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Abstract: 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.

***Highlights (for review)**

Highlights

- Terbuthylazine (TBA) reduced phytosiderophores (PS) release in Fe deficient barley.
- TBA treated iron deficient barley showed reduced expressions of PS related genes.
- Reduced cysteine (Cys) levels in TBA treated samples explained the lower PS release.
- The interferences on Cys content were due to TBA interference on ATPS and OASTL.

***Manuscript**

Title page

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Terbuthylazine affects phytosiderophores release in barley by disturbing sulfur metabolism

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Abstract

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.

KEYWORDS: terbuthylazine; iron deficiency; phytosiderophores; cysteine; sulfur assimilation.

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 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 50-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 (*Oryza sativa* 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 hours of light/light, 23/19°C) composed as follow: 2 mM $Ca(NO_3)_2$ x $4H_2O$, 0.5 mM $MgSO_4$ x $7H_2O$, 0.7 mM K_2SO_4, 0.1 mM KCl, 0.1 mM KH_2PO_4, 1 µM H_3BO_3, 0.5 µM $MnSO_4$ x H_2O, 0.5 µM $CuSO_4$, 0.5 µM $ZnSO_4$ x $7H_2O$, 0.01 µM and $(NH_4)_6Mo_7O_{24}$ x $4H_2O$. At the first visible symptoms of chlorosis (day 14), some tanks were added with solutions containing 1.0 mg L^{-1}

of terbuthylazine, while other tanks were untreated and left as controls. At 24, 48 and 72 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 hrs after the treatment) to measure 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 ontinuous aeration. The amount of PS exuded was then quantified by a colorimetric method (Cu-CAS assay - LOQ for PS determination = 30 mM) (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 mg) 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 x 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, 5 mM $MgCl_2$, 1 mM EDTA, 10% (v/v) glycerol, 0.1% (v/v) Triton X-100, 5 mM DTT, 1 mM PMSF, and 1% (w/v) PVPP. The homogenate was filtered and then centrifuged at 4°C for 5 min at 1000 x g. The supernatant was collected and desalted at 4°C on a Sephadex G-25 column (PD-10, Pharmacia, Uppsala, Sweden) pre-equilibrated with the extraction buffer without Triton X-100. The desalted extract was centrifuged at 4°C for 5 min at 30 000 *g*. The resulting supernatant was frozen in liquid nitrogen and stored by freezing (-80°C), until use for in vitro enzyme assays.

The extractable ATP sulfurylase (ATPS; EC 2.7.7.4) activity was determined by the bioluminescence technique as described in Ciaffi et al. (2013). O-acetylserine(thiol) lyase (OASTL; EC 4.2.99.8) activity was assayed by detecting cysteine production as described by Ciaffi et al. (2013). Quantitative protein determination of the root extracts was estimated by the protein-binding Coomassie brilliant blue G-250 dye method, with bovine serum albumin (BSA) as standard (Bradford, 1976).

2.6. Determination of shoot and root iron concentration

Plants were harvested at 24, 48 and 72 h after the TBA treatment. Roots and shoots were separated, weighed and ovendried at 60 °C until constant weight was reached. Roots and shoots were microwave digested with concentrated nitric acid (65% v/v, Carlo Erba) using a single reaction chamber (SRC, UltraWAVE, Milestone Inc., Shelton, CT, USA). Iron concentration was then determined by inductively coupled plasma-optical emission spectroscopy (ICP-OES, Spectro Ciros CCD, Spectro, Germany).

2.7. Statistical analysis

Each reported value represents the mean ± standard deviation (SD) of data from four independent experiments on at least three biological replicates per experiment. For the determinations of shoot and root length and plant weights twenty replicates were used. Statistical analyses of data were carried out by ANOVA tests and significant differences were established by Duncan's tests at $P < 0.05$.

3. Results

3.1. Length and weight of shoots and roots, and chlorophyll content in barley plants treated or not with TBA

Terbuthilazine affected the growth of seedlings of barley already within the first 24 h after the treatment; in particular, some significant reductions in both shoots and roots length and weight have been ascertained (Table 1). In particular, at 24, 48 and 72 h after the treatment, the shoot length of the TBA treated samples was reduced by 12.9, 25.0 and 24.6%, respectively, with respect to the untreated Fe-deficient samples. Shoot fresh weight was also significantly reduced by the herbicide treatment during all the experimental period (9.3, 36.1 and 29.7% at 24, 48 and 72 h, respectively; Table 1). The same occurred to the root length, which was reduced by 14.7 and 34.1%, at 48 and 72 h after the treatment, respectively. Finally, the TBA treatments reduced the roots' fresh weight at 72 h after the treatment (12.3%) with respect to the untreated controls.

