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Lotus japonicus plants of the Gifu B-129 ecotype subjected to alkaline stress improve their Fe^{2+} bio-availability through inoculation with *Pantoea eucalypti* M91

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ABSTRACT

Inoculation assays with *Pantoea eucalypti* M91 were performed on *Lotus japonicus* ecotype Gifu. Under alkaline conditions, this ecotype is characterized by the development of interveinal chlorosis of the apical leaves due to low mobilization of Fe^{2+}. Inoculation with *P. eucalypti* M91, a plant growth-promoting bacterial strain capable of producing pyoverdine-like and pyochelin-like siderophores under alkaline growth conditions, alters the root, resulting in a herringbone pattern of root branching. Additional features include improvement in Fe^{2+} transport to the shoots, acidification of the hydroponic solution of the plant cultures, and an accompanying increase in the efficiency of the PSII parameters. In addition, there was an increase in the expression of the *FRO1* and *IRT1* genes, accompanied by a significant increase in FRO activity. Results showed that *P. eucalypti* M91 has a beneficial effect on the Fe acquisition machinery of Strategy I, as described for non-graminaceous monocots and dicots, suggesting its potential as an inoculant for legume crops cultivated in alkaline soils.

1. Introduction

Calcareous soils contain high concentrations of HCO_3^- and CO_3^{2-} that increase soil pH and limit Fe solubility. Plants require Fe for the proper functioning of multiple metabolic and enzymatic processes, such as those related to oxygen and electron transport, nitrogen fixation, DNA and chlorophyll biosynthesis, and photosynthesis (Eichert et al., 2010; Ivanov et al., 2012). Fe deficiency therefore induces certain conditions, particularly in young leaves, including interveinal chlorosis, which results from low concentrations of photosynthetic pigments, chlorophylls, and carotenoids (Abadia et al., 2000). As a result, photosynthetic rate, as well as light absorption, photosystem II (PSII), and RuBisCo efficiencies are markedly reduced (Larbi et al., 2006). These alterations have been reported previously in model and crop species of the *Lotus* genus (Babuin et al., 2014; Paz et al., 2012). Fe deficiency is a serious economic problem that severely affects crop quality and yields and requires expensive corrective methods to resolve. However, there is also an inexpensive, environmentally friendly biotechnological solution in which plants are inoculated with growth-promoting bacterial strains (Eichert et al., 2010).

Plants have developed several strategies to cope with Fe deficiency. In Strategy I, which occurs in non-graminaceous monocots and dicots, several physiological and morphological processes are used to increase Fe uptake (Ramírez et al., 2008; Romera et al., 2011; Ivanov et al., 2012; Wang et al., 2012). First, the surround-

Abbreviations: PSII, photosystem II; FRO, ferric reductase oxidase; IRT, iron regulated transporter; PGPB, plant growth promoting bacteria; CAS, chrome-azurol S; RFP, red fluorescent protein; DW, dry weight; PI, performance index; TT, topological tendency; RU, relative units; ANOVA, analysis of variance; VOC's, volatile organic compounds; IAA, indole-3-acetic acid.

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ing rhizosphere is acidified via proton extrusion by a root plasma membrane-localized proton ATPase in order to increase the solubility of Fe^{3+} complexes, after which Fe^{3+} is reduced to Fe^{2+} by ferric reductase oxidase 1 (FRO1). Subsequently, Fe^{2+} ions are taken up into root cells by the divalent iron regulated transporter 1 (IRT1) (Jain et al., 2014). Resulting morphological modifications include changes in root architecture, with enhanced development of lateral roots and differentiation of specialized transfer cells for increased surface area and improved performance (Guerinot and Yi, 1994). Meanwhile, Strategy II, or the chelation strategy, applies to graminaceous plants (Singh et al., 2013). In this case, extruded phytosiderophores chelate Fe^{3+}, after which the resulting complexes are imported by the surrounding roots (Guerinot and Yi, 1994; Romera et al., 2011; Ivanov et al., 2012). In nature, plants which are unable to adopt either of these strategies, or which have acquired them only partially, are classified as Fe-inefficient plants, and eventually develop interveinal chlorosis of the apical leaves in environments with low Fe content (Bacaicoa and García-Mina 2009; Von Wirén et al., 1993).

A wide variety of bacterial species broadly classified as Plant Growth Promoting Bacteria (PGPB) are capable of colonizing the rhizosphere and activating a mechanism similar to that of graminaceous plants growing in calcareous soils. Under the appropriate conditions, they synthesize and release siderophores, increasing and regulating Fe bio-availability. As a result, these PGPBs promote plant growth, improve the nutrition of the host plant, and protect it from biotic and abiotic stress factors (Zhang et al., 2009; Castagno et al., 2011; Zamioudis et al., 2013; Castagno et al., 2014; Jin et al., 2010). As a result, their role in plant nutrition has been studied in-depth (Crowley et al., 1988). Furthermore, many PGPBs exert other beneficial effects on plants, since they are able to solubilize phosphates and other micronutrients (Radzki et al., 2013). For example, *Pantoea eucalypti* M91 was isolated from alkaline soils and showed the ability to solubilize phosphates and Fe, serving as a PGPB for *Lotus tenuis* under both greenhouse (Castagno et al., 2011) and outdoor conditions (Castagno et al., 2014).

