in soil, due to its low solubility and dissolution kinetics, Fe availability for plants is very limited, especially in alkaline and calcareous soils (Mengel, 1994; Shenker and Chen, 2005). Fe is essential for, but not only, chlorophyll synthesis; as a result, insufficient Fe in leaves causes low chlorophyll levels and the consequent yellowing of leaves (Chatterjee et al., 2017; Lucena, 2000). As a consequence, many crop yields are negatively affected and impaired by low Fe bioavailability in soils (Martins et al., 2017).

The most common practice employed to overcome Fe deficiency in plants is the application of synthetic Fe chelates, mostly aminopolycarboxylates (APCAs) compounds, such as ethylenediaminedi(o-hydroxyphenylacetic) acid (*o,o*-EDDHA). Among them, *o,o*-EDDHA/Fe(III) chelate is the most effective Fe fertilizer in neutral and alkaline soils (Bin et al., 2016; García-Marco et al., 2006). In general, these compounds are strong chelating agents but they are not biodegradable (Bucheli-Witschel and Egli, 2001). So, they accumulate in the

environment and their effects on metal bioavailability is of growing environmental concern (Nowack, 2008). In addition, only cash crops can be treated with *o,o*-EDDHA/Fe(III) and other synthetic chelates owing to their prices.

Therefore, the study of environmental-friendly alternatives is important. Even though many recent works have been conducted in this perspective (Ferreira et al., 2019; López-Rayo et al., 2019; Martins et al., 2018; Nikolić, 2018; Santos et al., 2016), the search of alternative pathways for producing environmental-friendly chelators for Fe fertilization still continues an open issue and deserves further research.

In this respect, one alternative may be the use of siderophores. These compounds are low weight molecules (between 500 to 1500 dalton) produced biologically with a great affinity (1:1 stability constants of Fe(III)-siderophores complexes range from 10^{23} to 10^{50}) and selectivity to bind Fe(III). Siderophores are produced by a wide range of microorganisms, such as bacteria, fungi or cyanobacteria, as well as by some graminaceous plants (Hider and Kong, 2010). Being naturally produced, siderophores have the advantage of being better biodegradable than synthetic APCAs (Crumbliss and Harrington, 2009; Fazary et al., 2016; Pierwola et al., 2004). Moreover, literature describes the production of a significant amount of siderophore(s), when microorganisms were grown in an iron-deficient mineral medium (Santos et al., 2014; Tschierske et al., 1996). These facts, together with others that demonstrated that microbial cultures containing siderophores were able to supply Fe to plants (Chen et al., 2000; Hördt et al., 2000; Yehuda et al., 1996) when cultivated in hydroponic

medium, suggest that siderophores may have potential to substitute the synthetic APCAs traditionally used for Fe fertilization in calcareous soils.

However, the direct application of microbial cultures containing siderophores for fertilizing soils raises several problems. The handling (transport and storage) and direct application of the supernatants of the microbial culture containing siderophore(s) for fertilizing soils is not a feasible solution due to the large volumes needed. Moreover, as it was mentioned above, siderophores are natural compounds and, thus, potentially biodegradable; therefore, a long storage period of the supernatants of these cultures will certainly lead to partial or complete degradation of the siderophores with a consequent decrease of its concentration. To overcome these problems, an innovative approach, based on the preparation of freeze-dried iron products from the supernatants of microbial cultures, can be a solution to be directly applied on calcareous soils after a previous appropriate rehydration process.

In previous chapter (Chapter 3), it was demonstrated that the medium filtrates of two cultures of *Azotobacter vinelandii* and *Bacillus subtilis* bacteria, grown in iron-deficient mineral medium, revealed high efficient complexation capacity for Fe(III) at pH 9.0. These results are encouraging and prompted us to investigate the potential of these three cultures as alternative environment-friendly chelators for Fe fertilization. Therefore, in the present chapter, the freeze-dried products of the three medium microbial filtrates were prepared and, subsequently, their potential for correcting iron deficiencies of soybean plants grown in calcareous soil conditions was checked. For this purpose, their interactions with soil components and soil, their stability, and their efficiency in mending IDIC in soybean plants grown in calcareous soil were studied

2. Materials and methods

2.1. Microorganisms used and growth conditions

In this study, three bacteria strains were used: *Azotobacter vinelandii* Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSM) 2289, *Bacillus megaterium* American Type Culture Collection (ATCC) 19213 and *Bacillus subtilis* DSM 10. *A. vinelandii* was grown in Burk´s medium (BM) (Newton et al., 1953) whereas *B. megaterium* and *B. subtilis* were grown in a mineral minimal medium (MM). Media compositions were the same as described in Chapter 3.

2.2. Optimization of freeze-dry conditions

Due to the inherent logistical problems of using high amounts of filtrate solution, a solid-state concentrated product is desirable. The lyophilization of the filtrate solution was the chosen method for this purpose. As freeze-dried filtrates were highly hygroscopic, a caking agent was needed.

Corn starch was used as anti-caking agent for stabilizing the freeze-dried end product. The optimal quantity of corn starch to be added to the filtrate medium was studied. For this end, small filtrate samples with varied concentrations of corn starch were freeze-dried and the resulting powder was evaluated for its consistency and hygroscopicity.

2.3. Preparation of the freeze-dried product

Bacterial filtrates were produced as previously described (Chapter 3). Shortly, bacteria were grown in Fe depleted media until siderophore

concentration was stable (72, 48, and 48 h for *A. vinelandii B. megaterium* and *B subtilis*, respectively). Bacterial biomass was pelleted by centrifugation, and the supernatant was filtered through a 0.45 µm pore size filter. The resulting filtrate was stored at -20 ºC until use. For soil applications, the resulting filtrate was mixed with corn starch (5 and 15 $g.L^{-1}$ for *B. subtilis* and *A. vinelandii*, respectively, *B. megaterium* was not applied), which was used as anti-caking agent. Then, the mixture was freeze-dried (Labconco FreeZone 2.5 L coupled with a VacuuBrand RC 6 pump, USA). The resulting powder was homogenized and stored at 4 ºC until use.

2.4. Preparations of iron siderophore solutions

2.4.1. Solution and soil interaction assays

To study the suitability of each bacterial media for Fe chlorosis remediation in soil applications, an iron-siderophore solution (ISS) was prepared by re-hydrating the previously obtained powders to the initial concentration of siderophore. Then, a soluble salt of Fe(III), $FeCl_3.6H_2O$ (Sigma), was added to the maximum complexation capacity at pH = 9.0 (*A. vinelandii* ≈ 188 $\mu mol.L^{-1}$, B. megaterium ≈ 280 $\mu mol.L^{-1}$ and *B. subtilis* ≈ 225 $\mu mol.L^{-1}$) (Chapter 3). During the process, the pH of the solution was kept between 7.0 and 9.0 and the final pH was set at 9.0. The solution was allowed to settle overnight and filtered (0.45 µm pore size filter). The initial Fe concentration was then measured by atomic absorption spectroscopy with flame atomization (AAS-FA) using a Perkin-Elmer AAnalyst 400 spectrometer (Norwalk, CT, USA).

2.4.2. Application in calcareous soil and soybean response

For soybean chlorosis amendment, a more concentrated ISS was used. Therefore, ISS were produced by dissolving the powder, obtained previously, in deionized water (20 and 120 $g.L^{-1}$ for *B. subtilis* and *A. vinelandii*, respectively) and a soluble salt of Fe(III), $FeCl_3.6H_2O$, was added to achieve 120 and 110 $mg.L^{-1}$ of Fe for *B. subtilis* and *A. vinelandii*, respectively. In the process, the pH of the solution was kept between 7.0 and 9.0 and the final pH was set at 9.0. The solutions were allowed to settle overnight and filtered (0.45µm pore size filter). The initial Fe concentration was then measured in a similar way as described in section 2.4.1.

2.5. Equilibrium and stability of Fe-chelates in soil

To understand the suitability of each ISS for Fe chlorosis amendment of soybean plants in soil applications, a sequence of tests was performed.

The first test addressed the effect of pH solution and the presence of Ca^{2+} on the ISS stability. Five sets of replicates (three per set), corresponding to the pH values tested, were prepared by mixing 5 mL of ISS with 5 mL of a buffered solution (10 $mmol.L^{-1}$ HEPES, 10 $mmol.L^{-1}$ MES, 10 $mmol.L^{-1}$ CAPS and 10 $mmol.L^{-1}$ CAPSO) of 100 $mmol.L^{-1}$ $CaCl_2$. The tubes were covered with aluminium foil to prevent light exposure. Then, the pH was adjusted to 7.0, 7.5, 8.0, 8.5 and 9.0 respectively. The tubes were shaken (150 rpm) in an orbital shaker (Grant Ols200, UK) at 25 ºC for 3 days. At the end, samples were filtered (0.45 µm pore size filter), acidified with HNO_3 (1 %) and the soluble Fe was determined as described above.

The second test consisted on the interaction study of ISS with different soils and soil components. For this purpose, 25 mL of each ISS was added to two types of soil (5.0 g): a sandy clay soil from Picassent (Valencia, Spain) and a standard soil prepared in our lab, as described by Álvarez-Fernández et al. (1997); individual soil constituents were also tested, as described elsewhere (Álvarez-Fernández et al., 1997): $CaCO_3$ (2.0 g), Ca – montmorillonite (0.5 g), goethite (0.25 g), ferrihydrite (0.25 g) and organic matter (0.5 g). Tubes were covered with aluminium foil to prevent light exposure and each experimental condition was conducted in triplicate. Samples were shaken (150 rpm, 1 h) in an orbital shaker (Grant Ols200, UK) and then left to stand for 3 days at 25 ºC. At the end, samples were filtered (0.45 µm pore size filter), acidified with HNO_3 (1 %) and the soluble Fe was determined by AAS, as described above.

The third and last test was conducted using a mixture of the soils with calcareous sand (50/50 %), for a total of 5 g. To the mixture, 0.5 mL of ISS was added, intercalating with 0.5 mL of deionized water, before and after (for a total of 1.5 mL added). This resulted in a more reactive condition as the soil:solution ratio was higher [soil:solution ($^{g}/_{ml}$) = 3.33] compared to the one used in the previous experiment [soil:solution ($^{g}/_{ml}$) = 0.20]. Samples were kept in the dark at 25 ºC for 3 days and the moisture level of the soils was checked regularly and maintained by adding water when necessary. Three replicates were done for each ISS. At the end of the 3 days, the soluble Fe was extracted by adding 10 mL of water to each sample, which was then manually shaken and filtered. Then, the soluble Fe was determined by AAS-FA, as described above.

2.6. Evaluation of the soybean response to Fe chelates application in calcareous soil

2.6.1. Experimental setup

Firstly, *Glycine Max* cv. seeds were germinated in perlite on sterile trays placed in distilled water. Trays were kept at 30 ºC and 60 % relative humidity for four days and water level inside trays was monitored to prevent dryness.

Seedlings were then examined, selected and transferred to a tray, with a perforated plate floating on top of nutrition solution [macronutrients (mmol.L^{-1}) 1.0 $Ca(NO_3)_2$, 0.9 KNO_3, 0.3 $MgSO_4$, and 0.1 KH_2PO_4; buffered micronutrients (µmol.L^{-1}) 2.5 $MnSO_4$, 1.0 $CuSO_4$,10 $ZnSO_4$, 1.0 $NiCl_2$, 1.0 $CoSO_4$, and 115.5 Na_2EDTA; other micronutrients (µmol.L^{-1}) 35 NaCl, 10 H_3BO_3, and 0.05 Na_2MoO_4. The pH was buffered with HEPES 0.1 mmol.L^{-1} and adjusted to 7.5 with KOH 1.0 mol.L^{-1}] (Nadal et al., 2012), and left for 7 days. Then, plants were transferred to the experiment pots.

Methacrylate cylinders (7 cm diameter, 16 cm high) were used as pots, coated with aluminium foil in the outside to protect the content from light and a piece of mesh on the bottom to allow aeration. A mixture of sandy clay soil (70 %) from Picassent (Valencia, Spain) and calcareous sand (30 %) in a total of 0.6 kg was used to fill the pots. Two days prior to the plants transfer, pots were irrigated to 80 % of the soil water capacity, and henceforth, watered with sufficient solution to preserve it. The irrigation was conducted using a pH buffered nutrition solution containing 0.1 g.L^{-1} of $CaCO_3$ and 0.1 g.L^{-1} of $NaHCO_3$ (pH = 8.0 - 8.5). The pots were placed on petri dishes plates in order to control lixiviation. The experiment was carried out in a growth chamber

(Dycometal-type CCKF 0/16985) using a daily cycle of 16 h/ 8 h (day/night); at day: 25 ºC and a relative humidity of 40 %; at night: 20 ºC with a relative humidity of 60 %.

A total of six pot sets, were prepared: one without any treatment (negative control, C-), one for *o,o*-EDDHA (Positive control, C+), two for *A. vinelandii* ISS (A) and two for *B. subtilis* ISS (B). Each pot set had five pots, totalling five pots for negative control, five for positive control, and ten for *A. vinelandii* [A and A(2)] and *B. subtilis* ISS [B and B(2)], respectively, according to the scheme presented in Figure 15. To each pot (unless negative control pots), enough volume of chelate or ISS solution was applied in order to have 2.5 mg of Fe(III) per kg of soil. At the 15th day after the first treatment applications, to one set (five pots) of each *A. vinelandii* and *B. subtilis* sets [A(2) and B(2), respectively], a second treatment was applied (Figure 15). Plants were harvested 7 and 21 days after treatment (DAT) application. Major nutrients (K^+, Mg^{2+}, PO_4^{3-}, NO_3^-, NH_4^+, Mn^{2+} and Ca^{2+}) present in ISS were quantified and balanced by the addition of appropriate nutrient solutions in order that the same quantity of major nutrients was present in all pot sets.

2.6.2. Plant and soil analysis

The level of plant “greenness” was carried out using a soil and plant analyser development (SPAD) meter (Minolta SPAD-502; Minolta, Osaka, Japan). This equipment allows the evaluation of the chlorophyll index of plant leaves without damaging the leaf. SPAD index was evaluated in all leaf stages from the cotyledons to the last developed plant stage.

