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Genotype Specific Responses to *Bacillus* spp. Inoculation in Lowland Rice (*Oryza sativa* L.) under Iron Toxicity

Dissertation

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Gold is for the mistress – silver for the maid –
Copper for the craftsman cunning at his trade!
“Good!” said the Baron, sitting in his hall,
“but iron – cold iron – is master of them all”
Rudyard Kipling

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Table of Content

List of Abbreviations

ACC	1-aminocyclopropane-1-carboxylic acid
ANOVA	Analysis of variance
APX	Ascorbate peroxidase
CAS	Chrome azurol S
CAT	Catalase
CFU	Colony forming unit
DPD	Dipyridyl
DW	Dry weight
FOC TR4	*Fusarium oxysporum* f.s*P. cubense* Tropical Race 4
FOS	*Fusarium oxysporum* f.s*P. strigea* strain FK3
GPM	General purpose medium
GR	Glutathione reductase
GSH	Reduced glutathione
GSSG	Oxidized glutathione
L1	Leaf 1
L2	Leaf 2
L3	Leaf 3
L4	Leaf 4
LSD	Least significant differences
NA	Nicotianamine
PDA	Potato dextrose agar
PRX	Guaiacol peroxidase
ROS	Reactive oxygen species
SDT	Sodium dithionite
SES	Standard evaluation system for rice
SOD	Superoxide dismutase
TSA	Tryptic soy agar
TSB	Tryptic soy broth

List of Figures

List of Tables

Summary

Amidst a growing global population, limited arable land, and higher pressure from both abiotic and biotic stressors in a shifting climate, there is a need for enhancing yields through sustainable agricultural practices, and new, more tolerant cultivars. In recent decades, employing microbial inoculants as biofertilizers and biopesticides has gained growing popularity.

Yield reductions ranging from 16-78%, and sometimes complete crop failure, can occur in in lowland rice cultivation systems where high iron concentrations in the soil solution lead to excess iron uptake by the plants. Twenty to 60% of the rice growing area of sub-Saharan Africa is affected by iron toxicity. Development of iron-tolerant cultivars has lagged, largely due to gaps in understanding the genotypic adaptation mechanisms to this stress. Furthermore, effects of the microbiome on such stress responses are often overlooked. Although there have been previous reports on growth promoting effects of bacteria inoculation in lowland rice under iron toxicity, these studies were focused on plant growth promotion and mineral nutrient uptake.

The primary aim of this dissertation was to assess the effects of *Bacillus* spp. inoculation on different lowland rice cultivars under iron toxicity, with emphasis on genotypic shoot tolerance strategies. Physiological, biochemical, and molecular mechanisms underlying genotypic responses to *Bacillus* inoculation were investigated and potential overlaps with responses to biotic stressors explored.

Within the framework of this dissertation, three lowland rice cultivars, were inoculated with three *Bacillus* isolates (two *B. pumilus* isolates, one *B. megaterium* isolate) and exposed to 1000 ppm Fe^{2+} in the nutrient solution. The three cultivars were selected because they differ in their tolerance against iron toxicity, with one being sensitive, one a tolerant excluder (tolerance through minimizing iron uptake), and one a tolerant includer (tolerance of high iron concentrations in the plant). At day eight of stress exposure, the effects of *Bacillus* inoculation on tolerance against iron toxicity were evaluated by leaf symptom scoring. The effects of bacteria on the progression of leaf bronzing were then related to specific tolerance mechanisms, such as shoot iron content, iron allocation within the shoot, ROS scavenger enzyme activity, and the expression of genes related to iron toxicity tolerance. Furthermore, the effects of inoculation on brown spot disease development seven days after infection with *Bipolaris oryzae* were also assessed by leaf symptom scoring. All three *Bacillus* isolates were characterized for their ability to solubilize Zn and P, production of auxin, siderophores, and HCN, the presence of ACC deaminase activity, and *in vitro* inhibition of fungal growth.

Effects of *Bacillus* inoculation on iron toxicity tolerance were found to depend on the cultivar x *Bacillus* isolate combination. While leaf symptom expression was ameliorated in the inoculated sensitive cultivar, the tolerant excluder cultivar generally developed stronger symptoms of iron toxicity when inoculated with *Bacillus*. No significant effects of *Bacillus* inoculation on the tolerance against iron toxicity were found in the tolerant includer cultivar. The beneficial outcomes of bacterial inoculation on plant stress tolerance are often credited to the bacteria's plant growth promoting properties. However, we did not find a clear association between plant growth and tolerance to iron toxicity. Furthermore, the *Bacillus* isolates did not display ACC deaminase activity nor the ability to solubilize Zn or P. Auxin production was only notable in *B. megaterium*, the isolate with least effects on both tolerance to iron toxicity and brown spot disease development. Siderophore production was found in *B. pumilus* D7.4 but only under low iron supply. Instead of plant growth, iron homeostasis as well as the interconnection between iron homeostasis and the immune response of lowland rice seem to be affected by bacterial inoculation. For the first time it was shown that *Bacillus* inoculation can directly affect tolerance against iron toxicity in lowland rice through inducing the production of ferritin in the young leaf blades of the sensitive cultivar. NO produced by *B. pumilus* Ni9MO12 is hypothesized to be involved in the signaling cascade leading to *OsFER* expression. Activity of ROS scavenger enzymes of the ascorbate-glutathione redox cycle were not affected by *Bacillus* inoculation in the leaf blades. In the tolerant excluder cultivar, an alteration in iron distribution within the shoot of *B. pumilus* Ni9MO12 inoculated plants, is assumed to cause the decline in tolerance.

In conclusion, it was shown that *Bacillus* inoculation can influence iron toxicity tolerance in lowland rice. The results underline the significance of the interaction between rice genotypes and bacteria isolates. Furthermore, *Bacillus* inoculation did not promote plant growth, instead, distinct adaptation mechanisms within the shoot tissue were triggered to allow for increased tolerance of high iron concentrations in the leaves. The signaling cascades involved might be linked with biotic stress responses. Understanding such intricate mechanisms is vital for improving plant productivity. While inoculants composed of single microbial isolates may not meet the anticipated outcomes for practical application in sustainable agriculture, they offer a valuable laboratory tool for investigating genotypic plant tolerance to various abiotic and biotic stresses and the role of the microbiome within. New breeding approaches that consider genotypic traits essential for obtaining a beneficial microbiome might accelerate the creation of more tolerant cultivars.

Zusammenfassung

Angesichts der wachsenden Weltbevölkerung, der begrenzten Anbauflächen und des zunehmenden Drucks durch abiotische und biotische Stressfaktoren in einem sich wandelnden Klima besteht die Notwendigkeit, die Erträge durch nachhaltige landwirtschaftliche Praktiken und neue, tolerantere Sorten zu steigern. In den letzten Jahrzehnten hat der Einsatz von mikrobiellen Inokulanten als Biodünger und Biopestizide an Popularität gewonnen.

In Anbaugebieten von Nassreis, in denen hohe Eisenkonzentrationen in der Bodenlösung zu einer übermäßigen Eisenaufnahme durch die Pflanzen führen, kann es zu Ertragseinbußen zwischen 16 und 78 % und manchmal zu einem vollständigen Ernteausfall kommen. Zwanzig bis 60 % der Reisanbauflächen in Afrika südlich der Sahara sind von Eisentoxizität betroffen. Die Entwicklung eisentoleranter Reissorten erfolgt nur langsam, was vor allem auf das mangelnde Verständnis der genotypischen Anpassungsmechanismen an diesen Stress zurückzuführen ist. Außerdem werden die Auswirkungen des Mikrobioms auf di pflanzliche Stressreaktionen oft übersehen. Es gibt zwar frühere Berichte über wachstumsfördernde Wirkungen der Inokulation mit Bakterien in Nassreis unter Eisentoxizität, doch konzentrierten sich diese Studien auf die Auswirkungen auf das Pflanzenwachstum und die Aufnahme von Mineralstoffen.

Das Hauptziel dieser Dissertation war es, die Auswirkungen der Inokulation mit *Bacillus* spp. auf verschiedene Nassreissorten unter Eisentoxizität zu bewerten, wobei der Schwerpunkt auf genotypischen Spross-Toleranzstrategien lag. Die Mechanismen, die den genotypischen Reaktionen auf die *Bacillus*-Inokulation zugrunde liegen, wurden untersucht und mögliche Überschneidungen mit den Reaktionen auf biotische Stressfaktoren erforscht.

Im Rahmen dieser Dissertation wurden drei Nassreissorten mit drei *Bacillus*-Isolaten (zwei *B. pumilus*-Isolate, ein *B. megaterium*-Isolat) beimpft und 1000 ppm Fe^{2+} in der Nährlösung ausgesetzt. Die drei Sorten wurden ausgewählt, weil sie sich in ihrer Toleranz gegenüber Eisentoxizität unterscheiden, wobei eine Sorte empfindlich ist, eine Sorte ein toleranter „Excluder“ (Toleranz durch Minimierung der Eisenaufnahme) und eine Sorte ein toleranter „Includer“ ist (Toleranz gegenüber hohen Eisenkonzentrationen in der Pflanze). Am achten Tag der Stressexposition wurden die Auswirkungen der *Bacillus*-Inokulation auf die Toleranz gegenüber der Eisentoxizität durch eine Bonitur der Blattsymptome analysiert. Die Auswirkungen der Bakterien auf das Fortschreiten der Symptomentwicklung wurden dann mit spezifischen Toleranzmechanismen in Verbindung gebracht, wie z.B. dem Eisengehalt des

Sprosses, der Eisenverteilung innerhalb des Sprosses, der Aktivität von Enzymen zum Abfangen von Sauerstoffradikalen und der Expression von Genen, die mit der Toleranz gegenüber Eisentoxizität zusammenhängen. Darüber hinaus wurden die Auswirkungen der Inokulation auf die Entwicklung der Braunfleckenkrankheit sieben Tage nach der Infektion mit *Bipolaris oryzae* anhand der Bewertung der Blattsymptome beurteilt. Alle drei *Bacillus*-Isolate wurden auf ihre Fähigkeit zur Solubilisierung von Zn und P, die Produktion von Auxin, Siderophoren und HCN, das Vorhandensein von ACC-Deaminase-Aktivität und die in-vitro Hemmung des Pilzwachstums untersucht.

Die Auswirkungen der *Bacillus*-Inokulation auf die Eisentoxizitätstoleranz hingen von der Kombination Reissorte x *Bacillus*-Isolat ab. Während sich die Ausprägung der Blattsymptome bei der inokulierten sensitiven Sorte verringerte, entwickelte die tolerante „Excluder"- Sorte im Allgemeinen stärkere Symptome der Eisentoxizität, wenn sie mit *Bacillus* beimpft wurde. Bei der toleranten "Includer"-Sorte wurden keine signifikanten Auswirkungen der *Bacillus*-Inokulation auf die Toleranz gegenüber Eisentoxizität festgestellt. Die positiven Auswirkungen der bakteriellen Inokulation auf die Stresstoleranz der Pflanzen werden häufig auf die wachstumsfördernden Eigenschaften der Bakterien zurückgeführt. Wir konnten jedoch keinen eindeutigen Zusammenhang zwischen Pflanzenwachstum und Toleranz gegenüber Eisentoxizität feststellen. Darüber hinaus zeigten die drei *Bacillus*-Isolate weder ACC-Deaminase-Aktivität noch die Fähigkeit, Zn oder P zu solubilisieren. Auxinproduktion war nur bei *B. megaterium* deutlich vorhanden, dem Isolat mit den geringsten Auswirkungen auf die Toleranz gegenüber Eisentoxizität und die Entwicklung der Braunfleckenkrankheit.

Produktion von Siderophoren wurde bei *B. pumilus* D7.4 festgestellt, allerdings nur bei geringer Eisenversorgung. Anstelle des Pflanzenwachstums scheinen der Eisenhaushalt sowie die Verbindung zwischen Eisenhaushalt und der Immunantwort von Nassreis durch die bakterielle Inokulation beeinflusst zu werden. Zum ersten Mal wurde gezeigt, dass die Inokulation mit *Bacillus* die Toleranz gegenüber Eisentoxizität bei Nassreis direkt beeinflussen kann, indem die Produktion von Ferritin in den jungen Blattspreiten der sensitiven Sorte angeregt wird. Es wird angenommen, dass das von *B. pumilus* Ni9MO12 produzierte NO an der Signalkette beteiligt ist, die zur *OsFER*-Expression führt, während die Aktivität der Enzyme des Ascorbat-Glutathion-Redoxzyklus in den Blattspreiten durch die *Bacillus*-Inokulation nicht signifikant beeinflusst wurde. Bei der toleranten Excluder-Sorte wird eine Veränderung der Eisenverteilung im Spross der mit *B. pumilus* Ni9MO12 inokulierten Pflanzen als Ursache für den Rückgang der Toleranz angenommen.

Abschließend wurde gezeigt, dass die *Bacillus*-Inokulation die Toleranz gegenüber Eisentoxizität bei Nassreis beeinflussen kann. Die Ergebnisse unterstreichen die Bedeutung der Wechselwirkung zwischen Reisgenotypen und Bakterienisolaten. Darüber hinaus förderte die *Bacillus*-Inokulation nicht das Pflanzenwachstum, sondern es wurden verschiedene Anpassungsmechanismen im Sprossgewebe ausgelöst, die eine erhöhte Toleranz gegenüber hohen Eisenkonzentrationen in den Blättern ermöglichen. Die beteiligten Signaltransduktionsketten überlappen möglicherweise mit denen biotischer Stressantworten. Das Verständnis solch komplizierter Mechanismen ist für die Verbesserung der Pflanzenproduktivität von entscheidender Bedeutung. Auch wenn Inokulate, die aus einzelnen mikrobiellen Isolaten bestehen, möglicherweise nicht die erwarteten Ergebnisse für die praktische Anwendung in der nachhaltigen Landwirtschaft liefern, bieten sie doch ein wertvolles Instrument für die Untersuchung der genotypischen Pflanzentoleranz gegenüber verschiedenen abiotischen und biotischen Stressfaktoren und der Rolle des Mikrobioms darin. Neue Züchtungsansätze, die genotypische Merkmale berücksichtigen, die für den Aufbau eines vorteilhaften Mikrobioms wesentlich sind, könnten die Entwicklung toleranterer Sorten beschleunigen.

1 General Introduction

With climate change, abiotic and biotic stresses are increasingly negatively affecting the productivity of agroecosystems worldwide. Human population is predicted to reach 9.7 billion by 2050 and arable land is limited (FAO, 2016; Rodriguez and Durán, 2020). In order to ensure global food security, yields must be increased. Between the 1940s and the late 1970s the Green Revolution increased agricultural production worldwide, particularly in developing countries. The introduction of high-yielding cultivars and new management practices came with a significant increase in the global production of staple foods like rice, wheat, and maize (Pingali, 2012). However, the Green Revolution also had its drawbacks, such as environmental degradation, loss of biodiversity, increased socioeconomic disparities, and health concerns through the overuse of chemical fertilizers and pesticides. In many parts of the world, stagnation, or diminishing returns in crop yields for several staple crops have been observed, partly due to these drawbacks that led to pest resistance, soil degradation or water scarcity (as a result of over-extraction of groundwater for irrigation). Furthermore, technologies of the Green Revolution were not developed for production in marginal environments subjected to abiotic stresses such as drought or flooding (Pingali, 2012). A new Green Revolution which aims at increasing agricultural productivity under unfavorable environmental conditions through sustainable and ecologically friendly intensification is needed (Backer et al., 2018). The application of bacterial and fungal inoculants as biofertilizers and biocontrol agents has attracted attention in recent decades and the market share of these alternatives to conventional fertilizers and chemical pesticides is expected to increase (O'Callaghan et al., 2022). However, many of the commercially available products fail to perform under field conditions. One of the reasons being the lack of knowledge of the underlying mechanisms of genotypic interaction between crop cultivars and specific microorganisms.

1.1 Rice (*Oryza sativa L.*) Production

Rice is a major staple food in Asia, Latin America, the Caribbean, and in Africa, and its consumption is expected to increase, largely due to population growth (OECD, 2022). Whereas rice production is a driver of climate change, due to its high methane emissions in flooded rice paddies, it is also one of the crops most susceptible to the impacts climate change, such as higher global temperatures (Peng et al., 2004). Droughts, floods, extreme temperatures, and the intrusion of saltwater into the irrigated fields, can lead to complete crop failures. Some areas are prone to become unsuitable for rice cultivation (Turral et al., 2011). Rice is cultivated by 144 million smallholder farmers around the world (IRRI, 2023). And despite the FAO's prediction that, on a global scale, decreasing harvests in some areas, such as China, Vietnam, and Brazil, will be offset by gains in India and African countries (OECD, 2022), yield losses caused by abiotic and biotic stressors risk the livelihoods of those smallholder farmers.

Figure 1.1: Rice fields in Madagascar where iron toxicity in the paddy fields leads to yield reductions. Orange coloring of the leaves are first symptoms of iron toxicity in rice.

1.2 Iron Toxicity

Iron toxicity constitutes a major abiotic stress in lowland rice production systems, causing yield loses from 16-78% (Audebert and Fofana, 2009; Becker and Asch, 2005), and in severe cases even complete yield losses might occur (Sahrawat, 2005). It is estimated that around 20% to 60% of the rice growing area of sub-Saharan Africa is affected by iron toxicity (Kirk et al., 2021).

Paddy rice fields, especially in tropical ferruginous or ferritic soils, are prone to develop iron toxicity because waterlogging leads to poor oxygen circulation. Under anaerobic conditions, ferric iron (Fe^{3+}), along with NO_3, Mn^{4+}, and SO_4, acts as electron acceptor for microbial respiration, resulting in increased concentrations of ferrous iron (Fe^{2+}) (Becker and Asch, 2005). Especially in acidic soils, precipitation of Fe^{2+} is slow. The soil Fe^{2+} concentrations at which iron toxicity symptoms in the plant can be observed greatly differ depending on the site and cultivar. Twenty to 2500 mg kg^{-1} are reported as the critical range in rice cultivation (Becker and Asch, 2005), which indicates that iron toxicity within the rice plant is also influenced by factors other than pH and iron concentration alone (Becker and Asch, 2005). The availability of other nutrients and the accumulation of hydrogen sulfide and organic acids have been suggested to play a role (Becker and Asch, 2005). Ferrous iron present in the soil solution first needs to cross the oxidizing threshold of the rhizosphere. Rice plants can transport atmospheric oxygen from the leaves down to the rhizosphere through the aerenchyma. This results in a redox potential in the rhizosphere that is higher than the surrounding soil. Certain bacteria, known as lithotrophic bacteria, utilize Fe^{2+} as an electron donor and can transform Fe^{2+} into Fe^{3+}, or $Fe(OH)_3$. This transformed Fe^{3+} creates an iron-rich coating around the plant roots (Emerson et al., 1999). Fe^{2+} that is not fixed on the root surface is absorbed into the root cortex. From there, it can either move directly to the xylem via the apoplastic route or cross through the Casparian strip into the symplast. Once in the xylem, driven by transpiration, Fe^{2+} travels upwards towards the leaf (Shrestha et al., 2015), where it can potentially rejoin the symplast. Ferrous iron plays a crucial role in the synthesis of chlorophyll, plant respiration, and the photosynthetic electron transport chain (Müller et al., 2015). Nonetheless, it also facilitates the Fenton reaction, leading to the production of reactive oxygen species (ROS). These ROS can cause irreversible harm to cellular components and potentially trigger oxidative damage in plants (Frei et al., 2016; Onyango et al., 2019). Disruption of the cellular redox equilibrium can impact the plant's morphology, physiology, and biochemistry. This results in leaf bronzing, reduced tillering and in extreme cases in plant death (Audebert, 2006; Onyango et al., 2018). Beyond the direct detrimental effects of elevated iron levels in plant tissues, iron toxicity is a multi-nutritional disorder. It can impede the uptake of other essential nutrients like Ca^{2+}, Mg^{2+}, Mn, N, P, and K, leading to their deficiencies in plants (Majerus et al., 2007; Mehraban et al., 2008; Sahrawat, 2005). Reduced absorption of potassium for instance can further intensify iron toxicity in plants. Research by Wu et al. (2019) revealed that the potassium transporter *OsAKT1* plays a role in the internal movement of iron in plants, influencing their tolerance to excessive iron. It is hypothesized that when there is a drop in the internal potassium concentration, the plasma membrane becomes more polarized. This polarization is thought to elevate the transfer of Fe^{2+} to the xylem, facilitating its movement from the roots to the shoots.

1.3 Iron Toxicity Symptoms

Initial responses of rice plants to elevated iron levels include leaf rolling and the closure of stomata, actions that help avert the production of H_2O_2. The onset of toxicity is marked by a bronze coloration of leaves, typically beginning in older leaves which have higher transpiration rates (Yamanouchi & Yoshida, 1981). The visible symptoms can differ among rice varieties; while some display an orange coloring, others manifest brown deletions (Figure 1.2 A-C). In cases of extreme toxicity, these symptoms can encompass the entire transpiring leaf (Fairhurst et al., 2007). Iron toxicity can severely limit plant growth, leading to decreased root mass (Fageria, 1988)(Figure 1.2 D-F) and affecting tillering, including a reduction in the proportion of productive tillers (Cheema et al., 1990). When iron toxicity emerges in the latter part of the vegetative phase or the initial reproductive stages, it correlates with a reduction in panicles for each hill (Thakur et al., 2010) and heightened spikelet infertility (dos Santos et al., 2020). There is also a postponement in flowering and maturity, and in cultivars that are highly sensitive, flowering may be completely inhibited. Following the booting stage, root development ceases, leading to the aging and deterioration of the aerenchyma.

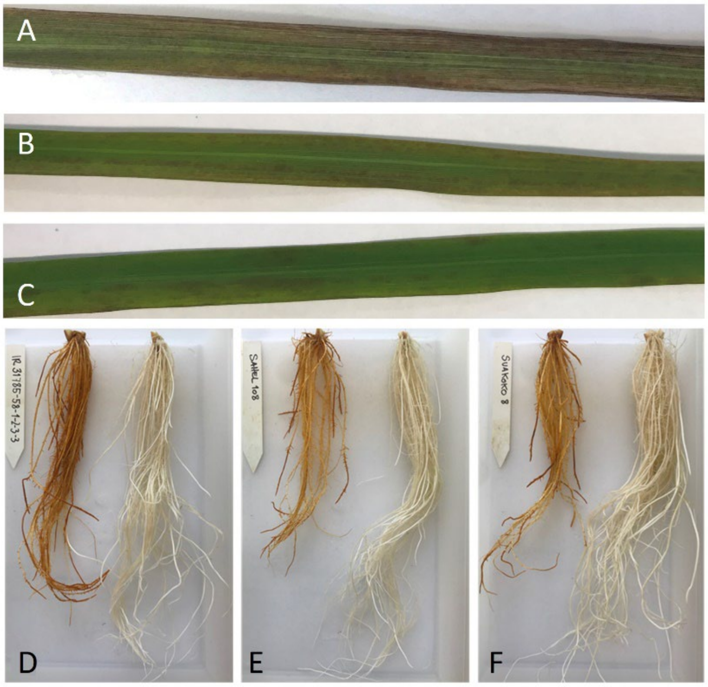

Figure 1.2: Symptoms of iron toxicity in three different lowland rice cultivars. A: severe leaf symptoms in the blade of sensitive cultivar IR31875, B: development of orange coloring of the leaf blade in Sahel 108, C: weaker leaf symptoms in the tolerant cultivar Suakoko 8. D-F: comparison of roots of IR31875 (D), Sahel 108 (E), and Suakoko 8 (F) grown under iron toxicity (on the left side of each photo) and roots grown under normal growth conditions (on the right side of each photo). Under iron toxicity, root growth becomes stunted and iron plaques cover the root surface.

1.4 Adaptation Strategies to Iron Toxicity

It is crucial for plants to maintain a delicate balance of iron within their tissues to ensure the proper functioning of metabolic processes. Rice, in particular, has evolved multiple strategies to manage both iron toxicity in the soil and elevated iron concentrations within its tissues (Figure 1.3). Becker and Asch (2005) have classified these adaptation strategies into three major types:

Strategy I (excluder/avoidance) refers to the ability of rice plants to minimize iron uptake. This involves the conversion of Fe^{2+} to its oxidized state on the root's surface, aided by the molecular oxygen that is transported from the atmosphere through the plant's aerenchyma to the roots (Becker and Asch, 2005). The onset of anoxic conditions stimulates this process by boosting ethylene production (Kawase, 1981). The efficiency of iron oxidation in the root area varies based on the rice plant's growth phase and the expansion rate of its root system. Other elements, like ethylene concentration within the plant tissues, might indirectly influence this iron restriction mechanism. Additionally, certain genes linked to iron uptake have been identified that are less active in some iron-tolerant excluder cultivars (Aung and Masuda, 2020).

Strategy II (inclusion/avoidance) pertains to mechanisms that include iron chelation by nicotianamine, restricting iron from the symplast by fixing it in the leaf apoplast, storing iron as ferritins, compartmentalizing iron in the vacuole (Briat et al., 2010; Majerus et al., 2007, Aung et al., 2019), and translocation (Audebert and Sahrawat, 2000; Aung et al., 2020), possibly through Fe^{2+} binding by ferritin in the xylem, which is then stored in stem tissues (Silveira et al., 2009). The apoplast's pH level plays a role in determining iron's movement within the leaves. A more acidic environment promotes the absorption of Fe^{2+}, while an alkaline environment limits Fe^{2+}'s mobility and supports its oxidation (Becker and Asch, 2005). Oxidizing Fe^{2+}, especially evident in iron-tolerant cultivars with higher apoplast pH (Nikolic and Römheld, 2001), typically results in the formation of non-diffusible compounds. This, in turn, limits the effectiveness of the membrane-bound ferric-chelate reductase (Li et al., 2019; Peng and Yamauchi, 1993).

Strategy III (inclusion/tolerance) refers to the ability of plant tissues to manage elevated Fe^{2+} iron levels in the leaf tissue and remain unharmed. Tolerance is the result of enzymatic neutralization of radicals formed via the Fenton reaction which is fueled by Fe^{2+} in the symplast. Compounds that neutralize these radicals, such as cytosolic ascorbate or glutathione, combined with specific enzyme functions, counteract oxidative stress (Fang et al., 2001; Wu et al., 2017; Lu et al., 2007; Smirnoff, 1998; Kang and Saltveit, 2002). Central to the regulation of plant redox balance is the glutathione-ascorbate cycle (Halliway-Foyer-Cycle). In this cycle, Ascorbate peroxidase (APX) plays a crucial role by converting H_2O_2 to H_2O, while simultaneously turning ascorbate into monodehydroascorbate (MDHA). This then breaks down into ascorbate and DHA, with the latter being a substrate for dehydroascorbate reductase (DHAR). This enzyme simultaneously oxidizes glutathione (GSH). With NADPH assisting, oxidized glutathione (GSSG) is then transformed back into GSH by glutathione reductase (GR) (Noctor et al., 2012).

The above adaptation mechanisms can occur individually or simultaneously in tolerant cultivars (Engel et al., 2012). This often depends on growth stage (Rajonandraina et al., 2023). However, locally adapted, traditional varieties, are low-yielding compared to modern high-yielding cultivars. This is partially due to rice breeding efforts not being focused on the selection for tolerance against iron toxicity (Kirk et al., 2021) but also because these mechanisms and the factors which affect them are still not fully understood.

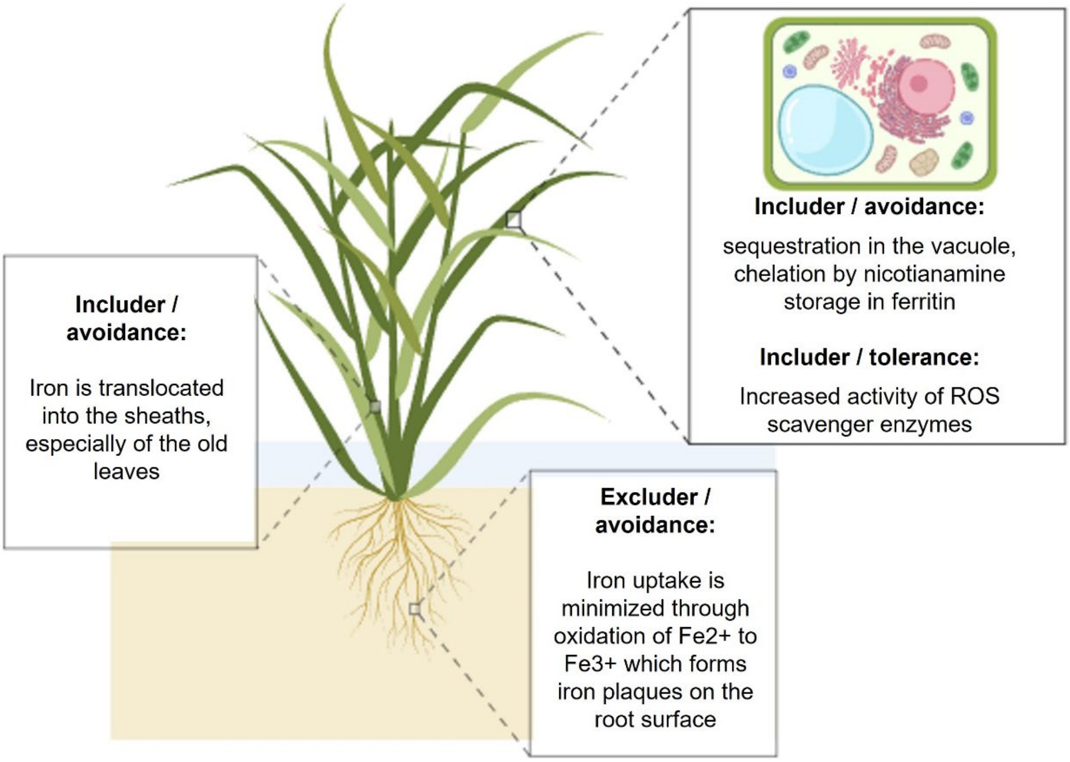

Figure 1.3: Different mechanisms of tolerance against iron toxicity. At the root surface, Fe^{2+} is oxidized and this way prevented from excess uptake (Excluder / avoidance). On the shoot level, iron is translocated into the sheaths, preferably of the older leaves (Includer / avoidance). Within the leaf tissue, iron is either sequestered into the vacuole, chelated by nicotianamine or stored in ferritins (Includer / avoidance). Increased activity of ROS scavenger enzyme can lead to increased tolerance to high iron concentrations (Includer / tolerance) (graphic created with BioRender.com).

