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Ecotoxicological Assessment of a Glyphosate-Based Herbicide in Cover Plants: *Medicago sativa* L. as a Model Species

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Featured Application: This study is a starting-point to better understand the effects of GLY-contaminated soil on non-target plants, since very little is known about this subject.

Abstract: Despite the several innovations that have been incorporated in agriculture, the use of herbicides, especially glyphosate (GLY), is still the major tool for weed control. Although this herbicide has a notable worldwide representation, concerns about its environmental safety were recently raised, with a lot of divergence between studies on its non-target toxicity. Therefore, it is of utmost importance to understand the risks of this herbicide to non-target plants, including cover crop species, which have a crucial role in maintaining agroecosystems functions and in preventing soil erosion. Thus, this work aims to evaluate the growth and physiological responses of a cover plant species (*Medicago sativa* L.) exposed to increasing concentrations of a GLY-based herbicide (GBH), particularly focusing on the oxidative metabolism. The growth of roots and shoots was affected, being this effect accompanied by a rise of lipid peroxidation, suggesting the occurrence of oxidative stress, and by an activation of the antioxidant (AOX) system. Indeed, the results showed that adverse effects are visible at active ingredient concentrations of 8.0 mg kg^{-1}, with the lowest EC_{50} being 12 mg kg^{-1}, showing that GBH-contaminated soils may pose a risk to the survival of non-target plants in the most contaminated areas. Overall, these findings proved that GBH greatly impairs the growth of a non-target plant, strengthening the need of additional studies to unravel the real risks associated with the over usage of this pesticide, since there is an evident lack of studies performed with contaminated soils.

Keywords: herbicides; alfalfa; oxidative stress; antioxidant system; reactive oxygen species

1. Introduction

Plant protection products, also referred to as pesticides, are widely used in agriculture in order to improve productivity, prevent crop loss or yield reduction, and control disease vectors or agents.

However, it is known that only a small portion of the applied pesticides reach the target pests, while the remainder will end up in soil or will have the potential to move to other environmental compartments, including ground and surface waters [1,2]. Nevertheless, the mobility of these contaminants in the environment depends on several biotic and abiotic variables, and their physical–chemical properties. Thus, depending on the persistence of each substance, soil contamination can occur, thereby affecting soil quality, compromising its ability to perform its functions and leading to an irreversible degradation of this non-renewable resource [3–7]. For this reason, concerns about the use of pesticides are increasing, and the most controversial at the moment is probably glyphosate (GLY), a post-emergence and systemic herbicide of broad spectrum (non-selective). Applied to the foliage of weeds, GLY is absorbed by the leaves and is rapidly translocated in the plant through the phloem, particularly accumulating in meristems (root and shoot apex). Right after its discovery in the 1970s, GLY quickly became the most applied herbicide worldwide and, in 2014, the volume applied was sufficient to treat between 22% and 30% of globally cultivated cropland [8]. Despite its great efficiency, several concerns about this herbicide were recently raised, related to the divergence between scientific studies regarding its toxicity to non-target organisms [9,10]. Another factor that may turn difficult to evaluate the real impacts of GLY on the environment is that GLY commercial formulations not only contain GLY, but also substances such as polyethoxylated amine (POEA) surfactants [11]. It is known that the first generation of POEA surfactants present in Roundup® were markedly more toxic than GLY, but since the mid-1990s, these compounds were progressively replaced by other POEA surfactants, ethoxylated etheramines, which exhibit lower non-target toxic effects [11]. However, the composition of non-active ingredients in a GLY-based herbicide (GBH) is not fully known, and while a recent study pointed to a lower toxicity for earthworms of the GBH compared with the active ingredient (a.i.) itself, Pochron et al. [9] in another study concluded the opposite regarding *Dimorphandra wilsonii* seed germination [12]. Thus, GLY can be considered an old pesticide, but an emergent problem.

In areas in which high extensions of land are dedicated to intensive agriculture, the dispersion of GLY in the environment can be a serious problem of diffuse contamination, particularly due to its tendency to adsorb to solid particles [13,14]. Depending on climactic conditions (especially temperature and humidity), the removal of GLY from soils can be reduced, resulting in its accumulation [15]. This accumulation and dispersion through the environment, due to its non-selectivity [16,17], can cause damage to plants that are not targeted, affecting a great number of species that account both directly and indirectly for soil biodiversity. From the available data, it was suggested that GLY negative effects on plant growth and development substantially exceed the effects triggered by its mode of action as it can induce several metabolic and physiological disorders, favoring the occurrence of oxidative stress as an indirect consequence [18]. Indeed, when plants are exposed to stress factors, such as soil contamination, oxidative stress occurs due to an overproduction of reactive oxygen species (ROS) [19,20]. Therefore, given their higher sensibility, ROS, along with oxidative stress parameters (e.g., lipid peroxidation), can be used as exposure biomarkers, allowing an early warning and sensitive evaluation of plant physiological status, representing a potential tool to phytotoxicity studies [21]. Although ROS are important signaling agents, high levels of these compounds can easily become phytotoxic, damaging proteins, lipids, carbohydrates, and nucleic acids. By influencing the cellular gene expression pattern, ROS are involved in many processes, such as growth, cell cycle, abiotic stress responses, pathogen defense and systemic signaling and development. Thus, in order to maintain the redox homeostasis of the cell, plants possess a powerful antioxidant (AOX) system, composed of both enzymatic and non-enzymatic mechanisms [22]. It is the joint action of these players that prevent the occurrence of redox disorders in the cell, by directly neutralizing the toxic effects of ROS and/or by preventing their overaccumulation. However, depending on the plant species, the magnitude of stress and the exposure period, the AOX system may not be able to efficiently counteract ROS-induced toxicity, leading to the establishment of an oxidative stress condition [20].