Lotus japonicus and *L. tenuis* are both legume species of the genus *Lotus*. *L. japonicus* is primarily of interest as a model plant for physiological and genetic studies, particularly those relating to the mechanisms involved in stress tolerance (Escaray et al., 2012; Bordenave et al., 2013; Babuin et al., 2014). In particular, *L. japonicus* is most commonly used in the laboratory as the Gifu B-129 (Gifu) or Miyakojima (MG-20) ecotype. Previous work has shown that they exhibit contrasting behavior under similar alkaline stress conditions, with MG-20 being tolerant and Gifu being sensitive (Babuin et al., 2014). To corroborate this observation, we adopted similar criteria, which have been valuable in evaluating other *Lotus* species under salt stress (Sanchez et al., 2011). Klein et al. (2012) also confirmed comparatively better performance of the MG-20 ecotype, in that case under low Fe concentrations. Considering these results, the principal aim of this research was to evaluate Fe^{2+} uptake by the Gifu ecotype in the presence of *P. eucalypti* M91, in the hopes of developing strategies for improved nutrition under alkaline stress.

2. Materials and methods

2.1. Bacterial strain, growth conditions, and siderophore production

P. eucalypti M91 (Castagno et al., 2011) was grown in TY media (Sperry and Wilkins, 1976). Siderophore production was determined in Chrome-azurol S (CAS) media as per the Universal Chemical Assay (Schwyn and Neilands, 1987). A bacterial suspension that was cultured for 24 h was spotted on a CAS plate and incubated at $28 \pm 1\,^{\circ}C$ for 48 h. The production of siderophores was indicated by the formation of an orange to yellow halo around the colonies. The siderophore types were assessed in the supernatant of samples that had been cultured for 72 h in minimal media (Carrillo and Peralta, 1988) containing either no Fe source or an $FeCl_3$ supplement (6 or 60 μM). The absorbance spectra were recorded using a Synergy H1 multi-mode reader, and revealed the presence of two maxima (355 and 405 nm) that correspond to pyochelin (Xiao and Kisaalita, 1995) and pyoverdine (Yang et al., 2011). The fluorescence emission spectra revealed maxima at 430 and 460 nm for excitation wavelengths of 355 and 405 nm, respectively, values that are consistent with pyochelin and pyoverdine (Yang et al., 2011; Dumas et al., 2013).

2.2. Plant material, plant growth conditions, and experimental design

Seeds of the *L. japonicus* Gifu ecotype were scarified with sulfuric acid (98%), washed in distilled water, and placed in Petri dishes containing water-agar (0.8%). The dishes were incubated in a growth chamber until cotyledons were observed using a 16/8 h photoperiod at 24/19 °C and 60/80 ± 5% relative humidity under fluorescent light bulbs providing 200 μmol photons $m^{-2}\,s^{-1}$. The same environmental conditions were used for all the treatments described below. During these treatments, plants were irrigated with half-strength Hoagland's nutrient solution (Hoagland and Arnon, 1950) composed of 3 mM KNO_3, 2 mM $Ca(NO_3)_2{\cdot}4H_2O$, 1 mM $MgSO_4{\cdot}7H_2O$, 0.5 mM $NH_4H_2PO_4$, 50 μM $NaFeO_8EDTA{\cdot}2H_2O$, 4.5 μM $MnCl_2{\cdot}4H_2O$, 23 μM H_3BO_3, 0.16 μM $CuSO_4{\cdot}5H_2O$, 0.09 μM $ZnSO_4{\cdot}7H_2O$, and 0.06 μM $Na_2MoO_4{\cdot}2H_2O$.

To determine total Fe content, gene expression, and root morphology, seedlings were transferred to their individual cylindrical pots (5.8 × 20 cm; v = 0.53 dm^3) containing sand that had been autoclaved twice. Experiments were completely randomized and tested, with four sets of treatments for control, alkalinity, control + inoculation, and alkalinity + inoculation. Alkalinity trials included 10 mM $NaHCO_3$ (pH 8.3). Inoculation with a *P. eucalypti* M91 bacterial suspension (1×10^8 cells per mL) was done during the transplant period. All seedlings were first irrigated for 1 week, followed by 3 week under the respective treatment. The irrigation system was as described by Paz et al. (2012).

For Fe^{2+} content, growth promotion, acidification of the hydroponic solution, and root FRO activity measurement, seedlings that had been cultivated in sand and irrigated with the nutrient solution for 15 days were removed and their roots rinsed with sterile deionized water, after which each seedling was transferred to a beaker containing 150 mL of sterile, half-strength Hoagland's nutrient solution. The nutrient solution was replaced every 3 days. The roots were protected from light by wrapping the beakers in black paper. All seedlings were grown for 1 week, followed by 2 week under the respective treatment. The bacterial suspension was added to the nutrient solution at the beginning of the solution culture and refreshed whenever the nutrient solution was replaced.

2.3. Evaluation of endophyte colonization capacity

In order to evaluate the endophytic bacteria on the roots and shoots of the plants, *P. eucalypti* M91 was designed to express the red fluorescent protein (RFP). The traceable strain was generated by four-parental mating conjugation, using the donor strain *Escherichia coli XL1-Blue* containing the plasmid *pBK-miniTn7*-Plac-rfp-GenR (Koch et al., 2001). In order to evaluate endophytic colonization, epifluorescence microscopy was used. During harvest, plants were divided into roots and shoots. Samples were analyzed with a Nikon Eclipse E600 microscope equipped with a