Possible changes in chlorophyll contents in response to the TBA-induced stress were assessed in both treated and untreated Fe deficient leaves of barley by a SPAD meter (Fig. 1). The leaf chlorophyll level was significantly reduced in the TBA treated samples at 48 and 72 h after the treatment (19.6 and 18.8%, respectively).

3.2. Fe concentration in barley shoots and roots

Fe concentration was evaluated in shoots and roots of barley treated with TBA at 1.0 mg L^{-1} (Table 2) as means of the values obtained at 24, 48 and 72 h after the treatment. The herbicide strongly affected the Fe translocation in treated plants. In particular, in TBA treated roots the Fe concentration almost doubled (+62%) with respect to the controls, while in TBA treated shoots it decreased by 36% with respect to the respective controls.

The shoot to root ratio increased by 254% following the TBA treatment, clearly indicating that the herbicide impaired the root-to-shoot transfer of Fe in barley plants (Table 2).

3.3. Phytosiderophores release from TBA treated and control barley plants

Root exudates were collected from TBA treated and untreated barley plants grown in hydroponic solutions under Fe deficiency. The collected exudates were then analyzed in order to determine the PS concentration released by the plants (Fig. 2). Phytosiderophores release increased during the 72 h in control plants as a response to Fe shortage. TBA treated plants showed strong reductions of the PS release (Fig. 2); in fact, the concentration of PS released by TBA stressed roots decreased starting from 48 h after the treatment (-50.8%) and reached the lowest value of 0.25 μmol g^{-1} RFW (-81.5%) at 72 h after the treatment, with respect to the controls.

3.4. Expression analysis

In order to understand at molecular level how TBA could interfere with the Fe acquisition process, the expression of several genes involved in PS secretion (*HvTOM1*) (Nozoye et al., 2011), in Fe-PS uptake (*HvYS1*) (Ueno et al., 2009) and in PS biosynthesis (*HvNAS3*, *HvNAS4*, *HvNAS6*, *HvNAS7*, *HvNAAT-A*, *HvDMAS*) (Nagasaka et al., 2009) was investigated by RT-PCR in roots of barley plants at 24 h after TBA exposure. The application of TBA down-regulated the expression of *HvTOM1*, a gene coding for the transporter directly involved in the PS efflux and *HvYS1*, coding for the major Fe-PS complex transporter involved in the primary Fe acquisition by barley roots (Fig. 3) (Nozoye et al., 2011; Ueno et al., 2009). Treatment with TBA also elicited a decreased expression of those genes involved in the synthesis of PS (Fig. 3). These genes include NA synthase genes (*HvNAS3*, *HvNAS4*, *HvNAS6* and *HvNAS7*), a gene coding for nicotianamine aminotransferase (*HvNAAT-A*), and the DMA synthase (*HvDMAS*) gene.

3.5. Cysteine and GSH contents in barley shoots of plants treated or not with TBA

The concentration of Cys and GSH was determined in Fe deficient barley shoots treated with or without TBA at 24 and 72 h after the treatment (Fig. 4(a) and (b)). The Cys concentration decreased in the TBA treated samples already at 24 h after the treatment (-25.3%); this negative effect was continuous until 72 h after the treatment when the TBA treated samples evidenced a stronger decrease of Cys concentration (-46.6%).

Differently from Cys the concentration of GSH was unaffected during the first 24 h after the treatment, while it was significantly reduced at 72 h after the TBA treatment when the reduction in the content of the tripeptide was of 28.7% in the treated samples, with respect to the untreated controls.

3.6. Activities of enzymes involved in sulfate assimilation

The activities of key enzymes of the reductive S assimilation pathway were measured in barley roots at 24, 48 and 72 h after TBA application. The activity of ATPS increased during the first 24 h by the TBA treatment, which was even greater at 48 h representing an approx. 40% increase compared to control plants; at 72 h the enzyme activity of TBA-treated roots decreased (19% lower with respect to the control) (Fig. 5(a)).

The activity of OASTL decreased with the time of TBA exposure (Fig. 5(b)). In particular, between 24 and 48 h, root OASTL activity decreased 40 and 70%, respectively, and also dropped by more than 70% between 48 and 72 h (Fig. 5(b)).

4. Discussion

Iron (Fe) deficiency is a widespread problem in agriculture with negative effects on crop productions. Specific soil characteristics are the main cause for low Fe availability to plant roots (Marschner et al., 1986). In this scenario, the present study documents the effects of the herbicide terbuthylazine (TBA) on phytosiderophore (PS) release and sulfur (S) metabolism of Fe deficient barley plants. To date, no pieces of information are available on the effects of triazine herbicides on plants' capacity to release PS and their link with sulfur metabolism, which regulates and influences the capability of Strategy II plants to cope with Fe-shortage.

