



Institute of Crop Science and Resource Conservation (INRES)

**Effects of Rhizobacteria on iron uptake
and root iron plaque formation in lowland rice under conditions
of iron toxicity**

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Bonn, den 15. September 2009

Sunilda Terre Fornies

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Dedication

Para Misi, por estar ahí y para mi padre, por enseñarme a luchar sin tregua.

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List of Abbreviations

FAO	Food and Agricultural Organisation of the United Nations
GA	Gibberellin
IAA	Indole-3-acetic Acid
IKP	I Kong Pao
INGER	International Network for Genetic Evaluation of Rice
IRRI	International Rice Research Institute
NA	Nicotianamine
Ni ₉ MO ₁₂	<i>Bacillus pumilus</i>
Ni ₅ SO ₁₁	<i>Bacillus megaterium</i>
PGPR	Plant Growth-Promoting Rhizobacteria

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Abstract

Under iron toxic conditions, roots of lowland rice take up excess amounts of ferrous iron, which is translocated via the xylem with the transpiration stream to the leaves. High concentrations of iron in the leaves result in the formation of free radicals which damage cell components, in particular membranes. The rice plant has developed several mechanisms to prevent this nutritional disorder, such as iron storage in different forms and tissues, partitioning among roots, leaves and stems, and exclusion at the root surface by oxidizing Fe(II) into a root surface plaque of Fe(III) compounds. Although the oxygen diffusing through the aerenchyma to the roots is responsible for the oxidation, some bacteria endemic to rice may affect the iron uptake by the plant and the oxidation power of the roots. The aim of the research presented in this thesis was to study the effect of 4 root-associated strains of bacillus (*B. megaterium*, *B. pumilus*, and two un-identified isolates of *Bacillus*) on the iron uptake by the plant and on Fe(III) deposition at the root surface in five lowland rice genotypes.

Two experiments were run. The first one combined the five genotypes I Kong Pao, IR31785, ITA 306, TOX4004, WITA 7 with the bacteria PA31, PB32 and *B. megaterium*. The second one combined the genotype I Kong Pao with four bacillus strains, the three used in the first experiment and *B. pumilus*. Plants were inoculated with bacteria and subjected to two iron treatments (0 and 1000 mg L⁻¹ Fe(II)). In both experiments plants were hydroponically grown for 6 weeks. Additionally, plants had their roots either cut or uncut in the second experiment. Nitrogen gas was pumped into the cultural solution to maintain reduced conditions. Toxicity symptoms were scored visually; Fe uptake and partitioning within plant organs, and iron plaque formation were determined by chemical analysis.

All four strains improved plant height of most of the genotypes as they are plant growth-promoting rhizobacteria. *B. megaterium* reduced both Fe uptake and leaf symptoms, probably due to an increase in phosphorus solubility in the culture solution. The isolate bacillus spp. (PB32) increased both Fe uptake and leaf iron toxicity symptoms whereas the isolate bacillus spp. (PA31) increased iron plaque formation and leaf symptoms, which could be related to a secretion of volatile compounds and auxins by PB32 and PA31 respectively. *Bacillus pumilus* affected iron partitioning in the plant by increasing the iron concentration in the roots and decreasing the concentration in the shoots. This is probably related to the production of gibberellins by this bacterium. Iron plaque formation was more pronounced when the roots were cut. The increased formation of iron plaque may be due to improved bacterial penetration, facilitated by the cutting of the roots

The results imply that bacillus spp. produce growth-promoting hormones, affect the ratio of gibberellins to auxines, and help the solubility of micronutrients; therefore these bacterial isolates play an important role in the development of the plant and are promising candidates for ameliorating the performance of rice under conditions of iron toxicity. Possible mechanisms of bacterial action related to iron toxicity are discussed.

1. Introduction

Rice is one of the most important cereals worldwide with a production of approximately 650 million tonnes in 2007. It is grown in 114 countries. Asian farmers account for 90% of the total production with China and India growing more than half of the total crop. The most cultivated rice species in Africa is *Oriza glaberrima* Steud while in Asia it is *Oriza sativa* L. Although both of them are very similar, any hybrid between them remains sterile (Chang, 1976). Paddy rice was the most produced commodity after cow milk in 2007, with China, India and Indonesia being among the most important producer countries (FAO, 2009).

Rice is mainly grown in two different ecosystems as lowland (flooded) rice and upland (dry) rice, and the factors determining the different environments are soils, water regime, drainage, temperature and topography (Fageria et al., 2008). In upland culture rice is depending on rain water, the root remains mostly oxidized, and the yield is unstable and low. Lowland culture implies flooding, reduced root during most of the growing stage and a stable and higher yield (Fageria et al., 1997). The common practice cultivation of lowland rice consists of flooding the field up to 0.10-0.15 m height when the seedlings are between 25 and 30 days old, with the flooding being maintained until approximately one week before harvesting (Fageria et al., 2008). This study focuses on lowland rice since iron toxicity can only appear in this type of cultivation.

Paddy fields undergo physico-chemical changes when flooded. Amongst the most important changes are the reduction of both ferric iron and ferric sulphate, which in the reduced form may directly or indirectly affect growth and development of the rice plant (Jacq et al., 1986).

The iron content in soils ranges from 0.5 to 5%, as it is one of the major constituents of soils (Ma and Nomoto, 1996). Iron plays a major role in biological redox reaction as well as in electron transport, DNA synthesis, and nitrogen fixation and is therefore an essential element for living organisms (Laulhere and Briat, 1993). When the iron content of the leaves reaches high concentrations, iron is deposited and develops brown spots scattered in the leaf area, which may turn the whole leaf brown in severe cases (Tanaka et al., 1966). Excess of iron uptake reacts with the oxygen which goes through the aerenchyma of the plant. This catalyses the production of free radical species in the leaves causing iron toxicity, which can be extremely toxic and cause a decrease in photosynthetic activity (da Silveira et al., 2007). The threshold level for iron of rice leaves has been shown to be 300 ppm, however, the critical iron content leading to iron toxicity symptoms (known as bronzing) depends on variety and other factors (Tanaka et al., 1966).

A natural way of reducing an excessive iron uptake in rice plants occurs by depositing iron at the root surface. These depositions are iron plaque or coatings formed due to oxygen release from rice roots under anaerobic conditions (Armstrong, 1967). However, at high iron concentrations in the soil, the depositing capacity in the plants cannot prevent that iron enters in excessive amounts via the root (Tanaka et al., 1966).

Iron toxicity is widespread in the humid tropical regions of Asia, Africa, and South America, limiting the potential area for rice production, especially in Central and Western Africa (Sahrawat, 2004). Iron toxicity can strongly reduce yields and therefore there is a need to find a way to help the rice plant to face the excessive iron uptake.

Bacteria of the genus *Bacillus* are very important in agricultural applications, since they enhance plant growth. Several mechanisms for the phytostimulatory activity of this group of plant growth promoting bacteria have been suggested (Ali et al., 2009). Experiments by Asch and Padgham (2005) used the same bacteria as in this work to investigate if root-associated bacteria endemic to rice could mitigate the effects of iron toxicity in rice. In addition, *Bacillus* spp. have several growth promoting traits such as production of phytohormones (Ali et al., 2009). Padgham et al. (2005) showed the efficacy of *Bacillus megaterium* as a bio-control agent against *Meloidogyne graminicola* after inoculation of rice seedlings with this bacterium.

2. Hypotheses and Objectives

Hypothesis

Rhizobacteria of the genus bacillus isolated from the rice rhizosphere could help the plant to adapt to excess of iron in the soil. These bacteria live in association with the plant roots and a symbiotic relationship could ameliorate the damage provoked by an excess of iron in the media.

Objectives

The overall objective of this work is to assess the influence of rhizobacteria on iron uptake and root iron plaque formation in lowland rice subjected to iron toxicity, to find out and understand the effects of the bacteria on the rice plants after inoculating the plants with *Bacillus*.

The specific objectives are:

1. To investigate whether bacteria mitigate iron toxicity symptoms and influence plant height and tillering in different rice genotypes
2. To quantify the effect of the bacteria on iron uptake and distribution in the plant organs in different rice genotypes
3. To study the effect of bacillus spp. on iron plaque formation

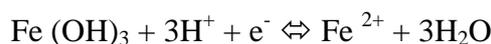
3. State of the art

3.1. Iron in the soil

3.1.1. Distribution and forms of iron in nature

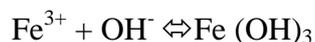
Iron is the fourth most common element in the earth crust (Emerson and Moyer, 1997) Due to their high iron content and low pH, Ultisols, Oxisols and acid sulphate soils are most likely to induce iron toxicity in plants (Sahrawat, 2004). Iron is also widely present in the lateritic soils of West Africa (Audebert, 2006). Lower concentration of Si, Mn and K and higher concentration of total sulphides (especially H₂S) were shown in soils involved in producing bronzing symptoms in rice as compared to soils not involved in producing bronzing (Foy *et al.*, 1978). Lowland-rice yields are reportedly affected by ferrous iron concentration in the soil solution that ranges from 10 to more than 2000 mg per liter (Becker and Asch, 2005).

At a low pH, reduction of ferric (Fe³⁺) into ferrous iron (Fe²⁺) takes place by undergoing the following chemical process:



In most lowlands in Western Africa it was observed that when natural drainage was hampered in rice fields, the complete seasonal waterlogging favored iron toxicity conditions (Audebert, 2006)

The iron precipitation in the nutrient solution occurs at low pH. If the pH in the soil solution increases, the soluble ferrous iron in acidic conditions is oxidized into ferric iron which precipitates (Macfie and Crowder, 1987) and is not available for the plant any more. Therefore, plants will release chelating agents (in grasses the so-called phytosiderophores) into the rhizosphere to take up the iron from the soil (Fageria *et al.*, 2008). The following chemical process takes place during the iron oxidation:



Factors enhancing iron solubility

In the soil iron is mostly present in its oxidized form (ferric iron) having a low solubility in water (Briat and Lobréaux, 1997; Fageria *et al.*, 2008). Römheld and Marschner (1983) observed that a considerable amount of this iron appears as complexed iron, depending on both the redox potential and the pH of the soil. The valence of iron and its uptake by the plant is affected by oxidation-reduction processes (Fageria *et al.*, 2008). Plants take up iron in the form of Fe (II) although some plant species also take up Fe (III) (Marschner, 1986). Plants

can also take up iron in the form of chelates. In fact, the predominant forms of soluble iron in nutrient solutions and soils are either chelates of Fe (III) or less often chelates of Fe (II). In aerated systems the concentrations of ferric and ferrous iron are below 10^{-15} M (Marschner, 1986) whereas chelates are more abundant.

The presence of rice roots in the soils favors microbial processes, especially iron reduction. This can be due to the root debris and/or the exudation of carbohydrates that provide a higher energy input, and thus stimulate the microbial activity and anaerobic respiration which lead to denitrification and/ or ferric iron reduction into ferrous iron in the absence of oxygen (Prade *et al.*, 1988). The release of organic compounds depends on plant age, species and genotype within species, as well as on soil type and properties (Fageria and Stone, 2006). Additionally, as a result of the chelators (low molecular organic compounds) exudated by roots and microorganisms in the rhizosphere, concentration of soluble iron in the rhizosphere could be higher than in the bulk soil (Römheld and Marschner, 1983).

While iron toxicity occurs mainly in flooded slightly acidic lowland soils, iron deficiency is common in sandy, calcareous and alkaline upland soils (IRRI, 2003), where the transport of iron by mass flow to the roots is also low and iron may not be well diffused in the rhizosphere (Marschner *et al.*, 1989). Therefore, the iron availability to the plant depends on the rhizosphere conditions rather than on the bulk soil.

Plants most easily extract iron from large particle soils since small particle soils can better adsorb iron. Therefore, Macfie and Crowder (1987) concluded that the capacity of plants to extract iron in the soils is highest in sandy soil, followed by sandy loam and finally organic soils.

3.1.2. Potential iron toxicity

When a significant amount of ferric iron is transported from upper levels of the landscape to the lowlands and stored in the soil solution and the environment additionally becomes reductive due to the absence of oxygen, iron toxicity may occur (Audebert, 2006). This iron may be already in the soil solution or may be transported through subsurface runoffs from adjacent slopes. In the soil solution, the concentration of ferrous iron to develop iron toxicity in rice appears to vary with the pH of the solution (Sahrawat, 2004). However, a critical concentration of ferrous iron that can cause toxic effects in lowland rice has been established from 10 to 500 mg per liter, depending largely on the presence of reduction products and on the nutrient status of the plant (Sahrawat, 2004; Bode *et al.*, 1995)

Audebert (2006) studied soils at several sites in Guinea, Ivory Coast and Nigeria characterized by a high iron concentration and an elevated carbon content used for the metabolism of microflora. He showed that although the soils had a normal pH for rice cultivation, oxygen content and redox potential affected the appearance of iron toxicity.

Acidification of soils has the positive effect of solubilising nutrients like Phosphorus, whose availability is limited (Begg *et al.*, 1994). Nevertheless, antagonistic effects on the uptake of nutrients like Zn and K can result from a high iron concentration in the soil, provoking nutrient imbalance and decreasing the oxidizing capacity of the roots. The uptake of Mn and P will also be adversely affected when the level of iron in the soil solution is high (Tanaka *et al.*, 1966, Majerus *et al.*, 2007). Applying nutrients like K, P (Fageria *et al.*, 2008) and Zn, helps the plant to deal with iron toxicity and increases rice yield on iron-toxic soils (Sahrawat, 2004).

3.2. Iron in the plant

3.2.1. Iron uptake pathway

Basically, once the iron is taken up by the roots, it is sequestered in the cells or complexed to avoid damage to the cell caused by free radicals generated by Fe^{2+} in the Fenton reaction. There are two pathways of ions across the cortex towards the stele (Marschner, 1986). Either ions pass through the apoplast (intercellular spaces and cell walls in the rhizodermis and cortex) or through the symplast where ions flow from cell to cell through the plasmodesmata. Nevertheless, for apoplastic transport, the casparian band forms a strict barrier and to move through this site, all ions must enter the symplast (Stephan, 2002). However, in the iron uptake, the mechanism of iron uptake from leaf apoplast into the leaf symplast is not well understood (Nikolic and Römheld, 1999).

Iron can be taken up by the roots either in its ferrous or in its ferric form. Ferrous iron is present in the protoxylem, and may or may not enter the root with the help of a carrier. The ferrous iron absorbed by the root is oxidized into ferric iron at the junction of the protoxylem and metaxylem (Brown, 1978) and translocated to the leaf apoplast through the metaxylem with the help of citric acid (Tiffin, 1970). Previous studies showed that citrate is a natural carrier of Fe and this carrier moves in the xylem and is able to bind and translocate iron (Tiffin, 1970). Thus, ferric iron citrate complex is the major form of iron in the xylem. Tiffin (1966) observed that citric acid appeared in plants in which xylem exudates had high concentrations of iron.

Other studies showed a symplastic pathway of iron, in which iron passes through the plasmodesmata and is transported in chelated form, so the intracellular environment is protected against the reactive species during the symplastic transport (Stephan, 2002). In this process, chelators like the amino acid nicotianamine are required (Stephan and Scholz, 1993). Nicotianamine is a metabolite in the biosynthesis of phytosiderophores in Poaceae and is easily transported among the different plant organs. The Fe(II)-nicotianamida complex is oxidized to Fe(III) in the symplast of the root and then transported through the xylem to the leaves. An experiment with BPDS (Fe²⁺ scavenger) showed that a step at the plasma membrane of the leaf cells is required, involving the reduction of ferric iron in the process of iron uptake into the leaf symplast (Nikolic and Römheld, 1999). However, this process is disturbed under iron toxicity conditions.

Nicotianamine was found in both phloem sap and xylem exudates of *Ricinus communis* seedlings (Stephan and Scholz, 1993), indicating that NA probably circulates in the plant and could play a role in the transport through the phloem and through the xylem (Stephan and Scholz, 1993), which also suggests the presence of iron in the phloem.

Nikolic and Römheld (1999) showed that pH in leaf apoplastic fluid of faba bean neither depended on high bicarbonate concentrations in the nutrient solution nor on the iron nutritional status. Considering that the apoplast pH ranges between 5.0 and 6.5 in most plant species, their study also showed that a pH higher than 6.0 in the leaves produced a considerable decrease of iron uptake into the leaf symplast.

3.2.2. Functions of iron

Due to its properties and functions; iron plays an important role in most of the basic redox reactions for oxygen production and consumption, and in several enzymatic systems such as prosthetic groups like cytochromes (Audebert, 2006). It is also involved in vital enzymatic reactions, which are necessary for nitrogen fixation and DNA synthesis among others.

The cytochrome acts as electron transporter and the cytochrome oxidase is involved in the terminal step of the respiration chain (Audebert, 2006). Iron can be considered as a principal component of chloroplasts since it plays a role in the porphyrin structure of chlorophyll. It is also required for protein synthesis and can be stored as phytoferritin in the plant cells of the stoma. In this way, the plant has Fe reserves in leaves to be used by developing plasmids during the photosynthesis (Marschner, 1995; Audebert, 2006)

3.2.3. Effects of iron deficiency

Plants have developed several adaptation mechanisms in response to iron-deficiency stress, allowing the mobilization of iron in the rhizosphere. Previous studies (Römheld and Marschner 1986b) showed that cucumber and barley expressed two different mechanisms in response to iron deficiency. It was seen that roots of barley in an iron deficient culture medium, released exudates identical to aminoacids classified as phytosiderophores, which were able to chelate the iron of Fe^{3+} hydroxide when this was supplied to the medium solution. Thus, the iron-deficient barley plants took up the ferric iron solubilised by phytosiderophores at a rate 10^2 to 10^3 times higher than the iron from synthetic Fe^{3+} chelates.

