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# A Comparative Study on the Interference of Two Herbicides in Wheat and Italian Ryegrass and on Their Antioxidant Activities and Detoxification Rates

Daniele Del Buono,* Gerardina Ioli, Luigi Nasini, and Primo Proietti

Dipartimento di Scienze Agrarie e Ambientali, Università degli Studi di Perugia, Borgo XX Giugno 74, 06121 Perugia, Italy

**ABSTRACT:** A study was carried out to compare the effects of treating wheat (*Triticum aestivum*) and Italian ryegrass (*Lolium multiflorum*) with atrazine and fluorodifen. The herbicides interfered with photosynthesis and dark respiration, depending on the species. Atrazine decreased photosynthesis in both species and dark respiration in wheat, while fluorodifen caused decrements of photosynthetic activity of wheat. Antioxidant enzymes, such as ascorbate peroxidase (APX), dehydroascorbate reductase (DHAR), and glutathione reductase (GR), were generally more active in untreated and treated wheat with respect to Italian ryegrass, which explains why oxidative damage, expressed as malondialdehyde (MDA) content, was only found in ryegrass. Investigations on the activity of herbicide-detoxifying enzyme, glutathione *S*-transferase (GST), and on the accumulation and persistence of the herbicides in the plants showed higher detoxification rates in wheat than in the grass.

**KEYWORDS:** wheat, Italian ryegrass, herbicides, antioxidant enzymes, detoxification rate, photosynthesis, dark respiration, lipid peroxidation

## INTRODUCTION

Numerous abiotic and biotic stresses can cause the production of reactive oxygen species (ROS) in cells, resulting in oxidative stress. ROS include superoxide $O_2^-$, singlet oxygen $^1O_2$, hydrogen peroxide $H_2O_2$ and hydroxyl radical $^{\bullet}OH$.[1] These species can cause injuries to cells, even death; in fact they are very reactive toward chlorophylls, proteins, lipids and DNA.[2] However, the production of ROS is a fact in organisms living in aerobic environments: they are produced through normal metabolism, as byproduct of the electron transport chains of chloroplasts and mitochondria, and by peroxisomes, oxidases and peroxidase.[3] In addition, they can be produced in response to stressing agents, such as drought, chilling and herbicides.[4] The ability of a plant to control and remove these oxidants is closely connected with its stress tolerance.[5] In fact, these stresses cause the up- or down-regulation of hundreds of genes and plants have developed strategies to minimize ROS toxicity, based on the induction of a variety of antioxidant enzymes and on the increase in the synthesis of reducing molecules which remove the oxidants nonenzymatically.[5] These organized antioxidant defense mechanisms cooperate in scavenging ROS and in maintaining the physiological redox status; damages to cells emerge when the production of ROS exceeds the antioxidant defense.[6]

Herbicides can cause significant ROS formation which can seriously injure plants and crops. Nonetheless, herbicide resistance has been mainly attributed to the detoxificative metabolism and/or alterations of the target sites of the herbicide's action.[7] However, it has been reported that some species are resistant to herbicides because of their antioxidant activities.[8] In spite of this, comparative studies on the effects of herbicides on crops and the respective infesting weeds are scarce, as well as on their antioxidant metabolisms and detoxification rates, even though this could evidence important differences among crops and weeds, useful for the understanding of the plant-stress tolerance.

In this context, wheat and one of its more problematic weeds, Italian ryegrass, were submitted to a comparative study to evaluate the differences in the responses of these plants to two ROS-determining herbicides, atrazine and fluorodifen. The effects of the two herbicides on photosynthesis and on some antioxidant enzymes were investigated. In particular, ascorbate peroxidase (APX; EC 1.11.1.11), catalase (CAT; EC 1.11.1.6), glutathione reductase (GR; EC 1.6.4.2) and dehydroascorbate reductase (DHAR; EC 1.8.5.1) were investigated in response to the two herbicide treatments. These enzymes are essential in controlling and removing the cellular hydrogen peroxide which arise from oxidative perturbations: in fact, APX catalyzes the reduction of $H_2O_2$ to water by using ascorbic acid (AA) as the reducing agent. CAT, mainly involved in the metabolism of long chain fatty acids in peroxisomes, transforms hydrogen peroxide to water and oxygen;[9] DHAR reduces the dehydroascorbate produced by APX, using reduced glutathione (GSH), which is then oxidized to GSSG. In order to continue its pivotal action, oxidized glutathione is reduced to GSH by GR which uses NADPH as reducing agent.[5,10] Furthermore, in order to evaluate the herbicide damage to membranes and then the effectiveness of the antioxidant activities, the malondialdehyde (MDA) content was investigated. MDA is a product of lipid peroxidation of membranes, and its content is considered a good indicator to evidence oxidative damages to cells.[2]