One group of plants that is particularly exposed to GLY contamination is cover plants, since they can be sown a few months after the herbicide application, during the off-season. In crops, such as vines,

they can be sown between the lines and left as a green cover. They are of extreme importance to the management of soil erosion, fertility and quality, as well as crop yield [23,24]. Indeed, the European Commission established that the maintenance of permanent grassland areas is one of the actions that every EU country and all farmers must put in place, if they want to be rewarded for the protection of natural resources [25]. Thus, by affecting cover plants, GLY may jeopardize the balance of the ecosystem in which they are inserted. An example of a cover plant is *Medicago sativa* L., commonly known as alfalfa, a perennial leguminous, belonging to the family Fabaceae and subfamily Faboideae, well known by its ability to improve both soils' structure and biochemical activity [26]. This cover crop has the potential to establish symbiotic relations with N_2-fixing bacteria, thus increasing its growth and development, while contributing for the enrichment of soils with nitrogen compounds [27,28].

Since little is known about the potential phytotoxicity of GLY contaminated soil, particularly in non-target species, the aim of this work is to unravel the effects of soil contamination by this herbicide on the growth and redox homeostasis of a cover plant species, *Medicago sativa*. By combining biometrical and biochemical approaches, this study will focus not only on the effects of a GBH (GLY-based herbicide) on the development and growth performance of *M. sativa*, but also on the assessment that whether its toxicity is mediated by the occurrence of oxidative stress.

2. Materials and Methods

2.1. Preparation of the Artificial Soil

The substrate used in this work consisted in an artificial soil composed of 70% (m/m) sand, 20% (m/m) kaolin and 10% (m/m) peat [29]. The pH_{KCl} of the soil (1:5 m/v) was adjusted to 6.0 ± 0.5 by the addition of calcium carbonate ($CaCO_3$), whenever necessary.

2.2. Glyphosate (GLY) Concentrations Tested

The herbicide Roundup UltraMax® (Bayer, Germany), acquired from a local supplier, was used in this study. From the commercial formulation (360 g L^{-1} GLY as potassium salt), a stock solution was prepared and a series of sequential doses of the GBH was applied, ranging from 0 to 40 mg kg^{-1} of the active ingredient (a.i.), with a dilution factor of 1.5, giving rise to the following concentrations: 40; 27; 18; 12; 8.0 mg kg^{-1}, which were tested together with a GBH-free control (CTL). The concentrations were chosen based on the results of a previous published work conducted by the team [30,31], and reflect not only data concerning GLY contamination levels in soils, but also the recommended applied doses for agricultural practices.

2.3. Plant Material and Growth Conditions

The seedling emergence and seedling growth test, performed according to the OECD (Organisation for Economic Co-operation and Development) protocol for terrestrial plants [3], was carried out in plastic pots containing 200 g of artificial soil, to which the solutions with the desired GLY concentrations were added. Maintenance of soil moisture was ensured by the presence of a pot with distilled water placed at the base of the soil pots with soil, and by using a cotton rope to ensure the capillarity rise of the water. Twenty seeds of *Medicago sativa* var. Dimitra, acquired from Flora Lusitana Lda (Cantanhede, Portugal), were placed in each pot, after sterilization with 70% (*v/v*) ethanol (7 min) and 20% (*v/v*) commercial bleach (5% active chloride; 7 min), followed by washing with deionized water. To ensure the availability of nutrients, a commercial fertilizer (EcoGrow, NPK 3-6-7) was added at the start of the test. A negative control (CTL; absence of contaminant) was also prepared, obtaining a total of 24 pots (four replicates for each treatment). The assay began when 50% of the seeds from the CTL germinated. In each pot, only eight plants were kept, avoiding intraspecific competition. The plants germinated and grew in a growth chamber with controlled temperature (21 °C), photoperiod (16 h light/8 h dark) and photosynthetically active radiation (120 μmol m^{-2} s^{-1}). After 21 days of growth, plants from each replicate were collected, used for the estimation of biometric parameters and then shoots were frozen

in liquid nitrogen and stored at −80 °C until analyses. The assay lasted 21 days post-germination, since, at this point, *M. sativa* individuals are in their vegetative phase before flowering. Moreover, this period did not conditionate the acquisition of fresh material for the biochemical analyses.

2.4. Analysis of Biometric Indicators

The biometric analysis was performed as described in the OECD protocol for seedling emergence and seedling growth test [32]. Eight plants from each replicate of every experimental group were used. After root and shoot separation, root length, and shoot height were measured, and the fresh masses of roots and shoots were registered.

2.5. Determination of Physiological Endpoints

Total chlorophylls (a + b) and carotenoids were extracted in 80% (*v/v*) acetone and quantified by spectrophotometry, as described by Lichetenthaler [33]. The absorbances at 470, 647, and 663 nm were recorded, and the results obtained were expressed in mg g^{-1} fresh weigh (fw).

Total soluble protein content and glutamine synthetase (GS; EC 6.3.1.2) were extracted by homogenizing, on ice, frozen shoot samples in an extraction buffer, followed by a centrifugation at 4 °C for 20 min and 15,000× *g*. Afterwards, extracts were used to quantify the total soluble protein [34] and to determine GS activity by the transferase assay [35] by recording the absorbance at 500 nm. GS activity was calculated and expressed as nkat mg^{-1} protein.