There are two main strategies to increase the iron solubility in the rhizosphere of plants, both based on species differences. Römheld (1987) and Römheld and Marschner (1986a, 1986b) provide a very good description of these strategies:

Strategy I

Dicots and non-graminaceous monocots typically follow a strategy, where there is a reduction step by a plasma membrane-bound reductase of Fe^{3+} into Fe^{2+} and consequently chelate splitting, so that the iron can be transported across the plasma membrane of root cells (Römheld and Marschner, 1983). However, this reduction step is limited by the substrate added (i.e. ferric hydroxide or soluble iron chelate (FeEDDHA) (Marschner *et al.*, 1989). The enhancement of the reductase activity and of H^+ extrusion that occurs in the roots of plants which follow this strategy, is confined to the apical zones, which leads to an increase of the iron uptake in this area. In an experiment with cucumber to study the effect of the pH on the uptake of iron supplied as iron chelates, the rate of iron uptake by iron deficient plants rapidly declined due to the inhibitory action on the Fe^{3+} reductase at the root surface by the high pH when the pH of the nutrient solution was increased from 5.0 to 8.8 (Römheld and Marschner, 1986b). This is observed in calcareous soils, where high concentrations of HCO_3^- are available causing a strong buffer effect in the root free space of the plants, such that the capacity of the proton extrusion pump may not be sufficient to steepen the pH gradient enough to stimulate the reductase system in the root tips of plants grown on these soils (Römheld, 1987).

Strategy II

The iron uptake works via phytosiderophores, which are non-proteinogenic aminoacids released by the roots of grasses, allowing the plant to increase the use of insoluble Fe^{3+} hydroxide. Plants take up the complex formed by the phytosiderophores and the Fe^{3+}

hydroxide through a transport protein system. This transport is mediated by a translocator which is activated under iron deficiency and transports the Fe^{2+} across the plasma membrane (Marschner *et al.*, 1989). The release of phytosiderophores is regulated primarily strategy II (Römheld, 1987).

The typical steps of iron deficiency induced reductase activity and H^+ extrusion are generally absent in this strategy. Thus, no reduction step of Fe^{3+} into Fe^{2+} is required at the plasma membrane, for the preferential uptake of phytosiderophores by barley (neither for Fe-deficient nor Fe-sufficient barley plants) and other grasses. Another difference to strategy I is that the rate of iron uptake from the Fe^{3+} phytosiderophores by barley plants was by far less inhibited by high pH or high carbonate concentrations. Thus, when the substrate pH is in a range between 4 and 8, the release of phytosiderophores is barely affected (Marschner *et al.*, 1989).

The ability to use Fe^{3+} hydroxide under iron deficiency as a source for high rates of iron uptake has only been observed in graminaceous species (Römheld and Marschner, 1986b). The decay of Fe^{3+} chelates supplies the inorganic Fe^{3+} and makes it available for chelation by the root released phytosiderophores. The high concentration of Cu, Mn and Zn in the shoots of iron deficient barley plants, showed that mobilization of these metals was also enhanced by phytosiderophores and root exudates (Treeby *et al.*, 1989), which demonstrates that phytosiderophores can build-up complexes with other metals than iron and have different specificity for different micronutrient cations, and thus, a limited selectivity. Neither microbial nor synthetic phytosiderophores are as efficient as root released phytosiderophores in the uptake of iron into the plant. Furthermore, the high capacity of root exudates (i.e. phytosiderophores) for iron mobilization was corroborated by Awad *et al.* (1994) showing that exudates were able to mobilize iron in the rhizosphere over a distance of up to 4 mm from the root compartment. According to a rough calculation by Römheld and Marschner (1986b), the root takes up only 10% of the phytosiderophores as Fe equivalent, since microorganisms decompose the rest of the phytosiderophores.

3.2.4. Effects of iron excess

Free ferrous iron in plant cells can trigger the Fenton reaction and be deleterious for the cell (Becana *et al.*, 1998). When excess of ferrous iron reacts with reduced forms of oxygen, it accelerates the production of free radical species which are toxic and can damage all cellular components (Briat and Lobréaux, 1997; da Silveira *et al.*, 2007). However, in vivo experiments have demonstrated that OH radicals are formed during oxidative stress but plants

have natural antioxidant enzymes like catalases and peroxidases which are able to decompose H_2O_2 into water and O_2 (Becana *et al.*, 1998).

Nevertheless, under waterlogging cultivation, iron toxicity can appear in rice (da Silveira *et al.*, 2007). This is because in anaerobic soils, high concentrations of ferrous iron are excessively taken up by the plant and the spontaneous ferrous oxidation might be too slow to avoid an excessive uptake of iron, even if the level of oxygen supplied by the aerenchymatic transport is high (Bienfait *et al.*, 1985). An excessive ferrous iron uptake by the roots and its acropetal translocation through the xylem into the leaves have to take place for the expression of iron toxicity symptoms (Becker and Asch, 2005).

Iron toxicity has been reported to be widespread through West Africa as well as several Asian countries such as China, India, Indonesia, Thailand, Malaysia and the Philippines (Asch *et al.*, 2005). Rice yield losses due to this stress are related to the appearance of bronzing symptoms and can amount to 15% to 30% (Becker and Asch, 2005).

3.2.5. Iron toxicity

Symptoms of iron toxicity were described by Tanaka *et al.* (1966) as scattered reddish brown spots widespread over the lower leaves mainly, till the whole leaf turns brown and finally the lower leaves turn dark gray and die. The tips of the lower leaves of the rice plant turn yellow or even dark yellow to orange (Baruah *et al.*, 2001) with a lot of dark brown streaks in the severe cases, root systems may become short and highly branched acquiring a dark-orange coating of Fe and Mn oxides (Jugsujinda and Patrick, 1993). These brown spots developed faster in older leaves of the rice plant and the highest amount of iron appeared in these spots.

The bronzing of the leaves is the result of a nutritional disorder caused by an excess of water-soluble iron which is very common on severely acid soils. The symptoms develop faster during the ripening phase (from flowering to harvest) than during the early reproductive phase (Tanaka *et al.*, 1966) and the susceptibility to bronzing depends on plant nutritional status, age and the form of the iron added (Foy *et al.*, 1978). As a result of excess of iron in the growth medium, root and leaf development were retarded (Baruah *et al.*, 2001)

3.2.6. Iron excess avoidance by the plant

Plant tolerance to iron toxicity depends on plant species and genotypes within species (Sahrawat, 2004), as well as on external factors. Many studies have proved the efficiency of tolerant cultivars to prevent toxicities in unfavorable environments. The most prominent way to manage iron toxicity in the field is the use of tolerant rice germplasm (Becker and Asch,

2005). Hence, yield reduction of rice as a result of iron toxicity depends on the susceptibility or tolerance of these cultivars to iron (Fageria *et al.*, 2008). The tolerance shown to iron toxicity is expected to coincide with the one shown to waterlogging in soils (Foy *et al.*, 1978).

Therefore and in order to minimize the damage done by the free radicals and at the same time maintain the required iron steady levels for biosynthetic purposes, plants have developed some adaptation strategies. These strategies have been classified by Becker and Asch (2005) into three major types which comprise “includer” and “excluder” strategies as well as mechanisms of “avoidance” and “tolerance”. Plants using strategy I (exclusion/avoidance) prevent shoot and leaf damage by excluding ferrous iron at the root level through a) oxidation of iron in the rhizosphere and plaque formation at the root surface (this study is based in this strategy therefore it will be explained in more detail below) and b) root ion selectivity. In strategy II (inclusion/avoidance), plants take up ferrous iron into the roots but avoid tissue damage by a) compartmentation (active iron storage and immobilization in “dumping sites”), b) exclusion from the symplast and immobilization in the leaf apoplast. With strategy III (inclusion/tolerance) plants tolerate high concentrations of ferrous iron within the leaf cells, probably due to an enzymatic detoxification in the symplast.

Strategy Ia: Iron plaque formation at the root surface

In flooded soils, the ability of rice to survive is due to the transport of air via aerenchyma, which is released later on to the rhizosphere (Jacq *et al.*, 1991). Physiologically, cortical gas-phase diffusion in plants of waterlogged soil appeared to be the only significant way of aeration (Armstrong *et al.*, 1994). They suggested a gas-phase diffusion from shoot to root and a substantial radial oxygen loss from the root to the media. Gilbert and Frenzel (1998) suggested that rice roots are surrounded by an oxic layer that depends on bacterial colonization, age, etc. and has a variable extent. Therefore, roots had a more stable oxic-anoxic interface by their combination into a root mat. The oxygen released by the roots oxidized ferrous iron at the root surface into ferric compounds which are no longer available for the plant (Tanaka *et al.*, 1966). It allowed plaque formation on the root of wetland plants and was related to temperature, plant anatomy and photosynthetic biomass (Macfie and Crowder, 1987). Tanaka *et al.* (1966) showed that when the iron level in the media was low, most of the iron absorbed by the plants went from the roots to the growing leaf whereas when the iron level was high, there was a large amount of iron deposition on the root surface and the iron entering the plant after being absorbed into the root tips went to the older leaves.

In other experiments conducted under toxic conditions in paddy fields, Jugsujinda and Patrick (1993) observed that the root system of the rice plants had an orange coating of iron-oxide and the orangening symptoms of the rice plant were the result of a multiple nutritional stress after soil flooding (i.e. toxicity of Fe, Mn and Al and deficiency of Ca, P and K). Once the iron toxicity was alleviated in the roots, these recovered the usual white colour (Sahrawat, 2004). Ca, P, K, Mg and Mn deficiency decrease the iron-excluding power of roots influencing the plant's tolerance to iron toxicity (Sahrawat, 2004). P, K and Zn deficiencies are of special relevance for lowland rice (Yoshida, 1981). The iron plaque on plant roots can clearly affect the nutrient uptake by accumulating ions from the growth media (Zhang *et al.*, 1998). This is because iron plaque can act as a scavenger of metallic ions (including cations and anions as PO_3^{-4} , Zn^{2+} , Cu^{2+} and Cd^{2+}). A gradient of diminishing metal bioavailability to roots may be generated due to metal precipitation in the rhizosphere; while iron plaque on root surfaces may act as a barrier to nutrient uptake generating a deficiency (Batty and Younger, 2003). The co-precipitation of metals retained on these surfaces may facilitate the metal uptake to the plant (Machado *et al.*, 2005). Additionally, since the whole axis of the root is not covered by iron plaque, a pathway around the root tip may be available to nutrient uptake (Batty and Younger, 2003). Armstrong *et al.* (1994) observed that although the oxygen demand and diffusivity within individual tissue cylinders of maize roots is uniform, the radial oxygen profile differs a lot along the root. There was a decline in the potential oxygen supplying power of the cortex with distance from the shoot.

Römheld and Marschner (1986b) observed that at the root surface and in the apparent free space of the cortex, there was a presence of extracellular iron (i.e., precipitation of $\text{Fe}(\text{OH})_3$) which may act as readily accessible iron to be used by the phytosiderophores released by barley roots under iron deficiency.

It was observed in other experiments that the radial oxygen loss from individual rice roots was related to the age of the plants (Gilbert and Frenzel, 1998). Rice seedlings are more susceptible to sulphide toxicity than older rice plants since seedlings have a weak aerenchym formation in the primary roots. Older rice plants have a well established oxidative mechanism through the formation of new aerenchymatic roots (Jacq *et al.*, 1991). Gilbert and Frenzel (1998) suggested that brown-coloured roots covered with iron oxides, may have difficulties in the oxygen diffusion.

In wetlands, iron hydroxide plaques or coatings are frequently found in roots and the plaque deposition extent seems to be site-dependent and only predictable on an annual basis (Macfie

and Crowder, 1987). The iron oxyhydroxide deposits built up on the roots may act as a mechanism for the avoidance of iron toxicity by reducing the amount of iron entering the plant (Batty and Younger, 2003). Experiments with the aquatic plant *Lobelia dortmanna* showed a significantly lower radial oxygen loss from roots with iron plaques than from roots without them (Moller and Sand-Jensen, 2008). These experiments also observed that the iron plaques located in the outer epidermis layer did not appreciably increase the root diameter. Regarding the amount of iron plaque, a positive correlation to the concentration of $\text{Fe}(\text{OH})_3$ that was added to the nutrient solution was observed (Zhang *et al.*, 1998). Wetland studies with the species *Juncus effusus* and *Pontedaria cordata* showed no significant difference in the plaque accumulation rates. However, plaque accumulation is likely to be influenced by the differences between plant species in radial oxygen loss (Weiss *et al.*, 2005).

Macfie and Crowder (1987) studied iron plaque accumulation on roots of *Typha latifolia* L. genotypes in seven southern Ontario wetlands. Their results suggested that the differences in the levels of iron plaque were not due to different genetic strains but rather to environmental factors. However, Baruah *et al.*, (2001) showed that Fe-resistant genotypes had better leaf and root growth due to their ability to avoid an excess iron uptake by oxidizing and precipitating iron at the root surface.

Tanaka *et al.* (1966) also observed that at 200 ppm of Fe, the control plants showed no symptoms of iron toxicity, but serious symptoms appeared after cutting of the roots. Doing so impaired the plant's ability to deposit iron at the root, so iron could enter the plant more easily. This could indicate a close relationship between tolerance to iron toxicity and oxidizing power of roots. The fact that the plants under iron toxicity conditions have less Mn and K could impair their oxidizing power and make them even more susceptible to this toxicity (Tanaka *et al.*, 1966).

Strategy Ib: Iron selectivity at the root membrane

The regulation of metal uptake across the plasma membrane seems to be the primary control point for metal ion homeostasis (Rogers *et al.*, 2000). The ferrous iron able to pass the oxidative barrier of the rhizosphere may enter the root apoplast after passing the endodermis due to the Casparian band and load in the xylem (Becker and Asch, 2005).

To ensure the symplastic selectivity of ions that reach the xylem and later the shoot, the endodermis blocks the radial apoplastic pathway across the root (Yeo *et al.*, 1987). However, since this barrier is not perfect, an apoplastic flow may occur through the sites where the endodermis is ruptured with the development of each lateral root. In experiments with plants

grown in salinity conditions, the ion uptake increased with time as root selectivity became impaired because of a heterotrophic root, and the shoot was damaged because of salt accumulation.

Since this exclusion process highly depends on the concentration of ferrous iron, the environmental conditions and the growth stage of the plant, this mechanism is not considered to be efficient for seedlings or under conditions of persistent severe toxicity (Becker and Asch, 2005).

Strategy Ic: Organic acid secretion into the apoplast and the rhizosphere

The release of organic acids from the roots can be a response to environmental stresses like aluminium and iron toxicity and anoxia. These responses are highly plant-species and stress specific (Jones, 1998). For example, some crops like wheat and maize are able to release malate or citrate respectively into external solution and the apoplast upon exposure to high Al levels. Further experiments under conditions of aluminium toxicity showed that the tolerance mechanisms of the plants to this stress were related to a greater excretion of malate from the root apices (Andrade et al., 1997). Malate may immobilize Al in the rhizosphere and/or the apoplasm, diminishing its harmful effects on metabolic sites in the roots and protecting the plasma membrane. The excretion of these aluminium chelators could be a mechanism to prevent or ameliorate the effects of Al toxicity.

These organic acids will form complexes after leaving the roots making the roots more resistant to Al toxicity. It was therefore concluded that organic acids are able to complex metals in the solution, but the degree of complexation depends on several parameters such as the type and concentration of metal, the organic acid involved and the pH of the soil solution. Other authors proposed a symplastic tolerance mechanism developed by the weedy rice variety “Kumnung” (*Oryza sativa*) when it was grown in a medium with Pb toxic conditions (Yang *et al.*, 2000) but explained it as a non-strong mechanism.

Yang *et al.* (2000) studied rice varieties with high tolerance or sensitivity to lead. In the experiments, the plants in the Pb media, released organic acids like oxalate. The oxalate concentration in solutions in which the tolerant varieties were cultivated was higher than in the solution with the sensitive varieties. In the experiment, primary root seedlings were able to grow in solutions in which previously tolerant seedlings had been grown, while their root growth was inhibited in the solutions in which sensitive varieties had been before. They proposed the possibility that the Pb present in the previous solutions with the tolerant varieties had been turned into a form not available for the new seedlings. Furthermore, the varieties

were not different in their tolerance to Pb in the beginning, but the tolerant varieties developed adventitious roots once the Pb treatment was applied, while the sensitive varieties did not to develop adventitious roots. In an environment with iron toxicity, symptoms also varied within cultivars (Sahrawat, 2004).

Strategy IIa: Immobilization of iron in root and stem tissues

Once inside the xylem, the ferrous iron will follow an acropetal long-distance transport driven by the transpiration stream. However, part of the iron may be immobilized and deposited in “dumping sites” inside the plant or transported further and immobilized and deposited in stem/leaf tissues (Becker and Asch, 2005). In experiments with wild tomato plants, iron-containing particles appeared in chloroplasts and in vacuoles of root cells, indicating a “normal” deposition of these particles in tomato. The iron accumulation in the vacuoles may be due to the lower pH of the vacuolar sap compared to the cytoplasm (Liu et al., 1998).