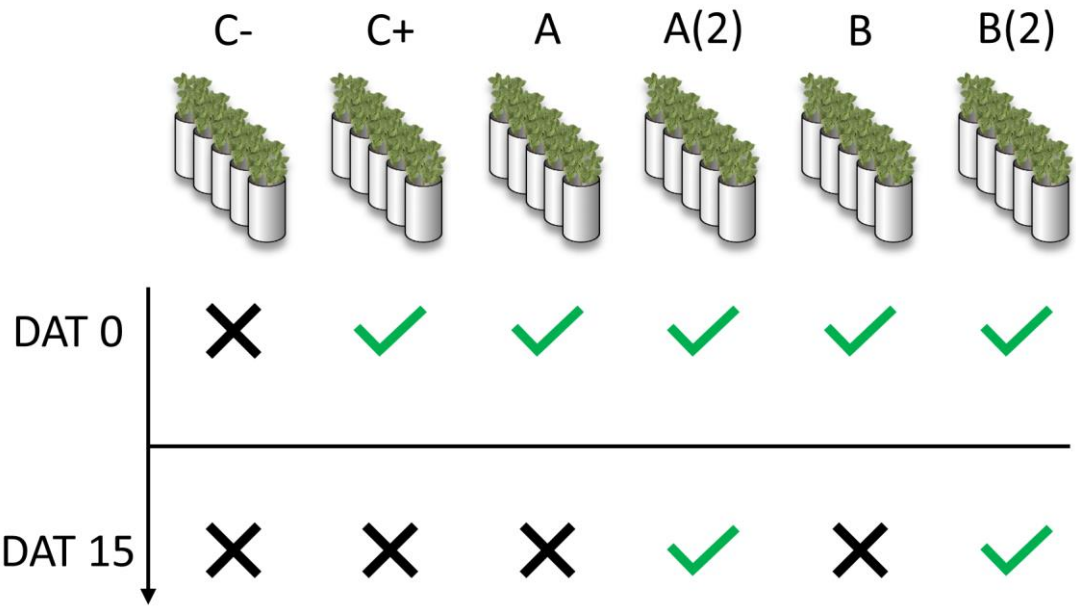

Figure 15. Treatment schedule for application of iron siderophore solutions (ISS) to chlorotic soybean in calcareous soils. DAT: Days after treatment; C-: Negative control; C+: positive control (o,o-EDDHA); A: A. vinelandii *ISS; B:B. subtilis ISS.*

At the seventh DAT, two plants shoots were harvested. At 21st DAT, the remaining plant was harvested, and all roots recovered from the soil. Plant tissues were washed with 0.1 % hydrochloric acid (HCl) and 0.01 % of a non-ionic detergent solution (Tween 80, Probus, Barcelona, Spain) to remove inorganic contaminants and dirt, and, subsequently, rinsed twice with distilled water. Then, the leaves, stems and roots were separated and dried in a force air oven at 60 ºC for 72 h. Once dried, the plant parts were weighted for the dry mass determination and calcinated at 480 ºC. Fe concentration was analysed by AAS-FA, as described above, after acid digestion of the resulting ashes with nitric acid (HNO_3) (Merck).

The remaining soil in each pot was also analysed for Fe speciation, namely, the soluble and available Fe concentrations. For this purpose, two

subsequent extraction steps were undertaken. Firstly, the aqueous soluble Fe fraction was extracted: the total soil content of the pot was submerged and mixed with 600 mL of distilled water and shaken for 10 minutes. Then, 40 mL of the mixture was centrifuged, the supernatant filtered (20 - 25 µm pore size filter) and acidified with HNO_3 (Merck). Secondly, the available Fe was extracted: 25 ml of an extractant solution [diethylenetriaminepentaacetic acid (DTPA) and ammonium bicarbonate] (Soltanpour and Schwab, 1977) was added to the remaining soil after the previous extraction and the mixture was shaken in an orbital shaker (180 rpm, 10 min). After centrifugation, the supernatant was filtered through a filter paper (20 - 25 µm pore size) and recovered. This procedure was repeated three times and the extracts were joined. Enough HNO_3 (65%) (Merck) was added to neutralize the present ammonium bicarbonate and, then, to acidify the solution; the final volume was set to 100 mL. For all samples, the concentration of Fe was analysed as described previously.

2.7. Data statistical analysis

All solution and soil interactions of ISS experiments were conducted at least twice, independently, with three replicates each, for a total of at least six samples. For soybean response to ISS application, each set was composed of five replicates. The SPAD indices were read in triplicate for each leaf stage, in all five replicates, for a total of up to 15 (or 45, for measurements before seventh day after treatment) measurements per level and per treatment. Unless stated otherwise, data is presented as mean ± standard deviation.

The statistical analysis was performed using SPSS software (Ver. 25, SPSS Inc., Chicago, USA). When comparisons were required, an analysis of

normality was first conducted using the Shapiro-Wilk test (a 99 % level of confidence was used), followed by a Levene test of homogeneity of variance (95 % confidence level). When normality and homogeneity of variance assumptions were verified, an ANOVA test was conducted to assess the significance of differences. A post-hoc test was also used, either a Dunnett t test (> negative control) or a Tuckey HSD test (95 % confidence level), to compare differences to the negative control or to create subgroups of samples, respectively. Otherwise, when the two assumptions failed, the non-parametric Kruskal-Wallis test was conducted with a post-hoc pairwise comparison for subgrouping of sample sets.

3. Results and discussion

3.1. Lyophilization optimization

The stability of the end product is important, and optimization efforts with that goal were conducted. The main issue of the freeze-dried product is its hygroscopicity. The product quickly reacts with air moisture and absorbs it, turning into a slime. The increased water content makes the product more susceptible to microbial degradation, for example. Therefore, an anti-caking agent, corn starch, was tested.

Increasing concentrations of corn starch were tested from 0.5 to 15 $g.L^{-1}$. For *A. vinelandii*, only samples with 15 $g.L^{-1}$ have shown to be moisture resistant, where all others still retained some degree of hygroscopicity. For *B. subtilis*, samples at 5 $g.L^{-1}$ were already moisture resistant. The resulting powder can be seen in Figure 16. This corn starch concentration was hereafter

used for all lyophilization procedures. For applications, the powder is dissolved in distilled water and the corn starch is removed from solution by filtration.

With this method, a more stable product is achieved due to lower moisture level of the sample. Also, by reducing the product to a powder, it is easier to transport and store than the equivalent supernatant. As an additional advantage, a product containing siderophores at high concentration can be obtained by this method since one can re-hydrate the powder in a lower volume than the original one.

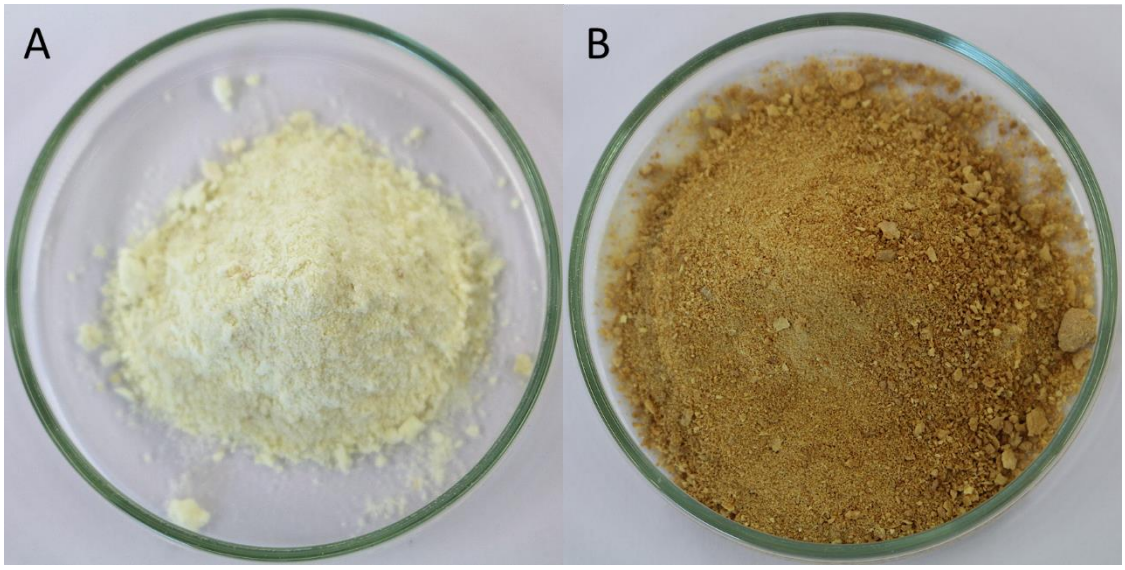

Figure 16. Samples of A. vinelandii (A) and B. subtilis (B) freeze-dried product with corn starch at concentrations of 15 and 5 g.L^{-1}, respectively.

3.2. Calcareous soil interactions

As a first step, the suitability of the ISS was evaluated for IDIC amendment and their properties were studied by performing solution and soil interaction studies. These assays provide an idea of how effective (or ineffective) a Fe-chelating solution agent might be. They may also provide information of

possible interactions, which may occur in the calcareous soils. These data are potentially useful for predicting the outcome in field application as well as will help on understanding the results obtained later.

3.2.1. ISS stability for soil application

The stability of ISS Fe-chelates in the presence of Ca(II) (as a solution of 50 mmol.L^{-1} $CaCl_2$) was evaluated at different pH values. The media obtained from *A. vinelandii* exhibited greater stability at three days than the one from *B. subtilis*. In the case of *A. vinelandii*, about 60 % of the initial Fe remained solubilized while less than 10 % was found for *B. megaterium* and *B. subtilis.* For each bacterial ISS, there was no substantial differences in the Fe-chelate stability efficiency within the pH range tested (Table 7).

In Chapter 3, it was verified that the siderophores produced by both bacteria evidenced high affinity for Fe, especially at neutral to alkaline pH. So, based on our previous results, a higher Fe complexation efficiency (namely with *B. megaterium* and *B. subtilis* ISS) would be expected in the presence of Ca(II) solution. The fact that part of Fe precipitated after three days may be due to one, or a combination, of the following reasons: 1) the simple inability of the ISS Fe-chelates to compete with the huge amount of Ca(II) ions present in solution; 2) degradation of the Fe-chelates over time; 3) the presence of a mix of different Fe-chelates with different stabilities; 4) co-precipitation phenomena that took out Fe from the solution.

The first hypothesis seems to be unlikely. Literature describes the values of the Fe-chelate constants for the main siderophores produced by *A. vinelandii*, and *B. subtilis*. For example, in the case of protochelin, the tri-catecholate

siderophore produced by *A. vinelandii*, the Fe formation constant is $10^{43.9}$, identical to that of ferrioxamine B (Cornish and Page, 1998), whereas for bacillibactin, the tri-catecholate produced by *B. subtilis*, the Fe formation constant is equally high ($10^{47.6}$) (Dertz et al., 2006). No formation constant was found for *B. megaterium*'s schizokinen, however, aerobactin, a di-hydroxamate similar to schizokinen has a formation constant of $10^{22.9}$ (Harris et al., 1979). To our knowledge, no formation constants for any of these siderophores with Ca(II) were described; therefore, a theoretical analysis of the competition of Ca(II) for the binding sites is not possible. Nonetheless, the catecholate moieties are very selective to hard trivalent metal ions (Hider and Kong, 2010), such as Fe(III), relatively to divalent ions as is the case of Ca(II). The decrease of selectivity from trivalent to divalent ions is notoriously visible in the decrease of the formation constants values from Fe(III) to Fe(II) (Hider and Kong, 2010). Based on all these facts, it seems that Ca(II) competition in solution should not be the major deterring factor.

Table 7. Influence of the pH on the stability of the ISS Fe chelates

Bacterial origin of ISS	**$Fe_{solublized}$ (%) vs pH [a]**				
	7.0	**7.5**	**8.0**	**8.5**	**9.0**
A. vinelandii	64.7 ± 6.2	59.2 ± 5.2	60.0 ± 3.9	56.2 ± 4.8	61.8 ± 5.2
B. megaterium	6.7 ± 0.7	4.8 ± 0.2	6.7 ± 0.3	7.2 ± 0.2	9.7 ± 0.9
B. subtilis	4.6 ± 0.5	4.7 ± 1.5	5.0 ± 1.3	5.5 ± 2.1	6.6 ± 5.6

[a] *The amount of Fe in solution was measured after three days of equilibrium and expressed in % relatively to the initial Fe added. The data corresponds to the mean values and standard deviations of two independent experiences performed in triplicate (n=6).*

On the other hand, the second possibility is somewhat feasible: siderophores, as natural products, are likely to be biodegradable. Also, the abiotic degradation of siderophores has also been reviewed (Harrington et al., 2015). As most factors involved in the abiotic degradation were not present, abiotic degradation is not expected. However, degradation by biotic or abiotic processes is a possibility and may explain part of the decrease in the Fe(III) chelation efficiency.

The third reason is also plausible since other different Fe-chelating molecules may be present at different quantities. Different media compositions are expected due to the different culture media initially used for growing the bacteria as well as different exudates produced by the bacteria. Different mild Fe chelating agents may be present, which are capable to complex Fe(III). One such example may be gluconic acid, resultant from the activity of glucose dehydrogenase, produced during the sporulation of *B. subtilis* (Fujita et al., 1977), which is known to complex Fe(III) (Sawyer, 1964). However, the resulting complexes are likely weak and lose their capacity when disturbed by the addition of high concentrations of Ca(II).

The occurrence of co-precipitation as a cause of Fe loss is also possible. In fact, during the experimental procedure, it was possible to see that upon the addition of Ca(II) to the *B. subtilis* ISS, a white-reddish precipitate was formed and a change in solution hue and saturation was also observed, indicating the precipitation of the iron previously present in solution. In *A. vinelandii* medium, such behaviour was not observed. Different concentrations of phosphate in ISS, as a result of different initial phosphate concentrations used in the two different growth media (higher in *B. subtilis* than in *A. vinelandii*), may explain the distinct

results. The presence of different organic exudates may also help to explain the discrepancies for the two ISS.

In order to have more clues regarding the interaction and properties of both media with soil, ISS of the three bacteria were tested in solution with soil and with the main components of the soil (Table 8).

