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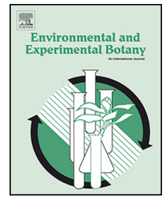

Effects of terbuthylazine on phytosiderophores release in iron deficient barley

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A B S T R A C T

One of the main purposes of modern agriculture is to raise crop production by means of a proper application of herbicides. Nonetheless, the use of herbicides is causing some concerns associated to their persistence and accumulation in the environment. Notwithstanding the relevance of these aspects, little or no attention has been paid to the interferences that these chemicals and their residues can exert on iron (Fe) mineral nutrition of plants. To this purpose, we studied the effect of terbuthylazine (TBA) on the Fe-acquisition processes of Fe-deficient barley plants, by investigating some aspects related to phytosiderophores (PS) exudation and sulfur (S) metabolism. Results showed that plant growth, chlorophyll content expressed as SPAD index, PS release and the expression of genes involved in their secretion, uptake and biosynthesis were negatively affected. This response was associated with reduced cysteine concentrations, and it suggests that the TBA interferences on Fe-acquisition in barley are the consequence of the induced changes in S metabolism.

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1. Introduction

Currently used herbicides consist mostly of synthetic compounds employed to control weeds (target organisms), with the objective to increase crop productivity. Over the last decades, the huge amount of these chemicals employed in agriculture has caused some major concerns due to their dispersion in the environment (Van Zelm et al., 2014). In addition, there are herbicides characterized by a slow degradability in soil (persistence) leading very often to the presence of numerous unwanted herbicide residues in the water–soil system also for prolonged periods (Chaudhry et al., 2001). Toxicity, mobility, persistence and over accumulation in the agricultural environment of these chemicals could represent a real risk also to other non-target plants (Magne et al., 2006). Triazines are a class of weed-killers widely used for various crops. They act by interrupting the photosynthetic electron transport at the level of photosystem II via inhibition of the activity of D1 protein (Cañero et al., 2011). For their very long persistence triazines can cause environmental pollution and contaminate non-target organisms (Delin and Landon, 2002; Gerard and Poullain, 2005). Only a limited number of studies has addressed the question if the exposure of crops (or non-target plants) to herbicides or their residues might affect plant growth by limiting the uptake and assimilation of mineral nutrients. Previous studies indicated that some herbicides can interfere with plant nutrition and in particular with zinc (Zn), copper (Cu), manganese (Mn) and iron (Fe) acquisition (Eker et al., 2006; Osborne et al., 1993; Rengel and Wheal, 1997). For instance, it has been demonstrated that frequent applications of *glyphosate* leads to the development of clear Fe chlorosis symptoms in treated crops (Bellaloui et al., 2009; Ozturk et al., 2008). The alteration of Fe uptake capacity of plants is of great importance, and of evident scientific interest, since Fe together with nitrogen (N) and phosphorous (P), is the most yield limiting crop nutrient in the world (Schachtman et al., 1998; Zhang et al., 2010). To cope with Fe shortage, monocots like barley (Strategy II plants) are characterized by biosynthesis and exudation of a huge concentration of phytosiderophores (PS) into the rhizosphere (Nozoye et al., 2011). This class of root exudates are characterized by a strong chelation affinity for Fe(III), making them very efficient in the mobilization of Fe from barley available soil sources (Schaaf et al., 2004). Once formed, the Fe(III)–PS complexes are then transported into root

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cells through specific transporters (Inoue et al., 2009). The synthesis of PS depends on the availability of methionine (Met), being the only precursor of the mugineic acid family of PS. Consequently, the PS biosynthesis is depending on the sulfur (S) assimilation rate in plants (Mori and Nishizawa, 1987). In fact, it has been widely demonstrated that interferences on this pathway or S deficits can strongly decrease the plant capacity to release PS in the rhizosphere (Astolfi et al., 2006; Zuchi et al., 2012).

Sulfur assimilation involves some sequential-operating enzymes which produce cysteine (Cys) as the first stable S containing compound (Davidian and Kopriva, 2010). Afterwards, the Cys serves as a S donor for the biosynthesis of the Met glutathione (GSH), vitamins and a variety of sulfur containing compounds which play major roles in the growth and development of plants in response to numerous biotic and abiotic stresses (Droux, 2004). The assimilatory reduction of sulfate in plants starts with ATP sulfurylase (ATPS, EC 2.7.7.4), which transforms sulfate in an activated ATP linked form (adenosine 5′-phosphosulfate), and *O*-acetylserine (thiol) lyase (OASTL, EC 4.2.99.8), which drives the final step in the assimilation of reduced sulfate and its incorporation in the Cys (Droux, 2004). Passing through the metabolism of S-reduced containing compounds, the capacity of monocotos to acquire Fe by means of PS is therefore deeply linked to the S metabolism (Aciksoz et al., 2011).

In the present work we investigated the effect of terbuthylazine (TBA) on the capacity of Fe-deficient barley to release PS. In particular, we examined whether changes in PS exudation could be related to interferences exerted by the chemical on S metabolism. Barley has been selected as a Strategy II plant releasing huge amounts of PS under Fe shortage, whereas terbuthylazine has been selected for its use in agriculture for different crops in order to control graminaceus weeds. In addition, TBA, because of its long persistence and wide use for crops (Borin et al., 2004), can reach also non-target crops with disturbing effects on their mineral nutrition.