The active iron could be stored in the form of ferritin (Becana *et al.*, 1998). Ferritins, called phytoferritins in the plants to differentiate them from animal ferritins, are ubiquitous iron storage proteins which can store up to 4500 iron atoms in a bioavailable and non-toxic form (Briat and Lobréaux, 1997). Studies conducted with two rice cultivars (*Oryza sativa* L.) proposed that since iron overload can induce expression of some plant Ferritin isoforms, storage of iron inside Ferritin could be connected with tolerance to excess of iron in some rice cultivars (da Silveira *et al.*, 2007). Ferritin buffers the ferrous ion concentration; the ferroxidase function of the protein moiety performs the oxidation of Fe^{2+} (Laulhere and Briat, 1993). Given the low redox potential of ferritin (-190 mV at pH 7), it seems reasonable that ferritin iron is Fe^{3+} (Harrison and Arosio, 1996). Since this protein was isolated in the presence of air, it is not clear if any iron is stored as Fe^{2+} in ferritin.

Briat and Lobréaux (1997) suggested that ABA could be involved in the ferritin synthesis in response to iron. An experiment with iron overloaded plantlets of maize increased ABA concentrations fivefold in roots and leaves, while an exogenous ABA treatment increased the abundance of ferritin mRNA. However, it was also seen that ABA alone could not increase the ferritin mRNA concentration, suggesting that another pathway is involved.

The efficiency of this process probably depends on the rate of acropetal transport of Fe^{2+} and may decline under conditions of high transpiration or when the storage capacity of the stem tissues is saturated, since in this case the iron surplus influx may reach the leaves. Additionally, there is no empirical evidence of genotypic variation (Becker and Asch, 2005).

Strategy IIb: Retention of iron in the leaf apoplast

Iron can be oxidized in the apoplast by precipitation as phosphate and hydroxide compounds (Stephan, 2002). This pool of iron in apoplastic space is not just an immobile result of the plant defense mechanisms against an excessive iron supply; it can also be used as storage iron to be mobilized when needed or under conditions of iron deficiency (Bienfait *et al.*, 1985; Stephan, 2002). This mobilization of iron in the leaves could be highly dependent on the apoplastic pH, which would regulate the influx of Fe^{2+} or Fe^{3+} in the leaves (Kosegarten *et al.*, 1999).

In addition, storage of iron may take place in the leaf cell vacuoles which by definition are leaf storage organs (Audebert, 2006). There, iron does not relocate during leaf senescence since it is a non-mobile element. Tolerant varieties appear to diminish the iron storage in their active leaves to maintain a better metabolic activity.

This iron storage may be in the form of phytoferritin. Those proteins accumulate in non-green plastids (amyloplasts, etioplasts and proplastids) and are found in tissues such as the seeds, root apex and shoots. Under iron overload conditions, ferritin is induced and accumulated in chloroplasts (Briat and Lobréaux, 1997).

Strategy III: Symplastic tissue tolerance

After having entered the symplast, Fe^{2+} will catalyze the production of radicals and active oxygen species (Becker and Asch, 2005). To prevent this, ferrous iron might be incorporated into ferritins (see strategy IIa) in the symplasm. This mechanism is based on avoiding the reaction between peroxides and iron by having high antioxidant levels and by subcellular compartmentation. The antioxidant content of young and healthy plants is enough to prevent free radicals from destroying them (Becana *et al.*, 1998). In plants exposed to a stressful situation, the formation rate of free radicals may exceed the plant's antioxidant capacity. However, some chelators like nicotianamine (one of the most important chelators involved in symplastic iron transport) can partially fulfill transport functions of iron in the symplast and prevent toxic effects of soluble ferrous ions (Liu *et al.*, 1998).

3.3. Rhizobacteria

To understand how soil biological processes are influenced by cultural practices and environmental factors, the species composition and structure of the microorganisms living in the rhizosphere have to be taken into account (Yang and Crowley, 2000). Distinct communities are associated with distinct root locations. However, some predominant bacterial

groups are present ubiquitously along the whole root. Flooded rice paddy soil can be divided into 3 compartments with different physiochemical conditions each: oxic surface soil, anoxic bulk soil and rhizosphere soil. Among these compartments, a wide range of functional groups of microorganisms drive the carbon, nitrogen, sulphur and iron cycle (Liesack et al., 2000).

3.3.1. Conditions favoring iron toxicity

After flooding of the rice fields, the oxygen is depleted in the soil due to the chemical oxidation reactions occurring there and oxygen consumption by the aerobic bacteria, reducing the pH of the environment. In reduced soils of lowland rice, Begg *et al.* (1994) found that up to 50% of oxygen consumption over a few days was microbial but this was depending on soil factors like organic matter and iron content. Organic matter aggravates the problem of iron toxicity by enhancing the reduction of Fe^{3+} in the soil and hampering the oxidizing capacity of the roots (Sahrawat, 2004). Under anoxic conditions of the soil, reduction reactions occur with the help of electron acceptors like nitrate and ferric iron (Liesack et al., 2000). Although the first electron acceptor reduced is nitrate, the remaining acidic conditions in the soil will lead to the reduction of Fe^{3+} favoring iron toxicity. In addition, the lower CO_2 consumption in the methanogenic zone resulted in a higher accumulation of siderite in soils with low organic carbon content (Liesack et al., 2000).

3.3.2. Conditions changed by the presence of rhizobacteria

Higher plants can affect the iron solubility in the rhizosphere directly by changing the redox potential, the pH and by releasing chelators through the roots, or indirectly by changing microorganism activity and population density in the rhizosphere (Marschner *et al.*, 1989). Root exudates by the plant and microorganisms colonizing its roots influence each other causing a variation in the plant's nutritional status. While root exudates alter the soil chemistry of the rhizosphere and serve as a selective substrate for bacteria and fungi living there, these microorganisms influence cell metabolism and plant nutrition in return (Jones, 1998; Yang and Crowley, 2000). Thus, organic acids released in large amounts into the rhizosphere can be expected to act as chemo attractants of flagellate bacteria towards the roots as well as to induce the growth of already-existing bacteria in the rhizosphere (Jones, 1998). Several studies have been run about the ability of flagellate bacteria to respond chemotactically to organic compounds. Although chemotaxis is a common feature within these bacteria, the way in which they are attracted to those compounds is different from one bacterium to another (Zheng and Sinclair, 1996).

In a study with soybean exudates and *Bacillus megaterium* strain B153-2-2, Zheng and Sinclair (1996) observed that at an optimal pH of 6-7 and an optimal temperature of 25 to 30°C, *B. megaterium* showed a significant chemotactic response to seed exudates. However, both motility and chemotactic response were significantly reduced at pH beyond 7; the optimal temperature for cell motility was about 20°C. The chemotactic response of this strain was in general greater to seed exudates than to root exudates. While *B. megaterium* showed higher motility and chemotactic response during growth stage, *Bacillus subtilis* cells had a higher chemotactic response and were more motile during the stationary stage.

At the same time, nutrient uptake and use efficiency in rice plants can be manipulated by inoculation with plant growth-promoting rhizobacteria (PGPR). In experiments with lowland rice, the addition of these bacteria resulted in growth promotion and higher accumulation of plant nitrogen (Biswas et al., 2000). Furthermore, the addition of plant growth-promoting bacteria or mycorrhizal fungi in the soil has been used in previous studies to diminish the negative effects of heavy metals uptake by plants (Burd et al., 1998). The production of the auxin Indole-3-Acetic Acid was increased in the rice root environment of lowland rice after inoculation with rhizobacteria promoting rice growth and seed production (Biswas et al., 2000).

Plant growth promoting bacteria which colonize the rhizosphere of rice roots have been used in this study to investigate a possible iron uptake reduction mediated by *Bacillus* spp.

3.3.3. Effects of rhizobacteria on iron availability

A large number of bacteria are able to produce siderophores in order to mobilize ferric iron. Studies showed that the proportion of phytosiderophores in the root exudates increases as iron deficiency stress becomes more severe, so that 50% of the root exudates are plant iron chelators (Yang and Crowley, 2000). However, plant iron nutritional status did not significantly influence the microbial community structures of the rhizosphere, when treatments of iron deficiency and iron sufficiency were compared, suggesting that the production of phytosiderophores and siderophores might cause consistent shifts in community structure but has minor effects on certain ubiquitous colonizers of the rhizosphere. The iron nutritional status of the plant explained around 20 to 40% of the total variation in the structure of the community. In this regard, the difference between root tips and older root parts must be considered; since the bacterial communities located at the root tips are most affected by the iron nutritional status of the plant, while the distribution of the species in the communities associated with the older root parts was more or less unchanged.

Unlike for gram-negative bacteria, information regarding the iron uptake system and transport in gram-positive bacteria is scarce (Heinrichs *et al.*, 2004). However, this has been improved due to an increase in knowledge of genome sequence information and its availability. Further information exists for bacterial siderophores. For example, the siderophore corynebactin which was identified first in *Corynebacterium glutamicum*, was recently detected in the well studied species *B. subtilis* (Hantke, 2004). In addition, *Bacillus megaterium* and some strains of *B. subtilis* were observed to produce the monohydroxamate siderophore schizokinen (Heinrichs *et al.*, 2004). The presence of these siderophores in the soil samples of rice fields was reported (Akers, 1983) after previous studies had shown that schizokinen was involved in the iron metabolism of the *Anabaena* sp. This suggests that schizokinen could also play a role in the iron acquisition and transport in *B. megaterium*.

For both ferric and ferrous iron, transport systems have been identified in bacteria (Hantke, 2004). The non-pathogenic *B. subtilis* has usually been used as a model of a gram-positive bacterium to investigate iron uptake systems (Heinrichs *et al.*, 2004), and its genome sequence appears to have numerous predicted iron uptake systems. The analysis of the *B. subtilis* genome shows several iron transporters which suggest that the bacteria may be able to use a large range of iron complexes. Many of these iron transporters are within a global iron-regulatory network controlled by the Fur homolog found in the bacteria. Fur-like proteins, which regulate iron in bacteria and have been found among others in *B. subtilis*, also regulate defense against oxidative stress (Touati, 2000). Cyanobacteria contain Fur-like proteins and since cyanobacteria diverged from purple bacteria before the development of an oxidizing atmosphere, this suggests the need of iron regulation even under anoxia conditions.

3.3.4. Effects of rhizobacteria on iron toxicity expression and plant ability to cope with Fe toxicity

For low pH environments, the importance of the microbial iron oxidation process that takes place in acidic environments has been recognized for years together with the existence of a polyphyletic group of lithoautotrophic microbes which utilize ferrous iron as an energy source. Studies to investigate bacterial toxin synthesis by iron showed that an iron-poor environment of the hosts limits the growth of the bacteria while it also serves as a signal to the bacteria for iron acquisition (Payne, 2003). Thus, in order to avoid bacterial cell damage, the iron uptake systems must be regulated.

Experiments with wetland plants showed that there is a large number of oxidizing bacteria associated with the root iron plaque (Emerson *et al.*, 1999). These bacteria found in the

rhizosphere include both acidophilic and neutrophilic iron oxidizers. Trolldenier (1988) studied the possible participation of bacteria in iron deposition. He showed that in root systems of rice plants grown in agar media containing insoluble or soluble ferrous compounds, iron oxidation appeared by the precipitation and accumulation of ferric oxide/hydroxide. Thus, spherical structures resembling bacterial colonies depositing ferric iron were observed in the oxidized rhizosphere of seedlings grown in agar and sand media inoculated with microorganisms of paddy soil.

Emerson and Moyer (1997) suggested that a population of unicellular prokaryotes even larger than the classic iron bacteria such as *Gallionella* and *Leptothrix*, might play a very important role in iron oxidation. They found two isolates of iron-oxidizing bacteria that utilize the energy obtained during the oxidation of Fe^{2+} into Fe^{3+} for growth at circumneutral pH which represent a new phenotypic group of bacteria. Preliminary evidence also suggested that both isolates create an extracellular matrix in which the iron oxide precipitation takes place. This oxidation process occurred within an oxic-anoxic transition zone and the results suggested that these bacteria were unable to use reduced organic carbon, converting them in true or obligate lithotrophs. The juxtaposition of oxic and anoxic conditions that takes place in the rhizosphere of wetland fields results in a dynamic Fe cycle where iron plaque is deposited and reduced simultaneously and alternatively (Weiss *et al.*, 2005). Hence, in the absence of radial oxygen loss in field conditions it was demonstrated that iron plaque is quickly reduced and solubilised.

Toutati (2000) suggested that Fur-like proteins coordinate the regulation of iron assimilation preventing acid and oxidative stress and are found in an increasing number of evolutionarily unrelated bacteria, thereby protecting cells against iron toxicity by allowing them to adapt their metabolism to the iron supply. Fur-like proteins which are involved in the regulation of iron in bacteria have been found in *B. subtilis*. Hence, the Fur regulatory functions which in the beginning were thought to be limited to control iron acquisition, appear to be more widespread suggesting a role for Fur as a general regulator. Fur will be inactivated under iron scarcity conditions (Toutati, 2000). *Bacillus subtilis* showed no significant differences in cell yields and growth rates between iron sufficient and iron deficient media (Peters and Warren, 1968). However, under conditions of iron deficiency, the capacity of the iron transport system of the bacteria was considerably increased.

4. Material and Methods

Between November 2007 and July 2008 two hydroponic experiments were conducted in the Greenhouse of the Institute of Crop Science and Resource Conservation (INRES), Department of Plant Nutrition, Germany. Both experiments were run for 6 weeks under tropical semi-controlled conditions.

The genotypes, all provided by the Plant Nutrition Institute at the University of Bonn, stem from breeding programs at the West Africa Rice Development Association, in Côte d'Ivoire (WITA 7) as well as in Senegal (I Kong Pao - IKP); at the International Institute for Tropical Agriculture (ITA306, ITA320 and TOX4004- 8-1-2-3) and at the International Rice Research Institute (IR 31785-58-1-2-3-3). These genotypes differ in their ability to cope with Fe stress. IKP and IR31785 are highly sensitive to excess of ferrous iron in the media, ITA 306 and ITA 320 are moderately tolerant and TOX 4004 and WITA 7 are tolerant genotypes.

For the first experiment, the six lowland rice genotypes of *Oryza Sativa* (I Kong Pao, IR 31785-58-1-2-3-3, ITA 306, ITA 320, TOX 4004-8-1-2-3 and WITA 7) were selected following previous experiments at the University of Bonn. This batch of seeds suffered from strong fungal infection and seeds were already stressed from the beginning of the experiments. ITA 320 hardly survived and therefore was not used for the statistical analysis. Due to the lack of seeds, they could not be replaced by healthy ones. In the second experiment, only the genotype I Kong Pao was used.

4.1. Definition of the treatments

Seven treatments were applied in the first experiment:

- 1) 0 ppm of iron without bacterial inoculation
- 2) 0 ppm of iron and inoculation with PA31
- 3) 0 ppm of iron and inoculation with PB32
- 4) 1000 ppm of iron without bacterial inoculation
- 5) 1000 ppm of iron and inoculation with PA31
- 6) 1000 ppm of iron and inoculation with PB32
- 7) 1000 ppm of iron and inoculation with *Bacillus megaterium* (Ni₅SO₁₁)

Six treatments were applied in the second experiment:

- 1) 0 ppm of iron without bacterial inoculation
- 2) 1000 ppm of iron without bacterial inoculation
- 3) 1000 ppm of iron and inoculation with PA31
- 4) 1000 ppm of iron and inoculation with PB32
- 5) 1000 ppm of iron and inoculation with *Bacillus megaterium*
- 6) 1000 ppm of iron and inoculation with *B. pumilus* (Ni₉MO₁₂)

The concentration of the bacteria inoculated was 6×10^6 cfu mL⁻¹ in each treatment. Due to the strong fungal infection, there were not enough seeds available to conduct the treatment with 0 ppm Fe and *B. megaterium* in the first experiment. Each treatment was added to a box in which plants were grown hydroponically. The treatments without iron addition were used to determine possible effects of bacteria on plant height and tillering. The treatments with 1000 ppm of Fe (II) were used to determine the iron concentration in the plant, iron partitioning, leaf scores symptoms and iron plaque (this was measured only in the second experiment) by the rice genotypes.

The Fe (II) contents in leaves, stems, and roots tissues were sampled separately and analyzed for iron content in the plant organ tissues with the help of the Dipyriddy method (Asch et al., 2005). In this method, samples undergo high pressure hot water digestion and are treated with Na-dithionite later on to reduce ferric into ferrous iron.

4.2. First experiment: Effects of *Bacillus* spp. inoculation on plant height and iron uptake

The first hydroponic experiment was conducted for 44 days. The bacterium was applied after 33 days to the nutrient solution and replaced by a solution containing Fe (II) 5 days later. Plant height, number of tillers and leaf score symptoms were recorded to find out if the bacterium mitigated the iron stress damage.

4.3. Second experiment: Effects of *Bacillus* inoculation on iron uptake and partitioning in the plant before and after cutting the root tips.

In the second hydroponic experiment, half of the plants had their root tips cut to investigate a possibly improved bacterial penetration. Plant height, tillering and leaf symptoms scores were also measured. To investigate whether the inoculated bacteria had favored an increase in the Fe (II) oxidation at the root surface, iron plaque was also evaluated. The duration of the

experiment was 44 days. The addition of the bacterial inoculation was 33 days after sowing and the iron was added to the culture solution 5 days thereafter. In addition, the roots were washed in a HCl solution 0.5N to determine the share of Fe precipitated on the root surface (iron plaque).

4.4. Plant material

Seeds were imbibed for 24 hours in a solution of 0.1M of 68% HNO₃ to improve germination rate. Afterwards, the seeds were washed with distilled water and germinated on water-soaked filter paper in Petri dishes for one week. Prior to transfer in hydroponic culture, germinated seeds were grown for 7 days in shallow trays of 50x30x4 cm in saturated, sandy soil.

