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Salicylic Acid Pre-Treatment Reduces the Physiological Damage Caused by the Herbicide Mesosulfuron-methyl + Iodosulfuron-methyl in Wheat (*Triticum aestivum*)

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Abstract: Chemical herbicides are the most common method of weed control in crops, but they can also negatively affect the host crops, such as wheat (*Triticum aestivum* L.). The damage caused to the crop plants is often temporary and minor, but sometimes, it can be more substantial, requiring remedial measures. Salicylic acid (SA) is a plant hormone widely used to promote plant growth and to mitigate oxidative stress through its exogenous application. We evaluated the role of exogenously applied SA (as a pre-treatment) in ameliorating the oxidative damage caused by the herbicide mesosulfuron-methyl + iodosulfuron-methyl in wheat plants. The herbicide disrupted the physiological function of plants by affecting several enzymatic antioxidants. The hydrogen peroxide (H_2O_2) and malondialdehyde (MDA) contents increased at herbicide concentrations higher than 18 g ai ha^{-1} compared with the untreated control. However, the SA decreased the H_2O_2 and MDA contents compared with plants that were not treated with SA prior to the herbicide application. The activity of superoxide dismutase (SOD) and polyphenol oxidase (PPO) enzymes increased with increasing rates of the herbicide, as well as over time, regardless of the SA treatment. The activity of catalase (CAT) increased up to the herbicide rate of 18 g ai ha^{-1} and then decreased at the higher rates, while SA pre-treatment enhanced the CAT activity. The activities of ascorbate peroxidase, peroxidase, and glutathione-S-transferase enzymes generally increased in response to the herbicide application and SA pre-treatment, but fluctuated across different days of sampling following the herbicide application. Herbicide stress also induced high levels of proline production in wheat leaves as compared with the untreated control, while SA pre-treatment decreased the proline contents. Overall, the pre-treatment with different concentrations of SA mitigated the herbicide damage to the physiological functions by regulating the enzymatic antioxidants.

Keywords: herbicide damage; salicylic acid; oxidative stress; *Triticum aestivum*; antioxidant enzymes; Atlantis; abiotic stress

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1. Introduction

Abiotic stresses are among the major constraints limiting crop productivity around the world [1,2]. Most abiotic stresses including sub- or supra-optimal temperatures, drought, salinity, and non-targeted herbicide application negatively affect the growth and productivity of food crops [2]. Chemical application is the most important weed control method in present-day farming, as it often provides effective and economical weed control when applied at the appropriate dose and growth stage [3,4]. The selectivity of herbicides relies on a few elements, including plant growth stage, application technique, and environmental conditions which directly affect absorption, metabolism, or translocation of herbicides [5–8].

Despite the selectivity of most herbicides, some chemicals can cause damage to the host crop, especially when used at higher label rates [9].

Different herbicides suppress plant growth through various physiological disruptions and are often classified based on their mode of action. Most herbicide groups cause damage to the plant by directly affecting their target site; however, reactive oxygen species (ROS) are often generated as a secondary effect once the target site is extremely affected [10]. Acetolactate synthase (ALS)-inhibiting herbicides are known to kill plants by inhibiting the ALS enzyme, which is required to produce the branched-chain amino acids that are crucial for plant growth and development [10]. It has been reported that ALS-inhibitor herbicides induce significant levels of oxidative stress, producing ROS [10]. This combination of ALS inhibition and ROS production causes significant plant growth suppression and/or death [1]. Overexpression of ROS also occurs under different stress situations; these are toxic molecules that can lead to oxidative harm to the cells. These molecules damage cell membranes by the peroxidation of membrane-forming lipids. Cell membrane damage leads to the leakage of cellular contents, which negatively affects major physiological functions, such as photosynthesis and the defense system [11]. Hence, the negative effects of high concentrations of herbicides on a plant may be related to decreased photosynthetic performance, inhibition of cell division, and oxidative damage triggering the plant defense system [11,12]. The magnitude of damage and defense response depend on the kind of plant, the selectivity of the herbicide, the concentration used, and other factors [12]. Therefore, monitoring the levels of ROS and antioxidant molecules can provide a good understanding of the negative impact and potential recovery in response to a stressor such as herbicide damage.

Plants possess indigenous cellular antioxidant mechanisms, including enzymatic and non-enzymatic molecules that capture ROS to regulate the physiological functions in the cells [10]. In particular, plant growth regulators are critical in complementing the developmental activities and plants response to the environment, especially in stressful situations [13]. These compounds act as messenger molecules for adapting to stress. Salicylic acid (SA) is one such vital compound which regulates major functions such as ion uptake, photosynthesis, and stress abatement [14]. This phenolic compound is able to induce and stimulate the plant protection system under biotic and abiotic stresses [15,16]. The exogenous application of SA has been effective in reducing the herbicide toxicity in crops. For instance, treatment with SA 24 h before paraquat application decreased the negative effects of paraquat on chlorophyll content and photosynthesis in a barley (*Hordeum vulgare* L.) crop [17].

Wheat (*Triticum aestivum* L.) is arguably the world's most significant crop, feeding billions of people. Non-targeted herbicide damage is a significant production constraint in wheat crops, especially in developing countries where farmers may use higher herbicide rates to achieve better weed control. Mesosulfuron-methyl + iodosulfuron-methyl (trade name: Atlantis) is a broad-spectrum, ALS-inhibiting herbicide which provides very effective control of many grasses, sedges, and broadleaf weeds in wheat [18,19]. However, this herbicide has been reported to cause significant growth suppression of wheat crops under certain conditions. The most obvious approach to manage this kind of damage is to apply the herbicide at the label rate and at the optimal growth stage (as recommended on the herbicide label). However, farmers sometimes have to apply herbicides under sub-optimal conditions due to various management and environmental constraints. Therefore, we evaluated the potential of SA as a pre-treatment in wheat to reduce the negative effects of mesosulfuron-methyl + iodosulfuron-methyl application at different rates. The activity of several antioxidant enzymes was observed in order to understand the dynamics of the ameliorative mechanism of the SA pre-treatment.

