ROS

RESEARCH ARTICLE

The Role of Antioxidants in the Protection of Plants against Inhibitors of Protoporphyrinogen Oxidase

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ABSTRACT | Protoporphyrin IX (proto), a photodynamic chlorophyll synthesis intermediate, is present in trace amounts in plant tissues. However, plants treated with protoporphyrin IX oxidase (PPO) inhibitors (e.g., acifluorfen-methyl) accumulate high levels of proto leading to the formation of reactive oxygen species (ROS) responsible for rapid light-dependent loss of cellular membrane integrity and subsequent cell death. We show that increasing the level of certain antioxidants protects plants against the herbicidal effect of PPO inhibitors. In particular, hydrophilic antioxidants such as reduced glutathione and ascorbic acid (ascorbate) had superior protective effects than the lipophilic antioxidant α-tocopherol. Conversely, inhibiting glutathione biosynthesis with 5 mM L-buthionine sulfoximine rendered plants more sensitive to acifluorfen-methyl. These reducing agents protect plants by quenching the ROS generated by the photoactivation of proto. The quenching of superoxide by ascorbate and reduced glutathione provided superior protection than quenching of hydrogen peroxide by ascorbate. Additionally, the absolute amounts of proto present in the tissues were also reduced in the presence of the more active antioxidants, suggesting that these molecules further protect plants by enhancing the degradation of proto.

KEYWORDS | Antioxidant; Herbicide; Porphyrin; Protoporphyrinogen oxidase

ABBREVIATIONS | AFM, acifluorfen methyl; DAB, 3,3′-diaminobenzidine; NBT, nitro blue tetrazolium; PPO, protoporphyrinogen oxidase; Proto, protoporphyrin IX; ROS, reactive oxygen species

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1. INTRODUCTION

The C-5 porphyrin pathway is ubiquitous to biological systems, with the tetrapyrrole backbone serving as a template for some of the most abundant pigments in nature. The tetrapyrrole ring can chelate metal ions (i.e., Fe, Mg, Co, Ni) that impart different biological functions to these structures [1]. In plants, the porphyrin pathway leads to the synthesis of hemes and chlorophylls, as well as to the formation of a host of biologically important cofactors [2]. Their critical functions are especially evident when enzymes of the pathway are artificially blocked, such as in loss-of-function mutants, or by chemical inhibitors [3, 4].

Consequently, the above pathway is under stringent molecular, biochemical, and physiological regulation, and requires light to be activated in the deetiolation process involving the switch from skotomorphogenesis to photomorphogenesis [5]. These mechanisms have evolved to protect plants from the harmful consequences of any unregulated extraplastidic accumulation of photodynamic intermediates [6]. Protoporphyrin IX (proto) is the last common precursor at the branching point of the tetrapyrrole pathway and the subsequent chelation of an iron or magnesium to either heme or chlorophyll synthesis, respectively. Protoporphyrinogen oxidase (PPO; EC 1.3.3.4), the enzyme catalyzing the oxidation of protoporphyrinogen IX (protogen) to proto, is the molecular target site for several herbicide classes (e.g., diphenyl ethers, oxadiazoles, phenylphthalimides, and triazolinones). Inhibition of this enzymatic step results in a rapid and unregulated cytosolic accumulation of proto, which is responsible for the photodynamic herbicidal action of PPO-inhibiting herbicides [7–12]. This unexpected accumulation of the product of the reaction catalyzed by the enzyme inhibited with the herbicide confounded scientists for years. But this is explained by the fact that inhibition of PPO deregulates porphyrin biosynthesis and causes the substrate (protogen) to leak out of the chloroplasts. Once in the cytosol, it is rapidly converted to the product (proto). Photoexcitation of proto present in the cytosol causes the formation of toxic reactive oxygen species (ROS) that destabilize membranes via lipid peroxidation [13]. This phenomenon is similar to patients afflicted by variegate porphyria (dysfunction in PPO activity) [1] and explained in greater detail in the literature (see review in [14]).

