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Research article

The characterization of the adaptive responses of durum wheat to different Fe availability highlights an optimum Fe requirement threshold

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Plant mechanisms responding to iron (Fe) deficiency have been widely described; it is well known that Strategy II plants, as durum wheat, cope with this stress by increasing both the synthesis and secretion of phytosiderophores (PS). The important contribution of the sulfate assimilatory pathway has been also demonstrated to improve Fe use efficiency in several grasses, such as maize, barley and wheat, most likely because PS are produced from nicotianamine, whose precursor is methionine.

Here, the physiological response of durum wheat (*T. durum* L.) plants - in terms of plant ionome, PS release, thiols content and S pathway-related enzymes — was investigated by gradually decreasing Fe availability that allowed the identification of three specific limit Fe concentrations: 75 μM, 25 μM and 0 μM Fe, *i.e.* the complete Fe deprivation. At each limit, plants begin to induce different and specific adaptive responses to improve Fe acquisition or to reduce the damage resulting from limited Fe availability.

The identification of the Fe availability level below which durum wheat plants start an expensive metabolic reorganization of S and several other elements, could be of benefit not only for an effective cultivation of the crop but also for the grain quality.

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1. Introduction

Iron (Fe) deficiency is a global abiotic stress in agricultural systems due to the low solubility of this micronutrient despite its total abundance in soils (Guerinot and Yi, 1994). Neutral and alkaline values of soil pH make the problem even worse. Moreover, since nearly one-third of the earth's surface consists of calcareous or alkaline soils, Fe deficiency results in serious threat to crop production affecting both their yields and nutritional values (Marschner, 2002; Valentinuzzi et al., 2015). These undesired effects are mainly ascribable to the impairment of fundamental cellular processes (such as respiration, chlorophyll biosynthesis, and photosynthetic electron transport) where Fe plays pivotal roles. Notwithstanding, plants have developed strategies aimed at coping with this nutritional disorder (Kim and Guerinot, 2007). In particular, graminaceous plants, such as durum wheat, increase the synthesis and release in the rhizosphere of ligands with high affinity for Fe, named phytosiderophores (PSs), concomitantly with an enhanced uptake of Fe^{III}-PS complexes via a specific transporter (YellowStripe1, YS1) (Marschner et al., 1986; Mori and Nishizawa, 1987; Murata et al., 2006). Among PS, a relevant role is played by those compounds owning to the family of mugineic acid, whose precursor is methionine (Mori and Nishizawa, 1987). Furthermore, it is well documented that one of the most striking adaptations to Fe shortage in grasses relies on the plant ability to modulate sulfate uptake and assimilation rates (Astolfi et al., 2004, 2006b; Ciaffi et al., 2013). This response was ascribed to the increased demand of reduced S for methionine and, consequently, PS synthesis induced by the Fe deprivation. It is interesting to note that all these studies were performed comparing plants adequately supplied with Fe with those grown without any addition of exogenous Fe-sources to the nutrient solution, even those scarcely available and/or soluble. This simplified experimental condition (inducing acute deficiency) doesn't allow the assessment of plant responses

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to the nutritional disorder when the availability of the micronutrient is increased, although of a small extent, as a consequence of the Fe mobilization process driven by root exudates (Mimmo et al., 2014; Pii et al., 2015b). For this reason, important traits of plant responses to sub acute or latent Fe deficiency could be underestimated or even not identified.

Fluctuations in Fe availability are not only translated into modulation of plant S metabolism both at transcriptional and physiological level, as previously described (Zuchi et al., 2009, 2012; Astolfi et al., 2004, 2012; Ciaffi et al., 2013), but also lead to the imbalances of other nutrients and non essential elements like titanium, aluminium, silicon and sodium (Pestana et al., 2012; Gattullo et al., 2016). In particular, a marked accumulation of Ca, Cu, Mn, and Zn has been determined in Fe deficient plants (Welch et al., 1993; Pii et al., 2015a; Astolfi et al., 2014; Tomasi et al., 2014). In this respect, studies based on molecular approaches have demonstrated that some transporters underlying the transmembrane Fe movements are also involved in the root uptake of other divalent cations (Cohen et al., 1998; Lux et al., 2011; Clemens et al., 2013; Uraguchi and Fujiwara, 2013; Astolfi et al., 2014). Interestingly, Pedas et al. (2008) have isolated and characterized an *IRT1*-like barley gene (*HvIRT1*) whose expression was induced not only by Fe deficiency but also by Mn shortage. Moreover, the HvIRT1 protein codified by this gene was described to be able to transport, besides Fe^{2+} and Mn^{2+}, also Zn^{2+} and Cd^{2+}. Despite many studies focused on shedding light on the plant strategies adopted to cope with Fe deficiency, very few attempts have been made to characterize how the adaptation to Fe shortage affects plant ionome (Baxter et al., 2008; Pii et al., 2015a). Furthermore, because these reports are based on comparing Fe sufficient and completely Fe deficient plants, their results do not help the understanding of the phenomena occurring in conditions closer to those of the rhizosphere in terms of both Fe availability and outcomes of soil-microorganism-root interactions.