2. Materials and Methods

2.1. Study Site and Treatments

A greenhouse study was carried out at the University of Mohaghegh Ardabili, Ardabil city (latitude: 38°12′39″ N, longitude: 48°17′36″ E, altitude: 1377 m a.s.l.), Iran, during 2019–2020. The experiment followed a completely randomized design, with a factorial arrangement using four replicates per treatment. The two treatment factors included: (i) SA pre-treatment as a foliar application (0.5 and 1 mM SA solution and a water spray as a control treatment); (ii) herbicide application (five rates including 4.5, 9, 18, 36, and 72 g active ingredient (ai) ha^{-1} and no application as a control treatment). These rates were chosen to evaluate a full spectrum, with rates below and above the recommended dose of 18 g ai ha^{-1}. The herbicide mesosulfuron-methyl + iodosulfuron-methyl (Atlantis OD, 10 g L^{-1} mesosulfuron-methyl + 2 g L^{-1} iodosulfuron-methyl + 30 g L^{-1} mefenpyr-diethyl) was obtained from Bayer CropScience, Persian AG, Tehran, Iran, and SA (Merck-818731, Darmstadt, Germany) was obtained from Asia Pajoohesh Company, Amol, Iran.

Plastic pots (diameter × height: 16 × 15 cm) were filled with soil collected from an agricultural field at the research station from a depth of 0–20 cm, where no pesticide or herbicide had been applied for more than ten years. Before sowing, the seeds of wheat cultivar *Gaskogen* were surfaced-sterilized for 10 min in a 5% sodium hypochlorite solution and were rinsed using distilled water. The seeds were placed on moist filter paper in the dark at 4 °C for pre-germination. These pre-germinated seeds were sown in pots, and a population of six plants per pot was maintained. The seedlings were grown in a controlled condition, with alternating day/night temperatures (22 ± 1 °C/15 ± 1 °C) and photoperiods (16/8; light/dark). The pots were manually irrigated regularly, as required. Herbicide was applied on the plants with 3 to 4 true leaves using a CO_2^- pressurized backpack sprayer, with 8002v flat fan nozzles delivering a spray volume of 150 L ha^{-1} at 300 kPa pressure. SA was applied 3 days prior to herbicide application onto the leaves/foliage using a simple atomizer spray bottle until the leaf surface was saturated. Plants were irrigated well 24 h before the SA application, and a light irrigation was again applied 24 h before the herbicide application. The normal irrigation schedule resumed 48 h after the herbicide application.

Several leaves were randomly selected from six plants growing in each pot to collect enough leaf tissue samples (3–4 g fresh weight per sample) at 1, 3, 5, and 7 days after herbicide treatment, and these samples were stored at −80 °C until biochemical analyses. These collection intervals were chosen based on the commonly observed timing of the activity of the herbicide mesosulfuron-methyl + iodosulfuron-methyl.

2.2. Antioxidant Analyses

2.2.1. Malondialdehyde and Hydrogen Peroxide Content

Fresh leaf tissue (0.5 g) was homogenized in 3 mL of 0.1% trichloroacetic acid (TCA) solution. The homogenate was centrifuged at 10,000× *g* for 30 min. The obtained supernatant was used for the determination of both hydrogen peroxide (H_2O_2) and lipid peroxidation levels.

Malondialdehyde (MDA) content was used as an indicator for lipid peroxidation, determined as 2-thiobarbituric acid (TBA) reactive metabolites, based on a method described by Heath and Packer [20]. A total of 2 mL of the supernatant was mixed with 2 mL of 0.5% TBA in 20% TCA. The mixture was incubated in a 95 °C boiling water bath for 30 min and then cooled on ice for 5 min. The final mixture was centrifuged at 10,000 rpm for 10 min. The absorbance of the supernatant was measured at wavelength A_{532} nm. The value for nonspecific absorbance at A_{600} nm was subtracted. The amount of MDA was expressed as nmol (g FW^{-1}) by using the extinction coefficient of 155 mM^{-1} cm^{-1}.

The endogenous H_2O_2 was measured spectrophotometrically at A_{390} nm. To determine the H_2O_2 level, the extracted solution was mixed with 100 mM potassium phosphate buffer and 1M potassium iodide (KI). The results were calculated using a standard curve, prepared with some concentration of H_2O_2 [21].

2.2.2. Extraction of Leaf Tissue for Determination of Enzyme Activity

Ground leaf tissues (0.1 g) were homogenized by adding 1.5 mL of cold potassium phosphate buffer (100 mM, pH 7.8). The homogenate was centrifuged at 10,000 rpm for 20 min at 4 °C. The supernatants were transferred to new centrifuge tubes, dropped into liquid nitrogen for quick freezing, and then store at −80 °C in a deep freezer for further analysis.

2.2.3. Ascorbate Peroxidase and Superoxide Dismutase Assays

Ascorbate peroxidase (APX) activity was determined by recording the decrease in absorbance at A_{290} nm using a protocol described by Nakano and Asada [22]. The reaction mixture contained 50 mM phosphate buffer (pH 7.0), 0.1 mM ethylene diamine tetra acetic acid (EDTA), 0.5 mM ascorbate, and 0.15 mM H_2O_2. One unit of APX activity was defined as the quantity of the enzyme necessary to decompose 1 µmol ascorbate min^{-1}, and the result was expressed as U mg^{-1} protein.

Superoxide dismutase (SOD) activity was assayed following the method of Chen and Zhang [23], with some modifications. The assay mixture contained 100 mM phosphate buffer (pH 7.8), 1 mM L-methionine, 750 µM nitro blue tetrazolium (NBT), and 20 µM riboflavin. One unit of SOD activity was defined as the enzyme activity that inhibited the photo-reduction of NBT to the formation of blue formazan by 50%, and the result was expressed as U mg^{-1} protein. The absorbance of the solution was measured at A_{560} nm.