4.5. Experimental set-up

4.5.1. Technical set-up

PVC pipes of 3.6 cm of diameter were cut in 9 cm long tubes. For each box, pipes were set together as a unit of 6x10 and fixed by melting the contact-side borders with a soldering iron. The pipe trays were constructed to support 6 different genotypes with 10 replications each. These 60 fixed pipes were introduced in rectangular boxes of 6.5 L, 26.5 cm width and 37 cm length. In the upper part of each 9cm long pipe, a half-split ceapren plug (3.5 cm in diameter, 3 cm high, supplied by Greiner) was inserted in order to fix each plant individually. The seed and the roots protruded from the ceapren plug, allowing only the root to be in contact with the nutrient solution

To induce anaerobic conditions in the above mentioned boxes, nitrogen gas was pumped through the nutrient solution. Two gas diffusers (3 x 25 cm, Long-Long, provided by Hobby, Dohse aquaristik KG) were placed on the bottom of each box. A nitrogen gas bottle (22 Mpa) was connected via plastic tubes to the gas diffusers, supplying N₂ for 15 min every 2 hours to the solution controlled by a magnetic valve. In this way, the oxidation of Fe²⁺ to Fe³⁺ was prevented in the nutrient solution (Figure 1).

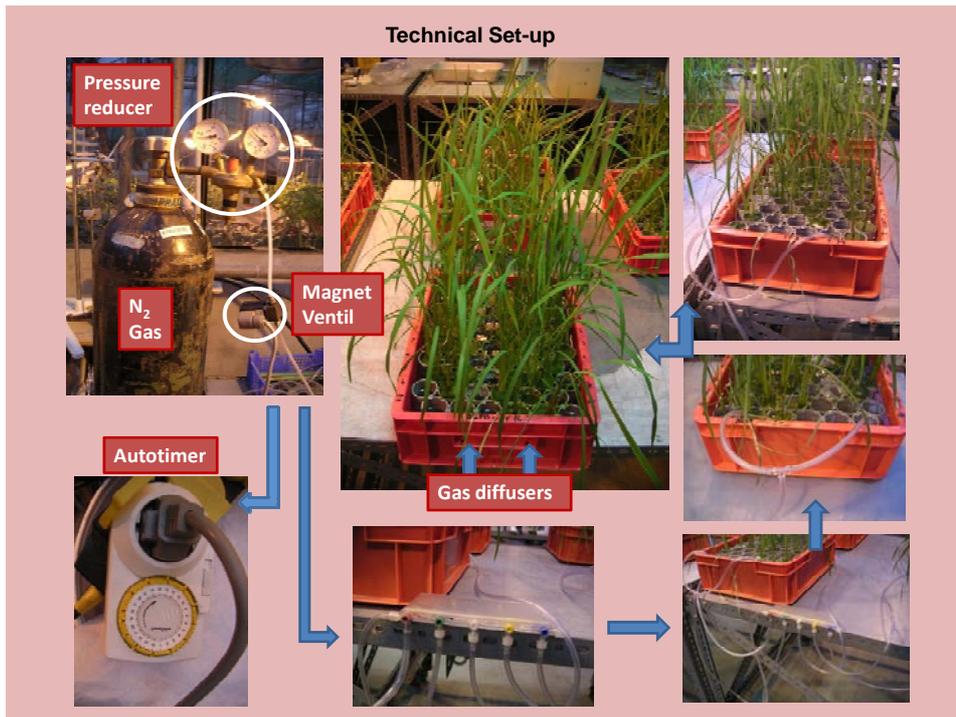


Figure 1: Schematic representation of the gas diffuser set-up of the experiment.

4.5.2. Set-up of the hydroponic culture

For the hydroponic culture, the temperature was set to 28 ± 2 °C during the day and 23 ± 2 °C during the night.

Plantlets were transplanted and each genotype was to a row of the PVC pipe trays placed in the boxes. These boxes were filled with Yoshida (Yoshida *et al.*, 1976). Before the onset of treatments, the solution was changed weekly gradually increasing in strength from 25% (after one week), to 50% (after six days) and to 100% (after four days). The Yoshida solution contains in full strength:

40mg L^{-1} N (NH_4NO_3), 10mg L^{-1} P ($\text{NaH}_2\text{PO}_4 \times 2\text{H}_2\text{O}$), 40mg L^{-1} K (K_2SO_4), 40mg L^{-1} Ca (CaCl_2), 40mg L^{-1} Mg ($\text{MgSO}_4 \times 7\text{H}_2\text{O}$), $0,5\text{mg L}^{-1}$ Mn ($\text{MnCl}_2 \times 4\text{H}_2\text{O}$), $0,05\text{mg L}^{-1}$ Mo ($(\text{NH}_4)_6 \times \text{Mo}_7\text{O}_{24} \times 4\text{H}_2\text{O}$), $0,2 \text{ mg L}^{-1}$ B (H_3BO_3), $0,01\text{mg L}^{-1}$ Zn ($\text{ZnSO}_4 \times 7\text{H}_2\text{O}$), $0,01\text{mg L}^{-1}$ Cu ($\text{CuSO}_4 \times 5\text{H}_2\text{O}$) und 2mg L^{-1} Fe ($\text{FeCl}_3 \times 6\text{H}_2\text{O}$).

The pH was weekly adjusted to 5.0 ± 0.2 with either NaOH 0.1M or HCl 1N.

4.6. Treatments

4.6.1. Growth of the bacteria and preparation of the iron and the bacterial solution

Four different strains of *Bacillus* were used in this study, namely PA31 (Figure 2), PB32, *Bacillus megaterium* (Ni₅SO₁₁) and *Bacillus pumilus* (Ni₉MO₁₂) (Figure 3). The isolates of Ni₅SO₁₁ and Ni₉MO₁₂ were originally isolated from rice roots in soils of Taiwan and the isolates 31Pa and 32Pb were isolated from rice seeds from Bangladesh.



Figure 2: PA31 under electronic microscope magnified 12000 times. Courtesy of Dr. Joachim Hamacher.



Figure 3: *Bacillus pumilus* under electronic microscope magnified 12000 times. Courtesy of Dr. Joachim Hamacher.

Bacteria were first cultivated in TSA (tryptic soy agar) for 24 hours at 28°C thereafter in TSB (tryptic soy broth) for 24 hours (Figure 4) such that bacteria grew fast enough to be sufficient for all the treatments. The bacteria suspended in ¼ strength Ringer solution (Figures 5 and 6) was added to the boxes containing 5 weeks old plantlets in 100% Yoshida solution.

Control treatments received only Ringer solution. The concentration of the bacteria was approximately 6×10^6 cfu mL⁻¹ in each box. Before adding the bacteria to the plantlets, the bacteria were multiplied in nutrient broth on a shaker table for 24 hours (Figure 2). Further details regarding this procedure, as well as the isolation, maintenance and multiplication of the bacteria are described in Paghdam and Sikora (2007). After five days the bacterial solution was replaced by a solution of FeSO₄. Iron sulphate was dissolved in Yoshida solution to a concentration of 1000 ppm of Fe²⁺ in the treatments with iron. Yoshida solution alone was prepared to give 0 ppm of Fe²⁺ for the control without iron. The Yoshida solution had a strength of 100% in both cases.



Figure 4: *Bacillus* spp. Grown in TSA and TSB at 28°C



Figure 5: *Bacillus* spp. suspended in 1/4 strength Ringer solution



Figure 6: *Bacillus* spp. Ready to be applied to the plants

4.6.2. Application of the nutrient solution, the bacteria and the Fe-solution

Bacteria were added to the boxes for which treatment was required (see in 3.1 the definition of the treatments). Five days after inoculation with bacteria, the nutrient solutions were replaced by the respective Fe-treatments (0 and 1000ppm) for six days to induce iron toxicity symptoms.

4.7. Sampling

After six weeks the rice plants were harvested. The roots of the plants subjected to 1000 ppm Fe were submerged for four minutes in scintillation vials containing 15 mL of 0.5M hydrochloric acid (HCl) to obtain the iron plaque, washed with distilled water, dried with paper towel, weighted, and stored in liquid nitrogen. The HCl washing solution was stored at 8°C, filtered and diluted to be analyzed with the atomic-absorption spectrometry (Perkin-ELMER AAS 1100B, Überlingen, Germany) in order to determine the iron plaque formation on the surface of the roots.

Roots from the non-iron toxicity treatment and stems and leaves from all treatments were collected, the fresh weight determined and stored in liquid nitrogen after the fresh weight had been determined. To estimate the iron distribution in the plant, leaves, stems and roots were sampled separately in paper bags. In the first experiment, several repetitions (groups of 2, 3 or 4) were placed in one paper bag, whereas plant organs were sampled individually (one repetition per bag) in the second experiment. Directly after harvesting and weighting, the samples were frozen with liquid Nitrogen and kept in a fridge at -20°C. Finally the samples were taken to a freeze-drier for drying in preparation for the analysis of iron.

4.8. Analytic methods

In order to investigate the effects of Fe²⁺ toxicity in lowland rice under the addition of bacteria, following data were recorded:

- Visual assessment of symptoms of iron toxicity in the leaves
- Iron content in the tissues of each organ of the plant; roots, leaves and stems
- Iron plaque formation on the root

4.8.1. Visual assessment

After 6 weeks, plants were visually scored and harvested. Iron toxicity symptoms were scored by assessing the percentage of leaf area affected by bronzing, one day before harvesting the plants. The percentage of bronzing covering the leaf area ranged from 0 to 10. We used an adapted version of the “Standard Evaluation System” for leaf blast (*Pyricularia oryzae*) lesion. This system was available at IRRI and was provided by the International Network for Genetic Evaluation of Rice (INGER; IRRI, 1996).

Table 1: Scoring of leaf area damage occurred due to the iron toxicity.

Scoring	Symptoms
0	No symptoms
1-2	Presence of the first symptoms till 20% of the leaf area damaged in the whole plant
3-4	First damage of the youngest leaves, strong damage of 1 to 2 older leaves, till 40% of the leaf area damaged in the whole plant.
5-6	Several young and old leaves are damaged, till 60% of the leaf area damaged in the whole plant.
7-8	Presence of the first dead parts of the leaves, high lost of photosynthesis. Strong damage till completely loss of the young leaves. Till 80% of the leaf area damaged in the whole plant.
9-10	Very strong damage, with dead parts or complete dead leaves. The whole plant may be dead. No more photosynthesis is possible at this point.

Height of the plant and number of tillers were also recorded.

4.8.2. Iron content in the plant

Roots, stems and leaves were left for 5 days in the freeze-drier after harvesting to obtain the dry matter. The Dipyridil method was used to analyze the iron content in plant tissues (Asch et al., 2005). Samples were ground in liquid nitrogen to prevent oxygen from entering the plant tissues (Figure 7). In this method (Figure 8), a small sample (around 0.1 g) is diluted into distilled water and left for one hour in the autoclave at 120°C. Then, the sample is centrifuged for 15 minutes at 3500 rpm. After removing the sample from the centrifuge, the sample is diluted first in a solution of Natrium dithionite 50 mM and second in a solution of 2, 2 Dipyridil 5 mM. Finally, the iron amount in the sample is measured in at a wavelength of 490 nm.



Figure 7: Grinding of the samples in liquid nitrogen previous to analyze them with the Dipyridil method (A: leaves; B: roots; C and D: stems)



Figure 8: Development of the analysis of the samples with the Dipyridill method (A: iron standards; B: trade with the samples diluted in 2,2 Dipyridil 5 mM; C: wavelength reader and D: computer program to calculate the amount of iron in the samples.

4.8.3. Statistics

The statistical analysis was done with SPSS version 17.0. The tests used were a Duncan Multiple Range Test at $p < 0.05$ and a T-test at $p < 0.05$ and 0.1 to show the levels of significance in the results.

5. Results

5.1. Effects of *Bacillus* spp. inoculation on plant height and iron uptake

5.1.1. Effect of Fe (II) stress on iron uptake and partitioning in leaves, stems and roots before and after the application of bacteria.

Leaf-, stem- and root tissues samples were analyzed for tissue iron content. Rice genotypes differed in their iron uptake after 1 week of exposure to Fe (II). The mean values of the results for iron concentration in the tissues of the roots and the level of significance are shown in Table 2. Leaf tissue iron concentration ranged from 0.33 to 0.51 mg g⁻¹ in the Fe (II) 1000 mg L⁻¹ treatment with the absence of bacteria, from 0.26 to 0.34 mg g⁻¹ in the Fe (II) 1000 mg L⁻¹ with addition of PA31, from 0.49 to 0.90 mg g⁻¹ in the Fe (II) 1000 mg L⁻¹ with addition of PB32 and from 0.21 to 0.46 mg g⁻¹ in the Fe (II) 1000 mg L⁻¹ with addition of Ni5SO11. The application of PA31 significantly reduced ($p < 0.05$) the iron concentration in leaves of the genotypes WITA 7 and ITA 306 with the application of PA31 compared to the Fe (II) control whereas leaf iron concentration was not significantly different in the other two genotypes. PB32 application to the iron treatment significantly increased Fe (II) uptake in IR 31785, IKP and TOX 4004 whereas no differences were observed in iron uptake in the other genotypes. With the application of *B. megaterium*, the iron concentration in the leaf tissues of ITA 306 was significantly reduced.

Table 2: Leaf tissue Fe concentration in 5 rice genotypes subjected to 0 and 1000 mg Fe (II) L⁻¹ in combination with or without *Bacillus* PA31 or PB32, or with *Bacillus megaterium* in 44 days old plants.

Genotype	Fe (II) concentration in leaf tissues (mg g ⁻¹)						
	Control 0 ppm	PA31 no Fe	PB32 no Fe	Control 10 ³ ppmFe	PA31 10 ³ ppmFe	PB32 10 ³ ppmFe	Ni5SO11 10 ³ ppmFe
WITA 7	0.26 ab	0.26 ab	0.08 a	0.51 d	0.32 bc	0.49 cd	0.46 cd
IR 31785	0.22 ab	0.19 ab	0.06 a	0.33 ab	0.38 b	0.77 c	0.43 b
IKP	0.25 ab	0.21 ab	0.05 a	0.48 b	0.35 ab	0.90 c	0.34 ab
TOX4004	0.21 a	0.05 a	0.16 a	0.39 a	0.31 a	0.81 b	0.21 a
ITA 306	0.25 a	0.16 a	0.26 a	0.51 b	0.26 a	0.56 b	0.27 a

Duncan Multiple Range Test at p < 0.05. Means in one column followed by the same letter are not significantly different at p < 0.05

The level of significance for the mean values of iron concentration in the tissue of stems is shown in Table 3. Independent of the genotype, application of 1000 ppm Fe (II) increased stems Fe concentration from 0.28 to 2.23 mg g⁻¹. Addition of PA31 resulted in stem Fe (II) concentrations of 0.35 to 0.66 mg g⁻¹ whereas the addition of PB32 resulted in stem Fe (II) concentrations of 0.51 to 0.90 mg g⁻¹ and the addition of Ni5SO11 resulted in concentrations that ranged from 0.51 to 1.00 mg g⁻¹ as shown in Table 3. Application of PA31 significantly reduced ($p < 0.05$) iron uptake in stems of the genotypes WITA 7, IKP, TOX 4004 and ITA 306 as compared to the control with 1000 ppm Fe whereas the iron uptake in IR 31785 was significantly increased. Similarly, application of PB32 reduced the iron concentration in stems of the genotypes WITA 7, IKP and ITA 306 whereas in IR 31785 it was significantly increased. With the application of *B. megaterium*, the iron concentration in the stems tissues of WITA 7, IKP, TOX 4004 and ITA 306 was significantly reduced whereas the iron concentration in IR 31785 was significantly increased.

Table 3: Stem tissue Fe concentration in 5 rice genotypes subjected to 0 and 1000 mg Fe (II) L⁻¹ in combination with or without *Bacillus* PA31 or PB32 or with Ni5SO11 in 44 days old plants.

Genotype	Fe (II) concentration stem tissues (mg g ⁻¹)						
	Control 0ppm Fe	PA31 no Fe	PB32 no Fe	Control 10 ³ ppmFe	PA31 10 ³ ppmFe	PB32 10 ³ ppmFe	Ni5SO11 10 ³ ppmFe
WITA 7	0.31 a	0.28 a	0.09 a	2.23 b	0.56 a	0.51 a	1.00 a
IR 31785	0.28 a	0.24 a	0.07 a	0.28 a	0.66 b	0.90 b	0.62 b
IKP	0.18 ab	0.15 ab	0.05 a	1.16 d	0.35 b	0.68 c	0.75 c
TOX4004	0.30 bc	0.05 a	0.17 ab	1.01 e	0.39 cd	0.57 d	0.53 d
ITA 306	0.24 a	0.31 ab	0.25 a	0.76 d	0.48 bc	0.70 d	0.51 c

Duncan Multiple Range Test at p < 0.05. Means in one column followed by the same letter are not significantly different at P < 0.05

The mean values for the iron concentration results in roots and the significance are presented in Table 4. Independent of the genotype, application of 1000 ppm Fe (II) increased from 4.30 to 5.65 mg g⁻¹ of Fe (II) in the absence of bacteria, from 4.22 to 5.13 mg g⁻¹ with the addition of PA31, from 10.94 to 16.41 mg g⁻¹ with the addition of PB32 and from 4.31 to 6.28 mg g⁻¹ with the addition of Ni5SO11. The treatment with PA31 and iron applied, showed no significant effects in the iron concentration of root tissues for any of the genotypes as compared to the control with 1000 ppm Fe. Similarly, the application of *B. megaterium* to the

control with 1000 ppm Fe did not significantly affect the iron concentration in the roots. Conversely, after application of PB32, root tissues of all genotypes presented significantly higher iron concentrations as compared to 1000 ppm Fe.