Results of our experiments show that length and weight of the shoots and roots were negatively affected by the herbicide during almost all the experimental period (Table 1). The same occurred to the root-to-shoot translocation of Fe in TBA-treated barley plants as already pointed out by previous authors (Eker et al., 2006) (Table 2). Also, the chlorophyll content expressed ad SPAD index was progressively reduced by the TBA treatment (Fig. 1): the interference on chlorophyll followed the negative trends evidenced by the reductions on the weight and length of the treated shoots and roots. These effects are a probable consequence of the intrinsic toxicity of the TBA; in fact, the primary target site of the triazines action is the photosynthetic electron transport (Del Buono et al., 2011). This interference results in a loss of plant photosynthetic activity and thus in a reduced net carbon (C) skeleton synthesis. Since a large amount of photosyntates are transferred from shoots to the roots (Mimmo et al., 2014), we hypothesized that TBA, because of its action on this primary target site, could reduce the amount of PS. To this purpose, we studied the PS exudation from roots of TBA-treated Fe-deficient barley plants (Fig. 2). The study showed that the PS release rate was significantly reduced by the TBA treatment already at 48 h after the treatment and the reduction was progressive and continuous up to the end of the experiment. The implication of this effect of TBA on the plant-exuding capacity is very important because it involves some severe consequences on

Fe nutrition. Therefore, we studied the expression of the genes involved in PS secretion, uptake and biosynthesis (Fig. 3). The expression levels of genes involved in PS synthesis and secretion in TBA treated plants are in agreement with the reduced PS release evidenced by our experiments (Fig. 3). Nonetheless, previous findings demonstrated that the expression of genes involved in the synthesis of nicotianamine (NA) and 20-deoxymugineic acid (DMA), which are the precursors of the mugineic acid family PS (MAs), is up-regulated by signals that respond to the limited Fe supply (Nagasaka et al., 2009). These genes include NA synthase genes (*HvNAS3*, *HvNAS4*, *HvNAS6* and *HvNAS7*), a gene coding for nicotianamine aminotranferase (*NAAT-A*), the DMA synthase gene (*DMAS1*), *HvYS1*, a gene coding for the major Fe-PS complex transporter involved in the primary Fe acquisition by barley roots (Ueno et al., 2009), and *HvTOM1*, a gene coding for the barley transporter directly involved in the MAs efflux (Nozoye et al., 2011). Interestingly, Fe-deficient barley plants treated with TBA reversed completely this trend by strongly reducing the expression of all the above mentioned genes. These findings suggest that TBA might play a regulatory role in Fe acquisition by directly targeting genes involved in PS biosynthesis.

Since a clear connection between S metabolism and PS release in Strategy II plants has been demonstrated (Astolfi et al., 2006; Ciaffi et al., 2013; Paolacci et al., 2014; Zuchi et al., 2012), we also evaluated if the reduced PS release could be related to changes in S metabolism. Normally, plants assimilate inorganic sulfate which is then reduced into sulphide and incorporated in cysteine (Cys), the central intermediate for most of the S containing compounds. In particular, Cys is used for the biosynthesis of methionine (Met), protein and glutathione (GSH) (Leustek et al., 2000). Methionine is the precursor of S-adenosyl methionine, which is in turn the precursor of PS (Hesse and Hoefgen, 2003). This finding evidences a deep connection between the S metabolism and the plant capacity to release PS. Therefore, we investigated the effect of TBA on Cys and GSH contents in order to better understand the nature of the negative interference exerted by TBA on S metabolism and consequently on the PS release. Data reported in Fig. 4(a) show that significant reductions in the Cys content were ascertained already at 24 h after TBA addition; the decreases were continuous and stronger reductions of the amino acid content was found at 72 h after the treatment. The effect on Cys indicates that TBA affected the assimilatory reduction of sulfate in barley. Given the central role of Cys in primary and secondary metabolism (Wirtz and Droux, 2005), it is evident that this interference compromises also the biosynthesis of other essential molecules, and, particularly, that of Met.

