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Research article

Plant-microorganism-soil interactions influence the Fe availability in the rhizosphere of cucumber plants

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ABSTRACT

Iron (Fe) is a very important element for plants, since it is involved in many biochemical processes and, often, for the low solubility of the natural Fe sources in soil, plants suffer from Fe – deficiency, especially when grown on calcareous soils. Among the numerous plant growth-promoting rhizobacteria (PGPR) that colonize the rhizosphere of agronomically important crops, *Azospirillum brasilense* has been shown to exert strong stimulating activities on plants, by inducing alterations of the root architecture and an improvement of mineral nutrition, which could result from an enhancement of ion uptake mechanisms as well as by increased bioavailability of nutrients. Some studies have also established that *A. brasilense* can act as biocontrol agent, by preventing the growth and/or virulence of phytopathogens, most likely through the production of microbial siderophores that sequester Fe from the soil. Despite microbial siderophores complexed with Fe could be an easily accessible Fe source for plants, the possible involvement of *A. brasilense* in improving Fe nutrition in plants suffering from the micronutrient deficiency has not been investigated yet. Within the present research, the characterization of the physiological and biochemical effects induced by Fe starvation and PGPR inoculation in cucumber plants (*Cucumis sativus* L. cv. Chinese Long) was carried out. The analyses of root exudates released by hydroponically grown plants highlighted that cucumber plants respond differently depending on the nutritional status. In addition, following the cultivation period on calcareous soil, also the root exudates found in the extracts suggested a peculiar behaviour of plants as a function of the treatment. Interestingly, the presence of the inoculum in soil allowed a faster recovery of cucumber plants from Fe-deficiency symptoms, i.e. increase in the chlorophyll content, in the biomass and in the Fe content of leaves. These observations might suggest a feasible application of *A. brasilense* in alleviating symptoms generated by Fe-limiting growth condition in cucumber plants.

1. Introduction

Soil microorganisms play a central role in the nutrient mineralization and transformation within the rhizosphere (Marschner et al., 2011). As well as plants, they are able to influence the availability of nutrients by modifying the neighbouring environment through the secretion of molecules with solubilizing, chelating, reducing and/or oxidizing capacities. However, the activities of microorganisms in soil are mainly restricted by carbon sources availability (De Nobili et al., 2001; Demoling et al., 2007) and, in this context, plants root exudates might represent an easily accessible source of nourishment for soil bacteria. Therefore, their concentration is much higher in the close proximity of the root than in the bulk soil (Badri et al., 2009; Badri and Vivanco, 2009; Glick, 2012). Beside concentration, also the structure of the rhizosphere microbial community differs from that of the bulk soil. This feature, together with the physical–chemical characteristics of soil, is thought to be the base of plant ability to shape the rhizosphere microbiome (Philippot et al., 2013). It is interesting to note that plants experiencing Fe-deficiency, besides inducing morphological and physiological modifications of their root systems, enhance root exudation in response to the nutritional stress, not exclusively in terms of quantity but also increasing the complexity of the molecular species released (Hinsinger et al., 2003). However, this

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response is highly variable depending on plant species and/or environmental conditions (type of substrate, soil chemical characteristics, temperature, CO_2 concentration and light conditions, Mimmo et al., 2011). Moreover, beside root exudation, dicots induce those activities underlying the Fe acquisition mechanism, namely the Fe(III) reduction carried out by the ferric chelate reductase oxidase (FRO), and the transport of Fe(II) across the PM membrane, that occurs through the iron-regulated transporter (IRT)-like protein (Connolly et al., 2003).

One of the best-studied aspects of the interaction between plant and rhizosphere microorganisms is their ability to induce positive effects on plant growth by enhancing the uptake of nutrients. For instance, rhizobacteria are able of increasing the mobility of Fe by releasing chelating compounds known as siderophores (Saha et al., 2013). Microbial siderophores (MSs) are generally low molecular weight compounds (<1000 Da) and display a very high affinity for Fe^{3+}, showing stability constants ranging from 10^{23} to 10^{52} (Glick, 2012). Several pieces of evidence have shown the direct benefits of MSs on plant mineral nutrition (Nagata et al., 2013; Sharma et al., 2003; Vansuyt et al., 2007), suggesting that the application of plant growth-promoting rhizobacteria (PGPR) as biofertilizers could be a promising fertilization practice.

Azospirillum brasilense is a PGPR naturally able to colonize agronomically important crops as wheat, tomato and strawberry (Bashan et al., 2004; Dobbelaere et al., 1999; Hadas and Okon, 1987; Pedraza et al., 2009). The most evident outcome of *A. brasilense* inoculation in plants is the increased growth of the root apparatus (Creus et al., 2005; Dobbelaere et al., 1999; Hadas and Okon, 1987; Levanony and Bashan, 1989; Molina-Favero et al., 2008; Okon and Kapulnik, 1986), that has been mainly attributed to the bacterial synthesis of bioactive molecules, such as phytohormones (Bashan and De-Bashan, 2010). In addition, the concomitant increase of the root volume and the presence of the bacteria lead to an enhanced content of a wide spectrum of nutrients in plant tissues (Bashan and De-Bashan, 2010).

