Effect of three Safeners on sulphur assimilation and iron deficiency response in barley plants.

Partecipanti (ordine da stabilire)

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1. INTRODUCTION

Safeners are a group of agrochemicals used in agriculture to enhance selectively the control of wild grasses by graminicides.[1,2] They act by reducing the toxicity of herbicides to crops, without decreasing their toxic efficacy in weeds.[3] When applied to crops, a safener is capable to shift the sigmoidal dose-response curve (of the species to the herbicide exposure) to higher herbicide rates, so permitting the achievement of a selective control of weeds with respect to the botanically related crops.[4] In the early stages of their utilization in agricultural practices, safeners were used in order to protect maize, sorghum and rice crops from the toxic effect of thiocarbamate and chloroacetanilide herbicides.[3] More recently, their use has been extended to winter cereal crops for their protection toward many other different classes of herbicides.[4] With respect to their mode of use, safeners can be applied directly to crop-cultivated fields, before herbicides treatments or, alternatively, concomitantly with the spraying of these latter.[3] In addition, a pre-treatment of seeds with safeners in aqueous solution before planting (seed safening) has been demonstrated to be very effective in its purpose, at least for some crops.

Safeners are member of diverse chemical groups; the main are chloroacetanilide derivatives, naphthopyranones, dichloromethyl acetals and ketals, oxime ether derivatives, thiocarbamates, phenyl pirazoles etc..[4] Since most of the commercially diffused herbicides are detoxified in plants by mean of various enzymes which can catalyze hydrolysis, oxidation and conjugation reactions, [5-7] safeners have been developed aiming at selectively increasing the activity of these detoxifying enzymes. Although the safening action has been observed in numerous monocot plants (as increased transcription of genes related to the defense response of plants, increased enzyme activity and enhanced herbicide tolerance), little is known about the mechanisms underlying this protective action of the safeners. However, general inductions of the main enzymes involved in herbicide detoxification have been observed in response to these chemicals. Particularly responsive to safeners are Cytochrome P450s oxidases, some esterases (carboxylesterase, acylamidase, etc.) and glutathione S-transferase (GST; glutathione(GHS)-mediated reactions).[8,9] This last class of enzymes

plays a very important role in herbicide detoxification in many species by catalyzing the conjugation of GSH with a variety of herbicides.[10,11] In addition to the GST induction, safeners can increase also the cellular GSH content, a molecule particular healthy/beneficial for plants for its involvement in many plant functions (like: protein synthesis, oxidative stress, toxic compounds detoxification, etc.).[9]

It is well known that the crop productivity in fields is strictly depending on an adequate mineral nutrition acquisition by plants; nutrient shortages can strongly reduce crop yields worsening often also their nutritional values.[12] Among the nutrient deficiencies, particularly impacting on crops is Fe shortage, especially when calcareous soils are considered.[13] In fact, despite the abundance of Fe in the geosphere, the insurgence of iron deficiency in plants can depend on soil characteristics as high pH values which can strongly limit the fraction of Fe available to plant roots.[14] To cope with this nutritional disorder, monocots (*Strategy II plant* species) enhance the release of Fe-chelating exudates (named phytosiderophores, PSs) able to solubilize Fe in the rhizosphere forming Fe-PSs complexes. These latter are then taken up by roots in their complexed form *via* a high affinity root uptake system.[13] In this respect, it has been recently demonstrated that herbicide treatments can affect the mechanisms activated by plants to cope with Fe deficiency interfering specifically on the functionality of these mechanisms.[15-17]

It has been well demonstrated that the efficacy of monocots plants like barley to respond to the Fe nutritional stress is heavily depending on sulfur reductive assimilation.[18-20] In fact, methionine (Met), a S-containing amino acid produced starting from cysteine (Cys), is the precursor for the PSs biosynthesis.[15] In this context, it is interesting to note that some safeners can have a positive effects on sulfur assimilation path inducing also the GSH biosynthesis.