1.5 Plant-Microbe Interaction

Plants grow in soil, a habitat they share with a myriad of other organisms. An estimated 10^{14} – 10^{16} bacteria and 10^{11}-10^{14} fungi live in per m^2 of soil in the temperate region (Gobat et al., 2004). Within this close proximity to each other, plants and microbes have developed strategies for their mutual benefit. Microbes solubilize nutrients from the soil, plants take them up and assimilate them to carbon metabolites by using the energy of the sun (Edwards et al., 2014). Five to 21% (and in some cases even up to 40% under certain conditions) of the plant's total photosynthetically fixed carbon are allocated to the root and released in form of root exudates - including sugars, amino acids, organic acids, and secondary metabolites - on which rhizobacteria feed (Edwards et al., 2014). This tight interaction has led a holistic view, where the plant and its associated microbiome are seen as a single entity. Applying non-pathogenic bacteria to the root zone can lead to manifold effects both within the plant and in the surrounding rhizosphere (Figure 1.4; Dimkpa et al., 2009). These can result in plant growth promotion under normal as well as under stress conditions. Recognizing the significance of advantageous microorganisms in enhancing a plant's tolerance to environmental stresses has stimulated the calls for a new Green Revolution, to be based on microbial inoculants and their metabolites, as well as on improving crops through the manipulation of the phytomicrobiome community structure (Backer et al., 2018). Nowadays, microbial additives, primarily composed of bacteria and fungi, are introduced to soil as substitutes for traditional inorganic fertilizers. They serve various purposes, such as biocontrol for combating pests and diseases, or for soil cleanup and improvement of soil properties (O'Callaghan et al., 2022). Different strains of the gram-positive, spore-forming *Bacillus* genus, are among the most commonly studied beneficial bacteria and different isolates have been commercialized. *Bacillus* spp. can be found ubiquitously in nature and many isolates are resistant against environmental stresses such as low nutrient availability or drought (Radhakrishnan et al., 2017). However, while these inoculants display positive outcomes in pot experiments, the same results are often not evident under field conditions. This discrepancy can be attributed to a limited understanding of the mechanisms underlying these beneficial effects, the inoculant's inability to establish within a natural environment, and insufficient understanding of the genotypic interactions between specific crop cultivars and the inoculant.

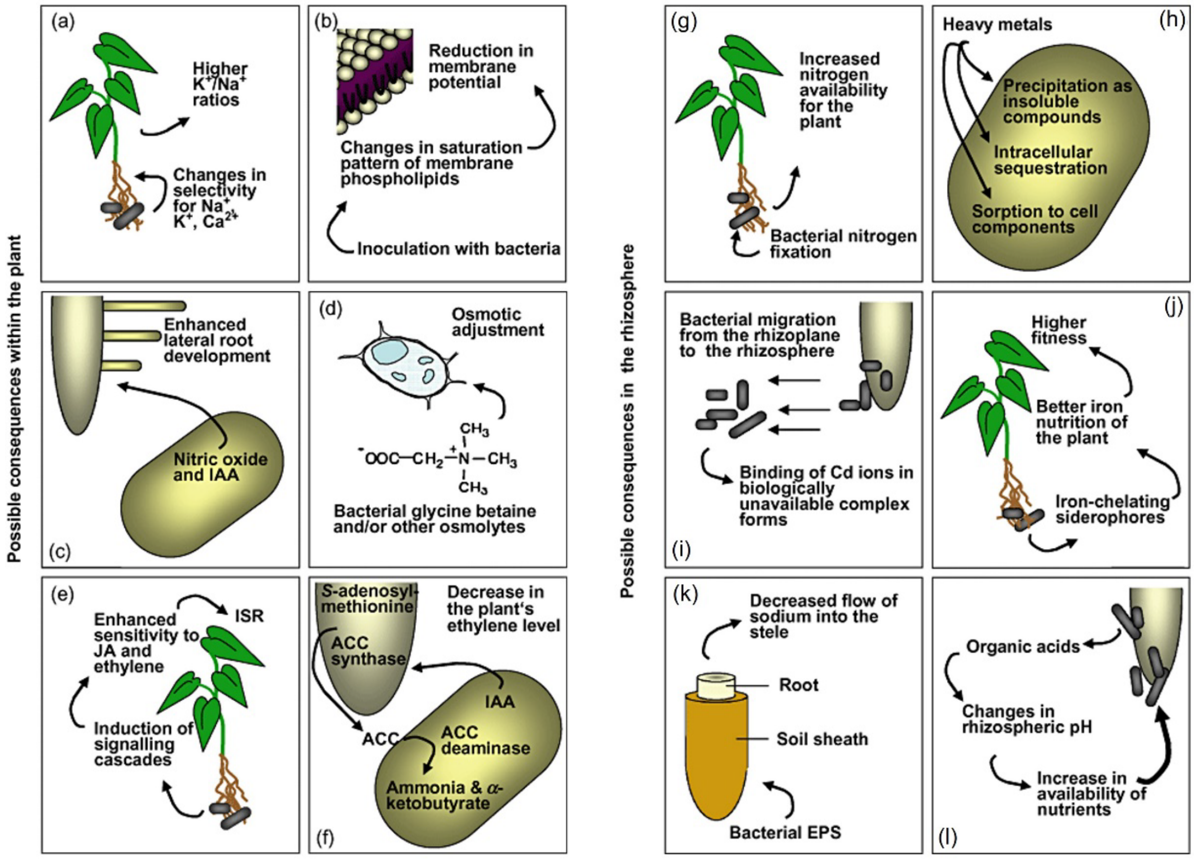

Figure 1.4: Proposed mechanisms underlying enhanced abiotic stress tolerance within the plant. Inoculation with non-pathogenic root zone bacteria can have various consequences within the plant [(a)-(f)] as well as in the rhizosphere [(g)-(l)]. Upon bacterial inoculation, the selectivity for Na^+, K^+ and Ca^{2+} is altered, resulting in higher K^+/Na^+ ratios. (b) Inoculation with rhizobacteria can lead to changes in membrane phospholipid content and alterations in the saturation pattern of the lipids. Membrane potential is, thus, reduced. (c) Nitric oxide and indole acetic acid (IAA) produced by bacteria promote lateral root development in the host plant, resulting in increased root surface area. (d) Bacteria-produced osmolytes, such as glycine betaine, can act synergistically with plant osmolytes, accelerating osmotic adjustment. (e) Inoculation with non-pathogenic rhizobacteria can induce signaling cascades that put the host plant in a ‘primed’ physiological state as part of a phenomenon of induced systemic resistance (ISR). (f) Bacterial 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase activity reduces ‘stress ethylene’ levels within the plant. Proposed rhizosphere-dependent mechanisms underlying enhanced abiotic stress tolerance by plants. (g) Host plant nitrogen uptake can be positively influenced by bacterial nitrogen fixation. (h) The mobility of heavy metals in contaminated soils can be significantly reduced through root zone bacteria. (i) Migration of bacteria from the rhizoplane to the rhizosphere plays a role in reducing plant uptake of Cd. (j) Iron–siderophores complexes can be taken up by the host plant, resulting in a higher fitness. (k) Bacterial exo-polysaccharides (EPS) lead to the development of soil sheaths around the plant root, which reduces the flow of sodium into the stele. (l) Root zone bacteria can influence pH and redox potential in the rhizosphere, for instance, through the release of organic acids. This can have positive effects on the availability of nutrients for the plant (from Dimkpa et al. 2009).

1.6 Use of Microbes in Rice Tolerance to Iron Toxicity

The tolerance mechanisms that rice employs under iron toxicity vary based on factors like the cultivar, stage of growth, the area where the rice is grown, the severity of the Fe toxicity, and the soil's physical and chemical properties. It is still largely unknown to what extent the microbiome of different rice cultivars differs under field conditions and to what extent it influences tolerance against iron toxicity in the respective cultivar. Based on the available knowledge, it is likely that some of the observed variability in the tolerance of individual rice cultivars to iron toxicity could be due to different microbiomes at different iron-toxic sites.

Prior research conducted by Le et al. (2015) and Terré et al. (2007) discovered both beneficial and adverse effects on iron toxicity tolerance in rice plants inoculated with *Bacillus* strains, in comparison to plants that were not inoculated.

1.7 Cross-Tolerance Against Biotic Stress

Plants frequently encounter multiple stresses simultaneously. Especially fungal diseases will be spreading more easily in future climate conditions (Stukenbrock and Gurr, 2023). Bacteria isolates which positively affect tolerance against both environmental and biological stresses, such as fungal pathogens, would be greatly beneficial (Dimpka et al., 2009).

Plant hormones are important components of plant stress responses and are often found to link abiotic and biotic stress signaling (Verma et al., 2016). Iron, as an essential mineral nutrient required for both photosynthesis and the production of chlorophyll also plays a key role in the plant's immune system (Schmid et al., 2020). Potential links between Induced Systemic Resistance (ISR) against pathogens and iron deficiency responses have been proposed earlier (Romera et al., 2019). Mainly because microbes known to induce physiological and morphological changes in dicot plant roots, typically caused by iron deficiency, can also activate ISR (Romera et al., 2019). However, not all responses to pathogen attack involve ISR. The most effective defense response against fungal pathogen attack is the hypersensitive response (HR) which involves cell death. There are several known forms of cell death in plants: apoptosis, necrosis, autophagy, and ferroptosis (Dangol et al., 2019). Ferroptotic cell death is iron-dependent and involves the accumulation of toxic lipid ROS, particularly lipid hydroperoxides (Dangol et al., 2019). Bacterial iron chelators have been shown to inhibit ferroptotic cell death (Yang and Stockwell, 2008). In rice, ferroptosis has been shown to be the mechanism of defense in avirulent infections with the fungal pathogen *Magnaporthe oryzae* (Dangol et al., 2019). Bacteria isolates affecting iron homeostasis in lowland rice under iron toxicity hold the potential of also affecting iron-related responses to biotic stresses.

1.8 Objectives

The overall objective of this thesis was to evaluate the effects of *Bacillus* spp. inoculation on different lowland rice cultivars under iron toxicity with focus on genotypic differences in regard to shoot tolerance mechanisms and interaction with distinct *Bacillus* isolates. Furthermore, possible mechanisms underlying the effects on the genotypic responses to *Bacillus* inoculation and possible signaling overlaps with responses to biotic stresses were also investigated. To that end, the study focused on the following specific objectives:

- Analysis of the effects of *Bacillus* spp. inoculation on leaf symptom expression and it's relation to iron concentration and distribution within the shoots of three lowland rice cultivars under iron toxicity.
- Analysis of the effects of *Bacillus* spp. inoculation on specific shoot tolerance mechanisms such as ROS scavenging enzyme activity and expression of genes involved in tolerance to iron toxicity.
- Characterization of the three *Bacillus* isolates in terms of plant growth promoting properties and evaluation of the effects of the three *Bacillus* isolates on biotic stress responses, using *Bipolaris oryzae* as an exemplary fungal pathogen.

1.9 References

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2 Effects of endophytic *Bacillus* spp. on accumulation and distribution of iron in the shoots of lowland rice grown under iron toxic conditions

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Keywords: *Bacillus* spp, iron toxicity, *Oryza sativa*, plant–bacteria interaction

Abstract

Background: The tolerance of plants against abiotic stresses can be greatly influenced by interaction with microbes. In lowland rice (*Oryza sativa*) production, iron toxicity of the soils constitutes a major constraint. Although there are tolerant cultivars, the mechanisms underlying the tolerance against excess iron are not fully understood. Even less is known about the role of microbes in the response to iron toxicity.

Aim: In the study presented here the effects of different *Bacillus* isolates on the accumulation and distribution of iron within the shoots of different rice cultivars grown under iron toxicity were analyzed.

Methods: Three lowland rice cultivars with contrasting tolerance to iron toxicity (IR31785-58-1-2-3-3, Sahel 108, Suakoko 8) were inoculated with three *Bacillus* isolates (two *B. pumilus* and one *B. megaterium*) and, after one week, exposed to excess iron (1,000 ppm) for 8 days. Tolerance was evaluated by leaf symptom scoring.

Results: Bacteria inoculation mitigated leaf symptoms in the sensitive cultivar IR31785-58-1-2-3-3 despite of no significant differences in shoot iron concentration between inoculated and non-inoculated plants. In the tolerant excluder cultivar Suakoko 8 leaf symptoms were exacerbated when inoculated with *B. pumilus* Ni9MO12. While total shoot Fe concentration was not affected in this bacteria x cultivar combination, the distribution of iron within the shoot was clearly disturbed. Tolerance to iron toxicity of the tolerant includer cultivar Sahel 108 was not affected by *Bacillus* inoculation.

Conclusions: In conclusion, our results show that *Bacillus* inoculation can affect the tolerance of lowland rice to iron toxicity and that the effects strongly depend on the bacteria x cultivar combination.

2.1 Introduction

Plant-microbe interactions are gaining increasing interest and the importance of a holistic view of the plant and its microbiome in future breeding strategies and agricultural management practices is becoming widely accepted (Busby et al., 2017; Kroll et al., 2017). The overall fitness of plants under stress conditions is greatly influenced by microbes such as ecto- and endophytic bacteria (reviewed e.g. Bakker at al., 2018; Dimkpa et al., 2009). Numerous reports have been published about the influence of bacteria on acquisition, uptake, and homeostasis of iron in rice and other plants under iron limited conditions (reviewed in Lurthy et al., 2021). However, little research has yet been trying to shed light on the effect of bacteria on the tolerance of rice against excess iron. Iron toxicity constitutes a severe stress in flooded (arable) soils, leading to significant economic losses in lowland rice cultivation in large parts of Asia, West and Central Africa, and in Brazil, (Aung and Masuda, 2020). Although gramineous monocots usually take up ferric iron (Fe III) in its chelated form, rice also takes up ferrous iron (Fe II) directly (Ishimaru et al., 2006), often leading to excess accumulation when the concentration in the soil is high. Elevated concentrations of $Fe2^+$ within the plant tissue catalyze the formation of reactive oxygen species (ROS) via the Fenton reaction. This can result in severe damage to cellular metabolism and membrane structures (Briat and Lobreaux, 1997; Thongbai and Goodman, 2000; Sahrawat, 2005). Therefore, iron homeostasis must be carefully preserved in plant tissues for them to retain metabolic functions and activities. Rice genotypes tolerant to iron toxicity have been shown to employ different adaptation mechanisms, which may be active individually or in combination (Becker and Asch, 2005; Engel et al., 2012b). One strategy to tolerate high levels of ferrous iron in the soil is through decreasing iron uptake either by formation of iron plaques on the root surface or through downregulation of genes involved in iron uptake (= excluder type) (Aung and Masuda, 2020). Other strategies allow for tolerance of higher iron content within the plant: a) through deposition of iron in specific tissues such as roots or old leaves, chelation, and sequestration to the vacuole (= includer / avoidance type) (Majerus et al., 2007; Briat et al., 2010; Aung et al., 2019; Kar et al., 2021), or b) through enzymatic detoxification of iron-induced radical formation in both symplast and apoplast (= includer / tissue tolerance type) (Becana et al., 1998; Fang et al., 2001; Wu et al., 2017). Furthermore, dilution of iron within the plant through increased biomass production has been suggested to enhance tolerance to iron toxicity (Audebert and Fofana, 2009; Onyango et al., 2019). Regardless of the adaptation mechanism, the ability of different lowland rice cultivars to withstand toxic concentrations of soluble iron in the soil further depends on numerous environmental parameters, such as geographic region, soil type, cropping season, severity, and duration of iron toxicity occurrence but also on agricultural management practices. Stress symptom severity may even differ within a single field (Becker and Asch, 2005; Kirk et al., 2022). Soil microbial activity can enhance iron toxicity through reduction and mobilization of Fe-oxides in the soil (Becker and Asch, 2005). However, De Souza et al. (2015) reported ameliorating effects of rhizobacteria on rice grown under iron toxic conditions. Their study mainly focused on the isolation and characterization of bacteria from different environments and the effects they have on plant growth and nutrient uptake in two rice cultivars subjected to excess iron. The aim of our study was to analyze the effect of endophytic bacteria on tolerance to iron toxicity in terms of iron content, concentration, and distribution within the shoot as mechanisms underlying natural adaptation strategies. To that end, three lowland rice cultivars with contrasting levels of tolerance to iron toxicity were inoculated with cell suspensions of three different *Bacillus* spp. Isolates, subsequently subjected to 1000 ppm Fe (II) for one week, and leaf symptom expression, concentration and partitioning within the shoots were analyzed.

2.2 Material and Methods

2.2.1 Plant Growth

The experiments were conducted under greenhouse conditions with 12h light/dark period with light intensity of 450-550 μmol m^{-2} s^{-1}. The first experiment was carried out in February/March (spring screen, further called S1) with mean temperatures of 23/26°C (night/day) and 70-73% relative air humidity. The second experiment was carried out in September/October (autumn screen, further called S2) with mean temperatures of 27/29°C (night/day) and 45-55% relative air humidity. The experiments were laid out in a randomized complete block design with two iron treatments (0 Fe and 1000 ppm Fe), four bacteria treatments (No bacteria, *B. pumilus* D7.4, *B. megaterium*, and *B. pumilus* Ni9MO12), and 6 and 12 replications in S1 and S2, respectively. The set up was as described in Hartmann and Asch 2018. Three rice varieties, namely IR31785-58-1-2-3-3 (further IR31785), Suakoko 8, and Sahel 108 with known levels of tolerance to iron toxicity (Engel et al., 2012b; Onyango et al., 2020), were selected for this study. Rice seeds were germinated for 48 hours in a humid box before they were transferred into fine sand. When the 3rd leaf was emerging, seedlings were transferred to the hydroponic system consisting of 7-L containers (Eurobox, Auer, Germany, 600 mm x 400 mm x 15 mm) fitted with a rack of PVC tubes of 4 cm diameter and 12 cm length. Plants were grown in adequate Yoshida nutrient solutions (25% week 1, 50% week 2, and 100%, week 3) (Yoshida et al., 1971) for three weeks before being subjected to further treatments. Nutrient solution was renewed weekly.

2.2.2 *Bacillus* Inoculation

Three different *Bacillus* isolates (*Bacillus pumilus* D7.4, *Bacillus pumilus* Ni9MO12 (rif.res.) and *Bacillus megaterium*) isolated from rice seedling roots (Le et al. 2015, Padgham and Sikora, 2007) were used. For each iron treatment. Bacteria were grown on tryptic soy agar (TSA) for 24 h at 28°C. Single colonies were picked and propagated in liquid tryptic soy broth (TSB) at 28°C with shaking at 125 rpm. After overnight incubation, bacteria were harvested by centrifugation at 3,000 g for 20 min, and resuspended in ¼ strength Ringer Solution to a concentration of approx. 7×10^{7} CFU ml^{-1}. 28 days after sowing, the bacteria solution was added to plant nutrient solution (100% Original Yoshida) to a final concentration of approx. 10^{6} CFU ml^{-1}. *Bacillus* containing nutrient solution was left for one week before being exchanged.

2.2.3 Iron Treatment

At 35 days after sowing, Fe toxicity was induced by exchanging the nutrient solution to 100% Original Yoshida supplemented with iron II sulfate heptahydrate ($FeSO_4*7H_2O$) to a final concentration of 1000 ppm Fe. pH was adjusted to 5.5 only at the start of the treatment but not during its course. To prevent oxidation of ferrous to ferric iron, N_2 was percolated through the nutrient solution for 15 min every other hour using a standard aquarium percolator (Hobby LongLong, Aquaristikshop, Germany). Reductive conditions due to hypoxia were monitored using a redox electrode (Hanna Instruments, Germany). Non-iron treated plants were also subjected to N_2 percolation as described for iron-treated plants.

2.2.4 Plant Harvest

At eight days of Fe treatment, plants were harvested. In S1 plants were separated into roots and shoots, in S2 plants were separated into roots, sheaths and blades of single leaves, and dead leaf material. Numbering of the leaves started with the youngest fully expanded leaf = leaf 1, the second youngest fully expanded leaf = leaf 2 and so on. Samples were oven-dried at 70°C until constant weight and dry weights were determined.

2.2.5 Leaf Symptom Scoring

After seven days of stress exposure, leaf bronzing was assessed visually on fully expanded leaves for the entire plant based on the Standard Evaluation System for scoring for leaf blast lesions provided by the International Network for the Genetic Evaluation of Rice (IRRI - INGER, 1996), adapted to iron toxicity symptoms as described earlier (Asch et al., 2005; Engel et al., 2012).

2.2.6 Iron Analysis

The plant material was oven dried until constant weight for 2 days at 70 °C in a drying oven (ULM500, Memmert, Germany). The dry samples were milled in 20 ml scintillation vials (Nerbe, Germany), using ceramic milling balls. Iron Analysis was carried out according to Hartmann and Asch (2018). Extraction of ferrous iron was done in 500 mM sodium dithionite (SDT) using a Fast Prep (Fast Prep-24, MP Biomedicals, USA). Analysis was performed by adding 500 mM SDT and 10 mM dipyridyl (DPD). Color intensity was measured after 10 min at 490 nm (Infinite© 200pro, Tecan Trading AG, Switzerland). Fe II and Fe III dilution series were used as standards, with Fe III standard serving to ensure that the concentration of reducing agent was adequate.

2.2.7 Data Analysis

Relative changes in dry weights were calculated by dividing dry weights of the non-bacteria inoculated plants by the values of the corresponding dry weights of bacteria-inoculated plants. For analyzing the effect of bacteria inoculation on leaf bronzing scores, iron content, iron concentrations, the share of iron stored in sheaths one-way ANOVAs were carried out per cultivar. Two-factorial ANOVA was carried out for investigating the effects of cultivar, bacteria and bacteria x cultivar interaction on iron content in different organs. Skewness and Kurtosis were checked prior to performing analysis of variance (ANOVA). When normality tests failed, data was transformed by using ln function in excel. When ANOVA results were significant, post-hoc Dunnett's tests were used to compare each of the bacteria treatments with the non-inoculated control. ANOVAs and post-hoc Tukey and Dunnett's tests were performed in Rstudio (version 4.0.3 "Bunny-Wunnies Freak Out"). Microsoft Excel was used LSD tests, SigmaPlot 14.0 (Systat Software, Inc.) was used for Student's t-tests (used when only one particular bacteria treatment was compared to the non-inoculated control), and graphs.

2.3 Results

2.3.1 Leaf Scores

The effects of bacteria inoculation on leaf bronzing scores were highly comparable between the two experiments ($r = 0.92$, Figure 2.6) and differed between cultivars (Figure 2.1). Inoculation with *B. pumilus* D7.4 significantly mitigated leaf symptom expression in the naturally sensitive cultivar IR31785 (Dunnett's test, $\alpha = 0.05$), lowering the mean leaf bronzing score from 5.88 to 2.88 in the first screen (S1) and from 6.71 to 4.1 the second screen (S2). The tolerant includer cultivar Sahel 108 had a mean leaf bronzing score of 3.75 in S1 and 4.04 in S2 when iron stressed without bacteria inoculation. No significant changes in these leaf bronzing scores were observed for plants inoculated with *B. pumilus* D7.4 under iron toxic conditions (Dunnett's test, α = 0.05). *B. pumilus* D7.4 inoculated, iron-stressed plants of the tolerant excluder cultivar Suakoko 8 displayed significantly stronger leaf symptoms as compared to the non-inoculated, iron-stressed plants (Dunnett's test, α = 0.05), increasing the mean leaf bronzing scores from 3.6 to 4.5 in S1 and from 3.7 to 5.1 in S2. Inoculation with *B. megaterium* led to a reduction in mean leaf bronzing score by 1.7 to 4.3 in S1 and by 1.1 to 5.6 in S2 in IR31875 (Dunnett's test, not significant, α = 0.05), while *B. megaterium* inoculated Suakoko 8 plants showed a mean leaf bronzing score of 5.1 in S1 (+1.5 difference to non-inoculated, iron-stressed plants, Dunnett's test, $p \leq 0.05$) and 4.4 in S2 (+0.75 difference to non-inoculated, iron-stressed plants, Dunnett's test, not significant, α = 0.05). In Sahel 108, *B. megaterium* inoculated plants showed no significant differences in leaf bronzing scores as compared to the non-inoculated, iron-stressed plants. IR31875 inoculated with *B. pumilus* Ni9MO12 had a mean leaf bronzing score of 4.9 in S1 (- 1 difference to non-inoculated, iron-stressed plants, Dunnett's test, not significant, α = 0.05) and 5.0 in S2 (-1.75 difference to non-inoculated, iron-stressed plants (Dunnett's test, $p \leq 0.01$). *B. pumilus* Ni9MO12 inoculation of Suakoko 8 led to an increase in mean leaf bronzing score to 4.6 (+1 difference to non-inoculated, iron-stressed plants, Dunnett's test, $p \leq 0.05$) in S1 and to 4.4 (+0.75 points difference to non-inoculated, iron-stressed plants, Dunnett's test, not significant, α = 0.05) in S2. No significant effects of *B. pumilus* NiMO12 inoculation on leaf bronzing scores were seen in Sahel 108.

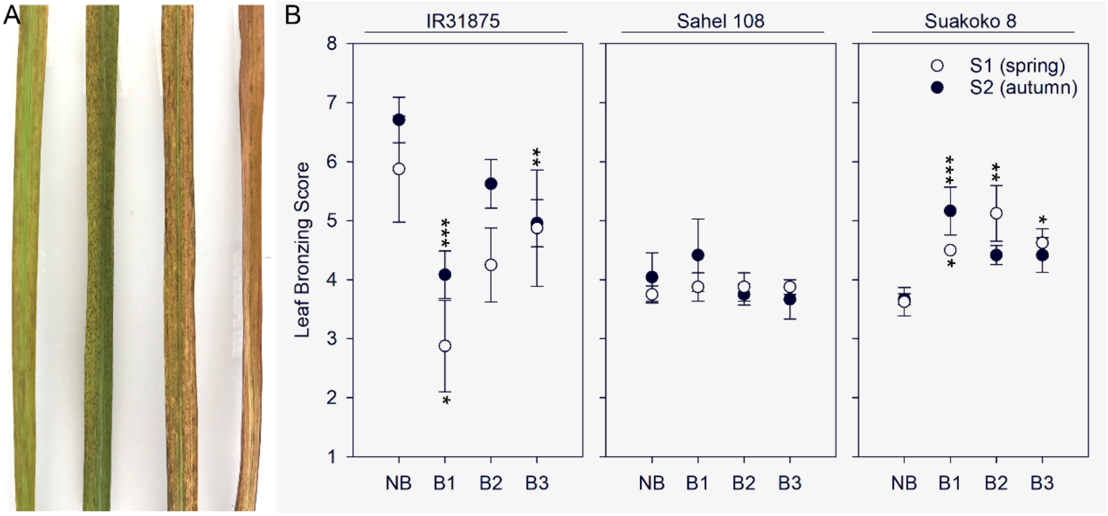

Figure 2.1: **A:** Rice leaves differently affected by bronzing. **B:** Effect of bacteria inoculation on leaf symptom scores after 8 days of iron treatment. Asterisks show significant differences to non-inoculated plants within each cultivar (Dunnett's test, * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$) NB = no bacteria, B1 = *B.pumilus* D7.4, B2 = *B.megaterium*, B3 = *B.pumilus* Ni9MO12 rif. res.