Table 8. Influence of soil and soil components on the stability of ISS Fe chelates

	Bacterial origin of ISS		
	A. vinelandii	***B. megaterium***	***B. subtilis***
Solid phase (in solution)	**$Fe_{solublized}$ (%)[a]**		
Montmorillonite-Ca	50.7 ± 4.1	0.8 ± 0.5	8.7 ± 5.3
Peat	77.1 ± 5.3	17.21 ± 8.5	50.4 ± 10.7
Goethite	99.2 ± 7.0	4.3 ± 2.1	38.8 ± 9.6
Ferrihydrite	78.8 ± 9.8	1.6 ± 1.3	80.0 ± 7.4
Calcium carbonate	92.5 ± 6.5	4.5 ± 5.9	13.4 ± 5.4
Picassent soil	53.3 ± 12.5	0.4 ± 0.2	10.8 ± 3.5
Standard soil	31.2 ± 3.6	0.3 ± 0.3	9.4 ± 4.7
Solid phase (wet condition)			
Picassent soil	36.9 ± 7.9	9.1 ± 3.4	16.6 ± 6.3
Standard soil	41.5 ± 17.6	10.8 ± 3.1	8.7 ± 3.0

[a] *The amount of Fe in solution was measured after three days of equilibrium and expressed in % relatively to the initial Fe added. The data corresponds to the mean values and standard deviations of two independent experiences performed in triplicate (n=6).*

Again, different bacteria media have shown a different outcome. Whereas *A. vinelandii* ISS has shown lower retention profiles, *B. megaterium* and *B.*

subtilis ISS showed greater losses by the end of the three days experiment. This behaviour was visible with the soil components as well as with the soils.

Interestingly, the behavior of the three ISS in the presence of $CaCO_3$ mimics the profile recorded in the previous experiment. While, in the case of *A. vinelandii* medium, an average of 92 % of the Fe initially added was recovered at the end of the experiment, in the case of *B. subtilis* medium, only 13 % of Fe was recovered and in the case of *B. megaterium* only about 4.5 % was recovered. The lower precipitation of Fe observed for both *A. vinelandii* and *B. subtilis* ISS, compared to the previous experiment, may be related to the lower concentration of Ca(II) in solution. While in the previous experiment, $CaCl_2$ was dissolved to 50 mmol L^{-1}, the latter was conducted in the presence of less amount of soluble $CaCO_3$. Based on the solubility product of $CaCO_3$ ($10^{-8.48}$) (Martell and Smith, 2004), the concentration of free Ca soluble under these conditions should be under 5 mmol.L^{-1}, which is substantially lower than the value previously used (50 mmol.L^{-1}). Therefore, it is plausible that the greater precipitation previously observed on *B. subtilis* ISS is correlated with the formation or presence of inorganic Ca precipitates, such as calcium phosphates, or other inorganic/organic compounds. *B. megaterium* lower recovery of Fe may instead be a result of adsorption to $CaCO_3$ phenomena instead. In any case, in all solutions, Ca-Montmorillonite was the soil component that evidenced higher retention; at the third day, 49.3, 99.2 and 91,3 % of iron was retained in the case of *A. vinelandii*, *B. megaterium* and *B. subtilis* ISS, respectively. This observation is in agreement with the observations that siderophores produced by microorganisms are adsorbed in the clays (Siebner-

Freibach et al., 2004). The substantial difference of adsorption between both may be due to the difference of ionic composition of the media, which was shown to influence the adsorption of siderophores to montmorillonite-Ca (Siebner-Freibach et al., 2004). Regarding the iron (oxy)hydroxides tested, the interaction of ferrihydrite with *A. vinelandii* and *B. subtilis* media resulted in similar retention rates (approximately 80 % of Fe remained soluble at the end of the third day experiment), while *B. megaterium* ISS lost most iron. Hydroxamate siderophores, such as schizokinen, are known to adsorb to minerals (Harrington et al., 2015) which may be responsible for the loss of Fe from solution in *B. megaterium* ISS case. In contrast, goethite had different behaviours with ISS interaction: for *A. vinelandii* ISS, an average of 99 % Fe was found in solution while only 39 % was found in the case of *B. subtilis* ISS, and *B megaterium* had the lowest quantity found, with only 4 %. In fact, for some *A. vinelandii* ISS samples, the content of Fe dissolved quantified at the end of the experiment was higher than in the beginning. These substantial differences may be the result of two actions taking place: adsorption of siderophores to the minerals surface and promotion of mineral dissolution by siderophores and other organic acids present in solution (Kraemer, 2004). The presence of organic acids can be also an important factor on the dissolution of goethite (Vinet and Zhedanov, 2011).

In a previous work, our research group has carried out similar tests with two synthetic siderophores, one being azotochelin, a catecholate siderophore produced by *A. vinelandii* (Ferreira et al., 2019). Regarding the stability of Fe(III) in the presence of Ca(II) solution, our results are in fair agreement with those obtained for azotochelin-Fe(III) solution. Azotochelin evidenced lower

interactions with the different soil components than *A. vinelandii*'s ISS. The differences found may be attributed to a different siderophore composition and/or the presence of other organic and inorganic compounds in solution. The variances in the siderophore composition can justify some differences in the adsorption profiles; however, the presence or absence of other organic molecules justifies better the discrepancies. For example, ferrihydrite is more soluble than goethite, and more easily dissolved by siderophores when acting alone (Kraemer, 2004) whereas goethite dissolution may be improved by the presence of organic acids (Vinet and Zhedanov, 2011), which would justify the increase in *A. vinelandii* ISS case. On the contrary, the presence of different electrolytes in *A. vinelandii* ISS (and the absent in azotochelin study) may be a key element in stabilizing the adsorbed siderophores on the other solid phases tested.

Compared to other synthetic chelating agents, such as *N,N'*-bis(2-hydroxyphenyl)ethylendiamine-*N,N'*-diacetic acid (HBED), *N,N'*-bis(2-hydroxy-5-methylphenyl)ethylendiamine-*N,N'*-diacetic acid (HJB) or *o,o*-EDDHA (López-Rayo et al., 2009), both *A. vinelandii* ISS and particularly both *Bacillus* ISS underperform under similar conditions. However, compared to other natural based chelating agents, namely lignosulfonates, amino acids, gluconates, and humates (Lucena et al., 2010), and despite the slightly worse stability that *A. vinelandii*'s ISS evidenced in Ca(II) alkaline solutions, these compounds underperform when in contact with soils or soil components compared to *A. vinelandii*'s ISS.

3.2.2. Chelate stability in soil

With the purpose to mimic the soil irrigation under agronomic conditions, a smaller volume of chelate solution may be applied to simulate soil experiments. This set up was considered by many authors as a more ideal experimental setting (Hernández-Apaolaza and Lucena, 2011; Limousin et al., 2007). The lower solution/soil ratio is preferred because it allows a better observation of the chelates retention, which are difficult to study when high solution/soil ratios are used (Hernández-Apaolaza and Lucena, 2011). The results obtained for both bacterial ISS can be seen at Table 8 [see Solid phase (wet condition)]

At the end of the three-day experiment, higher losses in soluble Fe were found for *B. megaterium*'s ISS, and then for *B. subtilis'* ISS, compared to *A. vinelandii's* ISS: about 37, 17 and 9 % of initial Fe was recovered in Picassent soil for *A. vinelandii*, *B. subtilis* and *B. megaterium* ISS, respectively, while about 41, 9 and 11 % were recovered for the standard soil. The results obtained with this setup mimic those found for the solution experiments described earlier. Contrary to what was observed with azotochelin-Fe solutions (Ferreira et al., 2019), not only no substantial difference between both soils under test was found for *A. vinelandii* ISS, but also no substantial decrease was found when moving to the more intimate soil interaction. For both *Bacillus* ISS, the results obtained for this experiment were also in loose agreement with those obtained with the previous solution experiments. The results from all ISS seem to indicate that no major changes on the adsorption profile occurs when the solution/soil ratio is changed.

The higher retentions on the standard soil may be a result of the finer gain of the soil and high clay composition. Despite the low Fe recovery results, the

average Fe concentration found in the soil solution in all experiments was found to be in the range between 10^{-4} to 10^{-5} mol.L^{-1}. These concentrations can be considered suitable for soil application since they are within the required Fe concentration range described for plants (10^{-4} to 10^{-9} mol.L^{-1}) (Kim and Guerinot, 2007). However, the very weak results of *B. megaterium* ISS, compared to other two tested ISS, and compared to other chelating agents found in literature, raise serious doubts about its capability to correct IDIC. Therefore, *B. megaterium* was discarded from further tests, and only *A. vinelandii* and *B. subtilis* ISS were further tested.

3.3. Application of iron siderophore solution in calcareous soil and soybean response

3.3.1. Dry plant weight evaluation

At the moment of sampling, plants were sectioned into their different main organs, namely leaves, stems and roots (these last only at the final sampling at 21 DAT). Then, after drying, plant parts were weighted and kept for subsequent determinations (Table 9).

Data from the sampling conducted at the 7th day after treatment (DAT 7) shows no significant difference between the various treatments and the negative control. The stem average weights were particularly even. The leaves average values were even between controls and *A. vinelandii* ISS treated plants while *B. subtilis* ISS treated plants had slightly higher dry weights. However, the scenario changed radically by the 21st day after treatment (21 DAT). Although again, no statistically significant differences ($p > 0.05$) in weight were found, two sets of data can be identified: *o,o*-EDDHA and *A. vinelandii* ISS treated plants

vs negative control and *B. subtilis* ISS treated plants. At 21 DAT, in the first group, the values measured for leaves, stems and shoots were around 0.6, 0.4 and 1.0 g, respectively. However, roots on average had higher dry weights on *A. vinelandii* ISS treated plants than on *o,o*-EDDHA ones. On the other hand, the second group averaged at about 0.4, 0.3, and 0.7 g for leaves, stems and shoots, respectively.

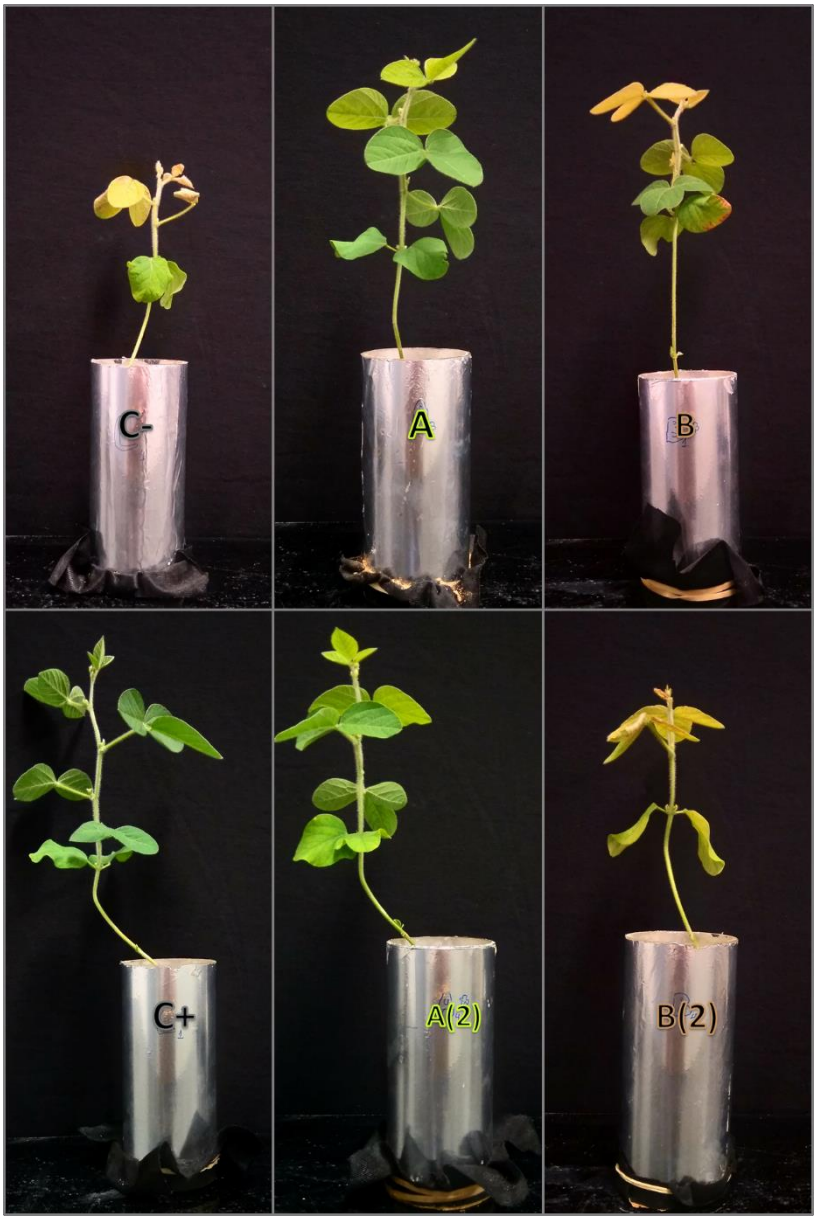

Figure 17. Photographs of representative plants at the 21st day after treatment (DAT 21). Negative control (C-); positive control (C+; EDDHA); Azotobacter vinelandii ISS (A) and Bacillus

subtilis (B) ISS. A(2) and B(2): second application of Azotobacter vinelandii ISS (A) and Bacillus subtilis (B) ISS at 15th day after treatment (DAT 15).

Table 9. Dry weight (g) of the different plant organs versus different treatments [a]

Treatment	Plant organ	Days after treatment		
		7	21	Difference[b]
No Fe (C-)	Leaves	0.38 ± 0.05	0.46 ± 0.16	0.27 ± 0.15
	Stems	0.30 ± 0.05	0.36 ± 0.11	0.21 ± 0.10
	Roots	-	0.58 ± 0.25	-
	Shoots	0.68 ± 0.10	0.82 ± 0.27	0.48 ± 0.24
	Total	-	1.40 ± 0.51	-
o,o-EDDHA (C+)	Leaves	0.36 ± 0.10	0.62 ± 0.10	0.44 ± 0.15
	Stems	0.24 ± 0.08	0.41 ± 0.08	0.29 ± 0.11
	Roots	-	0.56 ± 0.10	-
	Shoots	0.60 ± 0.17	1.03 ± 0.18	0.73 ± 0.25
	Total	-	1.59 ± 0.26	-
A. vinelandii	Leaves	0.38 ± 0.16	0.60 ± 0.09	0.40 ± 0.14
	Stems	0.27 ± 0.09	0.42 ± 0.06	0.27 ± 0.09
	Roots		0.63 ± 0.08	
	Shoots	0.65 ± 0.25	1.02 ± 0.14	0.67 ± 0.22
	Total	-	1.66 ± 0.20	-
A. vinelandii (2)[c]	Leaves	-	0.62 ± 0.10	0.44 ± 0.14
	Stems	-	0.40 ± 0.05	0.28 ± 0.06
	Roots	-	0.67 ± 0.04	-
	Shoots	-	1.02 ± 0.14	0.72 ± 0.20
	Total	-	1.69 ± 0.15	-
B. subtilis	Leaves	0.47 ± 0.17	0.44 ± 0.05	0.19 ± 0.14
	Stems	0.28 ± 0.06	0.33 ± 0.03	0.20 ± 0.02
	Roots	-	0.55 ± 0.13	-
	Shoots	0.75 ± 0.21	0.77 ± 0.08	0.39 ± 0.15
	Total	-	1.31 ± 0.19	-
B. subtilis (2)[c]	Leaves	-	0.50 ± 0.11	0.28 ± 0.09
	Stems	-	0.33 ± 0.06	0.18 ± 0.06
	Roots	-	0.46 ± 0.10	-
	Shoots	-	0.83 ± 0.16	0.46 ± 0.12
	Total	-	1.29 ± 0.25	-

[a] The data corresponds to the mean values and standard deviations of at least 5 plants measured in triplicate (n=15).

