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Interference of three herbicides on iron acquisition in maize plants

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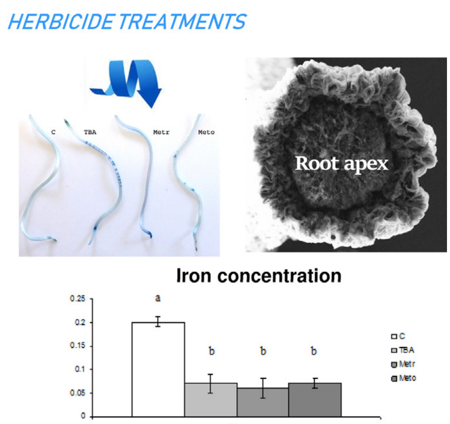
HIGHLIGHTS

- Herbicides can be hazardous to the environment and non-target organisms.
- Terbuthylazine, metribuzin and metolachlor reduced Fe content in maize roots.
- Herbicides damaged root extremities as evidenced by Evans Blue Test.
- Phytosiderophores rate release was decreases by herbicides' treatments.
- SEM-EDX showed as treatments reduced Fe, Cu, Zn and Mn contents in root apices.

GRAPHICAL ABSTRACT



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ABSTRACT

The use of herbicides to control weed species could lead to environmental threats due to their persistence and accumulation in the ecosystems and cultivated fields. Nonetheless, the effect of these compounds on plant mineral nutrition in crops has been barely investigated. This study aimed at ascertaining the effect of three herbicides (*S*-metolachlor, metribuzin and terbuthylazine) on the capacity of maize to acquire iron (Fe). Interferences on plant growth and reductions on the Fe contents were found in the plants treated. Furthermore, root cell viability and functionality losses were ascertained following the treatments, which, in turn, decreased the amount of phytosiderophores (PSs) released by the roots. An investigation carried out in greater depth on root apices of treated plants using an FE-SEM (Scanning Electron Microscope) coupled with EDX (Energy Dispersive X-ray) indicated that the reductions on Fe content started in this part of the roots. Lastly, decreases were found also in copper (Cu^{+2}), zinc (Zn^{+2}) and manganese (Mn^{+2}) content in root apices.

1. Introduction

Modern agriculture needs high crop productivity in order to cope with the increasing food demand, and this in order to meet the necessities of the growing world population (Del Buono et al., 2016). In cultivated fields, weeds compete with crops for

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nutrients and other resources (Gaba et al., 2017). In order to manage this situation, herbicides are used worldwide for crop protection against weeds (Mimmo et al., 2015). However, the massive use of these chemicals can cause resistance in weeds and affect the environment (Gaba et al., 2017). In particular, they can impact on non-target organisms and bring about hazards to human and animal health (Boily et al., 2013; van der Meulen and Singh Chauhan, 2017). Some of these chemicals can also accumulate and persist in soils (Arias-Estévez et al., 2008; Magne et al., 2006), and thus could represent a risk for crops in the following cultural cycle (Del Buono et al., 2015).

Despite their selectivity in targeting weeds, some herbicides can be phytotoxic to crop species at morphological, physiological and biological levels (Vercampt et al., 2017). Recently, some authors found significant interferences of herbicides on plant mineral nutrition. For example, glyphosate was shown to be able to reduce the uptake of certain micronutrients in soybeans (Zobiole et al., 2010) and sunflower (Eker et al., 2006). In particular, some of these studies showed reductions on iron (Fe) assimilation and distribution in various crop species (Bartucca et al., 2017; Bellaloui et al., 2009; Del Buono et al., 2015; Eker et al., 2006; Ozturk et al., 2008). In general, interferences on the capacity of plants to acquire Fe can result in Fe-chlorosis. This disorder is characterised by interveinal to complete yellowing and browning of leaves which could, in extreme situations, lead to plant death (Singh and Dayal, 1992). Fe has a fundamental role in photosynthesis, DNA and chlorophyll biosynthesis, mitochondrial respiration and nitrogen fixation (Nagajyoti et al., 2010). This element is also a key constituent of many important proteins such as ferredoxin, catalase, peroxidase and superoxide dismutase (Nagajyoti et al., 2010). Therefore Fe is a limiting factor for plant biomass production and quality (Briat et al., 2015).

Fe is acquired by plants through root-uptake mechanisms which take place in the rhizosphere. For instance, in graminaceous plants (*Strategy II* plants), Fe uptake is based on the biosynthesis of Fe^{3+} chelating compounds, called phytosiderophores (PSs) (Hell and Stephan, 2003). These compounds, which belong to the mugineic acids (MAs) family, are released by roots directly into the rhizosphere, where they form chelates with the nutrient (Colombo et al., 2014). The chelates are then imported into roots by specific transporters (Ma et al., 1995). In graminaceous plants, PS biosynthesis and secretion into the rhizosphere is markedly increased in Fe-deficiency conditions, (Nagasaka et al., 2009). The release of PSs is also reported to be enhanced by Cu^{+2}, Zn^{+2} and Mn^{+2} deficiency (Römheld, 1991). It has been hypothesized that PSs are involved in the acquisition of the abovementioned cations with which they form stable chelates (Römheld, 1991).