2.2.4. Peroxidase and Catalase Assays

Peroxidase (POD) activity was assayed at 470 nm using guaiacol as the substrate, based on the method described by Chen and Zhang [23]. One unit of POD was defined as the amount of enzyme that increases 0.01 min^{-1} of absorbance due to the oxidation of guaiacol at 470 nm per minute, and the result was expressed as U mg^{-1} protein.

Catalase (CAT) activity was measured by monitoring the decrease in absorbance at 240 nm in a 100 mM potassium phosphate buffer (pH 7.0) containing 15 mM H_2O_2 and 50 µL extracted leaf tissue, using the method of Chen and Zhang [23]. One CAT unit was defined as the amount of enzyme necessary to decompose 1 µmol min^{-1} H_2O_2, and the result was expressed as U mg^{-1} protein under the above-mentioned assay conditions.

2.2.5. Glutathione-S-Transferase and Polyphenol Oxidase Assays

The activity of glutathione-S-transferase (GST) was measured using the method of Chen and Zhang [23]. The activity was estimated by monitoring the increase in absorbance at A_{340} nm per min, which is related to the rate of the 1-chloro-2, 4-dinithrobenzene (CDNB) conjugation with glutathione. The reaction mixture consisted of 0.1 M K-phosphate buffer (pH 6.5) containing 5 mM reduced glutathione, 1.5 mM CDNB, and 50 µL crude enzyme. Enzyme activity was expressed in terms of specific activity (U mg^{-1} protein).

Polyphenol oxidase (PPO) activity was determined by the method of Kar and Mishra [24]. For this purpose, 1.5 mL of Tris buffer was mixed with 0.4 mL of pyrogallol and 0.1 mL of enzyme extract left for 5 min at 25 °C in a bain-marie. One unit of PPO activity was defined as the amount of enzyme that caused a change in absorbance of 0.001 O.D. per min. Activity was determined by monitoring the increase in absorbance at A_{420} nm. Finally, the amount of enzyme was calculated in terms of absorption as U mg^{-1} of protein.

2.2.6. Proline Content

The proline content was quantified according to the methodology described by Bates et al. [25], with some modifications. The results are expressed in micromoles of proline g^{-1} FW by designing a proline standard curve with known concentrations. Approximately 0.1 g of leaf tissue was homogenized in 3% aqueous sulfosalicylic acid (2 mL), and the homogenate was centrifuged at 8000 rpm for 10 min. One mL of supernatant was reacted with acid ninhydrin (1.25 g ninhydrin in 30 mL glacial acetic acid and 20 mL 6 M phosphoric acid) and glacial acetic acid (1 mL each) in a test tube and incubated in a boiling water bath

for 1 h, and the reaction was terminated in an ice bath. After cooling, 2 mL toluene was added and mixed thoroughly by vortex mixing for 15–20 s. The chromophore containing toluene was elicited from the hydrous phase, warmed to room temperature, and the absorbance was read at A_{520} nm using toluene as a blank.

2.3. Statistical Analysis

All of the results for different parameters reported in this study are the means of four replicates for each treatment, with ± standard deviation. The differences between the mean values of different herbicide rates and of each rate of herbicide under different concentrations of SA were determined according to the Duncan's and Independent Samples *t*-tests, respectively, using the software SPSS 18.0 (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Malondialdehyde and Hydrogen Peroxide Contents

The MDA and H_2O_2 contents increased with increasing herbicide rates, without considering the effect of SA treatment (Figures 1 and 2). The highest amounts of MDA (0.39 µmol g^{-1} FW) and H_2O_2 (15.19 µmol g^{-1} FW) were observed in the leaves sprayed with 72 g ai ha^{-1} of herbicide (Figures 1 and 2). MDA and H_2O_2 content decreased by exogenous SA treatment in the untreated control, and as well as in the herbicide treatment samples. MDA and H_2O_2 contents increased over time, with the highest contents in leaves sampled at 5 and 7 days after herbicide application, regardless of the SA pre-treatment (Figures 1 and 2).

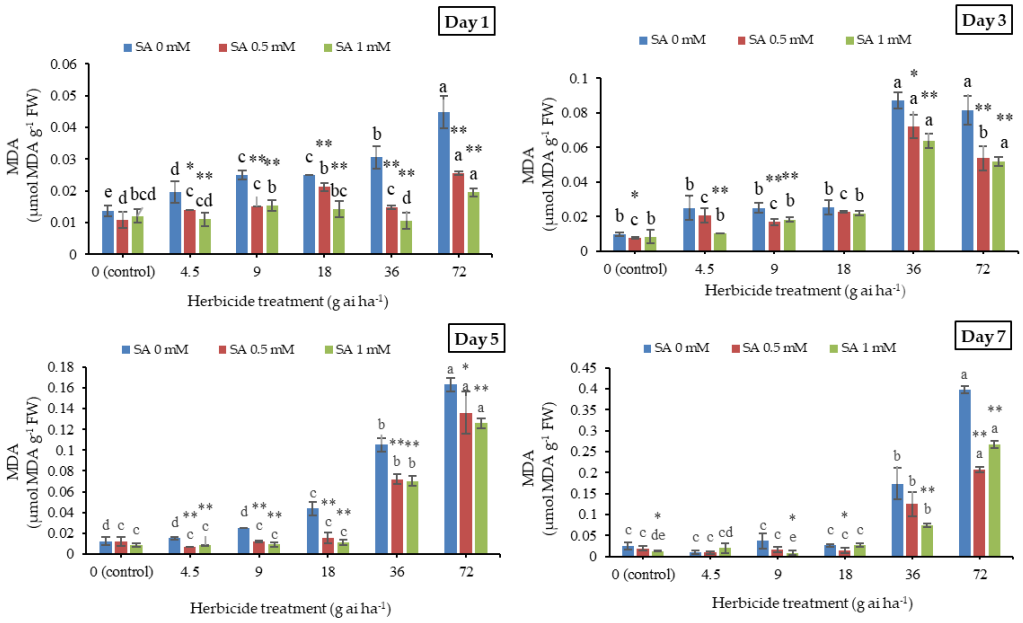

Figure 1. Changes in malondialdehyde (MDA) content in the leaf tissues of wheat at 1, 3, 5, and 7 days after herbicide application at different rates. The leaves were pre-treated with different concentration of salicylic acid (SA) 3 days prior to herbicide application. The bars represent the treatment mean ± SD (n = 4), and the bars with different lower-case letters were significantly ($p \leq 0.05$) different, according to the Duncan's test. Asterisks indicate a significant difference between the herbicide only and herbicide with each concentration of SA, according to the Independent Samples *t*-test.

