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16

RHIZOSPHERE BACTERIA *BACILLUS* STRAINS IN MITIGATION OF BIOTIC AND ABIOTIC STRESSES IN RICE UNDER OXIC AND ANOXIC CONDITIONS

T.T.H. Le[1], J. Padgham[2], S.T. Fornies[3], Hartmann, J.[1] and F. Asch[1]

[1]University of Hohenheim, Stuttgart, Germany Institute of Crop Production and Agroecology in the Tropics and Subtropics Crop Waterstress Management in the Tropics and Subtropics
[2]International START Secretariat, 2000 Florida Ave NW, Washington, DC 20009
[3]Bundesanstalt für Landwirtschaft und Ernährung, 53179 Bonn, Germany
**Email:huong@uni-hohenheim.de*

ABSTRACT

Rice is the single most important staple crop in the world, especially in Asian countries where it accounts for more than 90% (450.6 million tons) of the world production (FAO, 2013). However, rice production systems suffer from a multitude of constraints. The rice root-knot nematode, Meloidogyne gramicola is an important pest in several rice growing areas in Asia while iron toxicity, caused by the excessive ferrous iron (Fe^{2+}) in the soil, is one of the major environmental factors limiting production of lowland rice worldwide. The use of microorganisms to tackle pest and disease problems or nutrient disorders in crop production is not new. However, studies on microbes that have mitigation effects on both abiotic and biotic stresses are not well documented. Furthermore, the rice plant, with its intermittent growth stages under both anoxic and oxic condition requires specific antagonists that can survive and thrive under both conditions.

Several Bacillus strains, isolated from seeds and roots of rice have demonstrated antagonistic activities against the rice root-knot nematode Meloidogyne graminicola. Treatment with Bacillus bacteria under oxic conditions in greenhouse reduced galling severity caused by of M. graminicola by up to 30%. Studies on the modes of action of the isolate Bacillus megaterium against M. graminicola revealed that the bacteria reduced nematode penetration and host finding ability.

When subjected to high iron concentration (Fe^{2+}, 1000 mg/L) in the hydroponic solution, rice seedlings inoculated with Bacillus strains showed lower level of iron toxicity compared to non- treated plants. Application of Bacillus bacteria also reduced leaf Fe content and altered Fe partitioning in plant tissues. The bacterial *isolates clearly showed a differentiated interaction with the individual rice genotypes. The mechanisms of the bacteria and their metabolites alone or in combination with specific rice genotypes that lead to the observed positive effects are being investigated by a series of morphological (growth rate, visual scoring, iron plague) and physiological (phytohormone synthesis, enzyme activities and antioxidant levels) assessments.*

Key words: Root-knot nematode, *Meloidogyne graminicola*, iron toxicity, leaf scoring, iron partitioning.

INTRODUCTION

Rice is the single most important staple crop in the world providing food for more than half of the world population. Irrigated and rainfed low land rice are the most common production systems in Asia, covering more than 90% of rice cultivation area while upland rice constitutes 60% of the rice area in Africa (FAOSTAT, 2009).

These rice production systems suffer from a multitude of constraints that are often related to the cropping system as such, climatic, and geographic conditions. Many species of nematodes are associated with rice but only a few are considered as economically important including the rice root-knot nematode *Meloidogyne graminicola* (Bridge et al. 2005) that has been reported to cause significant yield losses of 20-50% in many upland rice production areas. The nematode possesses the capacity to infect, survive, and re-infect the rice root as soils fluctuate between oxic and anoxic states (Bridge et al. 2005). Different management strategies have been used to control the nematode but the degree of success is usually limited and may vary substantially due to site specific and management and resource limitations (Bridge et al. 2005). In the past few decades, biological control has been considered as a promising alternative to expensive and toxic nematicides, limited and inadequate cultural control practices and the lack of resistant varieties (Sikora 1992).

Iron toxicity is a major nutrient disorder of lowland rice which affects about 55% of rice growing areas worldwide. It is frequently reported to cause significant yield loss ranging between 15-30% and can lead to complete crop failures under severe conditions (Becker & Asch 2005). Iron toxicity occurs when rice takes up excess amounts of ferrous iron (Fe^{2+}). This can damage cell components and cell membrane and impair essential physiological processes due to the accelerated production of free radicals (Thongbai & Goodman 2000).