Conversely, the TBA effect on GSH levels was less marked, even though its content was significantly reduced in the treated samples at 72 h after the treatment (Fig. 4(b)). This result should be discussed by considering the role of this non protein thiol in the detoxification of toxic

xenobiotics, including herbicides (Del Buono et al., 2007; Del Buono and Ioli, 2011). In fact, GSH can react enzymatically or not with toxic substrates to give conjugation products which are usually less toxic and mobile than the precursors (Del Buono et al., 2007; Del Buono and Ioli, 2011). In addition, as a consequence of the inhibition of photosystem II, triazines produce high amounts of oxidants; GSH is thereby consumed by cells not only to detoxify the herbicide, but also to remediate to its oxidative damages. Finally, the Cys drop impaired the plant's capacity to renew the cellular stock of GSH contributing to its decrease observed at 72 h after the treatment.

We also measured the activity of two important enzymes involved in the S reductive assimilation to deeply investigate the negative effect of TBA on the Cys synthesis: OASTL, which catalyzes the final step of the synthesis of Cys and ATPS, which initiates the S assimilatory metabolism by adenylation of sulfate (Leustek et al., 2000). The activity of OASTL was strongly reduced already at 24 h after the treatments (Fig. 5(b)), suggesting that TBA reduced Cys synthesis by decreasing this enzyme activity (Fig. 4(a)). Some studies have paid a particular attention to the regulation of OASTL in plants exposed to different abiotic stresses, because of its key-role in controlling the cellular content of Cys. In particular, some experiments conducted on crop legumes evidenced that OASTL was induced by cadmium (Cd), lead (Pb) and arsenic (As), while copper (Cu) decreased it (Pajuelo et al., 2007). Also other authors confirmed the stress-related responsiveness of this enzyme: salt stress and Cd enhanced the OASTL activity in Chlamydomonas reinhardtii, while As reduced it (Vega et al., 2005). The importance of OASTL in plant stress-related responses has also been evidenced by some researchers studying the production of transgenic plants overproducing OASTL (Sirko et al., 2004; Youssefian et al., 2001). Finally, our experiments show that the strong reduction in the Cys content was due to the interference exerted by TBA on OASTL activity.

The other enzyme investigated was ATPS. Its activity increased significantly during the first 48 h, but decreased at 72 h after the TBA treatments (Fig. 5(a)). The induction observed during the First 48 h is most likely due to a high S demand to produce GSH for detoxification and antioxidant responses, necessary to overcome the toxic effects of TBA. ATPS has in fact been indicated as an enzyme responsive to stresses (Heiss et al., 1999; Yatusevich et al., 2010). For instance, it has been reported that this enzyme was induced in *Brassica juncea* plants in response to Cd exposure (Heiss et al.,1999). In particular, ATPS induction was functional to the high demand of S containing compounds for the heavy-metal-detoxification (Heiss et al., 1999; Yatusevich et al., 2010). At last, this finding could indicate that upon TBA exposure, barley plants activated ATPS during the first 48 h in order to overcome the stress; afterwards, the enzyme activity declined because of the impaired capacity of the plant to synthesize Cys which compromised all the S assimilatory pathway.

In conclusion, the study presented here is the first experimental evidence highlighting that a herbicide as TBA is capable to interfere with the release of PS, which play a fundamental role in the Fe acquisition of iron deficient Strategy II plants. This impairment has been confirmed by the genes expression analysis involved in the PS synthesis, release and uptake. The cause of this disturbance has been attributed to the plant stress-related responses regarding the S metabolism and in particular the synthesis of Cys the keymolecule for the production of PS and other S containing compounds. On a wider scale, the results here reported point out the importance of the negative consequences of herbicide accumulation in soil with some major implications on crop productions and quality.

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FIGURE LEGENDS

Fig. 1 - Soil plant analysis development (SPAD) units in shoots of control and TBA treated (1.0 mg L^{-1}) barley plants at 24, 48 and 72 h; data are means ± SD (n= 5).

Fig. 2 - Phytosiderophores amounts released by roots of control and TBA treated (1.0 mg L^{-1}) barley plants at 24, 48, 72 h; data are means ± SD (n= 4).

Fig. 3 - Expression analysis by RT-PCR of genes coding for phytosiderofore efflux (*HvTOM1*) and ferric-phytosiderofore influx (*HvYS1*) transporters (a) and for nicotianamine synthase (*HvNAS3, HvNAS4, HvNAS6 and HvNAS7*), nicotianamine aminotranferase (*NAAT-A*) and 2'deoxymugineic acid synthase (*DMAS1*) (b) in roots of barley seedlings treated with TBA at 1.0 mg L^{-1} (T), compared with untreated samples (C) at 24 hours after the treatment. Actin cDNA was also amplified as internal control.

Fig. 4 - Cysteine **(**a**)** and glutathione contents **(**b**)** determined in shoots of control and TBA treated (1.0 mg L^{-1}) barley plants at 24 and 72 h; data are means ± SD, (n= 3).