2.3.2 Biomass Accumulation

Due to differing light and temperature conditions, plants in S2 were generally larger and had more tillers than plants of S1. In non-inoculated plants after 8 days of iron treatment, above ground biomass accumulation and tiller number did not differ from plants that received neither iron treatment nor *Bacillus* inoculation. Root biomass, however, was significantly reduced in response to iron treatment in non-inoculated plants (Table 2.1). In order to distinguish growth promoting bacterial effects from other growth-independent bacterial effects we plotted the relative changes in dry weight between non-inoculated plants and inoculated plants in Figure 2.2. Values larger than 1 indicate positive whereas values smaller than 1 depict negative bacterial effects on biomass accumulation. In general, and in both screens, only few growth promoting effects of bacteria inoculation were observed for any bacteria x cultivar combination. *B. pumilus* D7.4 tended to reduce growth under non-iron toxic conditions and to increase growth under iron toxic conditions by about 10-20%. Inoculation with *B. megaterium* generally reduced growth of both roots and shoots in all cultivars, whereas the effects of an inoculation with *B. pumilus* Ni9MO12, varied strongly between the two screens for all cultivars. In S1 growth under non-iron toxic conditions was strongly and significantly reduced whereas in S2 no effect was observed. For Sahel 108 growth was promoted under iron toxic conditions but reduced under non-toxic conditions in the same order of magnitude as in IR31785. Equally no effect was seen in S2. In Suakoko 8 inoculation with *B. pumilus* Ni9MO12 resulted on average in a 10% better growth in S1 and in on average 20% growth reduction in S2.

Table 2.1: Mean effects of iron treatment across three different lowland rice cultivars on biomass traits in the two experiments S1 and S2. Values are means, ± standard error, n = 12 (S1), n = 36 (S2). Different letters indicate significant differences between iron treatments per trait, overall cultivars without bacteria treatment (Least Significant Difference, LSD, $p \leq 0.05$).

	S1							S2						
	Iron treatment [ppm]							Iron treatment [ppm]						
Trait	0			1000			LSD	0			1000			LSD
Dry weight shoot [g]	0.86	±0.07	A	0.82	±0.07	a	0.22	1.31	±0.06	A	1.29	±0.06	A	0.18
Dry weight root [g]	0.46	±0.03	A	0.32	±0.02	b	0.08	0.71	±0.03	A	0.53	±0.04	B	0.10
Tiller number	3.92	±0.09	A	3.67	±0.1	a	0.5	5.94	±0.33	A	5.89	±0.34	A	0.94

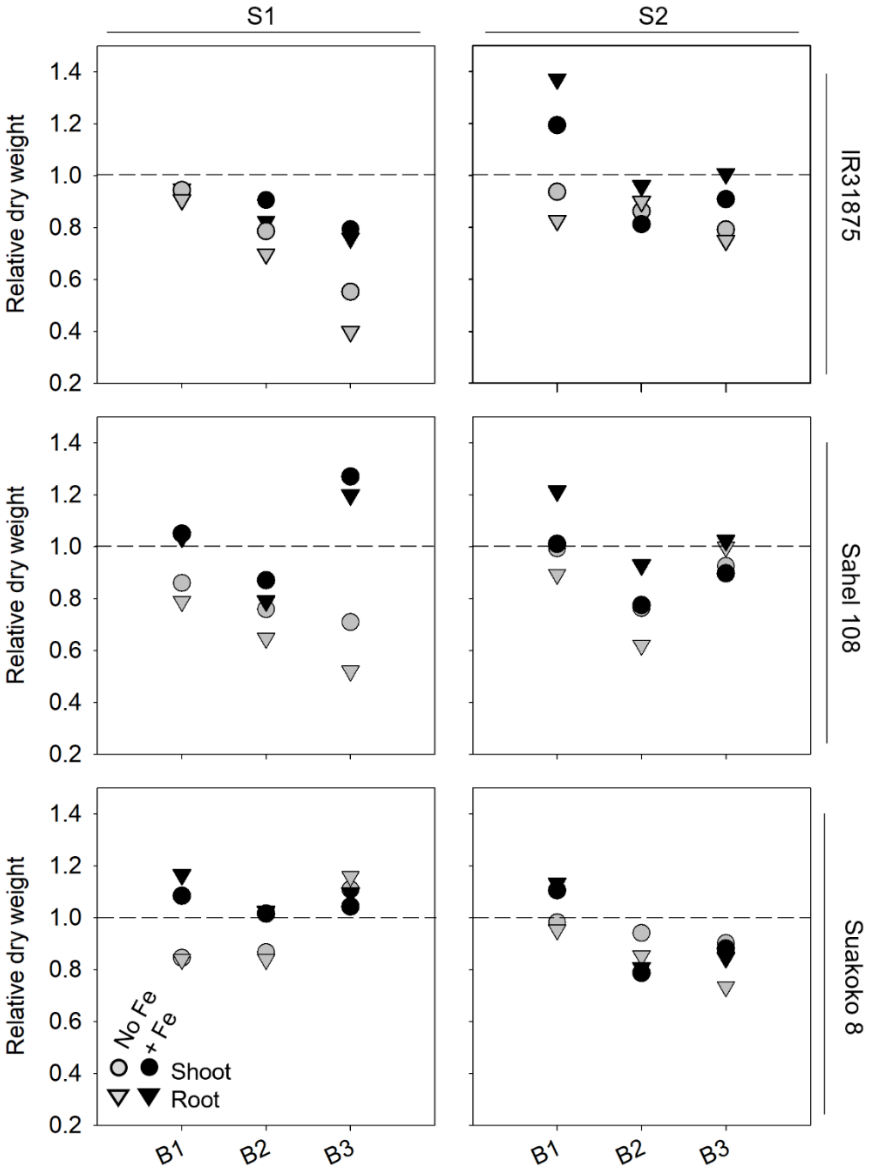

Figure 2.2: Effect of bacteria inoculation on biomass accumulation. Graphs show the changes in dry weight relative to non-inoculated plants. No Bac = no bacteria, B1 = *B.pumilus* D7.4, B2 = *B.megaterium*, B3 = *B.pumilus* Ni9MO12 rif res. S1= Screen 1 carried out in spring, S2= Screen 2 carried out in autumn.

2.3.3 Relation Between Shoot Iron and Leaf Bronzing Scores

Leaf symptom score was used as an indicator for tolerance to iron toxicity and related to total shoot iron content and total shoot iron concentration (Figure 2.3). Differences in shoot iron content and shoot iron concentration between non-inoculated, iron-stressed plants and inoculated, iron-stressed plants per cultivar x bacteria combination were in most cases not statistically significant (Dunnett's test, $\alpha \leq 0.05$). In general, the tendencies of numerical differences, either increase or decrease, were comparable between experiments. No general correlation between leaf bronzing score and shoot iron content and concentration was found for any of the three cultivars. Effects of bacteria inoculation on leaf symptom scores varied between bacteria x cultivar combinations. In Sahel 108 no bacterial effects on leaf symptom scores were observed (Fig 2.3 C, D). In both screens, inoculation with *B. pumilus* D 7.4 significantly decreased the leaf bronzing scores in IR31875 with only non-significant changes in shoot iron content and concentration, whereas in Suakoko 8 the leaf bronzing score significantly increased, accompanied by a non-significant increase in shoot iron concentration and an increase in shoot iron content, which was numerical in the first and significant in the second experiment (Dunnett's test, $\alpha = 0.05$) (Fig 2.3 E, F). Similar but less pronounced effects on leaf symptoms scores were observed for inoculations with *B. megaterium* and *B. pumilus* Ni9MO12, (Fig 2.3 A, B and E, F) whereas inoculations had non-significant effects on shoot iron content or concentration (Dunnett's test, $\alpha = 0.05$).

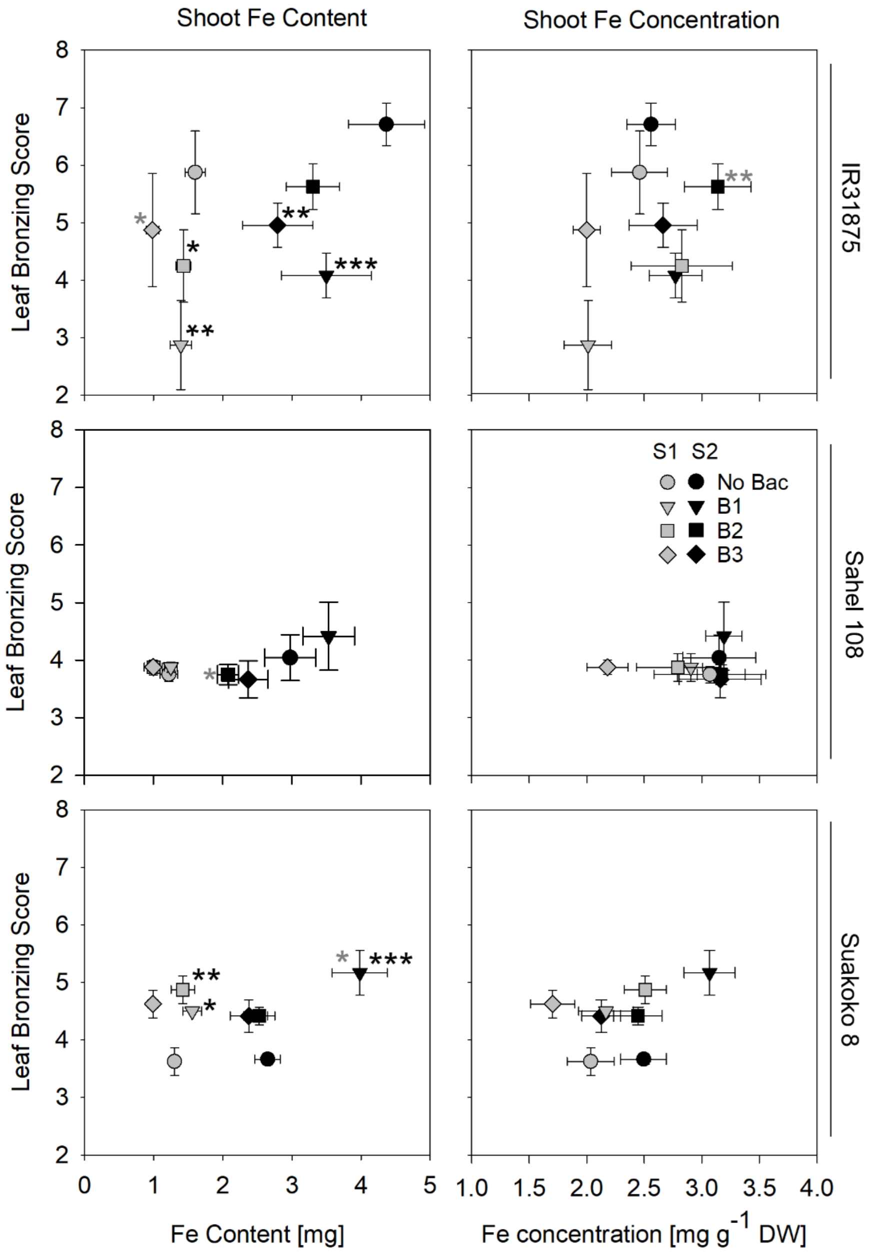

Figure 2.3: Leaf symptom scores of plants grown under iron toxic conditions in relation to average total shoot iron content and average shoot iron concentration [mg g-1 DW]. Asterisks show significant differences to non-inoculated plants within each cultivar (Dunnett's test, * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$) black asterisk = score (only shown in graphs on the left), grey asterisk = Fe content and Fe concentration, respectively. No Bac = no bacteria, B1 = *B.pumilus* D7.4, B2 = *B.megaterium*, B3 = *B.pumilus* Ni9MO12 rif. res. S1= Screen 1 carried out in spring, S2= Screen 2 carried out in autumn.

2.3.4 Distribution within the Shoot

In S2 the distribution of iron within the shoot was analyzed. A two-factorial analysis of variance (ANOVA) was carried out to analyze the effects of cultivar, bacteria inoculation, and their interaction on iron content within single organs. Results are shown in Table 2.2.

Table 2.2: Two-factorial analysis of variance (ANOVA) for iron content in the leaf blades, sheaths, dead tissue, and total shoot of iron treated plants. Abbreviations Cult= Cultivar, Bac = bacteria, df= degrees of freedom, MS = mean square, Sign = significance, n.s. = not significant, * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$

Iron Content	Effect	df	MS	*p*	Sign
Blade	Cult	2	1.64	<0.01	**
	Bac	3	0.99	0.12	n.s.
	Cult x Bac	6	0.98	0.44	n.s.
Sheath	Cult	2	0.17	0.04	*
	Bac	3	0.22	<0.01	**
	Cult x Bac	6	0.15	0.01	*
Dead Tissue	Cult	2	1.30	<0.05	*
	Bac	3	2.64	<0.001	***
	Cult x Bac	6	0.63	0.05	*
Total Shoot	Cult	2	7.66	0.02	*
	Bac	3	11.1	<0.001	***
	Cult x Bac	6	2.85	0.17	n.s.

Iron content of total shoot, dead tissue, and sheaths were statistically significantly affected by both cultivar and bacteria inoculation whereas content in blades was significantly affected by cultivar but not by bacteria inoculation. Interaction of cultivar: bacteria treatment did significantly affect iron content in sheaths and dead tissue but not total shoot iron content and iron content in blades. Mean iron contents of leaf blades, sheaths, dead tissue, and total shoot in plants grown under iron toxic conditions under different bacteria inoculations are shown in Table 2.3. Bacteria inoculation effects on the iron content of individual organs varied between the genotypes when compared to the non-inoculated control. Whereas in most cases interactions were not significant, there was for each rice genotype a different bacteria strain that showed strongest influence on the iron content of one or more than one organ. In IR31785 inoculation with *B. pumilus* Ni9MO12 most strongly decreased shoot iron uptake by about 36% and highly significantly decreased iron content of dead tissue by about 53% (Dunnett's test, $\alpha \leq 0.05$), and inoculation of IR31875 with *B. megaterium* significantly decreased total shoot iron content by about 28%, content in the sheaths by approximately 55 %, and content in the dead leaves by approximately 47% (Dunnett's test, α ≤ 0.05). In Sahel 108 inoculation with *B. megaterium* most strongly, although only numerically, reduced iron content of leaf blades, sheaths, dead tissue, and total shoot by 20%, 24%, 37%, and 30%, respectively (Dunnett's test, α ≤ 0.05). In Suakoko 8 none of the bacteria inoculation reduced tissue iron accumulation as compared to the non-inoculated control, but inoculation with *B. pumilus* D7.4 significantly increased iron content of leaf blades, sheaths, , and total shoot by 69%, 44%, and 50% respectively (Dunnett's test, α ≤ 0.05). Iron content was also reduced in dead tissue of *B. pumilus* D7.4 inoculated Suakoko 8, this 46% increase was not statistically significant though. The share of leaf iron content located in the blades in non-inoculated, iron stressed IR31785 was 56% (0.6 mg of 1.07 mg) and thus 11% higher than in the tolerant genotypes Sahel 108 and Suakoko 8 (45%). Inoculation with *B. pumilus* Ni9MO12 highly significantly increased this share under iron toxic conditions in Suakoko 8 to 56% (0.51 mg of 0.91 mg) (p = 0.002, t-test, α ≤ 0.05), whereas inoculation effects on the share of iron in the leaf blades were minor and not significant in the other cultivars.

Table 2.3: Shoot iron content of iron treated plants. Mean values and standard errors are given. Asterisks show significant differences to non-inoculated plants within each cultivar (Dunnett's test,. = p ≤ 0.1, * = p ≤ 0.05, ** = p ≤ 0.01). Ɨ Difference between total shoot and the sum of leaf blade, sheath and dead tissue arise from iron in leaf 0 (not fully expanded) which is not included in the table.

		Total Iron Content [mg]							
		Organ							
Cultivar	Bacteria Treatment	Leaf Blade		Sheath		Dead Tissue		Total Shoot[Ɨ]	
IR31875	No Bacteria	0.60	±0.06	0.47	±0.08	3.08	±0.46	4.37	±0.55
	B.pumilus D7.4	0.87	±0.25	0.63	±0.06	1.83	±0.36.	3.50	±0.65
	B.megaterium	0.77	±0.08	0.73	±0.07**	1.62	±0.26*	3.30	±0.39*
	B.pumilus Ni9MO12	0.73	±0.15	0.50	±0.05	1.45	±0.34**	2.79	±0.51
Sahel 108	No Bacteria	0.62	±0.10	0.75	±0.07	1.45	±0.26	2.97	±0.37
	B.pumilus D7.4	0.59	±0.04	0.71	±0.07	2.12	±0.34	3.53	±0.37
	B.megaterium	0.50	±0.08	0.57	±0.05	0.92	±0.09	2.08	±0.16
	B.pumilus Ni9MO12	0.58	±0.09	0.62	±0.09	1.04	±0.15	2.37	±0.29
Suakoko 8	No Bacteria	0.42	±0.03	0.52	±0.05	1.57	±0.16	2.65	±0.19
	B.pumilus D7.4	0.71	±0.1**	0.75	±0.06*	2.30	±0.28	3.98	±0.4*
	B.megaterium	0.54	±0.07	0.50	±0.05	1.37	±0.18	2.53	±0.23
	B.pumilus Ni9MO12	0.51	±0.03	0.40	±0.08	1.38	±0.23	2.37	±0.27

2.3.5 Iron Distribution within Living Leaves

Under iron toxic conditions, the share of iron stored in the leaf sheaths increased with leaf age in all three cultivars and all bacteria treatments (Figure 2.4). Without bacteria inoculation, up to 75% of the iron taken up into the leaf were stored in the sheaths of the oldest leaves, but the differences in distribution of iron within leaves of different ages did not statistically differ between cultivars (LSD, Least Significant Difference, $p \leq 0.05$, data not shown). Leaf 3 (L3) was the oldest living leaf found in all bacteria x cultivar combinations, a living Leaf 4 (L4) was only found in some plants. The largest numerical differences between cultivars – non-inoculated, iron-stressed – were seen in the second youngest fully expanded leaves (L2) with on average 46% of iron stored in the sheaths in IR31875, 52% on average in Sahel 108, and 56% on average in Suakoko 8. The effect of bacteria inoculation on the distribution of iron within the leaves varied between cultivar, bacteria isolate, and leaf age. Inoculation with *B. pumilus* D7.4 (B1 in Fig. 2.4) only numerically affected the share of iron in the sheaths of all three cultivars tested. *B. megaterium* inoculation (B2 in Fig. 2.4) significantly decreased the share of iron in the sheaths of L2 in Suakoko 8 by 11% (Dunett's test, $\alpha \leq 0.05$). Differences found in other leaves and cultivars were numerical only. *B. pumilus* Ni9MO12 inoculation (B3 in Fig. 2.4) led to a significantly lower share of iron in the sheaths of L2 of Sahel 108 and Suakoko 8, as compared to the non-inoculated, iron-stressed plants (-9.5%, Dunett's test, $p \leq 0.05$, and -18.2%, Dunett's test, $p \leq 0.01$, respectively), leading to a share of 38% of leaf iron in the sheaths of L2 in Suakoko 8. In this cultivar, inoculated with *B. pumilus* Ni9MO12, a significant reduction of iron share in the sheaths was also found in L3 (Dunnett's test, $p \leq 0.05$). Only 48 % of leaf iron were found in the sheaths of L3 of the inoculated, iron-stressed plants, compared to 70% in the sheaths of L3 of the non-inoculated, iron-stressed plants. Although no statistical comparison of L4 was possible due to the limited number of non-inoculated plants possessing a living L4, the percentage of iron stored in the sheaths of L4 of *B. pumilus* Ni9MO12 inoculated, iron-stressed Suakoko 8 was found to be very low with an average of 40%.

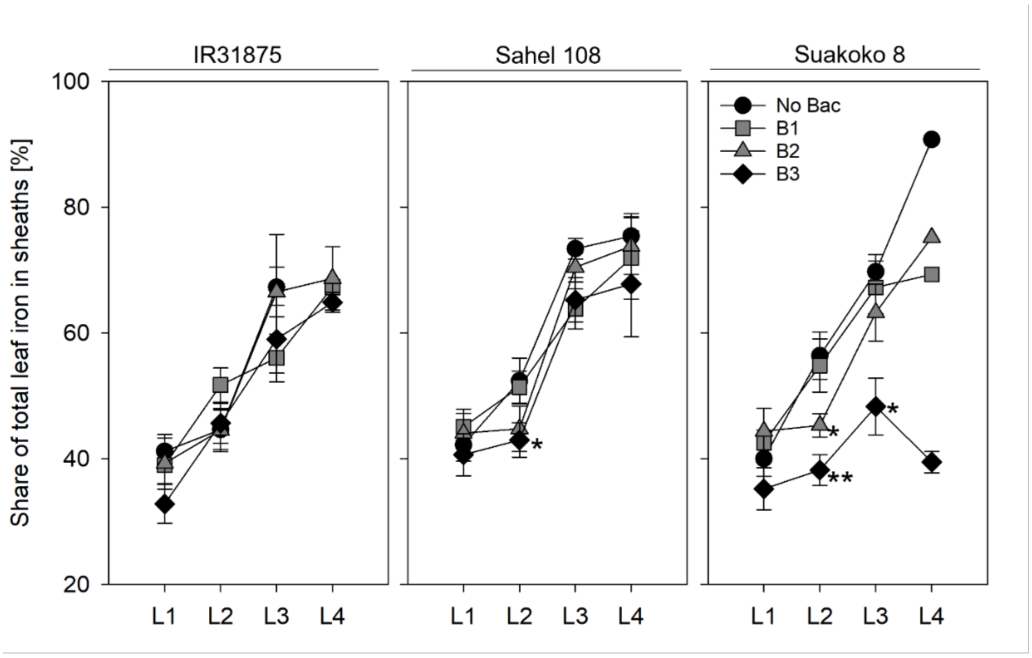

Figure 2.4: Effect of bacteria inoculation of the share of total leaf iron in the sheaths of different leaf ages of plants grown under iron toxic conditions. L1 = leaf 1 (youngest fully expanded leaf), L2= leaf 2 (second youngest fully expanded leaf), L3 = leaf 3, L4= leaf 4. Asterisks show significant differences to non-inoculated plants within each cultivar (Dunett's test, * = $p \leq 0.05$, ** = $p \leq 0.01$). No Bac = no bacteria treatment, B1 = *B.pumilus* D7.4, B2 = *B.megaterium*, B3 = *B.pumilus* Ni9MO12 rif. res.

2.3.6 Iron Concentration within the Leaves

Although iron concentrations in the sheaths of iron-stressed Sahel 108 were numerically higher than in the other two cultivars, differences between cultivars in iron concentration in blades and sheaths of L1-L3 under iron-toxic conditions without bacteria inoculation were not statistically significant (LSD, $\alpha \leq 0.05$). In young leaves, iron concentration in the blades of non-inoculated, iron-stressed plants, was generally higher than in the respective sheaths (Figure 2.5). This was found for all three cultivars tested. With increased leaf age, the ratio of iron concentration in the blades to iron concentration in the sheaths changed because of a steady increase in iron concentration in the sheaths. While non-inoculated, iron-stressed IR31875 did not have living L4, in non-inoculated, iron-stressed Sahel 108, the mean iron concentration in the sheaths of L4 was found to be 5.4 times higher than in the sheaths of L1. In Suakoko 8, the mean iron concentration in the sheaths of L4 was found to be 9.2 times higher than in the sheaths of L1. *Bacillus* inoculation did not significantly change the ratio of iron concentration in the sheaths of L4 to iron concentration in the sheaths of L1 in iron-stressed Sahel 108 (Dunnett's test, $\alpha \leq 0.05$). For iron-stressed, Suakoko 8, statistical analysis was not possible as only one non-inoculated plant had a living L4. However, while in Suakoko 8 inoculated with *B. megaterium*, a steady increase in iron concentration from L1 to L4 was found, in *B. pumilus* inoculated plants, iron concentration in the sheaths of L4 was lower than in the respective L3. While only one plant inoculated with *B. pumilus* D7.4 had a fourth leaf, the low iron concentration shown for the sheaths of L4 of *B. pumilus* Ni9MO12 inoculated Suakoko 8 is the mean value from 5 plants.

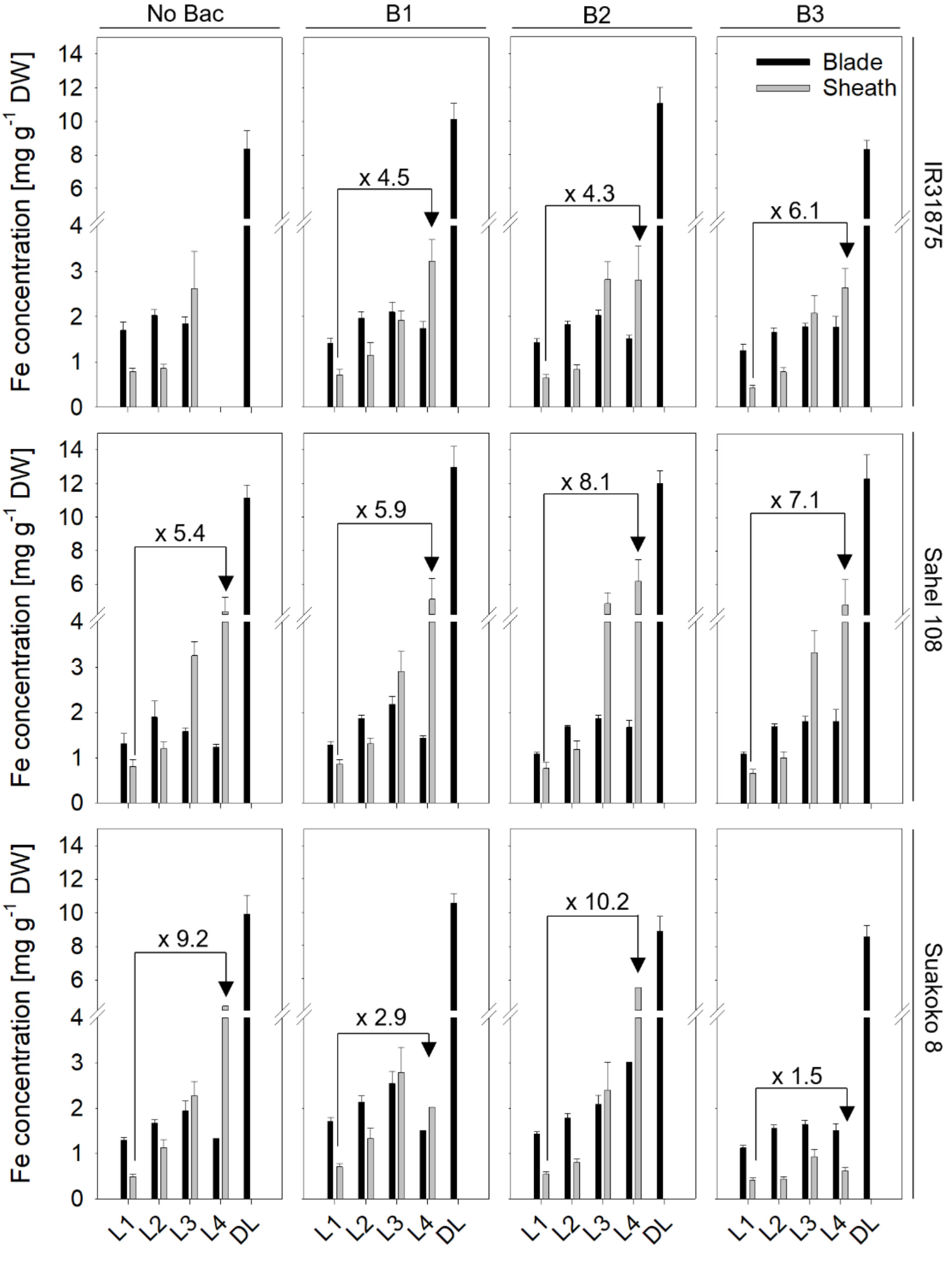

Figure 2.5: Iron concentration in the blades and sheaths of leaves of different ages of plants grown under iron toxic conditions. The numbers above the arrows show the ratio of iron concentration in the sheaths of L4 to the iron concentration in the sheaths of L1. L1= youngest fully expanded leaf, L2 = second youngest fully expanded leaf, etc. DL = dead leaves No Bac = no bacteria, B1 = *B.pumilus* D7.4, B2 = *B.megaterium*, B3 = *B.pumilus* Ni9MO12 rif. res.

2.4 Discussion

It has been shown earlier that different bacteria strains interact with different rice genotypes in various ways by changing the growth patterns of the rice seedlings under non-stressed conditions (Seerat et al., 2019). In the current study, *Bacillus* spp., originally isolated from rice rhizospheres, interacting with rice genotypes contrasting in iron toxicity tolerance under iron toxic conditions, resulted in strong effects on the rice plants which differed according to the combination of *Bacillus* strains and rice genotype. In the naturally sensitive cultivar IR31785, a reduction of iron toxicity symptoms in the leaves was observed, whereas in the tolerant cultivar Suakoko 8 symptom expression was exacerbated in inoculated plants, and no significant effects of inoculation with bacteria on the leaf symptom scores were observed for the tolerant includer type Sahel 108. In order to elucidate potential causes for differential leaf symptom expression following the inoculation with various bacteria strains we investigated the effect of bacteria inoculation on plant biomass accumulation, shoot iron content, and iron distribution in the shoot.