[b] Average difference of dry mass in plants from 7th day till 21st day.

[c] Treatments with (2) represent plants with a second application performed fifteen days after initial treatment

The plant mass development can be clearly seen when calculating the difference between DAT 7 and DAT 21 plant mass. On average, leaves, stem and shoots of the plants treated with *o,o*-EDDHA and *A. vinelandii* ISS have grown more 0.2, 0.1 and 0.3 g, respectively, than the negative control and *B. subtilis* ISS treated plants. Moreover, 21 days after treatment application, plants supplied with Fe-*A. vinelandii* ISS or Fe-o,o-EDDHA fertilizers were greener than the negative control (Figure 17). On the other hand, plants of the negative control clearly evidenced chlorosis and had a significant impaired plant development (Figure 17).

3.3.2. Soil and plant analyzer development (SPAD)

The evolution of plant "greenness" was read using the chlorophyll meter SPAD in all leaf stages after the cotyledons to the last developed plant stage. The results for plant stage two can be seen in Figure 18. This stage was chosen because all plants had it fully developed at the start of the trial (DAT 0) and had relatively similar initial values between treatments; therefore, a better comparison of the treatment effect is achievable. As can be seen in Figure 18, plants treated with *o,o*-EDDHA and *A. vinelandii* ISS developed higher levels of chlorophyll than those of the negative control and plants treated with *B. subtilis* ISS. An ANOVA analysis revealed that this difference was significant ($p < 0.05$) from the eight day after treatment (DAT 8) onward until the end of the experiment. A Dunnett t post-hoc test also showed that for all days after DAT 8, *o,o*-EDDHA and *A. vinelandii* ISS treated plant sets were statistically different ($p < 0.05$) than the negative control (Figure 18). These results are comparable to those obtained by Martins et al. (2018) and Ferreira et al. (2019) while testing

synthetic siderophores (or analogues thereof) on hydroponic conditions and calcareous soils, respectively.

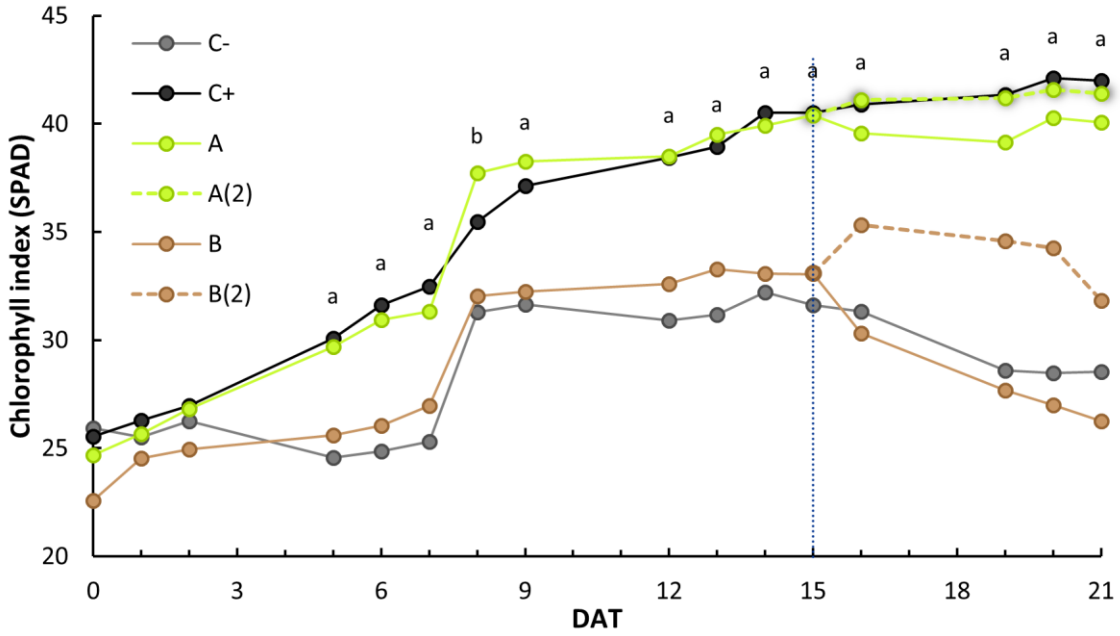

Figure 18. Stage 2 leaves average SPAD index evolution on the course of the 21 days after treatment(s) (DAT). At DAT 15, a second treatment was applied to half of A and B plants [designated as A(2) and B(2), respectively], splitting both sets (n=10) into two sets (n=5). Each plant SPAD was read in triplicate. Different letters denote a significant difference of SPAD levels within each treatment (95 % confidence interval), as shown by Dunnett t test (> negative control)]: a = both o,o*-EDDHA (C+) and A. vinelandii ISS treated plants [A and A(2)] were statistically different (p < 0.05) than the negative control (C-); b = Only A. vinelandii ISS treated plants were statistically different (p < 0.05).*

Further examination was conducted on data from 21 DAT, which allowed to examine the influence of each treatment on each stage level. A quick analysis of Figure 19 allows us to draw three different patterns: *o,o*-EDDHA's (positive control) leaf stages showed no statistically significant differences within all leaf stages; the negative control and both *B. subtilis* ISS treated plants had a sharp decrease from the second to the forth leaf stages whereas fifth stage remained equally low as forth; both *A. vinelandii* ISS treated sets had a

moderate decrease in SPAD from second to forth/fifth leaf stages (Figure 19). Unless for *o,o*-EDDHA, all other treatments had shown a decrease in the effectiveness over time, in particular *B. subtilis* ISS. With the use of *o,o*-HBED and *o,o*-EDDHMA Fe chelates, similar SPAD profiles to those of *A. vinelandii* ISS were obtained for the last fully developed trifoliate 28 DAT (Bin et al., 2016). In this study, authors reported that both chelates performed good, with just slightly lower performance than *o,o*-EDDHA. On the contrary, the effectiveness of *B. subtilis* ISS was comparable to the one registered for the negative control.

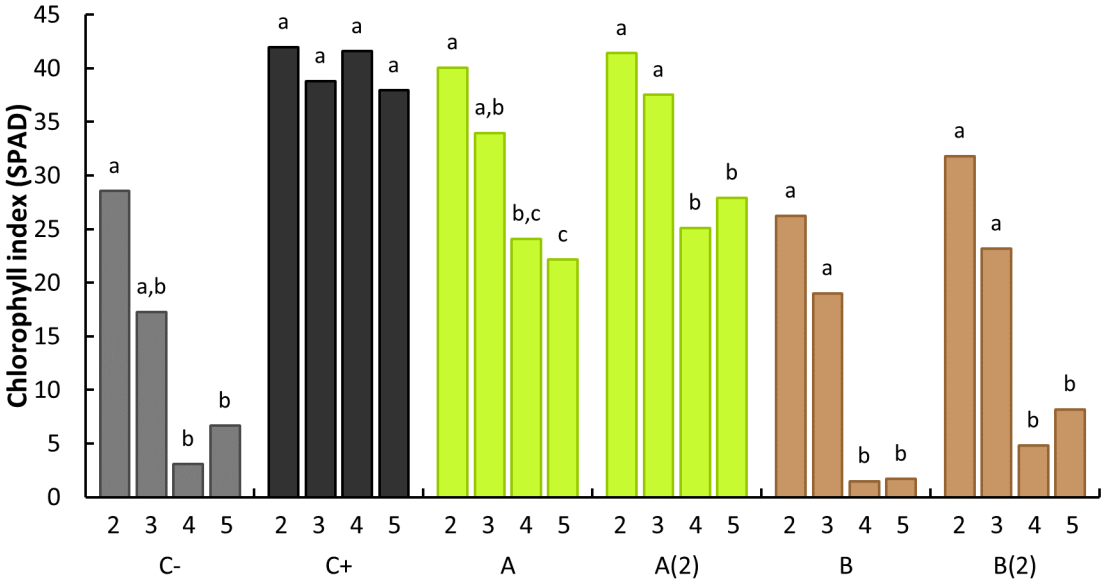

Figure 19. Intra-treatment leaf stage (from 2nd to 5th stage) comparison of the SPAD average read at DAT 21. Different letters denote a significant difference of SPAD levels within each treatment (95 % confidence interval) as shown by Tuckey HSD test. For intra-leaf stage treatment comparison, please check Figure 1S in supplementary material. C-: no iron treatment (negative control); C+: EDDHA (positive control); A: A. vinelandii ISS; B: B. subtilis ISS. A(2) and B(2) represent plants with a second application performed 15th day after the first treatment (n=5).

Moreover, the intra-stage treatment statistical analysis, presented in Figure 20, upholds the previous assumption. In a consistent manner, *B. subtilis*

ISS treated plants were grouped with the negative control in all stages whereas *A. vinelandii* ISS treated plants were paired with *o,o*-EDDHA set in the second and third stages and grouped alone in the fourth and fifth stages (Figure 20). A common result for both bacterial ISS corresponds to the apparent loss of Fe provision efficiency over time (*B. subtilis* much more than *A. vinelandii*). In the data obtained by Yehuda et al. (2000) for rhizoferrin Fe chelates in nutrient solution, it was possible to observe a relationship between chelate concentration and a decrease in SPAD readings in latter leaf stages. The results obtained in our study, in particular in *B. subtilis* ISS, may be due to biodegradation of the siderophore(s) and/or insufficient Fe provided in the solution. Conversely, the *o,o*-EDDHA exerts its effect throughout the entirety of the trial due to its persistence whereas possible degradation decreases the effect of bacterial ISS over time.

Comparing *A. vinelandii* ISS results with those of *B. subtilis'*, the first evidenced to be more stable than the last. Besides possible biodegradation of the siderophore(s), soil adsorption phenomena may also contribute for decreasing the availability of Fe-siderophores in both cases; in fact, previous experiments have demonstrated high levels of Fe retention to the soils in the case of *B. subtilis* ISS while less Fe adsorption was recorded for *A. vinelandii* ISS (Table 8). On a final note, the second application of ISS has resulted in little effect. Schenkeveld et al. (2010) have demonstrated that when Fe chelates (*o,o*-EDDHA in their case) were applied to chlorotic plants, some time was needed for SPAD values increase significantly. Therefore, the lack of clear effect of the second application may be due to the short time that plants were allowed to recover from chlorosis.

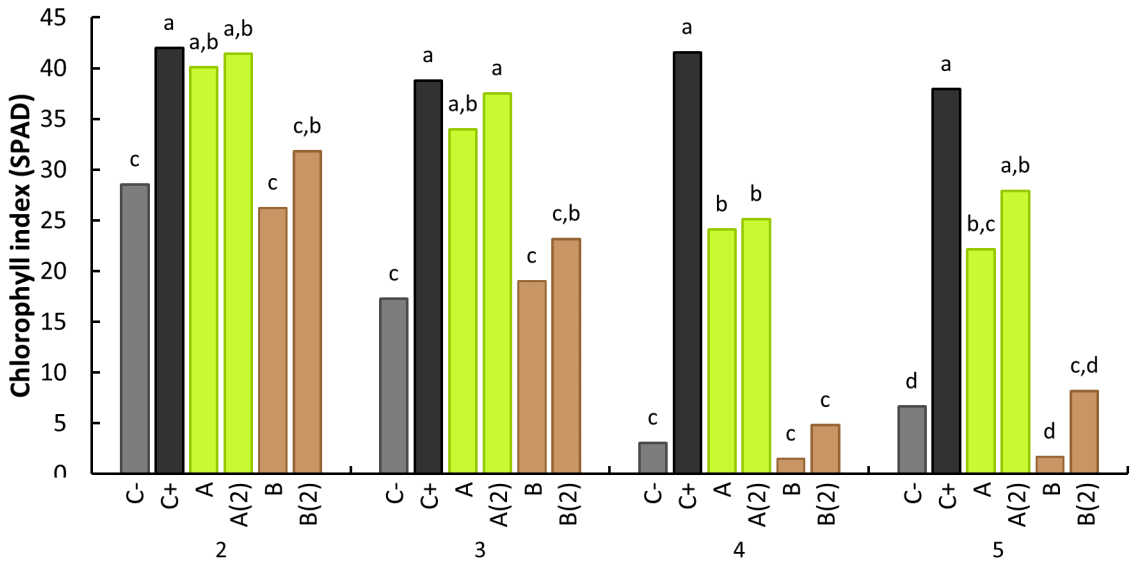

Figure 20. Intra-leaf stage (from 2nd to 5th stage) treatment comparison of average SPAD read at DAT 21. Different letters denote a significant difference of SPAD levels within each leaf stage within 95% confidence interval, as shown by Tuckey HSD test. C-: no iron treatment (negative control); C+: EDDHA (positive control); A: A. vinelandii ISS; B: B. subtilis ISS. Treatments with (2) represent plants with a second application performed 15th day after the first treatment (n=5).

The SPAD results (Figure 18) are in agreement with those obtained for plant dry mass (Table 9). Therefore, the comparable development of plants treated with *o,o*-EDDHA and those treated with *A. vinelandii* ISS lead us to conclude that *A. vinelandii* ISS had a positive effect on the plant development and chlorosis amendment. On the other hand, the comparable behaviour of *B. subtilis* ISS treated plants with the one recorded for the negative control, evidenced that *B. subtilis* ISS had no significant effect on the plant growth of chlorotic soybean plants. This might be due to the effect of Fe-chelates in *A. vinelandii* ISS being able to deliver iron to soybean plants during the first days after application. This has resulted in an accumulation of enough Fe in the early developed and developing leaves to allow chlorophyll production in these leaves. However, sometime after the application, Fe-chelates in *A. vinelandii*

ISS lose efficiency, and the Fe uptake was lower. The newly developed leaves had a lower Fe supply and developed less green (see Figure 22 and following section for clarification).