Plants have evolved a number of mechanisms to cope with oxidative stresses. In particular, processes contributing to photosynthesis are particularly prone to generating ROS. Consequently, chloroplasts possess multiple physiological (non-enzymatic) and biochemical (enzymatic) components that act in concert to quench ROS and protect the cell from oxidative damage [15–18]. We already mentioned that inhibition of PPO causes a massive deregulation of the chlorophyll biosynthesis resulting in accumulation of the highly photodynamic proto intermediate in the cytoplasm. There have been reports of increased glutathione levels in plants treated with PPO inhibitors [19, 20]. Plants that are naturally sensitive to such inhibitors do not have that same capacity to cope with the oxidative stress caused by the accumulation of proto [12, 21]. Furthermore, transgenic rice plants overexpressing herbicide-resistant PPO are not affected by the herbicides and they normally do not need to enhance their antioxidant defense response mechanisms [22, 23].

In addition to their protective roles against the oxidative stress caused by ROS, thiol-containing antioxidants such as reduced glutathione (GSH) and cysteine have been associated with in vitro enzymatic degradation of proto [24, 25]. This process may be involved in protection against the photodynamic damage caused in the presence of PPO inhibitors as has been reported with common chickweed (*Stellaria media* Vill.) [26].

We report the protective effect of antioxidants on the damages caused by PPO inhibitors by manipulating the amount of GSH and ascorbate in the experimental system. The protective effect is associated with quenching of ROS. The effect of these compounds on the accumulation of proto also suggests

that their mechanism(s) of protection also involves enhanced degradation of proto.

2. MATERIALS AND METHODS

2.1. Measurement of Cellular Damage Caused by Acifluorfen-Methyl (AFM)

Cucumber seedlings (*Cucumis sativus* L. "Long Green Improved") used for the leakage, proto accumulation, and ROS studies were grown in a greenhouse maintained at 25 ± 2°C and exposed to natural sunlight. AFM-induced electrolyte leakage was determined using 7-day-old cucumber cotyledons as described before [27]. Briefly, for each assay, 50 cotyledon discs (4 mm in diameter) (approximately 0.2 g of fresh weight) were floated on 5 ml of a medium containing 10 g/L sucrose and 1 mM 2-(*N*-morpholino)ethanesulfonic acid (MES, pH 6.5) with or without the test compounds in 6-cm diameter disposable Petri dishes. Cellular damage was determined by measuring electrolyte leakage into the bathing medium with a conductivity meter. Conductivity was monitored for 20 h in darkness, followed by 8 h of continuous light (at a photon flux density of 375 µmol m^{-2} s^{-1}). Results are expressed as a change in the conductivity after initial measurement at the beginning of the dark period. All treatments for electrolyte leakage measurements were performed in 5 replications. The data presented is a representative example of the experiment. Each point is the mean of 5 measurements and the error bars represent the standard deviation.

2.2. Effects of Antioxidants

The effects of 5 mM GSH, ascorbate, and α-tocopherol on membrane integrity and accumulation of proto were determined by adding one of these antioxidants to both AFM-treated and untreated solutions. Measurement of membrane integrity was done as described previously [27] except that the cotyledons discs were floated on the medium with 5 mM of the respective antioxidant with or without AFM. The effect of 5 mM L-buthionine sulfoximine was also tested as described before [27]. Finally, accumulation of proto was measured in the cotyledon discs after the overnight incubation but before exposure to light.

2.3. Quantitative Proto Analysis

Proto was extracted by homogenizing 25 cotyledon discs in 2 ml of basic methanol (9:1 vol/vol MeOH:0.1 M NH_4OH) for 30 s using a polytron tissue homogenizer (Polytron PT3100, Elkhart, Indiana, USA). The extracts were sonicated for 15 min in a water bath sonicator. Plant debris were pelleted by centrifugation at 13,000 *g* for 15 min. The extract was placed in a high-performance liquid chromatography (HPLC) vial. The pellet was resuspended in 1 ml of basic methanol, vortexed, and centrifuged again. The supernatant was combined with the previous 2 ml. The amount of proto was determined by HPLC analysis. The HPLC consisted of Waters Associates components: Model 717 autosampler, Model 600 controller, and Models 470 fluorescence and 996 photodiode spectrophotometric detectors. The column was a 300 × 3.9 mm (i.d.) µ-Bondapak C18 reversed-phase column. The solvent system was a linear gradient from 70 to 100% HPLC-grade methanol over 10 min and maintained at 100% methanol for 10 more min and then re-equilibrated to initial conditions before the next injection. The injection volume was 50 µl. Proto was quantified based on a 4 point calibration curve obtained using proto standard as described before [25].