2.4.1 Effect of *Bacillus* Inoculation on Biomass Accumulation

To distinguish the effects of bacteria inoculation from environmental effects, the two screens were carried out during German spring in February/March (S1), and S2 during German autumn in September/October (S2) resulting in different growth conditions regarding day length, temperature, and VPD. As a result of the different environmental conditions, plant growth differed between the two screens. Plants grown in autumn were generally larger than those grown in spring. Independent of differences in growth between the two experiments reported here, the relative effects of bacteria treatments on the leaf bronzing scores were highly comparable between the two screens (Fig. 2.1, Fig 2.6). The effects of bacteria inoculation on biomass accumulation on the other hand varied between bacteria strains, cultivars, iron treatment and experiments. The hypothesis that positive effects of bacteria inoculation on leaf symptom expression arise from a dilution effect of tissue iron concentration due to increased biomass accumulation, cannot be confirmed, as no correlation between plant size and leaf score was found. Our results show that firstly, plant growth as an indicator for tolerance to iron toxicity should be viewed critically and secondly, many bacteria with true stress mitigating effects may remain undetected when only screening for plant growth promoting properties.

2.4.2 Effect of *Bacillus* Inoculation on Shoot Iron Content and Concentration

Lower iron concentrations in the shoot could also be a consequence of lower total shoot iron contents either due to a decreased uptake or a reduced root-to-shoot translocation. De Souza et al. (2015) reported reduced shoot iron concentrations in iron-stressed rice inoculated with bacteria of different genera, including one *Bacillus* s*p.* isolate. In contrast, our results show that bacteria effects on shoot iron content and concentration differs between cultivars, bacteria isolates, and bacteria x cultivar combinations. Engel et al. (2012) linked bronzing scores to leaf iron concentrations with higher leaf iron concentrations resulting in higher leaf bronzing scores. Inoculation with different strains of *B. pumilus* resulted in contradicting results. Where, compared to the non-inoculated plants, a significantly higher iron content in the shoot of *B. pumilus* D7.4 inoculated Suakoko 8 resulted in exacerbated leaf symptoms, the inoculation with *B. pumilus* Ni9MO12 on the other hand resulted in lower leaf iron concentrations but higher leaf bronzing scores. In IR31875 inoculation with *B. pumilus* D7.4 led to significantly lower leaf bronzing scores but shoot iron concentration was not significantly affected. Only in IR31875 inoculated with *B. pumilus* Ni9MO1 reduction in leaf symptoms could be linked to lower shoot iron concentrations compared to non-inoculated plants (Fig. 2.3). In Sahel 108 *Bacillus* inoculation affected neither shoot iron concentrations, nor leaf symptom scores.

2.4.3 Effect of *Bacillus* Inoculation on Iron Distribution within the Shoot

Since the effect of bacteria inoculation on leaf symptom score could not simply be explained by total shoot iron content or concentration, the leaves of the larger plants in the spring experiment (S2) were dissected into single organs upon harvest in order to analyze the distribution of iron within the shoot. Our results confirmed previous reports which found larger shares of Fe partitioned to stem tissues, especially in the older leaves of tolerant cultivars. (Aung et al., 2018; Engel et al., 2012b). Inoculation of the three contrasting rice cultivars with *Bacillus* spp. differently affected the overall distribution of iron within the shoot: Total iron contents of sheaths and dead tissue were affected by cultivar, bacteria, and cultivar x bacteria interaction, while an effect in total iron content of blades was only found for cultivar (Table 2.2). The importance of the distribution of iron within the shoot becomes apparent in Suakoko 8 inoculated with *B.pumilus* Ni9MO12. Both total shoot iron content and total shoot iron concentration were lower in these plants as compared to non-inoculated, iron-stressed plants (Fig. 2.3). At the same time, leaf symptoms were significantly exacerbated. In the non-inoculated, iron-stressed Suakoko 8 the absolute iron content in sheaths was higher than in blades and the share of iron in sheaths increased with leaf age (Table 2.3 and Fig.2.4). In the inoculated, iron-stressed plants however, both total iron content in sheaths as well as the share of iron in sheaths of old leaves was decreased (Table 2.3 and Fig.2.4). Engel et al. (2012b) suggested balancing the share of Fe in the blades and sheaths to be a tolerance mechanism of includer genotypes. In agreement with this, iron concentration in the sheaths as well as the share of leaf iron stored in the sheaths of the tolerant includer Sahel 108 steadily rises with leaf age (Fig. 2.4 and 2.5). However, our results show that this balance is also important in excluder genotypes such as Suakoko 8 and that alteration of iron distribution within the shoot, i.e. decreased iron partitioning into the old stems, can therefore be hypothesized to be the cause for decreased tolerance to iron toxicity in Suakoko 8 inoculated with *B. pumilus* Ni9MO12. Iron concentration within the blades did not differ between inoculated and non-inoculated Suakoko 8 under iron toxic conditions. The differences in share result from a lower concentration of iron in the older sheaths (Table 2.3). It can be assumed that not the transport into the blades is disturbed but rather the deposition of excess iron into the old sheaths decreased i.e. through increased transport of iron from mature to growing leaves. The metal chelator nicotianamine (NA) and the Fe-NA transporter *OsYSL2* have been shown to be involved in internal iron transport (Aung et al., 2019; Koike et al., 2004; Yoneymama, 2021). However, further in-depth studies will be necessary to understand iron translocation within the shoot under iron toxic conditions and the effect endophytic bacteria can have on these transport mechanisms. The effects of bacteria inoculation on the expression of different candidate genes are currently being investigated. Under iron deficiency, Lurthy et al. (2020) reported differential impacts on iron status and root to shoot allocation of iron in pea inoculated with bacterial siderophores (ferripyoverdines from different pseudomonads), depending on the pea cultivar x bacteria combination. In the study presented here, we found a very strong effect of inoculation with *Bacillus pumilus* on iron distribution within the shoots of the tolerant cultivar Suakoko 8: iron concentration in the sheaths did not increase with leaf age as seen in the non-inoculated plants. No such effect was seen in Suakoko 8 inoculated with *B. megaterium*, or in the tolerant cultivar Sahel 108 inoculated with either one of the three *Bacillus* isolates. Distribution within the living shoot of non-inoculated, iron-stressed IR31875 was difficult to compare to *Bacillus*-inoculated plants as these plants showed severe symptoms and leaves older than L3 had already died off by the time of harvest and the largest share of shoot iron was found in dead tissue.

2.4.4 Possible Mechanisms Underlying Bacteria Effects on Tolerance to Iron Toxicity

Using the three different defense strategies identified in lowland rice – I. excluder; II. includer/avoidance III. includer tolerance (Becker and Asch, 2005) – as a basis for developing hypotheses, our results suggest that the mitigation of leaf symptoms in *Bacillus* inoculated IR31875 does not relate to tolerance mechanisms underlying the excluder type, but rather encompass mechanisms which lead to increased tolerance to the natural iron concentration in the shoot. This could either be achieved by so-called “avoidance” or through increased “tissue tolerance”.

Increased “tissue tolerance” can also be brought about by increased antioxidant enzyme activity and ROS scavenging (Briat et al., 2010; Kar et al., 2021; Stein et al., 2014; Tadaiesky et al., 2020; Wu et al., 2017). Endophytic bacteria could affect this tolerance mechanism by either stimulating the plant antioxidant apparatus in rice (Liu et al., 2020) - or through bacterial ROS scavenging activity (Sessitsch et al., 2012). “Avoidance” involves enhanced chelating of ferrous iron by NA, its exclusion from the symplast through immobilization in the leaf apoplast, its storage as ferritins or its sequestration to the vacuole (Aung et al., 2018; Majerus et al., 2007; Kar et al., 2021). Investigations into altered expression of genes involved in these pathways, such as *OsFER*s, *OsVIT2*, and *OsNAS3* are currently underway. Another form of “avoidance” mechanism involves the partitioning of iron into older sheaths, which seems to be disturbed in Suakoko 8 inoculated with *B. pumilus*. Apart from iron transporters, such as *OsYSL2*, the potassium transporter *OsAKT1* has been reported to be involved in the translocation of iron within the plant, affecting the tolerance to iron toxicity (Wu et al., 2019) In our experimental set up plants were grown in original Yoshida solution supplying the plants with sufficient, readily available nutrients (Yoshida et al., 1971), however, rhizobacteria have been shown before to alter uptake of N, P, and K in rice under normal and iron toxic conditions (Dimpka et al. 2009, De Souza et al., 2015) and an impact of *Bacillus* on the uptake and or translocation of nutrients requires further investigation.

2.5 Conclusions

In conclusion, further investigation is needed to decipher the mechanisms underlying altered shoot iron content, concentration, and distribution as well as physiological responses to iron toxicity in contrasting lowland rice cultivars inoculated with *Bacillus* spp. The study presented here, focused on iron distribution within the shoot. Alterations of morphological, physiological, and molecular nature will also have to be studied in the shoot, as well as in the root as the entry site into the plant for both endophytic *Bacillus* and iron. Owing to the method of iron extraction used in our study, it is not possible to distinguish between iron deposited in plant tissues and iron within the endophytic bacteria. Understanding the movement and location of bacteria in the tissue as well as different metabolic capacities of the endophytes within the iron stressed plants will be vital to fully understand plant-bacteria interactions.

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Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Conflicts of Interest

The authors declare no conflict of interest.

Supplementary Material

Following additional supporting information can be found online in the Supporting Information section at the end of this article [see Figure 2.6 below].

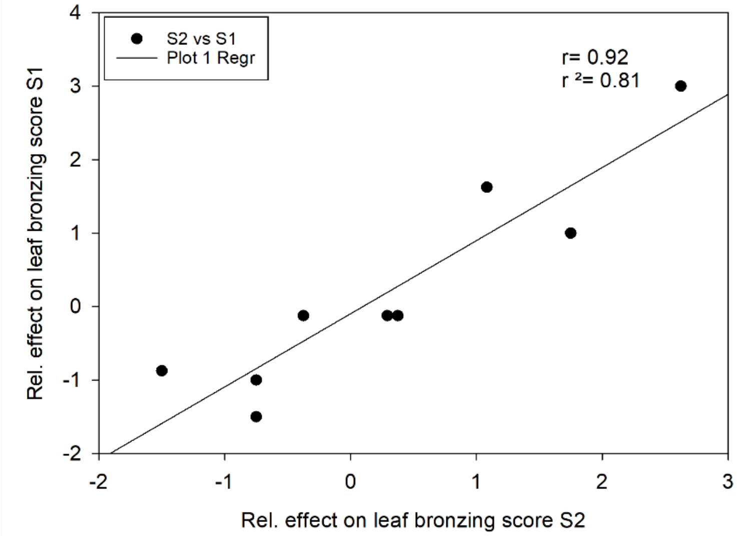

Figure 2.6: Comparison of relative effects of bacteria inoculation on leaf bronzing scores in the two experiments. S1= Screen 1 carried out in spring, S2= Screen 2 carried out in autumn.

2.5.1 References

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3 Role of *Bacillus* spp. Plant Growth Promoting Properties in Mitigating Biotic and Abiotic Stresses in Lowland Rice (*Oryza sativa* L.)

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Keywords: PGPR; iron toxicity; brown spot disease; *Bipolaris oryzae*; abiotic and biotic stress; ACC deaminase; auxin; nutrient solubilization

Abstract

The ability of microorganisms to promote plant growth and mitigate abiotic and biotic stresses makes them an interesting tool for sustainable agriculture. Numerous studies aim to identify new, promising bacteria isolates. Traditional culture-based methods, which focus on selecting microorganisms with plant-growth-promoting traits, such as hormone production, nutrient solubilization, and antifungal properties, are widely used. This study aims to investigate the role of plant-growth-promoting properties in bacteria-mediated stress mitigation and the suitability of traditional culture-based methods as a screening tool for the identification of beneficial bacteria. To this end, we tested three endophytic *Bacillus* isolates, which have previously been shown to affect tolerance against iron toxicity in lowland rice, (a) for their effect on the resistance against brown spot disease, and (b) for plant-growth-promoting traits using common culture-based methods. Both *B. pumilus* isolates inhibited fungal growth in vitro and reduced brown spot disease in two of three rice cultivars in planta, although they tested negative for all plant-growth-promoting traits. While *B. megaterium* was negative for ACC deaminase activity and nutrient solubilization, it exhibited auxin production. Nevertheless, *B. megaterium* did not suppress brown spot disease in any of the three rice cultivars. This study shows that bacteria do not necessarily have to possess classical plant-growth-promoting properties in order to be beneficial to plants, and it emphasizes the limitation of common culture-based methods in effectively identifying beneficial bacteria. Moreover, our results highlight the significance of the interaction between bacteria and plant cultivars in determining the beneficial effects of *Bacillus* spp. on plants under biotic or abiotic stresses.

3.1 Introduction

The use of beneficial microorganisms in sustainable agriculture has become increasingly popular. Endo- and ectophytic bacteria have been frequently shown to pro-mote plant growth and/or increase plant tolerance against abiotic or biotic stressors (Dimpka et al., 2009). Positive effects of bacterial inoculants on plant stress responses can be either direct—e.g., through bacterial phytohormone production or solubilization of nutrients—or indirect—e.g., by activation of plant transcription factors through bacterial metabolites (Dimpka et al., 2009; Glick, 2012). Over the last decades, a broad range of bacterial inoculants have been described for their beneficial effects in numerous plant species (O'Callaghan et al., 2022). The most common approach for screening beneficial bacteria relies on culture-based techniques. Bacteria isolated from different sources (e.g., soil or seed coats) are cultivated on various media to identify those with plant-growth-promoting traits. Bacteria showing these effects are generally known as plant-growth-promoting rhizobacteria (PGPR). Screening for bacteria strains that improve tolerance against abiotic stresses and resistance against biotic stressors often focuses on such PGPR. Plant-growth-promoting traits include the ability to solubilize tricalcium phosphates and zinc, 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase activity, or siderophore and auxin production (Glick, 2012; Glick, 2014). Through microbial solubilization of phosphates and zinc, these nutrients also become available to the plant, and hence, plant growth can be increased (Glick, 2012). Plant growth reduction as a response to abiotic stress is mediated through the plant hormone ethylene (Glick, 2012). However, bacterial ACC deaminase can counteract this effect by utilizing ACC, a precursor in the ethylene synthesis pathway, as substrate. This enzymatic activity decreases plant ethylene levels and subsequently alleviates growth inhibition (Glick 2014). Moreover, siderophores released by bacteria can enhance iron uptake by plants, and auxin produced by bacteria can alter the expression of genes involved in hormone production, plant defense pathways (Spaepen et al., 2013), and root growth (Vacheron et al., 2013). Bacteria isolates lacking these essential traits are typically excluded from further evaluation.

Various biotic and abiotic stresses constrain lowland rice production worldwide. In 1942/1943, an epidemic outbreak of brown spot disease in the area of Bengal in West India caused yield losses of up to 90%. This led to the devastating Bengal famine (Padmanabhan, 1973). Brown spot disease is caused by the fungus *Bipolaris oryzae* (telemorph: *Cochliobolus miyabeanus*). To this day, the fungal pathogen poses a threat to rice production. A recent study found symptoms of brown spot disease in almost 80% of the 179 rice fields investigated in western Burkina Faso (Barro et al., 2021). One of the major abiotic stresses of lowland rice is iron toxicity. Excess uptake of iron caused by high concentrations of reduced iron (Fe II) in the soil damages the plant tissue, which, in turn, can result in complete yield losses (Becker and Asch, 2005). Symptoms of brown spot disease and iron toxicity are similar to each other. Necrotic lesions develop on the leaf blades and leaf sheaths, reducing photosynthesis rates. In severe cases, entire leaves and, eventually, entire plants die. *Bacillus* spp. (consisting of two isolates of *Bacillus pumilus* and one isolate of *Bacillus megaterium*) used in this study were originally isolated from roots of lowland rice. These specific isolates were selected for their ability to reduce susceptibility to root knot nematode (Padgham and Sikora, 2007). In a previous study, we demonstrated that inoculation with these isolates also affects the tolerance of various lowland rice cultivars to iron toxicity (Weinand et al., 2023). The objectives of the present study are (1) to investigate whether the effects of bacterial inoculants on different rice cultivars under abiotic and biotic stresses are likely to be caused by traits commonly associated with plant-growth-promoting processes, such as phytohormone production and solubilization of nutrients, and (2) to investigate if traditional culture-based methods are a suitable screening tool for the identification of bacteria isolates with the potential to mediate stress tolerance in plants. To this end, the effects of three *Bacillus* isolates on the sensitivity of the different rice cultivars to brown spot disease were evaluated in planta by leaf symptom scoring, as well as in vitro by testing for direct inhibitory effects on fungal growth and HCN production. Furthermore, culture-based methods were applied to test for plant-growth-promoting properties such as the production of indole compounds (IC), ACC deaminase activity, siderophore production, and the ability to solubilize zinc and tricalcium phosphates.

3.2 Materials and Methods

3.2.1 Microorganisms

Three *Bacillus* isolates (*Bacillus pumilus* D7.4; *B. pumilus* Ni9MO12 (rif.res.), hereinafter *B. pumilus* Ni9MO12; and *B. megaterium*) isolated from roots of rice seedlings (Padgham and Sikora 2007, Le et al. 2015) were obtained as a frozen stock in 30% (v/v) glycerol from the Institute of Crop Sciences and Resource Conservation, Department of Plant Health, Nematology and Soil Ecosystems Laboratory, University of Bonn. *Pseudomonas protegens* type strain CHAO (DSM 19095) and *Pseudomonas brassicacearum* 3Re2-7 (DSM 32001) served as reference strains in *in vitro* assays. *P. protegens* CHAO was used as a positive control due to its known inhibitory effects on mycelial growth on *Fusarium sp.*, its siderophore production, and its nutrient solubilization ability. *P. brassicacearum* was used due to its ACC deaminase activity. The *Bipolaris oryzae* strain was kindly provided by BASF, Germany, and was multiplied on PDA. *Fusarium oxysporum* f.sp. *strigea* (FOS) strain FK3 was obtained as a frozen stock in 30% (v/v) glycerol from the Department of Agricultural Sciences, University of Hohenheim; *Fusarium oxysporum* f.sp. *cubense* Tropical Race 4 (FOC TR4) VCG 01213/16 was obtained as a frozen stock in 30% (v/v) glycerol from the Department of Plant Pathology, Stellenbosch University, South Africa. FOS, a mycoherbicide against witchweeds (*Striga hermonthica*); FOC TR4, the causative agent of Fusarium wilt in banana; and *Bipolaris oryzae* were cultured on potato dextrose agar (PDA, Roth, Karlsruhe, Germany).

3.2.2 Plant Growth Conditions

Three rice varieties, namely, IR31785-58-1-2-3-3 (hereinafter IR31785), Suakoko 8, and Sahel 108 were selected for this study because of their known interaction with the three *Bacillus* isolates under iron toxicity (Weinand et al., 2023). Rice seeds were germinated and grown in a high-humidity plastic box on filter paper soaked in dH_2O. When the 3rd leaf emerged, seedlings were transferred to a hydroponic system consisting of 7 L containers (Eurobox, Auer, Germany, 600 mm × 400 mm × 15 mm) fitted with a rack of 60 PVC tubes of 4 cm diameter and 12 cm length. Plants were grown for 21 d under greenhouse conditions with a 12 h light/dark period, mean temperatures of 25/30 °C (night/day), 50–60% relative air humidity, and a light intensity of 450–550 μmol m^{-2} s^{-1} before being subjected to further treatments. Yoshida nutrient solution (Yoshida et al. 1976) was renewed at weekly intervals with pH adjusted to 5.5. To adapt the plants to the nutrient solution, 25% strength was used during the first week, 50% strength during the second week, and full-strength nutrient solution until the end of the experiment.

3.2.3 *Bacillus* Inoculation

B. pumilus D7.4, *B. megaterium*, and *B. pumilus* Ni9MO12 were grown on tryptic soy agar (TSA) for 24 h at 28 °C in the dark. Single colonies were picked and propagated in liquid tryptic soy broth (TSB) at 28 °C on a shaker at 125 rpm. After overnight incubation, bacterial cells were harvested by centrifugation at 3000× g for 20 min, washed once with 1/4 strength Ringer's solution, and resuspended in 1/4 strength Ringer's solution to a final concentration of approx. 7×10^7 CFU mL^{-1}. Twenty-eight days after sowing, the bacteria solutions were added to the plant nutrient solution to a final concentration of approx. 10^6 CFU mL^{-1}. Control plants (non-inoculated) received the same volume of 1/4 strength Ringer's solution. The experiment was laid out in a randomized complete block design with 6 replicates.

3.2.4 *Bipolaris oryzae* Inoculation

At 35 days after sowing, plants were inoculated with *Bipolaris oryzae*. To prepare the inoculum, PDA colonized with *Bipolaris oryzae* were rinsed twice with 20 mL of sterile dH_2O, and the mycelium, including conidia, was scraped off completely. The resulting biomass suspension was then filtered through two layers of sterile gauze. Conidia concentration was microscopically determined using a Fuchs–Rosenthal counting chamber and adjusted at 5.1 × 10^4 conidia mL^{-1}. Rice plants were sprayed with 40 mL of the conidia suspension per box using a chromatography nebulizer. Control plants were sprayed with an equal volume of dH_2O. Subsequently, the inoculated plants were placed on wet fleece inside of a previously moistened humidity chamber.

3.2.5 Scoring of Disease Symptoms

Leaf symptoms were visually assessed 7 days after pathogen inoculation on fully expanded leaves for the entire plant based on the Standard Evaluation System for Rice (SES) (Table 3.1) (IRRI, 2013).

Table 3.1: Leaf symptom scoring according to the Standard Evaluation System for Rice (SES).

Scale (Affected Leaf Area)	
1	No incidence
2	Less than 1%
3	1–3%
4	4–5%
5	11–15%
6	16–25%
7	26–50%
8	51–75%
9	76–100%

3.2.6 Plant Harvest and Biomass Determination

For determining the dry weight, three plants from each treatment were randomly selected, separated into roots and shoots, and dried for 2 d at 70 °C in a drying chamber (ULM500, Memmert, Schwabach, Germany).

3.2.7 Detection of Siderophores

For testing the ability of *Bacillus* isolates to produce siderophores, CAS blue agar assay was used. CAS agar was prepared as described in detail in Glick (2014). Because *Bacillus* spp. are Gram-positive, they were first grown overnight on TSA plates supplemented with 0, 5, 10, and 50 ppm Fe as described above and subsequently topped with 12 mL of CAS blue agar as described by Pérez-Miranda et al. (2007). The cultures were then inoculated at 25 ± 1 °C for 21 d. Siderophore production became visible by formation of orange halos around bacterial colonies.

3.2.8 Measurement of Indole Compounds

For measurement of indole compounds produced, the method described by Sarwar et al. (1992) was used. Bacteria isolates were grown in a general-purpose medium (GPM) containing 1.5 g L^{-1} glucose, 0.5 g L^{-1} ammonium sulphate, 0.5 g L^{-1} potassium hydrogen phosphate, 0.5 g L^{-1} peptone from meat, and 0.1 g L^{-1} magnesium sulphate heptahydrate at a pH 7.3, with supplementation of 0.1% L-tryptophan and different concentrations of $FeSO_4$ (0, 2.5, and 5.0 ppm). Incubation was carried out at 28 °C on a shaker at 150 rpm for 4 d. Cultures were then centrifuged for 30 min at 3000× g. Supernatants were subsequently sterilized by passing through 0.2 µm syringe filters. Salkowsky reagent was prepared by mixing 750 µL of 0.5 M $FeCl_3$ with 25 mL dH_2O and carefully adding 15 mL of 90% H_2SO_4. Equal volumes of sample and Salkowsky reagent (10 mM ferric chloride in 35% chloric acid) were mixed and incubated for 30 min in the dark. A standard curve of IAA was generated using serial twofold dilutions (100, 50, 25, 12.5, 6.25, and 3.125 µM) of an IAA standard in GPM. GPM without IAA served as a blank. A separate standard curve was generated for each Fe supplementation, and the resulting equations (0 ppm Fe: $y = 0.0054x + 0.1338$; 2.5 ppm Fe: $y = 0.0058x + 0.135$; 5 ppm Fe: $y = 0.0056x + 0.1454$, respectively) were used for the calculation of indole compounds in the samples. Absorbance was measured at 530 nm with an Infinite 200 PRO plate reader (Infinite© 200pro, Tecan Trading AG, Männedorf, Switzerland). Aliquots of 100 µL per overnight culture were plated on TSA plates in triplicate and incubated for 12 h at 28 °C. Bacterial colonies were then counted to calculate IAA production per colony forming unit (CFU).

3.2.9 ACC Deaminase Activity

Bacterial isolates were tested for their ACC deaminase activity as described in Nascimento et al. (2019) with minor modifications. *Bacillus* isolates and *Pseudomonas protegens* were grown in Minimal DF (Dworkin and Foster) salts medium (Dworkin and Foster, 1958) containing 4.0 g L^{-1} KH_2PO_4, 6.0 g L^{-1} Na_2HPO_4, 0.2 g L^{-1}, $MgSO_4 \times 7H_2O$, 2.0 g L^{-1} glucose, 2.0 g L^{-1} gluconic acid, and 2.0 g L−1 citric acid with the following trace elements: 1 mg L−1 FeSO4 × $7H_2O$, 10 mg L^{-1} H_3BO_3, 11.19 mg L^{-1} $MnSO_4 \times H_2O$, 124.6 mg L^{-1}, $ZnSO_4 \times 7H_2O$, 78.22 mg L^{-1} CuSO4 × $5H_2O$, and 10 mg L^{-1} MoO_3; adjusted to pH 7.2. Each isolate was grown in Minimal DF salts medium supplemented with one of the following: (a) no source of nitrogen (negative control medium), (b) 2.0 g NH4Cl as nitrogen source (positive control medium), or (c) 3.0 mM ACC as sole source of nitrogen (ACC test medium). Isolates which showed no growth in the negative control medium but growth in both the positive control medium and the ACC test medium were considered positive for ACC deaminase activity.

3.2.10 HCN Production Assay

HCN production was determined using a modified version of the procedure originally described by Lorck (1948). An amount of 5 mL of liquid TSB medium supplemented with 4.4 g L^{-1} of glycine for the stimulation of HCN production was inoculated with 100 µL fresh overnight bacterial culture in TSB. A sterile strip of Whatman filter paper (approx. 1 cm × 5 cm) was soaked in 0.5% picric acid in 2% sodium carbonate and then placed inside each of the culture flasks (not reaching the liquid) and fixed with the flask's lid. The flasks were closed with parafilm and incubated for 10 days at 30 °C shaking at 150 rpm. HCN production was detected via a change in color of the filter paper strip from yellow to brownish.

3.2.11 Zinc Solubilization Assay

Bacterial isolates were tested for their ability to solubilize zinc. To that end, they were grown on Tris-mineral agar medium (Gandhi et al., 2016) containing 10.0 L^{-1} g D-glucose, 1 g L^{-1} $(NH_4)SO_4$, 0.2 g KCl, 0.1 g L^{-1} K_2HPO_4, 0.2 g L^{-1} $MgSO_4$, and 15 g L^{-1} agar. The medium was supplemented with either 1.244 g L^{-1} ZnO or 1.728 g L^{-1} $ZnCO_3$. An amount of 1 µL of overnight culture (in TSB) was spotted on the plates and incubated at 28 °C for 10 d in the dark. A clear halo zone was formed around colonies of zinc-solubilizing bacteria.

3.2.12 Phosphate Solubilization Assay

Pikovskaya's Agar (Pikovskaya and Pikovskaya, 1948) was used for testing the ability of bacteria to solubilize phosphate. The medium contained 10 g L^{-1} D-glucose, 5 g L^{-1} Ca3(PO4)2, 0.5 g L^{-1} $(NH_4)SO_4$, 0.5 g L^{-1} NaCl, 0.1 g $MgSO_4 \times 7H_2O$, 0.5 g L^{-1} yeast extract, 0.002 g L^{-1} $MnSO_4 \times H_2O$, 0.002 g L^{-1} $FeSO_4 \times 7H_2O$, and 15 g L^{-1} agar. An amount of 1 µL of overnight culture (in TSB) was spotted on the plates and inoculated at 28 °C for 10 d in the dark. A clear halo zone was formed around colonies of phosphate-solubilizing bacteria.