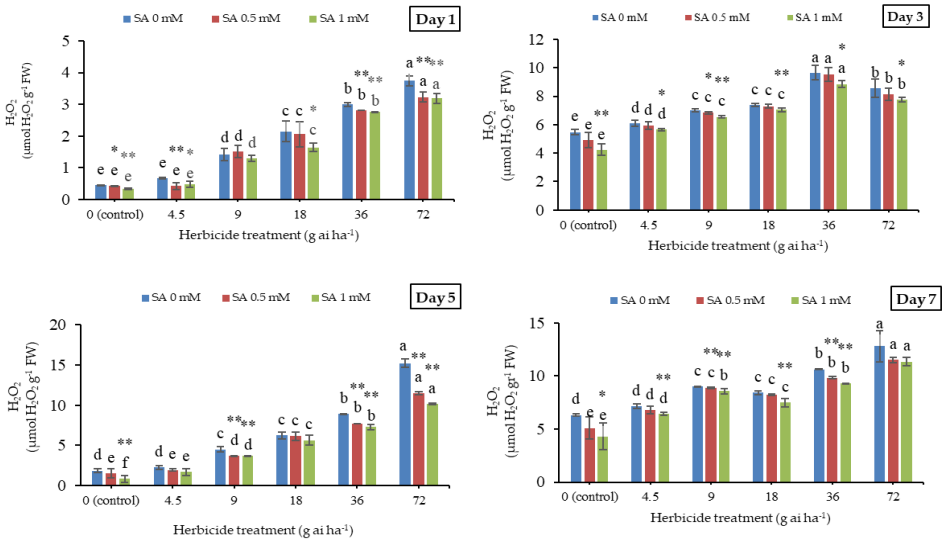

Figure 2. Changes in hydrogen peroxide (H_2O_2) content in the leaf tissues of wheat at 1, 3, 5, and 7 days after herbicide application at different rates. The leaves were pre-treated with different concentration of salicylic acid (SA) 3 days prior to herbicide application. The bars represent the treatment mean ± SD (n = 4), and the bars with different lower-case letters were significantly ($p \leq 0.05$) different according to the Duncan's test. Asterisks indicate significant difference between the herbicide only and herbicide with each concentration of SA, according to the Independent Samples t-test.

3.2. Superoxide Dismutase Activity

The SOD activity was higher in herbicide treatments compared to the untreated control under all levels of SA treatment (Figure 3).

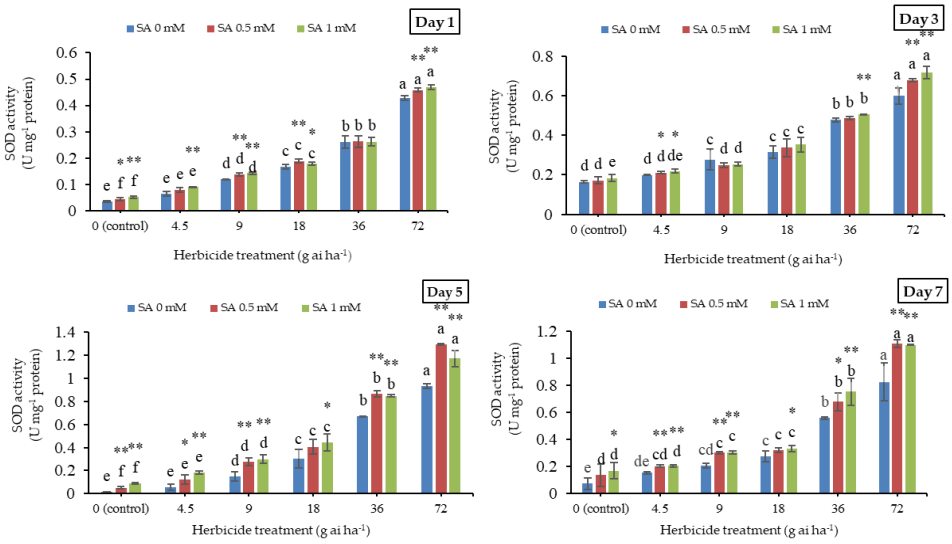

Figure 3. Changes in superoxide dismutase (SOD) activity in the leaf tissues of wheat at 1, 3, 5, and 7 days after herbicide application at different rates. The leaves were pre-treated with different concentrations of salicylic acid (SA) 3 days prior to herbicide application. The bars represent the treatment mean ± SD (n = 4), and the bars with different lower-case letters were significantly ($p \leq 0.05$) different, according to the Duncan's test. Asterisks indicate significant difference between the herbicide only and herbicide with each concentration of SA, according to the Independent Samples t-test.

The highest SOD activity (1.29 U mg^{-1} protein) was recorded for the highest rate of herbicide (72 g ai ha^{-1}) treatment in plants which received SA pre-treatment. Overall, SOD

activity was significantly greater in the group of plants which was pre-treated with SA compared with the non-SA treated group (Figure 3). Similarly, SOD activity increased over time, with higher activity at later sampling intervals.

3.3. Ascorbate Peroxidase Activity

The APX activity increased, without considering the SA treatment, especially at higher herbicide treatments (36 and 72 g ai ha^{-1}) at all sampling intervals (Figure 4). The APX activity reached its peak at the 7th day of sampling within the herbicide treatment range of 18–72 g ai ha^{-1} and the pre-treatment of 0.5 mM SA. (Figure 4).

