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Research article

Characterization of plant growth promoting traits of bacterial isolates from the rhizosphere of barley (*Hordeum vulgare* L.) and tomato (*Solanum lycopersicon* L.) grown under Fe sufficiency and deficiency

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ABSTRACT

Plant Growth Promoting Bacteria (PGPB) are considered a promising approach to replace the conventional agricultural practices, since they have been shown to affect plant nutrient-acquisition processes by influencing nutrient availability in the rhizosphere and/or those biochemical processes determining the uptake at root level of nitrogen (N), phosphorus (P), and iron (Fe), that represent the major constraints for crop productivity worldwide. We have isolated novel bacterial strains from the rhizosphere of barley (*Hordeum vulgare* L.) and tomato (*Solanum lycopersicon* L.) plants, previously grown in hydroponic solution (either Fe deficient or Fe sufficient) and subsequently transferred onto an agricultural calcareous soil. PGPB have been identified by molecular tools and characterized for their capacity to produce siderophores and indole-3-acetic acid (IAA), and to solubilize phosphate. Selected bacterial isolates, showing contemporarily high levels of the three activities investigated, were finally tested for their capacity to induce Fe reduction in cucumber roots two isolates, from barley and tomato plants under Fe deficiency, significantly increased the root Fe-chelate reductase activity; interestingly, another isolate enhanced the reduction of Fe-chelate reductase activity in cucumber plant roots, although grown under Fe sufficiency.

1. Introduction

In the last years much attention has been paid to the solution of a very important world issue: how to feed the growing world in a sustainable way (Zhang et al., 2010). The exponentially increasing human population is coupled to an increased use of chemical fertilizers aimed at enhancing crops productivity. However, the excessive use of these unsustainable approaches has a negative impact on human and environment health (Glick, 2012). Thus, the exploitation of the intrinsic biological potential of soil ecosystem might represent a sustainable solution. The employment of Plant Growth Promoting Bacteria (PGPB) (Bashan and Holguin, 1998) is considered a promising approach to replace the conventional agricultural practices, in terms of chemical fertilization and control of pathogenic agents (Bashan, 1998). Several studies demonstrated the different effects of these microorganisms on the abiotic and biotic mechanisms of soil and plants ecosystem (Saharan and Nehra, 2011). PGPB can influence, both directly and indirectly, plant growth and root development, through different activities, which can occur either individually or synergistically (Pii et al., 2015a). Indeed, PGPB have been shown to affect plant nutrient acquisition processes by influencing nutrient availability in the rhizosphere and/or those biochemical processes governing nutrients uptake at root level. PGPB enhance the uptake of different nutrients, like nitrogen (N), through nitrogen fixation (Zahran, 1999), or potassium, by solubilization of immobilized forms in the soils (Parmar and Sindhu, 2013). In addition, they are involved in the increasing of iron (Fe) uptake, that along with phosphorus (P) and N, represents one of the major constraints for crop productivity worldwide (Schachtman et al., 1998; Zhang et al., 2010). Iron has two stable oxidation states, Fe^{III} and Fe^{II}, with the last form being more soluble than the other one. However, in the presence of oxygen, Fe^{II} is rapidly oxidised to Fe^{III} that might precipitate, having a pH-dependent solubility (Mimmo et al., 2014). Since the majority of

Abbreviations: PGPB, plant growth promoting bacteria; B, barley; T, tomato; FS, Fe sufficiency; FD, Fe deficiency; FCR, ferric chelate reductase.

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agricultural soils in the Mediterranean area features an alkaline pH, the bioavailable Fe fraction results lower than that required for an optimal plant growth. Under these Fe-limiting conditions, PGPB might increase the mobility and bioavailability of insoluble Fe by the production of low molecular weight organic compounds, called microbial siderophores (MSs). They chelate Fe with high affinity, making it available for both themselves and plants (Colombo et al., 2013). There are some pieces of evidence showing that several microorganisms, such as *Pseudomonas fluorescens* (Saravanakumar and Samiyappan, 2007), *Pseudomonas chlororaphis* (Sharma and Johri, 2003) and *Azotobacter vinelandii* (Kraepiel et al., 2009), are able to improve Fe uptake in plants by MSs production.

PGPB play also an important role in the P assimilation pathway. In general, the content of P in soil is quite large, but the monobasic $H_2PO_4^-$ (P_i), which is the predominant form absorbed by roots, is very reactive and precipitates readily with Ca in calcareous soils (Schachtman et al., 1998), and with Fe^{3+} and Al^{3+} in acidic soils (Mehta et al., 2014). PGPB solubilize and mobilize inorganic and organic P by the release of organic acids (ligand exchange reactions) (Kaur and Reddy, 2013) and phosphatase enzymes (Sharma et al., 2011), respectively, thus significantly contributing to plants P supply (Calvo et al., 2014).

Furthermore, it is very well known that PGPB possess metabolic pathways for the biosynthesis of phyto-regulators, such as indole-3-acetic acid (IAA), cytokinins (CKs), gibberellins (GAs), and ethylene (ET) that may affect the morphogenetic processes in plants. In particular, IAA is a plant hormone involved in several mechanisms, such as promotion of cell elongation and cell division, apical dominance, root development, differentiation of vascular tissue, ethylene biosynthesis, phototropism (Sachdev et al., 2009; Overvoorde et al., 2010). Several studies demonstrated that the IAA-producing microorganisms are able to regulate root development and morphology (Aloni et al., 2006). With respect to plant mineral nutrition and in particular Fe, it is well known that dicots acquire Fe via an Fe^{III}-reduction-based mechanism involving at the plasmalemma ferric-chelate reductase (FCR) and an Fe^{II}- transporter (IRT) (Marschner, 2011). In this context, it is interesting to note that exogenous application of IAA to plants significantly enhanced FCR activity (Schmidt, 1994; Li and Li, 2004; Chen et al., 2010).