3.2.13 In Vitro Antifungal Activity

For antifungal activity assays, nutrient agar medium (1 g L^{-1} peptone, 1 g L^{-1} beef extract, 0.5 g L^{-1} NaCl, and 15 g L^{-1} agar) was used. A 5 mm diameter plug of actively growing fungal pathogen was placed in the center of each petri dish. An amount of 5 µL of fresh bacteria overnight cultures (in TSB) was spotted 3 cm away from the fungal disc on two opposite sides. Controls were inoculated with TSB without bacteria. Plates were incubated at 28 °C for 5–10 days in the dark. Growth of the fungus was observed daily. The diameter of the fungal growth between the bacteria colonies was measured, and the percentage of growth inhibition relative to control plates was calculated as follows:

$$\text{Inhibition rate (\%)} = 100\,\frac{(C - B)}{C}$$

where B and C are the fungal colony diameter in the presence and absence of the bacteria, respectively (Zygadlo et al., 1994).

3.2.14 Data Analysis

For analyzing the effect of bacterial inoculants on leaf spot development among cultivars, two-factorial ANOVA with a post hoc Dunnett's test was carried out. To test the effect of bacteria inoculation within each cultivar, single-factor ANOVAs with post hoc Dunnett's tests were performed. ANOVAs and post hocs were carried out in R studio version 4.0.3 "Bunny-Wunnies Freak Out" (R Foundation for Statistical Computing, Vienna, Austria). Normal distribution of data was tested by checking skewness and kurtosis in Microsoft Excel. SigmaPlot 14.0 (Systat Software, Inc. San Jose, CA, USA) was used for graphs.

3.3 Results

3.3.1 Effect of *Bacillus* spp. On Brown Spot Disease

Inoculation with *Bacillus* spp. Showed a highly significant effect on leaf symptom score among cultivars. The effects differed significantly between cultivars, and a significant interaction between bacterial isolates and rice genotype was found (Figure 3.1). When comparing the bacteria-inoculated plants of each cultivar to the noninoculated controls of the same cultivar, *B. pumilus* D7.4 and *B. pumilus* Ni9MO12 significantly suppressed brown leaf spot disease in IR31875 and Suakoko 8 but not in Sahel 108. However, *B. megaterium* inoculation did not reduce disease symptoms on *Bipolaris oryzae*-infected plants of all three cultivars tested.

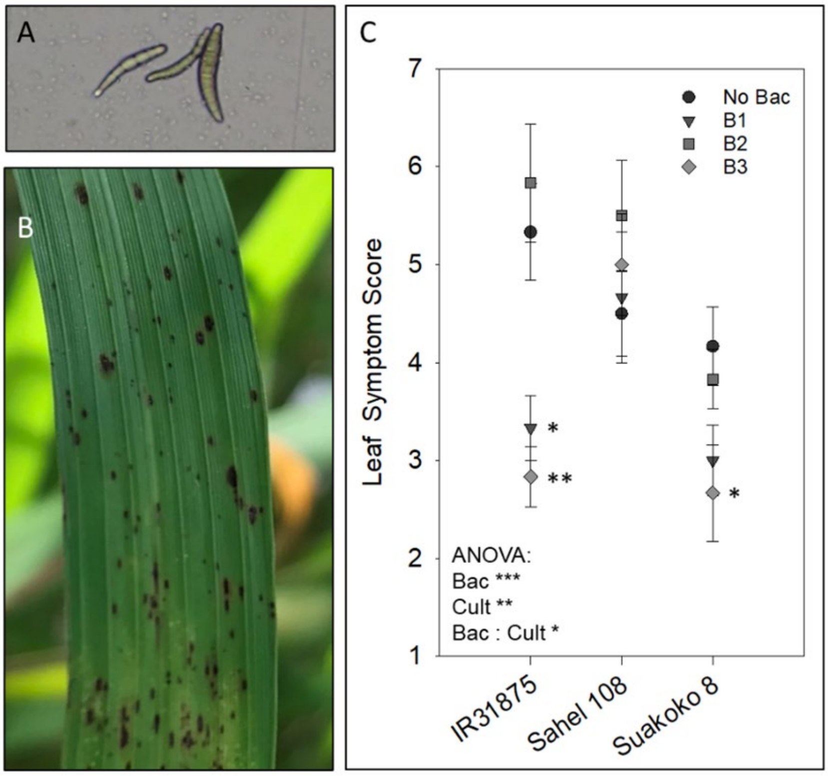

Figure 3.1: Effect of bacterial inoculation on brown spot disease. (A) *Bipolaris oryzae* conidia used for the infection of rice plants. (B) Rice leaf showing typical symptoms of brown spot disease. (C) The effect of bacterial inoculation on the brown leaf spot disease on three different rice cultivars. Asterisks near data points indicate significant differences compared to the noninoculated control (Dunnett's test, $\alpha = 0.05$). ANOVA = two-factorial ANOVA with bacteria (Bac) and cultivar (Cult) as factors. No Bac = no bacteria treatment, B1 = *B. pumilus* D7.4, B2 = *B. megaterium*, B3 = *B. pumilus* Ni9MO12 rif. res.

3.3.2 HCN Production

The results obtained from HCN assay revealed that filter paper strips obtained from *Pseudomonas* culture tubes resulted in changing the color from yellow to brownish, a clear indication for HCN production. In contrast, filter paper strips incubated in the culture flasks of the three *Bacillus* isolates did not show any change in color after 10 days of incubation, and hence, they did not produce HCN (Figure 3.6).

3.3.3 Antifungal Activity

Direct inhibitory effects of the three *Bacillus* isolates on fungal growth of different species were tested in vitro. In the presence of CHAO, mycelial growth of F. oxysporum FOS and FOC was inhibited by 56% and 100%, respectively (Figure 3.2 D, I). The same bacterial strain suppressed the mycelial growth of *Bipolaris oryzae* by 60% (Figure 3.2N). Coculturing of *B. pumilus* D7.4 on the agar plates inhibited the mycelial growth of FOS, FOC, and *Bipolaris oryzae* by 60%, 88%, and 72%, respectively (Figure 3.2 A, F, K). Similarly, the presence of *B. pumilus* Ni9MO12 resulted in 60%, 80%, and 72% mycelial growth inhibition of FOS, FOC, and

Bipolaris oryzae, respectively (Figure 3.2 C, H, M). However, cocultivation of agar plates with *B. megaterium* did not significantly affect the mycelial growth of both Fusarium oxysporum formae speciales tested (Figure 3.2 B, G). Nevertheless, mycelial growth of *Bipolaris oryzae* was retarded by 44% in the presence of *B. megaterium* (Figure 3.2L).

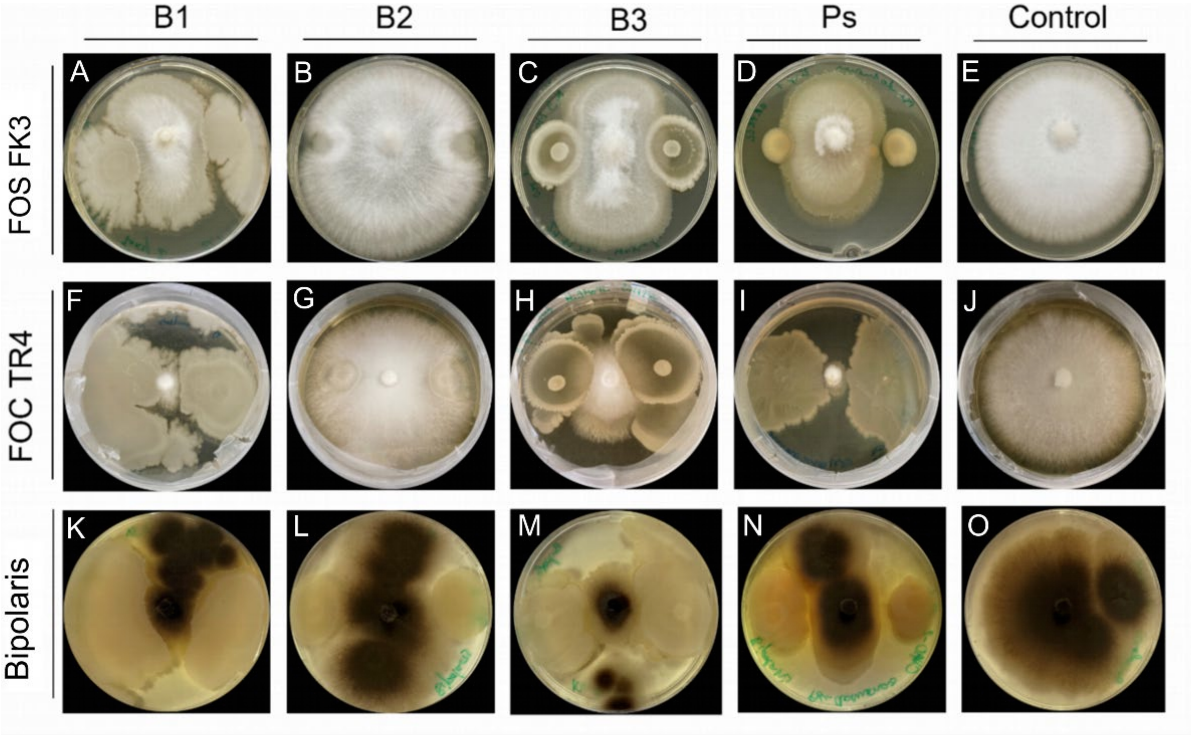

Figure 3.2: Antagonistic effects of *Bacillus* spp. on mycelial growth of *F. oxysporum* and *Bipolaris oryzae*. Bacterial cell suspension in sterile dH_2O was spotted 3 cm apart on either side of the fungal agar plug, which had been placed in the middle of the plate. A-E: FOS FK3 co-cultured with B1, B2, B3, Ps, control, respectively. F-J: FOC TR4 co-cultured with B1, B2, B3, Ps, control, respectively. K-O: Bipolaris co-cultured with B1, B2, B3, Ps, control, respectively. B1 = *B. pumilus* D7.4, B2 = *B. megaterium*, B3 = *B. pumilus* Ni9MO12, Ps = *Pseudomonas protegens* CHAO, Control = dH_2O, FOS FK3 = *Fusarium oxysporum* f.sp. strigea strain FK3, FOC TR4 = *Fusarium oxysporum* f.sp. cu-bense Tropical Race 4, Bipolaris = *Bipolaris oryzae*.

3.3.4 Effects of *Bacillus* spp. on Plant Growth

Plant growth was evaluated using biomass determination of both shoots and roots. In the cases of IR31875 and Sahel 108, shoot biomass of plants not infected with the pathogen did not differ between bacteria-inoculated and noninoculated plants (Figure 3.3). Root biomass in noninfected and *B. pumilus* D7.4-inoculated IR31875 was also not affected, whereas in *B. pumilus*, D7.4-inoculated, noninfected Sahel 108 root biomass was reduced by 15% compared to the noninoculated, noninfected controls (Figure 3.3). Pathogen-infected plants of IR31875 and Sahel 108 accumulated approx. 20% less root biomass when inoculated with *B. megaterium* and about 40% less when inoculated with *B. pumilus* Ni9MO12 compared to the noninoculated, pathogen-infected controls. Non-pathogen-infected Suakoko 8 inoculated with *B. pumilus* D7.4

accumulated approx. 40% more root and shoot biomass compared to the noninoculated control. *B. megaterium* inoculation of noninfected Suakoko 8 strongly doubled root biomass but decreased shoot biomass by about 10%. Effects of *B. pumilus* Ni9MO12 inoculation on biomass of roots and shoots of Suakoko 8 were similar to those recorded in IR31875 and Sahel 108. While shoot biomass was about 10% less than in the non-inoculated, noninfected control, root biomass was reduced by approximately 35%. With the exception of higher root biomass in *B. megaterium*-inoculated Suakoko 8, all differences between bacteria-inoculated, noninfected plants and non-bacteria-inoculated, noninfected plants were not statistically significant at $p < 0.05$. In *Bipolaris oryzae*-infected IR31875, *Bacillus* inoculation in general led to a larger biomass compared to the *Bipolaris oryzae*-infected, noninoculated plants. This effect was stronger in the roots than in the respective shoots. In *Bipolaris oryzae*-infected, *B. megaterium*-inoculated plants, root biomass more than doubled and shoot biomass was 60% larger compared to the noninoculated, *Bipolaris oryzae*-infected plants.

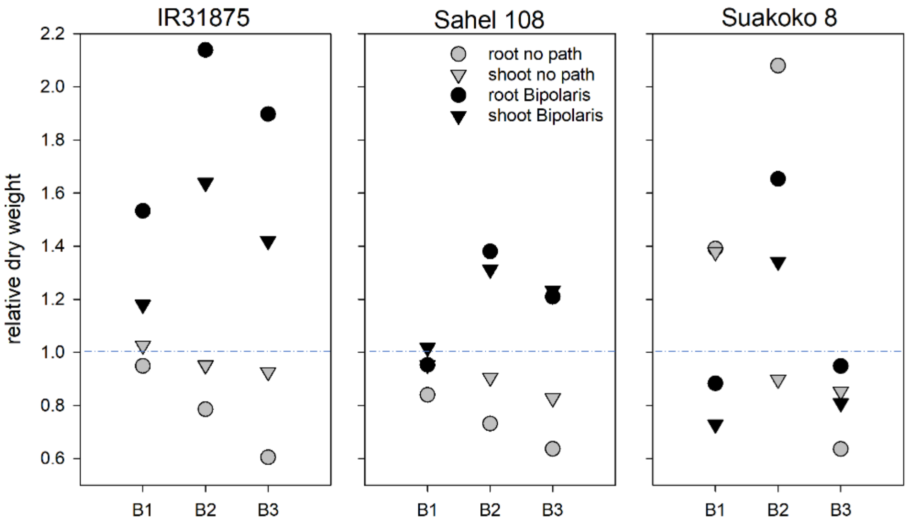

Figure 3.3: Effect of *Bacillus* inoculation on dry weight of roots and shoots of *Bipolaris oryzae*-infected and noninfected plants. B1 = *B. pumilus* D7.4, B2 = *B. megaterium*, B3 = *B. pumilus* Ni9MO12, no path = no pathogen.

3.3.5 Siderophore Production

Siderophore production was monitored over three weeks. Siderophore production by *P. protegens* CHAO was so strong that the blue CAS agar started to turn orange after only 2 h and had completely turned orange after one week (Figure 3.4). An orange halo started forming around the colonies of *B. pumilus* D7.4 after 24 h; for *B. pumilus* Ni9MO12, the halo became visible after one week at room temperature; and for *B. megaterium*, a small halo started forming after three weeks at room temperature. When grown on TSA supplemented with different concentrations of FeSO4 (5 ppm, 10 ppm, and 50 ppm Fe), for all bacteria tested, a small halo around the colonies only started to develop three weeks after plates were covered with CAS agar, with no differences in halo sizes between *Pseudomonas* and *Bacillus* isolates on any of the iron-supplemented plates (Figure 3.7).

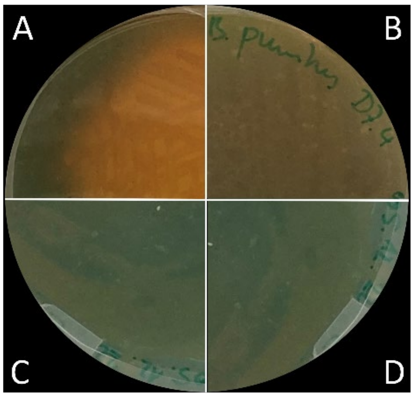

Figure 3.4: Siderophore production after 24 h. Bacteria were grown on TSA plates overnight then topped with CAS agar and incubated for 24 h. Bright orange color shows siderophore production by *Pseudomonas protegens* CHAO (A). Orange color development was also visible on *B. pumilus* D7.4 plates (B), while plates with *B. megaterium* (C) and *B. pumilus* Ni9MO12 (D) did not show any color change.

3.3.6 Auxin Production

B. megaterium showed the highest concentrations of indole compounds in the culture supernatants, with up to 33.8 µg mL^{-1} in the medium without iron supplementation, 11.4 µg mL^{-1} in medium supplemented with 2.5 ppm iron, and 15.8 µg mL^{-1} in medium supplemented with 5 ppm Fe (Table 3.2). *B. pumilus* D7.4 showed the lowest concentrations of IAA in the culture supernatants, less than 0.5 µg mL^{-1} in all three media. *B. pumilus* D7.4 showed similar concentrations of indole compounds in the supernatant as *B. megaterium*: 25.6 µg mL^{-1} in the non-iron-supplemented medium and around 11 µg mL^{-1} in the media supplemented with 2.5 ppm and 5 ppm Fe.

Table 3.2: Production of indole compounds in liquid cultures of *B. pumilus* D7.4, *B. megaterium*, and *B. pumilus* Ni9MO12 bacteria grown in a general-purpose medium (GPM) for 4 d at 28 °C and shaking at 125 rpm. The medium was supplemented with 0 ppm, 2.5 ppm, and 5.0 ppm Fe in the form of FeSO4. Values show the mean of three independent cultures with the standard error of the mean.

	Total Indole Compounds μg mL^{-1}		
	0 ppm Fe	**2.5 ppm Fe**	**5.0 ppm Fe**
B. pumilus D7.4	6.43 ± 0.28	3.83 ± 0.15	7.07 ± 0.57
B. megaterium	33.88 ± 1.01	11.4 ± 0.55	15.82 ± 1.54
B. pumilus Ni9MO12	28.69 ± 0.07	10.04 ± 0.74	8.49 ± 0.81

3.3.7 ACC Deaminase Activity

Ethylene plays an important role in the stress responses of plants. Through the activity of ACC deaminase, bacteria can alter ethylene levels and affect their tolerance against stress. Therefore, we tested the *Bacillus* isolates for their ACC deaminase activity. All three *Bacillus* isolates showed growth in the NH_4Cl-supplemented medium, while only *P. brasscicacearum* were able to grow in medium with ACC as the only source of nitrogen. None of the three *Bacillus* showed any ACC deaminase activity (Figure 3.8).

3.3.8 Zinc and Phosphate Solubilization

Zinc is an essential nutrient for plants, and rice grown in iron-toxic soils often shows symptoms of zinc deficiency. *P. protegens* CHAO developed clear halos around the colonies on both plates containing ZnO (Figure 3.5 D) and $ZnCO_3$ (Figure 3.5 H) as the only source of zinc. However, neither on $ZnCO_3$ nor on ZnO did *Bacillus* isolates show any Zn-solubilizing activity (Figure 3.5 A–C, E–G).

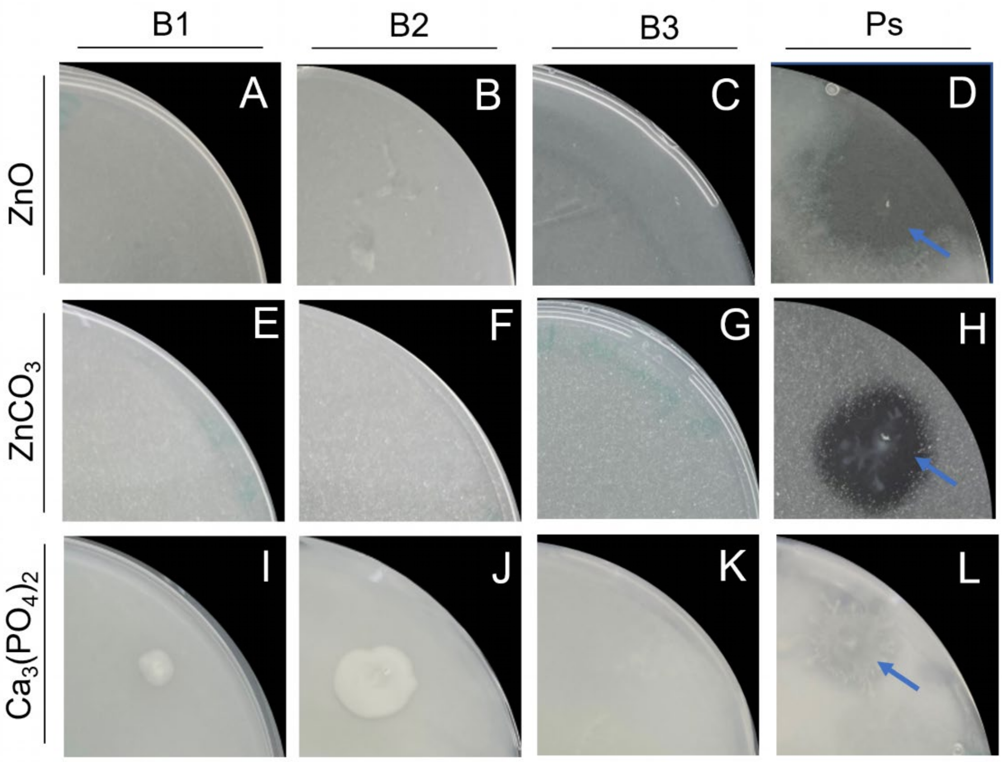

Figure 3.5: Nutrient solubilization by the different bacterial isolates. (A–D) Agar plates containing ZnO as sole Zn source; (E–H) Agar plates containing ZnCO3 as sole Zn source; (I–L) Pikowskaya agar plates containing Ca3(PO4)2 as sole P source. B1 = *B. pumilus* D7.4, B2 = *B. megaterium*, B3 = *B. pumilus* Ni9MO12, Ps = *Pseudomonas protegens* type strain CHAO. Blue arrows indicate halo zones around *P. protegens* colonies.

In order to test the ability of the bacteria to solubilize $Ca_3(PO_4)_2$, *Bacillus* isolates as well as the *Pseudomonas* control strain were grown on Pikowskayas agar plates. *B. pumilus* Ni9MO12 did not grow on Pikowskayas agar (Figure 3.5 K). *B. pumilus* D7.4 and *B. megaterium* grew but did not show any clear halo around the colonies (Figure 3.5 I, J). Only on plates with *P. protegens* did the medium become clearer around the colonies after 10 days of inoculation (Figure 3.5 L).

3.4 Discussion

Bacillus spp. are amongst the most common bacteria studied for plant growth promotion and biocontrol properties (Tsotetsi et al., 2022). Recently, we were able to show that *B. pumilus* D7.4, *B. megaterium*, and *B. pumilus* Ni9MO12 affect tolerance against iron toxicity in different rice cultivars (Weinand et al., 2023). In the present study, the effect of *Bacillus* spp. on the ability of different rice cultivars to withstand biotic stressors was tested in planta. Bacterial inoculation resulted in highly significant suppression of brown spot disease incited by *Bipolaris oryzae* (Figure 3.1). Similar to the previously described effects on the tolerance against iron toxicity (Weinand et al., 2023), the effects of inoculation in any of the three isolates on the expression of brown spot disease in rice cultivars differed between the isolates and between cultivars. Significant suppression of leaf spot was observed in IR31875 and Suakoko 8 inoculated with *B. pumilus* isolates but not in Sahel 108 inoculated with any of the isolates (Figure 3.1). Under iron toxicity, genotypic differences in tolerance mechanisms may account for the varied effects of *Bacillus* inoculation on plant fitness across the different treatment × cultivar combinations (Weinand et al., 2023). Resistance mechanisms against fungal diseases also differ between cultivars. These differences can arise due to genetic variations and the presence of specific defense mechanisms in the respective cultivar. Specific genes may encode proteins involved in recognizing and responding to fungal invasion, such as pathogen recognition receptors (PRRs) or proteins involved in defense signaling pathways. In addition, phytohormones, such as salicylic acid, jasmonic acid, and ethylene play crucial roles in regulating plant defense responses. The balance and interaction of these hormones can differ among cultivars, influencing their resistance against specific fungal pathogens. These genotypic differences could also account for the differences seen in the effect of bacteria inoculation on brown spot disease development. The ability to produce HCN is often included in studies screening for beneficial bacteria (Ahmad et al., 2008; Anwar et al., 2016). None of the isolates tested in our study showed any HCN production, and it can be concluded that the effect of bacteria inoculation on brown spot manifestation in rice is not due to the production of HCN. Nevertheless, the role of HCN in bacterially mitigated biocontrol has been questioned before, as concentrations of bacterial HCN in the rhizosphere have been shown to be too low to exhibit any detectable effect (Rijavec and Lapanje, 2016). Among the three *Bacillus* isolates tested, *B. megaterium* showed the least inhibitory effect on the mycelial growth of different fungi on agar plates (Figure 3.2). This was consistent with *in planta* results. Suppression of leaf spot disease was not significant in any of the three rice cultivars when inoculated with *B. megaterium*. Both *B. pumilus* isolates showed strong inhibitory effects on fungal growth in vitro. Inoculation with these two isolates also decreased the disease severity in IR31875 and Suakoko 8. However, there was no observable effect on disease severity in Sahel 108. This leads to the hypothesis that the beneficial effects of *B. pumilus* inoculation are not solely attributed to the direct inhibition of fungal growth, but rather, other mechanisms, probably the in-duction of resistance, may be involved. Another plausible explanation could be that Sahel 108 is unable to interact with any of the three *Bacillus* isolates tested. Results from our previous study (Weinand et al., 2023) strongly support the latter hypothesis: inoculation with *Bacillus* affected the tolerance of IR31875 and Suakoko 8 against iron toxicity, while no effects were observed in Sahel 108. To gain a deeper understanding of the underlying mechanisms governing the interaction between bacterial isolates and different rice genotypes, further research is warranted. The potential of *Bacillus* isolates to promote plant growth was also analyzed in planta. Under regular growth conditions—without being subjected to the biotic stress-or—plants did not exhibit increases in biomass either of the root or of the shoot, with the exception of Suakoko 8. In this particular cultivar, plants inoculated with *B. pumilus* D7.4 showed numerically increased growth, while those inoculated with *B. megaterium* displayed significantly larger root biomass compared to the noninoculated, non-infected control group (Figure 3.3). The effect of bacteria inoculation on the growth of *Bipolaris oryzae*-infected plants differed between bacteria isolates and rice cultivars. In the case of IR31875, bacteria inoculation positively affected the growth of roots and shoots. However, these effects were not significant. The effects of bacteria inoculation on *Bipolaris oryzae*-infected Sahel 108 were only small and not significant. *B. pumilus* D7.4 and *B. pumilus* Ni9MO12 inoculation of Suakoko 8 infected with *Bipolaris oryzae* resulted in numerically lower biomasses compared to the noninoculated controls. This was despite the beneficial effects on brown spot disease of the two isolates in this par-ticular rice cultivar. Only inoculation with *B. megaterium* led to significantly larger root and shoot biomass of *Bipolaris oryzae*-infected compared to non-bacteria-inoculated *Bipolaris oryzae*-infected Suakoko 8. However, this *Bacillus* isolate did not demonstrate any significant effects on leaf spot disease development. Effects of *Bacillus* inoculation on the disease reduction on the three lowland rice cultivars does not seem to be related to biomass accumulation. This is consistent with observations we previously described for plants grown under iron toxicity. The effects of *Bacillus* inoculation on biomass ac-cumulation of the three rice cultivars grown under iron-toxic conditions could not be correlated to the effects of *Bacillus* inoculation on leaf symptoms scores (Weinand et al., 2023). Moreover, the effects of bacterial inoculation on the growth of the three cultivars appeared to be influenced by varying environmental conditions and may differ between experiments (Weinand et al., 2023). The ability of bacteria to solubilize nutrients is considered a plant-growth-promoting trait that is routinely tested in studies aiming to identify bacteria which can neutralize certain environmental stresses (Dimkpa et al., 2009). Iron toxicity is a multinutrient disorder that often leads to deficiencies in phosphate and zinc (Becker and Asch, 2005). We tested *Bacillus* isolates for their ability to solubilize zinc and phosphate. In the hydroponic system used for testing the effect of bacteria inoculation on the tolerance of different rice cultivars against iron toxicity and brown spot disease, plants were supplied with sufficient amounts of nutrients. However, the pH of the nutrient solution decreased due to root activity. Phosphate availability decreases at low pH and is related to the reduction of iron in the soil (Kirk et al., 2021). P deficiency in rice leads to an increased exudation of organic metabolites which, in turn, increases the availability of P through microbial reduction of iron phosphates (Becker and Asch, 2005). Under iron toxicity, plaques of iron form on the root surface of lowland rice. This can impair the uptake of zinc, leading to Zn deficiency (Becker and Asch, 2005). None of the three *Bacillus* isolates tested in this study showed the ability to solubilize phosphate or zinc in vitro. Whether nutrient uptake of rice plants could be improved by inoculation with any of the three *Bacillus* isolates would require further in planta investigations. Siderophores serve as high-affinity iron chelators, binding to iron ions and forming stable complexes that can be taken up by the producing organism. Under iron-limited conditions, bacterial siderophores have been shown to improve iron acquisition in rice and other plant species (Dimkpa et al., 2009). The role of bacterial siderophores and their potential significance under iron toxic soil conditions remains unknown. However, it has been demonstrated that bacterial siderophores play a crucial role in disease suppression. *Pseudomonas* fluorescens mutants that lost their ability to produce siderophores also lost their potential to suppress leaf blast in rice (De Vleesschauwer et al., 2008). It is believed that siderophores trigger unknown signals in the roots to be transmitted to the shoots leading to ET/JA-dependent priming of plant defenses (Aznar et al., 2015). The abilities of the three *Bacillus* isolates to produce siderophores were assessed under different iron concentrations. Out of the *Bacillus* isolates tested, only *B. pumilus* D7.4 clearly showed siderophore production under normal growth conditions (Figure 3.4). Siderophore production of *B. pumilus* Ni9MO12 only became visible after 3 weeks, possibly when iron in the growth medium became so limited that siderophore production was induced or because Fe^{3+} was oxidized due to other bacterial activity. Unsurprisingly, with increasing iron concentrations in the growth medium, siderophore production of all strains tested was suppressed (Figure 3.7). As *B. pumilus* D7.4 produced siderophores under normal growth conditions, it is possible that these siderophores play a role in the suppression of brown spot disease in rice either by triggering signals that lead to a priming of plant defenses or by depriving pathogens from iron, leading to a phenomenon described as "nutritional immunity" (Backer et al., 2018). To our knowledge, the involvement of siderophores in suppression of brown spot disease has not been demonstrated before. Therefore, further research is needed to test this hypothesis thoroughly and establish any potential relationship between siderophores and the suppression of brown spot disease. Based on our results, we can exclude the possibility that any of the *Bacillus* isolates used in our study affect iron acquisition of rice through siderophore production under iron-toxic conditions. Our results, however, do not rule out that other alterations in iron homeostasis could be behind the effects of *Bacillus* on the plant's response to iron toxicity and infection with *Bipolaris oryzae*. Local accumulation of Fe within the plant can be toxic to the invading pathogen (Herlihy et al., 2020). This strategy requires targeted transport of iron. Previous studies have shown iron transporters of the NRAMP and the YSL families to be involved in the immune response of rice infected with *Magnaporthe oryzae* (Sánchez-Sanuy et al., 2022). These transporters are needed for the transport of iron within the plant and were hypothesized to be affected by *B. pumilus* inoculation in Suakoko 8 (Weinand et al., 2023). Preliminary results showed that iron might also be involved in the suppression of brown leaf spot disease by *Bacillus* isolates. Perls-DAB staining of leaf blades revealed spots of iron ac-cumulation in plants inoculated with *B. pumilus*, while in the noninoculated plants, no such iron accumulation was observed (Weinand et al., 2021). Further investigation is needed to decipher the role of iron translocation and sequestration in the plant's defense against both *Bipolaris oryzae* infection and iron toxicity and the possible effects of *Bacillus* inoculation on these mechanisms. Cultures of *B. megaterium* contained the highest concentrations of indole com-pounds of all three *Bacillus* isolates tested. The highest concentration was released in *B. megaterium* cultures without iron supplementation. The same was true in case of *B. pumilus* Ni9MO12. Similarly, the concentration of indole compounds in the supernatants of cultures without iron supplementation was the highest. *B. pumilus* D7.4 only produced small amounts of auxin, which were at the limit of detection, despite generally showing the strongest growth in the medium used. In contrast, *B. megaterium* and *B. pumilus* Ni9MO12 showed the least growth without Fe supplementation but produced the highest amount of indole compounds. In media supplemented with iron, their aux-in production decreased, whereas growth was stronger. These results suggest that bacterial isolates which grow well in the medium used for screening for auxin production are likely to produce smaller amounts of indole compounds despite their general ability to do so. *B. megaterium* showed the highest level of auxin production among the isolates tested. Additionally, this particular isolate exhibited a growth-promoting effect on both the roots and the shoots of Suakoko 8 (Figure 3.2) when compared to the non-inoculated, iron-stressed plants. Both findings are consistent with previous research demonstrating that bacterial auxin production has been shown to promote the growth, particularly of roots, of various plant species (Backer et al., 2018). However, among the three isolates tested here, *B. megaterium* is the one that showed the least effect on the tolerance of lowland rice cultivars against iron toxicity (Weinand et al., 2023) and the least effect on resistance of lowland rice against brown spot disease. Auxin does not seem to be involved in the signaling cascades triggered by *Bacillus* inoculation. Although the smallest of the plant hormones, ethylene has been shown to play a pivotal role as a signaling molecule in the plant's response to both abiotic and biotic stresses (Dubois et al., 2018). Upon exposure to abiotic stress conditions or pathogen attack, plants increase the production of ethylene, which leads to a promotion of growth and elongation of the cells of young leaves. 1-aminocyclopropane-1-carboxylate (ACC) is the immediate precursor of ethylene. ACC can be hydrolyzed and degraded into α-ketobutyrate and ammonia by the enzyme ACC deaminase. Many PGPR have been shown to possess ACC deaminase activity. In this way, they can decrease the ethylene level in plants, which, in turn, mitigates growth inhibition under stress (Glick, 2014). This phenomenon is widely referred to as "plant growth promotion". For accumulative stresses, such as iron toxicity or salinity, promotion of plant growth could be useful due to dilution effects; for deficit stresses, such as drought or fungal pathogens, promotion of plant growth could have negative effects, for it would use up energy needed for stress responses. We therefore suggest that it is important to strictly discriminate between actual growth promotion and mitigation of growth inhibition. Ethylene also plays a role in the tolerance of lowland rice against iron toxicity. It induces the production of aerenchyma, a gas-conducting tissue, through which molecular oxygen is channeled from the atmosphere through the stems into the roots and in-to the rhizosphere, where the oxygen leads to the oxidation of Fe^{2+} (Becker and Asch, 2009). However, none of the three *Bacillus* isolates tested in this study showed any ACC deaminase activity. The effects they have on the suppression of brown leaf spot disease and tolerance against iron toxicity may not be caused by directly decreasing the ethylene level. Earlier reports have also indicated that ethylene does not appear to have any influence on the resistance or susceptibility of rice against brown spot disease. However, the defense mechanisms against this disease involve signaling through abscisic acid (ABA) (De Vleesschauwer et al., 2010, Sharma et al., 2013). ABA is also a key player in the response to abiotic stresses. It has been shown to be involved in the plant responses to drought, cold, salinity, and heavy metals (Hu et al., 2020). Inoculation of rice with an ABA-producing, seed-borne endophytic *Bacillus amyloliquefaciens* isolate RWL-1 led to increased salt tolerance of the plants (Shahzad et al., 2017). Further investigation into possible ABA production of the three *Bacillus* isolates described here and/or into indirect effects of inoculation on ABA signaling in the different rice cultivars under iron toxicity and after infection with *Bipolaris oryzae* is still needed.