3.3.3. Fe content in soybean and soil Fe speciation

Dry plant matter was subjected to incineration and the resulting ashes were digested in HNO_3. Metal content was subsequently measured. Data from Fe content in leaves, stems and shoots, per plant, are presented in Figure 21.

Plants treated with *o,o*-EDDHA evidenced the highest Fe content among all treatments, within both leaves and stem. Conversely, on leaves, both negative control and *B. subtilis* ISS treated plants showed the lowest amount of Fe. In stems, all but *o,o*-EDDHA, were equally low. Using the pairwise comparisons of the non-parametric Kruskal-Wallis test (since no homogeneity of variance was found in the samples), significant differences raised between *o,o*-EDDHA and negative control in leaves and shoots. Also, using the same test, differences in all tested tissues between both *B. subtilis* samples and *o,o*-EDDHA were found. From this pairwise comparison, two sub sets always resulted: one sub-set containing *o,o*-EDDHA and another one containing negative control and B. subtilis. *A. vinelandii* samples were placed in both subsets, as it can be seen in Figure 21.

Considering the dry mass (Table 9) and chlorophyll content (Figure 18) results, the plant Fe values found in *A. vinelandii* ISS (Figure 21) seems particularly low when compared with those obtained for *o,o*-EDDHA. At a median of about 20 µg of Fe per plant they were lower than previously reported for the siderophore azotochelin synthetically produced in lab (Ferreira et al.,

2019). Considering that the effectiveness of siderophores in providing Fe to strategy 1 and 2 plants has been demonstrated before (although for shorter periods of time) (Chen et al., 2000; Yehuda et al., 1996), it is unlikely that the reason for the lower amount of Fe in plants was due to incompatibility of siderophores with plants. Given the evolution of the SPAD values registered in the different plant growth stages (Figure 19) one possible explanation can be that later leaf stages had less iron content than first ones (see Figure 22 for clarification). Since all the leaf dried mass was ground and homogenized, the low content of Fe present in the upper part of plant (leaves and shoots) decreased the overall leaf and shoot Fe content.

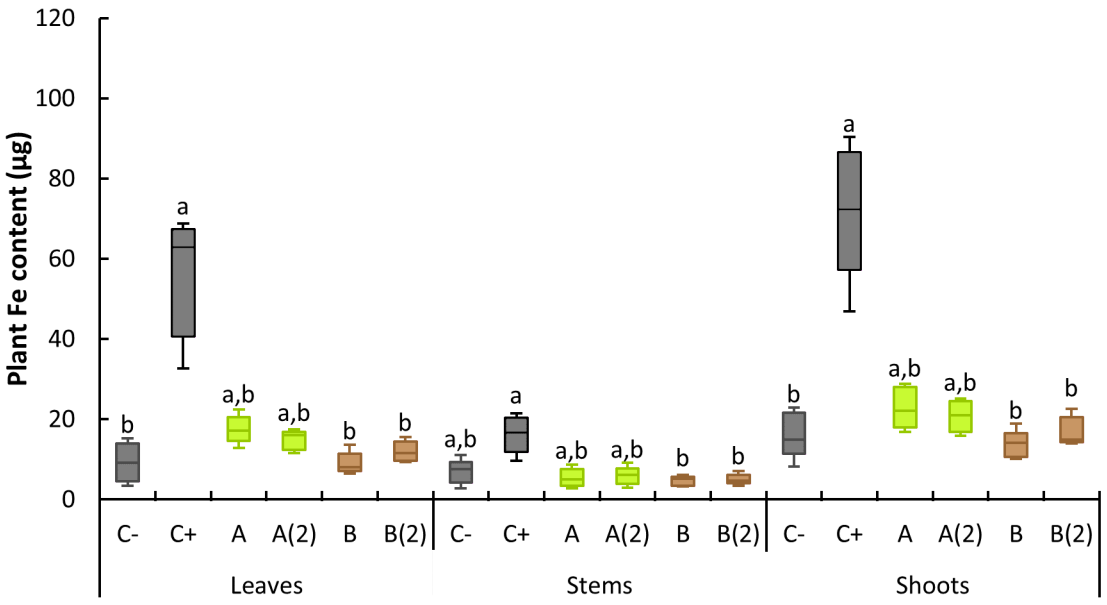

Figure 21. Boxplot representation of Fe content per plant found at DAT 21. C-: no iron treatment (negative control); C+: EDDHA (positive control); A: A. vinelandii ISS; B: B. subtilis ISS. Different letters denote a significant difference of Fe content levels for each organ within 95 % confidence interval, as shown by the pairwise comparisons of the non-parametric Kruskal-Wallis test (n=5).

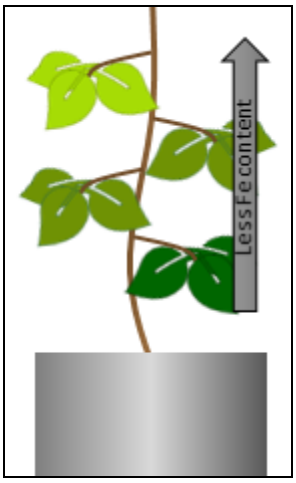

Figure 22. General model of leave SPAD and Fe content progression on Azotobacter vinelandii ISS treated plants.

Nonetheless, the initial quantities of Fe taken by the plant were apparently sufficient for the initial recovery of soybean plants and chlorophyll production. In order to obtain information about the translocation of Fe from the stem to leaves, a ratio between the Fe content determined in the leaves and in the stem was calculated (Table 10). Higher values of the translocation ratios can be related to faster Fe uptake by leaves: as Fe moves from the transport organ (stem) into leaves, less is found in the first and more in the latter, increasing the ratio value. In average, *o,o*-EDDHA presented the highest ratio, followed by the values found for *A. vinelandii* and *B. subtilis* treatments. The negative control presented the lowest value. The value found for *o,o*-EDDHA was higher than those obtained by Ferreira et al. (2019) but of the same order of magnitude of those calculated from the data obtained by Gonzalo et al. (Gonzalo et al., 2013). By using an ANOVA, followed by a Tuckey HSD test, two sets were found: plants with high translocation ratios [*o,o*-EDDHA, A, A(2) and B(2)] and those with lower translocation ratios [C-, A(2), B and B(2)]. However, differences in translocation ratios within both *A. vinelandii* and *B. subtilis* treated

plants [A vs A(2) and B vs B(2)] were due to differences in leaf Fe content, and not because of Fe content in stem, as it remained similarly low (Figure 21). We can conclude that, in the both cases, Fe translocation from stem to leaves occurs and, therefore, any Fe absorbed by roots shall, in theory, reach leaves easily. The *B. subtilis* treated plants had a lower ratio in result of the overall lower Fe uptake and consequent translocation of Fe to leaves.

Table 10. Average leaves Fe content to stem Fe content ratio in different treatments.

Treatment	$Fe\ content_{leaves}/Fe\ content_{stem}$ [a]
Without Fe (C-)	1.52 ± 0.72 b
o,o-EDDHA (C+)	3.72 ± 1.72 a
A. vinelandii	3.66 ± 1.43 a
A. vinelandii (2)	2.87 ± 1.15 a,b
B. subtilis	1.99 ± 0.55 b
B. subtilis (2)	2.48 ± 0.55 a,b

[a] *Average ratio of Fe content found in leaves to Fe content found in stem for each treatment at DAT 21. Different letters denote a significant difference of ratio within 95 % confidence interval, as shown by Tuckey HSD test. Treatments with (2) represent plants with a second application at 15th day after first treatment.*

The analysis of the soil Fe speciation may shed some clues on the outcomes of treatments applied. Two fractions were analysed: the water soluble phase (WSP) and the DTPA extracted (available Fe) phase (DEP) (Table 11).

Table 11. Average of Fe concentration in soil (µg g-1) in two different phases for different treatments

Treatment	Fe concentration (µg.g^{-1})[a]	
	WSP	**SEP**
No Fe (C-)	0.03 ± 0.04 b	7.08 ± 0.70 c
o,o-EDDHA (C+)	0.68 ± 0.03 a	6.80 ± 0.41 c
A. vinelandii	0.02 ± 0.04 b	7.29 ± 0.91 c
A. vinelandii (2)	0.01 ± 0.03 b	8.41 ± 0.82 b,c
B. subtilis	0.00 ± 0.03 b	10.58 ± 2.39 b
B. subtilis (2)	0.00 ± 0.04 b	15.70 ± 2.11 a

[a] *Water soluble phase (WSP) and Soltanpour extraction phase (SEP). Different letters denote a significant difference of ratio within 95 % confidence interval, as shown by Tuckey HSD test. Treatments with (2) represent plants with a second application performed15th day after the first treatment. Average ± standard deviation (n=5).*

For WSP, significant amounts of Fe were found only in the case of soils treated with *o,o*-EDDHA, with an average of 0.68 mg.kg^{-1}. All other soils had very limited or null concentrations found (Table 11). These results are similar to those found recently by Martín-Fernández et al. (2017), where only *o,o*-EDDHA presented a high quantity of soluble Fe in soil whereas for other biodegradable organic chelating agents tested was less. On the other hand, for both SEP controls and *A. vinelandii* ISS treated soils, low amounts of Fe were extracted (between 6.80 and 8.41 mg.kg^{-1}). Considering the value of the negative Fe control (C-), most Fe present likely resulted from the dissolution of Fe oxy-hydroxides, which were dissolved by DTPA solution. The slightly larger quantities found for *A. vinelandii* ISS treated soils may be due to some

adsorption phenomena of the Fe-chelates, as it was witnessed in the soil interaction assays. A significant different profile was observed with both *B. subtilis* ISS treated soils, where higher quantities of Fe were extracted. These differences are consistent with the proposition that *B. subtilis'* siderophores (or Fe chelates thereof) adsorb to the soil phases and remain bio-unavailable. Consequently, Fe is not provided to plants and, thus, there is development of chlorosis, as it was observed for *B. subtilis* ISS treated soybean plants (Figure 17), with less Fe in tissues (Figure 21).

4. Conclusions

In this work, the ability of three new ISS fertilizer products, prepared from the filtrate cultures of *A. vinelandii*, *B. megaterium* and *B. subtilis,* for amending IDIC of soybean plants grown in calcareous soils was evaluated.

Preliminary studies on ISS interactions with soils and soil components reveals that all three ISS interacted differently. *A. vinelandii* ISS had the best interaction behaviour, with lower amounts of Fe lost to soil in the three day experiments. *B. subtilis* had the second best result, although with appreciable losses of Fe to soil. *B. megaterium* had even lower final Fe concentrations at the final of the three day experiments with soils and its components. For this reason, *B. megaterium* was discarded from soil applications to correct chlorosis in soybean.

Plants treated with *A. vinelandii* iron fertilizer developed a dry mass comparable to that of *o,o*-EDDHA and a significant increase of the SPAD levels when compared to the negative control plants. Conversely, plants treated with

B. subtilis iron fertilizer had a response (dry mass and SPAD) comparable to the one registered for the negative control. However, Fe content found in *A. vinelandii* and *B. subtilis* treated plant's leaves and shoots were not as high as *o,o*-EDDHA treated plants. *A. vinelandii* plants had higher Fe content than *B. subtilis* plants. Therefore, a higher efficiency for correcting IDIC was observed when *A. vinelandii* iron fertilizer was used comparatively to *B. subtilis* one. These results may be a consequence of the respective soil interactions as *B. subtilis* iron fertilizer demonstrated higher degrees of instability in solution and in soil. These claims are also supported by the soil analysis at the end of the soybean pot experiments. In conclusion, the overall results pointed out that the freeze-dried product prepared from *A. vinelandii* has potential for application in IDIC amendment in calcareous soils.

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Chapter 5

Biodegradability, theoretical and calculated data

1. Introduction

Current methods for correction of IDIC of plants are based on the application of synthetic aminopolycarboxilic acids (APCAs). However, these compounds are not readily biodegradable. [Compounds are only considered “Readily Biodegradable” when they have the ability to biodegrade to their natural state, when subjected to sunlight, water and microbial activity, from 60 – 100 % in 28 days (OECD, 1992), and in turn, according to the United Nations (UN), “Biodegradation is the process by which organic substances are decomposed by micro-organisms (mainly aerobic bacteria) into simpler substances such as carbon dioxide, water and ammonia” (UN DESA, 1997)]. Consequently, they may pose risks of increased metal leaching, eutrophication of water sources and toxicity to aquatic biota (Bucheli-Witschel and Egli, 2001; Schmidt and Brauch, 2006). For this reason, the search for new chelating agents to combat IDIC, particularly on calcareous soils, must focus both on the character of biodegradability as well as in their iron chelating capacity. Siderophores, which are natural iron chelating agents produced by microorganisms (Fazary et al., 2016b), are good candidates to either replace or complement synthetic chemicals. Siderophores are produced by microorganisms (such as bacteria and fungi) as a mean of scavenging bio-unavailable iron from low soluble Fe(III) environments, such as alkaline soils (Hider and Kong, 2010). Given their natural source, and wide usage and uptake by other organisms (Fazary et al., 2016b; Harrington et al., 2015; Hider and Kong, 2010), it is expected that these compounds are readily biodegradable. In the past, some siderophores (desferrioxamines) have already been shown to biodegradable (Fazary et al., 2016a; Martínková et al., 2009; Pierwola et al.,

2004). However, in the overall, data about this issue is scarce and limited only to a few specific siderophores.

Biodegradation processes are dependent of the interaction of a given molecule with microorganisms and their respective enzymatic machinery. While there are no doubts regarding the existence of microorganisms interaction with siderophores (Harrington et al., 2015), metabolic data analysis is lacking. This analysis may be key to assess the theoretical biodegradability of siderophores produced by studied microorganisms. Besides metabolic pathways analysis, siderophore structure analysis may also shed some light into the degree of biodegradability of each siderophore. Computational models look for key groups within tested structures and use them to compute the probability of biodegradability. Some models also provide time frames for biodegradation.