In this study the effect of three herbicides on the capacity of maize (*Zea mays*, L. a *Strategy II* plant) to acquire Fe was investigated. The experiments were carried out with plants grown in Fe-sufficiency conditions. The herbicides selected were terbuthylazine (TBA), metribuzin (Metr) and *S*-metolachlor (Meto). They are applied to maize fields all over the world to control a wide range of annual and perennial grasses and broadleaf weeds. They were selected for this study also for their long persistence in the environment, which can result in their accumulation in the soil. The excessive or inappropriate use of these chemicals has in fact resulted in their frequent detection in the environment at concentrations above the European limits (Del Buono et al., 2016; Jaikaew et al., 2015; Lopez-Piñero et al., 2014). TBA and Metr, which belong respectively to the *s*-triazine and triazinone families of herbicides, act by interrupting the photosynthetic electron transport chain at the level of photosystem II (Godinho de Almeida et al., 2017; Mimmo et al., 2015). Meto, a member of the chloroacetanilide chemical family, inhibits plant growth by disturbing long-chain fatty acid biosynthesis (Copin et al., 2016).

Therefore, in order to reach the aim proposed, maize plants were treated with TBA, Metr and Meto, and plant biomass, chlorophyll concentration, root health status and functionality, Fe accumulation in roots and shoots and PS secretion in roots were assessed. As stated in the literature, the apex is the most active part of the root in nutrient absorption, as well as in the excretion of exudates (Uren and Reisenauer, 1988). For this reason Fe was quantified on the sub-apical cross sections of maize roots using a Scanning Electron Microscope (SEM) equipped with EDX (Energy Dispersive X-rays). Cu, Zn and Mn were also quantified in treated samples. To our knowledge, this is the first study aimed at investigating the effect of these herbicides on the Fe acquisition process in maize plants.

2. Materials and methods

2.1. Plant material and growth conditions

Maize (*Zea mays* L.) seeds were soaked in ultrapure water and left in continuous agitation for 8 h. Then they were placed in Petri dishes, with ultrapure water added, and kept in the dark. After 4 days, the seedlings were transferred into a growth chamber (12/12 h of light/dark, 23/19 °C) and placed in tanks containing a continuously aerated hydroponic solution composed as follows: 2 mM $Ca(NO_3)_2\ 4H_2O$, 0.5 mM $MgSO_4\ 7H_2O$, 0.7 mM K_2SO_4, 0.1 mM KCl, 0.1 mM KH_2PO_4, 1 μM H_3BO_3, 0.5 μM $MnSO_4\ H_2O$, 0.5 μM $CuSO_4$, 0.5 μM $ZnSO_4\ 7H_2O$, 0.01 μM $(NH_4)_6Mo_7O_{24}\ 4H_2O$ and 100 μM Fe-EDTA. Two weeks after sowing, plants were treated with 2.0 mg L^{-1} of TBA or Metr or Meto (Sigma Aldrich, St. Louis, MO, USA), while other samples were untreated and left as controls. The applied concentration of the herbicides (2.0 mg L^{-1}) was chosen to reach high field rates (usually employed to manage problematic weeds), and/or simulate situations of herbicide accumulation in soils. Plants were collected at 24, 48 and 72 h after the treatment, and length and weight of shoots and roots were assessed. SPAD index (SPAD-502 Plus, Konica Minolta, Japan) was measured to estimate the chlorophyll concentration in leaves. SPAD measurements were taken for the third leaf of each plant, 5–10 cm from the bottom, midway between the midrib and the leaf margin.

2.2. Determination of shoot and root Fe concentration

Plants were harvested at 24, 48 and 72 h after the treatments. Roots were separated from shoots and washed with water. Both the roots and shoots were then oven dried at 60 °C and microwave digested with 8.0 mL nitric acid (65% v/v, Carlo Erba) and 2.0 mL hydrogen peroxide (30% v/v) using a microwave system (ETHOS One, High-Performance Microwave Digestion System, Milestone Inc, Sorisole, Bergamo, Italy). Iron concentration was determined by Atomic Absorption Spectroscopy (AA-680 Series, Shimadzu, Kyoto, Japan).

2.3. Evans Blue Test

Cell death in roots of controls and herbicide-treated samples was evaluated using the Evans Blue Test. 72 h after the treatments, collected roots were washed with water for 10 min, separated from leaves and placed into Petri dishes; roots were then stained for 1 min with 0.5% (w/v) Evans blue (Sigma, USA) at room temperature and under stirring. These samples were then washed several times with ultrapure water until the solution became colourless. Images were captured using a digital camera.

2.4. Collection of exudates and PS quantification

Plants were harvested at 24 and 48 h after the treatments. After accurate washing of the roots, 5 plants/sample were placed into beakers containing 15 mL of ultrapure water. Root exudates were collected in the morning, starting 2 h after the onset of the light period in the growth chamber (Aciksoz et al., 2011). The duration of the sampling was 5 h (Del Buono et al., 2015). The amount of PS exuded was then quantified by a colorimetric method, the Cu-CAS assay (Shenker et al., 1995). The Cu–CAS reagent was composed of a 40 mM MES buffer (adjusted at pH 5.7 with 0.1 M NaOH) containing 200 μM $CuCl_2$ and 210 μM CAS. Various volumes of the root exudates collected were transferred into cuvettes (5.0 mL) and Cu–CAS reagent was added. Once mixed, the absorbance (Abs) of the cuvettes was recorded spectrophotometrically at 582 nm, and PS concentration in each sample was estimated (Shenker et al., 1995).