3.5 Conclusions

When searching for beneficial bacteria from any given microbiome, methods are needed for narrowing down the number of isolates. However, our results show that beneficial bacteria cannot always be detected by traditional culture-based methods. Some beneficial isolates might not possess any of the traits selected for, while others might only display them under certain environmental conditions. The selection of beneficial microorganisms for use in sustainable agricultural practices should, therefore, be based on the comparison of natural microbiomes from tolerant and sensitive cultivars rather than on "plant growth promoting" traits alone. Furthermore, the effect of bacterial inoculation strongly depends on the rice genotypes. Efforts to develop artificial microbiomes should go hand in hand with breeding strategies that consider the genotypic ability to acquire—and sustain—beneficial microbiomes.

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Conflicts of Interest: The authors declare no conflicts of interest.

Supplementary Materials: The following supporting information can be downloaded at: www.mdpi.com/xxx/s1 [see Figures 3.6 to 3.8 below].

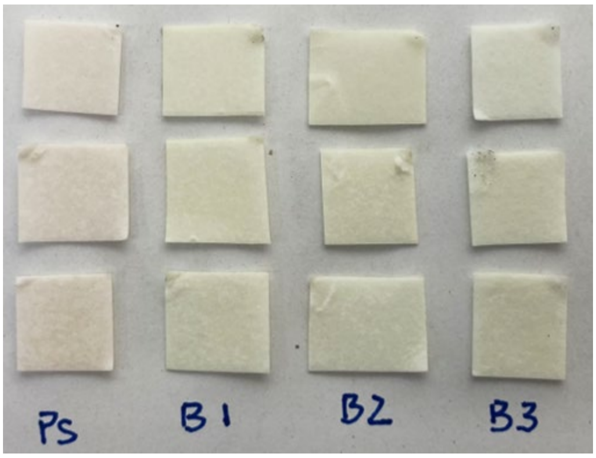

Figure 3.6: HCN production. Color of Whatman filter paper changed from yellow to brownish in culture flasks with Pseudomonas protegens growing in TSB supplemented with glycine. Filter papers stayed yellow in flasks with *Bacillus* cultures in TSB supplemented with glycine and in medium without bacteria inoculation. Ps = Pseudomonas protegens CHAO; B1 = *B. pumilus* D7.4; B2 = *B. megaterium*; B3 = *B. pumilus* Ni9MO12.

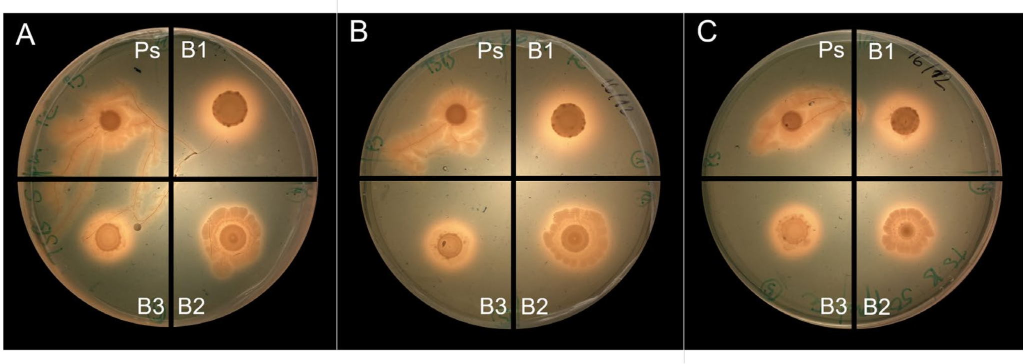

Figure 3.7: Siderophore production after 21 days on medium supplemented with FeSO4. Orange halos developed around all colonies independent of the concentration of supplemented iron and the bacteria isolate. A = TSA supplemented with 5 ppm Fe, B = TSA supplemented with 10 ppm Fe, C = TSA supplemented with 50 ppm Fe. Ps = Pseudomonas protegens CHAO; B1 = *B. pumilus* D7.4; B2 = *B. megaterium*; B3 = *B. pumilus* Ni9MO12.

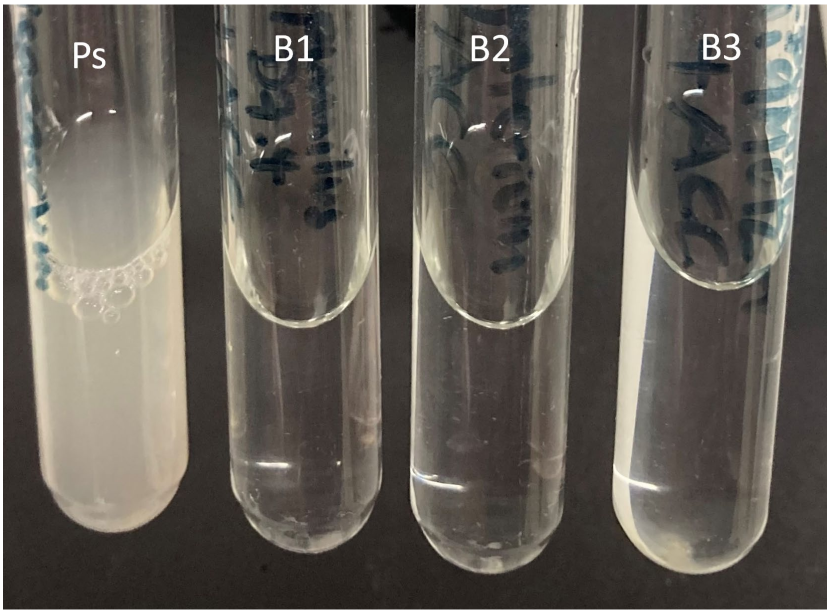

Figure 3.8:. ACC deaminase activity. Bacteria growth in minimal DF salts medium with ACC as sole source for nitrogen. Only Pseudomonas protegens CHAO was able to grow in this medium. *Bacillus* isolates showed no growth and are therefore considered negative for ACC deaminase activity. Ps = Pseudomonas protegens CHAO; B1 = *B. pumilus* D7.4; B2 = *B. megaterium*; B3 = *B. pumilus* Ni9MO12.

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4 Effects of *Bacillus* spp. Inoculation on Suggested Shoot Tolerance Mechanisms in Lowland Rice (*Oryza sativa* L.) Grown Under Iron Toxicity

This chapter is submitted to the Journal of Plant Nutrition and Soil Sciences as:

Weinand, T.; Asch, J..; Asch, F. Effects of *Bacillus* spp. Inoculation on Shoot Tolerance Mechanisms in Lowland Rice (*Oryza sativa* L.) Grown Under Iron Toxicity

Keywords: *Oryza sativa* L., iron toxicity, *Bacillus spp*., ROS scavenger enzymes; ferritin; *OsFRO1*; *OsNRAMP6*

Background: In areas of lowland rice production, high iron concentrations in the soil often lead to yield reductions. Local adapted varieties possess different adaptation mechanisms, which, however, are not fully understood. Previous studies have shown that endophytic bacteria can influence plant tolerance to abiotic stresses, including iron toxicity.

Aim: This study aims at analyzing the effects of different *Bacillus* isolates on distinct shoot tolerance mechanism in different rice cultivars grown under iron toxicity.

Methods: Three lowland rice cultivars, varying in their tolerance against iron toxicity (IR31785-58-1-2-3-3, Sahel 108, Suakoko 8), were inoculated with three *Bacillus* strains (two of *B. pumilus* and one of *B. megaterium*). One week after *Bacillus* inoculation plants were subjected to high iron levels (1,000 ppm) for 7 days. Leaf symptom scoring was used to assess tolerance levels. Activities of ascorbate peroxidase (APX), glutathione reductase (GR), catalase (CAT), superoxide dismutase (SOD), and guaiacol peroxidase (PRX) were measured by spectrophotometric assays. Expression of genes related to iron toxicity (*OsFER* , *OsFRO*1, OsNRAMP6) was determined by RT-qPCR. Bacterial production of NO was evaluated by measuring nitrite levels in the culture supernatants.

Results: In general, iron toxicity affected the activities of APX, GR, CAT, and PRX but not SOD activity. Only PRX activity in response to iron differed between cultivars with a significantly stronger increase in IR31785-58-1-2-3-3. Inoculation with *B. pumilus* Ni9MO12 led to higher activity of CAT in the leaf sheaths of all cultivars and an increase in GR activity in the sheaths which was significantly higher in Suakoko 8. In the young leaf blades of IR31785-58-1-2-3-3 expression of *OsFRO*1 and OsNRAMP6 was not significantly affected by *Bacillus* inoculation, whereas accumulation of *OsFER* mRNA was significantly higher in iron-stressed, B. pumilius Ni9MO12 inoculated plants compared to non-inoculated, non-iron-stressed plants.

Nitrite concentration as an indicator for NO production was increased in *B. pumilus* Ni9MO12 culture supernatants.

Conclusion: Our results show that in the sensitive cultivar IR31875-58-1-2-3-3 tolerance to iron toxicity increases when inoculated with *B. pumilus* Ni9MO12 due to higher levels of ferritin expression. NO production by the *Bacillus* isolate might confer the promotion of *OsFER* gene expression in the inoculated plants.areas of lowland rice production, high iron concentrations in the soil often lead to yield reductions. Local adapted varieties possess different adaptation mechanisms, which, however, are not fully understood. Previous studies have shown that endophytic bacteria can influence plant tolerance to abiotic stresses, including iron toxicity.

4.1 Introduction

Endophytic bacteria are known to be able to affect plant tolerance against abiotic stresses, such as drought, salinity, or iron toxicity (Dimpka et al., 2009; Weinand et al., 2023a). Often, stress mitigation by bacteria is attributed to bacterial traits generally referred to as plant growth promoting properties. These include the production of auxin or nutrient solubilization ability (Dimpka et al., 2009). However, not all bacteria found to affect plant stress responses, do possess such properties (Weinand at al., 2023b). The mode of action underlying the effects of such bacteria are mostly unknown. Iron toxicity is one of the major constraints to lowland rice production worldwide and occurs in highly weathered tropical soils (Becker and Asch, 2005). Rice, being a gramineous plant, mainly takes up iron as chelated $Fe^{3+.}$ However, rice also possesses transporters for the uptake of Fe^{2+} (Ishimaru et al., 2006), which can lead to an excess uptake of iron when Fe^{2+} concentrations in the soil solution are high. Once transported into the shoot, Fe^{2+} reacts with H_2O_2, creating highly reactive hydroxyl radicals. These damage cell membranes, which becomes visible as brown lesions on the leaves. In severe cases, entire leaves and eventually entire plants die. Yield losses between 15-78% , sometimes even complete yield failure, can be the consequence (Becker and Asch, 2005). Cultivars tolerant to iron toxicity have been shown to employ different mechanisms of adaptation. Although not fully understood, they are generally categorized into 3 different types. Types 1 and 2 are so-called ‘includer types’ which have evolved mechanisms to tolerate high iron concentrations within the plant tissue. Type 1 includers tolerate excess iron through internal ‘avoidance’, where iron is being partitioned into older leaves and there preferentially into leaf sheaths. This way, young leaves, which have the highest photosynthesis rates, are being protected. Internal avoidance can also take place by sequestering ferric iron into the vacuoles, or by chelation of iron by the storage protein ferritin (Aung and Masuda., 2020).

Among the genes which have been shown to play a role in the sequestration of iron in the cell are *OsFRO1* and *OsNRAMP6*. *OsFRO1* encodes for a ferric reductase oxidase, which reduces ferric iron into ferrous iron. It has been shown to be located in the vacuolar membrane and its expression is downregulated in old leaves under high iron concentrations (Li et al., 2019b) It is believed that it functions by making iron stored in the vacuole available for transport into the cytosol. RNAi lines with downregulated expression of *OsFRO1* showed to be more tolerant to iron toxicity (Li et al., 2019b). *OsNRAMP6* (natural resistance-associated macrophage protein 6) is one of eight members of a metal transporter protein family in rice. Members of this protein family have been shown to play an important role in regulating Fe transport at sub-cellular level as well as the transport to vacuoles and plastids. Out of the eight family members, only expression of *OsNRAMP6* showed to be upregulated under high iron concentrations (Finatto et al., 2015).

In the rice genome two ferritin genes have been identified: *OsFER1* and *OsFER2* (Gross et al., 2003). Ferritin expression has been shown to be induced by high iron concentrations in both monocot and dicot plants (Aung and Masuda, 2020). In rice, accumulation of ferritin under iron toxicity was first documented in the African rice species *Oryza glaberrima* Steud. (Majerus et al., 2007). Silveira et al. (2009) reported a differential ferritin protein accumulation in two *Oryza sativa* cultivars, which differed in their tolerance against iron toxicity.

Type 2 includer have developed an increased ‘tissue tolerance’. Tissue tolerance is suggested to be achieved by stronger activity of reactive oxygen species (ROS) scavenger enzymes (Engel et al., 2012). A major pathway within the plant’s ROS scavenging system is the ascorbate-glutathione redox cycle, also referred to as Foyer-Halliwell-Asada cycle (Foyer and Kunert, 2024).

Ascorbate peroxidase (APX) functions in reducing H_2O_2 to H_2O by oxidizing ascorbate to monodehydroascorbate (MDHA), which disproportionates to ascorbate and DHA. DHA is the substrate of dehydroascorbate reductase (DHAR) which concomitantly oxidizes glutathione (GSH) to GSSG. Using NADPH as a co-factor, GSSG, in turn, is reduced to GSH through the enzymatic activity of glutathione reductase (GR) (Noctor et al., 2012).

Type 3 cultivars employ a mechanism of ‘exclusion’. Atmospheric oxygen is transported from the shoot to the root through the rice aerenchyma. This oxygen is released into the rhizosphere where it oxidizes Fe^{2+} to Fe^{3+}. These Fe^{3+} ions are not taken up by the plant but instead deposited on the root surface in form of iron plaques (Becker and Asch, 2005).

In a previous study we analyzed if effects of inoculation with endophytic *Bacillus* isolates on leaf symptom scores of three different lowland rice cultivars grown under iron toxic conditions were caused by altered iron partitioning within the shoots (Weinand et al., 2023a). Although we found some differences between non-inoculated and inoculated plants, the effects differed between cultivars, *Bacillus* isolates, and their interaction. In the sensitive cultivar IR31785-58-1-2-3-3, bacteria inoculation mitigated leaf symptoms despite of no significant differences in shoot iron concentration between inoculated and non-inoculated plants. *Bacillus* inoculation, therefore, seemed to increase "tissue tolerance/avoidance" in IR31785-58-1-2-3-3, either through increased ROS scavenging activity or through enhanced chelation or intercellular sequestration of iron. The aim of the study presented here was to elucidate putative mechanisms underlying the previously reported increased tolerance of *Bacillus*-inoculated IR31785-58-1-2-3-3. We established three different hypotheses: I) *Bacillus* inoculation leads to increased scavenging of ROS, II) *Bacillus* inoculation affects iron sequestration within the cell, and III) *Bacillus* inoculation positively affects the production of ferritin. To test hypothesis I), the activity of four ROS scavenger enzymes – namely superoxide dismutase (SOD), catalase (CAT), APX, GR as well as guaiacol peroxidase (PRX) – were compared in leaf sheaths and blades of three cultivars (IR31785-58-1-2-3-3, Sahel 108, Suakoko 8) with and without iron stress, with and without *Bacillus* inoculation. Accumulation of H_2O_2 in the leaf blades of IR31785-58-1-2-3-3 subjected to 1000 ppm iron, with and without *Bacillus* inoculation was visualized by *in situ* staining with 3,3'-Diaminobenzidine tetrahydrochloride (DAB). In order to test hypothesis II), the expression of genes involved in intercellular iron sequestration – namely *OsFRO1*, and *OsNRAMP6* – was analyzed in the young leaf blades of IR31785-58-1-2-3-3 subjected to 1000 ppm iron with and without *B. pumilus* Ni9MO12 inoculation. Hypothesis III) was tested by analyzing the expression of *OsFER* genes in the young leaf blades of IR31785-58-1-2-3-3 subjected to 1000 ppm iron with and without *B. pumilus* Ni9MO12 inoculation. Furthermore, the three *Bacillus* isolates were tested for their ability to produce nitric oxide, an important signaling molecule in plant stress responses, which has been shown to regulate ferritin production in *Arabidopsis thaliana*.

4.2 Material and Methods

4.2.1 Plant Growth

Three rice varieties, namely, IR31785-58-1-2-3-3 (hereinafter IR31785) obtained from the International Rice Research Institute (IRRI), Philippines, Suakoko 8 obtained from Africa Rice, Benin and Sahel 108 obtained from IRRI, Philippines, were selected for this study because of their known interaction with the three *Bacillus* isolates under iron toxicity (Weinand et al., 2023a). Rice seeds were germinated and grown in a high-humidity plastic box on filter paper soaked in dH2O. When the 3rd leaf emerged, seedlings were transferred to a hydroponic system consisting of 7 L containers (Eurobox, Auer, Germany, 270 mm x 170 mm x 115 mm) fitted with a rack of 24 PVC tubes of 4 cm diameter and 12 cm length. Plants were grown for 21 d in growth chamber with a 12 h light/dark period, temperatures set to 25/20 °C (day/night), 40-50% relative air humidity, and a light intensity of 450–550 µmol m^{-2} s^{-1}

before being subjected to further treatments. Yoshida nutrient solution (Yoshida et al., 1971) was renewed weekly with pH adjusted to 5.5. To adapt the plants to the nutrient solution, 25% strength was used during the first week, 50% strength during the second week, and full-strength nutrient solution until the end of the experiment.

4.2.2 *Bacillus* Inoculation

Three different *Bacillus* isolates (*Bacillus pumilus* D7.4, *Bacillus pumilus* Ni9MO12 (rif.res.) and *Bacillus megaterium*) isolated from rice seedling roots (Padgham and Sikora, 2007) were used. Bacteria were grown on tryptic soy agar (TSA) for 24 h at 28°C. Single colonies were picked and propagated in liquid tryptic soy broth (TSB) at 28°C shaking at 125 rpm. After overnight incubation, bacteria were harvested by centrifugation at 3,000 g for 20 min, washed once, and resuspended in ¼ strength Ringer Solution to a final concentration of approx. 7 x 10^7 CFU ml^{-1}. 28 days after sowing the bacteria solution was added to plant nutrient solution to a final concentration of approx. 10^6 CFU ml^{-1}.

4.2.3 Iron Treatment

At 35 days after sowing, Fe toxicity was induced by adding iron II sulfate heptahydrate ($FeSO_4*7H_2O$) to a final concentration of 1000 ppm Fe to the nutrient solution. pH was adjusted to 5.5 only at the start of the treatment but not during its course. To prevent oxidation of ferrous to ferric iron, N_2 was percolated through the nutrient solution for 15 min every other hour using a standard aquarium percolator (Hobby LongLong, Aquaristikshop, Germany). Reductive conditions due to hypoxia were monitored using a redox electrode (Hanna Instruments, Germany).

4.2.4 Leaf Symptom Scoring

Leaf bronzing was assessed visually on fully expanded leaves for the entire plant based on the Standard Evaluation System for scoring for leaf blast lesions provided by the International Network for the Genetic Evaluation of Rice (IRRI - INGER, 1996), adapted to iron toxicity symptoms as described earlier (Asch et al., 2005). This was done on day eight of stress exposure for the enzyme analysis experiment and on day seven of stress exposure for the gene expression experiment.

4.2.5 Enzyme Activity Assays

At day eight of iron exposure the second youngest fully developed leaves of the main tiller were harvested and separated into leaf blades and leaf sheaths. They were then cut into small pieces and around 0.02g of fresh material was filled into reaction tubes on ice. APX analysis was performed immediately, plant material for other enzyme measurements were stored at -20°C until analysis (which was performed within a few weeks to avoid any storage effects on enzyme activity (Hartmann and Asch 2019)). A detailed protocol is given in Mondal et al. (2023). Enzyme activity was determined spectrophotometrically using an Infinite© 200pro (Tecan Trading AG, Switzerland). Absorbance was measured for APX, CAT, GR and PRX for 180 s at 290, 240, 340 and 470 nm, respectively. Enzyme activities were calculated based on the slope. For determination of SOD activity exposure to a strong light source (LED growth lamp, spLED) is required prior to measurement. Initial absorbance was measured at 560 nm after 60s of light exposure, second measurement was performed after another 5 min of exposure to light. Calculation of activity was based on the amount of inhibition as compared to a standard curve (dilution series of SOD standard S9697, Sigma Aldrich; Hartmann & Asch, 2019).

4.2.6 *In situ* H_2O_2 Detection

In situ detection of H_2O_2 was carried out as described by Wu et al (2017). The second youngest fully developed leaves were harvested, washed with 0.05% Triton-X-100, rinsed 3 times in $_dH_2O$. The leaves were then submerged in 3,3'-Diaminobenzidine tetrahydrochloride (DAB) solution (0.5 mg mL^{-1} DAB in Millipore water, pH 3.8) and incubated for 12 hours in the dark. Afterwards, DAB solution was removed and the leaves washed 3 times with dH2O. Water was removed and leaves fully submerged in bleaching solution (glycerol : lactic acid : ethanol, 1 : 1 : 4). When bleaching was sufficient, leaves were rinsed in dH2O and photos taken with a camera (Canon EOS 600D).

4.2.7 Gene Expression Analysis

Total RNA was extracted from leaf blades using TRIzol® reagent (Invitrogen™, Life Technologies, Darmstadt, Germany) combined with the direct-zol RNA Kit (Zymo, Freiburg, Germany) according to the manufacturer's instructions. RNA concentration and purity were checked on an Infinite© 200pro, (Tecan Trading AG, Switzerland) using a NanoQuant Plate (Tecan Deutschland, Crailsheim, Germany). cDNA was synthesized from approximately 1 µg of total RNA using the QuantiTect® Reverse Transcription Kit (Qiagen, Hilden, Germany). qPCRs were conducted using Luna® Universal qPCR Mastermix (New England Biolabs, Frankfurt, Germany) on a StepOne qPCR instrument (Applied Biosystems™, Life Technologies, Darmstadt, Germany). A melt curve was performed after the last amplification cycle in order to ensure primer dimer formation did not occur.

Expression of *OsFER*, *OsFRO1*, and *OsNRAMP6* were analyzed in the blades of the youngest fully developed leaves of IR31875 without iron treatment and *Bacillus* inoculation, as well as in iron-treated plants with and without *Bacillus* inoculation. Pooled samples of 3 biological replicates were used and 3 technical replicates analyzed. Primers used were either taken from the literature (with modifications) (Li et al., 2019b; Peris-Peris et al., 2017), or designed using Primer3web Software (Whitehead Institute for Biomedical Research, Cambridge, USA). Primer sequences are shown in Table 4.3 (supplementary material).