2.6. Quantifiaction of Oxidative Stress Biomarkers

The assessment of lipid peroxidation (LP) was performed as described by Heath and Packer [36], by the quantification of malondialdehyde (MDA). Briefly, plant samples were homogenized in 0.1% (*w/v*) trichloroacetic acid (TCA) and subsequently centrifuged (5 min; 10,000× *g*). Afterwards, the extracts were incubated with a mixture of 0.5% (*w/v*) thiobarbituric acid (TBA) and 20% (*w/v*) TCA for 30 min at 95 °C. At the end, the absorbances of each sample was read at 532 and 600 nm. After this step, the absorbance values of 532 nm were subtracted from those obtained at 600 nm to eliminate the effects of unspecific turbidity. The molar extinction coefficient (ε = 155 mM^{-1} cm^{-1}) was used to calculate MDA levels and the results were expressed as nmol g^{-1} fw.

The determination of hydrogen peroxide (H_2O_2) was performed according to the procedure described by Jana and Choudhuri [37]. Upon the homogenization of shoot aliquots in 0.1% (*w/v*), TCA and centrifugation (6000× *g*; 25 min), the obtained plant extracts were combined with a mixture containing 0.1% (*w/v*) $TiSO_4$ in 20% (*v/v*) H_2SO_4. Finally, the absorbance at 410 nm of each sample was recorded and the H_2O_2 levels were determined using the molar extinction coefficient of 0.28 μM^{-1} cm^{-1}. Results were expressed in nmol g^{-1} fw.

2.7. Analysis of the AOX Response

In order to determine the total antioxidant capacity (TAC) and the total phenolics, the procedure described by Zafar et al. [38] was followed. Firstly, frozen shoot samples were extracted in 80% (*v/v*) methanol followed by a centrifugation at 2500× *g*, for 10 min. Regarding TAC, upon dilution of the extracts (1:5), these were mixed with a reaction solution (0.6 M H_2SO_4, 4 mM ammonium molybdate and 28 mM sodium phosphate), incubated at 95 °C for 90 min, and cooled on ice. After that, the absorbance was read at 695 nm. TAC levels were obtained from a calibration curve obtained with dilutions of a standard solution of ascorbic acid (AsA) and the results expressed in mg equivalents of AsA g^{-1} fw. Concerning phenolics, their quantification was performed by a colorimetric assay using the Folin–Ciocalteu reagent. Absorbance was registered at 725 nm and total phenols concentrations were calculated from a calibration curve, prepared with dilutions of a gallic acid solution. The results were expressed in mg of gallic acid g^{-1} fw.

The extraction and quantification of proline (Pro) was performed as previously described by Bates et al. [39], using the ninhydrin-based colorimetric assay. Samples were homogenized in 3% (*w/v*)

sulphosalicylic acid and centrifuged (500× *g*; 10 min). Then, the extracts were incubated, under acid conditions, with a ninhydrin solution for 1 h at 96 °C. At the end, the absorbance of each sample was read at 520 nm and Pro content was obtained from a calibration curve obtained with known Pro concentrations, and the results were expressed as μg g^{-1} fw.

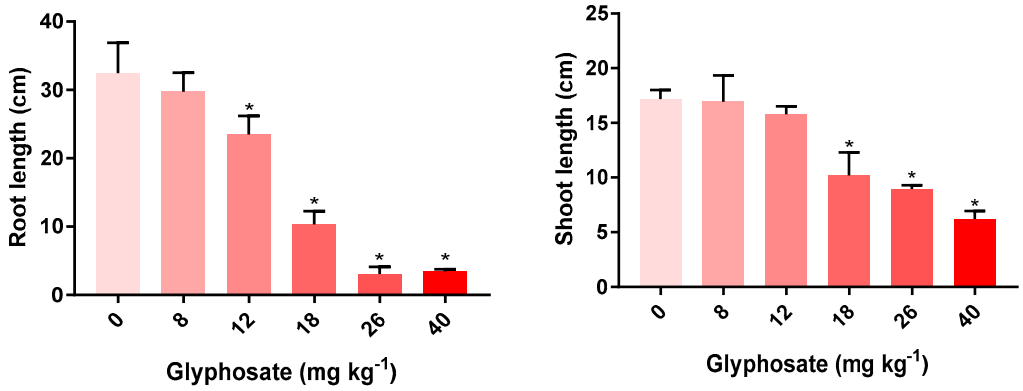
2.8. Statistical Analyses

All endpoints were evaluated using at least three replicates per treatment and the results were expressed as mean ± standard deviation (SD). The effects of the herbicide on the parameters previously mentioned were evaluated using one-way analysis of variance (ANOVA) after checking the homogeneity of variances by the Levene Test. Whenever $p \leq 0.05$, the post-hoc Dunnett's test was used to compare the mean of each group with the CTL. The EC_{50} (concentration of GLY expected to have an effect in 50% of test organisms) and the corresponding 95% confidence limits (95% CL) for the biometric parameters, were estimated with a non-linear least-squares regression adjustment. All statistical procedures were performed in GraphPad Prism 8.

3. Results

3.1. Biometric Parameters of M. sativa

As shown in Figures 1 and 2, the application of a GBH had a negative impact in both root and shoot length and biomass. By analyzing Figure 1a, it is possible to notice that there was a significant decrease in root length ($F(5, 16) = 106.8$; $p \leq 0.05$) for concentrations above the second lowest, with a monotonic dose–response relationship. Between 12 and 18 mg kg^{-1} of the a.i. there was a drastic reduction in root length—the inhibition values rose from 27% to 68% comparatively to the CTL group, in which the EC_{50} was estimated to be 16 mg kg^{-1} (95% CL: 14–19). Regarding shoot length, despite the observed decrease as the concentration increased, significant differences ($F(5, 16) = 36.21$; $p \leq 0.05$) were only recorded when plants were exposed to the highest doses of GBH (18, 26 and 40 mg kg^{-1} of the a.i.), with inhibition values up to 64% in relation to the CTL. Nevertheless, a similar EC_{50} was estimated (16 mg kg^{-1} of the a.i.; 95% CL: 14–22).