Considering all these premises also in relation to the wide use of safeners currently in agriculture, in the present work the effect of three herbicide safeners (mefenpyr-diethyl (Mef), fenchlorazole-ethyl (Fen) and dichlormid (Dic)) on the mechanisms adopted by monocots to cope with Fe limited availability, have been studied. To this purpose, barley plants as representative of monocots were

used. Fen and Mef are normally employed for winter cereal crops, and specifically for barley safening, while Dic is mainly used for maize.[1,4] Therefore, we monitored in safened barley plants the activity of ATPS sulpurylase (ATPS, EC 2.7.7.4) and O-acetylserine (thiol) lyase (OASTL, EC 4.2.99.8). ATPS and OASTL are important enzymes for their crucial-role in the pathways of the reductive sulphate assimilation.[21] Specifically, ATPS catalyzes the incorporation of sulphate into an activated ATP linked form (adenosine 5'-phosphosulfate). On the other hand, OASTL is responsible for the incorporation of sulfur into Cys.[21] Finally, we assessed in safeners-treated barley plants the contents of Cys and GSH. Since Cys is a biosynthetic precursor of PS, we monitored the PS rate release in treated plants. At last, some experiments were carried out in order to ascertain eventual effect of safeners on plant capacity to uptake iron.

2. MATERIAL AND METHODS

2. Materials and methods

2.1. Plant material and growth conditions

Barley (*Hordeum vulgare* L., research line Europa) seeds were soaked in ultrapure water and left in continuous agitation for 8 hours. Then, they were placed in Petri dishes, added of ultrapure water and kept in the dark for 4 days. Afterwards, the seedlings were positioned in a growth chamber (12/12 hours of light/dark, 23/19°C). Plants were grown in Fe deficiency conditions in a continuously aerated hydroponic solution composed as follows: 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 $(NH_4)_6Mo_7O_{24}$ x $4H_2O$ and 1 µM Fe-EDTA. Growth mediums were renewed every 4 days. At the appearance of the first symptoms of chlorosis (day 14), the nutrient solutions were renewed and Mef, Fen and Dic were added at the concentration of 1.0 mg L^{-1} (treated), which corresponds to about their field dosage. Other tanks were untreated

and then left as controls (control). At 24, 48 and 72 hours after the beginning of safener's treatment, SPAD index (SPAD-502 Plus, Konica Minolta, Japan) was measured on the shoots of treated and untreated plants in order to evaluate the chlorophyll concentrations. The measurements were performed using the first leaf of each plant, 5-10 cm from the bottom, midway between the midrib and the leaf margin. In each time, after the measures of SPAD, some plants were harvested and collected in order to measure length and weight of shoots and roots and for the following analysis.

2.2. Enzyme extraction and assay

Root tissues (ca. 1 g FW) of control and treated plants were powdered in a pre-chilled mortar under liquid nitrogen and homogenized in 3 mL of a cold extraction buffer 50 mM HEPES-KOH (pH 7.4) containing 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 then filtered and centrifuged at 4°C for 5 min at 1,000*g*. The supernatant was 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*. Finally, the resulting supernatant was frozen in liquid nitrogen and stored by freezing (-80°C) until use for in vitro enzyme assays.

The extractable ATP sulphurylase activity was determined by the bioluminescence technique according to Ciaffi et al. [22] O-acetylserine(thiol)lyase activity was assayed by detecting cysteine production as described by Ciaffi et al. [22] Protein content of roots extracts was determined by the protein-binding Coomassie brilliant blue G-250 dye method, using bovine serum albumin (BSA) as standard.[23]

2.3. Cysteine and glutathione contents

In plant tissues collected as previously described Cys and GSH were quantified by reversed-phase HPLC.[24] Briefly, 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*. Aliquots of 280 µL

of the supernatant were added of 420 µL of 200 mM CHES (pH 9.0) containing 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 for 15 min. The reactions were stopped by adding 760 µL of 0.25% (v/v) methanesulfonic acid. HPLC separation and determination of the derivatized thiols was done in accordance with Zechmann et al.[24]

2.4. Root exudates collection and PS quantification

In order to quantify the amount of PS exuded, after accurate root washing, 3 plants/sample were placed into beakers containing 20 mL of ultrapure water, under continuous aeration. Root exudates were collected for 5 hours, starting 2 hours after the beginning of the photoperiod. The amount of PS exuded was quantified by a colorimetric method (Cu-CAS assay) according to Shenker et al.[25]

2.5. Determination of shoots and roots Fe concentration

At harvest, plants were deeply washed, and then roots and shoots were separated and oven-dried at 60°C. When a constant weight was reached, all plant parts were microwave digested (ETHOS One, High Performance Microwave Digestion System, Milestone Inc, Sorisole, Bergamo, Italy) with 8 mL of nitric acid (65% v/v, Carlo Erba) and 2 mL of H_2O_2 (30% v/v). Iron concentration was determined by atomic absorption spectophotometer (AA-680 Series - Shimadzu, Kyoto, Japan).