In addition, to ascertain the contribution of the detoxification metabolism to plant tolerance, glutathione *S*-transferases (GST; EC 2.5.1.18), a class of enzymes very active in herbicide detoxification, were investigated for both plants.[11] Finally, the

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accumulation and persistence of the herbicides in shoots of wheat and Italian ryegrass were determined and correlated to the detoxification rates and to the interference with photosynthesis and levels of MDA.

Atrazine and fluorodifen were chosen because they act by determining ROS formation. In particular, atrazine, of the triazine family, inhibits photosystem II by blocking electron transfer to the plastoquinone pool, leading to the production of triplet chlorophyll and $^1O_2$, which cause oxidative stress.[12,13] Fluorodifen, of the diphenyl ether family, acts by interfering with the activity of protoporphyrinogen IX oxidase (PROTOX), the final enzyme in the tetrapyrrole biosynthesis pathway, before it branches to heme or chlorophyll.[14] The inhibition of PROTOX results in the accumulation of protogen IX, which leaks out of the plastid and begins a series of nonenzymatic oxidations which give rise to oxidative stress.[15]

## MATERIALS AND METHODS

**Chemicals and Apparatus.** Atrazine, fluorodifen, 1-chloro-2,4-dinitrobenzene, ascorbate, dehydroascorbate, hydrogen peroxide, nicotinammide adenine dinucleotide phosphate, reduce glutathione reduced, oxidized glutathione, and SPE Florisil cartridges were supplied by Sigma Aldrich (St. Louis, MO). Acetonitrile, ethyl acetate, methanol, *n*-hexane and water were all of analytical grade and were purchased from BDH (Poole, U.K.). All other reagents were of ACS grade. A Perkin-Elmer series 410 HPLC, equipped with an LC 95 UV and a C-18 column (4.6 mm i.d.; 25 cm length), was employed for the determinations of herbicide residues. A UV/vis Thermo Spectronic Genesys 10 Bio spectrophotometer was used for the enzyme assays.

**Plant Material and Growth Conditions.** Seeds of *Lolium multiflorum*, 'Bofur' (Italian ryegrass) (30 g), and seeds of *Triticum aestivum*, 'Eridano' (wheat) (30 g), were germinated in plastic pots (0.08 $m^2$) containing quartz sand prewashed with a solution of hydrochloric acid (10%, v/v) and sterilized with a solution of NaClO (5%, w/v). Seedlings were grown in a growth chamber in the dark at 18 °C (relative humidity 80%). After two days, the seedlings were submitted to day−night conditions (12 h of light at 23 °C, light intensity 200 $\mu$mol $m^{-2}$ $s^{-1}$, and 12 h of darkness at 21 °C) and watered daily. For each species, when the seedlings were 7 days old, the pots were divided into three groups: one group was left as the control, and the others were treated with atrazine and fluorodifen, at the recommended field rates, which were 14 mf/flat for both the chemicals. For the determination of photosynthetic activity, MDA and enzyme activities, samples were collected 24, 48, and 72 h after the treatments; for the determination of herbicide residues, shoots were collected 24, 48, 72, 96, and 144 h after the treatments.

**Evaluation of $CO_2$ Gas Exchange.** Gas exchanges were determined using a portable ADC LCA-3 gas exchange analyzer (Analytical Development Company Ltd., Hoddesdon, U.K.) and a Parkinson-type assimilation chamber. Leaves were enclosed in the chamber and exposed perpendicularly to the light source. The flow rate of dried air passing through the chamber was kept at 5 $cm^3$ $s^{-1}$. During gas exchange measurements, the external $CO_2$ concentration was about 450 $cm^3$ $m^{-3}$ and the air temperature inside the leaf chamber was about 1 °C higher than that in the atmosphere.

