

Article

# Time-Dependent Effects of Bentazon Application on the Key Antioxidant Enzymes of Soybean and Common Ragweed

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**Abstract:** The presence or absence of light is one of the most significant environmental factors affecting plant growth and defence. Therefore, the selection of the most appropriate time of application may maximize the benefits of photosynthetic inhibitors. In this work, the concentration and daytime or night-time-dependent effects of bentazon were tested in soybean and common ragweed. The recommended dose (1440 g ha<sup>-1</sup>) and also half the recommended dose significantly reduced the maximum quantum yield ( $F_v/F_m$ ) and increased H<sub>2</sub>O<sub>2</sub> levels in common ragweed. Interestingly, bentazon did not change  $F_v/F_m$  in soybean. The activity of superoxide dismutase changed in a dose-dependent manner only in common ragweed. The activity of ascorbate peroxidase, catalase and glutathione S-transferase (GST), as well as the contents of ascorbate (AsA) and glutathione (GSH) did not change significantly in this plant species. In soybean, alterations in H<sub>2</sub>O<sub>2</sub> levels were lower but GST and APX activity, as well as AsA and GSH levels were higher compared to common ragweed. At the same time, the rate of lipid peroxidation and ion leakage increased upon bentazon, and were higher in the light phase-treated leaves in the case of both plant species. These results can contribute to optimizing the effects and uses of herbicides in agriculture.

**Keywords:** antioxidants; Basagran<sup>®</sup>; chlorophyll *a* fluorescence parameters; dark; herbicide; light; night; reactive oxygen species

## 1. Introduction

Soybean (*Glycine max* L.) is a very significant crop of the developing world because this plant is an essential source of protein for animal feeds and many packaged meals [1]. USA (119.51 million tons), Brazil (114.59 million tons) and Argentina (54.97 million tons) were the most important soybean producers in 2017 (FAOSTAT; <http://www.fao.org/faostat/en>). Sustained and increased productivity, improved quality of soybeans, (bio) controlled weed and pest management and new, sustainable and environmentally friendly methodology and technology are needed in the future [2–4]. The results of basic and applied researches can serve sustainable agriculture management especially in the case of using herbicides that protect the ecosystem and biodiversity from excessive and harmful application.

Bentazon (3-isopropyl-1*H*-2,1,3-benzothiadiazin-4(3*H*)-one 2,2-dioxide; CAS number: 25057-89-0) is the main component of the mercantile herbicide Basagran<sup>®</sup> (BASF Chemicals, Ludwigshafen, Germany) and belongs to the benzothiadiazoles chemical group. Basagran<sup>®</sup> is a contact and selective herbicide applied to limit broad-leaved and some grassy weeds in pre- and post-emergence on

soybean [5–7]. Among many weeds of soybean, common ragweed (*Ambrosia artemisiifolia* L.) is one of the most significant [7–9]. Interference of common ragweed with the growth of other crops results in variable yield losses depending upon the density, the type of infested plant species and the time of emergence relative to the harvested plant [9]. It was observed that common ragweed is very competitive in soybean culture and was able to cause ca 70% yield losses [10]. Therefore, the optimization of the treatments against common ragweed plants is essential to control yield losses of soybean culture.

In sensitive plant tissues, bentazon inhibits the photosynthetic electron flow in chloroplasts thus reducing the photosynthetic activity. Bentazon competes with quinone B ( $Q_B$ ) for its binding site in the reaction centre of Photosystem II (PSII), thus decreasing the rate of the electron flow as  $Q_A$  is unable to reoxidize. Light-induced PSII blockage by bentazon promotes the generation of triplet-chlorophyll ( $^3\text{Chl}$ ) and the formation of singlet oxygen ( $^1\text{O}_2$ ) upon reaction with molecular oxygen [11–13]. Bentazon-promoted oxidative stress damages the proteins and membranes of photosynthetic cells and induces cell death in weeds [12,14–16]. At the same time, bentazon seems to induce oxidative stress expressed as enhanced free radical production and lipid peroxidation, as well as significant induction of components of the antioxidant system in crops, respectively [15,16]. Crops survival under stress conditions depends on the plant defence system via xenobiotic detoxification in tolerant species, a process which may go on in parallel with cell defence reactions to alleviate oxidative stress depending on the antioxidant capacity [17,18]. Thus investigation and understanding of the role of the antioxidant system in herbicide-tolerant crops such as soybean could provide relevant information on practical purpose.