2.5. Elemental quantification by EDX

Maize plants were collected at 72 h after the treatments. Roots were washed with water and separated from shoots. The apical part of the secondary roots was cut approximately 0.5 cm from the apex and fixed with 5% glutaraldehyde in sodium cacodylated buffer 0.075 M (pH 7.0). After 24 h, dehydration was done with a series of washings in ethanol solutions: 2 times for 10 min with 25% ethanol, 2 times for 10 min with 50% ethanol, 2 times for 10 min with 75% ethanol, 2 times for 10 min with 95% ethanol and 7 times for 8 min with 100% ethanol. Samples were dried to critical point (Critical Point Drying, CPD), hand-cut in sections of 3 mm and fixed on glass slides. The slides were positioned on stubs. The quali-quantitative element composition was determined in the core of root apices using a Field Emission Scanning Electron Microscope (FE-SEM) LEO 1525 ZIESS equipped with an energy-dispersive x-ray detector (EDX) (Bruker Quantax). A sample area of 6400 μm^2 was selected to acquire EDX spectra. The detection time was 30 min.

2.6. Statistical analysis

Each reported value represents the mean ± standard deviation (SD) of data from three independent experiments on three biological replicates per experiment. The length and weight of shoots and roots and chlorophyll concentration were determined using 20 plants (average of 5 measurements/plant). Statistical analyses of data were carried out by ANOVA tests and significant differences were established by Duncan's test at $P < 0.05$.

3. Results

3.1. Length and weight of shoots and roots, and SPAD values in maize plants

In plants subjected to TBA treatment, reductions in plant growth were not found during the first 48 h after the treatments. On the contrary, growth of both shoots and roots was inhibited at 72 h after the treatment. The length and weight of the aerial parts were reduced by 17.1% and 38.4% respectively, compared to untreated samples. Root length and fresh weight were reduced by 21.2% and 21.3%, respectively (Table 1). No significant effects in biomass production resulted from treatment with Metr (Table 1). Lastly, plants treated with Meto did not show growth inhibition during the first 48 h after the treatment. Differently, Meto interfered with plant growth at 72 h after the treatment. More specifically, there were reductions of 7.7% and 27.2% in shoot length and fresh weight, respectively (Table 1).

TBA and Metr caused a reduction in the chlorophyll concentration (determined as SPAD units) of 18.5% and 18.3% at 72 h respectively, compared to untreated samples (Table 2). On the other hand, no significant differences in SPAD values were found in Meto-treated plants in comparison with untreated samples (Table 2).

Table 1
Shoots and roots length (cm) and fresh weight (g) of maize plants, treated or not (C) with the herbicides (terbuthylazine - TBA, metribuzin – Metr and Metolachlor - Meto). Data are means ± SD (n = 20).

	24 h	48 h	72 h
Length of shoots (cm)			
C	31.09 ± 1.31a	34.46 ± 3.20a	40.30 ± 3.20a
TBA	29.73 ± 2.53a	32.54 ± 3.71a	33.40 ± 3.18b
Metr	29.91 ± 2.43a	34.91 ± 3.05a	38.09 ± 3.44a
Meto	30.69 ± 3.30a	35.54 ± 2.33a	37.18 ± 3.22b
Fresh weight of shoots (g)			
C	1.46 ± 0.22a	1.77 ± 0.30a	2.50 ± 0.32a
TBA	1.52 ± 0.29a	1.52 ± 0.31a	1.54 ± 0.31b
Metr	1.51 ± 0.33a	1.74 ± 0.31a	2.09 ± 0.33a
Meto	1.43 ± 0.32a	1.75 ± 0.22a	1.82 ± 0.30b
Length of roots (cm)			
C	18.54 ± 1.61a	19.91 ± 2.30a	22.21 ± 2.62a
TBA	16.78 ± 2.29a	17.21 ± 1.52a	17.50 ± 2.02b
Metr	17.92 ± 2.56a	18.46 ± 2.40a	20.93 ± 3.04a
Meto	19.87 ± 2.39a	19.57 ± 2.77a	21.13 ± 2.99a
Fresh weight of roots (g)			
C	0.62 ± 0.03a	0.65 ± 0.04a	0.75 ± 0.08a
TBA	0.57 ± 0.04a	0.57 ± 0.03a	0.59 ± 0.06b
Metr	0.64 ± 0.06a	0.66 ± 0.08a	0.70 ± 0.09a
Meto	0.60 ± 0.06a	0.61 ± 0.04a	0.71 ± 0.08a

For each column, means followed by different letters are significantly different at $P < 0.05$.

3.2. Fe concentration in maize shoots and roots

Fe concentration was determined in shoots and roots of maize plants harvested and collected at 24, 48 and 72 h after the treatments. No significant differences were found in the aerial Fe concentration in maize plants treated with TBA and Metr during the entire experimental interval. Conversely, in Meto-treated samples the nutrient content in shoots decreased at 72 h after treatment. In this case, the Fe concentration was reduced by 25.0% compared to untreated controls (Fig. 1). Differently, the Fe content of the roots was generally strongly affected by all the three herbicides during the entire experimental period (Fig. 1). TBA decreased Fe content in roots at 48 and 72 h after the treatment by 23.0% and 38.0%, respectively, in comparison with untreated plants (Fig. 1). In Metr- and Meto-treated plants, decreases in Fe concentration in roots were found at all the experimental intervals. In the first case (Metr), the reductions were higher during the first hours after treatment, and then gradually diminished. More specifically, Fe decreases of 51.0%, 45.9% and 25.8% were found at 24, 48 and 72 h respectively, compared to untreated control plants (Fig. 1). Treatment with Meto

Table 2
Estimated chlorophyll concentration (SPAD Units) in maize shoots treated or not (C) with the herbicides (terbuthylazine - TBA, metribuzin – Metr and Metolachlor - Meto). Data are means ± SD (n = 20).