Fig. 5 - ATPS **(**a**)** and OASTL **(**b**)** activities determined in roots of control and TBA treated (1.0 mg L^{-1}) barley plants at 24, 48 and 72 h; data are means ± SD, (n= 3).

TABLES

Table 1: Length and weight of shoots and roots of control and TBA treated 1.0 mg L^{-1}) barley plants at 24, 48 and 72 h. For each column, means followed by different letters are significantly different at $P \leq 0.05$ (n=20).

	24 h	48 h	72 h
Shoot length (cm)			
Control	24.7 a	27.6 a	29.2 a
+TBA	21.5 b	20.7 b	22.0 b
Shoot fresh weight (g)			
Control	0.32 a	0.36 a	0.37 a
+TBA	0.29 b	0.23 b	0.26 b
Root length (cm)			
Control	15.5 a	17.7 a	22.0 a
+TBA	15.4 a	15.1 b	14.5 b
Root fresh weight (g)			
Control	0.099 a	0.100 a	0.106 a
+TBA	0.102 a	0.101 a	0.093 b

Table 2: Iron concentration in shoots and roots of barley treated with TBA at 1.0 mg L^{-1}, compared with untreated samples together with the root to shoot ratio. Concentrations are means of values obtained at 24, 48 and 72 h of the treatment, means followed by different letters are significantly different at $P \leq 0.01$ (n=3).

	$[Fe]_{roots}$	$[Fe]_{shoots}$	Root/shoot
	$\mu g\ g^{-1}$	$\mu g\ g^{-1}$	
Control	4.43 b	4.70 a	0.964 a
+TBA	7.21 a	3.02 b	2.47 b