Table 4: Root tissue Fe concentration in 5 rice genotypes subjected to 0 and 1000 mg Fe (II) L⁻¹ in combination with or without *Bacillus* PA31 or PB32, or with *Bacillus megaterium* in 44 days old plants.

Genotype	Fe (II) concentration in root tissues (mg g ⁻¹)						
	Control 0 ppmFe	PA31 no Fe	PB32 no Fe	Control 10 ³ ppmFe	PA31 10 ³ ppmFe	PB32 10 ³ ppmFe	Ni5SO11 10 ³ ppmFe
WITA 7	0.38 a	0.36 a	0.45 a	4.99 a	5.12 a	10.94 b	4.31 a
IR 31785	0.24 a	0.29 a	0.24 a	4.30 b	4.69 b	11.88 c	6.28 b
IKP	0.28 a	0.18 a	0.25 a	5.35 a	4.22 a	12.62 b	4.68 a
TOX4004	0.30 a	0.22 a	0.27 a	5.65 b	4.34 b	16.41 c	5.20 b
ITA 306	0.40 a	1.39 a	0.29 a	4.30 b	4.68 b	14.09 c	4.59 b

Duncan Multiple Range Test at p<0.05. Means in one column followed by the same letter are not significantly different at P < 0.05

The mean values for the total iron concentration by the rice genotypes are presented in Table 5. Results are the sum up of the three plant organs roots, stems and leaves.

Table 5: Total Fe uptake in 5 rice genotypes subjected to 0 and 1000 mg Fe (II) L⁻¹ in combination with or without *Bacillus* PA31 or PB32, or with *Bacillus megaterium* in 44 days old plants.

Genotype	Fe (II) concentration in all tissues (mg g ⁻¹)						
	Cont 0ppm Fe	PA31 no Fe	PB32 no Fe	Control 10 ³ ppmFe	PA31 10 ³ ppmFe	PB32 10 ³ ppmFe	Ni5SO11 10 ³ ppmFe
WITA 7	0.95	0.90	0.62	7.73	6.00	11.94	5.77
IR 31785	0.74	0.71	0.36	4.91	5.72	13.55	7.32
IKP	0.71	0.55	0.35	7.00	4.92	14.20	5.78
TOX4004	0.81	0.32	0.60	7.05	5.04	17.79	5.94
ITA 306	0.88	1.87	0.80	5.57	5.41	15.35	5.37

5.1.2. Effect of the bacteria on plant height, on number of tillers and on iron toxicity symptom expression

Generally, the application of either *Bacillus* strain PA31 or PB32 in the absence of Fe (II) promoted plant height, as shown in the mean values of the results in Table 6. *Bacillus* PB32 induced a greater effect in plant height than PA31. In the 44 days old plants, the height measurements ranged from 13.62 to 16.33 cm in the control without iron, from 14.31 to 16.95 cm with addition of PA31 and from 16.21 to 18.24 cm with addition of PB32. In the treatments with application of iron, plant height ranged from 12.69 to 16.05 cm, from 14.94 to 17.55 cm with addition of PA31, from 13.11 to 16.4 after addition of PB32 and from 13.36 to 16.7 in the treatment with addition of Ni5SO11.

In the treatments without addition of 1000 ppm Fe, application of PB 32 resulted in the tallest plants as compared to the control; the plant height of the genotypes WITA 7, IR 31785, TOX 4004 and ITA 306 was significantly increased. After inoculation of the plants with PA31, plant height was not significantly different from the control group.

The addition of 1000 ppm Fe in the absence of bacteria, significantly reduced plant height in the genotypes WITA 7 and TOX 4004 as compared to the control.

In the presence of bacteria, 1000 ppm Fe (II) with PA31 significantly increased plant height in the genotypes WITA 7, IKP, TOX 4004 and ITA 306 as compared to the control with 1000 ppm Fe, and in the genotypes WITA 7 and ITA 306 as compared to the control in the absence of iron. In contrast, the addition of 1000 ppm Fe with PB32 did not induce any significant difference in plant height as compared to the 1000 ppm Fe control whereas it significantly decreased plant height in the genotypes WITA 7 and TOX 4004 as compared to the control in the absence of iron. After application of *B. megaterium* in combination with 1000 ppm Fe, plant height in the genotypes WITA 7 and TOX 4004 was significantly reduced as compared to the control. The results and their level of significance are presented in Table 6.

Table 6: Plant height of 44 days old plants in 5 rice genotypes subjected to 0 and 1000 mg Fe (II) L⁻¹ in combination with or without *Bacillus* PA31 or PB32, or with *Bacillus megaterium*.

Genotype	Plant height (cm)						
	Control 0 ppmFe	PA31 no Fe	PB32 no Fe	Control 10 ³ ppmF	PA31 10 ³ ppmF	PB32 10 ³ ppmF	Ni5SO11 10 ³ ppmFe
WITA 7	15.36 b	15.93 b	17.07 c	13.93 a	17.00 c	14.00 a	13.36 a
IR 1785	15.85 a	16.85 ab	17.10 b	16.05 ab	16.95 ab	16.40 ab	16.65 ab
IKP	16.30abc	16.95 bc	17.65 c	15.45 ab	17.55 c	15.15 a	16.70abc
TOX 4004	16.33 c	16.83 c	18.24 d	13.94 ab	17.11 cd	15.00 b	13.50 a
ITA 306	13.62 ab	14.31 bc	16.21 d	12.69 a	14.94 c	13.11 a	13.37 ab

Duncan Multiple Range Test at p<0.05. Means in one column followed by the same letter are not significantly different at p < 0.05

The mean tiller number values and the level of significance are shown in Table 7 for the five genotypes.

With bacteria applied in the absence of iron, all the genotypes had a higher number of tillers. Application of PA31 significantly increased the number of tillers in the genotype TOX 4004. Application of PB32 significantly increased the number of tillers in the genotypes WITA 7, IKP, TOX 4004 and ITA 306.

With addition of 1000 mg L⁻¹ Fe (II), the number of tillers in WITA 7 was significantly reduced as compared to the control. After application of PA31, the number of tillers in WITA 7 and ITA 306 were significantly increased as compared to the 1000 ppm Fe (II) control. After inoculation with PB32 or with *B. megaterium*, no significant changes in the number of tillers appeared as compared to the 1000 ppm Fe (II) control.

Table 7: Tillering in 44 days old plants in 5 rice genotypes subjected to 0 and 1000 mg Fe (II) L⁻¹ in combination with or without *Bacillus* PA31 or PB32, or with *Bacillus megaterium*.

Genotype	Number of tillers						
	Control no Fe	PA31 no Fe	PB32 No Fe	Control 10 ³ ppmFe	PA31 10 ³ ppmFe	PB32 10 ³ ppmFe	Ni5SO11 10 ³ ppmFe
WITA 7	1.57 bc	1.86 c	2.29 d	1.14 a	2.43 d	1.50 abc	1.29 ab
IR 31785	1.60 a	1.90 a	1.80 a	1.60 a	2.10 a	1.50 a	1.50 a
IKP	1.30 abc	1.90 c	2.60 d	1.20 ab	1.80 bc	1.00 a	1.10 a
TOX4004	1.00 a	1.44 b	1.99 c	1.00 a	1.00 a	1.00 a	1.00 a
ITA 306	1.13 a	1.13 a	2.00 b	1.13 a	2.00 b	1.11 a	1.00 a

Duncan Multiple Range Test at p<0.05. Means in one column followed by the same letter are not significantly different at P < 0.05

The leaf bronzing symptoms were scored 44 days after sowing. The mean values and the level of significance are presented in Table 8. Scores ranged from 0.87 to 4.00 in 1000 ppm Fe, from 2.11 to 4.78 with addition of PA31, from 1.00 to 4.80 with addition of PB32 and from 0.75 to 3.60 with addition of Ni5SO11.

In the absence of bacteria, the application of 1000 ppm Fe to the rice plants caused the strongest leaf bronzing symptoms in the sensitive genotypes IR31785 and IKP. With inoculation of PA31, leaf symptoms scores were significantly increased in the genotypes WITA 7, TOX 4004 and ITA 306 as compared to the 1000 ppm Fe (II) control. With inoculation of PB 32, the leaf bronzing scores were significantly increased in the genotypes WITA 7, IKP and TOX 4004. With the application of *B. megaterium*, the leaf symptoms scores significantly increased in the genotype WITA 7 as compared to the 1000 ppm Fe (II).

Table 8: Leaf symptoms score in 44 days old plants in 5 rice genotypes subjected to 0 and 1000 mg Fe (II) L⁻¹ in combination with or without *Bacillus* PA31 or PB32, or with *Bacillus megaterium*.

Genotype	Leaf symptoms score						
	Control no Fe	PA31 no Fe	PB32 no Fe	Control 10 ³ ppmFe	PA31 10 ³ ppmFe	PB32 10 ³ ppmFe	Ni ₅ SO ₁₁ 10 ³ ppmFe
WITA 7	0a	0a	0a	2.57 b	3.71 d	4.62 e	3.14 c
IR 31785	0a	0a	0a	3.50 bc	4.10 c	4.10 c	3.40 b
IKPo	0a	0a	0a	4.00 b	3.50 b	4.80 c	3.60 b
TOX4004	0a	0a	0a	1.67 b	4.78 d	4.22 c	1.67 b
ITA 306	0a	0a	0a	0.87 b	2.11 c	1.00 b	0.75 b

Duncan Multiple Range Test at $p < 0.05$. Means in one column followed by the same letter are not significantly different at $P < 0.05$

5.2. Effect of *Bacillus* inoculation on iron uptake and partitioning in the plant before and after cutting the root tips.

5.2.1. Effect of Fe (II) stress on iron uptake and partitioning in leaves, stems and roots

44 days old plants of IKP were exposed to 0 mg Fe (II) L⁻¹, 1000 mg Fe (II) L⁻¹ and to 1000 mg Fe (II) L⁻¹ + PA31/ PB32/ *Bacillus megaterium*/ *Bacillus pumilus* in the nutrient solution. Results are shown in Table 9. The application of PB32 significantly reduced ($p < 0.05$) Fe (II) tissue concentration in the cut roots with an iron concentration of 15.36 mg g⁻¹ as compared to the uncut roots with an iron concentration of 13.67 mg g⁻¹.

In the stems, the application of 1000 mg Fe (II) L⁻¹ and *Bacillus megaterium* significantly reduced ($p < 0.05$) iron concentration in the stem tissue to 1.75 mg g⁻¹ for the plants with uncut roots whereas the iron concentration in the stems for the plants with cut roots was 2.50 mg g⁻¹. The Fe (II) concentration in the tissue of the leaves of the cut plants was significantly higher ($p < 0.1$) as compared to the uncut control after application of following treatments; When 1000 mg Fe (II) L⁻¹ were applied, iron concentration in the leaf tissues of plants with uncut roots was 0.79 mg g⁻¹ whereas the iron concentration in the leaves of plants with cut roots was 1.20 mg g⁻¹; When 1000 mg Fe (II) L⁻¹ and *Bacillus pumilus* were applied, the leaf tissues of the plants with uncut roots had a Fe (II) concentration of 0.93 mg g⁻¹ whereas the iron concentration in the leaves of plants with cut roots was 1.15 mg g⁻¹.

Table 9: Root, stem and leaf tissue Fe concentration in I Kong Pao rice genotypes with uncut or cut root tips, in 44 days old plants subjected to 0, 1000 mg Fe (II) L⁻¹ and to 1000 mg Fe (II) L⁻¹ in combination with PA31/ PB32/ *Bacillus megaterium*/ *Bacillus pumilus*

Treatment (Fe)	Fe (II) concentration in plant tissues (mg g ⁻¹)					
	Roots		Stems		Roots	
	Uncut	Cut	Uncut	Cut	Uncut	Cut
Control+ 0 ppm	0.57 n.s. a	0.65 n.s. a	0.25 n.s. a	0.25 n.s. a	0.22 n.s. a	0.20 n.s. a
Control+ 10 ³ ppm	12.71 n.s. bc	12.56 n.s. bc	3.12 n.s. c	3.66 n.s. c	0.79 * b	1.20 * b
PA31+ 10 ³ ppm	10.68 n.s. b	11.29 n.s. b	4.63 n.s. d	4.38 n.s. d	1.36 n.s. cd	1.24 n.s. b
PB32+ 10 ³ ppm	15.36 ** d	13.67 ** c	3.24 n.s. c	3.44 n.s. c	1.41 n.s. d	2.05 n.s. c
Ni ₅ SO ₁₁ + 10 ³ ppm	11.71 n.s. b	11.65 n.s. b	1.75 ** b	2.50 ** b	0.93 n.s. bc	1.04 n.s. b
Ni ₉ MO ₁₂ + 10 ³ ppm	14.09 n.s. cd	13.22 n.s. bc	2.83 n.s. c	2.45 n.s. b	0.93 * bc	1.15 * b

**;* significantly different from the non-cut control at $p < 0.05$ and 0.1 , respectively; ns = non-significant. (two-sided Student's *t*-test)

The mean values for the total iron concentration by the rice plants with uncut and cut roots are presented in Table 10. Results are the sum up of the three plant organs roots, stems and leaves. The percentages of iron share in each organ are also represented there, showing the iron partitioning along the plant.

Table 10: Root (R), stem (S) and leaf (L) tissue Fe concentration in mg g⁻¹ and % of I Kong Pao rice genotypes with uncut or cut root tips, in 44 days old plants subjected to 0, 1000 mg Fe (II) L⁻¹ and to 1000 mg Fe (II) L⁻¹ in combination with PA31/ PB32/ *Bacillus megaterium*/ *Bacillus pumilus*

	Treatment (Fe) - Uncut roots											
	0 ppm Fe		Control10 ³ ppm		PA31+10 ³ ppm		PB32+10 ³ ppm		Bmeg+10 ³ ppm		Bpum+10 ³ ppmFe	
	mg g ⁻¹	%	mg g ⁻¹	%	mg g ⁻¹	%	mg g ⁻¹	%	mg g ⁻¹	%	mg g ⁻¹	%
L	0.22	21.44	0.79	4.75	1.36	8.15	1.41	7.03	0.93	6.44	0.93	5.21
S	0.25	23.92	3.12	18.76	4.63	27.78	3.24	16.18	1.75	12.18	2.83	15.85
R	0.57	54.62	12.71	76.47	10.68	64.06	15.36	76.77	11.71	81.37	14.09	78.93
T	1.05		16.62		16.67		20.01		14.39		17.85	

	Treatment (Fe) - Cut roots											
	0 ppm		10 ³ ppm		PA31+10 ³ ppm		PB32+10 ³ ppm		Bmeg+10 ³ ppm		Bpum+10 ³ ppm	
	mg g ⁻¹	%	mg g ⁻¹	%	mg g ⁻¹	%	mg g ⁻¹	%	mg g ⁻¹	%	mg g ⁻¹	%
L	0.20	18.04	1.20	6.87	1.24	7.35	2.05	10.68	1.04	6.84	1.15	6.83
S	0.25	22.88	3.66	21.01	4.38	25.91	3.44	17.94	2.50	16.44	2.45	14.57
R	0.65	59.07	12.56	72.11	11.29	66.72	13.67	71.37	11.65	76.7	13.22	78.58
T	1.10		17.41		16.92		19.16		15.19		16.82	

5.2.2. Iron plaque formation

Fe (II) inside the roots and Fe (III) coatings on the root surface were measured. Results are presented in Table 11. For the uncut treatment, the iron concentration inside the root ranged from 10.68 to 15.36 mg g⁻¹ and from 11.29 to 13.67 for the cut treatment. After application of 1000 ppm Fe in combination with PB32, the iron concentration inside the root tissues was significantly lower ($p < 0.05$) in the cut roots as compared to the uncut ones.

For the iron plaque on the roots, the iron concentration in the root tissues of the uncut roots ranged from 44.75 to 65.44 mg g⁻¹ whereas the concentration in the tissues of the cut roots ranged from 46.20 to 113.69 mg g⁻¹. The application of 1000 ppm Fe with PB32 significantly reduced the iron plaque amount in the uncut roots as compared to the cut roots. Also, the application of 1000 ppm Fe with *Bacillus megaterium* significantly reduced ($p < 0.1$) the iron plaque amount in the uncut roots as compared to the cut roots.

Table 11: Root tissue Fe concentration and iron plaque in I Kong Pao rice genotypes with uncut and cut root tips, in 44 days old plants subjected to 0, 1000 mg Fe (II) L⁻¹ and to 1000 mg Fe (II) L⁻¹ in combination with PA31/ PB32/ *Bacillus megaterium*/ *Bacillus pumilus*

Treatment (Fe)	Fe (II) concentration inside vs. outside the roots (mg g ⁻¹)			
	Iron inside the roots		Iron plaque	
	Uncut	Cut	Uncut	Cut
Control+0 ppm	0.57 n.s.	0.65 n.s.	0 a	0 a
Control+10 ³ ppm	12.71 n.s.	12.56 n.s.	60.10 n.s. bc	45.35 n.s. b
PA31+10 ³ ppm	10.68 n.s.	11.29 n.s.	65.44 n.s. c	72.94 n.s. c
PB32+10 ³ ppm	15.36 **	13.67 **	48.90 ** bc	55.48 ** b
Ni ₅ SO ₁₁ +10 ³ ppm	11.71 n.s.	11.65 n.s.	44.75 * b	58.49 * bc
Ni ₉ MO ₁₂ +10 ³ ppm	14.09 n.s.	13.22 n.s.	54.08 n.s. bc	46.20 n.s. b

**;* significantly different from the non-cut control at $p < 0.05$ and 0.1 , respectively; ns = non-significant. (two-sided Student's *t*-test)

The amount of iron inside the roots and in the iron plaque is in Table 12. The percentages of iron share are also represented there, showing the iron distribution inside and on the roots.