To this purpose, we monitored the effect of the herbicide on plant growth and chlorophyll content, on the amount of PS released by roots and on expression of genes involved in PS secretion (*HvTOM1*), in Fe–PS uptake (*HvYS1*) and in PS biosynthesis (*HvNAS3*,*HvNAS4*, *HvNAS6*, *HvNAS7*, *HvNAAT-A*, *HvDMAS*) (Nagasaka et al., 2009; Nozoye et al., 2011; Ueno et al., 2009). Furthermore, the impact of TBA on S assimilation pathway was evaluated by measuring Cys and GSH contents and the activity of ATPS and OASTL in Fe-deficient barley plants treated with the chemical.

2. Material and methods

2.1. Plant material and growth conditions

Barley (*Hordeum vulgare* L. cv Europa) seeds were germinated in Petri dishes, added of ultrapure water. After 4 days, the seedlings were transferred and grown in a continuously aerated hydroponic solution (12/12 h of light/light, 23/19 °C) composed as follows: 2 mM $Ca(NO_3)_2 \times 4H_2O$, 0.5 mM $MgSO_4 \times 7H_2O$, 0.7 mM K_2SO_4, 0.1 mM KCl, 0.1 mM KH_2PO_4, 1 μM H_3BO_3, 0.5 μM $MnSO_4 \times H_2O$, 0.5 μM $CuSO_4$, 0.5 μM $ZnSO_4 \times 7H_2O$, 0.01 μM and $(NH_4)_6Mo_7O_{24} \times 4H_2O$. At the first visible symptoms of chlorosis (day 14), some tanks were added with solutions containing 1.0 mg L^{-1} of TBA (the treatment was based on the recommended field application rate), while other tanks were untreated and left as controls. At 24, 48 and 72 h after the treatment, plants were collected and the length and weight of shoots and roots were assessed. SPAD index was measured (at 24, 48 and 72 h after the treatment) to evaluate the chlorophyll content in the shoots (SPAD-502 Plus, Konica Minolta, Japan) (Soil and Plant Analysis Development). Measurements were taken on the first leaf of each plant, 5–10 cm from the bottom, midway between the midrib and the leaf margin. The measurement were then transformed into chlorophyll content (Markwell et al., 1995).

2.2. Root exudates collection and PS quantification

Barley plants were collected at 24, 48 and 72 h after the treatment with TBA in order to determine the amount of PS released. Briefly, root exudates were collected from plants starting 2 h after the beginning of the photoperiod. After accurate washing of the roots, 3 plants/sample were placed into beakers containing 15 mL of ultrapure water. Roots exudates were collected for 5 h under continuous aeration. The amount of PS exuded was then quantified by a colorimetric method (Cu-CAS assay – LOQ for PS determination = 30 μM) (Shenker et al., 1995).

2.3. RNA extraction and expression analysis by semi-quantitative RT-PCR

Total RNA was extracted from roots of barley plants collected 24 h after the TBA treatment, using the TRIzol Reagent System (Invitrogen, Grand Island, NY), according to the manufacturer's instructions. The extracted RNA was treated with RNase-free DNase I (Promega, Madison, WI), in agreement with the manufacturer's protocol. Total RNA samples were quantified by spectrophotometry for absorbance at 260 nm and adjusted to equal concentration. The integrity of RNA samples was assessed by electrophoresis on 1.2% (w/v) agarose gels. RNA (1 μg) was reverse-transcribed by the M-MLV (H−) Reverse Transcriptase (Invitrogen, Life Technologies, NY, USA), to synthesize the first-strand cDNA and the final cDNA was diluted 1:5 in RNase free water.

Polymerase chain reactions (PCRs) were performed by the HotMasterMix System (Eppendorf, Hamburg, Germany), using 1 μL of the diluted RT reaction and primer pairs designed to specifically detect and amplify genes involved in barley PS biosynthesis (HvNAS3, HvNAS4, HvNAS6, HvNAS7, HvNAAT-A, HvDMAS), PS secretion (HvTOM1), and Fe^{3+}–PS complexes selective uptake (HvYS1) (Astolfi et al., 2014). Actin was also amplified as internal control. The PCR products were resolved electrophoretically on 1% (w/v) agarose gels and stained with ethidium bromide. Each semi-quantitative RT-PCR experiment was independently repeated in triplicate to test the amplification reproducibility.

2.4. Cysteine and glutathione contents

Cys and GSH were separated and quantified by reversed-phase HPLC after derivatization with monobromobimane (Zechmann et al., 2005). In detail, 60 mg of shoot tissue was pulverized and extracted in 2.0 mL 0.1 M HCl with 60 mg of polyvinylpolypyrrolidone. The extracts were then centrifuged at 10,000 × *g*, after that, aliquots of 280 μL of the supernatant were added of 420 μL of 200 mM CHES (pH 9.0) with 70 μL 5 mM DTT. The derivatizations of GSH and Cys were carried out by incubating the above solutions with 50 μL 8 mM monobromobimane, at room temperature and for 15 min; the reactions were stopped by adding 760 μL of 0.25% (v/v) methanesulfonic acid. Separation and determination of the derivatized thiols was done in accordance with Zechmann et al. (2005).

2.5. Enzyme extraction and assay

Barley root tissue (ca. 1 g FW) was powdered in a pre-chilled mortar under liquid N_2 and then homogenized in 3 mL of a cold extraction buffer (pH 7.4), prepared by adding 50 mM HEPES–KOH,

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