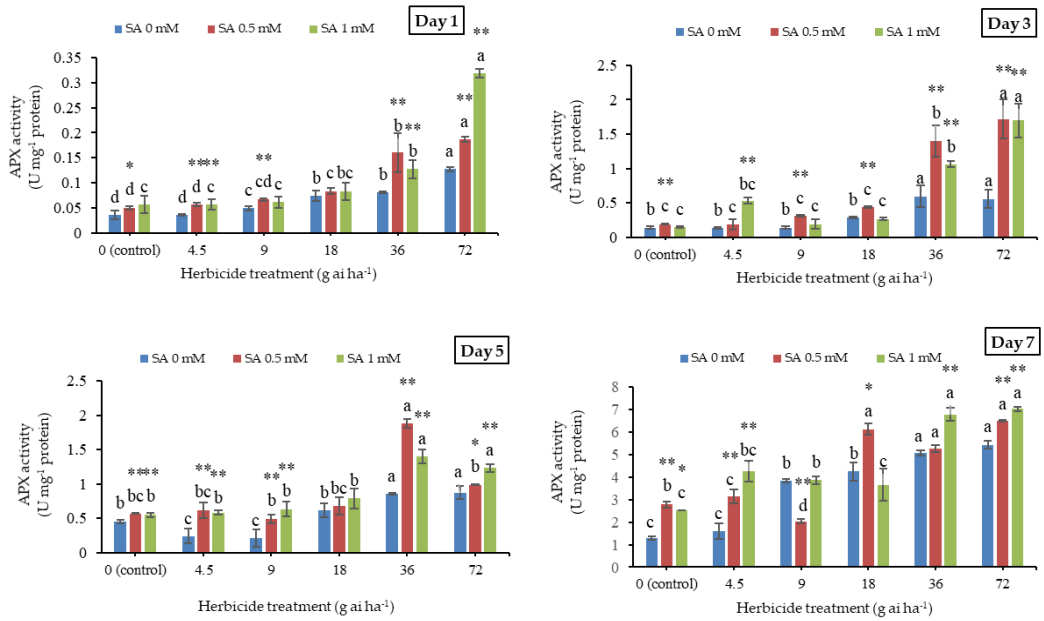

Figure 4. Changes in ascorbate peroxidase (APX) activity in the leaf tissues of wheat at 1, 3, 5, and 7 days after herbicide application at different rates. The leaves were pre-treated with different concentration of salicylic acid (SA) 3 days prior to herbicide application. The bars represent the treatment mean ± SD (n = 4), and the bars with different lower-case letters were significantly ($p \leq 0.05$) different, according to the Duncan's test. Asterisks indicate significant difference between the herbicide only and herbicide with each concentration of SA, according to the Independent Samples t-test.

3.4. Peroxidase Activity

The POD activity also rose with increasing rates of herbicide, peaking at later sampling intervals (Figure 5). The highest value (29.98 U mg^{-1} protein) was observed at the herbicide rate of 36 g ai ha^{-1} in leaf samples collected 5 days after herbicide application (Figure 5).

3.5. Catalase Activity

The CAT activity in the leaves was enhanced due to herbicide application at a range between 4.5 to 18 g ai ha^{-1}, regardless of SA pre-treatment (Figure 6).

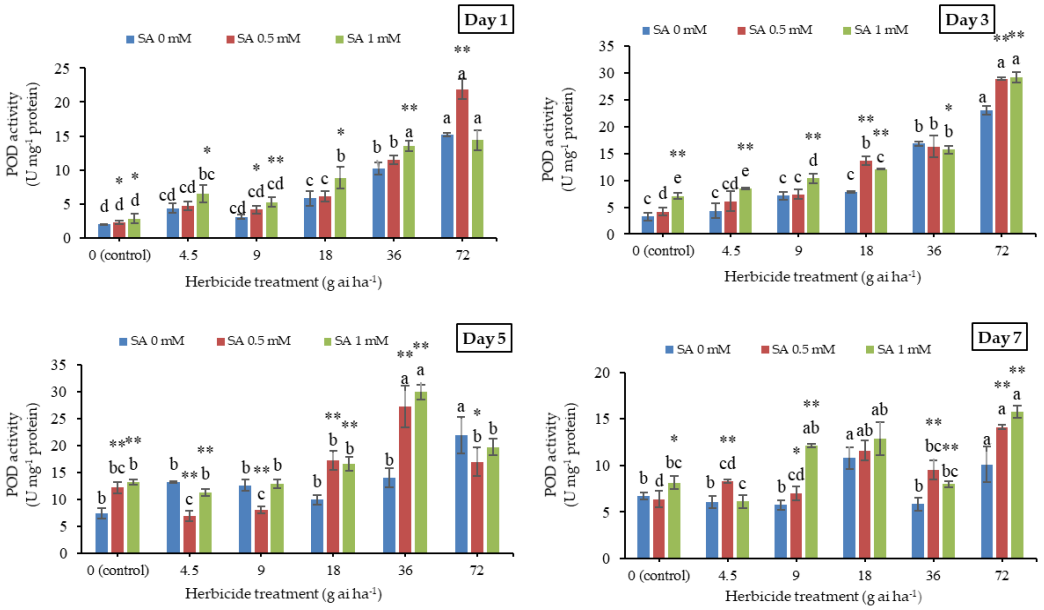

Figure 5. Changes in peroxidase (POD) activity in the leaf tissues of wheat at 1, 3, 5, and 7 days after herbicide application at different rates. The leaves were pre-treated with different concentration of salicylic acid (SA) 3 days prior to herbicide application. The bars represent the treatment mean ± SD (n = 4), and the bars with different lower-case letters were significantly ($p \leq 0.05$) different, according to the Duncan's test. Asterisks indicate significant difference between the herbicide only and herbicide with each concentration of SA, according to the Independent Samples t-test.