2.6. 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 concentration in maize plants

Results of the present work show that plant biomass production and tissues morphology were substantially unaffected by the presence of safeners in the growth medium. In fact, as reported in Table 1, length and fresh weight of shoots and roots of barley plants growth in Fe deficiency and exposed to Mef, Fen and Dic at the concentration of 1.0 mg L^{-1} were generally not affected by safeners, as compared with untreated controls (Table 1). The only exception was plants treated with Mef at 72 hours after the treatment where a root fresh weight increase of 9.4%, was recorded. Also the chlorophyll concentration in leaves, evaluated by a SPAD meter, was unaffected by the safener's exposure of barley plants.

3.2. Activity of ATPS and OASTL

With respect to ATPS, increases in the enzymatic activity were recorded during the whole experimental period when barley plants were treated with Mef (Figure 2). In particular, as compared to control-plant extracts, inductions of 14.8, 89.2 and 88% were found at 24, 48 and 72 hours after the treatments, respectively. Also in the case of Fen, enhancements of ATPS activity of 20.7 and 61.1% were found at 48 and 72 hours after the treatment. Similarly also in plants treated with Dic the ATPS activity increased of 17.2 and 47.5%, after 48 and 72 hours of the plant exposure to the safener, respectively.

For its involvement in sulphate reductive assimilation, in the present work it has been decided to monitor the activity of OASTL in response to the safeners' treatments. At 24 hours after the treatments both the three safeners decreased the enzyme activity, with the following rank: Fen (-15.7%) > Dic (-38.2%) > Mef (-25.7%) (Figure 3). At 48 hours after the treatments Fen was still exerting a depressing effect on OASTL activity (being -19.7%, with respect to control samples). Differently, Dic treated barley recovered and showed the same activity of untreated controls, while

Mef slightly stimulated the enzymatic activity (+13%). At 72 hours after the treatments all the three safeners exerted an inductive effect on OASTL activity and the inductions were as follows: Fen (101.7%) > Mef (71.3%) > Dic (25.2%).

3.3. Cysteine and GSH contents in barley shoots of plants treated or not with safeners

The concentrations of Cys and GSH were investigated in Fe deficient barley plants treated with the three safeners, and collected at 24 and 72 h after the treatments. Data reported in Figure 4 show that Cys concentration increased in Mef treated sample at 24 and 72 hours after the treatments and the increases were of 68.2 and 109.5%, respectively. On the other hand, the other two safener, while they did not modify the Cys content at 24 hours after the treatments, they increase of 53.0 and 67.5% the concentration of this amino acid in the samples, respectively.

With regard to GSH content, Mef induced the tripeptide content of 42.2 and 73.9% in both the time sampling considered, respectively (Figure 5). No effects were recorded when the plants were treated with Fen and Dic for 24 hours. On the other hand, after 72 hours of exposure to these two safeners, the GSH content increased of 15.3 and 17.0%, respectively.

3.4. Phytosiderophores release

As expected, the PS release by control barley plants increased during the experiment period as the consequence of Fe starvation (Figure 6). As reported in the Figure 6, plants treated with the three safeners did not show any significant difference in PS rate release at 24 hours after the treatments, as compared to untreated controls. On the contrary, prolonging the exposure to the safeners, the PS release was enhanced in all three treatments. In fact, starting from 48 hours after the treatment, the amount of phytosiderophores exuded by root were increased of 127.3%, 63.4% and 84.5% for Met, Fen and Dic, respectively, as compared with control plants. At 72 hours after the exposure to the safeners, this stimulatory effect of safeners was still present with increases of 62.9, 37 and 53.3% for Mef, Fen and Dic, respectively.

3.5. Fe concentration in maize shoots and roots

In this work, being the nutrient solution supplied with very low amount of Fe (1 uM), the plants were grown essentially in Fe deficiency, independently of the presence of safeners. Therefore, Fe plant tissue concentration was assessed in shoots and roots of barley harvested at 24, 48 and 72 hours after the Mef treatment. No significant differences in Fe contents were found in shoots of treated plants, along the whole experimental, with respect to the untreated controls (data not reported). With regard to roots, at 24 and 48 hours after the treatment no variations on Fe concentrations were found in the treated samples, with respect to untreated controls. On the other hand, differences in roots iron content were found in maize plants treated with Mef at 72 hours after the treatment. In fact, the safener provoked an increase of 35.7% on root Fe content, with respect to untreated barley controls.