Three leaf samples (about 6 and 11 $cm^2$ for Italian ryegrass and wheat, respectively) per treatment were randomly sampled from the plastic pots 24, 48, and 72 h after treatment, and the gas exchange was determined in the morning from 9:00 to 11:00 (incoming photosynthetic photon flux density about 200 $\mu$mol $m^{-2}$ $s^{-1}$). The rate of $CO_2$ exchange was determined, first in the light, to measure net photosynthesis, and then in the dark, by covering the assimilation chamber with a black cloth screen (the temperature in the chamber decreased about 1.5 °C), to measure dark respiration rate. Measurements were recorded under steady-state conditions. Net photosynthesis and dark respiration rate are expressed on a leaf area basis.

**Enzyme Extraction.** Samples (2.0 g) were ground to a fine powder in liquid nitrogen. The powder was then suspended in an extraction buffer (1/5, w/v) composed of 100 mM Tris-HCl (pH 7.5), containing 2 mM EDTA, 1 mM dithiothreitol and 1.5% (w/v) polyvinylpolypyrrolidone. After filtration through layers of muslin, the homogenate was centrifuged at 10000*g* for 20 min and the supernatant was submitted to activity determinations of GST, APX, CAT and GR. For the extraction of DHAR, the powders were suspended in an extraction buffer (1/5, w/v) composed of 100 mM phosphate buffer, 2 mM ascorbic acid, 1 mM EDTA, 2.5% (v/v) glycerol and 2% (w/v) polyvinylpolypyrrolidone. After filtration, the suspension was centrifuged as described above.

**Determination of Enzyme Activities.** The APX activity was determined by adding 50 $\mu$L of 10 mM ascorbate and 50 $\mu$L of 30 mM $H_2O_2$ to a solution containing 800 $\mu$L of 50 mM $KH_2PO_4/K_2HPO_4$ (pH 7.0) and 50 $\mu$L of enzymatic extract. The amount of ascorbate oxidized was determined spectrophotometrically at 290 nm at 30 °C for 60 s.[16]

The CAT activity was determined by adding 300 $\mu$L of 30 mM $H_2O_2$ and 100 $\mu$L of enzymatic extract to 3 mL of 50 mM $KH_2PO_4/K_2HPO_4$ buffer (pH 7.0). The amount of $H_2O_2$ consumed was determined spectrophotometrically at 240 nm at 30 °C for 60 s.[17]

The DHAR activity was determined by adding 50 $\mu$L of 100 mM GSH, 50 $\mu$L of 4 mM dehydroascorbate mM and 50 $\mu$L of enzyme extract to 1600 $\mu$L of 100 mM $KH_2PO_4/K_2HPO_4$ buffer (pH 7.0) containing 1 mM EDTA. The amount of ascorbic acid increase was evaluated spectrophotometrically at 265 nm and 30 °C for 60 s.[18]

The GR activity was determined by adding 50 $\mu$L of 20 mM oxidized glutathione (GSSG) to 800 $\mu$L of 100 mM Tris-HCl (pH 8.0), containing 50 $\mu$L of 4 mM NADPH and 100 $\mu$L of enzyme extract. The amount of NADPH oxidized was evaluated spectrophotometrically at 340 nm at 30 °C for 3 min.[19]

The GST activity was determined by adding 25 $\mu$L of 40 mM CDNB to a solution containing 900 $\mu$L of 0.1 M $KH_2PO_4/K_2HPO_4$ buffer (pH 6.5), 25 $\mu$L of enzymatic extract and 50 $\mu$L of 0.1 M GSH (pH 7.0). The amount of conjugate formed by reaction between GSH and CDNB was determined spectrophotometrically at 340 nm at 35 °C for 60 s.[20]

The protein content of each sample was determined according to the procedure of Bradford.[21]

**MDA Content.** MDA content was measured according to Meng et al.[22] Shoots of Italian ryegrass and of wheat (0.5 g) were ground to a fine powder using a mortar and pestle. The powders were suspended in 1% (w/v) trichloroacetic acid and centrifuged at 12000*g* for 12 min. The supernatant was incubated at 95 °C for 30 min, then cooled for 2 min in an ice bath and centrifuged at 15000*g* for 10 min. The MDA content was expressed as $C = 6.45(A_{532} - A_{600}) - 0.56A_{450}$, where $C$ is the concentration of MDA in $\mu$M $L^{-1}$, and $A_{532}$, $A_{600}$ and $A_{450}$ represent the absorbances at 532, 600, and 450 nm, respectively.