On the basis of these premises, in the present work durum wheat was chosen as a model plant to characterize the development of the adaptive response to changing Fe availability by exposing plants to a wider range of conditions, from complete deprivation to adequate supply (0, 1, 5, 25, 50, and 75 μM Fe). In particular, we will discuss the effect of Fe nutrition on total S and thiols accumulation rate and on the activity of S pathway-related enzymes (ATPsulfurylase and *O*-acetylserine(thiol)lyase), at both root and shoot level, and relate changes in S accumulation and assimilation rate to the development of plant physiological responses to Fe deficiency (i.e. PS secretion). In addition, using inductively coupled plasma-optical emission spectroscopy (ICP-OES), the relative contributions of the plant ionome in the response mechanisms adopted by wheat plants to cope with this environmental constraint, are also presented.

The identification of the Fe availability level below which durum wheat plants start an expensive metabolic reorganization of sulfur and several other elements, could be of benefit not only for an effective cultivation of the crop but also for the grain quality.

2. Materials and methods

2.1. Plant growth conditions

Durum wheat (*T. durum* L. cv. Svevo) seeds were germinated on wet filter paper in the dark at 20 °C. Four days later, homogenous seedlings were transplanted to plastic pots containing the nutrient solution (NS) (12 seedlings/2.2 L) as described by Zuchi et al. (2012) and supplemented with six different concentrations of Fe(III)-EDTA (0, 1, 5, 25, 50, and 75 μM). Seedlings were kept under controlled conditions in a growth chamber with a day/night cycle of 14/10 h at 27/20 °C air temperature, 80% relative humidity, and 200 μmol m^{-2} s^{-1} light intensity. The use of black plastic pots has guaranteed the limitation of photochemical reduction phenomena of the micronutrient in the nutrient solution (Zancan et al., 2006). Shoot and root samples were collected eight days after the transfer to the NS.

2.2. Collection of root washings and quantification of PS

Iron-chelating compounds secreted by wheat roots were analyzed determining the PS concentration in root washings, as previously described (Zuchi et al., 2012). Briefly, the plants were removed from the NS 2 h after the onset of the light and the roots were thoroughly rinsed twice for 1 min in deionised water. Root systems of three plants from each condition (about 0.250 g root fresh weight/plant) were immersed into 30 mL deionised water for 3 h under continuous aeration. Thereafter, 10 mg Micropur L^{-1} (Roth, Karlsruhe, Germany) was added to prevent PS microbial degradation. The PS concentration in root washings was measured by using the Fe-binding assay, according to Reichman and Parker (2006).

2.3. ICP-OES analysis

To measure mineral elements concentrations, shoot and root tissues were oven-dried at 80 °C until constant weight was reached and thereafter digested with concentrated ultrapure HNO_3 (65% v/v, Carlo Erba, Milano, Italy), using a Single Reaction Chamber (SRC) microwave digestion system (UltraWAVE, Milestone, Shelton, CT, USA). The elements concentration was subsequently analyzed by ICP-OES (Spectro Arcos, Spectro, Germany). Elements quantifications were carried out using certified multi-element standards (CPI International, https://cpiinternational.com). The limits of detection for each element are reported as follow: Al 6.7 mg L^{-1}, B 1.8 mg L^{-1}, Ba 0.1 mg L^{1}, Ca 2.0 mg L^{-1}, Cu 3.0 mg L^{-1}, Fe 0.4 mg L^{-1}, K 2.0 mg L^{-1}, Li 0.1 mg L^{-1}, Mg 3.0 mg L^{-1}, Mn 0.2 mg L^{-1}, Mo 6.0 mg L^{-1}, Na 1.0 mg L^{-1}, P 4.0 mg L^{-1}, S 4.0 mg L^{-1}, Si 12.0 mg L^{-1}, Sr 0.1 mg L^{1}, Ti 1.3 mg L^{-1}, and Zn 0.2 mg L^{-1}. Tomato leaves (SRM 1573a) and spinach leaves (SRM 1547) have been used as external certified reference material.

2.4. Non-protein thiols extraction and determination

Water soluble non-protein thiol compounds were determined colorimetrically with 5,5′ dithio-bis-(2-nitrobenzoic acid) (DTNB), following the procedure reported by Ciaffi et al. (2013). Briefly, both shoots and roots (1 g fresh weight) were ground in liquid N_2 and extracted in 3 mL of a solution composed of 80 mM trichloroacetic acid (TCA), 1 mM ethylenediaminetetraacetic acid (EDTA), 0.15% (w/v) ascorbic acid, and 10% (w/v) polyvinylpolypyrrolidone (PVP). After a centrifugation step (30 min at 4000 g and 4 °C), the supernatants were collected and the concentrations of DTNB-reactive compounds were measured spectrophotometrically by reading the A_{415}.

2.5. Enzyme extraction and assays

Both root and shoot tissues (1 g fresh weight) were powdered in a pre-chilled mortar with liquid N_2. Cold extraction buffer, containing 50 mM HEPES-KOH (pH 7.4), 5 mM $MgCl_2$, 1 mM EDTA, 10% (v/v) glycerol, 0.1% (v/v) Triton X-100, 5 mM dithiothreitol (DTT), 1 mM phenylmethylsulphonyl fluoride (PMSF), and 1% (w/v) polyvinylpyrrolidone (PVP), was added in a ratio of 1:7 and 1:3 (w/v) for shoots and roots, respectively. Following extraction steps were performed according to the method described by Ciaffi et al. (2013).

The activity of ATP sulphurylase (ATPS; EC 2.7.7.4) and *O*-

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