2.4. ROS Analysis

The 3,3′-diaminobenzidine (DAB) staining solution was prepared by adding 0.1 g of DAB in 200 ml of distilled water adjusted to pH 3.8 with HCl [28]. The nitro blue tetrazolium (NBT) staining solution was prepared by adding 0.1 g of nitro blue tetrazolium chloride, 13.6 g of potassium phosphate monobasic (KH_2PO_4) (50 mM), and 1.3 g of sodium azide (10 mM) in 200 ml distilled water [29]. For the DAB and NBT assays, which were developed to quantify H_2O_2 and superoxide, respectively, cucumber cotyledon discs were exposed to 100 µM AFM for 16 h in darkness followed by 1, 2, and 4 h exposure to light. The discs were placed in beakers containing 50 ml of either the DAB or NBT staining solution on a shaker and under mild vacuum for 10 min and shaken without vacuum for 1 h two times. Afterward, the solutions were discarded and replaced with 70% ethanol, placed on a water bath and brought to a boil. The ethanol was changed three times as the solutions turned dark to remove chlorophyll. The discs were

TABLE 1. Electrolyte leakage and levels of proto in cucumber cotyledons after 24 h exposure to 50 µM acifluorfen-methyl either alone or with 5 mM of the antioxidants

Treatment	Proto accumulation (pmol/g FW)[1]	$Leakage_{max}$ (% of total)[2]
Control	24.1 ± 3.5[a]	6[a]
AFM	403.4 ± 35.6[d]	87[d]
AFM + GSH	203.5 ± 38.4[b]	37[a]
AFM + ascorbate	337.5 ± 38.9[c]	49[c]
AFM + α-tocopherol	302.0 ± 13.1[c]	84[d]

Note: [1]Data are expressed as means ± SD (n =5). Numbers followed by the same letter are not different at $p < 0.05$ according to Duncan multiple range test. [2]Total leakage was measured after boiling samples. $Leakage_{max}$ is leakage after 8 h of light exposure/total leakage following boiling of samples. FW denotes fresh weight.

placed on white filter papers and scanned in 600 dpi color. The intensity of the stains was measured and quantified using the record measurement function of Adobe Photoshop CS3 (San Jose, CA, USA). Briefly, images of the stained tissues were converted to gray scale and inverted. Care was taken to ensure the images were not overexposed in order to reliably quantify the intensity of the pixels. A total of 8 replicates for each treatment were measured and adjusted by removing background values.

2.5. Statistical Analyses

All experiments consisted of three observations and each experiment was repeated in time. All data represent means pooled over all experiments ± standard deviations (SD). Graphs were generated with Sigma Plot 12 (Build 12.0.0.182, Systat Software, Inc., San Jose, CA, USA). The data were subjected to analysis of variance, and the means were separated with Fischer's LSD at $p = 0.05$ using the Agricolae module on R platform (version 3.3.3, R Foundation for Statistical Computing, Vienna, Austria). The Pearson correlation coefficient between proto accumulation and conductivity was calculated using the statistical module of Sigma Plot.

3. RESULTS AND DISCUSSION

ROS accumulating during abiotic stresses are not necessarily a symptom of cellular dysfunction. These might act as signals to adjust the cellular machinery to cope with less than optimal growth conditions [30]. However, plants are generally very sensitive to uncontrolled accumulation of ROS. Consequently, plants have developed elaborate chemical enzymatic and non-enzymatic antioxidative systems to cope with stress-induced ROS bursts [15–18].

3.1. Physiological Response to the PPO Inhibitor AFM

The mode of action of PPO inhibitors involves the light-dependent loss of membrane integrity [31]. This is due to the peroxidation of the lipid component of membranes by ROS generated by photoenergized proto that accumulates in the cytoplasm. This unexpected accumulation of the product of the reaction being inhibited has been briefly described in the Introduction section and in great detail elsewhere [14]. AFM, an herbicide that inhibits PPO activity, was used to analyze the physiological response caused by the herbicide in cucumber cotyledons. As expected, proto levels in untreated tissues are very low (**Table 1**). Consistent with the mechanism of action of PPO inhibitors, AFM caused a dramatic increase in the amount of proto in the treated cucumber cotyledon discs, relative to the untreated samples (**Table 1**). The herbicide treatment caused the rapid loss of membrane integrity (as measured in electrolyte leakage) in tissues exposed to light (**Figure 1**). In contrast, no electrolyte leakage was evident in control samples.