**Determination of Atrazine and Fluorodifen in Shoots of Wheat and Italian Ryegrass.** The herbicide residues were determined according to the method of Del Buono et al.[23] Briefly, the shoot samples (1 g) were powdered in liquid nitrogen and extracted with methanol (w/v, 1:5); the resulting suspension was filtered, dried under vacuum and rinsed with 2 mL of *n*-hexane. The solution was then charged on a SPE cartridge Florisil column (1000 mg/6 mL, 170 $\mu$m, 80 A), preactivated with *n*-hexane, washed with *n*-hexane and then recovered with ethyl acetate/*n*-hexane (v/v, 2:3). The recovered fractions were then evaporated to dryness, rinsed with 1 mL of methanol and subjected to HPLC analysis.[23] To validate the method, recovery tests were performed for both herbicides, by adding adequate amounts of chemicals to the plant samples (1.0 g) in order to give the concentrations

**Table 1. Photosynthetic Activity and Dark Respiration of Shoots of Wheat and Italian Ryegrass 24, 48, and 72 h after Treatment, with Respect to Untreated Plants[a]**

| | leaf net photosynthesis and dark respiration ($\mu$mol of $CO_2$ $m^{-2}$ $s^{-1}$) | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | wheat | | | | | | italian ryegrass | | | | | |
| | net photosynth | | | dark respirn | | | net photosynth | | | dark respirn | | |
| | control | atrazine | fluorodifen | control | atrazine | fluorodifen | control | atrazine | fluorodifen | control | atrazine | fluorodifen |
| 24 h | 1.35 a | −0.52 b | 1.15 a | −1.35 a | −0.51 b | −0.82 b | 4.55 a | −0.61 b | 4.64 a | −1.51 a | −1.32 a | −1,42 a |
| 48 h | 1.92 a | 0.22 b | 2.13 a | −1.43 a | −0.43 b | −0.77 c | 4.89 a | −1.04 b | 4.42 a | −1.68 a | −1.56 a | −1.36 a |
| 72 h | 1.46 a | 0.00 b | 1.25 a | −1.07 a | −0.90 a | −0.94 a | 4.45 a | −1.35 b | 4.05 a | −1.32 a | −1.67 a | −1.06 a |

[a] Data represent the means of triplicate determinations. In each row, for each species, for net photosynthesis and dark respiration, means followed by the same letters are not significantly different at $P \leq 0.05$.

**Table 2. Ascorbate Peroxidase Activity Determined in Shoots of Wheat and Italian Ryegrass Treated with Atrazine and Fluorodifen, 24, 48, and 72 h after Treatment, with Respect to Untreated Samples[a]**

| | APX (nmol $s^{-1}$ mg of protein$^{-1}$) | | | | | |
|---|---|---|---|---|---|---|
| | wheat | | | Italian ryegrass | | |
| | control | atrazine | fluorodifen | control | atrazine | fluorodifen |
| 24 h | 15.7 a | 22.0 b | 22.9 b | 12.5 a | 15.0 b | 13.9 c |
| 48 h | 18.4 a | 23.5 b | 20.2 c | 11.3 a | 14.2 b | 15.3 c |
| 72 h | 17.2 a | 18.0 a | 17.2 a | 11.6 a | 12.2 a | 14.0 b |

[a] The data represent the means of triplicate determinations. For each species, means within the same row followed by the same letter are not significantly different from the control, at the 5% level using the Student *t* test.

**Table 3. Catalase Activity Determined in Shoots of Wheat and Italian Ryegrass Treated with Atrazine and Fluorodifen, 24, 48, and 72 h after Treatment, with Respect to Untreated Samples[a]**

| | CAT (nmol $s^{-1}$ mg of protein$^{-1}$) | | | | | |
|---|---|---|---|---|---|---|
| | wheat | | | Italian ryegrass | | |
| | control | atrazine | fluorodifen | control | atrazine | fluorodifen |
| 24 h | 0.10 a | 0.10 a | 0.11 a | 0.13 a | 0.13 a | 0.11 a |
| 48 h | 0.12 a | 0.11 a | 0.12 a | 0.10 a | 0.10 a | 0.11 a |
| 72 h | 0.13 a | 0.12 a | 0.14 a | 0.12 a | 0.13 a | 0.13 a |

[a] The data represent the means of triplicate determinations. For each species, means within the same row followed by the same letter are not significantly different from the control, at the 5% level using the Student *t* test.

of 0.2, 0.5, 2.0, and 10.0 mg $kg^{-1}$. The recovery tests gave satisfactory results for the residue determinations in both plants (data not shown); in fact, they ranged from 80 to 100%, at the concentrations of 0.2, 0.5, 2.0, and 10.0 mg $kg^{-1}$.