Fig. 1

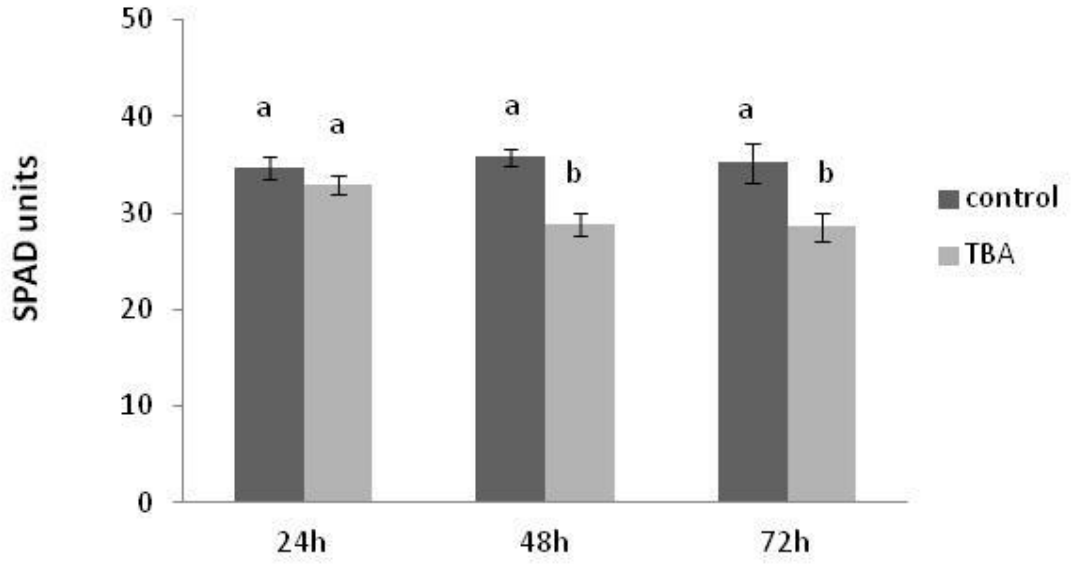


Fig. 2

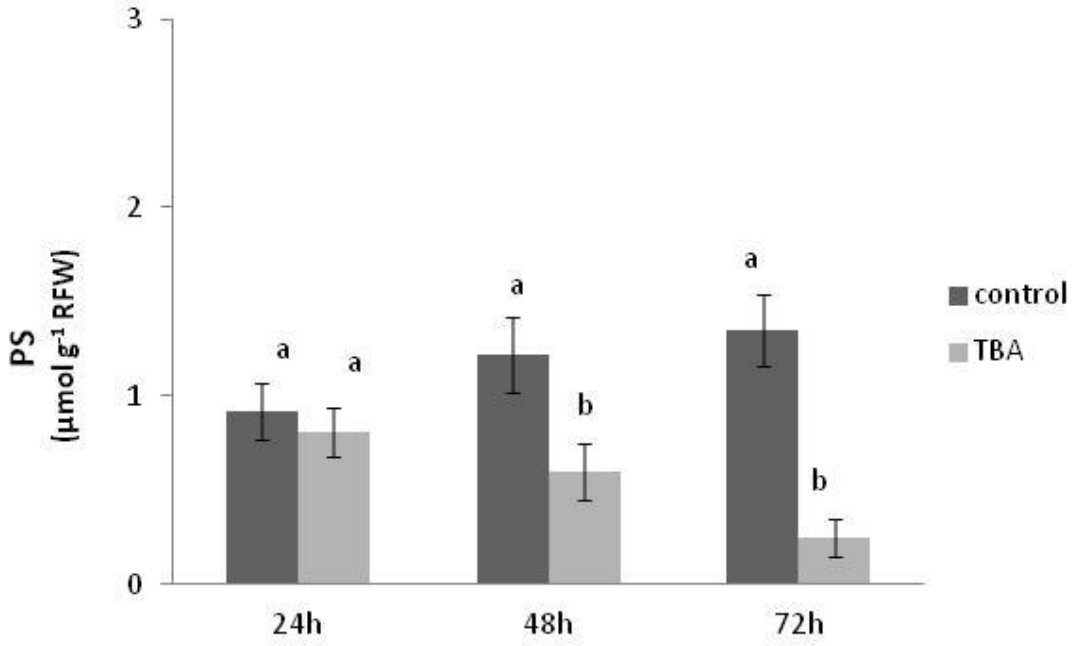
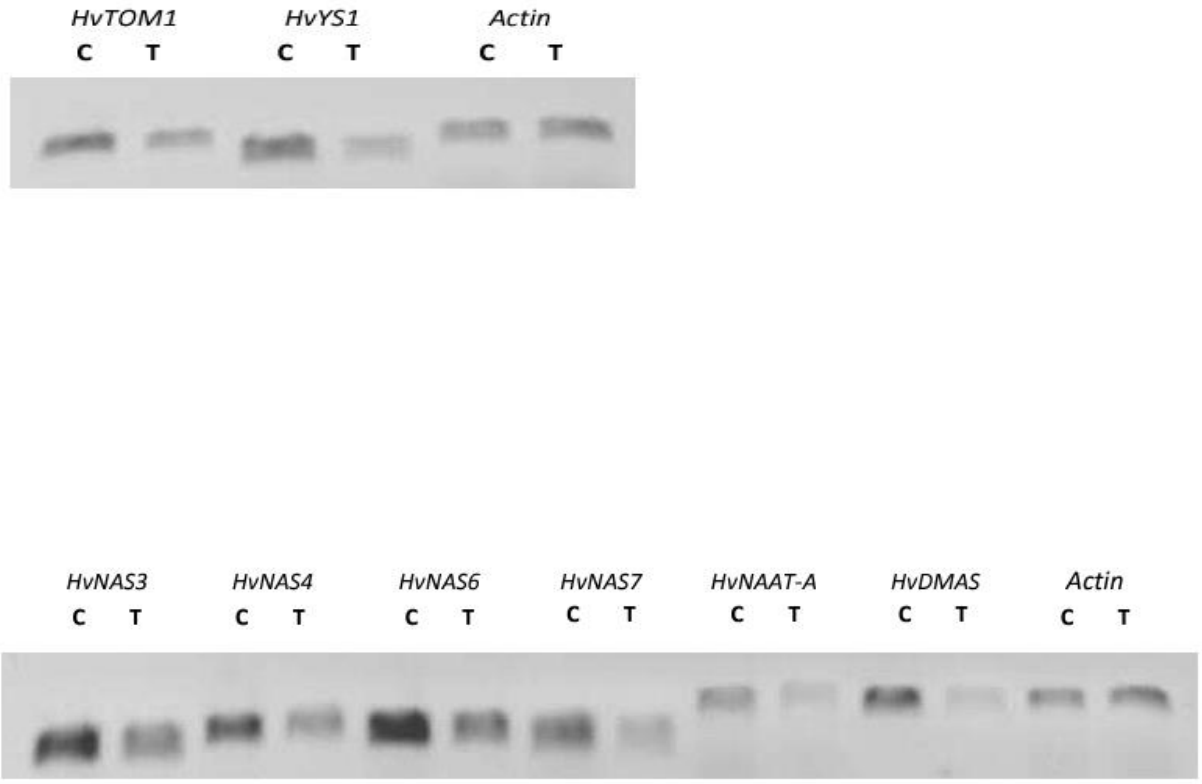