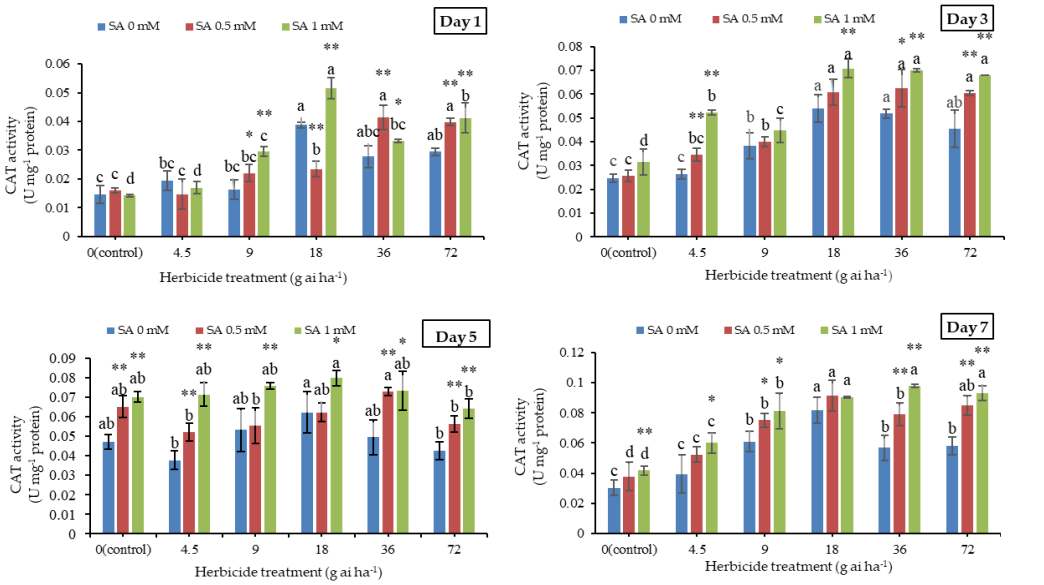

Figure 6. Changes in catalase (CAT) activity in the leaf tissues of wheat at 1, 3, 5, and 7 days after herbicide application at different rates. The leaves were pre-treated with different concentration of salicylic acid (SA) 3 days prior to herbicide application. The bars represent the treatment mean ± SD (n = 4), and the bars with different lower-case letters were significantly ($p \leq 0.05$) different, according to the Duncan's test. Asterisks indicate significant difference between the herbicide only and herbicide with each concentration of SA, according to the Independent Samples t-test.

The CAT activity decreased when the herbicide rate exceeded 18 g ai ha^{-1}. The SA pre-treatment (both rates) also improved the CAT activity in the leaves when exposed to the herbicide compared to the untreated leaves (Figure 6). The CAT activity was lower in

the leaves sampled just 1 day after herbicide application, but it was similar for the later sampling intervals (Figure 6).

3.6. Glutathione S-Transferase Activity

The activity of GST increased up to the herbicide dose of 36 g ai ha^{-1}, but decreased at 72 g ai ha^{-1}, regardless of SA pre-treatment (Figure 7). The highest GST activity (0.201 U mg^{-1} protein) was observed at the 7th day after the 36 g ai ha^{-1} treatment in the SA pre-treated group (Figure 7).

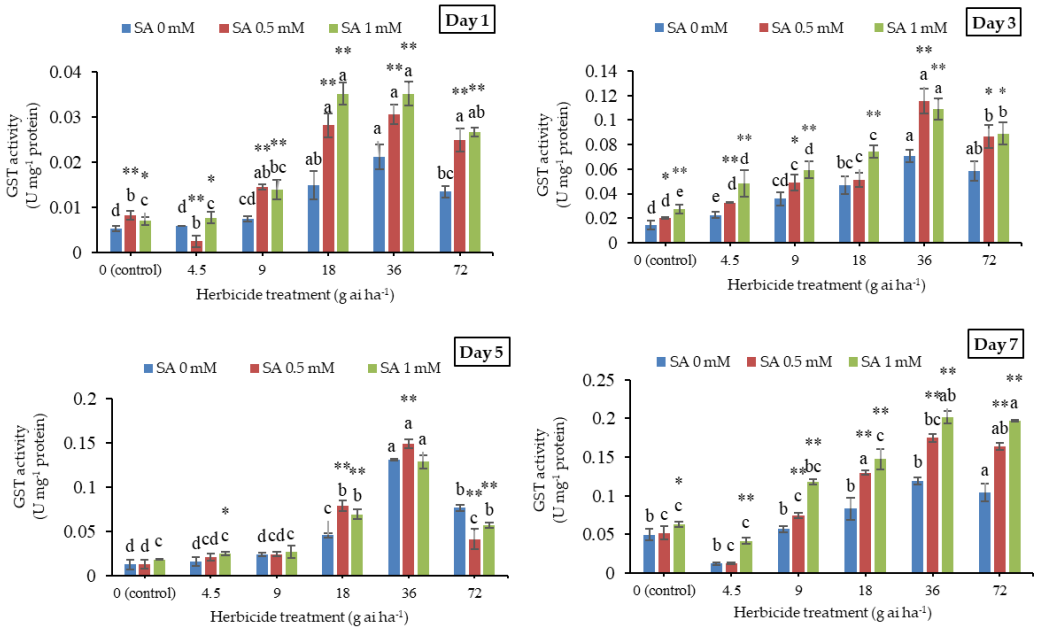

Figure 7. Changes in glutathione S-transferase (GST) activity in the leaf tissues of wheat at 1, 3, 5, and 7 days after herbicide application at different rates. The leaves were pre-treated with different concentration of salicylic acid (SA) 3 days prior to herbicide application. The bars represent the treatment mean ± SD (n = 4), and the bars with different lower-case letters were significantly (emphp ≤ 0.05) different, according to the Duncan's test. Asterisks indicate significant difference between the herbicide only and herbicide with each concentration of SA, according to the Independent Samples t-test.

3.7. Polyphenol Oxidase Activity

The PPO activity exhibited a pattern similar to the SOD activity (Figure 8). Herbicide applications at higher rates of 36 and 72 g ai ha^{-1} increased the PPO activity 2–3 folds, as compared to that in the untreated control (Figure 8). Overall, SA pre-treatment did not exhibit much difference across herbicide treatments or sampling intervals. The leaves sampled at 5 and 7 days after herbicide application showed higher PPO activity (Figure 8).