## RESULTS

**Photosynthetic Activity and Dark Respiration.** Table 1 reports data of photosynthetic activity and dark respiration for leaves of wheat and Italian ryegrass treated with atrazine and fluorodifen and the respective controls. In wheat, atrazine inhibited photosynthetic activity, but reduction in net photosynthesis (which is given by the difference between the total amount of photosynthesis and the respiration) was lessened because of the contemporary reduction in dark respiration, which, however, recovered 72 h after treatment. Fluorodifen treatment did not interfere with net photosynthesis, but dark respiration was slightly decreased 24 and 48 h after treatment.

In Italian ryegrass, atrazine caused an inhibition in the fixation of $CO_2$ and net photosynthesis was always negative, decreasing from 24 to 72 h after treatment. On the contrary fluorodifen did not influence net photosynthesis, nor did the treatment significantly influence dark respiration.

**Activity of Antioxidant Enzymes in Untreated and Treated Wheat and Italian Ryegrass.** Table 2 reports the effect of the herbicides on the activity of ascorbate peroxidase. For both species, there were significant increases in the enzyme activity in response to the treatments. In particular, atrazine caused a significant increase in APX activity 24 and 48 h after the treatments; the increases were 40 and 28% for wheat and 20 and 26% for Italian ryegrass, respectively. Fluorodifen also significantly increased the APX activity of the two species. In particular, in wheat the APX activity increased 46 and 10% 24 and 48 h after treatment, while in the shoots of Italian ryegrass the increases were 11, 35, and 21% 24, 48, and 72 h after treatment, respectively. There were no significant differences in catalase activity (Table 3).

Regarding dehydroascorbate reductase (DHAR) activity (Table 4), atrazine caused significant increases of 20, 15, and 21%, 24, 48, and 72 h after treatment, respectively; regarding Italian ryegrass, atrazine caused a 15% increase in the above enzyme activity only 24 h after the treatment. Fluorodifen increased the enzyme activity in both species; in particular, increases of 9, 11, and 45% were determined in wheat 24, 48, and 72 h after treatment; while in Italian ryegrass, there were increases of 8 and 19% 24 and 48 h after treatment, respectively.

Both herbicide treatments caused increases in GR activity only at 24 h after treatments, and they were 36% for wheat and 27 and 72% for seedlings of Italian ryegrass treated with atrazine and fluorodifen, respectively (Table 5).

Concerning GSTs, the plants exhibited different responses to the treatments. In particular, in wheat, atrazine caused significant decreases in GST activity of 37 and 26%, 24 and 48 h after treatment; also fluorodifen decreased the GST activity in wheat 15%, 24 h after treatment, respectively.

**Table 4. Dehydroascorbate Reductase Activity Determined in Shoots of Wheat and Italian Ryegrass Treated with Atrazine and Fluorodifen, 24, 48, and 72 h after Treatment, with Respect to Untreated Samples[a]**

| | DHAR (nmol $s^{-1}$ mg of $protein^{-1}$) | | | | | |
|---|---|---|---|---|---|---|
| | wheat | | | Italian ryegrass | | |
| | control | atrazine | fluorodifen | control | atrazine | fluorodifen |
| 24 h | 4.6 a | 5.5 b | 5.0 c | 3.9 a | 4.5 b | 4.2 b |
| 48 h | 4.6 a | 5.3 b | 5.1 b | 3.6 a | 3.5 a | 4.3 b |
| 72 h | 4.7 a | 5.7 b | 6.8 c | 3.7 a | 3.6 a | 3.7 a |

[a] The data represent the means of triplicate determinations. For each species, means within the same row followed by the same letter are not significantly different from the control, at the 5% level using the Student *t* test.