Given the importance of the biodegradable character of new chelating agents to combat IDIC, the tools described above will be used, in combination with known data for other siderophore, to assess the likely biodegradability of each siderophore produced by the two tested bacterial strains tested in soil, *Azotobacter vinelandii* and *Bacillus subtilis*, to further evaluate their suitability for soil application and IDIC correction.

2. Studied microorganism's siderophores

2.1. *Azotobacter vinelandii*

As previously described, the siderophore complex of *A. vinelandii* has been extensively studied in the past. *A. vinelandii* produces a total of six

siderophores: 2,3-dyhydroxybenzoic acid (2,3-DHBA); aminochelin; azotochelin; protochelin; azotobactin and vibrioferrin (Figure 3) (Baars et al., 2016).

With exception of Vibrioferrin, all siderophores are catecholates, possessing at least one catechol moiety. Protochelin, azotochelin and aminochelin share some structural similarities. Indeed, it is proposed that protochelin is the result of the condensation of azotochelin and aminochelin, via an amide bound (Cornish and Page, 1995). Therefore, the biodegradation pathway studies of protochelin, aminochelin and azotochelin shall be deeply linked.

2.2. Bacillus subtilis

For *B. subtilis*, the production of two siderophores has been described: 2,3-dihydoxylbenzoylglycine, also known as itoic acid (Ito and Neilands, 1958) and its trimeric ester, named bacillibactin (Figure 4) (May et al., 2001). Although not described, the production of 2,3-DHBA is also very likely, as it has been described in other *Bacillus* strains (Wilson et al., 2006), as well as it is one of the precursors of itoic acid (Figure 4). Therefore, all possible siderophores from *B. subtilis* are catecholates.

3. Estimated aerobic biodegradability

Chemical and biological computational models are a useful tool for a first level assessment on chemical and biological properties of unknown molecules. The environmental Protection Agency (EPA) of USA has developed a suite of physical/chemical property and environmental fate estimation programs named EPI (Estimation Programs Interface) Suite™. It is comprised of several program

models for various environmental related subjects (US-EPA, 2012). Of this list, the BIOWIN™ program is of relevance for our study, as it is used for the estimation of aerobic and anaerobic biodegradability of organic chemicals, using 7 different models (US-EPA, 2012).

Models 3 and 4 estimate the time required for "complete" ultimate and primary biodegradation, respectively. Primary biodegradation is defined as the transformation of a parent compound to an initial metabolite while ultimate biodegradation is the transformation of a parent compound to basic compounds: carbon dioxide, water, mineral oxides of any other elements present in the test compound, and new cell material (US-EPA, 2012). Considering that we are evaluating the environmental fate of compounds produced, absorbed and used by microorganisms, the analysis of the time required for the metabolite acquisition form parent compounds (model 4) is more meaningful than the total primary biodegradation (model 3), and was therefore used (Table 12). It is also slightly more accurate, according to the software manual, with 83.5 % of the predictions proving to be correct (US-EPA, 2012). Model 3 is, as discussed below, a key element for the final assessment by BIOWIN™ and is therefore also presented (Table 12).

Models 5 and 6 are based on the Ministry of International Trade and Industry (MITI) Biodegradation Probability Model, as described by Tunkel et al. (2000). They evaluate the potential biodegradability as a Boolean result: either "readily degradable" or "not readily degradable" (US-EPA, 2012). As previous models, one uses a linear approach (model 5) whereas the other uses a non-linear method (model 6). Like previous models, it also uses molecular fragments in model to predict biodegradability. However, some changes were made: MITI

models provide fuller characterization of alkyl chain length and branching, replacing the original C4 terminal alkyl group fragment with a fragment set consisting of -CH3, -CH2 (both linear and ring types), -CH (both linear and ring types), and -C=CH (alkenyl hydrogen) (US-EPA, 2012). The resulting linear and nonlinear regression models accurately classified 81% of the chemicals in an independent validation set (Tunkel et al., 2000).

Table 12. Predicted biodegradability by BIOWIN™ in EPI Suite™ for all described produced siderophores of A. vinelandii and B. subtilis. Results from models 3, 4 and 5, and final remark are presented.

		BIOWIN™ model			
Bacteria	**Siderophore**	**Biowin3: Ultimate Biodeg.**	**Biowin4: Primary Biodeg.**	**Biowin5: MITI Linear Biodeg**	**Final***
A. vinelandii	Vibrioferrin	Weeks	Hours-Days	Positive	Yes
	Azotobactin	Recalcitrant	Hours	Negative	No
	Protochelin	Months	Days	Negative	No
	Azotochelin	Weeks	Days	Negative	No
	Aminochelin	Weeks	Days	Positive	Yes
	2,3-DHBA	Weeks	Days-Weeks	Positive	Yes
B. subtilis	Itoic Acid	Weeks	Days	Positive	Yes
	Bacillibactin	Months	Hours-Days	Positive	Yes

**final remark calculated from Biowin3 and Biowin5 results, as described in the BIOWIN™ manual (US-EPA, 2012). 2,3-DHBA: 2,3-dyhydroxybenzoic acid.*

Based on the data collected at Table 12, different biodegradation time frames were estimated for different siderophores using model 4: the smaller and simpler the structure, the longer the time frame. For example, the whole amino-,azoto-, and proto- chelin family of siderophores are estimated to be degraded

into basic metabolites in a matter of days, while the more structurally complex azotobactin is estimated to be so in a matter of hours. The same pattern is found with *B. subtilis* siderophores estimated times: bacillibactin < 2,3-dihydoxylbenzoylglycine < 2,3-DHBA (Table 12). This model predicts the degradation of the parent compound into metabolites, and therefore, larger molecules with more target sites for degradation are more likely to suffer breakdown faster. In contrast, in model 3, the opposite observation in made: the larger the structure, the longer the time needed for the complete biodegradation. This is expected, as larger molecules will need more time to be fully converted into its mineral basic elements, such as CO_2 and H_2O. The MITI based method (Biowin5) has predicted all *B. subtilis* siderophores to be biodegradable whereas half of *A. vinelandii*'s siderophores were predicted to not be biodegradable (azotochelin, protochelin and azotobactin, Table 12).

Using the data from Biowin3 and Biowin5 models, BIOWIN™ attributes a final remark to each tested compound. This automatic evaluation by BIOWIN™ balances information obtained from these two different models (time and likelihood of biodegradation) for a more robust evaluation. All siderophores are classified as biodegradable except azotochelin, protochelin and azotobactin (Table 12). The model that, in the end, dictates the different end results is MITI model (Biowin5). On the fragments/parameters evaluated by the model Biowin5, the molecular weight (MW) of tested molecules seems to be one of the underlaying reason for the negative result. For example, when comparing aminochelin (MW = 224.26) with azotobactin (MW = 1394.34), despite the latter having many more moieties passive of biodegradation (such as amide, ester, aromatic and aliphatic alcohols, etc, and in greater quantity than aminochelin,

its MW nullifies the positive effect of these moieties in the model, ending in a “not readily degradable” evaluation. Also, azotochelin and protochelin had very low quantities of moieties for the model evaluation, which decreases the likelihood of a “readily degradable” result. Probably, for this reason, the three siderophores (azotobactin, azotochelin and protochelin) with the highest MW to number of moieties passive of biodegradation ratio, had negative results.

However, a mathematical model is not enough as it does not take into consideration the structure of the molecules as a whole. Also, different models use different criteria for the estimation of biodegradation which raises some contradictions (for example, Biowin4 vs Biowin5). For this reason, an evaluation of possible biodegradation pathways and end metabolites is needed.

4. Structure evaluation and metabolic pathways evaluation

The analysis of the siderophore structure, paired with the study of possible catabolism pathways present in microorganisms may shed clues about the biodegradability of siderophores. For this end, not only bibliography was used, but also online comprehensive databases, such as the Kyoto Encyclopedia of Genes and Genomes (KEGG) (Kanehisa and Goto, 2000), were used.

Given that these molecules are produced, secreted and used by microorganisms (Fazary et al., 2016b; Harrington et al., 2015; Hider and Kong, 2010), the issue of microbial intake of siderophores is not debatable. In fact, many studies discuss the use of foreign siderophores by microorganisms (Champomier-Vergès et al., 1996; Niehus et al., 2017; Villa et al., 2014). The

fact that most microorganisms are able to use siderophores is already a hint of their biodegradability.

4.1. 2,3-Dihydroxybenzoic acid

As discussed above, most siderophores produced by the two microorganisms contain catecholates groups (Figure 3 and Figure 4). Not only that, 2,3-DHBA is present at least in the *A. vinelandii* group of siderophores. Given the likelihood of 2,3-DHBA resulting as a metabolite to most, if not all, catecholate siderophores, 2,3-DHBA biodegradation will be discussed first.

The analysis of KEGG databases reveals the presence of two main alternative metabolic pathways responsible for the degradation and reutilization of 2,3-DHBA, as part of the benzoate degradation pathways: One where 2,3-DHBA is firstly decarboxylated to catechol (Kanehisa Laboratories, 2018a), which in turn is broken down into cis,cis-muconate and then further processed into succinyl-CoA and finally enter the citrate cycle (Kanehisa Laboratories, 2018a; Martínková et al., 2009). The second alternative pathway is the degradation of DHBA into pyruvate and acetaldehyde (Figure 23). Pyruvate is the end product of the glycolysis, whereas the acetaldehyde can be turned into acetyl-CoA and enter the citrate cycle (Kanehisa Laboratories, 2018a).

It is worth noting that this second pathway has some alternatives itself, namely the degradation of 2-Hydroxymuconate semialdehyde into 4-hydroxy-2-oxopentanoate (3 and 5, in Figure 23). Also, both pathways are linked, as catechol can be converted to 2-hydroxymuconate semialdehyde by action of a catechol 2,3-dioxygenase (Kanehisa Laboratories, 2018a). Based on the

pathways found, we believe it is very likely that 2,3-DHBA is target of biodegradation and reutilization by microorganisms.

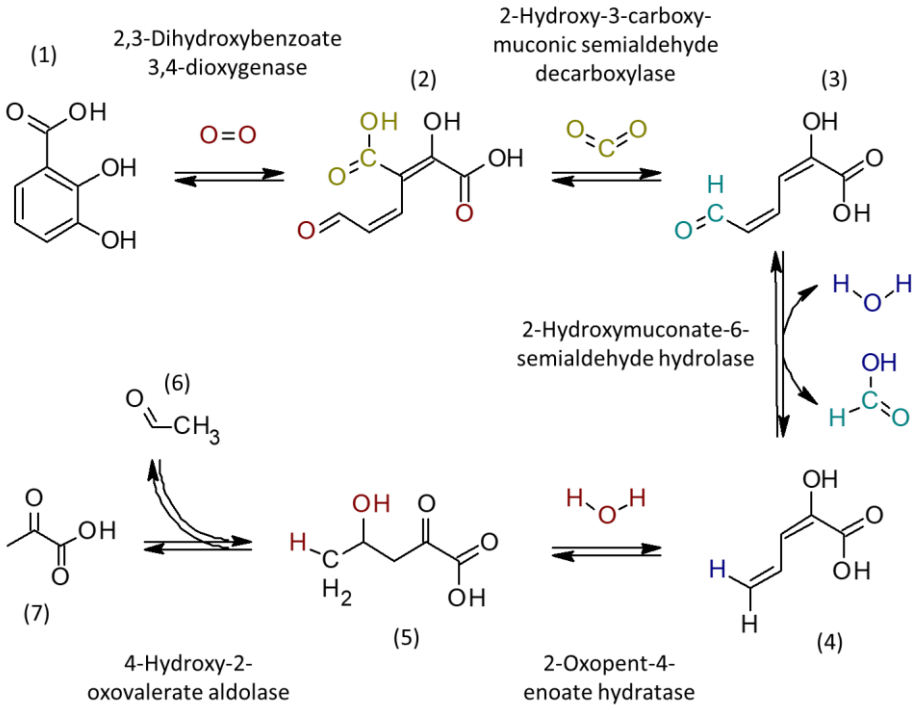

Figure 23. Degradation pathway of 2,3-DHBA into acetaldehyde and pyruvate. Colours indicate atoms involved in different steps. Enzyme names represented besides each step (1) = 2,3-DHBA; (2) = 3-Carboxy-2-hydroxymuconate semialdehyde; (3) = 2-Hydroxymuconate semialdehyde; (4) = 2-Hydroxy-2,4-pentadienoate; (5) = 4-Hydroxy-2-oxopentanoate; (6) = Acetaldehyde; (7) = Pyruvate. (Kanehisa Laboratories, 2018a)

The enzymes referenced in Figure 23 are found in soil bacteria, which further supports the likely biodegradability of 2,3-DHBA. For example, 2,3-dihydroxybenzoate 3,4-dioxygenase was isolated from *Pseudomonas fluorescens* and *P. putida* (Schomburg and Stephan, 2006). 2-Oxopent-4-enoate hydratase was also found in *Pseudomonas* species (Harayama et al., 1989). Although these strains had not been described to produce pure catechol siderophores, the capability to acquire, degrade and use other bacterial siderophores, grants an advantage for the development of the bacteria. Being

able to degrade and recycle endogenous and exogenous siderophores as carbon and nitrogen sources is an existing and sound strategy (Harrington et al., 2015; Niehus et al., 2017). It is reasonable to believe that more soil microorganisms, involved in producing and using siderophores have these same mechanisms for 2,3-DHBA degradation and recycling.

4.2. Amino-, Azoto-, and Protochelin

Aminochelin, azotochelin and protochelin are structurally related siderophores produced by *A. vinelandii*. Their structural similarity results from fact that all have one or more, rather prominent and isolated 2,3-DHBA group(s) (in red, Figure 3). The presence of several amide bounds is also characteristic of all three (in violet blue, Figure 24). In fact, it is speculated that protochelin is the result of the condensation of azotochelin and aminochelin, via one amide bound (Cornish and Page, 1995).

These amide bounds are likely targets for biodegradation reactions in all three siderophores, as they may be targeted by proteases. Proteases are a large family of hydrolytic enzymes capable of breaking amide bounds, widely found in living organisms. For example, a recent genomic study of soil rhizosphere population evidenced the presence of neutral metallo-proteases and alkaline metallo-proteases in several genus, such as *Pseudomonas*, or *Dickeya* (Baraniya et al., 2016). Other study also reported the production of extracellular proteases on several PGPR strains (Pereira and Castro, 2014), which may indicate that protease-promoted degradation of siderophores may not be exclusive of intracellular environments.