Fig. 3


HvTOM1
C T
HvYS1
C T
Actin
C T
HvNAS3
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HvNAS4
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HvNAS7
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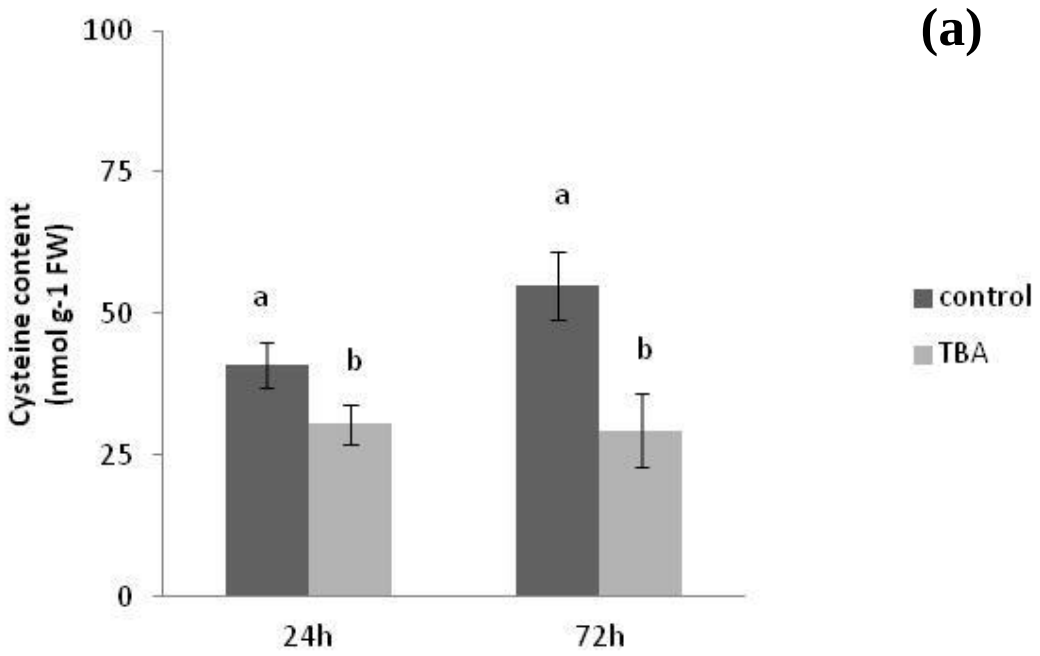
HvNAAT-A
C T
HvDMAS
C T
Actin
C T