SPAD Units	24 h	48 h	72 h
C	33.08 ± 3.41a	35.89 ± 4.17a	40.93 ± 4.03a
TBA	32.38 ± 3.14a	34.86 ± 3.25a	33.34 ± 3.01b
Metr	31.98 ± 2.83a	33.12 ± 2.19a	33.44 ± 3.10b
Meto	32.45 ± 3.01a	34.45 ± 3.18a	37.56 ± 4.12a

For each column, means followed by different letters are significantly different at $P < 0.05$.

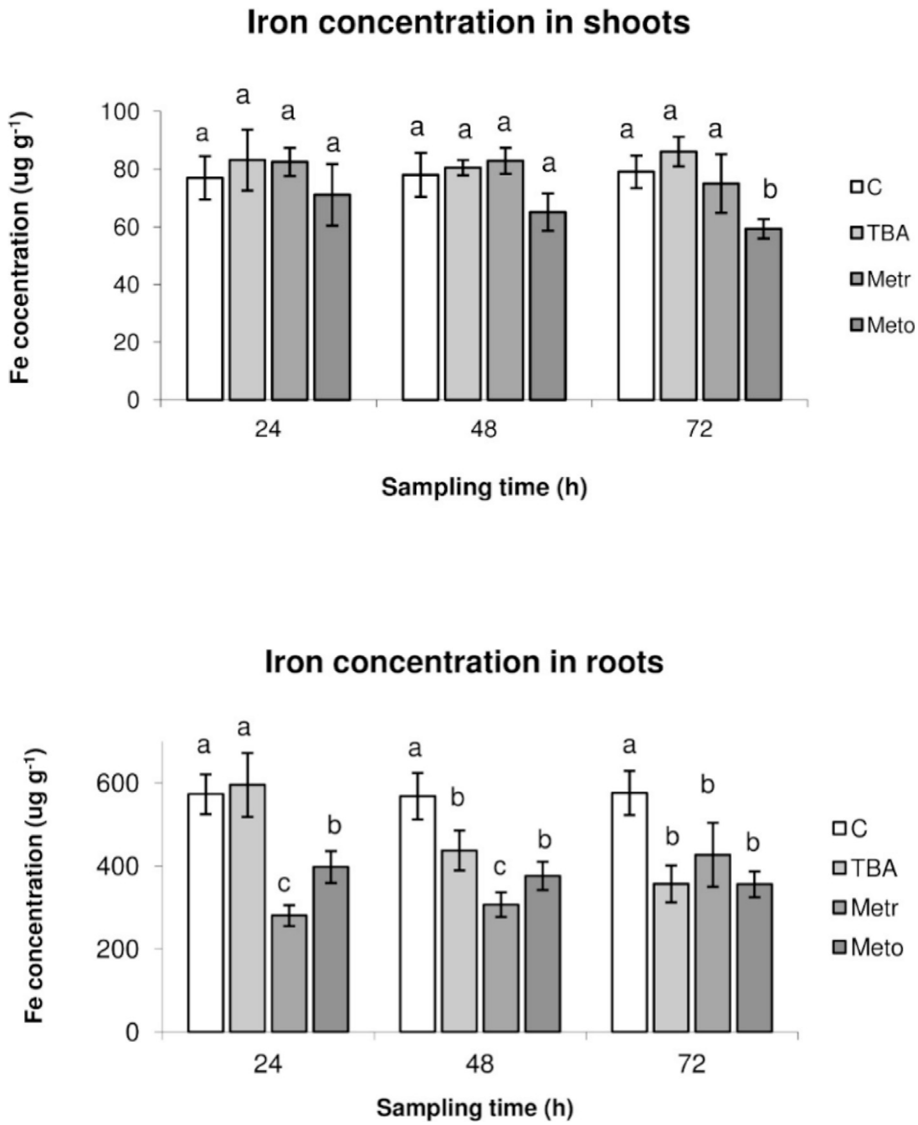

Fig. 1. Fe concentration (μg g^{-1} DW) in maize shoots (top of the figure) and roots (bottom of the figure) treated or not (C) with herbicides terbuthylazine (TBA), metribuzin (Metr) and metolachlor (Meto). Data are means ± SD (n = 3).

caused reductions in Fe concentration in roots that remained constant during the entire experimental period, with decreases of 30.6%, 33.8% and 38.2% at 24, 48 and 72 h after the treatment, compared to untreated controls (Fig. 1).