4. DISCUSSION

Herbicide safeners protect crops form herbicides damage, without reducing their efficacy against target weeds. The physiological and biochemical mechanisms underlying the safening actions have stimulated an intense research activity in order to elucidate the mode of action of these molecules, and to manipulate the mechanisms which contribute to enhance herbicide selectivity and resistance in crops.[4] To date, about the 30% of herbicides are sold in formulations containing a safener.[26] However, despite their wide use in agriculture, there are no literature reports addressing the question whether safeners can affect the mechanisms activated by plants for Fe acquisition, in spite of some recent studies have documented that herbicides can impair Fe mineral nutrition in crops.[15,17]

For these reasons, we carried out some experiments in order to investigate the effect of three herbicide safeners, namely mefenpyr-diethyl (Mef), fenchlorazole-ethyl (Fen) and dichlormid (Dic),

on the mechanisms activated by barley plants in Fe deficiency. Results here reported show that biomass accumulation and tissues morphology, both at the root and leaf level, were unaffected by the three safeners, with exception of a slight root-fresh-weight increase found at 72 hours for Mef treated plants (Table 1). The safeners were also ineffective to modify at the leaf level chlorophyll concentrations, which maintain essentially the same values along the treatment, independently of the safeners considered (Figure 1). The data of these physiological parameters indicate that Mef, Fen and Dic did not cause any appreciable stress symptoms, along the entire experimental period, and this in accordance with the scarce or absent phytotoxicity often described for safeners with respect to crops.[27]

Safeners act as "bioregulators" and are able to enhance the herbicide detoxification.[28] In order to be effective, these chemicals must interact with plant defense systems and induce both defensive genes and the activity of herbicide-detoxifying enzyme.[29] At last, some herbicide safeners can increase the content of endogenous sulfur containing molecules.[3] In this context, it is well demonstrated that sulphur metabolism is closely related to the capacity of monocots to acquire Fe and to cope with the Fe shortage.[18-20] To ascertain if Mef, Fen and Dic can exert a similar positive effect on sulfur containing molecules in barley plants, we assessed the activity of the two key-enzymes, which control the reductive sulphate assimilation in plants, ATPS and OASTL. ATPS is responsible of the transformation of sulphate in an ATP activated linked form, while OASTL catalyzes and regulates the crucial step of the conversion of o-acetyl-serine (OAS) in Cys. The intake flux of reduced sulfur in plants is regulated by the coordinated action of these two enzymes. With regard to ATPS, starting from 24 hours after the treatment, Mef strongly induced the enzyme activity in treated barley plants (Figure 3). Fen and Dic stimulated the enzyme activity later with respect to Mef, and of more modest entities. However, all the safeners were very effective in inducing the ATPS activity. The responsiveness of this enzyme to abiotic stresses has been reported in many studies.[30] In general, ATPS induction is functional to meet the high sulfur demand for the heavy-metal-detoxification.[31] For instance, Heiss et al. [30] found in *Brassica juncea* ATPS inductions in response to cadmium (Cd)

exposure. Furthermore, very recent findings have pointed out as terbuthylazine, a triazine herbicide, can impair the activity of the ATPS enzyme, so determining some severe reductions on thiols pool.[15] The second enzyme here investigated was OASTL; from the activity of this enzyme depends the cellular content of Cys. In this case, different trends were found in response to the exposure to the different safeners. Data reported in Figure 3 show that shortly after the exposure of the plants to the safeners (24 hours), Mef, Fen and Dic significantly reduced the OASTL activity; afterwards, prolonging the treatments up to 48, the enzyme activity recovered in Mef-treated plants, where reached values significantly higher than controls plants. At the end of the experiment (72 hours), all the three safeners were able to induce strong increases in OASTL activity. This trend is surely curious; however, the initial decreases of OASTL activity should be seen as the earlier response to the safeners treatments. In fact, safeners act as modulators of genes coding for plant defense enzymes[32,33] and it has been suggested that safener-mediated induction of detoxifying enzymes is part of a more general plant response to stress, which includes three major steps: stress recognition, signal transduction and defense gene activation.[34] In particular, Droog [35] reported that safeners initially induce oxidative bursts which provoke some membrane damages, the generation of hydrogen peroxide (H_2O_2) and lead to the activation of defensive genes.[35] For this reason, immediately after the treatments, safeners cause the same oxidative stress as that caused by herbicides. Afterwards, the intrinsic toxicity of herbicides, which rapidly targets specific site of action and injuries plants, while the inertness of safeners to plants does not cause toxicity to target crops. On this context, the decreases of OASTL activity found during the first hours after the treatments are the consequence of an unspecific related response of plant to stress; afterwards, the safening action prevailed, then the chemicals activated the defensive responses and induced the OASTL activity. In accordance with our results, some investigations have already evidenced not only the susceptibility of OASTL to certain different abiotic stresses, but also its specific response related to the nature of the stressor.[36,37]