The rice plant has developed several adaptation strategies to cope with and to survive severe iron toxic environments namely 1) exclusion/ avoidance; 2) inclusion/ avoidance and 3) inclusion/ tolerance (Becker & Asch 2005). However, these strategies are dependent on genotypes, plant developmental stages and climatic and geographic conditions. Among the currently available management options, employing ferrous iron-tolerant cultivars is considered the most cost-effective measure but the genotypic mechanisms of iron toxicity tolerance remain unclear up to date (Engel et al. 2012).

The beneficial effects of root-associated bacteria in biologically controlling soil borne pathogens have been well established. Many bacterial species have been evaluated for their antagonistic activity against a wide range of plant parasitic nematodes and their modes of action have been demonstrated: parasitism, interference with nematode-host recognition, competition for nutrients and induced systemic resistance (Sikora & Hoffmann-Hergarten 1993; Hasky-Günther et al. 1998; Siddiqui & Shaukat 2002). In many cases, antibiotics or the toxic secondary metabolites produced during fermentation processes show nematicidal activity.

Conversely, little is known about how these beneficial microorganisms affect responses of plants to iron stresses as well as their activity under anoxic conditions. During the course of biological studies of the rhizobacteria against *M. graminicola*, the *Bacillus* strains endemic to rice displayed an ability to grow under anaerobic conditions (Padgham & Sikora 2007). This finding, in combination with the current knowledge on the multiple effects of root associated bacteria on plant health (see reviews from Dimpka et al. 2009 and Rejeb et al. 2014) suggested an evaluation to see whether these root-associated bacteria could be used to mitigate the effects of iron toxicity symptoms in lowland rice. The present paper aims to demonstrate the multiple effects of the rhizosphere *Bacillus* strains on the rice root-knot nematode and the tolerance of rice under iron toxicity conditions.

MATERIALS AND METHODS

Biocontrol of Bacillus strains toward M. graminicola under oxic conditions

Four bacterial strains, *Bacillus megaterium*, *B. subtilis* and two isolates of *Bacillus* sp. isolated from seminal roots of Bangladesh and Taiwanese *Oryza sativa indica* lowland rice genotypes were tested for their biocontrol activity against *M. graminicola*.

The rice variety BR11, an irrigated and susceptible rice variety to *M. graminicola* from Bangladesh was used in this experiment.

The *Bacillus* strains were cultured on TSA (Tryptic Soy Agar) for 24 hours. The bacteria were collected by scraping bacterial biomass with a sterile Drisgalski spatula and mixed with 1 ml sterilized methyl cellulose (2%). Surface sterilized seeds (95% ethanol, 30 s and 2.5% NaOCl, 10 min) were coated with the bacterial substrate for 30 min and then planted in experimental pots containing 250 cm^3 sterilized sandy soil (v/v=2:1) with Yoshida nutrient solution at pH 5 as a nutrient source (Yoshida 1976). The average concentration of *Bacillus* strains for inoculation was approximately 10^7cfu per seed, as determined by dilution plating. The seedlings were grown under greenhouse conditions (temperature 27^{o}C ± 5, 15h light period) in the Section of Nematology in Soil Ecosystems, Phytomedicine, INRES, University of Bonn.

Newly hatched second stage juveniles (J2) of *Meloidogyne graminicola* were introduced around the root zone at a density of 500 J2 per seedling at 2 weeks after planting. Ten days later, the experiment was harvested and the number of galls per gram of root was determined.

To investigate the modes of action of the rhizobacteria toward *M. graminicola*, only the isolate *B. megaterium* was selected. The experiment was carried out in the same manner as the first experiment, but the infection with nematodes was evaluated by both galling severity and nematode penetration, which was determined by staining the rice roots with acid fuchsin 1% and then counting the nematode inside the root using a binocular.