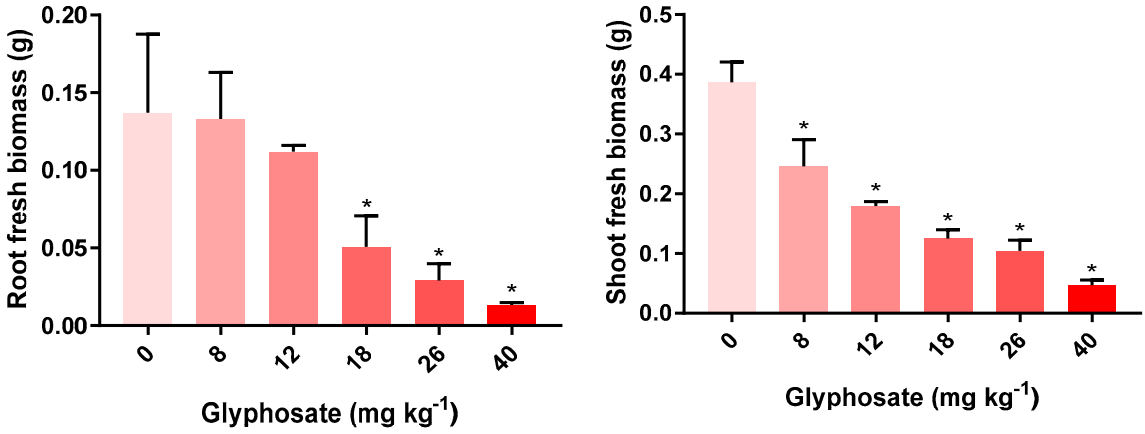


(**a**) (**b**)

Figure 1. Average root (**a**) and shoot (**b**) lengths of *M. sativa* plants, 21 days after exposure to different concentrations of glyphosate (GLY). Error bars correspond to the standard deviation. Statistically significant differences compared to the control (CTL; no GLY), considering $p \leq 0.05$, are marked with a * above bars.

Regarding fresh biomass (Figure 2), both roots and shoots were affected by GBH exposure in a concentration-dependent manner. Despite both organs exhibiting the same global trend, the results point towards a higher sensitivity of shoots when compared with roots. In fact, while in shoots, all concentrations are statistically different from the CTL ($F(5, 15) = 92.02$; $p \leq 0.05$)—reaching inhibition

values ranging from 36% to 88%, in roots biomass—significant differences ($F(5, 16) = 16.02$; $p \leq 0.05$) were only detected upon exposure to a.i. concentrations of 18, 26 and 40 mg kg^{-1}, with reductions of about 62, 79 and 90%, respectively. The highest effects observed in shoots are translated into differences in the EC_{50} values obtained. For root fresh biomass, the estimated a.i. concentration was 15 mg kg^{-1} (95% CL: 12–22), whereas for the shoot fresh biomass it was 12 mg kg^{-1} (it was only possible to calculate the lower limit of the CL, which was 8.5).

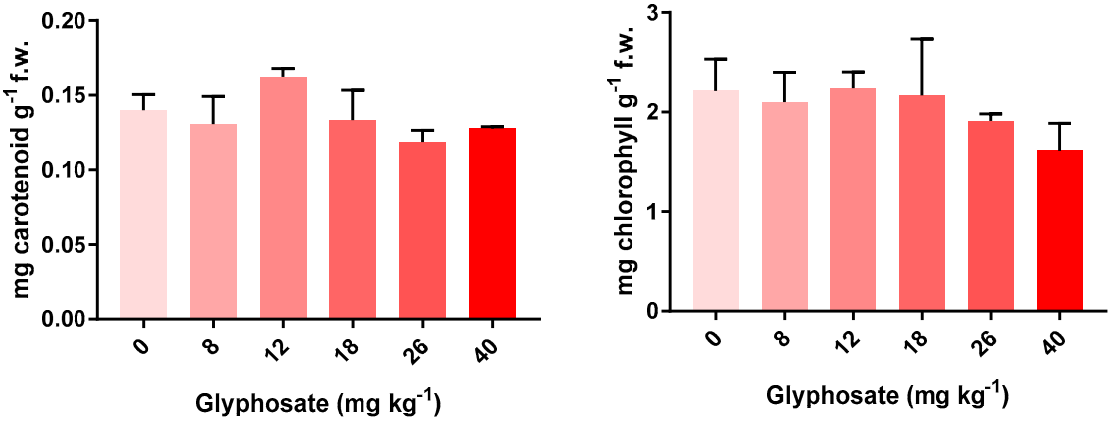


(**a**) (**b**)

Figure 2. Average biomass of roots (**a**) and shoots (**b**) of *M. sativa* plants, 21 days after exposure to increased concentrations of GLY. Error bars correspond to the standard deviation. Statistically significant differences compared to the CTL, considering $p \leq 0.05$, are marked with a * above bars.