4.2.8 Indirect Measurement of Nitric Oxide

For the detection of bacterial nitric oxide (NO) production, the Griess reagent (Griess, 1879) was used. The assay is based on the measurement of nitrite (NO_2^-) as one of two primary, stable and nonvolatile breakdown products of NO. Bacteria were grown for 2 days in liquid TSB medium at 28°C without shaking. Of the cultures, 2 ml were centrifuged for 20 min at 10,000 g and supernatants used for analyzes. For the modified protocol following solutions were prepared: 1: $NaOHPO_4$ buffer containing of 0.8% NaOH (w/v), 0.1% KH_2PO_4 (w/v), 0.5% Triton X (v/v), 0.5% Ethanol (v/v). 2. Hydraz solution containing of 0.75% $H_6N_2O_4S$ (v/w), 0.0025 ‰ $CuSO_4$, and 3: Color solution containing of 15% concentrated H_3PO_4 (v/v), 0.1% N-1-Naphthylethylenediamine di-hydrochloride (NED) (w/v), 1% Sulfanilamide (w/v). In a 96 well plate 110 µl $NaOHPO_4$ buffer was first mixed with 20 µl of Hydraz solution, then 20 µl of Color solution, and lastly 150 µl of sample were added. A reference curve with a range of concentrations between 0-100 µM NO_2^- was generated by testing 6 serial twofold dilutions of a nitrite standard in TSB medium. Color formation was measured after 45 min incubation in the dark at 40°C, photometrically at 550nm using the Infinite© 200pro (Tecan Trading AG, Switzerland).

4.2.9 Data Analysis

For enzyme analysis, outliers were removed by 1st and third quartile method. When data failed skewness and kurtosis tests it was transformed using ln function. Prior to analysis of variance (ANOVA), Shapiro-Wilk test was carried out to ensure normal distribution of the data. Multifactorial ANOVA was used to analyze the effects of iron treatment, cultivar, *Bacillus* inoculation, organ, and their interactions. Two factorial ANOVA was used to analyze the effect of *Bacillus* inoculation, cultivar and their interaction, within the different organs under iron toxic conditions. Dunnett's test was used for post-hoc testing when comparing different *Bacillus* treatments to the non-inoculated controls, Tukey's test when comparing different cultivars with each other.

Expression of the gene of interest (GI) in each sample was normalized to the expression of ubiquitin (*OsUBQ*). Fold gene expression was calculated per sample repeat as:

$$2^{-\,(\Delta\text{CT GI treatment- mean }\Delta\text{CT GI control})}$$

Standard error was calculated from the mean of calculated fold changes. Dunnett's test was applied to analyze statistical differences to control plants (non-iron treated, non-*Bacillus* inoculated) using ΔCT values. Excel (Microsoft, Redmond, USA) was used to calculate fold gene expression, for other statistical analysis and graphs SigmaPlot 14.0 (Systat Software, Inc. San Jose, USA) was used.

4.3 Results

4.3.1 Leaf Scores

Leaf symptom scoring was carried out in both experiments. Results for plants used for enzyme analysis are shown in Table 4.1. *Bacillus* inoculation decreased the leaf symptoms in IR31875 from 5.6 in the non-inoculated plants to approx. 4 when inoculated with *B. pumilus* and to approx. 3 when inoculated with *B. megaterium*. In Sahel 108 scores remained unaffected when inoculated with *B. pumilus* and were decreased from 3.5 to around 3.2 when inoculated with *B. megaterium*. Leaf scores of Suakoko 8 inoculated with *Bacillus* were increased compared to the non-inoculated plants. With the strongest negative effect found for inoculation with *B. pumilus* Ni9MO12 with a leaf symptom score of 5 compared to 3.7 in the non-inoculated plants. IR31875 plants used for gene expression analysis had a leaf score of 4 when not inoculated and a score of 2 when inoculated with *B. pumilus* Ni9MO12 (data not shown).

Table 4.1: Leaf symptom scores after 7 days of exposure to 1000 ppm Fe in the nutrient solution. ± = Standard error of the means. No Bac = Non-inoculated.

	Cultivar		
Bacteria Treament	IR31875	Sahel 108	Suakoko 8
No Bac	5.67 ±0.38	3.50 ±0	3.67 ±0.21
B.pumilus D7.4	4.00 ±0.37	3.50 ±0.18	4.33 ±0.28
B. megaterium	3.17 ±0.11	3.17 ±0.11	4.00 ±0.18
B. pumilus Ni9MO12	4.17 ±0.21	3.67 ±0.28	5.00 ±0

4.3.2 Enzyme Activity

ROS scavenger enzyme activities in blades and sheaths of the second youngest fully expanded leaves were analyzed. At first, changes in enzymatic activity upon iron treatment in all three cultivars without any bacteria inoculation were analyzed (Figure 1). Glutathione reductase (GR) activity changes upon iron treatment were significantly dependent on the organ, with increased activity in the sheaths but decreased activity in the blades, of the 2nd youngest fully developed leaves. Ascorbate peroxidase (APX) activity was found to be highly significantly increased in both the blades and sheaths of plants treated with 1000 ppm Fe^{2+}. APX activity in the blades was generally significantly higher than in the sheaths, both under normal and under iron toxic conditions. Iron treatment significantly affected catalase (CAT) activity, here too, activity was significantly higher in the blades than in the sheaths under both treatments. In contrast, no significant effect of iron treatment was found for the activity of superoxide dismutase (SOD) in both the blades and the sheaths. Again, activity differed significantly between organs, with higher activity in the blades than in the sheaths. Cultivars did not differ in the activities of GR, APX, CAT, and SOD under iron toxic conditions. Activity of guaiacol peroxidase (PRX) was highly significantly affected by iron treatment und significantly affected by iron treatment x cultivar interaction. Whereas in all three cultivars PRX activity was significantly increased in blades and sheaths of iron-stressed plants, this increase was significantly higher in IR31875 compared to Sahel 108 (Tukey test, $p = 0.013$) and numerically higher compared to Suakoko 8 (Tukey test, $p=0.082$).

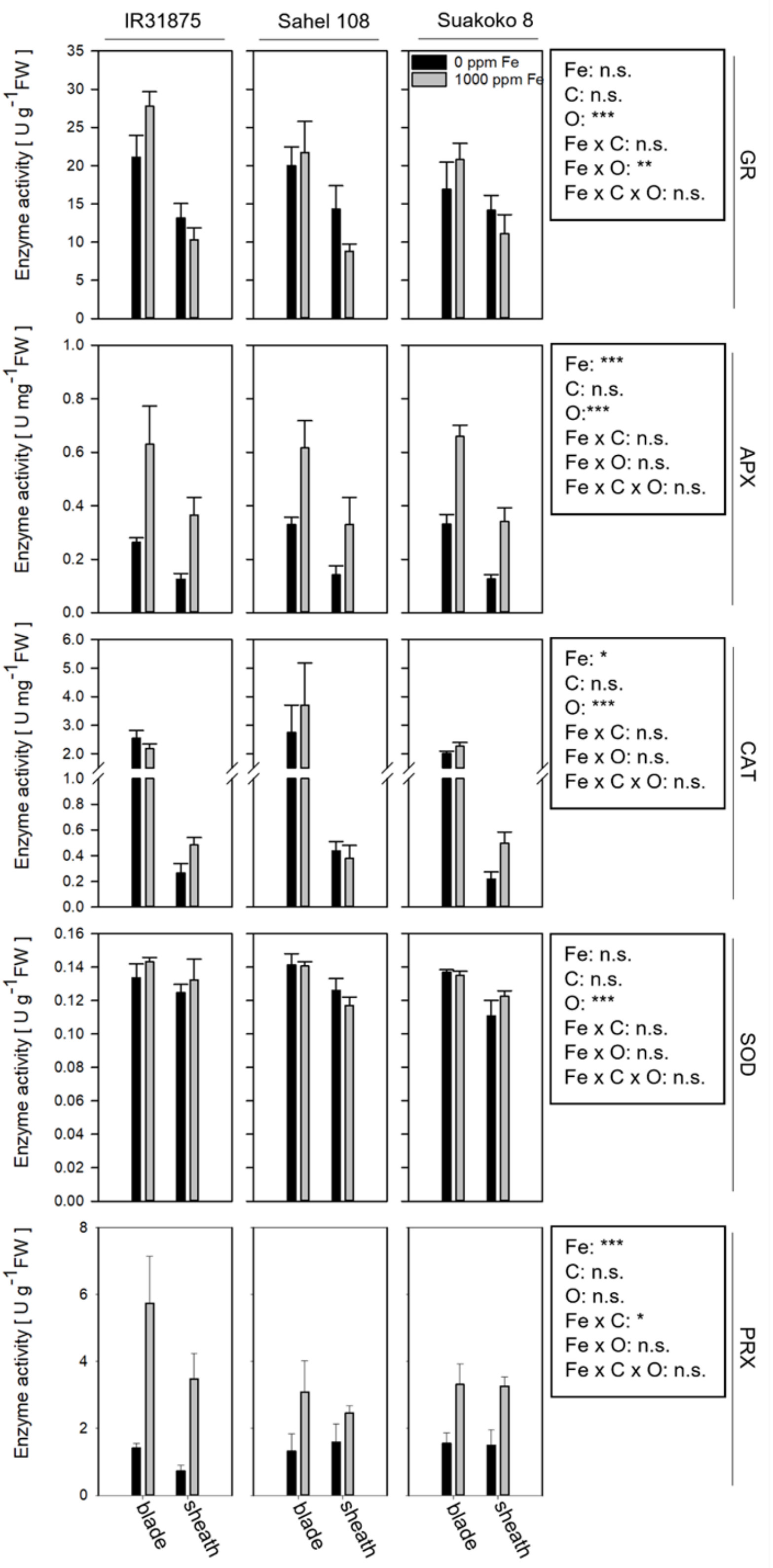

Figure 4.1: Activity of enzymes involved in ROS scavenging in the second youngest leaves of three different cultivars under normal growth conditions and under iron toxicity., 0 ppm = Original Yoshida no supplemented iron, 1000 ppm = Original Yoshida with supplementation of 1000 ppm iron, GR = glutathione reductase, APX = ascorbate reductase, CAT = catalase, SOD = superoxide dismutase, PRX = guaiacol peroxidase, C = cultivar, O = organ, n.s.= not significant, * = $p \leq 0.05$;**= $p \leq 0.01$ ***= $p \leq 0.001$ (multi-factorial ANOVA). Error bars = standard error of the mean.

To investigate the effects of *Bacillus* inoculation on the activities of the selected ROS scavenger enzymes, two-factorial ANOVAs were carried out for each organ (blades and sheaths) with *Bacillus* isolate and cultivar being factors (Table 2). Within the blades, CAT activity was significantly affected by *Bacillus* inoculation but not by cultivar. Neither did the effects of *Bacillus* inoculation dependent on the cultivar. Post-hoc testing showed that the decrease in CAT activity in *B. pumilus* Ni9MO12 inoculated plants - compared to the non-inoculated plants – was statistically significant (Dunnett's test, $p = 0.03$). *Bacillus* inoculation was found to highly significantly affect SOD activity in the blades ($p \leq 0.001$), post hoc testing, using Dunnett's test (which compares each treatment with the control but not with other treatments) showed that none of the *Bacillus* treatments differed significantly from the non-inoculated controls. Cultivar had no significant effects on SOD activity in the blades, and the effects of *Bacillus* inoculation on SOD activity in the blades were not dependent on the cultivar. No significant effects of *Bacillus* inoculation on the activities of GR, APX, and PRX were found in the leaf blades of iron-stressed plants, neither for the factors (bac and cult) nor for their interaction.

Within the sheaths, GR activity significantly differed dependent on *Bacillus* inoculation and cultivar, furthermore, the effects of both bacteria and cultivar were significantly dependent on each other (two-factorial ANOVA, $p \leq 0.05$). Post-Hoc testing, showed significant differences between the different bacteria treatments (Tukey test, $p \leq 0.05$) but no significant differences between *Bacillus*-inoculated and non-inoculated plants (Dunnett's test; $p \leq 0.05$). A significant difference between cultivars was found when inoculated with *B. pumilus* Ni9MO12, with Suakoko 8 displaying a significantly higher GR activity in the sheaths than the other two cultivars (Tukey test, $p \leq 0.05$). CAT activity in the sheaths was also significantly affected by *Bacillus* inoculation. Post-hoc testing revealed that activity was significantly increased in *B. pumilus* Ni9MO12 inoculated plants (Dunnett's test; $p \leq 0.05$) but did not differ between cultivars. Enzyme activities of APX, SOD, and PRX did not differ in the sheaths between cultivars or *Bacillus* treatments.

Table 4.2: Analysis of variance (ANOVA) for activities of glutathione reductase (GR), ascorbate peroxidase (APX), catalase (CAT), superoxide dismutase (SOD), and guacaiol peroxidase (PRX) in the leaf blades and sheaths (=organs) of the second youngest fully developed leaves. Cultivars tested: IR31875, Sahel 108, Suakoko 8. Abbreviations: df= degrees of freedom, MS = mean square, Sign = significance.

	Source of Variation	DF	GR			APX			CAT			SOD			PRX		
			MS	P	sign	MS	P	sign	MS	P	sign	MS	P	sign	MS	P	sign
Blades	Bac	3	0.12	0.25	n.s.	0.008	0.75	n.s.	0.052	0.04	*	0.0004	0.001	***	2.43	0.19	n.s.
	Cultivar	2	0.14	0.20	n.s.	0.010	0.62	n.s.	0.005	0.73	n.s.	0.0000	0.914	n.s.	0.04	0.97	n.s.
	Bac x Cultivar	6	0.05	0.77	n.s.	0.011	0.78	n.s.	0.008	0.82	n.s.	0.0001	0.175	n.s.	1.33	0.48	n.s.
Sheaths	Bac	3	47.93	0.05	*	0.004	0.80	n.s.	1.580	0.03	*	0.0004	0.13	n.s.	2.41	0.09	n.s.
	Cultivar	2	79.29	0.02	*	0.032	0.08	n.s.	0.154	0.72	n.s.	0.0001	0.68	n.s.	0.14	0.87	n.s.
	Bac x Cultivar	6	43.76	0.03	*	0.007	0.68	n.s.	0.796	0.16	n.s.	0.0002	0.44	n.s.	1.76	0.14	n.s.

4.3.3 *In situ* H_2O_2 Staining

After six days of exposure to 1000 ppm Fe^{2+}, DAB staining showed on average less accumulation of H_2O_2 in the youngest fully developed leaf blades of *Bacillus*-inoculated IR31875 compared to the non-inoculated plants (Figure 2).

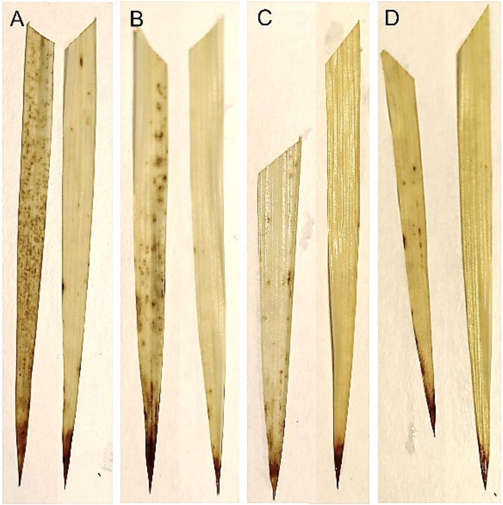

Figure 4.2: *In situ* DAB staining of H_2O_2. Photographed are leaf blades of the youngest fully expanded leaves of IR31875 grown under iron toxicity (1000 ppm Fe^{2+}) with and without *Bacillus* inoculation. To the left are blades representing plants of each treatment with most leaf symptoms, to the right, leaf blades representing plants of each treatment with least leaf symptoms. A = Non-inoculated, B = *B. pumilus* D7.5 inoculated, C = *B. megaterium* inoculated, D = *B. pumilus* Ni9MO12 inoculated.

4.3.4 Gene Expression Analysis

Changes in gene expression were analyzed in the leaf blades of the second youngest fully expanded leaves (Figure 4.3). A 2.4 fold increase in ferritin mRNA accumulation was found in non-inoculated IR31875 plants after seven days of exposure to 1000 ppm Fe^{2+}, compared to non-inoculated IR31875 grown under normal conditions. IR31785 plants inoculated with *B. pumilus* Ni9MO12 were found to have a highly significant (t-test, $p \leq 0.0001$) 64.4 fold increase in ferritin mRNA abundance after 6 days of iron stress, compared to the non-inoculated, non-iron stressed control. Accumulation of *OsFRO1* was 1.2 fold higher in the non-inoculated, iron stressed plants, compared to the non-inoculated, non-stressed plants and 7.1 fold higher in the *Bacillus*-inoculated, iron stressed plants. None of these differences were found to be statistically significant. A 3.6 fold increase in *OsNRAMP6* mRNA was found in the non-inoculated, iron-stressed plants compared to the non-iron-stressed plants. In *Bacillus*-inoculated IR31875 grown under iron toxic conditions, *OsNRAMP6* transcript abundance was 18.5 fold higher compared to the non-inoculated, non-iron stressed plants.

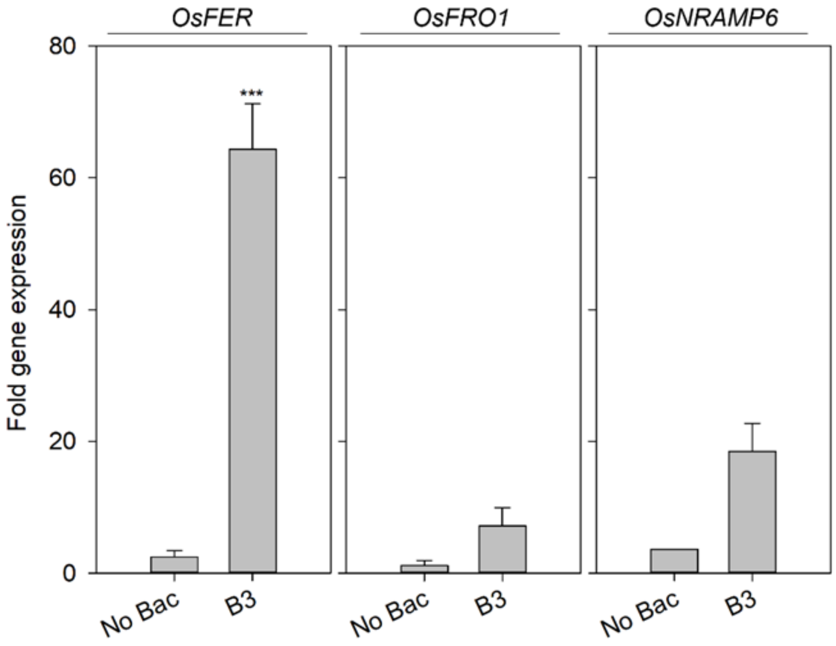

Figure 4.3: Expression of *OsFER*, *OsFRO1*, and *OsNRAMP6* in IR31875 under iron toxic conditions (1000 ppm Fe). Fold change in gene expression of non-inoculated, iron-stressed plants (No Bac) and *B. pumilus* Ni9MO12 inoculated, iron-stressed plants as compared to the non-inoculated, non-iron stressed IR31875. Asterisks show significant differences in gene expression between non-inoculated, iron-stressed plants and inoculated, iron stressed plants. *** = $p \leq 0.001$ (Dunnett's test). No Bac = non-inoculated, B3 = *B. pumilus* Ni9MO12 inoculated. Error bars = standard error of the mean.

4.3.5 NO Production

NO production was measured indirectly through photometric detection of nitrite in the supernatants of bacteria cultures (Figure 4.4). No nitrite was detected in the supernatant of *B. pumilus* D7.4 and *B. megaterium*. In the supernatants of *B. pumilus* Ni9MO12, nitrite was detected. In normal growth medium, 2.32 mmol OD_{600}^{-1} was measured. When supplemented with 5 ppm Fe2+, concentration of nitrite in the bacteria culture increased to 2.44 mmol OD_{600}^{-1}, and a supplementation with 10 ppm Fe^{2+} lead to a further increase to 2.59 mmol OD_{600}^{-1}. The differences in nitrite concentrations between the three different iron supplementations (0, 5, 10 ppm) were not statistically significant (t-test, $\alpha \leq 0.05$).

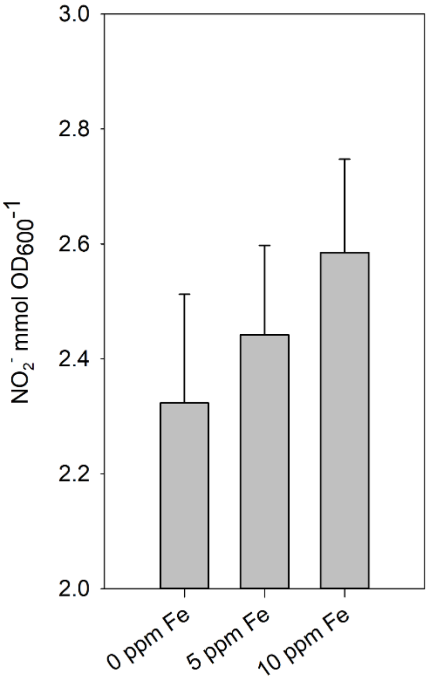

Figure 4.4: Nitrite concentration in supernatants of overnight cultures of *B. pumilus* Ni9MO12 rif res. grown in TSB supplemented with different iron concentrations. 0 ppm Fe = no additional iron added to the growth medium, 5 ppm Fe = TSB supplemented with 5 ppm Fe, 10 ppm = TSB supplemented with 10 ppm Fe. Iron was added in the form of $FeSO_4$. n= 3. Error bars = standard error of the mean.

4.4 Discussion

In a previous study (Weinand et al., 2023a) differences in the effects of *Bacillus* inoculation on the tolerance against iron toxicity of three lowland rice cultivars were found. In the naturally sensitive cultivar IR31875, *Bacillus* inoculation led to a mitigation of leaf symptoms. However, no correlation between shoot iron concentration and leaf symptom scores were found. Instead, inoculation of IR31875 with *Bacillus* seems to increase the cultivar's ability to tolerate high iron concentrations in the leaf tissue. Tissue tolerance is a phenomenon seen in so called 'includer-type' cultivars. Underlying mechanisms are not fully understood, but they include increased activities of ROS scavenger enzymes, sequestration of iron into the vacuoles, or chelation of iron by ferritin (Aung and Masuda, 2020). We therefore postulated three hypotheses: I) *Bacillus*-inoculated plants display higher ROS scavenging, II) *Bacillus* inoculation affects iron sequestration within the plant cell, and III) *Bacillus* inoculation positively affects the production of ferritin. The effects of *Bacillus* inoculation on leaf symptom scores of IR31875, Sahel 108, and Suakoko 8, were consistent with previous findings (Weinand et al., 2023a). To test the first hypothesis, we analyzed the activities of ROS scavenging enzymes as a response to iron toxicity with and without *Bacillus* inoculation. Furthermore, we performed in-situ analysis of H_2O_2 accumulation in the leaf blades of iron-stressed IR31875 with and without *Bacillus* inoculation.

Before investigating the effect of *Bacillus* inoculation on the selected ROS scavenger enzymes, we analyzed genotypic differences in enzymatic activity changes in response to iron toxicity in non-*Bacillus*- inoculated plants. The strongest increase in activity as a response to high iron concentration in the nutrient solution were found for APX and PRX, which are both haem containing enzymes (Noctor et al., 2012). However, changes in enzyme activity under iron toxicity were not found to be dependent on the cultivar. Only for PRX the increase of PRX activity under iron stress was stronger in the sensitive cultivar IR31875 than in the tolerant cultivars Sahel 108 and Suakoko 8. This suggests that tolerance against iron toxicity in the tolerant cultivars is achieved upstream of PRX and does not seem to involve the Foyer-Halliwell-Asada pathway . It is well established in the literature that this ascorbate/glutathione cycle regulates ROS accumulation in each cellular compartment, with the exception of the apoplast/cell wall (Foyer et al., 2020; Foyer et al., 2024), which is why we selected these enzymes for our analysis. However, in the apoplast the enzyme ascorbate oxidase (AO) regulates the redox status (Pignocchi, C. and Foyer, C. H., 2003). Wu et al. (2017) have shown that this enzyme plays a role in the tolerance of a lowland rice genotype subjected to high iron concentrations. Increased expression of glutathione-S-transferase under high iron concentration in tolerant cultivars also suggests involvement of this enzyme into tolerance mechanisms (Wairich et al. 2021). To gain insights into the role of these enzymes in the response to iron toxicity in the three genotypes used in this study, further investigations are needed. Furthermore, other, non-enzymatic, low molecular compounds such as α-tocopherol, carotenoids, phenolics, flavonoids, and proline have been shown to maintain redox homeostasis under abiotic stress conditions (Das, K. and Roychoudhury, A., 2014). Wu et al. (2017) reported though that despite increased expression of genes involved in the biosynthesis of such compounds in the roots of lowland rice grown under iron toxicity, no significant differences in expression levels occurred between tolerant and sensitive cultivars. Only one gene of the carotinoid biosynthesis pathway showed increased expression in the tolerant genotype. However, because carotenoids have been shown to only detoxify singlet oxygen but not others, the authors suggest no direct link to the mechanism underlying tolerance in the cultivar they tested.

Microbial inoculation has been shown to mitigate drought stress in rice through increased activity of PRX, APX, and GR enzymes and over-expression of SODs, APX, and CAT (Singh et al. 2020). A lack of genotypic differences in enzymatic activities in response to iron toxicity as described above does not rule out an effect of *Bacillus* inoculation on these enzymes. Therefore, changes in activities of GR, APX, CAT, SOD, and PRX in the leaves of *Bacillus* inoculated plants under iron toxic conditions were analyzed. Again, no genotypic differences were found. When inoculated with *Bacillus*, PRX activity no longer differed between Sahel 108 and IR31875. PRX activity in the blades and sheaths of IR31875 was lower when *Bacillus* inoculated than in non-inoculated plants. The heme-containing guaiacol peroxidase reduces surplus H_2O_2 during normal metabolism as well as under stress conditions (Das and Roychoudhury, 2014). Leaf bronzing was decreased in *Bacillus* inoculated IR31875 while PRX activity was also decreased. Increased H2O2 levels in *Bacillus* inoculated plants compared to non-inoculated plants, as confirmed by in situ H_2O_2 staining in the leaves (Figure 2), must be caused by a mechanism upstream of PRX.

Literature shows contradictory results for activities of the enzymes tested in this study in response to iron toxicity. A possible explanation could be that enzyme activities greatly vary in a spatial-temporal manner. Onyango et al. (2020) measured the activities of SOD, APX, GR, and PRX in rice exposed to high iron concentrations. Although they report increased activities of all enzymes in iron-treated plants, their heat map of enzyme activities is more complex. Depending on the variety and the time point of sampling (days after stress application), activity of SOD in the iron stressed plants can be higher or lower than in the non-treated controls. Multiple SOD genes which are located in different cellular compartments, requiring different co-factors was suggested by Wu et al. (2017) a possible cause for differing results. This raises the question if measuring SOD activity can give conclusive results at all.

In IR31875 inoculation with *B. pumilus* Ni9MO12 did not reduce iron concentrations in the shoots under iron toxicity (Weinand et al., 2023a). Since the reduced leaf symptoms in *Bacillus* inoculated IR31875 could not be explained by increased activity of the ROS scavenger enzymes as postulated in hypothesis I), we tested hypothesis II): *Bacillus* inoculation affects intercellular iron sequestration. *OsFRO1* encodes for a ferric iron reductase which is localized in the vacuolar membrane. It is believed that through its action, ferric iron stored in the vacuole becomes available in the cytoplasm either through Fe^{2+} or chelated Fe^{2+}transporters (Li et al. 2019b). RNA interference (RNAi) lines with downregulated expression of *OsFRO1* showed to be more tolerant to iron toxicity, while plants overexpressing *OsFRO1* were more sensitive, the RNAi plants reduced the amount of Fe2+ available for cytoplasm, to alleviate Fe excess toxicity (Li et al. 2019b). Under iron toxicity, iron concentrations in the young leaf blades of IR31785 inoculated with *B. pumilus* Ni9MO12 did not to differ from the iron-stressed, non-inoculated plants, whereas leaf bronzing was repressed in the *Bacillus* inoculated plants (Weinand et al., 2023a). Li et al. (2019b) found expression of *OsFRO1* to be inhibited in the old, but not in the young leaves of rice plants grown under high iron concentrations. We, therefore, tested if inoculation with *Bacillus* negatively affected expression of *OsFRO1* in the young leaf blades of IR31875 grown under iron stress. This way, less iron would be transported from the vacuole into the cytosol, possibly alleviating iron toxicity. However, our results show, that in the leaf blades of iron-stressed plants inoculated with *B. pumilus* Ni9MO12, expression of *OsFRO1* was higher than in the non-inoculated, iron-stressed plants. The differences between mRNA abundancies were not significant though and too little is known about the regulation of *OsFRO1* gene expression to speculate about the reason for this increase. What can be ruled out is an inhibitory effect of *Bacillus* inoculation on the expression of *OsFRO1*. It is therefore unlikely that *OsFRO1* is involved in the mechanism that leads to an increased tolerance against iron toxicity in *Bacillus* inoculated IR31875.