Fig. 4


(a)
Cysteine content (nmol g-1 FW)
100
75
50
25
0
a
b
a
b
24h
72h
control
TBA

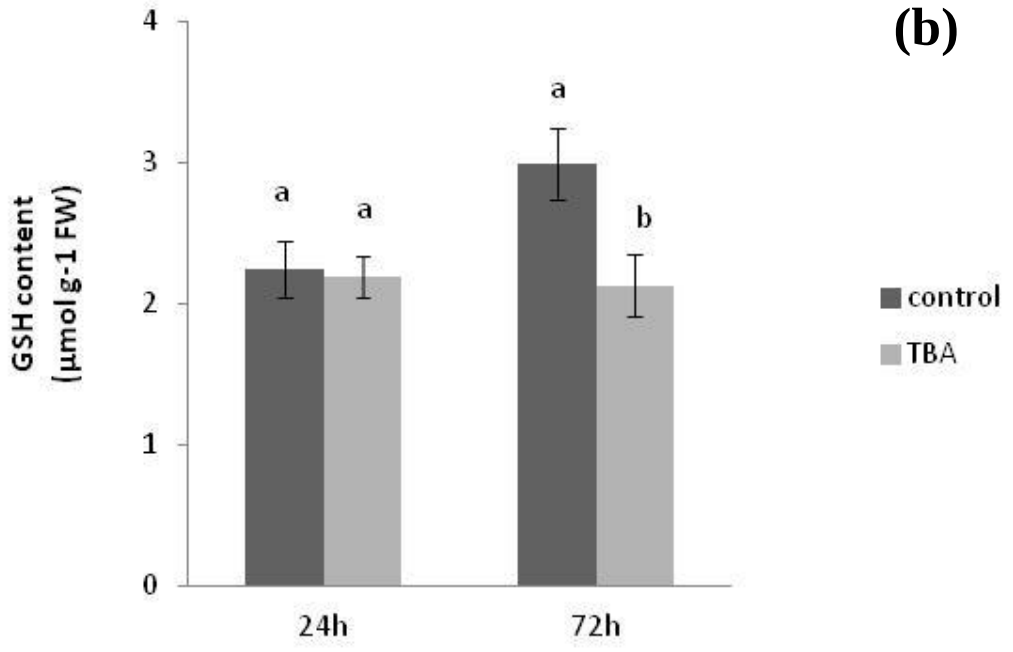
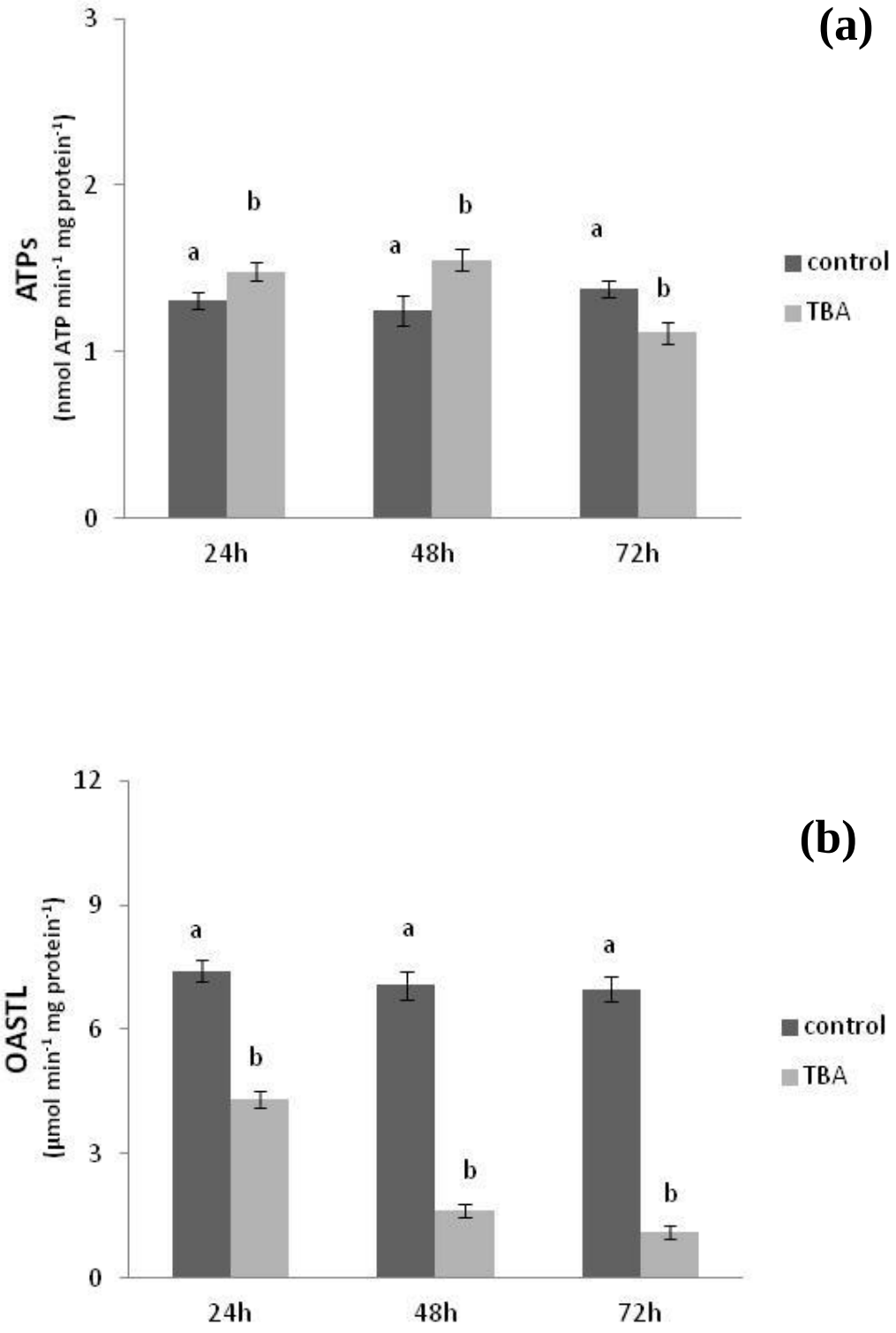

(b)
GSH content (µmol g-1 FW)
4
3
2
1
0
a
a
a
b
24h
72h
control
TBA

Fig. 5


(a)
ATPs
(nmol ATP min-1 mg protein-1)
3
2
1
0
a
b
a
b
a
b
24h
48h
72h
control
TBA
(b)
OASTL
(µmol min-1 mg protein-1)
12
9
6
3
0
a
b
a
b
a
b
24h
48h
72h
control
TBA

SUITABLE GRAPHIC FOR PUBLICATION IN TABLE OF CONTENTS (TOC)