Table 12: Root tissue Fe concentration and iron plaque in I Kong Pao rice genotypes with uncut and cut root tips, in 44 days old plants subjected to 0, 1000 mg Fe (II) L⁻¹ and to 1000 mg Fe (II) L⁻¹ in combination with PA31/ PB32/ *Bacillus megaterium*/ *Bacillus pumilus*

Treatment (Fe)	Uncut roots				Total Fe mg g ⁻¹
	iron inside the root		iron plaque		
	mg g ⁻¹	%	mg g ⁻¹	%	
Control+10 ³ ppm	12.71	17.45	60.1	82.54	72.81
PA31 + 10 ³ ppm	10.68	14.02	65.44	85.97	76.12
PB32 + 10 ³ ppm	15.36	23.9	48.9	76.09	64.26
<i>B. megaterium</i> + 10 ³ ppm	11.71	20.74	44.75	79.25	56.46
<i>B. pumilus</i> + 10 ³ ppm	14.09	20.66	54.08	79.33	68.17

Treatment (Fe)	Cut roots				Total Fe mg g ⁻¹
	iron inside the root		iron plaque		
	mg g ⁻¹	%	mg g ⁻¹	%	
Control+10 ³ ppm	12.56	21.68	45.35	78.31	57.91
PA31 + 10 ³ ppm	11.29	13.40	72.94	86.59	84.23
PB32 + 10 ³ ppm	13.68	19.77	55.48	80.22	69.16
<i>B. megaterium</i> + 10 ³ ppm	11.65	16.61	58.49	83.38	70.14
<i>B. pumilus</i> + 10 ³ ppm	13.22	22.24	46.20	77.75	59.42

5.2.3. Visual assessment

44 days old plants subjected to 0, 1000 mg Fe (II) L⁻¹ and to 1000 mg Fe (II) L⁻¹ + PA31/ PB32/ *Bacillus megaterium*/ *Bacillus pumilus*. Results are presented in Table 13. Plant height ranged from 15.73 cm to 17.72 in the plants with uncut roots whereas it ranged from 15.5 to 17.08 in the plants with cut roots. The number of tillers ranged from 3.00 to 3.17 in the plants with uncut roots and from 3.00 to 3.33 in the plants with cut roots. The symptoms score in the leaves of plants with uncut roots ranged from 4.00 to 5.50 whereas in the leaf of plants with cut roots it ranged from 3.33 to 5.67.

Application of 1000 ppm Fe in combination with PA31 significantly increased ($p < 0.1$) the height of plants with uncut roots as compared to the plants with cut roots. Tiller number was not significantly affected.

After visual assessment of the symptoms, the solution combining Fe (II) and *Bacillus megaterium* significantly increased ($p < 0.1$) the symptoms in the leaves of plants with uncut roots as compared to the leaves of plants with cut roots.

Table 13: Height, number of tillers and leaf symptoms score in 44 days old plants of I Kong Pao genotypes with uncut and cut root tips subjected to 0, 1000 mg Fe (II) L⁻¹ and to 1000 mg Fe (II) L⁻¹ in combination with PA31/ PB32/ *Bacillus megaterium*/ *Bacillus pumilus*

Treatment (Fe)	Plant height (cm)		Number of tillers		Leaf symptoms score	
	Uncut	Cut	Uncut	Cut	Uncut	Cut
Control+ 0 ppm	17.72 n.s. b	17.08 n.s. b	3.00 n.s. a	3.00 n.s. a	0 a	0 a
Control+ 10 ³ ppm	15.73 n.s. a	15.77 n.s. a	3.17 n.s. a	3.33 n.s. a	4.33 n.s. bc	4.50 n.s. c
PA31+ 10 ³ ppm	16.23 * ab	15.50 * a	3.00 n.s. a	3.17 n.s. a	5.50 n.s. c	5.33 n.s. cd
PB32+ 10 ³ ppm	17.03 n.s. b	16.28 n.s. ab	3.17 n.s. a	3.00 n.s. a	5.33 n.s. c	5.67 n.s. d
Ni ₅ SO ₁₁ + 10 ³ ppm	16.52 n.s. ab	16.35 n.s. ab	3.17 n.s. a	3.17 n.s. a	4.00 * b	3.33 * b
Ni ₉ MO ₁₂ + 10 ³ ppm	16.35 n.s. ab	15.73 n.s. a	3.00 n.s. a	3.00 n.s. a	4.40 n.s. bc	5.67 n.s. d

**;* significantly different from the non-cut control at $p < 0.05$ and 0.1 , respectively; ns = non-significant. (two-sided Student's *t*-test)

6. Discussion

Due to reducing conditions in the root environments, iron uptake by the rice plants under conditions of iron excess may have severe consequences for yield. Iron plaque formation may act as an avoidance mechanism of this abiotic stress (Tanaka et al., 1966), however, it may not be sufficient to prevent excessive iron uptake and the subsequent damage to the plant. Thus, enhancing this avoidance mechanism with the aid of microorganisms could be a helpful tool. Root hairs participate in nutrient and water uptake and increase the absorptive surface of the root system. Their development is very sensitive to biotic and abiotic stimuli (López-Bucio et al., 2007), such as both iron excess in the media and inoculation with plant growth promoting bacteria. These bacteria have been proven to affect the plant metabolism in a direct way by producing substances that are usually only available in short supply (Hernández et al., 2009). Some are able to fix atmospheric nitrogen, to solubilize iron and phosphorus, or to produce plant hormones like auxins, cytokinins and gibberellins. They can also improve the tolerance of plants to pesticide load, drought, salinity, and metal toxicities (Hernández et al., 2009). As such, they may affect the response of rice plants to an environment with iron excess. Several *Bacillus* spp. isolated from the plant rhizosphere may play a role in the plant's tolerance against iron toxicity.

6.1. Symptom scores, plant height and tillering

In the first experiment, the strongest symptom expression after application of the treatment of 1000 ppm Fe, appeared in IR31785 and I Kong Pao confirming previous reports on the sensitivity of these two genotypes and the relative tolerance of WITA7, TOX4004 and ITA 306 to iron toxicity (Table 8).

The strongest symptoms of iron toxicity appeared with the application of 1000 ppm Fe in combination with PA31 and PB32 in both experiments, with the exception of IKP inoculated with PA31 (Table 8). This exception may have been due to the stress caused by the fungal infection during germination, thus, interfering with our treatments. This biotic stress should be kept in mind for the analysis of the results in the first experiment.

The higher leaf symptoms score after inoculation with PA31 may be related to auxin production by this bacterium. Schopfer et al. (2002) have shown apoplastic production of O_2^- by a plasma-membrane-bound NADH oxidase to be promoted by auxins. Part of the O_2^- is converted into H_2O_2 . In rice, an excess of ferrous iron in the leaves would react with H_2O_2 following the Fenton reaction and, therefore, the concentration of OH would increase in the

leaves, leading to increased iron toxicity symptoms. Schopfer et al. (2002) also proposed that peroxidase bound to cell wall polymers can use these reactive oxygen intermediates (O_2^- and H_2O_2) for generating OH close to load-bearing sites in the polysaccharide wall matrix. Other authors (Jiang et al., 2003) proposed that a shift in the auxin maximum correlates with a change in the redox status. The redox status becomes more oxidizing towards which the auxin maximum has been shifted (the proximal meristem), whereas it becomes less oxidizing in the region from which the auxin maximum has been displaced (the quiescent center). This would explain why roots under iron toxicity stress and inoculated with PA31 showed the highest measurements of iron plaque. Taken together these results provide strong evidence that PA31 could produce high amounts of auxin.

The strong symptoms appeared after inoculation with PB32 of plants subjected to 1000 ppm Fe (Table 8) were expected since the presence of these bacteria in the nutrient solution produced the highest iron concentration in the plants of both experiments (Tables 5 and 10). The total iron concentration in the plants of the first experiment inoculated with PB32 and subjected to 1000 ppm Fe was twice as high or even higher compared to the non-inoculated 1000 ppm Fe treatment. Regarding the second experiment, the total iron concentration was around 20% higher for uncut and 10% higher for cut roots plants as compared to the non-inoculated 1000 ppm Fe treatments respectively. Because of this, a higher iron concentration was also found in the leaves, and symptoms score also increased.

In the first experiment, no difference was found in symptoms of iron toxicity between the plants subjected to 1000 ppm Fe and inoculated with *B. megaterium* compared to the 1000 ppm Fe control except for the genotype WITA 7 for which symptoms were significantly worse for those plants inoculated with *B. megaterium* (Table 8). In the second experiment, the only significant reduction in symptoms of plants subjected to 1000 ppm Fe and inoculated with *B. megaterium* compared to the control occurred for the leaves of plants with cut roots (Table 13). These plants showed a significant reduction in the symptoms score, probably because cutting the roots increased the bacterial penetration in the plant, suggesting a mechanism of this bacterium to reduce the iron concentration inside the plant. Several studies showed the production of siderophores by *B. megaterium*, which would have helped the plant to alleviate the symptoms of iron toxicity. Further details on siderophore production by *B. megaterium* and a reduction in the concentration of iron culture solution due to mediation by *B. megaterium* can be found in section 6.2. The small reduction of the symptoms is probably related to the reduced iron concentration in the media and therefore to a reduced iron uptake by the plant after inoculation with *B. megaterium*.

In general, all the bacteria applied improved shoot height (Tables 6 and 13) probably because these bacteria produce plant hormones which promote plant growth. In fact, many bacillus spp. are plant growth-promoting rhizobacteria (this issue will be explained in more detail in section 6.2). PA31 was most successful in promoting height in the first experiment (Table 6), where plant height was significantly increased in all genotypes compared to the 1000 ppm Fe control, except for IR31785. PA31 also significantly increased the number of tillers in the genotypes WITA 7 and ITA 306 (Table 7). In the second experiment, inoculation with PB32 significantly increased plant height as compared to the 1000 ppm Fe control (Table 13). Cutting the roots to improve bacterial penetration did not affect plant height. As to why PA31 was the best one in promoting plant height in our first experiment instead of PB32 (Table 6), which was the best one in experiment two, we again attribute these differences to the fact that after inoculation with PB32 in plants subjected to 1000 ppm Fe, plants in the first experiment dramatically took up more iron than plants in the second experiment.

The application of *B. megaterium* to solubilize P was used in other experiments conducted with chickpea to study the effect of combined application of Rhizobium, the PSB *B. megaterium* sub. Sp. Phosphaticum Strain-PB and *Trichoderma* spp. on growth, nutrient uptake and yield (Rudresh et al., 2005). Plant height was the highest and significantly higher in the treatments in which PSB was included together with *Rhizobium*, *T. harzianum* and rock phosphate under glasshouse and field conditions respectively, compared to the treatments in which *Rhizobium* was inoculated without the PSB *B. megaterium*. These results may suggest an explanation for the results obtained in our experiments regarding the height measurements for the plants subjected to 1000 ppm Fe and inoculated with *B. megaterium*. In the first experiment, both sensitive varieties, IR31785 and IKP, and the moderately tolerant ITA 306, increased their height as compared to the 1000 ppm Fe control (Table 6). Therefore it would be interesting to measure the phosphate concentrations in further experiments to confirm this possibility. In the second experiment, the inoculation with *B. megaterium* to plants subjected to 1000 ppm Fe had a positive effect on the plants' height. The height of plants with cut and uncut roots were the highest and second highest respectively as compared to the control plants with 1000 ppm Fe applied (Table 13).

Several studies have included *B. megaterium* as plant growth-promoting rhizobacteria (Cakmakci et al., 2006; López-Bucio et al., 2007). Experiments conducted with *Arachis hypogaea* L. and inoculation of *B. megaterium* GPS 55 to study seedling emergence, growth and yield of field-grown groundnut showed that the bacteria promoted all of them, including shoot and root length (Kishore et al., 2005). In addition, *B. megaterium* stimulated bean

plants' growth both in soil and in vitro and *Arabidopsis* growth in vitro, suggesting that this bacterial strain probably works as a novel plant growth-promoting rhizobacteria (López-Bucio et al., 2007).

Inoculation with *B. pumilus* also increased plant height in plants with uncut roots and subjected to 1000 ppm Fe of our second experiment (Table 13). This bacterium was determined to be plant-growth promoting by Joo et al. (2005). Several gibberellins were identified after application of several *Bacillus* spp. in pepper; the content of gibberellins in control plants was very low compared to the gibberellins produced when the bacteria were present (Joo et al., 2005). Other experiments (Gutiérrez-Mañero et al., 2001) also demonstrated the effectiveness of this bacterium as a plant growth promoting rhizobacterium. They concluded that the combined results of a study with *B. pumilus* and *B. licheniformis* provide evidence that the induction of stem elongation promoted by the rhizobacteria may be mediated by bacterial gibberellins.

6.2. Iron uptake by plants

In the first experiment, the iron concentration in all genotypes was strongly increased after inoculation with PB32 in the presence of iron (Table 5) as compared to the 1000 ppm Fe control, which was also reflected in the iron concentration in the leaf (Table 2) and root tissues (Table 4). This higher concentration was also observed in the second experiment, but to a lower extent (Table 9). After inoculation with PB32 and in the presence of iron, iron concentration in plants with uncut roots was 20% higher and in plants with cut roots it was 10% higher than in plants non-inoculated and subjected to 1000 ppm Fe (Table 10).

The iron concentration in stem tissues after inoculation with each of the 3 bacteria (PA31, PB32 and *B. megaterium*) was significantly reduced in almost all the genotypes in the first experiment as compared to the plants subjected to 1000 ppm Fe (Table 3). Since neither PA31 nor PB32 are identified to species level, the discussion of these results must remain speculative. A possible mechanism could involve recirculation of iron from shoot to root, especially given that the amount of iron in the roots was at least double after inoculation with PB32 as compared to the other three treatments with 1000 ppm Fe (Table 4) regardless of bacterial inoculation. Hell and Stephan (2003) discussed such a mechanism. They found that Fe (II) iron was transported with the chelator nicotianamine in the phloem. Because of the physicochemical properties of these complexes, a shuttle function of nicotianamine chelating ferrous iron from ITP-bound Fe (III) during loading and unloading may occur and thereby a steady-state level of ferrous iron in the phloem is maintained. But nicotianamine can only

appear if either the plant or the bacteria release siderophores. In this case, given that there is excess of iron in the medium, it is unlikely that plants would release siderophores and it is therefore plausible that only PB32 would be responsible for their release. Nevertheless, the fact that plants were stressed due to the fungal infection, could have had a marked effect on the iron distribution along the plant, altering plant hormones and the signaling transport between shoot and root.

In contrast, the plants subjected to iron and inoculated with either of the two bacteria PA31 or *B. megaterium*, showed a reduced total iron uptake compared to the plants subjected to the 1000 ppm Fe treatment, with the exception of the sensitive genotype IR31785 (Table 5). This was also reflected in the iron concentration of the leaves, stems and root tissues, whose iron concentration was lower compared to the non-inoculated plant tissues subjected to 1000 ppm Fe in all the genotypes but for IR31785 (note as exception the root tissue of WITA 7, whose iron concentration was 5.12 mg g⁻¹ after inoculation with PA31 and subjected to 1000 ppm Fe as compared to 4.99 mg g⁻¹ in the presence of iron and without bacterial inoculation) (Table 4). IR31785 did not show any reduction in the iron concentration of the separate tissues, whereas all other genotypes, WITA 7, IKP, TOX 4004 and ITA 306 had lower iron concentrations in their tissues after the bacterial inoculation. In the second experiment, inoculation with *B. megaterium* reduced the Fe concentration in both, plants with uncut and cut roots, whereas inoculation with PA31 resulted only in reduced iron concentrations in plants with cut roots (Table 10). The iron concentration in plants with uncut roots after inoculation with PA31 and subjected to 1000 ppm Fe in the second experiment did not change as compared to the 1000 ppm Fe control (Table 10). In addition, iron concentration among the genotypes did not follow any systematic variation. After inoculation of the plants with *B. megaterium*, the concentration of iron in the plant was of 15.2 mg g⁻¹ for cut plants and of 14.4 mg g⁻¹ for uncut plants as compared to the plants subjected to 1000 ppm Fe without bacteria inoculation which iron concentrations of the plants were of 17.4 mg g⁻¹ and 16.6 mg g⁻¹ respectively (Table 10). Inoculation with *B. megaterium* resulted in the lowest Fe concentrations.

The lower concentration of iron in the plant could be explained by a removal of iron by the bacteria. This idea stems from experiments conducted to study iron adsorption by *Bacillus subtilis* in an aqueous concentration of 1 ppm Fe. Measurements of this experiment revealed that with a concentration of bacteria in the system of only 2g/L, 90 per cent of aqueous iron was removed from the solution by adsorption of iron onto the bacterial cell wall (Wightman and Fein, 2005). The authors concluded that the Fe-bacterial surface complex is by far more

stable than those involving other metals previously examined. The bacterial adsorption process plays a relevant role in the geochemical cycling of iron in bacteria-bearing environments by effectively concentrating iron, especially in environments where passive adsorption/ precipitation reactions may dominate like in groundwater aquifers (Wightman and Fein, 2005).