3.3. *Cell death in maize roots of herbicide treated samples*

As reported in the literature, Evans Blue is a reliable test for the determination of cell death and damage to cell membranes (Castro-Concha et al., 2006), as this method reveals the loss of plasma membrane integrity (Shaw and Hossain, 2013). Fig. 2 shows some roots representative of the damage caused by herbicide treatments. The test showed evidence of cell death in herbicide-treated maize roots, compared to untreated samples. In particular, TBA-treated roots showed extremities with extensive damaged areas. Metr-treated plants showed intense cell mortality in the apical part of the root. Lastly, Meto-treated roots showed longitudinally extensive cell mortality as well as tissue breakage (Fig. 2).

3.4. *Phytosiderophores release*

The PS release rate was measured in root exudates collected from control and herbicide-treated maize plants at 24 and 48 h after the treatments. In all the three cases, PS release was affected by the herbicide treatments, as shown in Fig. 3, during the entire experimental period. In TBA-, Metr- and Meto-treated plants, the PS release was respectively reduced by 47.6%, 40.7% and 36.8% at 24 h after herbicide treatment, and by 31.4%, 51.5% and 25.1% respectively at 48 h after the treatment.

3.5. *Fe concentration in root apices*

Given that the experiments showed decreases in Fe content, PS release and damage to roots following herbicide exposure, the apices of treated plants were investigated in depth with an FE-SEM equipped with EDX. The picture given in Fig. 4 indicates the section of the root samples where the studies were carried out. The

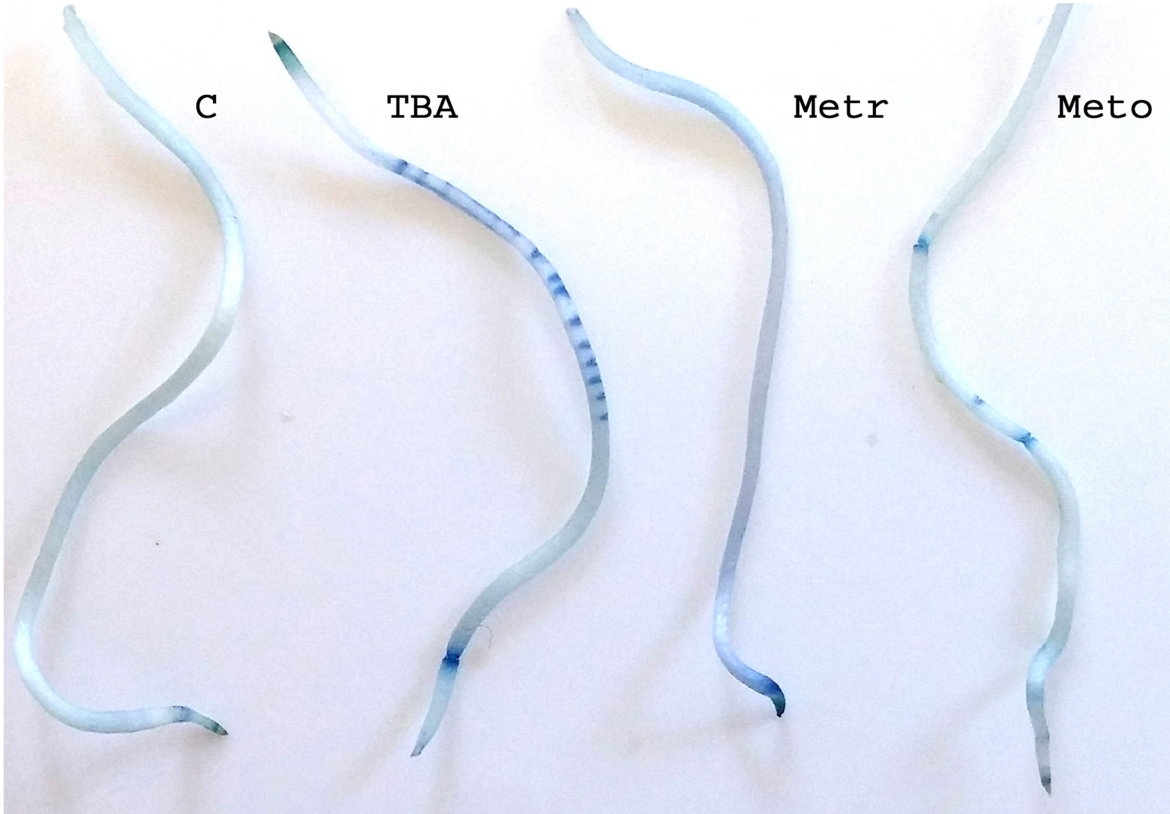

Fig. 2. Cell death and root damages, evidenced by Evans Blue Test, in untreated (C) samples and treated maize with terbuthylazine (TBA), metribuzin (Metr) and metolachlor (Meto) plants, at 72 h after the chemicals exposure.