3.2. Effect of Exogenous Antioxidants

As mentioned above, ROS are highly toxic, and plants have developed several protective mechanisms to reduce their half-lives within the cellular environ-

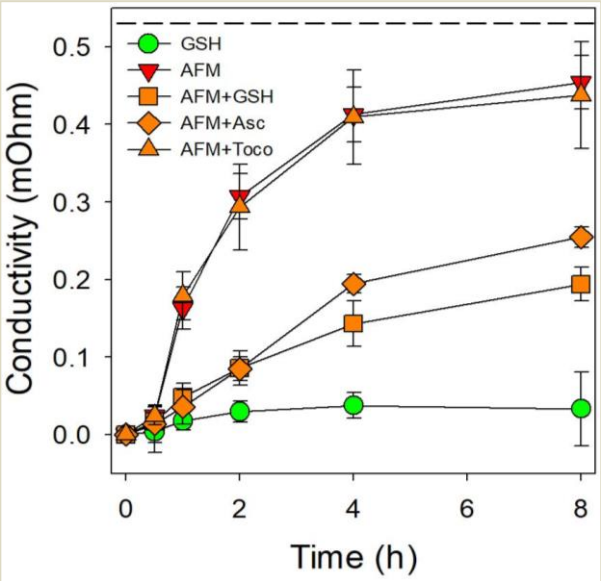

FIGURE 1. Electrolyte leakage caused by 50 µM acifluorfen-methyl (▼), and the effect of incubating the cotyledons in the presence of 5 mM GSH (■), ascorbate (◆), and α-tocopherol (▲). Control treatment was done with equivalent amount of solvent used with AFM-treated samples (●). The samples were incubated in the dark for 16 h before being placed in the presence of light (time 0). Data are expressed as means ± SD (n = 5). Dotted line represents maximum conductivity from boiled cotyledon discs. Asc, ascorbate; Toco, α-tocopherol.

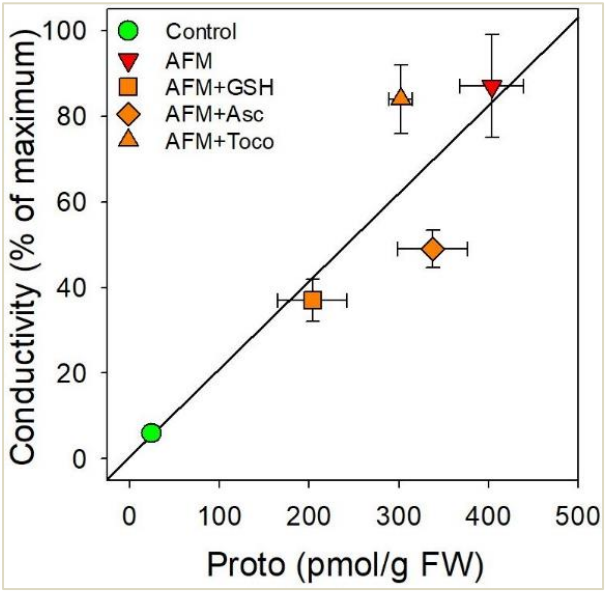

FIGURE 2. Correlation between proto accumulation caused by AFM's inhibition of PPO and loss of membrane integrity as measured by conductivity in the presence of various antioxidants. Asc, Toco, FW denote ascorbate, α-tocopherol, and fresh weight, respectively.

ment and avert physiological damage. The antioxidant system comprises numerous enzyme systems and many quenching compounds (e.g., ascorbate, GSH, and tocopherols). These important antioxidants are often compartmentalized and are involved in different mechanisms of protection [17, 32]. While little is known about the role of antioxidative protective mechanisms in cucumber, rice plants respond to PPO-inhibitors with increased activities of superoxide dismutase, catalase, ascorbate peroxidase, and glutathione reductase to cope with the ROS burst generated by the accumulation of photodynamic proto, but these changes are not sufficient to overcome ROS-induced lethality following exposure to these herbicides [23].

Our experiments showed that exogenously applied ascorbate and GSH protected plants from the oxidative damage caused by AFM (**Figure 1** and **Table 1**). On the other hand, exogenous application of α-tocopherol had no protective effect. The lack of response to α-tocopherol may be due to its highly lipophilic properties which may interfere with its uptake and/or cellular compartmentalization in the cucumber cotyledons. Interestingly, in addition to reducing electrolyte leakage, GSH and ascorbate also reduced the amount of proto accumulating in the treated plant tissues (**Table 1**). As reported by others [7–9], there was a significant positive relationship between accumulation of proto and electrolyte leakage as modulated by various antioxidants, with a Pearson correlation coefficient = 0.892, P value = 0.0417 (**Figure 2**).