Other studies by Kamaludeen and Ramasamy (2008) confirmed that microbial siderophores are used as metal chelating agents capable of regulating the iron availability in the rhizosphere of plants, helping the plants to alleviate the metal toxicity. The production of the siderophores schizokinen and ferrioxamine B by *B. megaterium* was demonstrated by Arceneaux *et al.* (1984). Although siderophores only have a weak affinity for ferrous iron, the production of ferric chelating siderophores by these bacteria should be considered for the removal of iron from the solution. Even though our experiment was done in anoxic conditions, the oxygen released by the aerenchyma of the rice plants increased the concentration of ferric iron by precipitating it at the root surface, a mechanism that has been well established in rice and demonstrated by several authors (Tanaka *et al.*, 1966; Sahrawat, 2004). Additionally, since the redox reactions of iron are dynamic, the reiterated oxidation of ferrous into ferric iron increased the amount of ferric iron available to build up siderophore complexes in the solution. These complexes could be taken up by the bacteria and therefore they could not be reduced into ferrous iron again. *B. megaterium* has a considerable amount of intracellular schizokinen that diffuses out to the surrounding medium (Hu and Boyer, 1996). Once the intracellular and extracellular levels of siderophore reach equilibrium, this diffusion stops.

In experiments which investigate the siderophore-mediated aluminium uptake by *B. megaterium*, the formation of schizokinen siderophores was usually repressed by the addition of 0.04 mg of iron per liter (Hu and Boyer, 1996), but increased levels of siderophores would appear with increased concentrations of added aluminium due to continued siderophore diffusion into the medium during the stationary phase. Other studies also pointed out that the activity of ferrisiderophore reductasa in *B. megaterium* was not significantly altered by the level of iron in the medium (Arceneaux and Byers, 1980). This enzyme participates in the reductive mechanism that makes ferrous iron available for cellular metabolism by removing iron from the ferrischizokinen siderophore in *B. megaterium*. It was postulated that the activity of this enzyme could form the link between the ferrisiderophore transport and iron assimilation into metabolic systems. In addition, experiments with rhizospheric bacteria under conditions of cadmium stress demonstrated their phytoprotective effect by binding cadmium ions in the media and therefore diminishing the amount of free irons entering the plant

(Pishchik *et al.*, 2005). To assess if *B. megaterium* reacts in the same way under conditions of iron toxicity, further experiments in this direction are needed.

Experiments with a newly identified strain of *B. megaterium* (UMCV1) in *Arabidopsis* showed that plant-growth promotion by this bacterium correlated with changes in the root-system architecture, in particular increased root-hair elongation and growth of lateral roots (López-Bucio *et al.*, 2007). The fact that bacterial inoculation inhibited cell elongation and meristematic activity in the primary root and promoted epidermal-cell differentiation in the experiments done by López-Bucio *et al.*, could explain why cutting the roots did not have a significant effect on the amount of iron accumulated in the cut roots as compared to the uncut roots, since the bacteria would have an effect on the architecture of the roots.

The inoculation of plants with *Bacillus pumilus* and subjected to 1000 ppm Fe had not effect on the total iron concentration in the plant as compared to *B. megaterium* (Table 10) but induced a change in the iron partitioning within the plant. The iron concentration in the roots increased and the iron concentration in the shoots decreased (Table 9). The amount of iron in roots of plants inoculated with *B. pumilus* and subjected to 1000 ppm Fe was of 14.1 mg g⁻¹ and 13.2 mg g⁻¹ in uncut and cut roots respectively as compared to the 12.7 mg g⁻¹ and 12.6 mg g⁻¹ found in uncut and cut roots respectively of plants subjected to 1000 ppm Fe and non-inoculated (Table 9). The mean iron concentration in the shoots of IKP was 2.8 mg g⁻¹ and 2.5 mg g⁻¹ with uncut and cut roots respectively as compared to 3.1 mg g⁻¹ and 3.7 mg g⁻¹ in the shoots of non-inoculated plants subjected to 1000 ppm Fe with uncut and cut roots respectively (Table 9).

Experiments with maize plants inoculated with *B. pumilus* to study heavy-metal extraction and uptake by plants growing on multi-metal-contaminated soils, showed that the bacteria significantly increased Zn, Pb, Cr and Cu accumulation in the roots of maize grown on tannery-effluent-polluted soil as compared to the non-inoculated control plants (Abou-Shanab *et al.*, 2008). Also, inoculation with PA31 did not change iron concentration in the plant. However, the amount of iron in the roots of uncut and cut plants after inoculation with PA31 and subjected to 1000 ppm Fe was 10.7 mg g⁻¹ and 11.3 mg g⁻¹ respectively as compared to 12.7 mg g⁻¹ and 12.6 mg g⁻¹ found in the roots of plants subjected to 1000 ppm Fe and non-inoculated (Table 9). These results show a modification in the partitioning pattern. This could be due to a change in the gibberellins and auxins balance, caused by *B. pumilus* and PA31. Raja *et al.* (2006) pointed out the importance of this balance for the regulation of cell development. Bacteria capable of producing high amounts of IAA probably inhibit plant

growth. The results of Raja et al. (2006) also indicated that in rice root environments, the microbial inoculation can change the IAA-like hormone level. Previous reports showed that auxins were present in the liquid culture media of a few PGPR strains such as *B. pumilus*, *B. pumilus* also produced high amounts of the C₁₉-GAs gibberellins (GA₁, GA₃, GA₄ and GA₂₀) as found in a study conducted with both plant-growth-promoting rhizobacteria *Bacillus pumilus* and *Bacillus licheniformis* isolated from the rhizosphere of alder (*Alnus glutinosa* [L.] Gaertn.) (Gutiérrez-Mañero et al., 2001). Studies by Aloni et al. (1988) indicated a role for gibberellic acid in the regulation of phloem loading and translocation rates, after observing a stimulatory effect on sucrose export out of attached *Vicia faba* source leaves. They proposed that the enhancement of phloem loading brought by the gibberellic acid may be mediated through a change in the H⁺ gradient across the plasmalemma. They also suggested that GA regulates the pH in the apoplast. Therefore, the GA amount in the plant should be measure in further experiments. Other authors pointed out that the production of gibberellins by PGPR is rare, with *B. pumilus* and *B. licheniformis* documented to produce them (Solano et al., 2008).

6.3. Iron plaque formation

The oxidizing power of roots may be a mechanism of lowland rice to avoid an excessive ferrous iron uptake by the plant. The oxygen transported towards the roots via aerenchyma precipitates Fe²⁺ into Fe³⁺ which accumulates as iron coatings on the root surface. In our second experiment, the iron plaque on the roots of the genotype I Kong Pao was measured.

After application of 1000 ppm Fe (II), the iron plaque on uncut roots amounted to 60.1 mg g⁻¹ and to 45.4 mg g⁻¹ on cut roots (Table 11), which confirms the precipitation of ferric iron at the root surface, diminishing the amount of iron entering the root. This mechanism was already proposed by Jacq et al. (1986) who suggested that plants are able to regulate iron influx probably by an oxidation in the rhizosphere with the help of the air conducting aerechym tissues. Although we have not measured iron plaque in our first experiment to discriminate iron plaque formation among genotypes, previous studies have proved that this process appeared to be more efficient in the resistant genotypes than in the sensitive ones (Majeurs et al., 2007). Other experiments by Asch et al. (2007) with 10 rice genotypes subjected to iron treatments of 0, 1000 and 1500 ppm, showed that genotypes differed significantly in the amount of iron plaque and the ratio between iron inside the root and iron plaque on the root. Additionally, in the experiments done by Asch et al. (2007), the sensitive rice genotype IR31785-1-2-3-3 presented higher iron content in the root and root iron plaque than the tolerant rice genotype ITA 320.

These results also show that the iron plaque on cut roots was 25% less than on intact roots. The reason for that could be that the oxidizing power of the roots diminished after cutting the root tips, reducing the plant capacity to precipitate ferrous iron at the root surface. These results are in agreement with studies by Tanaka and Navasero (1966). In these experiments, rice plants with cut roots developed severe iron toxicity symptoms after application of 200 ppm Fe indicating that by cutting the roots there is an impairment of the capacity to deposit iron at the root and therefore iron can enter the plant more freely. Since plants suffering from iron toxicity contain less manganese and potassium, they also propose that this nutritional disturbance could impair the oxidizing power of the roots. A higher proportion of iron was found inside the roots of the plants with cut roots compared to the plants with uncut roots, with percentages of iron of 21.7 and 17.5 respectively (Table 12), maybe due to the impaired oxidizing power of the roots. This would also be in agreement with Flessa and Fischer (1992), who showed that the oxidizing power of the root was concentrated at the root tip of rice.

Regarding the root, 22% of the total iron concentration was found inside the roots versus the 78% shared at the iron plaque. This is in line with St-Cyr and Campbell (1996) who showed that more iron, zinc and manganese were consistently found on the root than within the root tissue, in experiments with plants of *Vallisneria americana*. The percentage of iron outside the roots in the aquatic plant *Vallisneria americana* was equal to or higher than 91%. Other studies by Asch et al. (2007) with ten rice genotypes differing in their sensibility to iron toxicity and subjected to 0, 1000 or 1500 ppm of iron, demonstrated that in most cases, more than 50% of the root iron content is at the root surface instead of inside the roots and also that the major share of iron in the plant is in form of iron plaque. Both results are in agreement with the results obtained in our experiments.

Fe^{2+} can be oxidized either chemically or microbially in roots of lowland rice, resulting in an accumulation of $\text{Fe}(\text{OH})_3$ deposits or iron plaque (Becker and Asch, 2005). An experiment in which planted medium was inoculated with a suspension prepared from colored colony-like structures obtained from rhizosphere of rice grown in FeS- containing media, supported the assumption that iron is precipitated on bacterial colonies (Trolldenier, 1978). In our experiment, the application of the isolate PA31 significantly increased the amount of iron plaque on uncut and cut roots, resulting in the highest amount of iron plaque and suggesting the ability of this isolate to oxidize iron. In a review Liesack *et al.* (2000) suggested that the presence of iron-oxidizing bacteria in precipitates of ferric iron on aquatic plant roots may contribute to the precipitation of iron in the rhizospheric soil. With application of *B. megaterium*, the amount of iron plaque formation was significantly lower. One reason could

be that the ferrous iron concentration in the media would also be lower. This could be due to the siderophore production of the bacteria, which has already been explained above (see 5.2)

Inoculation of IKP subjected to 1000 ppm Fe (II) with *B. pumilus* produced an iron plaque formation on the roots of plants with uncut and cut roots that was very similar to the amount produced when the 1000 ppm of iron were applied to non-inoculated plants (Table 11). This may be due to the fact that this bacterium does not affect root system development, as shown by Probanza *et al.* (2002). In a plant biometrical study with *Pinus pinea* L. seedlings inoculated with *B. pumilus* CECT 5105, the main trend detected was that inoculated strains did not have an effect on the development of the root, even though the production of auxin-like compounds appeared.

7. Conclusions

1. With addition of the unidentified bacillus isolate PA31, the rice plants had a decreased iron concentration in the roots and increased it in the shoots and leaves of uncut plants, suggesting that this bacterium plays a role in iron partitioning in rice. This bacterium also increased iron toxicity symptoms and iron plaque.
2. Application of the unidentified bacillus isolate PB32 in the media had a deleterious effect in the rice plants subjected to an excess of iron, increasing the iron concentration in the roots. Further experiments with this bacterium under different degrees of iron availability in the rhizosphere of rice would be needed to quantify the effects of this bacterium on rice.
3. *Bacillus megaterium* can be used as promising bio-fertilizer since the addition of this bacterium to the medium resulted in lower iron concentration in the plant tissues and improved plant height. For the same reason, experiments with this bacterium in plants subjected to other toxicities, for example, aluminium should be studied in future research. In addition, siderophore secretion of this bacterium should be examined, since it could also play a role in phytoremediation of soils under iron toxicity.
4. The application of *Bacillus pumilus* increased plant height and the share of iron in the root tissues, potentially due to gibberellins secretion. Further experiments with different gibberellin to auxin ratios and their effects on root iron uptake would be needed to verify this assumption.
5. Other parameters to be considered in further experiments are H⁺ secretion by the plant roots after applying the bacteria, quantification of siderophores and ferric and ferrous iron inside the bacterial cell, and bacterial hormone production.

8. References

- Abou-Shanab, R.A. Ghanem, K. Ghanem, N. and Al-Kolaibe, A.** 2008. The role of bacteria on heavy-metal extraction and uptake by plants growing on multi-metal-contaminated soils. *World Journal of Microbiology and Biotechnology* 24, 253–262.
- Akers, H.A.** 1983. Isolation of the siderophore Schizokinen from soil of rice fields. *Applied and Environmental Microbiology* 45, 1704-1706.
- Ali, B. Sabri, A.N. Ljung, K. and Hasnain, S.** 2009. Quantification of indole-3-acetic acid from plant associated *Bacillus* spp. and their phytostimulatory effect on *Vigna radiata* (L.). *World Journal of Microbiology and Biotechnology* 25, 519-526.
- Aloni, B. Daie, J. and Wyse, R.E.** 1988. Regulation of apoplastic pH in source leaves of *Vicia faba* by gibberellic acid. *Plant Physiology* 88, 367-369.
- Andrade, L.R.M., Ikeda, M. and Ishizuka, J.** 1997. Stimulation of organic acid excretion by roots of aluminum tolerant and aluminium sensitive wheat varieties under aluminium stress. *Revista Brasileira de Fisiologia Vegetal* 9, 27-34.
- Arceneaux, J.E.L., Boutwell, M.E. and Byers, B.R.** 1984. Enhancement of copper toxicity by siderophores in *Bacillus megaterium*. *Antimicrobial Agents and Chemotherapy* 25, 650-652.
- Arceneaux, J.E.L and Byers, B.R.** 1980. Ferrisiderophore reductase activity in *Bacillus megaterium*. *Journal of Bacteriology* 141, 715-721.
- Armstrong, W., Strange, M.E., Cringle, S. and Beckett, P.M.** 1994. Microelectrode and modeling study of oxygen distribution in roots. *Annals of Botany* 74, 287-299.
- Asch, F., Aung, T. and Becker, M.** 2007. Root iron plaque formation as resistance mechanism to iron toxicity in lowland rice. Abstract. *Deutscher Tropentag*, October 9-11, 2007, Witzenhausen.
- Asch, F., Becker, M. and Kpongor, D.S.** 2005. A quick and efficient screen for resistance to iron toxicity in lowland rice. *Journal of Plant Nutrition and Soil Science* 168, 1-10.
- Asch, F. and Padgham, J.** 2005. Root Associated Bacteria Suppress Symptoms of Iron Toxicity in Lowland Rice. Abstract. *Deutscher Tropentag*, October 11-13, 2005 Stuttgart-Hohenheim.

- Asch, F., Ripken, C. and Becker, M.** 2005. A low cost miniature method to determine iron content in samples suitable for small research laboratories. Abstract. *Deutscher Tropentag*, October 11-13, 2005, Stuttgart-Hohenheim.
- Audebert, A.** 2006. Iron partitioning as a mechanism for iron toxicity tolerance in lowland rice. In: Audebert, A. (ed.), Narteh, L.T. (ed.), Kiepe P. (ed.), Millar D. (ed.), Beks B. (ed.). *Iron toxicity in rice-based systems in West Africa*. Cotonou : WARDA [Africa Rice Center], 34-46.
- Awad, F., Römheld, V. and Marschner, H.** 1994. Effect of root exudates on mobilization in the rhizosphere and uptake of iron by wheat plants. *Plant and Soil* 165, 213-218.
- Baruah, K.K., Nath, B.C. and Gogoi N.** 2001. Physiological and biochemical traits of rice (*Oryza sativa* L.) genotypes associated with tolerance to iron toxicity. In W.J. Horst et al., eds., *Plant nutrition – Food security and sustainability of agro-ecosystems*. Kluwer Academic Publishers, Dordrecht, 476-477.
- Batty, L.C. and Younger, P.L.** 2003. Effects of external iron concentration upon seedling growth and uptake of Fe and Phosphate by the common Reed, *Phragmites australis* (Cav.) Trin ex. Steudel. *Annals of Botany* 92, 801-806.
- Becana, M., Moran, J.F. and Iturbe-Ormaetxe, I.** 1998. Iron-dependent oxygen free radical generation in plants subjected to environmental stress: toxicity and antioxidant protection. *Plant and Soil* 201, 137-147.
- Becker, M. and Asch, F.** 2005. Iron toxicity in rice – conditions and management concepts. *Journal of Plant Nutrition and Soil Science* 168, 558-573.
- Begg, C.B.M., Kirk, G.J.D., Mackenzie, A.F. and Neue, H.U.** 1994. Root-induced iron oxidation and pH changes in the lowland rice rhizosphere. *New Phytologist* 128, 469-477.
- Bienfait, H.F.; Briel, W. and Mesland-Mul, N.T.** 1985. Free space of iron pools in roots. *Plant Physiology* 78, 596-600.
- Biswas, J.C., Ladha, J.K. and Dazzo, F.B.** 2000. Rhizobia inoculation improves nutrient uptake and growth of lowland rice. *Soil Science Society of America Journal* 64, 1644-1650.
- Bode, K., Döring, O., Lühje, S., Neue, H.U. and Böttger, M.** 1995. The role of active oxygen in iron tolerance of rice (*Oryza sativa* L.). *Protoplasma* 184, 249-255.