**Table 5. Glutathione Reductase Activity Determined in Shoots of Wheat and Italian Ryegrass Treated with Atrazine and Fluorodifen, 24, 48, and 72 h after Treatment, with Respect to Untreated Samples[a]**

| | GR (nmol $s^{-1}$ mg of $protein^{-1}$) | | | | | |
|---|---|---|---|---|---|---|
| | wheat | | | Italian ryegrass | | |
| | control | atrazine | fluorodifen | control | atrazine | fluorodifen |
| 24 h | 1.1 a | 1.5 b | 1.5 b | 1.1 a | 1.4 b | 1.9 c |
| 48 h | 1.1 a | 1.1 a | 1.2 a | 1.1 a | 1.0 a | 1.0 a |
| 72 h | 1.1 a | 1.0 a | 1.0 a | 1.2 a | 1.2 a | 1.1 a |

[a] The data represent the means of triplicate determinations. For each species, means within the same row followed by the same letter are not significantly different from the control, at the 5% level using the Student *t* test.

On the other hand, treatment of Italian ryegrass with atrazine caused significant increases in the GST activity that were 35, 103, and 24% 24, 48, and 72 h after treatment, respectively. In the case of fluorodifen, strong increases of the activity of the above enzyme were ascertained, and they were 97, 371, and 35%, 24, 48, and 72 h after treatment, respectively (Table 6).

**MDA Content in Untreated and Treated Wheat and Italian Ryegrass.** Determination of the malondialdehyde content for untreated samples and samples treated with atrazine and fluorodifen showed that herbicides did not cause significant changes in wheat, while there were significant changes in Italian ryegrass (Table 7). In fact, for samples of Italian ryegrass treated with atrazine, MDA increased 25, 31, and 10% 24, 48, and 72 h after treatment. In Italian ryegrass treated with fluorodifen, MDA increased 14 and 10% 48 and 72 h after treatment, respectively.

**Persistence and Accumulation of Atrazine and Fluorodifen in Shoots of Wheat and Italian Ryegrass.** Table 8 reports the data of persistence and accumulation of atrazine and fluorodifen in shoots of wheat and Italian ryegrass up to 144 h after treatment. There were significant differences in the persistence and in the amount of residues of atrazine and fluorodifen in wheat and Italian ryegrass. In fact, wheat had lower amounts of herbicide residues, which, as in the case of fluorodifen, were also less persistent. However, the atrazine residues in wheat reached the highest accumulation of 5.67 ppm at 48 h after treatment; in the case of fluorodifen the highest amount of residue of

**Table 6. Glutathione *S*-Transferase Activity Determined in Shoots of Wheat and Italian Ryegrass Treated with Atrazine and Fluorodifen, 24, 48, and 72 h after Treatment, with Respect to Untreated Samples[a]**

| | GST (nmol $s^{-1}$ mg of $protein^{-1}$) | | | | | |
|---|---|---|---|---|---|---|
| | wheat | | | Italian ryegrass | | |
| | control | atrazine | fluorodifen | control | atrazine | fluorodifen |
| 24 h | 2.7 a | 1.7 b | 2.3 c | 0.34 a | 0.46 b | 0.67 c |
| 48 h | 2.3 a | 1.7 b | 2.2 a | 0.35 a | 0.71 b | 1.65 b |
| 72 h | 2.4 a | 2.5 a | 2.3 a | 0.37 a | 0.46 a | 0.50 b |

[a] The data represent the means of triplicate determinations. For each species, means within the same row followed by the same letter are not significantly different from the control, at the 5% level using the Student *t* test.

**Table 7. Malondialdehyde Content Determined in Shoots of Wheat and Italian Ryegrass Treated with Atrazine and Fluorodifen, 24, 48, and 72 h after Treatment, with Respect to Untreated Samples[a]**

| | MDA ($\mu$M $L^{-1}$) | | | | | |
|---|---|---|---|---|---|---|
| | wheat | | | Italian ryegrass | | |
| | control | atrazine | fluorodifen | control | atrazine | fluorodifen |
| 24 h | 0.054 a | 0.055 a | 0.050 a | 0.68 a | 0.85 b | 0.64 a |
| 48 h | 0.053 a | 0.053 a | 0.050 a | 0.65 a | 0.85 b | 0.74 b |
| 72 h | 0.050 a | 0.057 a,b | 0.049 a | 0.67 a | 0.74 b | 0.74 b |

[a] The data represent the means of triplicate determinations. For each species, means within the same row followed by the same letter are not significantly different from the control, at the 5% level using the Student *t* test.