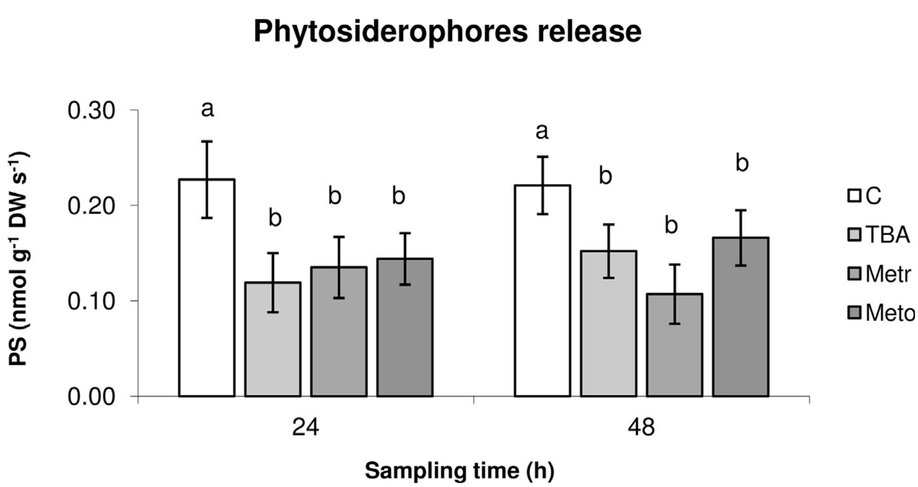

Fig. 3. Phytosiderophores amounts released by roots of control (C) and herbicide treated maize plants at 24 and 48 h after the treatments (terbuthylazine - TBA, metribuzin – Metr and Metolachlor - Meto). Data are means ± SD (n = 3).

determinations showed that the three herbicides strongly reduced Fe content in the apical root section (Fig. 5). The analytical quantification of the nutrient in the treated samples at 72 h after treatments showed that TBA, Metr and Meto reduced the Fe concentration by 65.0%, 70.0% and 65.0% (Fig. 5) respectively, compared to the untreated controls.

3.6. Cu, Zn and Mn concentration in root apices

Given the involvement of PS in plant Cu, Zn and Mn acquisition (Römheld, 1991), the same investigation was carried out for the abovementioned cations on root apices of herbicide-treated plants using the FE-SEM EDX. Similarly to that found for Fe, at 72 h after the treatment reductions in the apex contents of these elements were ascertained (Fig. 5). More specifically, the Cu, Zn and Mn contents were found to be decreased by 37.9%, 51.7% and 74.8% in TBA-treated plants, compared to controls. In Metri-treated plants, the Cu, Zn and Mn contents were found to be reduced by 65.5%, 48.3% and 52.1%, while in Meto-treated plants they were reduced by 44.8%, 36.8% and 67.2% compared to untreated samples (Fig. 5).

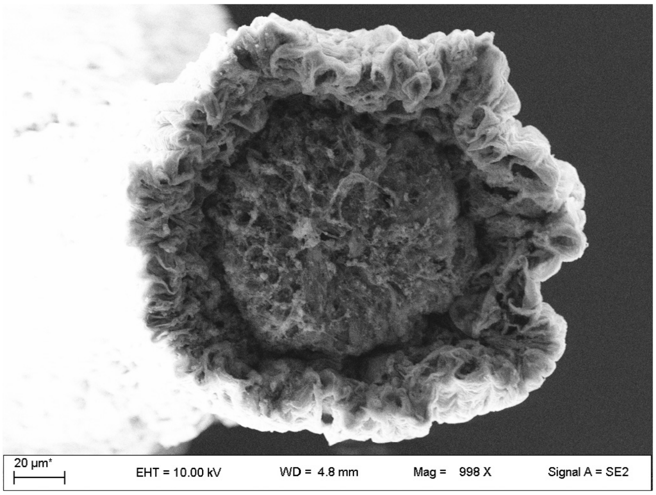

Fig. 4. Sectioned root apex utilized for the elemental quantification by FE-SEM equipped with EDX.

4. Discussion

The aim of this study was to point out any effects of three widely used herbicides for maize weeding paying particular attention to the plants' capacity to acquire Fe. First of all, growth parameters such as maize shoot and root length and weight were analyzed. Terbuthylazine (TBA) was found to be the most interfering herbicide; indeed, it was able to affect both aerial and root growth in maize. As shown by previous studies, this can be a common effect caused by the application of some herbicides, despite their selectivity towards crops (Nemat Alla et al., 2008). In our study, no effects were found on plant growth in samples treated with Metribuzin (Metr). Lastly, *S*-metolachlor (Meto) was able to affect only the shoot growth (72 h), but to a lesser extent if compared to TBA. Regarding SPAD index values, it was found that TBA and Metr-treatments affected the chlorophyll concentration (Table 2). All the aforementioned effects of TBA and Metr can be linked to their mechanism of action. In fact these herbicides act by interrupting the photosynthetic electron transport chain at the level of photosystem II, although targeting different sites (Godinho de Almeida