Since alterations on APTS and OASTL activities can interfere with the content of reduced thiols, and particularly with that of Cys, some other experiments were aimed at determining the content of Cys and GSH in treated plants. Data reported in figure 4, show that at the end of the experimental period, all the safeners were capable to significantly increase the Cys content, with respect to the untreated controls, following the order Mef > Dic > Fen. In addition, our results indicate that Mef stimulated the increase on Cys content earlier than the other two safeners. Considering the central role of Cys in plant metabolism,[38] increases on its content can stimulate the biosynthesis of other essential molecules, and, particularly, those of GSH, proteins and Met. It should be pointed out this beneficial effect to plants considering the protective role of GSH, mainly involved in herbicide detoxification and to cope with oxidative stress. With respect to Met, it should be highlighted that it is the only precursor of PSs and its availability can affect PSs biosynthesis and their release in the rhizosphere. Therefore, the successive experiments were aimed at ascertaining, in Fe starved barley plants, the effect of the three safeners on GSH content and on PSs rate release. Results of Figure 5 show a specific safener-related induction of GSH contents, with a strong increase in Mef-treated plants already after 24 hours of treatment with the safener. It is interesting to note that the other two safeners did not exert any appreciable effect on the GSH content. However, prolonging the exposure up to 72 hours with the safeners, the induction of GSH content was evident for all the treatments. As already stated, the main protective function of safeners in crops is to stimulate the herbicide metabolism by inducing detoxifying enzymes and increasing the GSH content which, in turn, can react enzymatically or not with toxic substrates to give GSH-herbicide conjugates, usually less toxic and mobile than the precursors.[39] In addition, some herbicides can target the photosynthesis producing thus high levels of oxidants; in this case GSH, for its strong reducing potential, is involved in coping and remediating with the oxidative perturbations.

Since graminaceous monocots plants under Fe deprivation exude huge amount of PSs in order to cope with the problem and to acquire enough nutrient for an equilibrate growth, we investigated the effect of safeners on PSs release. Figure 6 shows that no effects were found at 24 hours after the

safeners treatments, while elevated levels of PSs were released by safeners-treated plants after 48 and 72 hours of safener exposure. In particular, Mef was found to be the most effective at 48 hours of the treatment. These results are quite impressive particularly when considered in relation to the negative effects of herbicides on PSs release and, more in general, on Fe acquisition by roots.[15,17,40] This safener-mediated stimulatory effect on PSs release can be very beneficial in promoting Fe acquisition in plants suffering of Fe starvation. Finally, in this work it has been demonstrated for the first time that the safener action is not only restricted to the promotion of detoxifying pathways, but can act also at the level of the mechanisms induced in graminacoues monocots to cope with Fe shortage. In order to ascertain whether the increases in PSs release could enhance the amount of Fe adsorbed by treated plants, a conclusive experiments was carried out on Mef, for its general major effectiveness in stimulating the activity of enzymes involved in sulfur assimilation, Cys and GSH accumulation and PSs release. Data reported in Figure 7 clearly demonstrate that the treatment with this safener can induce higher accumulation of Fe in roots, despite the availability of the micronutrient in the nutrient solution was maintained at very low levels (1 μM Fe).

In conclusion the research carried out evidenced that the three safeners Mef, Fen and Dic can differently activate, in a specific-chemical manner, sulfur reductive metabolism so determining increases in Cys, GSH and PSs contents. This stimulatory action can regard the Fe content at root level, as ascertained in mefenpyr-diethyl treated barley. Finally, these results highlight new aspects regarding the safeners effect on the mechanisms activated by monocots to cope with Fe deficiency. Such stimulatory effects deserve attention not only for deeply elucidating the biochemical and physiological mechanisms involved in the safening action, but also for giving some useful indication and prospective finalized at improving the agricultural practices.

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