- Bosse, U. and Frenzel, P.** 1997. Activity and distribution of Methane-Oxidizing bacteria in flooded rice soil microcosms and in rice plants (*Oryza sativa*). *Applied and environmental microbiology* 63, 1199-1207.
- Briat, J.F. and Lobréaux, S.** 1997. Iron transport and storage in plants. *Trends in Plant Science* 2, 187-193.
- Brown, J.C.** 1978. Mechanism of iron uptake by plants. *Plant, Cell and Environment* 1, 249-257.
- Burd, G.I., Dixon, D.G. and Glick, B.R.** 1998. A plant growth-promoting bacterium that decreases nickel toxicity in seedlings. *Applied and Environmental Microbiology*. 64, 3663-3668.
- Cakmakci, R., Dönmez, F., Aydin, A. and Sahin, F.** 2006. Growth promotion of plants by plant growth-promoting rhizobacteria under greenhouse and two different field soil conditions. *Soil Biology and Biochemistry* 38, 1482-1487.
- Chang, T. T.** 1976. Rice. In N. W. Simmonds (ed.). *Evolution of crop plants*. Longman, London, 98-104.
- Da Silveira, V.C., de Oliveira, A.P., Sperotto, R.A., Espindola, L.S., Amaral, L., Dias, J.F., da Cunha, J.B. and Fett, J.P.** 2007. Influence of iron on mineral status of two rice (*Oryza sativa* L.) cultivars. *Brazilian Journal of Plant Physiology* 19, 127-139.
- Emerson, D. and Moyer, C.** 1997. Isolation and characterization of novel iron-oxidizing bacteria that grow at circumneutral pH. *Applied and Environmental Microbiology* 63, 4784-4792.
- Emerson, D., Weiss, J.V. and Megonigal, J.P.** 1999. Iron-oxidizing bacteria are associated with ferric hydroxide precipitates (Fe-Plaque) on the roots of wetland plants. *Applied and Environmental Microbiology* 65, 2758-2761.
- EPA,** 2003. Ecological soil screening level for iron. U. S. Environmental Protection Agency. Office of Solid Waste and Emergency Response, Washington, D.C.
- Fageria, N.K., Santos, A.B., Barbosa Filho, M.P. and Guimaraes, C.M.** 2008. Iron toxicity in lowland rice. *Journal of Plant Nutrition* 31, 1676-1697.
- Fageria, N.K. and Stone, L.F.** 2006. Physical, chemical and biological changes in the rhizosphere and nutrient availability. *Journal of Plant Nutrition* 29, 1327-1356.
- FAOSTAT** 2009. <http://faostat.fao.org>. Accessed September 20th, 2009.

- Flessa, H. and Fischer, W.R.** 1992. Plant-induced changes in the redox potentials of rice rhizospheres. *Plant and Soil* 143, 55-60.
- Foy, C.D., Chaney, R.L. and White, M.C.** 1978 The physiology of metal toxicity in plants. *Annual Review of Plant Physiology* 29, 531-535
- Gilbert, B. and Frenzel, P.** 1998. Rice roots and CH₄ oxidation: the activity of bacteria, their distribution and the microenvironment. *Soil Biology and Biochemistry* 30, 1903-1916.
- Gutierrez-Mañero, F.J.; Ramos-Solano, B.; Probanza, A.; Mehouchi, J.; Tadeo, F.R. and Talon, M.** 2001. The plant-growth-promoting rhizobacteria *Bacillus pumilus* and *Bacillus licheniformis* produce high amounts of physiologically active gibberelins. *Physiologia Plantarum* 111, 206-211.
- Hantke, K.** 2004. Ferrous iron transport. In Crosa, J.H., Mey, A.R. and Payne, S.M., eds., *Iron transport in Bacteria*, ASM Press, Washington, D.C., 178-184.
- Harrison, P.M. and Arosio, P.** 1996. The ferritins: molecular properties, iron storage function and cellular regulation. *Biochimica et Biophysica Acta – Bioenergetics*, 1275, 181.
- Heinrichs, D.E., Rahn, A.R., Dale, S.E. and Sebulsky, M.T.** 2004. Staphylococcus, streptococcus and bacillus. In Crosa, J.H., Mey, A.R. and Payne, S.M., eds., *Iron transport in Bacteria*, 387-401.
- Hell, R. and Stephan, U.W.** 2003. Iron uptake, trafficking and homeostasis in plants. *Planta* 216, 541-551.
- Hernandez, J.P., de-Bashan, L.E., Rodriguez, D.J., Rodriguez, Y. and Bashan, Y.** 2009. Growth promotion of the freshwater microalga *Chlorella vulgaris* by the nitrogen-fixing, plant growth-promoting bacterium *Bacillus pumilus* from arid zone soils. *European Journal of Soil Biology* 45, 88-93.
- Hu, X. and Boyer, G.L.** 1996. Siderophore-mediated aluminium uptake by *Bacillus megaterium* ATCC 19213. *Applied and Environmental Microbiology* 62, 4044-4048.
- IRRI**, 2003. Rice fact sheet Iron (Fe). International Rice Research Institute, Los Banos.
- IRRI – INGER.** 1996. Standard evaluation system for rice. 4th ed., International Rice Research Institute, Manila, The Philippines.

- Jacq, V., Prade, K. and Ottow, J.C.G.** 1986. Significance of iron and sulphate redox processes in flooded soils for the nutrition of rice. *Proceedings of the 13th Congress of the International Society of Soil Science*, 13-20.
- Jacq, V., Prade, K. and Ottow, J.C.G.** 1991. Iron sulphide accumulation in the rhizosphere of wetland rice (*Oryza sativa* L.) as the result of microbial activities. In Berthelin, J., ed., *Diversity of Environmental Biogeochemistry*, Elsevier, Amsterdam, 453-368.
- Jean, E.L.; Arceneaux, M.E.; Boutwell, M.E. and Byers, B.R.** 1984. Enhancement of copper toxicity by siderophores in *Bacillus megaterium*. *Antimicrobial agents and chemotherapy* 25, 650-652.
- Jiang, K.; Meng, Y.L. and Feldman, L.J.** 2003. Quiescent center formation in maize roots is associated with an auxin-regulated oxidizing environment. *Development* 130, 1429-1438.
- Jones, D.L.** 1998. Organic acids in the rhizosphere – a critical review. *Plant and Soil* 205, 25-44.
- Joo, G.J., Kim, Y.M., Kim, J.T., Rhee, I.K., Kim, J.H. and Lee, I.J.** 2005. Gibberellins-producing rhizobacteria increase endogenous gibberellins content and promote growth of red peppers. *The Journal of Microbiology* 43, 510-515.
- Jugsujinda, A. and Patrick, Jr. W. H.** 1993. Evaluation of toxic conditions associated with orange symptoms of rice in a flooded Oxisol in Sumatra, Indonesia. *Plant and Soil* 152, 237-243.
- Kamaludeen, S.P.B. and Ramasamy, K.** 2008. Rhizoremediation of metals: harnessing microbial communities. *Indian Journal of Microbiology* 48, 80-88.
- Kishore, G.K.; Pande, S. and Podile, A.R.** 2005. Phylloplane bacteria increase seedling emergence, growth and yield of field-grown groundnut (*Arachys hypogaea* L.). *Letters in Applied Microbiology* 40, 260-268.
- Kosegarten, H.U., Hofmann, B. and Mengel, K.** 1999: Apoplastic pH and Fe³⁺-reduction in intact sunflower leaves. *Plant Physiology* 121, 1069-1079.
- Laulhere, J.P. and Briat, J.F.** 1993. Iron release and uptake by plant ferritin: effects of pH, reduction and chelation. *Biochemical Journal* 290, 693-699.

- Liesack, W., Schnell, S. And Revsbech, N.P.** 2000. Microbiology of flooded rice paddies. *Federation of European Microbiological Societies, Microbiological Reviews* 24, 625-645.
- Liu, D.H., Adler, K. and Stephan, U.W.** 1998. Iron-containing particles accumulate in organelles and vacuoles of leaf and root cells in the nicotianamine-free tomato mutant *chloronerva*. *Protoplasma* 201, 213-220.
- López-Bucio, J; Campos-Cuevas, J.C.; Hernández-Calderón, E.; Velásquez-Becerra, C.; Farías-Rodríguez, R.; Macías-Rodríguez, L.I. and Valencia-Cantero, E.** 2007. *Bacillus megaterium* Rhizobacteria promote growth and alter root-system architecture through an auxin- and ethylene-Independent signaling mechanism in *Arabidopsis thaliana*. *Molecular Plant-Microbe Interactions* 20, 207-217.
- Ma, J.F. and Nomoto, K.** 1996. Effective regulation of iron acquisition in graminaceous plants. The role of mugenic acids as phytosiderophores. *Physiologia Plantarum* 97, 609-617.
- Machado, W., Gueiros, B.B., Lisboa-Filho, S.D. and Lacerda, L.D.** 2005. Trace metals in mangrove seedlings: role of iron plaque formation. *Wetlands Ecology and Management* 13, 199-206.
- Macfie, S.M. and Crowder, A.A.** 1987. Soil factors influencing ferric hydroxide plaque formation on roots of *Typha latifolia* L. *Plant and Soil* 102, 177-184.
- Majerus, V., Bertin, P. and Lutts, P.** 2007. Effects of iron toxicity on osmotic potential, osmolytes and polyamines concentrations in the African rice (*Oryza glaberrima* Steud.). *Plant Science* 173, 96-105.
- Marschner, H.** 1986. Mineral nutrition of higher plants (first edn.). Academic Press, London.
- Marschner, H.** 1995. Mineral nutrition of higher plants (second edn.). Academic Press, London.
- Marschner, H., Treeby, M. and Römheld, V.** 1989. Role of root-induced changes in the rhizosphere for iron acquisition in higher plants. *Zeitschrift für Pflanzenernährung und Bodenkunde* 152, 197-204.
- Moller, C.L. and Sand-Jensen, K.** 2008. Iron plaques improve the oxygen supply to root meristems of the freshwater plant, *Lobelia dortmanna*. *New Phytologist* 179, 848-856.

- Nikolic, M. and Römheld, V.** 1999. Mechanism of Fe uptake by the leaf symplast: Is Fe inactivation in leaf a cause of Fe deficiency chlorosis? *Plant and Soil* 215, 229-237.
- Padgham, J.; Le, H. and Sikora, R.** 2005. Opportunities for nematode biocontrol in lowland rainfed rice using bacterial endophytes. Abstract. *Deutscher Tropentag*, October 11 - 13, 2005, Stuttgart-Hohenheim.
- Payne, S.M.** 2003. Regulation of bacterial toxin synthesis by iron. In Burns, D.L., Barbieri, J.T., Iglewsk, B.H. and Rappuoli, R., eds., *Bacterial protein toxins*, 25-38. American Society for Microbiology Press.
- Peters, W.J. and Warren, R.A.** 1968. Phenolic acids and iron transport in *Bacillus subtilis*. *Biochimica et Biophysica Acta* 165, 225-232.
- Pishchik, V.N.; Vorob'eb, N.I. and Provorov, N.A.** 2005. Experimental and mathematical stimulation of population dynamics of rhizospheric bacteria under conditions of Cadmium stress. *Microbiology* 74, 725-740.
- Prade K., Ottow, J.C.G. and Jacq, V.** 1988. Excessive uptake (iron toxicity) by wetland rice (*Oryza sativa* L.) on an acid sulphate soil in the Casamance, Senegal. In: Dost, H., ed., *Selected Papers of the Dakar Symposium on acid sulphate soils*. International Institute for Land Reclamation and Improvement, Wageningen, 150-162
- Probanza, A., Lucas-García, J.A., Ruiz-Palomino, M., Ramos, B. and Gutierrez-Mañero, F.J.** 2002. *Pinus pinea* L. Seedling growth and bacterial rhizosphere structure after inoculation with PGPR *Bacillus* (*B. licheniformis* CECT 5106 and *B. pumilus* CECT 5105). *Applied Soil Ecology* 20, 75-84.
- Raja, P., Uma, S., Gopal, H. and Govindarajan, K.** 2006. Impact of bio inoculants consortium on rice root exudates, biological nitrogen fixation and plant growth. *Journal of Biological Sciences* 6, 815-823.
- Ramos-Solano, B.; Barriuso, J. and Gutierrez-Mañero, F.J.** 2008. Physiological and molecular mechanisms of plant growth promoting rhizobacteria (PGPR). In Ahmad, I.; Pichtel, J. and Hayat, S., eds., *Plant-Bacteria Interactions. Strategies and Techniques to Promote Plant Growth*. Wiley, Weinheim, 41- 54.
- Rich, J.J. and King, G.M.** 1998. Carbon monoxide oxidation by bacteria associated with the roots of freshwater macrophytes. *Applied and Environmental Microbiology* 64, 4939-4943.

- Rogers, E.E., Eide, D.J. and Guerinot, M.L.** 2000. Altered selectivity in an *Arabidopsis* metal transporter. *Proceedings of the National Academy of Sciences* 97, 12356-12360.
- Römheld, V.** 1987. Different strategies for iron adquisition in higher plants. *Physiology Plantarum* 70, 231-234.
- Römheld, V. and Marschner, H.** 1983. Mechanism of iron uptake by peanut plants. *Plant Physiology* 71, 949-954.
- Römheld, V. and Marschner, H.** 1986a. Mobilization of iron in the rhizosphere of different plant species. *Advances in Plant Nutrition* 2, 155-204
- Römheld, V. and Marschner, H.** 1986b. Evidence for a specific uptake system for Iron phytosiderophores in roots of grasses. *Plant Physiology* 80, 175-180.
- Rudresh, D.L., Shivaprakash, M.K. and Prasad, R.D.** 2005. Effect of combined application of *Rhizobium*, phosphate solubilizing bacterium and *Trichoderma* spp. on growth, nutrient uptake and yield of chickpea (*Cicer arietinum* L.). *Applied Soil Ecology* 28, 139-146.
- Sahrawat, K.L.** 2004. Iron toxicity in wetland rice and the role of other nutrients. *Journal of Plant Nutrition* 27, 1471-1504.
- Schopfer, P.; Liskay, A.; Bechtold, M.; Frahry, G. and Wager, A.** 2002. Evidence that hydroxyl radicals mediate auxin-induced extension growth. *Planta* 214, 821-828.
- St-Cyr, L. and Campbell, P.G.C.** 1996. Metals (Fe, Mn, Zn) in the root plaque of submerged aquatic plants collected in situ: Relations with metal concentrations in the adjacent sediments and in the root tissue. *Biogeochemistry* 33, 45-76.
- Stephan, U.W.** 2002. Intra- and intercellular iron trafficking and subcellular compartmentation within roots. *Plant and Soil* 241, 19-25.
- Stephan, U.W. and Scholz, G.** 1993. Nicotianamine: mediator of transport of iron and heavy metals in the phloem? *Physiologia Plantarum* 88, 522-529.
- Tanaka, A., Loe, R and Navasero, S.A.** 1996. Some mechanisms involved in the development of iron toxicity symptoms in the rice plant. *Soil Science and Plant Nutrition* 12, 32-38.
- Thompson, L. M. and Troeh, F.R.** 1973. *Soils and Soil Fertility*, 3rd edition. McGraw-Hill Book Company, New York.

- Tiffin, L.O.** 1966. Iron translocation. I. Plant culture, exudates sampling, iron-citrate analysis. *Plant Physiology* 41, 510-514.
- Tiffin, L.O.** 1970. Translocation of iron citrate and phosphorus in xylem exudates of soybean. *Plant Physiology* 45, 280-283.
- Touati, D.** 2000. Iron and oxidative stress in bacteria. Minireview. *Archives of Biochemistry and Biophysics*, 373, 1-6.
- Treeby, M., Marschner, H. and Römheld, V.** 1989. Mobilization of iron and other micronutrient cations from a calcareous soil by plant-borne, microbial, and synthetic metal chelators. *Plant and Soil* 114, 217-226.
- Trolldenier, G.** 1988. Visualization of oxidizing power of rice roots and possible participation of bacteria in iron deposition. *Journal of Plant Nutrition and Soil Science* 151, 117-121.
- Weiss, J.V., Emerson, D. And Megonigal, P.** 2005. Rhizosphere Iron (III) deposition and reduction in a *Juncus effusus* L.-Dominated wetland. *Soil Science Society of America Journal* 69, 1861-1870.
- Wightman, P.G. and Fein, J.B.** 2005. Iron adsorption by *Bacillus subtilis* bacterial cell walls. *Chemical Geology* 216, 177-189.
- Yang, C. and Crowley, D.E.** 2000. Rhizosphere microbial community structure in relation to root location and plant iron nutritional status. *Applied and Environmental Microbiology* 66, 345-351.
- Yang, Y.Y., Jung, J.Y., Song, W.Y., Suh, H.S. and Lee, Y.** 2000. Identification of rice varieties with high tolerance or sensitivity to lead and characterization of the mechanism of tolerance. *Plant Physiology* 124, 1019-1026.
- Yeo, A.R., Yeo, M.E. and Flowers, T.J.** 1987. The contribution of an apoplastic pathway to sodium uptake by rice roots in saline conditions. *Journal of Experimental Botany* 38, 1141-1153.
- Yoshida, S.** 1981. Fundamentals of rice crop science. International Rice Research Institute.
- Yoshida, S., Forno, D.A., Cock, J.H. and Gomez, K.A.** 1976: Laboratory manual for physiological studies of rice. (Third edn.) International Rice Research Institute, Los Baños, The Philippines

- Zhang, X., Zhang, F. and Mao, D.** 1998. Effect of iron plaque outside roots on nutrient uptake by rice (*Oryza sativa* L.) Zinc uptake by Fe-deficient rice. *Plant and Soil* 202, 33-39.
- Zheng, X.Y. and Sinclair, J.B.** 1996. Chemotactic response of *Bacillus megaterium* strain B153-2-2 to soybean root and seed exudates. *Physiological and Molecular Plant Pathology* 48, 21-35.

Annex

Curriculum Vitae

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