Mitigation of Bacillus strains against iron toxicity

Three *Bacillus* strains (*B. megaterium* and two *Bacillus* strains) were investigated for the interaction with six contrasting rice genotypes ITA 306, ITA 320, TOX 4004 -8-1-2-3, IR 31785-58-1-2-3-3, WITA 7, I Kong Pao under iron toxicity conditions. The genotypes TOX 4004 -8-1-2-3 (in short TOX 4004), IR 31785-58-1-2-3-3 (in short IR 31785) have been reported as tolerant and sensitive cultivars to iron respectively (Engel et al. 2012).

Rice was grown in a hydroponic system consisting of PVC tubes of 3.6cm of diameter and 9 cm length, assembled in a unit of 6x10 by melting the contact-side borders with a soldering iron. These assemblies were placed in a rectangular box measuring 26.5x37cm (6.5 L). In the upper part of each 9 cm long pipe, a half-split ceapren plug (3.5 cm diameter x 3 cm long from Greiner, Germany) was inserted in order to fix each plant individually. The rice roots protruded from the ceapren plug, allowing only the root to be in contact with the nutrient solution.

Rice seeds were pre-germinated on wet tissue paper before planting on washed fine sand for 7 days. The rice seedlings were then transferred to the hydroponic system containing quarter strength Yoshida nutrient solution. Bacteria were applied to the nutrient solution when plants were 3 weeks old as described by Padgham and Sikora (2007). The final concentration of bacteria in the nutrient solutions was approx. $5x10^6$cfu/ml.

Seven days after bacterial inoculation, the nutrient solution was replaced by the respective Fe-treatments (Fe^{2+}, 0 and 1000 mg L^{-1}) for 6 days. Two gas diffusers (3x25cm from Hobby Dohseaquari stik KG) were placed on the bottom of each box. Nitrogen gas was percolated through the nutrient solution for 15 minutes every 2 hours in order to prevent the oxidation of Fe^{2+} to Fe^{3+}.

Iron toxicity assessments

Five days after Fe application, plants were visually scored for the iron toxicity level by assessing the percentage of leaf area affected by bronzing. The leaf scoring ranged from 0 to 10 which 0 means no symptom while 10 means 100% affected, based on the "Standard Evaluation System" for leaf blast (*Pyricularia oryzae*" lesion from IRRI (Engel et al. 2012).

The experiment was harvested after 6 days and leaf tissues were destructively sampled for tissue iron analyses using high pressure acid digestion and AAS (Atomic Absorption Spectroscopy) (Engel et al. 2012).

Statistical analysis

All data were subjected to analysis of variance and mean treatment differences were estimated using a t-test. Mean comparisons were analyzed by Duncan Multiple Range Test when there were more than 2 treatments.

RESULTS

Biocontrol of Bacillus toward M. graminicola under oxic conditions

All four *Bacillus* isolates reduced galling severity caused by *M. graminicola* from 15-30% compared to the non-treated rice roots (Table 1).

Table 1. Effect of *Bacillus* application on root galling of rice seedlings caused by *M. graminicola.*

Treatment	Percent galling reduction (from control)
Bacillus sp. 1	15
Bacillus sp. 2	19
B. megaterium	26
B. pumilus	30

Further investigation revealed that the isolate *B. megaterium* reduced galling severity and nematode penetration significantly by 41% and 45% respectively (Fig. 1).

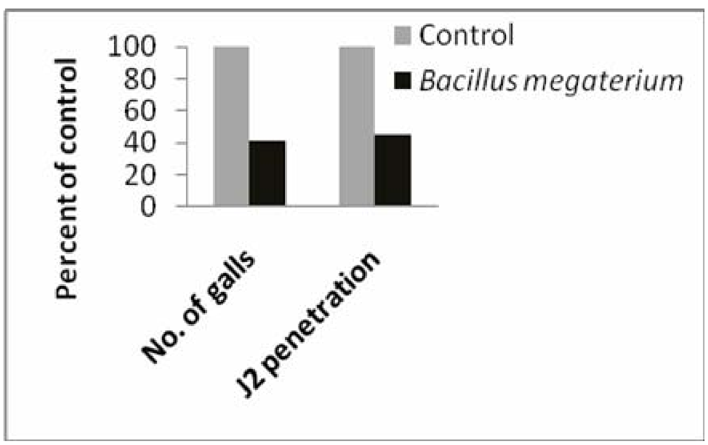

Fig 1. Effect of *Bacillus megaterium* application on root galling and J2 penetration of *Meloidogyne graminicola* in three weeks old rice seedlings.