Regarding this protease moderated siderophore degradation, the degradation of deferrioxamine B by proteases was described by Pierwola et al. (2004) whereas ferrichrome A degradation is promoted by a protease to a linear hexapeptide and then further degraded to free amino acids (Harrington et al., 2015). To the best of our knowledge, no description for similar degradations events for catecholates were described. However, we believe the likelihood of amide bounds on catecholates to be hydrolysed by proteases is high, due to the nearby structural simplicity which should not hinder protease activity. Hydrolase enzymes capable of hydrolysing amide bound sided by alkyls chains [Figure 24, amide (II)] or sided by a benzene ring [Figure 24, amides (I), (III) and (IV)] have also been described, such as the 6-aminohexanoate-oligomer exohydrolase (Kinoshita et al., 1981) or the acylagmatine amidase (Umezawa et al., 1973), respectively.

In Figure 24 we can see the likely degradation sub products of protochelin, azotochelin and aminochelin, based on the hydrolysis of the four amide bounds present in protochelin. The order by which amide groups are hydrolyzed does not necessarily need to be the one presented at Figure 24. However, regardless of the order of hydrolysis, the end metabolites shall remain the same: 2,3-DHBA (for all three), putrescine (for aminochelin and protochelin) and lysine (for protochelin and azotochelin). The degradation of 2,3-DHBA has been described in the previous section. The other two metabolites are both of biological relevance: putrescine takes part on many metabolic pathways, such as arginine and proline metabolism (Kanehisa Laboratories, 2018b) or glutathione metabolism (Kanehisa Laboratories, 2018c); and lysine is a biologically important amino acid. This means that the resources for the biodegradation

exists and the resulting metabolites are of relevant importance for basic biochemical processes within the cell.

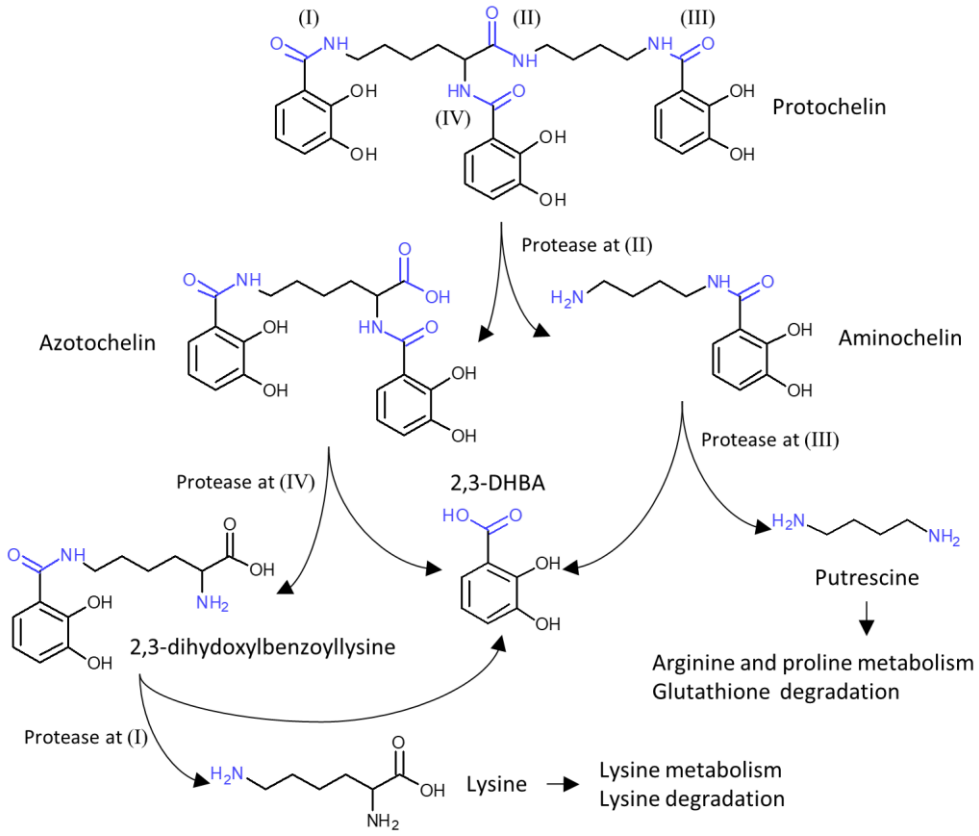

Figure 24. Structural analysis and likely degradation pathways of protochelin, azotochelin and aminochelin. Violet-blue, I to IV: amide (peptide) bounds and amine and carboxylic groups derived from amide bounds breaking. 2,3-DHBA: 2,3-dyhydroxybenzoic acid.

Interestingly, even if the degradation occurs outside the cell, or just partially, the resulting products still retain iron complexing capability. This is obvious on the degradation of protochelin into aminochelin and azotochelin and 2,3-DHBA, but degradation of azotochelin can also yields a similar molecule to aminochelin and itoic acid: 2,3-dihydrodybenzoyllysine (Figure 24). Although capable of complexing Fe(III), their efficiency should be lower due to the decreasing denticity effect. Nonetheless, the fact that the degradation products

are still capable to complex Fe(III) means that even when the degradation process begins, a limited iron complex capacity will still remain.

4.3. Azotobactin

The complex structure of azotobactin (Figure 25)(Page et al., 1991) might indicate a recalcitrant nature. However, its composition is easily broken down into smaller, biologically relevant, units. Azotobactin is composed of a polypeptide linked to a chromophore; therefore, breakdown of azotobactin is achievable by hydrolysis of the peptide bounds into singular amino acids via proteases (Figure 25). The peptide side chain is comprised of some ubiquitous amino acids, which should be promptly used by microorganisms, such as aspartic acid, serine, citrulline or homoserine. Depending on the type of azotobactin (δ- or D-), the terminal homoserine may or may not be in the lactone form (Baars et al., 2016).

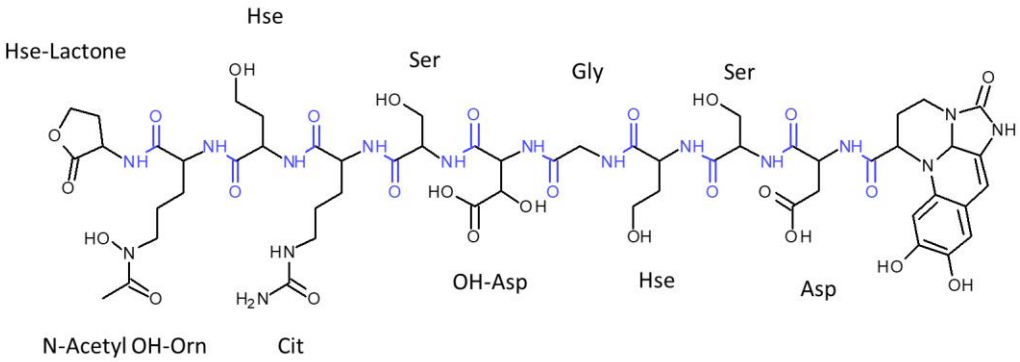

Figure 25. δ-Azotobactin structure with highlighted amide bounds and sidechain amino acid composition. Asp: Aspartic acid; Ser: Serine; Hse: Homoserine; Gly: Glycine; OH-Asp: Hydroxy-Aspartic acid; Cit: Citrulline; N-Acetyl OH Orn: N^5-Acetyl-N^5-Hydroxy-ornithine; Hse-Lactone: Homoserine lactonized. Violet: Amide bounds.

The conversion of homoserine-lactone into linear homoserine is carried out by conjugation with 5'-Methylthioadenosine, forming S-Adenosyl-L-

methionine (Figure 26). From S-Adenosyl-L-methionine, homoserine can be obtained either in linear or lactone form, by means of S-adenosyl-L-methionine hydrolase or adenosylmethionine cyclotransferase enzymes, respectively, both present in soil bacteria (Mazelis et al., 1965; Mudd, 1959a, 1959b).

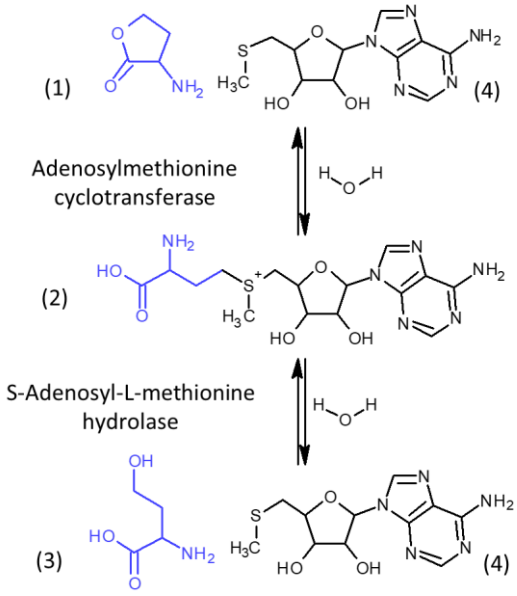

Figure 26. Interconversion of homoserine-lactone and homoserine via S-adenosyl-L-methionine. (1) = homoserine-lactone; (2) = S-adenosyl-L-methionine; (3) = homoserine; (4) = 5'-methylthioadenosine. Purple: homoserine (residue) (Mazelis et al., 1965; Mudd, 1959a, 1959b).

Other amino acids resulting from the breakdown of azotobactin can also be further processed into usable metabolites of bacteria. For example, hydroxyaspartic acid can be converted into glycine and glyoxylate, via an aldolase (Gibbs and Morris, 1964), or into oxaloacetate via an ammonia-lyase (Gibbs and Morris, 1965) (Figure 27).

In either case, all metabolites are biologically relevant. For example, oxaloacetate takes part in gluconeogenesis or citrate cycle while glyoxylate can

be integrated into the purine or into the glycine, serine and threonine metabolism (Kanehisa Laboratories, 2017).

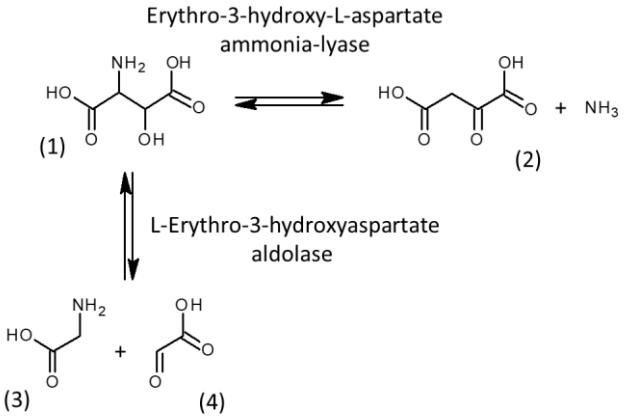

Figure 27. Catabolic pathways of hydroxyaspartic acid (1). (2) = oxaloacetate; (3) = glycine; (4) = glyoxylate. (Gibbs and Morris, 1965, 1964)

The N^5-acetyl-N^5-hydroxy-ornithine synthesis has been described in other siderophore producing microorganisms, such as *Aspergillus quadricinctus*, as a two-step process: ornithine is first hydrolyzed with a L-N^5-ornithine monooxygenase and an acetyl group is transferred via an appropriate transferase (Renshaw et al., 2002; Schwecke et al., 2006). N^5-acetyl-N^5-hydroxy-ornithine from azotobactin degradation can either be recycled by the microorganism into new metabolites (likely siderophores) or undergo a reversed pathway and be converted back into ornithine.

Regarding the dihydroxyquinoline chromophore from azotobactin, no pathways or information regarding its biodegradation were found. However, photodegradation of the chromophore is very likely given our observations of loss of fluorescent hue of *A. vinelandii* filtrates when exposed to direct sunlight, possible sign of degradation of the chromophore. It is also known to have several synthesis predecessors (Baars et al., 2016), which hints on possible

forms of degradation or interconversion to other chromophores for other siderophores. Also, some moieties present in the molecule are likely targets for degradation reactions, such as amide bounds, hydroxyls and amines.

4.4. Vibrioferrin

Vibrioferrin, the more recently described siderophore of *A. vinelandii* (Baars et al., 2016), is a citrate siderophore (Amin et al., 2009). The ester bound present in the molecule can be cleaved by a wide substrate specificity carboxylesterase, resulting in a citrate molecule and 2-hydroxy-1-{(2*S*)-1-[(2-hydroxyethyl)amino]-1-oxopropan-2-yl}-5-oxoproline (vibrioferrin residue, Figure 28). Such carboxylesterases have been described in soil bacteria, such as *B. subtilis* (Gangola et al., 2018).

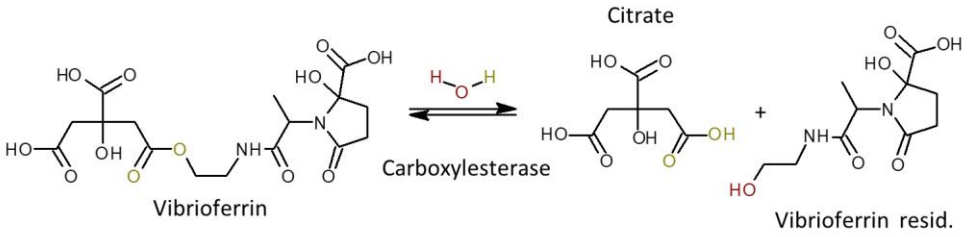

Figure 28. Structural analysis and likely hydrolysis of vibrioferrin. vibrioferrin resid.: 2-hydroxy-1-{(2S)-1-[(2-hydroxyethyl)amino]-1-oxopropan-2-yl}-5-oxoproline

The citrate molecule from this cleavage can be used in a wide array of pathways, most notoriously in the citrate cycle. To the best of our understanding, there is no metabolic pathways already described which would be capable of degrading the resulting vibrioferrin residue. However, the presence of amide bounds, acetyl and carbonyl moieties are possible targets for enzymatic degradation of the molecule. For example, part of the side chain

linked to the amino ring could be hydrolysed at the amide bound, resulting in an ethanolamine residue.

4.5. Bacillibactin and itoic acid

The siderophore bacillibactin (Figure 4) is described as being the cyclic trimeric ester of etoic acid (May et al., 2001). Therefore, the degradation pathways should present relevant similarities. The structure of bacillibactin has a radial symmetry, as a result of the ester condensation of etoic acid on a trithreonine ring (Figure 29).