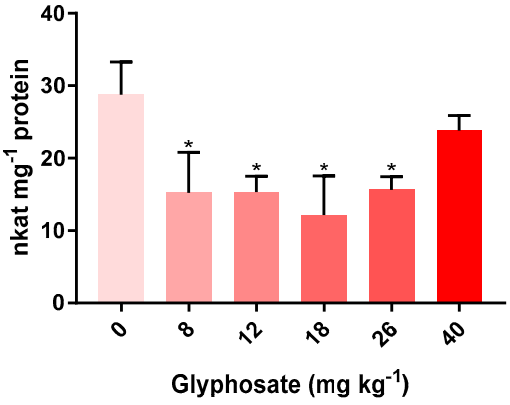
3.2. Physiological Parameters on *M. sativa*

For the photosynthetic pigments, the behavior was similar for both total chlorophylls and carotenoids (Figure 3a,b, respectively), as no significant statistical differences were registered among treatments and the CTL—$F(5, 12) = 2.072$; $p > 0.05$ for total chlorophylls and $F(5, 8) = 2.920$; $p > 0.05$ for carotenoids.



(**a**) (**b**)

Figure 3. *Cont.*



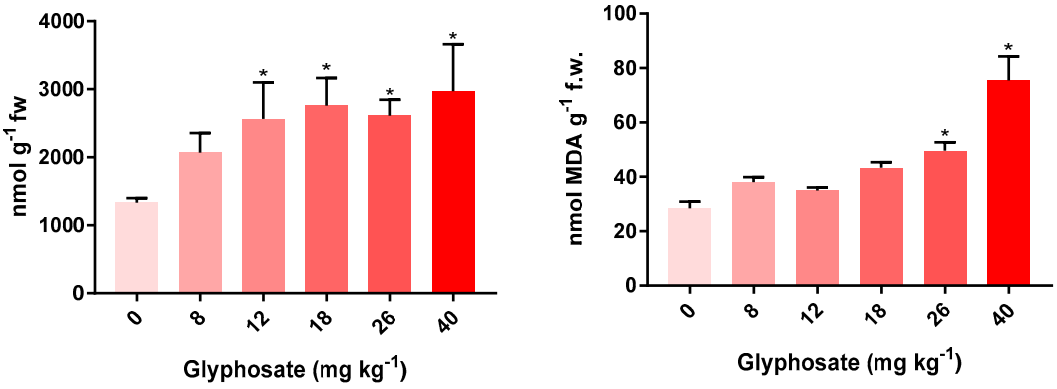
(**c**)

Figure 3. Average concentrations of carotenoid (**a**) and chlorophyll (**b**) and glutamine synthetase (GS) activity levels (**c**) in shoots of *M. sativa* plants 21 days after exposure to increased concentrations of GLY. Error bars correspond to the standard deviation. Statistically significant differences compared to the CTL, considering $p \leq 0.05$, are marked with a * above bars.

GS levels (Figure 3c) showed a different pattern from that of the photosynthetic pigments. Comparatively to the CTL, all GBH concentrations induced a significant reduction in GS activity levels ($F(5, 12) = 7.851$; $p \leq 0.05$). As can be observed in Figure 3c, when plants were exposed to a.i. concentrations between 8 and 26 mg kg^{-1}, decreases of around 50% were found in comparison with the CTL. Curiously, upon exposure to the highest concentration, GS levels became closer those registered for the CTL.

3.3. Oxidative Stress Biomarkers on *M. sativa*

The behaviors of the analyzed oxidative stress biomarkers, H_2O_2 and LP, are shown in Figure 4. In general, H_2O_2 levels rose along with the increase in GBH concentration (Figure 4a). However, significant differences ($F(5, 11) = 6.294$; $p \leq 0.05$) were only observed for concentrations higher than 12 mg kg^{-1}, compared to the CTL. A similar behavior was also observed for LP with MDA levels increasing in a concentration-dependent manner (Figure 4b). Despite this pattern, for LP, statistically significant differences from the CTL ($F(5, 30) = 13.37$; $p \leq 0.05$) were observed only at the highest a.i. concentrations (26 and 40 mg kg^{-1}).



(**a**) (**b**)

Figure 4. Average concentrations of H_2O_2 (**a**) and malondialdehyde (MDA) (**b**) in shoots of *M. sativa* plants 21 days after exposure to increased concentrations of GLY. Error bars correspond to the standard deviation. Statistically significant differences compared to the CTL, considering $p \leq 0.05$, are marked with a * above bars.

The AOX response, evaluated by assessing the TAC, total phenol content (TPC) and Pro levels, of *M. sativa* exposed to Roundup UltraMax® is presented in Figure 5. Regarding TAC (Figure 5a), although a tendency for enhanced values as the concentration of the GBH goes up, statistically significant differences ($F(5, 14) = 3.468$; $p \leq 0.05$) were only found when plants were exposed to 40 mg kg^{-1} of a.i., with an increase of about 75% above the CTL. On the other hand, TPC (Figure 5b) was reduced upon exposure to increased concentrations of the GBH, especially in the highest dose (decreases up to 36%). Indeed, significant differences ($F(5, 13) = 7.802$; $p \leq 0.05$) compared to the control were observed only for the higher concentration. Concerning Pro (Figure 5c), its content showed a similar pattern to that of TAC, with levels significantly higher ($F(5, 8) = 5.574$; $p \leq 0.05$) than the CTL (by threefold) only for the highest concentration of GLY.