Another gene known to be upregulated in rice subjected to iron toxicity is *OsNRAMP6* (Finatto et al., 2015, Wairich et al. 2021). This gene encodes for a protein that exists in two splice variants: l-NRAMP6 and s-NRAMP6 which are both plasma membrane-localized iron and manganese transporters. They have also been shown to play a role during infection with the fungal pathogen *Magnaporthe oryzae*, the causative agent of leaf blast in rice (Peris Peris et al., 2017). It was considered an interesting candidate gene due to its involvement in both iron transport as well as plant immunity (Peris Peris et al., 2017) because inoculation with *B. pumilus* Ni9MO12 does not only positively affect the tolerance of IR31875 against iron toxicity, but also increased the cultivar's resistance against brown spot disease (Weinand et al., 2023b). Our results show that under iron stress *OsNRAMP6* mRNA accumulation in the young leaf blades of *B. pumilus* Ni9MO12 inoculated IR31875 was higher than in the non-inoculated plants. However, the difference was not significant and due to the lack of knowledge on the regulation of *OsNRAMP6* expression it would be too speculative to ascribe a role in *Bacillus* mediated tolerance to iron toxicity based on these results.

Finally, we tested hypothesis III): *Bacillus* inoculation positively affects the production of ferritin. To that end, we investigated if inoculation with *B. pumilus* Ni9MO12 leads to changes in expression of *OsFER* genes, which encode for ferritin, an iron storage protein that can bind up to 4,000 iron atoms (Gross et al., 2003). Chelation of iron by ferritin was first shown to be part of a tolerance mechanism in the African rice *Oryza glaberrima* Steud. (Majerus et al., 2007) and later in *Oryza sativa* L. (Silveira et al., 2009; Stein et al., 2009). Our results show a highly significant 64.4 fold increase in *OsFER* expression in *B. pumilus* Ni9MO12 inoculated

IR31875 under iron toxic conditions, compared to non-inoculated, non-stressed plants. Expression of *OsFER* also increased slightly (2.4 fold) in iron-stressed, non-inoculated plants as compared to the non-inoculated plants grown under normal growth conditions. This suggests, that inoculation with *B. pumilus* Ni9MO12 induces *OsFER* gene expression in IR31875.

The exact mechanisms of iron-induced *OsFER* expression are yet unknown. High tissue iron concentrations have been shown to result in increased ABA concentration in plastids, which, in turn, induced ferritin expression in maize (Lobréaux et al., 1995). In rice, however, this was shown not to be the case (Stein et al. 2009). In Arabidopsis it has been shown that the signaling molecule NO acts downstream of high iron concentrations and upstream of a PP2A-type phosphatase to promote expression of *AtFer1*, the Arabidopsis homologue of the *OsFER* 1 gene (Arnaud et al., 2006). NO is a free radical with one unpaired electron, which can interact with dioxygen, superoxide, thiol groups, unsaturated fatty acids, and transition metals (Huang et al., 2019). Accumulation of NO seems a key component of Fe- and H_2O_2- induced oxidative damage in plants (Li et al., 2019a), however, it can react with glutathione to form S-nitrosoglutathione (GSNO), which, in turn, acts as a substrate for S-nitrosoglutathione reductase (GSNOR) (Astier et al., 2011). This enzyme has been shown to be involved in iron sensing at the root tip of rice plants (Li et al., 2019a). Through the action of GSNOR, iron-dependent production of NO and H_2O_2 is reduced. Higher expression of the GSNOR gene increases tolerance to iron toxicity, whereas mutants lacking the gene are more susceptible (Li et al., 2019a). Different *Bacillus* species have been shown to possess nitric oxide synthase-like (NOS-like) proteins (Adak et al.,2002). We therefore tested the ability of the three *Bacillus* isolates to produce NO under different iron supplementations by measuring nitrite as the stable product of NO in bacteria culture supernatants. While no nitrite was detected in the culture supernatants of isolates *B. pumilus* D7.4 and *B. megaterium*, culture supernatants of *B. pumilus* Ni9MO12 showed increased levels of nitrite. In bacteria, nitrite can also be a product of ammonium oxidation, with NO not always being a side product, dependent on the bacteria strain and the environmental conditions (Huang et al., 2019). However, the fact that the level of nitrite increased with increasing amounts of iron in the growth medium implies that *B. pumilus* Ni9MO12 could potentially exhibit NOS activity, as nonheme iron has been demonstrated to amplify the reaction rate of NOS (Perry and Marletta, 1998). Adak et al. (2002) further demonstrated that transient heme-Fe(II)O_2 serves as an electron acceptor during the formation of NO by *Bacillus* NOS. Under high iron concentrations NO produced by *B. pumilus* Ni9MO12 could possibly be a signal involved in the signaling cascade leading to higher *OsFER* 1 mRNA accumulation in the iron-stressed, inoculated plants. Higher levels of ferritin together with a higher activity of GSNOR in the inoculated plants could explain lower leaf bronzing scores and lower H_2O_2 accumulation in the leaf blades despite a significantly lower activity of PRX. Further investigations are needed to decipher a possible role of *Bacillus* derived NO, GSNO, and GSNOR in *Bacillus* mediated tolerance against iron toxicity.

4.5 Conclusions

Bacillus inoculation differently affects the tolerance against iron toxicity in different lowland rice cultivars. ROS scavenging through the ascorbate-glutathione redox cycle does not seem to play a role in increased tolerance against iron toxicity in *B. pumilus* Ni9MO12 inoculated IR31875. Instead, increased levels of ferritin most likely remove Fe^{2+} from the Fenton reaction, resulting in decreased production of hydroxyl radicals and subsequently reduced leaf bronzing. NO produced by *B. pumilus* Ni9MO12 might be involved in the signaling cascade leading to induction of *OsFER* expression in inoculated IR31875 under iron toxic conditions. Further research is needed into a possible role of S-nitrosation in signaling transduction affected by *Bacillus* inoculation.

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Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Conflicts of Interest

The authors declare no conflict of interest.

Supplementary Material

Following additional supporting information can be found online in the Supporting Information section at the end of this article [see Table 4.3 below].

Table 4.3: Primer sequences used for gene expression analysis.

Primer	Target gene	Sequence 5' - 3'	Reference
FRO1-F	Ferric Reductase Oxidase 1	GTC GAC GCG CAG TTC ATC AG	Li et al. 2019b
FRO1-R		GAA CAT GCC GGT CGC CTT CT	
NRAMP6-F	Natural Resistance-Associated Macrophage Protein	CAT AGC TGC GAT TGT ACT ATC ATT TG	Peris-Peris et al. 2017
NRAMP6-R		CGC GAT TCG GAT AAT AGA TGT TG	
UBI1-F	Ubiquitin	TTC CCC AAT GGA GCT ATG GTT T	Peris-Peris et al. 2017
UBI1-R		AAA CGG GAC ACG ACC AAG G	
FER-F	Ferritin 1 and 2	TCC GCC ATG CTT CCT CCT A	designed with Primer3
FER-R		CCT CGA ATG GCT GGA AGA CC	

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5 General Discussion

Iron toxicity constitutes a significant challenge to global lowland rice production systems. The progress in breeding new tolerant cultivars has been slow, partly due to an incomplete understanding of the mechanisms underlying tolerance against this stress (Kirk et al. 2022) and difficulties in evaluating germplasm under field conditions because of significant genotype-by-environment interactions (Rajonandraina et al., 2023). Over the last twenty years, there have been numerous attempts to pinpoint QTLs responsible for variances in iron toxicity tolerance, but these efforts have largely been unfruitful (Li et al. 2019a). Another factor adding to this might be the neglect of the microbiome's role in shaping the plant's responses to iron toxicity. As described in the previous chapters, inoculation using various *Bacillus* spp. isolates can alter genotypic responses to toxic iron concentrations in the nutrient solution. When using leaf symptom scoring as an indicator for the level of tolerance against iron toxicity, the effects of *Bacillus* inoculation differed depending on the cultivar x *Bacillus* isolate combination. While *Bacillus* inoculation significantly increased tolerance against iron toxicity in the susceptible cultivar IR31875, the tolerant excluder Suakoko 8 showed significantly more severe iron toxicity symptoms when inoculated with *Bacillus* spp., whereas no significant effects on symptom expression was found in the tolerant includer Sahel 108. Effects of bacteria inoculation on tolerance against iron toxicity have been described by De Souza et al. (2015), though the focus was on general effects like growth promotion and uptake of iron and other mineral nutrients. Within the framework of this dissertation, we aimed to shed light on the drivers of genotypic differences in leaf symptom expression in lowland rice inoculated with *Bacillus* spp.. Therefore, we focused on effects of *Bacillus* inoculation on specific tolerance mechanisms, such as shoot iron levels, iron allocation within the shoot, ROS scavenger enzyme activity, and the expression of genes related to iron toxicity tolerance.

5.1 Effects on Shoot Iron Content and Allocation

The most direct beneficial effect bacteria could have on lowland rice grown under iron toxicity, is to reduce Fe concentrations in the plant. Indeed, De Souza et al. (2015) observed lower shoot Fe concentrations in iron-stressed rice inoculated with bacteria from various genera, one of which was an isolate of *Bacillus* sp. Our findings indicate that the effect of *Bacillus* inoculation on shoot Fe content and concentration varies based on the plant cultivar, *Bacillus* isolate, and combinations of *Bacillus* isolate and cultivar. Engel et al. (2012) associated bronzing scores of leaves with their Fe concentrations, where leaves with higher Fe concentrations displayed increased bronzing scores. Yet, our results show different effects of *Bacillus* inoculation on whole shoot Fe content and concentration dependent on the cultivar x *Bacillus* isolate combination and no correlation to the effects on leaf symptom development. On the leaf level, our findings corroborate previous studies that showed a larger proportion of Fe is allocated to stem tissues, particularly in older leaves of iron-tolerant cultivars (Aung et al., 2018; Engel et al., 2012). We found that the inoculation of the three distinct rice cultivars with *Bacillus* spp. had different effects on Fe distribution within the shoot. The total Fe content in sheaths and dead tissues was influenced by cultivar, *Bacillus* isolate, and their interactions, while changes in the total Fe content of the blades were solely determined by cultivar. Engel et al. (2012) suggested that a harmonized Fe distribution between blades and sheaths is a characteristic of tolerance in includer genotypes. Our results emphasize the importance of this balance also in excluder genotypes like Suakoko 8. Within the shoots of Suakoko 8 inoculated with *Bacillus pumilus* Ni9MO12, a decrease in Fe concentration in older sheaths was found, while concentration within the leaf blades remained unchanged. But despite no differences in Fe concentrations in the leaf blades between inoculated and non-inoculated Suakoko 8, leaf bronzing was more severe in the inoculated plants. It seems plausible that the disparity in distribution found in *B. pumilus* inoculated Suakoko 8 is not caused by disturbed transportation to the leaf blades, but rather by reduced deposition of surplus Fe in older sheaths. The metal chelator nicotianamine (NA) the Fe-NA transporter *OsYSL2*, as well as the potassium transporter *OsAKT1* have been reported to play a role in Fe translocation within the plant. (Aung et al., 2019 ; Koike et al., 2004 ; Yoneymama, 2021 ; Wu et al., 2019). To better understand the influence of endophytic bacteria on these transporters, more comprehensive studies are required. Our results suggest a lack of involvement of bacterial siderophores in the translocation of Fe, as reported for pea under conditions of iron deficiency (Lurthy et al., 2020). Only *B. pumilus* D7.4 exhibited noticeable siderophore production under typical growth conditions. As Fe concentrations in the growth medium increased, siderophore production of all three *Bacillus* isolates diminished.

5.2 Effects on Shoot Tolerance Mechanisms

Increased ROS scavenging enzyme activity can lead to higher tolerance to iron toxicity in different cultivars (Briat et al., 2010; Stein et al., 2014; Wu et al., 2017). To explore if *Bacillus* can affect these mechanisms, activities of ROS scavenging enzymes which act within the ascorbate-glutathione cycle were assessed. As expected, activity of ascorbate peroxidase (APX) and guaiacol peroxidase (PRX) increased significantly under iron toxic conditions. Not so the activities of superoxide dismutase (SOD), catalase (CAT), and glutathione reductase (GR). Furthermore, no significant differences in enzyme activities of SOD, CAT, APX, and GR were found between the cultivars. Only the activity of PRX under iron stress was more pronounced in IR31875 compared to the more tolerant cultivars Sahel 108 and Suakoko 8. Iron toxicity in these two cultivars is possibly addressed upstream of PRX and does not seem to involve the ascorbate-glutathione redox cycle. No significant differences in alterations in the activities of GR, SOD, and APX between cultivars were detected in *Bacillus* inoculated plants. A significantly different genotypic response to *Bacillus* inoculation was observed for PRX activity. In *Bacillus*-inoculated IR31875, leaf bronzing decreased while PRX activity also diminished. Guaiacol peroxidase is a heme-containing enzyme that curtails excess H_2O_2 during regular metabolism and under stress (Das and Roychoudhury, 2014). Decreased leaf symptoms as well as reduced H_2O_2 in the leaf blades of *Bacillus* inoculated plants – as supported by *in vivo* staining – imply a mechanism upstream of PRX affected by *Bacillus*.

Upstream of PRX and the ascorbate-glutathione redox cycle is the generation of hydroxyl radicals through the Fenton reaction in which ferrous iron functions as an electron donor. The iron storage protein ferritin plays an important role in shoot tolerance because they can store up to 4,000 iron atoms, thereby preventing ROS generation through the Fenton reaction (Gross et al., 2003; Majerus et al., 2007) After 6 days of exposure to iron toxicity we found a highly significant 64-fold increase of *OsFER* mRNA accumulation in *Bacillus pumilus* Ni9MO12 inoculated IR31875 compared to the non-inoculated, non-iron stressed plants. In non-inoculated IR31875, *OsFER* mRNA accumulation was comparably marginal. This indicates that the increased tolerance of high Fe concentrations in the shoots of *B. pumilus* Ni9MO12 inoculated IR31875 is achieved through stimulation of ferritin production – at least in the leaf blades of the young leaves. While the underlying mechanism triggering *OsFER* expression in rice remain unclear, the signaling molecule NO has been shown to be involved in the signaling cascade, leading to increased expression of the Arabidopsis homologue, *AtFER1* (Arnaud et al., 2006). NO accumulation is crucial in oxidative damage induced by Fe and H_2O_2 in plants (Li et al., 2019a). Still, it can transform into S-nitrosoglutathione (GSNO), which then becomes a substrate for S-nitrosoglutathione reductase (GSNOR) (Astier et al., 2011). Through the action of GSNOR, iron-dependent production of NO and H_2O_2 is reduced (Li et al. 2019a). Higher activity of GSNOR would explain the lower levels of H_2O_2 despite reduced activity of PRX on the one hand, on the other hand, it would also lead to lower NO levels and therefore lower ferritin expression. Therefore, we explored if *Bacillus* derived NO could trigger *OsFER* gene expression. Prerequisite for this hypothesis being the ability of the *Bacillus* isolates to produce

NO. Different *Bacillus* species have been shown to possess nitric oxide synthase-like (NOS-like) proteins (Adak et al., 2002) and our results indicate that *B. pumilus* Ni9MO12 also does. Detailed studies, such as direct measurement of NO production, effect of NO inhibitors, measurement of GSNOR activity, etc., are required to pinpoint roles of *Bacillus*-produced NO, GSNO, and GSNOR in the *Bacillus*-mediated tolerance to iron toxicity. An enhanced ferritin production and GSNOR activity in inoculated plants could explain the diminished leaf bronzing and H_2O_2 accumulation, despite the notably reduced PRX activity.

Other possible effects of *Bacillus* inoculation on shoot tolerance mechanisms were also assessed. The vacuole acts as a storage compartment for iron and under normal growth conditions the ferric iron reductase 1 (FRO1) makes ferric iron stored within the vacuole accessible in the cytoplasm, either directly through Fe^{2}- or via chelated Fe^{2+}- transporters (Li et al. 2019b). Li et al. (2019b) observed that under high Fe concentrations, *OsFRO1* expression was reduced in older rice leaves, but remained unchanged in younger ones. *Bacillus*-induced suppression of *OsFRO1* expression in young leaf blades of IR31875 subjected to iron stress could lead to reduced iron transport from the vacuole to the cytosol, potentially mitigating iron toxicity. Yet, the results of this thesis show that *OsFRO1* expression in young leaf blades of *B. pumilus* Ni9MO12 inoculated plants, when iron-stressed, are only marginally elevated compared to those without inoculation under similar stress. Given the limited understanding of *OsFRO1* gene regulation, it would be too speculative to assign *OsFRO1* a role in the increased tolerance to iron toxicity observed in the *Bacillus*-treated IR31875. *OsNRAMP6*, with its two splice variants, encodes for Fe and manganese transporters located in the plasma membrane. They have been assigned a dual role in both Fe transportation and plant immunity (Peris Peris et al. 2017). Under iron stress, *OsNRAMP6* mRNA accumulation in young leaf blades of *B. pumilus* Ni9MO12 inoculated IR31875 was increased compared to non-inoculated plants. However, because the difference was not statistically significant and the current limited understanding of *OsNRAMP6* expression regulation, it is premature to attribute a definitive role to *OsNRAMP6* in *Bacillus*-mediated tolerance based on this data alone. Taken together, the increased tolerance of high Fe concentration in the young leaf blades of *B. pumilus* Ni9MO12 inoculated IR31875 seems to arise from an induction of ferritin synthesis rather than increased sequestration of iron within the vacuole. However, other genes, such as *OsVIT* and *OsNAS*, which been shown to be involved into vacuolar iron storage and iron chelation (Aung et al., 2018), respectively, could also be affected, particularly because their expression has been shown to be differentially regulated by *OsFER2* expression (Dangol et al., 2019).

5.3 *Bacillus*-mediated Cross Protection

Plants are often exposed to abiotic and biotic stresses simultaneously. Fungal pathogens result in global yield losses ranging from 10 to 23% (Stuckenbrock and Gurr 2023). One of the most important fungal pathogen in lowland rice production is *Bipolaris oryzae*, the causative agent of brown spot disease. To explore potential links in responses to iron toxicity and pathogen intrusion, the effects of the *Bacillus* isolates on *Bipolaris oryzae*, were evaluated both *in vitro* and *in planta*. Our results show that *Bacillus* inoculation leads to a marked reduction in brown spot disease *in planta*. Mirroring earlier findings regarding iron toxicity tolerance, the bacterial inoculation's effects on brown spot disease manifestation varied depending on the *Bacillus* isolate and the rice cultivar. Effects of *Bacillus* inoculation on the disease reduction on the three lowland rice cultivars does not seem to be related to biomass accumulation, aligning with our findings concerning plants under iron-toxic conditions. Direct inhibitory effects on fungal growth were found *in vitro*, however, other mechanisms leading to reduced disease development *in planta* could not be ruled out, because no effects of *Bacillus* inoculation were found in Sahel 108. The role of Fe, as an integral component of the plant's defense mechanism, in linking abiotic and biotic stress signaling is not well studied yet. There have been reports of potential connections between Induced Systemic Resistance (ISR) against pathogens and reactions to Fe deficiency (Romera et al., 2019). However, response to pathogenic attack does not always involve ISR. The hypersensitive response (HR) stands out as the most robust defense against fungal pathogen invasions, characterized by cell death. One form of cell death is the ferroptotic death which is reliant on Fe and is associated with the accumulation of toxic lipid ROS, especially lipid hydroperoxides (Dangol et al., 2019). In the context of rice, ferroptosis is recognized as a defensive mechanism against non-compatible infections by the fungus *Magnaporthe oryzae* (Dangol et al., 2019). It has been reported that deferoxamine (DFO), a bacterial Fe chelator can suppress ferroptotic death (Yang and Stockwell, 2008). The rice ferritin gene *OsFER2* on the other hand has been shown to positively regulate defense against *Magnaporthe oryzae* through positively regulating ferroptotic cell death (Nguyen and Bruns, 2022). Mechanisms of *Bipolaris oryzae* infection are less well studied. Recently, Thuy et al. (2023) found H_2O_2 accumulation to be involved in the plant's defense mechanism, suggesting that a hypersensitive response leads to cell death during incompatible infections. This defense response against fungal attack could possibly be affected by *Bacillus*-induced expression of *OsFER* or by siderophores produced by the *Bacillus* isolates. Furthermore, *B. pumilus* Ni9MO12 has been shown to produce NO, a molecule which has distinct functions in the interactions between host plants and fungal pathogens. In these interactions, the combined actions of NO and reactive oxygen species dictate successful plant colonization, cell death, and development of resistance (Hong et al., 2007). The findings presented here can be used as a basis for further investigations into the underlying mechanisms of *Bacillus*-mediated mitigation of brown spot disease in lowland rice. This could involve the analysis of *OsFER* gene expression in *Bipolaris oryzae* infected plants with and without *Bacillus* inoculation, or investigating if the ferroptosis inhibitor DFO counteracts the positive effects of *Bacillus* inoculation.

5.4 Plant Growth Promoting Rhizobacteria (PGPR)

The *Bacillus* isolates used in this dissertation, were shown to affect genotypic responses of lowland rice cultivars to both iron toxicity and *Bipolaris oryzae* infection. Bacterial isolates providing cross-protection against both biotic and abiotic stresses would be highly desirable for application in sustainable agriculture (Dimpka et al., 2009).

Positive effects of bacteria inoculation on plant stress tolerance are often attributed to their ability to promote plant growth, hence the term "plant growth promoting rhizobacteria (PGPR)" is commonly used in publications describing beneficial effects of bacteria inoculation on plants under various stress conditions. Within the frame of this dissertation, several experiments were carried out under varying growth conditions in terms of day length, temperature, and VPD. Due to these environmental variations, there was a noticeable difference in plant growth across experiments. However, despite the growth discrepancies between experiments, the relative effect of bacterial treatments on leaf bronzing scores was highly consistent. Conversely, the effects of bacterial inoculation on biomass accumulation differed depending on the bacterial isolates, rice cultivars, stress treatments, and specific experiments. In addition to measuring plant growth promoting effects *in planta*, the three *Bacillus* isolates were tested for traits generally considered plant growth promoting properties. These traits are routinely tested for in studies aiming to identify bacteria which can ameliorate certain environmental stresses (Dimpka et al., 2009). The ability of bacteria to solubilize nutrients such as zinc and phosphate is regarded beneficial for plant growth because both nutrients can become available to the plant through bacterial activity. Iron toxicity is a multi-nutrient disorder, often causing deficiencies in phosphate and zinc (Becker and Asch, 2005). Increased availability of Zn and P would therefore be highly beneficial. However, none of the three *Bacillus* isolates tested showed the ability to solubilize phosphate or zinc *in vitro.*

The plant hormone auxin plays an important role in plant stress responses and bacterial auxin production has been shown to promote the growth, particularly of roots, of various plant species

(Backer et al., 2018). The two *B. pumilus* isolates only showed minimal auxin production. *B. megaterium* exhibited the highest level of auxin production among the three isolates tested. Additionally, this particular isolate exhibited a significant growth-promoting effect on both the roots and the shoots of Suakoko 8 when compared to the non-inoculated, iron-stressed plants. However, among the three isolates tested, *B. megaterium* is the one that showed the least effect on the tolerance of lowland rice cultivars against iron toxicity overall experiments. Auxin is therefore unlikely to be involved in the signaling cascades triggered by *Bacillus* inoculation. Another important plant hormone, which plays a pivotal role in the plant's response to both abiotic and biotic stresses, is ethylene, the smallest among the plant hormones. When plants face stresses from the environment or pathogens, they produce more ethylene. This results in growth reduction. Through enzymatic action of bacterial ACC deaminase ethylene level in plants can be reduced, which consequently lessens growth constraints under stress. However, none of the three *Bacillus* isolates displayed any ACC deaminase activity. Furthermore, no correlation was found between plant size and leaf symptom scores in plants grown under iron toxicity. Taken together, these results suggest that the effects of *Bacillus* inoculation on iron toxicity tolerance are not related to plant growth promotion. Moreover, they highlight the limitations of culture-based methods for identifying new bacterial isolates for use in sustainable agriculture.

5.5 General Remarks on the Methodology

Hydroponic systems in general have their limitations when studying plant responses to nutrient deficiencies or toxicities. First of all, root growth in a hydroponic system is different than in soil, which can directly influence nutrient uptake. In a paddy rice field, the oxidation power of rice roots greatly affects the geochemical conditions of the rhizosphere, as well as the bulk soil. This also has great impact on the composition of the microbial community. Oxidized iron acts as an electron acceptor for microorganisms under anaerobic conditions of paddy rice fields. During the experiments described in the previous chapters, the nutrient solution was percolated with N_2 during Fe treatment in order to prevent oxidation of Fe (II). However, this possibly disturbs the natural oxidation zone in close proximity to the root surface. This oxidation zone differs between cultivars depending on the radial oxygen loss (ROL) of their roots (Onyango et al., 2020). Excluder cultivars which depend on the oxidation of Fe^{2+} on the root surface might not perform as well as in soil. Furthermore, the effects of bacteria inoculation on tolerance mechanisms involving Fe^{2+}oxidation in the rhizosphere will be ineffective. Although it has been shown that in hydroponics, plants dictate their microbiome composition (Lobanov et al., 2022), under field conditions, many factors, such as soil type, geographical location and cultivation practice also play a role (Edwards et al. 2014). Therefore, under field conditions, effects of inoculation with the three *Bacillus* isolates might differ from the ones described here.

5.6 Outlook

The application of bacterial and fungal inoculants as biofertilizers and biocontrol agents is often praised as the solution to closing yield gaps in an environmentally friendly, sustainable way. Numerous commercial products are already on the market. However, many of them fail to perform under field conditions (O'Callaghan et al., 2022). One of the reasons being the lack of knowledge on the underlying mechanisms of genotypic interaction between crop cultivars and specific microorganisms. Making use of microorganisms in sustainable agriculture can only be successful if mechanisms underlying plants stress tolerances are fully understood. Especially in the case of iron toxicity with genotypic differences and spatiotemporal changes in adaptation mechanisms, there are still large knowledge gaps which need to be filled.

Furthermore, inoculants consisting of single bacteria isolates might never fulfil their expectations for practical use in sustainable agriculture, because they fail to establish within the naturally given microbiome and many beneficial effects on plant performance have been shown to be dependent on the interaction between different bacterial and fungal isolates. As a result, current research often focuses on studying the effects of beneficial consortia (O'Callaghan et al., 2022), or even the development of whole new microbiomes (Mueller et al., 2021). Modern deep sequencing techniques and computational models will facilitate the identification of key organisms within a community context. New breeding strategies, which take into consideration plant traits responsible for the acquisition of a beneficial microbiome, could also accelerate the development of more tolerant cultivars. However, microbiomes greatly differ depending on environmental factors such as soil type and climatic conditions. The ability of single species to adjust to climate change is also dependent on interactions between microbial populations within the community structure (Jansson et al., 2020). Microbial growth as well as community structure can be affected by higher temperatures, elevated CO_2 levels of the atmosphere, and changes in precipitation (Cavicchioli et al., 2019). Interaction between plants and their microbiomes might also be affected by climate change due to plants changing their physiological responses to environmental stresses (Stuckenbrock and Gurr, 2023). Efforts are under way to predict changes in the metagenome of the soil microbiome under different climatic conditions (Jansson et al., 2019), and it will be essential to also model the effects of such changes on plant stress tolerance. Understanding of genotypic stress responses and possible effects of the microbiome on such responses will be essential for the development of more accurate models.

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6 General Conclusions

The results achieved within the framework of this thesis not only show that *Bacillus* inoculation can affect the tolerance against iron toxicity in lowland rice, they strongly emphasize the importance of genotypic interaction between cultivar and bacteria isolate. Furthermore, it was shown that different physiological responses were affected. Positive effects of *Bacillus* inoculation on the sensitive cultivar IR31875 were not caused by bacterial plant growth promotion. Instead distinct mechanisms within the shoot tissue were triggered to allow for increased tolerance of high Fe concentrations. It was shown for the first time, that *Bacillus* inoculation can promote the production of ferritin in lowland rice under iron toxicity, possibly through signaling cascades involving NO. Negative effects of *Bacillus* inoculation in the tolerant excluder type cultivar Suakoko 8 on the other hand are most likely caused by an altered distribution of iron within the shoot. Whether iron translocation into the old sheaths is disturbed or whether too much iron is transported back from old sheaths into new tissues needs to be further investigated. Severity of brown spot disease was lowered in *Bacillus* inoculated plants which could partially be attributed to direct inhibitory effects on fungal growth. However, changes in iron homeostasis, such as the promotion of ferritin production might represent overlaps of effects of *Bacillus* inoculation on responses to iron toxicity and response to *Bipolaris oryzae* attack.

Inoculation with single bacteria isolates is a useful tool for studying plant responses to various abiotic and biotic stresses under controlled conditions and the variation in these responses caused by bacteria action. Knowledge gained from such studies will be useful for future breeding efforts.