3.8. Proline Content

The proline content was higher in the herbicide-treated leaves when compared with the untreated control (Figure 9).

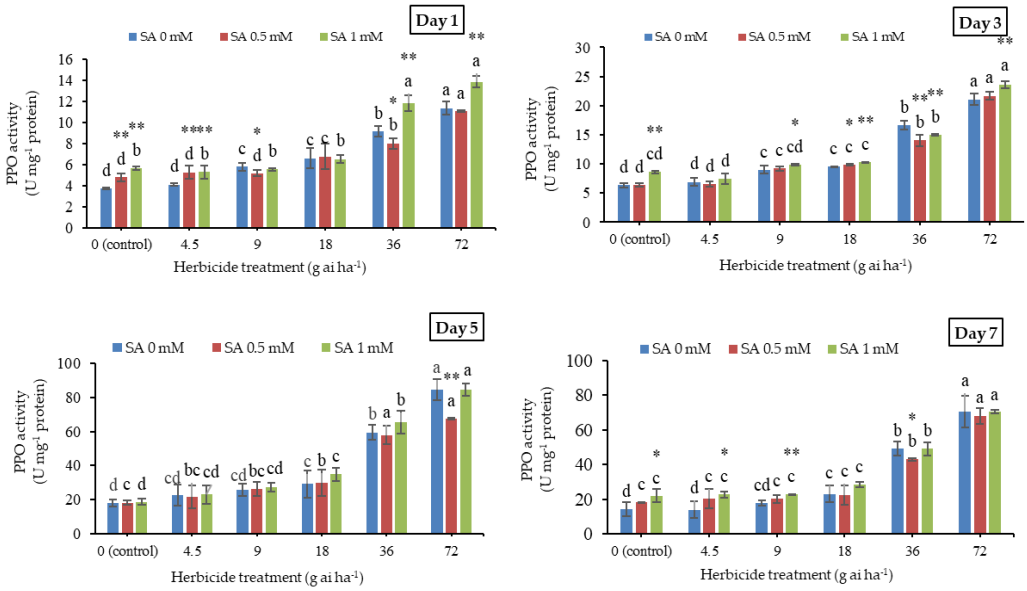

Figure 8. Changes in polyphenol oxidase (PPO) activity in the leaf tissues of wheat at 1, 3, 5, and 7 days after herbicide application at different rates. The leaves were pre-treated with different concentration of salicylic acid (SA) 3 days prior to herbicide application. The bars represent the treatment mean ± SD (n = 4), and the bars with different lower-case letters were significantly ($p \leq 0.05$) different, according to the Duncan's test. Asterisks indicate significant difference between the herbicide only and herbicide with each concentration of SA, according to the Independent Samples t-test.

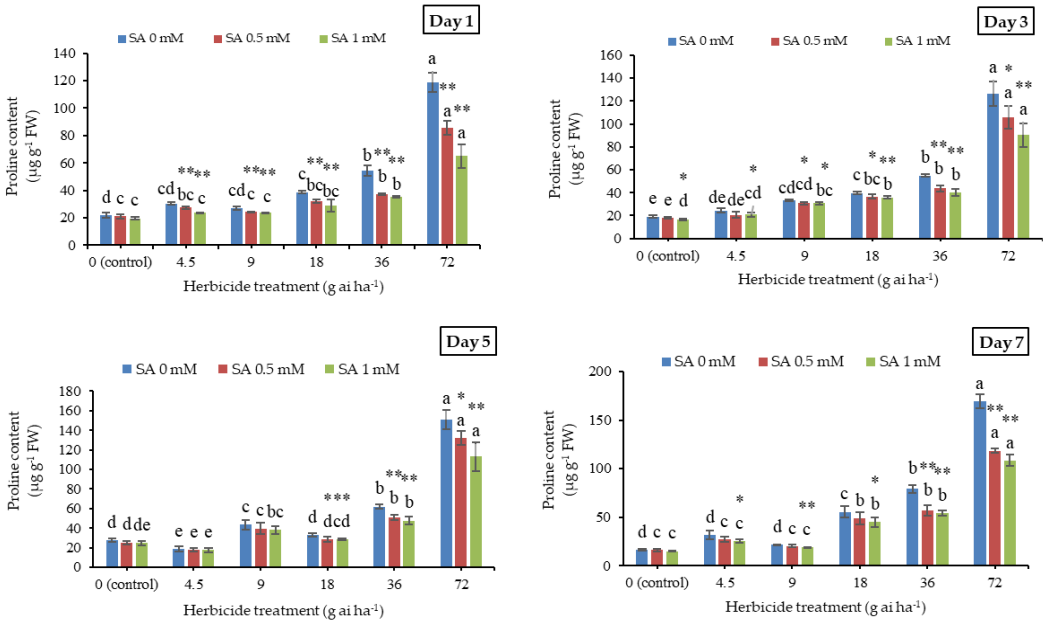

Figure 9. Changes in proline content in the leaf tissues of wheat at 1, 3, 5, and 7 days after herbicide application at different rates. The leaves were pre-treated with different concentration of salicylic acid (SA) 3 days prior to herbicide application. The bars represent the treatment mean ± SD (n = 4), and the bars with different lower-case letters were significantly ($p \leq 0.05$) different, according to the Duncan's test. Asterisks indicate significant difference between the herbicide only and herbicide with each concentration of SA, according to the Independent Samples t-test.

The highest proline content (169.16 µg g^{-1} FW) was observed at the herbicide rate of 72 g ai ha^{-1} at 7 days after application. The SA pre-treatment reduced the proline content across the treatment groups (Figure 9).