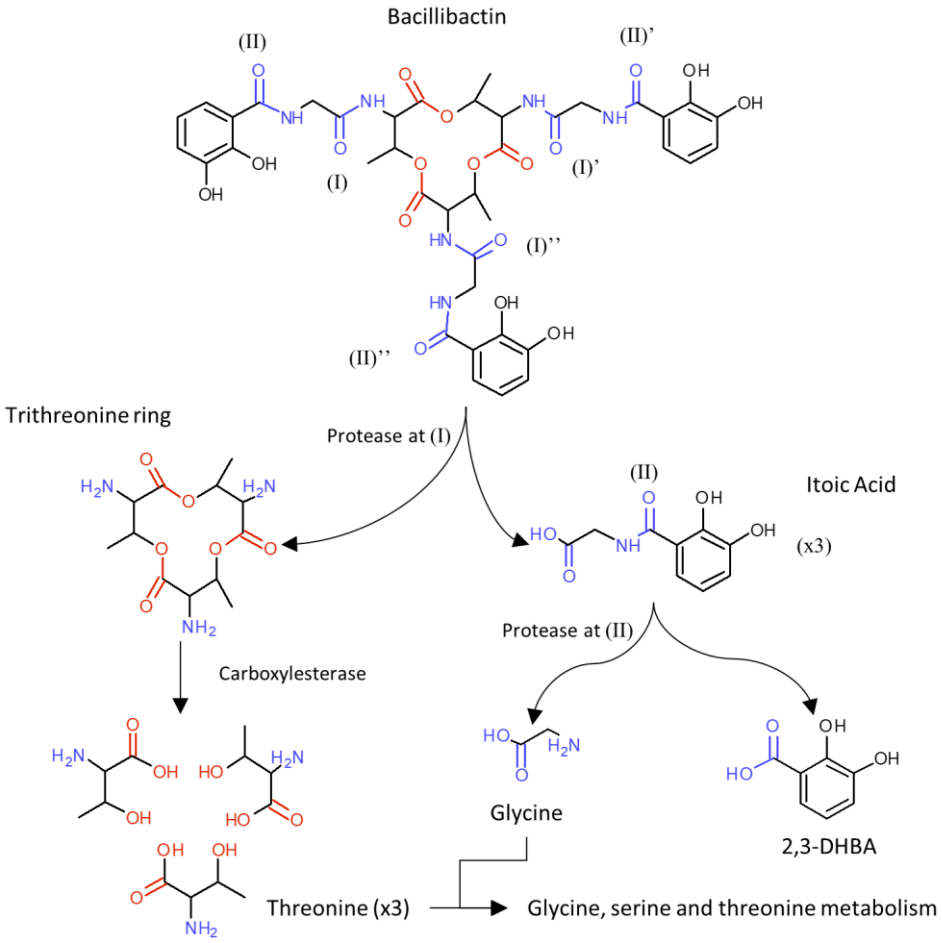

Figure 29. Structural analysis and likely degradation pathways of bacillibactin, and etoic acid. Violet-blue, I to IV: amide (peptide) bounds and amine and carboxylic groups derived from amide bounds breaking. Orange: ester bounds and carboxylic and alcohol groups derived from ester bonds.

As in protochelin case, the amide bounds are prone to be hydrolysed by protease (and the likelihood of the presence of such enzymes in soil have already been previously and thoroughly established above). Once more, the order of hydrolysis may be different than that shown in Figure 29; however, this should not change the final metabolites. One possible hydrolysis result is itoic acid, also a siderophore, which is described to be produced by *B. subtilis* (Raza et al., 2012). Its degradation by hydrolysis of its amide bound originates 2,3-

DHBA and glycine. The degradation of the first has been described above (see section 4.1), while the second can, for example, integrate the glycine (serine and threonine) metabolism (Kanehisa Laboratories, 2017). On the other hand, the trithreonine ring can be degraded by action of carboxylesterase. Bacteria, namely *E. coli*, have been described to be able to uptake and use trithreonine as a source of threonine (Barak and Gilbarg, 1975). However, the mechanism describing how trithreonine is converted to threonine is not discussed. Nonetheless, threonine is also a ubiquitous amino acid that is easily incorporated into different metabolisms (Kanehisa Laboratories, 2017).

Just as in *A. vinelandii*'s case, the resources for the possible biodegradation of siderophores produced by *B. subtilis* exists and the resulting metabolites are of relevant importance for basic biochemical processes within the cell.

5. Concluding remarks

In this chapter, the potential biodegradation of siderophores produced by *A. vinelandii* and *B. subtilis* was examined. Using EPA's EPI Suite™ and the known siderophore structures, the probability of biodegradation and time frame for that degradation were calculated. Also, based on known metabolic pathways, enzymes and siderophore structures, a critical analysis on likely paths of degradation and metabolites was conducted.

For most siderophores, at least one possible path for its degradation, which would result in meaningful metabolic residues, was found. The means were also discussed, and the presence of those means supported by findings in

soil bacteria: the bacteria responsible for the degradation of siderophores upon application in calcareous soils. The data calculated in EPI Suite™ reveals that most siderophores should be biodegradable, with exception of protochelin, azotochelin and azotobactin. However, the critical analysis of their structures seems to indicate otherwise. Both protochelin and azotochelin should be easily hydrolyzed by proteases, originating simpler metabolites that can be further degraded or incorporated into the bacteria metabolism (Figure 24). Azotobactin, possessing a linear peptide chain, is also likely to be degraded into amino acids and further processed or incorporated into the cell metabolism (Figure 25); however, for the chromophore, we did not found a sound degradation pathway or possibility.

Also, regarding the predicted time for degradation for the most critical siderophores, that is, the ones more likely to be present in bacterial media in our conditions and with best affinity for Fe(III), EPA's model predicts the time frame of biodegradation in days or hours-days (Table 12). This time frame is reasonably good for the objective at hand: amendment of IDIC in calcareous soil, while avoiding the negative effects of persistence in the environment. It is desirable that these siderophores are biodegradable, but at the same time, some degree of persistence is required, as to be able to perform their purpose of Fe delivery to plants. Furthermore, the degradation of some siderophores result in Fe(III) complexing compounds, which might further prolong the effect of the siderophore.

In summary, the joint analysis of siderophores produced by *A. vinelandii* and *B. subtilis* and calculated data by EPI Suit™ reveals that these siderophores are likely suitable for our proposed objective, as the likelihood of

their biodegradation within an adequate time-frame, is high. However, further biodegradability tests to validate this hypothesis are recommended.

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Chapter 6

Final conclusions and future prospects

1. Main Conclusions

The main objective of this work was to develop new environmentally-friendly alternatives to current APCAs used in agriculture to amend iron deficiency induced chlorosis (IDIC). As an alternative, siderophores, high Fe(III) affinity chelators produced by microorganisms, were considered. Different sub-objectives were completed in order to fulfill the main objective.

The first objective comprised the screening of bacteria in order to select those most promissory to produce siderophores. Bacteria were selected for their rapid growth rates, easiness of handling and versatility. Among bacteria, plant growth promoting rhizobacteria (PGPR) were a promising group of bacteria to look into. Their function, means of action and siderophore production were assessed and summarized in Chapter 2; bacteria handling safety concerns were always kept in mind and only the lowest risk bacteria were considered. From the available bacteria genera analyzed, *Azotobacter vinelandii*, *Bacillus megaterium*, *B.* subtilis, *Pantoea allii* and *Rhizobium radiobacter* species were selected for their described catechol and hydroxamate siderophores.

The growth and siderophore production of each of the selected species was monitored over time (Chapter 3). All bacteria but *A. vinelandii* were grown in a minimal mineral medium (MM); *A. vinelandii* was grown in the nitrogen free Burks medium (BM). All bacteria showed fast growth rates, reaching stationary phase under one day; *A. vinelandii* on the other hand, reached stationary phase by 72h only. The siderophore max concentration had a similar pattern, as it was found to be concomitant with the growth curve of the bacteria. Final average

concentrations of siderophore in culture ranges from 80 to 140 µmol.L^{-1} desferal equivalent, with *B. megaterium* and *P. allii* with the highest and lowest concentrations, respectively. The type of siderophores in culture were also of interest as catechol and hydroxamates types were desired. Both siderophores types were identified using the Arnow and Csaky tests: *B. subtilis* and *R. radiobacter* had positive results for catechol, *B. megaterium* and *P. allii* had positive results for hydroxamate while *A. vinelandii* had a positive result for both.

The determination of the bacterial siderophore media potential to complex Fe in alkaline solutions was the next objective. This was conducted carrying an adaptation of the EN15962 standard protocol, as explained in Chapter 3. The culture filtrates of *B. megaterium*, *B. subtilis* and *A. vinelandii* were the most effective in maintaining Fe(III) in solution at pH 9. High levels of efficiency (over 90 % Fe in solution) were achieved over the equivalent desferal concentration of siderophore, as read by CAS tests. In case of *B. subtilis*, 2.5 times more Fe was successfully dissolved, for an average of 225 µmol.L^{-1} soluble Fe, while in *B. megaterium,* 2 times more were successfully dissolved, for an average of 280 µmol.L^{-1} soluble Fe. *A. vinelandii* had a slightly different behaviour: it loses efficiency early on, however, is able to maintain high quantities of Fe (188 µmol.L^{-1}) in solution without much loss. Nonetheless, *P. allii* had a very weak complexation capacity while *R. radiobacter* had a complex capacity slightly worse than that of *A. vinelandii*. For this reason, and for the average concentration of siderophore in culture and easiness of growth and handling, *B. megaterium*, *B. subtilis* and *A. vinelandii* were selected for the next stages of the work.

Before testing the produced ISS for their potential of IDIC correction in calcareous soils, the assessment of its ability to maintain iron in solution, without being displaced by other cations or being retained by soil surfaces was evaluated. This was conducted by exposing ISS to Ca rich solutions as well as solutions containing alkaline soils or some of its relevant constituents. ISS had very different behaviors regarding their stability in alkaline solutions. *A. vinelandii* ISS significantly outperforms *B. megaterium* and *B. subtilis* ISS in all tests. For example, in the Ca displacement test, about 60 % of Fe was detected in solution in *A. vinelandii* ISS, only about 10% or less were found for both *Bacillus* ISS. This discrepancy was identified in the remaining tests: higher Fe losses were observed in both *B. megaterium* and *B. subtilis* ISS when exposed in solution to different soil components, such as montmorillonite, $CaCO_3$ or peat (organic matter) than *A. vinelandii* ISS. Soil samples in solution or in wet conditions also resulted in higher losses in *B. megaterium* and *B. subtilis* ISS than in *A. vinelandii* ISS. Given the weaker results of *B. megaterium* ISS, this was dropped in favor of *B. subtilis* and *A. vinelandii* ISS for the following tests.

Once the performance of each ISS in soil was known and understood, the efficiency for IDIC correction of each ISS was tested in chlorotic soybean grown in calcareous soil. For comparison, *o,o*-EDDHA was used as positive control and no Fe treatment was used as negative control. Different parameters were monitored and measured: dry plant mass, chlorophyll index (SPAD) and Fe content in plant tissue. *A. vinelandii* and *B. subtilis* ISS had opposite results to each other. While plant development (dry plant mass) and SPAD development of *A. vinelandii* ISS treated plants was similar to that of *o,o*-EDDHA treated plants, plants treated with *B. subtilis* ISS had a comparable performance to

those of the negative control. Moreover, although the initial development of SPAD in *A. vinelandii* ISS treated plants is comparable to that of *o,o*-EDDHA, in later stages a decreased effect is noticeable. This is possibly linked to the biodegradability of the ISS siderophores, which reduces the effectiveness over time. Analysis on Fe content in plant tissues reveals that *A. vinelandii* ISS treated plants had more Fe in tissues than those plants treated with *B. subtilis* ISS, but less than *o,o*-EDDHA plants. These may be consequence of the sought-after trait of biodegradability and might indicate that a change in treatment application methodology is needed to accommodate more biodegradable Fe chelating agents.

Finally, to elucidate the biodegradability of ISS composition, and more importantly, the siderophores that might be present, a comprehensive analysis of the literature and metabolic databases was undertaken, as seen in Chapter 5. Complementary, biodegradation estimation models, namely EPA's EPI Suite™, were ran. For all siderophores described to be produced by *A. vinelandii* and *B. subtilis* a complete degradation (or partial in case of vibrioferrin and azotobactin) pathway was found. These degradation pathways were supported on soil bacteria and their enzymes. Also, the described degradation results in relevant biological molecules that may be further processed by microorganism, providing the utility of the degradation of siderophores for those to perform it. Time wise, biodegradation estimation models place the biodegradation of the most relevant siderophores on the "days" time-frame. Given the objectives proposed, this time-frame is the most reasonable and desirable, enough time is provided for the action of siderophores to archive their intended function, with less risk of leaching and contamination of water sources.

In the overall, from the initially selected lot of bacterial strains, one, *A. vinelandii*, has shown great potential for IDIC correction in calcareous soils. Its filtrate composition has shown good siderophore production, good Fe complexation capability and easiness of production. Once combined with Fe to produce an ISS, it has shown the lowest interaction with soils and soil components from the tested ISS and was able to maintain adequate Fe levels in solution. When tested on soybean chlorotic plants grown in calcareous soil, an overall improvement in comparison to the negative control was observed, and in some regards, was comparable to the performance of *o,o*-EDDHA. The freeze-dried formulation was also successful in improving the logistical handling of the ISS as well shelf life storage, without compromising the original ISS Fe complexing properties.

2. Future prospects

Despite the good results obtained in this work, there is still much to do. The optimization of culture media composition and growth conditions for the optimal production of siderophore is one prospect of particular interest. Also, although strains as *R. radiobacter* have been dropped in earlier stages, due to the apparent lower performance of siderophore production and Fe complexation capacity, there is still the possibility that the application of ISS from these bacteria might have positive impacts on chlorotic plants, especially if new growth conditions results in improved siderophore production. The same can be said about other known bacteria not considered in this study, such as *Azospirillum* or *Pseudomonas* spp. Furthermore, the freeze-dried formulation

may also be subject to further optimization, namely in the formulation of the powder for easiness of re-hydration; end users should be able to prepare the ISS without the need of scientific equipment or elaborated procedures. Also, the siderophore biodegradability should be experimentally tested and quantified. For this end, protocols for isolation and purification of the different siderophores present in ISS should be created. This would allow to obtain meaningful quantities of pure siderophores to allow the test of biodegradability of said siderophores. Work on this subject in currently under way, and results are expected to come out soon.