Mitigation of Bacillus strains against iron toxicity under anoxic condition

On average, three bacterial strains significantly reduced leaf symptom score in all rice varieties. However, total Fe uptake was increased in all bacterial treated plants under induced iron toxic conditions (Fig. 2). There was a clear interaction between some bacteria and rice genotypes in this experiment. For example, Fe uptake increased in all bacterial treatments with two rice genotypes I Kong Pao and IR 31785 while the bacteria *Bacillus* sp. 2 only reduced total Fe uptake in the variety ITA 320 (Fig. 2).

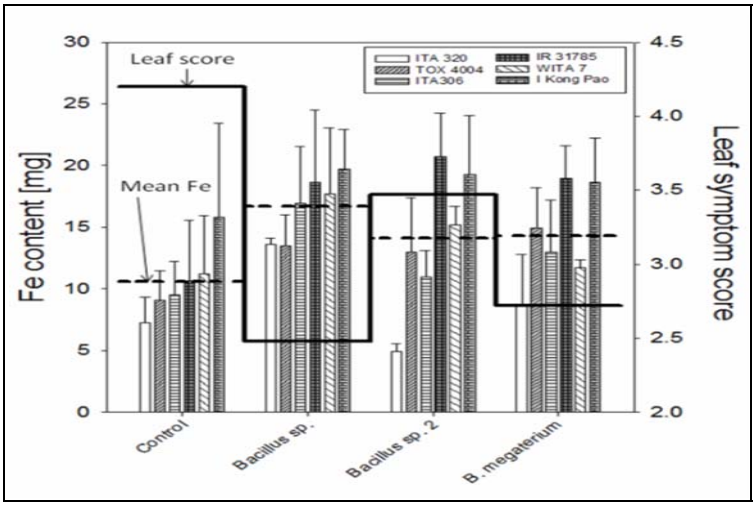

Fig 2. Leaf symptom score and total Fe uptake of 6 rice genotypes subjected to 1000 mg L^{-1} Fe treatment in combination with the application of four rhizobacteria *B. megaterium* and two *Bacillus* strains. The solid line indicates the mean symptoms score and the dotted line indicates the mean tissue Fe content across varieties. Error bars=Standard error of mean with n = 5.

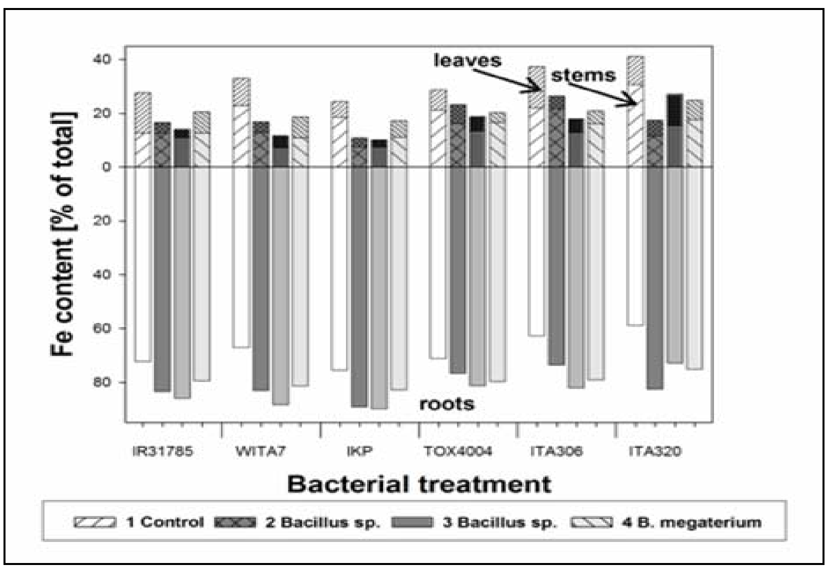

Fig 3. Iron partitioning of rice seedlings subjected to three *Bacillus* treatments under induced iron toxicity conditions (0 and 1000 mg $Fe^{2+}L^{-1}$).