On the base of these premises, in order to identify novel bacterial strains and their potentialities as biofertilizers, microorganisms from the rhizosphere of barley (*Hordeum vulgare* L.) and tomato (*Solanum lycopersicon* L.) plants have been isolated, identified and characterized. To this purpose, plants previously grown in hydroponic solution (either Fe deficient or Fe sufficient) were subsequently transferred to an agricultural calcareous soil (RHIZOtest system, Bravin et al., 2010). Selected bacterial isolates were tested for their PGPR traits in cucumber (*Cucumis sativus*).

2. Materials and methods

2.1. Soil samples

Seeds of barley (*Hordeum vulgare* L. cv. Europa) and tomato (*Solanum lycopersicum* L. cv. Marmande) were soaked for 24 h in an aerated 0.5 mM $CaSO_4$ solution and germinated for 4–5 days in the dark at 22 °C between two layers of filter paper moistened with 0.5 mM $CaSO_4$. Homogenous seedlings were then transferred to 6 L plant tanks containing the nutrient solution using the RHIZOtest system (ISO/CD 16198: 2011, Cirad, France) (Bravin et al., 2010), and were grown hydroponically under controlled conditions in a climatic chamber 14/10 h light/dark, 24/19 °C, 70% Relative Humidity and 250 μmol m^{-2} s^{-1} light intensity. Plants were cultured for 14 days in either Fe-free or Fe-supplemented nutrient solution. The composition of the nutrient solution was as follows: 2 mM $Ca(NO_3)_2$, 0.5 mM $MgSO_4$, 0.7 mM K_2SO_4, 0.1 mM KCl, 0.1 mM KH_2PO_4, 1 μM H_3BO_3, 0.5 μM $MnSO_4$, 0.5 μM $CuSO_4$, 0.5 μM $ZnSO_4$, 0.01 μM $(NH_4)_6Mo_7O_{24}$, 100 μM Fe(III)-EDTA. The nutrient solution was continuously aerated, changed three times a week and the pH was adjusted to 6.0 with 1 N KOH.

After 14 days of hydroponic culture, when the first symptoms of Fe deficiency were visible, a set of Fe-deficient and Fe-sufficient plants was transferred onto an agricultural calcareous soil, for 6 days using the plant-based biotest (RHIZOtest ISO/CD 16198: 2011) (Bravin et al., 2010). The experiments were performed in triplicate. The chemical-physical characteristics of the soil, collected from the South of Italy and used in the experiments are listed as follows: pH_{CaCl2} 7.72 C_{org} 0.86% (w/w), N_{tot} 1.16 g/kg, $CaCO_3$ 61.20% (w/w), CEC 20.40 $cmol_+$/kg, Ca 25.50% (w/w), K 1.18% (w/w), Fe 1.60% (w/w). A polyamide membrane with a 30 μm mesh enabled the physical separation between the root net and the soil. For the 6 days contact period, the soil was moistened at 70% of its water holding capacity (Bravin et al., 2010). At the end of the contact period, equal amounts of the rhizosphere soil coming from three different biological replicates were pooled to generate a unique representative sample (Canfora et al., 2014).

2.2. Isolation of PGPB

Microbial communities were desorbed from soil by sonication and bacteria were isolated by the standard dilution plating technique on Nutrient Agar supplemented with 0.01% (w/v) cycloheximide. Two-hundred colonies for each sample were screened and selected on CAS Agar plates (Schwyn and Neilands, 1987) on the basis of their capability to produce siderophores. The positive colonies were purified by three times subculture method on CAS Agar plates and stored in 20% (v/v) glycerol at −80 °C.

2.3. 16S rDNA gene sequencing and phylogenetic analysis

Genomic DNA extraction was carried out by thermal shock, heating the isolated colonies in 50 μl of distilled water at 95 °C for 15 min. The 16S rDNA gene PCR amplification was performed by the universal primers 357f and R1401, corresponding to the position 341–357 and 1385–1401, respectively, of the 16S rDNA of *E. coli* (Yu and Morrison, 2004). The PCR mix (50 μl) contained 50 pmol of each primer, 10 nmol of each 2′-deoxynucleoside 5′-triphosfate, 3 U of Taq DNA polymerase (EuroTaq; EuroClone), 2.5 mM $MgCl_2$, and 0.5–2 μl of template DNA. All PCR amplifications were performed using a MyCycler™ thermal cycler (Bio-Rad Laboratories Inc.). The 1060 bp amplicons were purified using the Wizard® SV Gel and PCR Clean-Up System kit (Promega) and sequenced from both ends by Eurofins Genomics (Milan, Italy).

16S rDNA sequences were aligned using the BLASTn tool (Camacho et al., 2009) against the NCBI database (www.ncbi.nlm.nih.gov) to identify the bacterial strains. The CRYEMA test (Deshwal and Chaubey, 2014) was also used to discriminate between *Agrobacterium* and *Rhizobium* genera.

The phylogenetic analyses of 16S rRNA sequences were carried out using the Seaview4 interface (Gouy et al., 2010). The sequences were aligned by ClustalW2 tool (Larkin et al., 2007) and the phylogenetic trees were built using the GTR coefficient (Tavare, 1986) and by the maximum-likelihood algorithms of PhyML software (Guindon et al., 2009). The clustering stability was evaluated by the bootstrap resampling method with 100 replicates (Felsenstein, 1985).