**Table 8. Residues of Atrazine and Fluorodifen in Shoots of Wheat and Italian Ryegrass[a]**

| | atrazine (ppm) | | fluorodifen (ppm) | |
|---|---|---|---|---|
| | wheat | Italian ryegrass | wheat | Italian ryegrass |
| 24 h | 1.38 a | 6.00 b | 1.64 a | 1.40 a |
| 48 h | 5.67 a | 5.90 a | 0.27 a | 1.17 b |
| 72 h | 1.94 a | 4.52 b | 0.20 a | 0.46 b |
| 96 h | 1.94 a | 4.22 b | 0.08 a | 0.36 b |
| 144 h | 0.35 a | 3.11 b | n.d. | 0.20 |

[a] For a herbicide, data of residues are compared between the two plant species; means within the same row followed by the same letter are not significantly different at the 5% level using the Student *t* test.

1.64 ppm was found at 24 h after treatment. Atrazine residues were found in wheat shoots throughout the entire experimental period; the lowest amount of 0.35 ppm was determined at 144 h after treatment. In the case of fluorodifen the residues were detectable in wheat shoots until 96 h after treatment, reaching the value of 0.08 ppm.

Concerning Italian ryegrass, atrazine and fluorodifen residues reached the highest levels of accumulation (6.00 ppm and 1.40 ppm, respectively) 24 h after treatments. Atrazine residues

were found throughout the entire experimental period, and the lowest amount of the chemical was 3.11 ppm, detected at 144 h after the treatment. Fluorodifen was detected up to 144 h after treatments, when it reached 0.20 ppm.

The overall average content of atrazine in wheat was 2.26 ppm, while the average content in Italian ryegrass was 4.75 ppm; the average content of fluorodifen in wheat was 0.44 ppm, while it was 0.72 ppm in Italian ryegrass.

## DISCUSSION

Data in Table 1 show that the effect of atrazine on photosynthesis of the two species was very severe. In fact, in wheat, it caused decreases in the rate of $CO_2$ fixation, and fixed $CO_2$ was similar to $CO_2$ produced by respiration; consequently net photosynthesis, calculated by the difference between the total amount of photosynthesis and the respiration, was around 0 $\mu$mol of $CO_2$ $m^{-2}$ $s^{-1}$. Also in the case of Italian ryegrass, $CO_2$ fixation was significantly reduced but net photosynthesis was negative. The net photosynthesis values in wheat treated with atrazine were slightly higher than those of Italian ryegrass due to the simultaneous reduction in dark respiration which was not affected in Italian ryegrass. The effect on photosynthesis can be explained on the basis of the interference of atrazine on the photosynthetic electron transport in the PS II. In fact, the herbicide binds to the D1 protein of the photosystem and it causes the block of the electron transfer to plastoquinone, which results in the inhibition of the photosynthetic pathways.[13,24] On the other hand, the reduction in dark respiration has already been documented for herbicide-treated wheat, as a plant response mechanism which is activated in turn to produce greater stress tolerance.[25] The data of Table 1 also indicate that, in response to fluorodifen, there was no significant change in the net photosynthesis of wheat, because dark respiration rate again decreased, while fluorodifen did not have effects on Italian ryegrass. The mechanism of action of fluorodifen is based on the inhibition of PROTOX, a key enzyme in the biosynthesis of hemes and chlorophylls;[14] this finding suggests that Italian ryegrass could have developed a defense mechanism based on the increase of PROTOX expression or on the target site insensitivity.[26,27]

However, the two herbicides cause the production of ROS, which are very toxic for the cells.[28] In order to remove ROS, cells dispose of antioxidant activities based on nonenzymatic and/or enzymatic systems.[29,30] In this context, very important antioxidant enzymes are ascorbate peroxidase, catalase, dehydroascorbate reductase and glutathione reductase.[10] APX and CAT are key enzymes in regulating the hydrogen peroxide content, while DHAR and GR are part of the ascorbate-glutathione scavenging cycle.[31] The cooperative functions of these antioxidant activities make it possible to scavenge the ROS and maintain the redox status of the cell.[32] Regarding wheat, Tables 2 and 4 show increases in the activities of APX and DHAR in response to both herbicides, and the extents of the increases were of similar magnitude in response to both chemicals. Furthermore, there were significant increases in the GR activity 24 h after the treatments, while the catalase activities remained unchanged (Tables 3 and 5). From these findings, it appears that wheat activated some of its antioxidant activities to counteract the oxidative stress caused by the two herbicides.