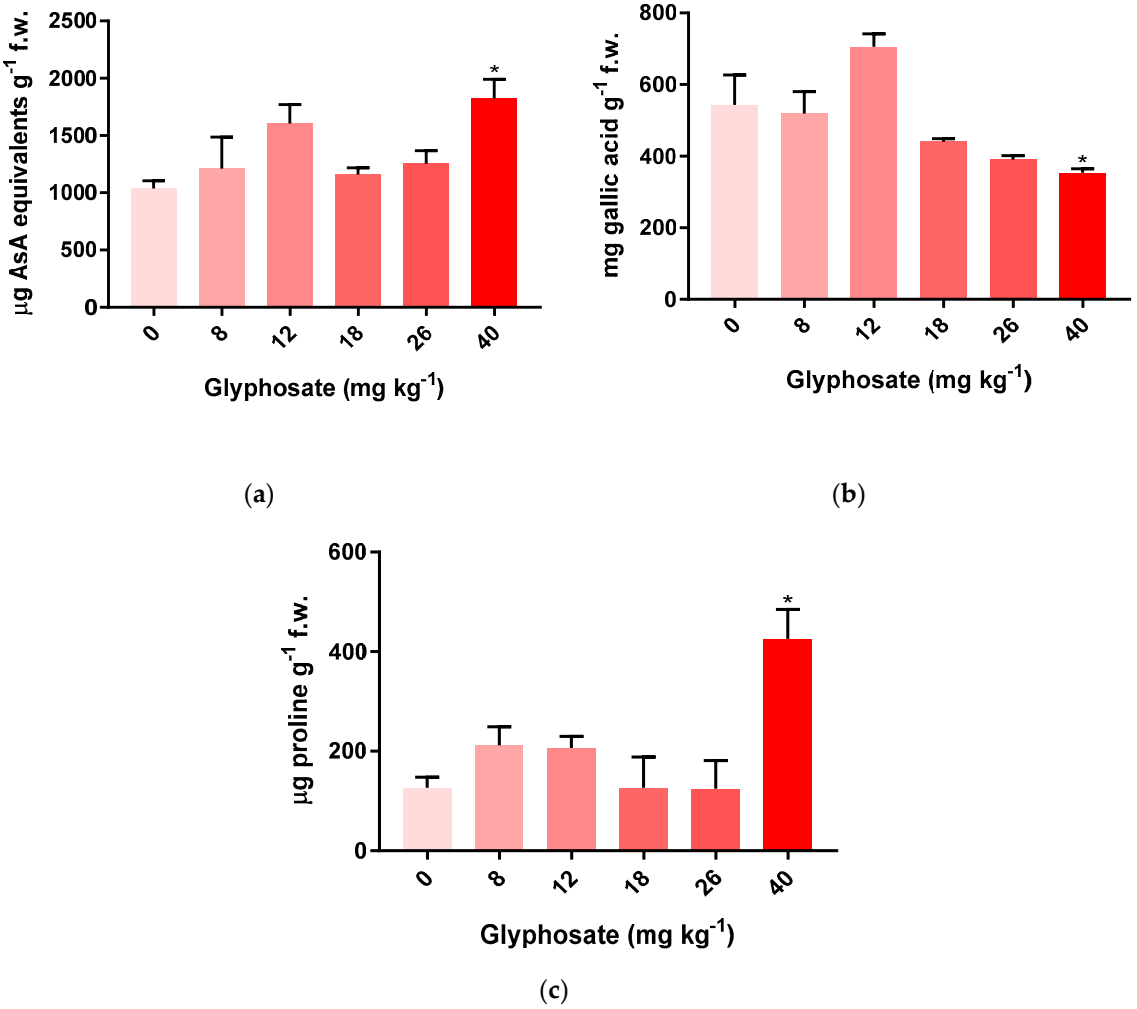


Figure 5. Effect of increased concentrations of GLY, on the AOX (antioxidant) system of M. sativa shoots after 21 days of exposure. (**a**) Total antioxidant capacity (TAC); (**b**) Total phenol content (TPC); (**c**) Proline (Pro). Error bars correspond to the standard deviation. Statistically significant differences compared to the CTL, considering $p \leq 0.05$, are marked with a * above bars.

4. Discussion

To date, little is known regarding the phytotoxicity of GLY-contaminated soils on non-target plants, including cover crop species, such as *M. sativa*. Although these plants are not intentionally treated with GLY, they can still be affected by its application through leaching, runoffs or even wind in the case of spraying. Moreover, GLY strongly adsorbs to solid particles [13,14] and accumulates in soils [15], resulting in a serious problem of diffuse contamination. Indeed, several studies were

conducted in order to determine GLY levels in soils around the world and despite many of them reporting levels lower than 3 mg kg^{-1} for agricultural soils or soil located nearby agricultural areas in South America [13,40–42] and Europe [7,43–46], other studies have reported values of 5.0 mg kg^{-1} in soybean-cultivated areas in Argentina [47], reaching concentrations as high as 40.6 mg kg^{-1} in olive groves from Greece [45] or even 608 mg kg^{-1} in a crop fields from Mexico [48]. Therefore, the main goal of the present study was to assess the effects of soil contamination by a GBH on the growth responses and redox homeostasis of alfalfa plants, at environmentally relevant concentrations of the a.i. In fact, despite recent studies having been conducted to evaluate the effects of GLY application in non-target plants, most of these works applied GLY as foliar spray [49–55] or as a supplement to the nutrient solution [12,56–60] rather than simulating soil contamination scenarios.