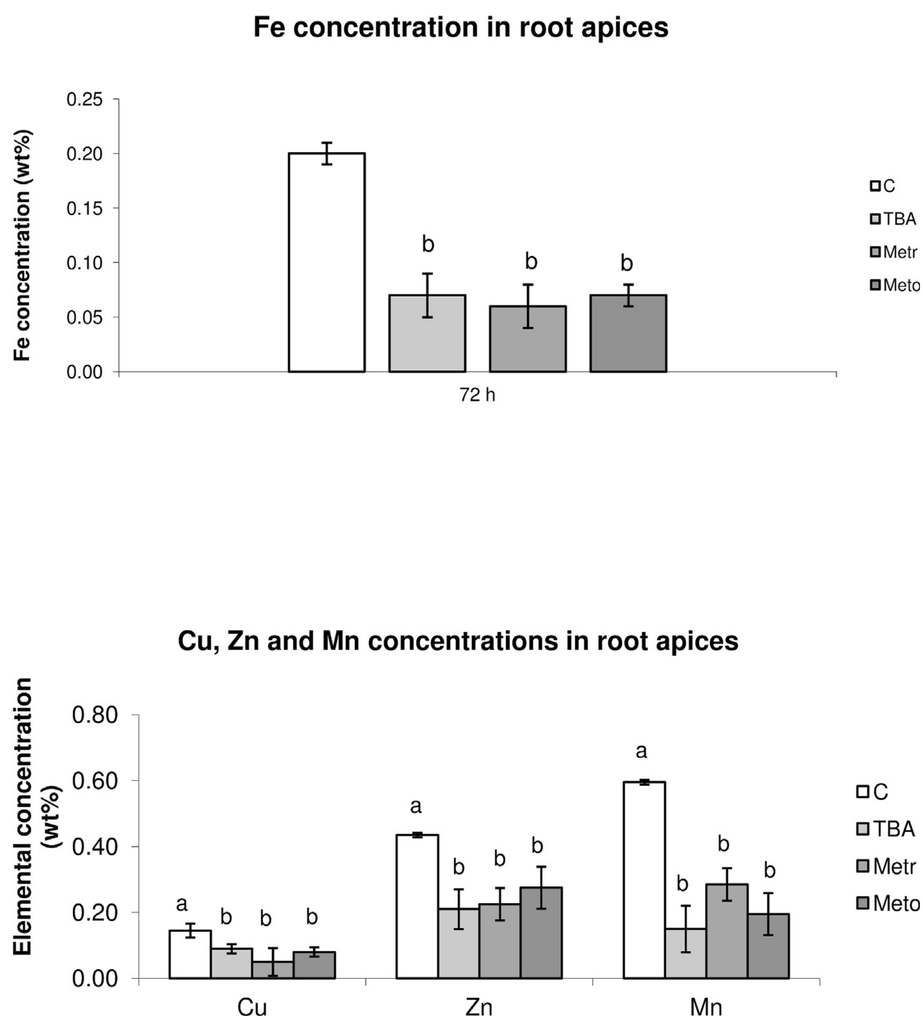

Fig. 5. The top of the figure shows the Fe concentration determined using SEM-EDX in the apical root sections of control (C) and herbicide treated maize plants (terbuthylazine - TBA, metribuzin – Metr and Metolachlor - Meto), at 72 h after the treatments. At the bottom of the figure, Cu, Zn and Mn contents (SEM-EDX) found at 72 h after the treatments are reported. Data are means ± SD (n = 3).

et al., 2017; Mimmo et al., 2015). More specifically, TBA inhibits the D1 protein (Mimmo et al., 2015) and Metr avoids the reduction of plastoquinone (Godinho de Almeida et al., 2017). As stated in some previous studies, there is a clear correlation between the photosynthetic rate and chlorophyll biosynthesis in plant species (Boussadia et al., 2011; Dawson et al., 2003). This link also explains the drop in chlorophyll caused by TBA and Metr. In the case of Meto, its mode of action, which is not directly related to photosynthesis, justifies the absence of interference on plant chlorophyll concentration (Table 2). Indeed, Meto acts by inhibiting the synthesis of long chain fatty acids and plant growth (Copin et al., 2016). We then determined the Fe concentration in the shoots and roots of untreated and herbicide-treated plants. Meto was found to be the only herbicide affecting Fe concentration in maize shoots (72 h after treatment) (Fig. 1). Conversely, all three herbicides greatly reduced Fe concentration in plant roots, starting to exert their interference in the first hours after the treatments (Fig. 1). Previous studies revealed disturbances caused by herbicides on Fe acquisition in plants. Most of them reported that glyphosate (Gly) was able to interfere on Fe-acquisition processes of dicotyledon (*Strategy I*) plants (Bellaloui et al., 2009; Eker et al., 2006; Zobiole et al., 2012). In particular, the authors showed that Gly decreased the Fe concentration in seeds and leaves of treated soybean, and this interference was attributed to the inhibiting effect of the herbicide on the activity of ferric chelate reductase (FCR) (Bellaloui et al., 2009). In addition, Gly reduced the Fe uptake and transport in sunflower, possibly for the formation of a Gly-metal complex (Eker et al., 2006). Finally, Zobiole et al. (2012) documented that increasing Gly rates and applications decreased the nutrient accumulation in plants and shoot biomass production. On the other hand, few studies showed reductions in Fe assimilation in herbicide-treated graminaceous (*Strategy II*) plants (maize and wheat), but these were generally carried out in Fe-deficiency (Bartucca et al., 2017; Del Buono et al., 2015). Nonetheless, the herbicides decreased plant growth, chlorophyll concentration, Fe content (Bartucca et al., 2017) and the expression of genes involved in Fe acquisition (Del Buono et al., 2015). However, to our knowledge, our findings are the first experimental evidence of interferences of the TBA, Meto and Metr on the capacity of mazie, grown in Fe sufficiency, to acquire this nutrient. In fact, there are no other reports in the literature documenting that these three herbicides can affect Fe content in maize. In order to clarify how these chemicals exerted this effect on Fe assimilation, root health status and their capacity to release PSs were assessed in treated plants. For this assessment, the Evans Blue Test was applied to study maize roots. This test, which allows the investigation of cell membrane integrity, gives information about root cell viability and functionality (Shaw and Hossain, 2013). The method applied indicated wide cell death in herbicide-treated roots compared to control samples. In particular, as shown in Fig. 2, TBA affected the root extremities; Metr instead caused damage which, however, was found to be confined to the root apex. Lastly, Meto-treated maize showed widespread damage and breakage along the root extremities. Based on these observations, we concluded that the treatments caused a lack of root functionality, and this altered the normal capacity of the species to uptake the nutrient. In accordance with these findings, other studies demonstrated that some herbicides can influence the plant capacity to uptake certain cations by depolarizing the plasma membranes of root cells (Wright, 1994). In addition, Robson and Snowball (1990) postulated that the reductions in plant capacity to acquire nutrients might result from impairments of root functions. Lastly, Dong et al. (1995) demonstrated that herbicides can interfere with root development by altering its geometry; as a result of this effect, plants showed a reduced capacity to assimilate nutrients (Dong et al., 1995).