Proto can be degraded by plant peroxidases in crude cucumber leaf extracts [24, 25, 33]. This reaction requires the presence of thiol-containing compounds such as GSH or cysteine. The involvement of peroxidases in chlorophyll catabolism has been suggested based on the characterization of a catabolite

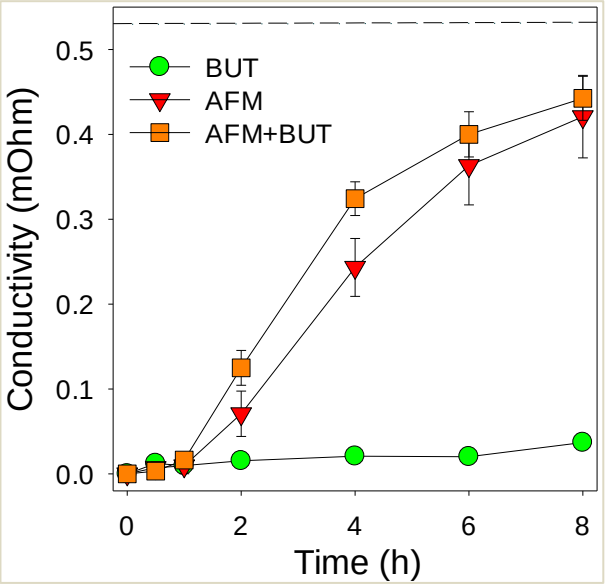

FIGURE 3. Effect of 5 mM L-buthionine sulfoximine (■) on electrolyte leakage caused by 50 µM acifluorfen-methyl (▼). Control treatment consisted of 5 mM L-buthionine sulfoximine without acifluorfen-methyl (●). The samples were incubated in the dark for 16 h before being placed in the presence of light (time 0). Data are expressed as means ± SD (n = 5). Dotted line represents maximum conductivity from boiled cotyledon discs. BUT, L-buthionine sulfoximine.

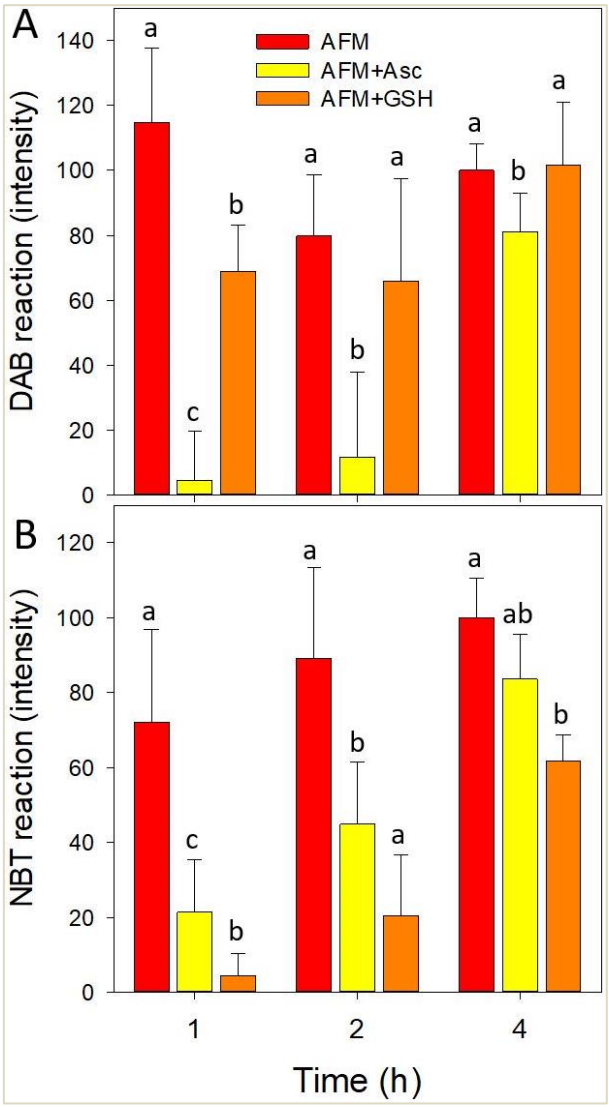

FIGURE 4. Quantitation of ROS generated in the plant tissue treated with 100 µM acifluorfen-methyl with or without antioxidants over 4 h exposure to light. (A) DAB and (B) NBT were used to visualize and quantify hydrogen peroxide and superoxide, respectively. Data are expressed as means ± SD (n = 8). Bars within each time-point with the same letter are not different at $p < 0.05$ according to Fischer's LSD.