The present study showed that, after 21 days of exposure, Roundup UltraMax® severely repressed the growth of *M. sativa* in a dose-dependent manner, inhibiting both organ elongation and biomass production. Actually, given the already accentuated reduction in shoot fresh weight upon exposure to the lowest concentration tested (8 mg kg^{-1} of a.i.), it can be suggested that even lower levels would be capable of impairing plant growth. When GLY is absorbed by the plant, it is translocated through vascular tissues, namely by phloem, reaching active metabolite sites, such as root and shoot meristems, following the same pathway as photoassimilates [18,61], which could explain the repression of shoot growth. The fact that GLY is an herbicide that inhibits an enzyme from the shikimate pathway, 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS; EC 2.5.1.19), can also explain the results obtained. EPSPS plays a role in the synthesis of the aromatic amino acids tryptophan, phenylalanine, and tyrosine that are crucial for the growth and survival of plants and which function as the precursors of many secondary metabolites, such as pigments, auxins and lignin [62]. As a result of the shikimic acid pathway being blocked, there will be an accumulation of shikimate in plant tissues, which will lead to a deficit in important end products, such as lignin, alkaloids and flavonoids, and a reduction in CO_2 fixation and biomass production in a dose-dependent manner [63]. The decrease in root and shoot length and biomass can also be due to the impact that GLY has on i) indole-3-acetic-acid (IAA) metabolism which is the main endogenous auxin in the plant, as well as on ii) the interference with plant-water relationships [30,58,64]. Another hypothesis that can explain these results is the fact that GLY can impair the absorption of several macro and micronutrients, such as Ca, Mg, N, P, Fe and Zn, among others, as reviewed by Gomes et al. [18].

Several studies were conducted in order to evaluate the phytotoxicity of GLY to non-target plants, such as: *Pisum sativum* (GLY or GBH, applied directly to the seeds or supplemented to the nutrient solution) [55,58]; *Hordeum vulgare* (GBH supplemented to a mixture of perlite:vermiculite (1:2)) [65]; *Solanum lycopersicum* (GLY applied by foliar spray) [54]; *Vigna radiata* (seeds treated with a GBH) [66]; *Fagopyrum esculentum* (GLY isopropylamine salt supplemented to the nutrient solution) [67]; *Lemna minor* (GBH supplemented to the nutrient solution) [68]; *Dimorphandra wilsonii* (seeds treated with a GBH or analytical grade glyphosate) [12]. Even though the experimental conditions of the previously mentioned studies were not similar to the present study, they all recorded a decrease in plant growth, demonstrating the negative effect that both GLY and GBH have on biometric indicators. Concerning GBH-contaminated soils, a similar decrease was also observed in the work of Soares et al. [30], in which tomato plants grew in an artificial soil contaminated by increasing a.i. concentrations (0, 10, 20 and 30 mg kg^{-1}). Their results showed significant statistical differences even at 10 mg kg^{-1}, a concentration pretty much identical to the lowest dose tested in this study.

Photosynthesis, one of the main biochemical processes occurring in photoautotrophic organisms, highly depends on light absorption by chlorophylls and carotenoids. The biosynthesis of these pigments, as well as fatty acids or amino acids, can be affected by GLY exposure [69]. As GLY is an EPSPS competitive inhibitor, it blocks the shikimate pathway, thus compromising the biosynthesis of secondary metabolites, such as quinones and photosynthetic pigments, all compounds involved in the photosynthetic metabolism [70]. Previous studies showed that GLY can impair plastoquinone synthesis, thereby contributing to a lower production of carotenoid precursors [50]. Regarding chlorophylls,

both GLY and GBH can also directly inhibit its biosynthesis, by reducing δ-aminolevulinic acid (ALA) levels, or increase chlorophyll degradation, as reported by several authors [50,55,71–74]. Based on these results, it was expected that we would observe a significant decrease in the levels of both chlorophylls and carotenoids. Indeed, even a previous work conducted with the same plant species but grown in perlite and quartz sand [75] reported that the foliar application of a GBH resulted in a reduction in the total photosynthetic pigments as the a.i. concentration increased. However, in the present study, the herbicide showed no effects on chlorophyll and carotenoid contents, despite the slightly lower contents observed when compared to the control group (except for 12 mg kg^{-1}). Thus, these results suggest that, at the tested doses, this herbicide did not negatively affect the photosynthetic pigments as also demonstrated in the study performed by Spormann et al. [65] with a GLY concentration of 30 mg kg^{-1}, applied in the form of Roundup UltraMax® and using a mixture of perlite:vermiculite as substrate. As discussed by Spormann et al. [65], these results could be explained by the lack of (aminomethylphosphonic acid) AMPA production in the artificial medium. Indeed, AMPA, the main metabolite formed upon GLY degradation, is considered as a potent phytotoxin, capable of competing with glycine and consequently inhibiting chlorophyll biosynthesis [76,77]. Thus, there are two hypotheses for the lack of negative effects due to GLY exposure on chlorophyll and carotenoid content: (i) the use of a standard artificial soil with low microbial activity, not allowing enough AMPA production to cause negative effects on biosynthesis of these pigments; (ii) the mode of application of GLY, which, in this study, was added to the soil contrasting to the majority of works which provided GLY as foliar spray. However, and regarding the former hypothesis, this does not mean that an enhanced effect on a natural soil with a more diverse and functionally active soil microbial community would certainly be expected, as the degradation rates of both GLY and AMPA are still not well studied.

As important as photosynthesis, the mineral nutrition of plants highly contributes to proper growth performance. However, the effect of GLY on plant mineral nutrition is yet to be fully understood [78]. Up to now, no consensus has been reached on the influence that GLY may bring on nutrient uptake, since the studies conducted so far point towards different result. While several authors reported a negative effect of GBH on plant's nutrient uptake [72,78–80], other studies concluded that this application does not affect the mineral status of the plants [81–83]. As reviewed by Duke et al. [83], these inconsistent results may be due to differences in the type of soil, climatic conditions, and/or GLY-resistant cultivars used. Aiming to assess the nutritional status of *M. sativa* under GLY exposure, the present study evaluated the activity of GS, an enzyme that is involved in the first step of ammonium ($NH4^+$) assimilation, not only that which is absorbed by roots, but also the one generated from photorespiration, proteolysis and processes that are increased by several stresses [84,85]. The results revealed that GS was dysregulated for almost all tested concentrations, indicating that, at least under the experimental conditions of the present work, GBH interfered with nitrogen (N) metabolism. Based on these findings, the hypothesis that GLY conditioned the physiological uptake of mineral nutrients, especially nitrogen (N), due to the formation of complexes making them unavailable for biological processes, arises [86]. Concerning N uptake, once again, results from different studies, all of them using GBH, are contradictory with no effect in field studies [87,88] and inconsistencies in greenhouse studies [78,79].