4. Discussion

Chemical weed control is an important crop management practice which ensures profitable crop production by providing effective control of weeds. Although there are many herbicide options for in-crop use due to selectivity of chemicals against weeds, certain herbicides can cause significant damage to crops, especially when used at higher rates. Herbicides often disrupt physiological processes in plants, resulting in significant growth suppression or mortality [26,27]. We tested the effect of a commonly used herbicide on wheat plants at different rates and studied how herbicide affects the antioxidant system in exposed plants. We established that higher rates of herbicide significantly disrupted the plant physiological processes, but pre-treatment (exogenous application) of plants with salicylic acid (SA) minimized these damaging effects. Hence, SA pre-treatment proved to be a promising practice to alleviate herbicide damage in sensitive wheat crops that are often exposed to higher rates of mesosulfuron-methyl + iodosolfuron-methyl.

Herbicide exposure often acts as an abiotic stress, resulting in the over-expression of reactive oxygen species (ROS) at the cellular level [9,28]. These ROS are detrimental to several physiological functions and therefore, cause abnormal growth and mortality of the plants. To prevent the potential damage by ROS, there is a cellular antioxidant system which includes different enzymes and non-enzymatic antioxidant molecules that effectively scavenge ROS to a certain extent [29,30]. However, the natural antioxidant system and its regulatory function is often not effective enough to overcome herbicide damage and/or other severe abiotic stresses. ALS-inhibiting herbicides form ROS in plants as a secondary effect, so they may exhibit a lag time between the time of treatment and the creation of oxidative stress [10].

Alterations in enzymatic antioxidants have been observed in several crops and weed species in response to herbicide exposure. In the first instance, the plants produce ROS, which cause lipid peroxidation and eventually, membrane damage [31–33]. We observed a similar response in the current study where lipid peroxidation increased with the increasing rates of herbicide application. This translated into higher levels of MDA and H_2O_2, especially at higher rates of herbicide (36–72 g ai ha^{-1}), and these levels increased over time (5 and 7 days after herbicide application). A similar increase in MDA and H_2O_2 contents was reported in a pea (*Pisum sativum* L.) crop exposed to the herbicide isoproturon, which is an ALS-inhibitor, similar to mesosulfuron-methyl + iodosulfuron-methyl (used in our study) [34]. Singh et al. [34] also reported the ameliorative effect of SA in pea plants that underwent oxidative stress due to isoproturon exposure. These remedial effects have been associated with the enhancement of enzymatic antioxidants under exogenous SA application [34].

The antioxidant enzymes measured in this study have been strongly associated with stress tolerance. For example, SOD dismutates $O_2^{\bullet -}$ to H_2O_2, avoiding membrane damage. We observed enhanced SOD activity, which was a response to the oxidative stress induced by physiological disruption in wheat after exposure to the herbicide. Similarly, certain enzymes, including POD and CAT, play critical roles in catalyzing the reactive H_2O_2 into H_2O or other nontoxic molecules [35]. For example, APX uses ascorbate as an electron donor, and this enzyme is widely distributed in different cellular entities and the cytosol [10]. Similarly, POD is involved in the biosynthesis of lignin, while it also consumes H_2O_2 to reduce the risk of oxidative damage [10]. The CAT enzyme facilitates the dismutation of H_2O_2 molecules to water and oxygen, and this enzyme is localized mainly in the peroxisomes [10]. We noticed a rise in APX and POD activities in the herbicide treatments, and more so in the plants that also received SA pre-treatment. This trend is similar to that found in a previous study with another ALS-inhibitor herbicide [34]. The CAT activity in this study increased up to the herbicide rate of 18 g ai ha^{-1}, but decreased at the higher rates. This was likely because of damage to the enzyme by high H_2O_2 levels or direct damage caused by herbicide residues. GST is an isozyme which reduces toxicity, mainly by improving the conjugation of glutathione to various substrates [36]. Our results depict a significant rise in GST activity under herbicide exposure. Similarly,

PPO activity increased over time in response to herbicide damage. PPOs are ubiquitous copper-containing enzymes which act as phenol oxidase in different plant species [37]. Phenolic compounds are oxidized by PPO, which is related to the antioxidant activity [38].

Non-enzymatic antioxidants, such as ascorbic acid, glutathione, and proline, are also crucial in capturing ROS, reducing oxidative damage, and restoring redox homeostasis [29,30]. These compounds reside in cell compartments and often help detoxify ROS [29,39]. Proline is an influential antioxidant, which avoids lipid peroxidation under stress conditions [29,30]. In this study, the proline content increased in response to herbicide application in a dose-dependent manner. Importantly, the proline content improved due to SA pre-treatment. Overall, SA application provided some protection to wheat plants through the accumulation of proline. Although plants activate their antioxidant defense system in response to abiotic stressors, such as herbicides, this natural defense system might not be effective to fully ameliorate the damaging effects of herbicides, so exogenous application of hormones such as SA help improve stress tolerance [40–42].

The results of this study show that rapid, disruptive physiological changes occurred in wheat leaves when exposed to different doses of a commonly used post-emergent chemical herbicide. However, SA treatment substantially mitigated the toxic effects caused by the herbicide [43]. The SA regulated several major antioxidants in plants, reducing the negative effects of the herbicide stress [44]. It has been suggested that such an ameliorative effect of SA is possible through the reduction of ROS production, which ultimately means less oxidative damage to the cell membrane [43,45]. Therefore, SA pre-treatment could improve cell membrane integrity and prevented herbicide molecules from entering the plant cells.

5. Conclusions

Clearly, mesosulfuron-methyl + iodosulfuron-methyl concentrations higher than the recommended label rate (18 g ai ha^{-1}) caused lipid peroxidation and disrupted the antioxidant system in wheat plants. The exposed plants then activated different antioxidative enzymes, including superoxide dismutase, polyphenol oxidase, catalase, ascorbate peroxidase, peroxidase, and glutathione-S-transferase to scavenge the reactive oxygen species. These negative effects were mitigated through an exogenous pre-treatment with salicylic acid. Therefore, the exogenous application of salicylic acid prior to herbicide application can be adopted to reduce its potentially toxic effects on wheat plants. However, the field-scale feasibility of this method requires further validation under field conditions, as well as an economic analysis of the treatment.

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