It was shown that the bacterial inoculations strongly altered iron partitioning in rice plant tissues. In general, bacterial applications increased root Fe retention while reduced iron uptake in the upper plant tissues (stem and leaf). Importantly, leaf Fe concentration was reduced in all rice varieties treated with the isolate *B. megaterium* (Fig. 3).

DISCUSSION

Treatment of rice plants with the rhizobacteria *Bacillus* resulted in reduction of galling formation by up to 30% and penetration by 45% compared with non-treated rice roots. The results demonstrated a biological potential of these bacteria against *M. graminicola*. Mode of action studies of the isolate *B. megaterium* revealed its ability to reduce nematode mobility and inhibit egg hatching (Padgham & Sikora 2007). Similar biocontrol activity and modes of action of the rhizobacteria *Bacillus* strains were also demonstrated in Basmati 370 rice cultivar (Pankaj et al. 2010). Moreover, some *Bacillus* strains displayed induced systemic resistance of plant when colonized the rhizoplane (Siddiqui & Shaukat 2002). These studies underline the great biological control potential of the rhizobacteria against the root-knot nematode in rice.

In the present iron toxicity study, all *Bacillus* isolates significantly reduced iron toxicity symptoms, indicating an overall migration effect. The *B. megaterium* strain also reduced leaf Fe content significantly in all cultivars. It has been known that high concentration of Fe (II) in the leaf enhances production of ROS (reactive oxygen species) which leads to irreversible destruction of cell membranes and cell functions and thus adversely affects photosynthesis (Thongbai & Goldman 2000). Therefore, leaf symptom score and leaf Fe content can be combined to identify avoidance mechanism as illustrated by Engel et al. (2012).

Next to reduction in leaf symptom scores, the rhizobacteria also caused changes in total iron uptake and altered iron partitioning. With the two sensitive rice cultivars IR 31785 and I Kong Pao, bacterial colonization resulted in significantly higher Fe uptake compared to the other varieties received the same bacterial treatments. Although iron uptake was increased in all treatments, the symptom of iron toxicity was milder in bacterial treated plants because most of the iron uptake was retained in root tissue, suggesting an inclusion/avoidance mechanism mediated by the bacterial inoculation (Becker & Asch 2005; Engel et al. 2012).

Rhizobacteria, including *Bacillus* species have been frequently reported to promote plant growth, increase water and nutrient uptake as well as enhance plant tolerance or resistance against pests and pathogens. The beneficial effects are enabled through production of secondary metabolites containing phytohormones, antioxidant enzymes and antioxidant molecules, changes in gene expression or induced systemic resistant of the host plant (Glick 2012; Dimpka et al. 2009) suggested that induction of systemic resistance in the host plant may be essential for the cross-tolerance between abiotic and biotic stress.

Similar to plants, iron plays an important role in metabolic activities of bacteria and iron homeostasis must be maintained. Rhizobacteria also produce siderophores that can chelate iron (III) in iron deficient environments or ferritin-like proteins (bacterioferritins, miniferritin and DNA protection) for iron sequestration under iron toxic conditions. In addition, important oxidative stress enzymes such as catalase, superoxide dismutase (SOD), peroxidase (POD) and polyphenol oxidases are found in bacterial metabolites and their roles are believed to be similar to those in plant (Cabiscol et al. 2000). Therefore, research on the physiological, morphological and molecular changes mediated by the rhizobacteria in rice under iron toxic environment is being undertaken in the Crop Water stress Management in the Tropics and Subtropics (University of Hohenheim, Germany) to understand the mechanisms through which the bacteria positively affect the rice plant. The present research not only demonstrated a potential of using rhizobacteria for the mitigation of both abiotic and biotic stresses in plants but also suggested that these root associated bacteria can be effectively used under anoxic environments.

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