Subsequently, the plants' capacity to release PS was investigated in herbicide-treated plants during the first hours 48 h after the treatments. PS, which are non-proteogenic aminoacids, are released by graminaceous plants, especially in iron-deficiency conditions, in order to acquire Fe (Nagasaka et al., 2009). PS exudation was found to be strongly reduced by the treatments, without significant differences among the chemicals investigated (Fig. 3). These interferences on PS release, with the altered functionality shown by the Evans Blue Tests, explain the reduced Fe concentrations found in the roots.

In order to investigate in depth the herbicides' effects on nutrient assimilation, we carried out further experiments on the root apex of maize plants. In fact, some authors reported that nutrient absorption by roots is highest at the level of the root apex, or at the as yet non-suberized part close behind the apex (Eshel and Beeckam, 2013; Russell and Clarkson, 1976). For example, Fang et al. (2007) demonstrated that the root tip of *Landoltia punctata* had the highest NH_4^+ and NO_3^- ion influx, which decreased at the distal elongation zone. Regarding Fe, root characteristics and functions, which impact the nutrient uptake in plants, are confined to their apical and sub-apical zones (Sijmons and Bienfait, 1986). In particular, the nutrient acquisition occurs in the root apices, particularly in the case of the less mobile elements, such as Fe (Uren and Reisenauer, 1988). At least, it should be mentioned as these parts of the roots are also considered the portions most active in the excretion and release of exudates (Uren and Reisenauer, 1988). Therefore, impairments of their functionality may alter the plant's capacity to release PS. In Fig. 4, an image capture, taken by an FE-SEM, gives an example of the apical root section utilized in our subsequent investigations. The Fe concentrations determined by using EDX showed that the three herbicides induced, in the treated apices, a strong reduction in the plant's capacity to assimilate the nutrient (Fig. 5). This is in accordance with our first findings, regarding the total Fe concentration ascertained in the whole roots (Fig. 1). Therefore, these results indicate that the herbicide interference on nutrient acquisition starts at the root tip. A very small number of studies reported the use of SEM EDX to investigate the capacity of plants to uptake nutrients following exposure to toxic xenobiotic compounds. However, regarding pesticides, only Lavid et al. (2001) demonstrated that by using SEM EDX the herbicide 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea reduced the accumulation of metals in the photosyntethic tissues of the leave of *Nymphaea*.

Since TBA, Meto and Metr were capable to decrease the Fe content in the apices of treated maize, the concentrations of Cu, Zn and Mn were investigated in root tips of plants exposed to the herbicides. In fact, it should be emphasized as in graminaceous species these elements, like Fe, are acquired thanks to phytosiderophores release (Römheld, 1991). Indeed, PSs permit to *Strategy II* plants to take up from soil Zn, Cu and Mn, in PS-chelated forms, despite this mobilization can be considered unspecific (Römheld, 1991). As shown in Fig. 5, the concentrations of Cu, Zn and Mn were reduced by all the treatments. Therefore, the decrease in PS release (Fig. 3) and the damages caused by TBA, Meto and Metr to root tips (Fig. 2) have led to reductions in the plant's capacity to assimilate these elements. Previous studies documented similar effects on Zn, Mn and Cu contents in crops caused by herbicide applications. For instance, a sulphonylurea herbicide – chlorsulfuron – decreased the net uptake of Cu, Zn and Mn in three wheat genotypes, regardless of the duration of exposure (Rengel and Wheal, 1997). Furthermore, the active compound affected also the root development (Rengel and Wheal, 1997). Finally, a study carried out on barley plants showed as metsulfuron-methyl (another sulphonylurea herbicide) decreased the contents of Zn, Mn, K and Cu, and interfered with root development (Pederson et al., 1994).

Nonetheless, this effect was considered temporary and related to the plant's capacity to detoxify the toxic xenobiotic compounds (Pederson et al., 1994).

5. Conclusions

In conclusion, our experiments showed alterations in root growth and loss of cell viability and functionality following the herbicide treatments. The release of PSs was also affected by the treatments, thus impairing the plant's capacity to assimilate Fe. A further in-depth analysis conducted on radical apices revealed that the inhibition of Fe assimilation begins here. In treated plants, root apices showed decreases in Fe content which affected the amount of the nutrient in the entire root. Regarding the other cations investigated (Cu, Zn and Mn), our experiments showed reductions in their concentrations in root apices. As with Fe, these interferences can be ascribed to a reduced absorption capacity seen in treated root apices.

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