[34], and an unknown enzymatic activity involved in porphyrin degradation has been reported [35]. Peroxidases can oxidize GSH [36] and generate highly reactive intermediates that may react directly with tetrapyrroles [37]. Proto may even be oxidized via a hydrogen peroxide mediated reaction similar to that reported to occur with heme [38]. Moreover, peroxidases can generate adducts by reacting with thiol-containing substrates and molecules containing accessible vinyl groups (e.g., proto) [39, 40].

The stimulation of proto degradation observed with ascorbate is similar to that reported for the thiol-dependent oxidation of other porphyrins by plant peroxidases (e.g., deuteroporphyrin) [24]. Additionally, ascorbate is a natural substrate of plant peroxidases [16]. As such, it may prevent peroxidases from utilizing the thiol substrates. In addition, ascorbate may act as a quenching agent by reducing any reactive radical intermediates involved in the degradation of proto.

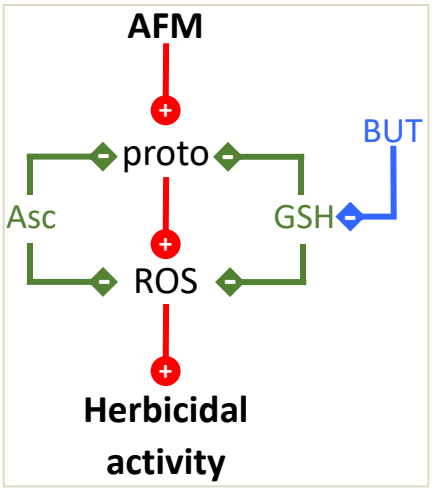

FIGURE 5. Relationship between the effect of the herbicide AFM causing accumulation of proto by inhibiting PPO (red arrows), which results in the light-dependent accumulation of ROS, and the protective influence of GSH and ascorbate on the various ROS forms (green arrows). Inhibitory effect of L-buthionine sulfoximine (BUT) on GSH is also depicted (blue arrow). Asc denotes ascorbate.

3.3. Effect of L-Buthionine Sulfoximine

L-Buthionine sulfoximine is a potent inhibitor of GSH synthesis via inhibition of gamma-glutamate-cysteine ligase, the enzyme required in the first step of GSH synthesis [41]. Consequently, plants exposed to L-buthionine sulfoximine have lower levels of endogenous GSH than control plants. Using this approach, we demonstrated that chemically-induced repression of GSH synthesis renders plants more sensitive to PPO inhibitors (**Figure 3**).

3.4. Modulation of Herbicide-Induced ROS Generation by Exogenous Antioxidants

As mentioned before, PPO-inhibiting herbicides cause proto accumulation, which is accompanied with ROS formation (**Figure 4A** and **4B**). The amount of ROS generated as a result of PPO inhibition by AFM was quantified using DAB and NBT stains. Ascorbate provided better quenching of hydrogen peroxide than GSH (**Figure 4A**). This effect was transient and ROS generation caused by proto accumulation overwhelmed the protective effect of the antioxidants by 4 h of light exposure. On the other hand, GSH provided superior quenching of superoxide, and the protection against these ROS was more prolonged, relative to ascorbate (**Figure 4B**).

4. CONCLUSION

In conclusion, the relationship between the light-dependent generation of ROS by PPO-inhibiting herbicides and the protective function of antioxidants can be summarized in **Figure 5**. AFM inhibits PPO and causes a rapid accumulation of proto. In the presence of light, this photodynamic tetrapyrrole intermediate generates both superoxide and hydrogen peroxide. These ROS react with lipids and lead to loss of membrane integrity. GSH seems to be mostly responsible for quenching superoxide whereas ascorbate can detoxify both superoxide and hydrogen peroxide but does not protect plants as efficiently as GSH. Most of the protection is associated with quenching superoxide. Quenching of hydrogen peroxide did not provide as much protection.

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The authors declare no conflicts of interest.

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