In the case of Italian ryegrass there were different responses which depended on the herbicide applied. In general, the APX and DHAR were less responsive with respect to that of wheat, while the GRs showed a very similar response to those of wheat. On the other hand, there were no significant changes in catalase activity for either herbicide on both species.

Data in Table 6 show that the MDA content significantly increased in shoots of Italian ryegrass in response to both the herbicides, while there was no effect in wheat. MDA is a good marker of the structural integrity of plant membranes because it indicates the level of damage to lipids for reactions of oxidation.[33,34] The higher content of MDA in Italian ryegrass, with respect to wheat, can be explained on the basis of a higher antioxidant activity of APX and of DHAR in wheat. In fact, the average specific activity of APX, in untreated samples, was higher in wheat with respect to the grass (+45%), as well as in the case of atrazine-treated samples (+53%). Also in the case of fluorodifen, by comparing the data of the specific activity of APX, wheat had a higher activity (+40%). In general a higher activity of APX in plant species has been proposed as a protective function in situation of oxidative stress.[35] Furthermore, the constitutive activity of DHAR of untreated samples and that of treated samples was significantly higher in wheat with respect to Italian ryegrass; the differences ranged from +25% in control samples to +45% in atrazine-treated samples. DHAR is a very important enzyme which supports the antioxidant activities of APX by regenerating its substrate, ascorbate. The homeostasis of antioxidant molecules in their reduced form is very important for plant survival and for maintaining the redox status of the cell.[36] Therefore, the higher activity of APX and DHAR in wheat with respect to Italian ryegrass suggests that the plant was more efficient in removing hydrogen peroxide and in the regeneration of reduced ascorbate, which was then available to remove oxidants. The above findings were in accordance with the increased of MDA ascertained in herbicide-stressed treated samples of Italian ryegrass.

The other enzyme investigated was glutathione *S*-transferase, which is directly implicated in the detoxification of herbicides and, furthermore, is often invoked in cell protection against oxidative stress. In fact, the GSTs are a family of enzymes present in all eukaryotes which constitute a large amount of the total soluble proteins, which permit the detoxification of a number of toxic organic compounds both endogenous and exogenous.[37] In particular they are very important for the resistance of plants to many classes of herbicides.[37−39] Furthermore, their involvement in the response to oxidative injuries has been documented, because they can also act as glutathione peroxidases in the reduction of lipid hydroperoxides of fatty acids, arising from oxidative perturbations.[40] Regarding the GST(CDNB) activity (Table 7), Italian ryegrass was found to be very responsive to the treatments, while wheat showed significant decreases in GST activity. Nonetheless, the above enzyme activity in untreated and treated wheat seedlings was significantly higher than that of Italian ryegrass. Therefore, in spite of a lower responsiveness, the GSTs of wheat were significantly more active with respect those of the ryegrass. In particular, untreated wheat shoots had a GST activity about 7-fold higher than that of untreated samples of Italian ryegrass; the samples treated with atrazine and fluorodifen showed a higher GST activity in wheat with respect to Italian ryegrass of 3.7- and of 2.4-fold, respectively. These observations are in agreement with the data of accumulation and persistence of the two chemicals in the plants (Table 8). Both herbicides had lower levels of residues in wheat, where they were about half of those in Italian ryegrass. This significant difference in the herbicide residues is in accordance with the higher GST activity

in wheat. Finally, the higher amount of residues found in shoots of Italian ryegrass was also in accordance with the higher damage to membranes, evidenced by the contents of MDA.

In conclusion, the species studied well-tolerated atrazine and fluorodifen, however, Italian ryegrass exhibited some symptoms of stress in terms of membrane oxidation (Table 7) and reduction in shoot length and fresh weight (data not shown), but these injuries were exerted at a sublethal level and they were explained on the basis of less efficient antioxidant activities and detoxification rates.

## AUTHOR INFORMATION

### Corresponding Author

*Tel: +39 075 585 6225. Fax: +39 075 585 6239. E-mail: delbuono@unipg.it.

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