In a vision of a more sustainable agriculture and for an integrated nutrient management (Adesemoye and Egamberdieva, 2013), the application of biological fertilizer in combination with chemical fertilizers could represent a valid opportunity. In order to explore the feasibility of this approach, in the present work the beneficial effects in the Fe acquisition by cucumber plants derivable from the treatment with *A. brasilense*, were evaluated. To this aim, in an agricultural calcareous soil either non-inoculated or inoculated with *A. brasilense*, Fe accumulation in tissues of cucumber plants (*Cucumis sativus* L. cv. Chinese Long) has been determined and compared with the root exudation pattern (quantity and quality) measured in water-extracted soil solution. A characterization, at the physiological and biochemical levels of Fe-sufficient and Fe-deficient plants grown in hydroponic solution prior the soil treatment, is also described in a time scale relating the exudation profile to that recorded in soil-grown plants.

2. Material and methods

2.1. Plant material and growing conditions

Cucumber plants (*Cucumis sativus* L. cv. Chinese long) 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. After soaking seeds in 0.5 mM $CaSO_4$ overnight, they were germinated on filter paper moistened with 0.5 mM $CaSO_4$ solution in darkness for 5 d (Nikolic et al., 2012). After the germination period, cucumber seedlings were transferred to a full-strength nutrient solution, either complete or Fe-free using a plant-based biotest (RHIZOtest ISO/CD 16198: 2011, Bravin et al., 2010). The composition of the hydroponic solution was as follows (mM): 2 $Ca(NO_3)_2$, 0.7 K_2SO_4, 0.1 KH_2PO_4, 0.1 KCl, 0.5 $MgSO_4$, and (μM): 10 H_3BO_3, 0.5 $MnSO_4$, 0.2 $CuSO_4$, 0.1 $ZnSO_4$, 0.01 $(NH_4)_6Mo_7O_{24}$, 80 Fe(III)-EDTA. In order to limit photo-chemical reduction phenomena of the micronutrient in the uptake solution (Zancan et al., 2006) added by the Fe-source, beakers were covered with black plastic foil during the entire experiment. The solution was continuously aerated and changed every two days; the pH was adjusted to 6 with NaOH 1 N.

Following 10 days cultivation in hydroponic solution, plants were transferred to an agricultural calcareous soil, sampled in the south of Italy, for 7 days. The soil was a silt loam with the following chemical-physical characteristics: pH_{CaCl2} 7.72 Corg 0.86% (w/w), Ntot 1.16 g/kg, $CaCO_3$ 61.20%, CEC 20.40 cmol+/kg, Ca 25.50% (w/w), K 1.18% (w/w), Fe 1.60% (w/w). For the 7 days contact period, the soil was moistened at 70% of its water holding capacity (Bravin et al., 2010).

2.2. Bacterial strain and soil inoculation

Azospirillum brasilense Cd (DSM-1843) was grown in LB medium (10 g L^{-1} tryptone, 5 g L^{-1} yeast extract, 10 g L^{-1} NaCl) at 30 °C with continuous shaking. At the onset of the stationary phase, cells were harvested by centrifugation for 15 min at 4500 × g and washed three times with a sterile saline solution (0.85% w/v NaCl). Bacterial suspension was used to inoculate the soil, to a final concentration of 10^6 cfu g^{-1} of soil. Control soil was treated with the same amount of sterile saline solution.

2.3. Collection of root exudates from hydroponically-grown plants and extraction of rhizosphere organic compounds from soil

During the hydroponic cultivation period, root exudates were collected at fixed time points (0, 2, 4, 5, 7, 9 and 10 days); Fe-deficient and Fe-sufficient cucumber plants were removed from the nutrient solution and the roots were washed three times in deionized water. The root systems from each plant pot, containing 10 seedlings/plants, were submerged separately into 20 mL of aerated double-distilled water (18.2 MΩcm^3) for 24 h. Trap solutions were freeze-dried and resuspended in double-distilled water before being further analysed.

Non-inoculated and inoculated soils have been collected after 7 days of soil-plant contact. Root exudates were determined in 1:4 (w/v) water extracts followed by centrifugation at 15000 × g (5 min). The supernatant was collected, filtered and freeze-dried before being further analysed (Mimmo et al., 2008).

2.4. Fe(III)-EDTA reduction by intact plants

The reduction of Fe(III)-EDTA by the root system of hydroponically grown cucumber plants was measured colorimetrically using bathophenantroline disulfonate (BPDS) (Dell'Orto et al., 2000). Briefly, roots of intact plants were incubated in the reagent solution containing 0.5 mM CaSO4, 10 mM MES NaOH (pH 5.5), Fe(III)-EDTA 0.25 mM and BPDS 0.6 mM in the dark at 25 °C. After 30–60 min the absorbance of the reagent solution was recorded at 535 nm and the concentration of Fe(II) was calculated on the base of the Fe(II)-$BPDS_3$ complex formed using the molar extinction coefficient of 22.1 mM^{-1} cm^{-1}.

2.5. Analyses of root exudates

The determination of the total chelating compounds were determined colorimetrically using a modified procedure of the spectrophotometric Chrome Azurol S (CAS) method (Shenker et al.,