As previously reviewed by Gill and Tuteja [22] and Soares et al. [20], plant development can be severely affected by various abiotic stressors, such as herbicide application, leading to an overproduction of ROS which, in its turn, will cause significant damage to cell structures, ultimately resulting in oxidative stress. In order to verify the occurrence of oxidative stress, H_2O_2 levels and LP degree, as a means to assess membrane damage, were evaluated. According to the results obtained, H_2O_2 accumulation was enhanced upon exposure to GBH, especially at levels of the a.i. higher than 12 mg kg^{-1}. However, when looking to LP results, MDA content was only increased in response to the two highest treatments (26 and 40 mg kg^{-1} of a.i.). Based on this behavior, one can suggest that ROS overproduction took place earlier than the observed membrane damage, this being possibly related

to the dual role played by ROS in plant cells. Indeed, H_2O_2, as with other ROS, can act as a signal molecule at low concentrations as it is involved in acclimation signaling, leading to plant tolerance to various biotic and abiotic stresses, becoming toxic above a certain threshold, capable of inducing programmed cell death [89]. Therefore, it can be hypothesized that, at lower GLY concentrations, H_2O_2 was involved in signaling mechanisms (with no LP increase), while at the highest concentrations (26 and 40 mg kg^{-1} of the a.i.), H_2O_2 accumulation started to induce oxidative damage, which is reflected by the occurrence of LP.

The induction of oxidative stress by GLY is described as one of its indirect effects on plant physiology, either by the overproduction of ROS or by a depletion of defense mechanisms [50]. Although not so explored as in target and resistant species, the influence of this herbicide on the redox status of non-target plants, including crops, willow and aquatic plants [30,49–52,54,55,65,86,90,91] is starting to gain attention. Corroborating the results of the present work, several studies reported an increase in H_2O_2 content and MDA levels in plants grown in GBH-contaminated solid substrate [65], or when GLY or GBH was supplied in nutrient solutions [50,52,55,90], or applied as foliar spray [49,51,54]. However, according to Moldes et al. [91] and Soares et al. [30], the exposure of soybean and tomato plants to GBH did not induce severe oxidative damage in leaves.

In order to defend themselves from oxidative damage caused by ROS, plants developed protective mechanisms by synthetizing enzymatic and non-enzymatic antioxidants [22]. In the context of this work, TAC, TPC and Pro levels were measured to assess the involvement of the non-enzymatic component of the AOX system in limiting GLY-induced stress. The results showed an increase in TAC and Pro levels only at the highest a.i. concentration (40 mg kg^{-1}). Since TAC gives a general idea regarding the cell's AOX status [92] and Pro acts as a strong AOX [22], the elevated TAC and Pro levels suggest that the AOX defense mechanisms were activated due to oxidative stress, but only at the highest concentrations of GLY. Thus, it can be hypothesized that *M. sativa* plants boosted the accumulation of Pro, along with other non-enzymatic players, to counteract the induced oxidative stress by this herbicide; however, bearing in mind that LP remained higher at the two highest concentrations, this response was not enough to counteract the harmful effects observed. Moreover, phenolic compounds, which are known to chelate metals, scavenge ROS and inhibit LP [93], were negatively affected by the presence of the herbicide, since reduced levels of these specialized metabolites were found in treated plants. This effect probably arises as a consequence of GLY-induced impairment of the shikimate pathway, once phenolic compounds are formed through this biosynthetic process [94], and is in accordance with the results obtained for LP.

Up to now, some studies were conducted in order to evaluate the AOX defense mechanisms of plant species exposed to both GLY and GBH [30,52,54,65,95]. These studies demonstrate that there is a dysregulation of the AOX defense system, with records of both increases and decreases in these mechanisms. Particularly, in the study of Soares et al. [30], performed with GBH-contaminated soils, it was observed that this formulation stimulated the AOX defense mechanisms of tomato shoots, at concentrations of 20 and 30 mg kg^{-1} of the a.i. This suggests that, like other environmental stresses, the response to herbicide application depends on several factors, such as the plant species, the concentration, and the mode of application. However, the results obtained in the present study are in line with those already published by other authors [30,52,54,65] indicating that the increase in Pro levels seems to be the most consistent signal of the activation of the AOX defense against GLY-induced stress, suggesting that this amino acid can be used as a biomarker of exposure to GLY.

5. Conclusions

Overall, it is possible to conclude that, after 21 days of exposure to a GBH, the growth and physiological performance of *M. sativa*, were negatively affected at the concentrations tested. The results also showed an activation of the AOX system, although its action was not enough to counteract the oxidative damage induced by an overproduction of ROS, ultimately leading to a decrease in this plant's growth. In the present work, adverse effects of GLY are visible at 8 mg kg^{-1} of the a.i., which

is a concentration much lower than the highest levels reported for European and South American soils. However, it should be noted that soil properties, such as soil organic matter content, may affect the behavior of GLY on soils. In addition, the type of formulation can also affect the toxicity, since the presence of surfactants may enhance the negative effects of the a.i. Thus, considering that plant responses to GLY can be species-specific and vary with distinct experimental conditions, it is of upmost importance to better understand the impacts of GLY-contaminated soils on the survival of non-target plants and subsequently on soil biodiversity, as well as developing new strategies to minimize its potential risks to agroecosystems.

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