Inhibition of chloroplastic electron transport, e.g., by herbicides, results in rapid (within a millisecond time range) and high production of reactive oxygen species (ROS) [11]. Biphasic ROS burst was described in the plant cell death process, where the first maximum of ROS was measured within minutes after the initial stimulus and the second, more intense burst occurred several hours later. The first ROS burst can originate from energy-organelle sources and both ROS maxima can be dependent on the basic antioxidant capacity and the activation of antioxidant enzymes [19]. ROS production and oxidative stress resulting in the rapid damage of lipids, carbohydrates, proteins and nucleic acids thus can inhibit active plant defence and induced cell death [20,21]. To control and minimize the toxic levels of ROS and to protect cells in the case of harmful oxidative stress conditions, plants possess several enzymatic and non-enzymatic antioxidants. Three key antioxidant enzymes play an essential role in these processes. One of the first is the superoxide dismutase (SOD; EC 1.15.1.1), which catalyses the dismutation (disproportionation) of  $\bullet\text{O}_2^-$  to  $\text{O}_2$  and  $\text{H}_2\text{O}_2$ . At the same time,  $\text{H}_2\text{O}_2$  can be degraded by catalase (CAT; EC 1.11.1.6) and ascorbate peroxidase (APX; EC 1.11.1.11), the second and third main antioxidant enzymes for  $\text{H}_2\text{O}_2$  [18]. Therefore, the activated antioxidant defence system can substantially and rapidly contribute to cell survival by alleviating the primary and secondary oxidative stress effects of herbicides besides activating other xenobiotic detoxification pathways in plants. This was confirmed in both weeds and soybean, where antioxidant enzymes were significantly activated after xenobiotic exposure such as oxyfluorfen [22], linuron [23], dimethenamid [23], glyphosate [24] and paraquat [25]. These results verified the presence of oxidative stress and the significance of antioxidants in defence reactions in the cells of both sensitive and tolerant plants.

Interestingly, the activity of these antioxidant enzymes is highly dependent on the circadian clock and the light or dark conditions [26]. It was earlier observed that the activity of SOD, CAT and APX, as well as the content of APX substrate ascorbate (AsA) rose during the light phase of the day and lowered during the night in various plant species. The highest activity at the end of the light phase can be twice as much as at dawn [27–31]. The other key enzyme, which plays a role in the detoxification of various xenobiotics is the glutathione S-transferase (GST) [32]. GST activity and glutathione (GSH) levels also display circadian- and light-dependent patterns with a maximum late in the light and early dark phase [32–35]. Therefore, the selected daytime or night-time, and the presence or absence of daylight can be fundamental in case of the herbicide application on crops and weeds. Consequently,

selection of the most appropriate time of application may maximize the benefits derived from bentazon, a photosynthetic inhibitor.

The main aim of our work is to detect and compare the daytime- or night-time-dependent effects of different concentrations of the photosynthesis inhibitor bentazon on the activity of photosynthesis and the key antioxidant enzymes (SOD, CAT, APX), levels of antioxidants (AsA, GSH) and GST in soybean and common ragweed plants. In the centre of these investigations was also the question whether bentazon application in lower concentration could provoke defence responses in the non-target soybean and cell death in the target common ragweed plants in different light or dark periods of the day.

## 2. Materials and Methods

### 2.1. Plant Materials

Seeds of soybean (*Glycine max* L. cv. Pannónia Kincse) and common ragweed (*Ambrosia artemisiifolia* L.) were germinated at 26 °C for 3 days in the dark. Six healthy soybean and twelve healthy common ragweed plants were grown in 4 kg mixture of 3:1 soil and sand (Florimo, MATÉCSA Kft., Kecel, Hungary) containing organic matter (70 m/m%), N (10,000 mg kg<sup>-1</sup>), P<sub>2</sub>O<sub>5</sub> (1000 mg kg<sup>-1</sup>); K<sub>2</sub>O (3000 mg kg<sup>-1</sup>), and pH 6.4 ± 0.5. They were irrigated every 3 days with 250 mL of  $\frac{1}{2}$  Hoagland nutrient solution (5 mM Ca(NO<sub>3</sub>)<sub>2</sub>, 5 mM KNO<sub>3</sub>, 1 mM KH<sub>2</sub>PO<sub>4</sub>, 2 mM MgSO<sub>4</sub>, 1 µM Fe-EDTA, 0.04 µM H<sub>3</sub>BO<sub>3</sub>, 14.48 µM MnCl<sub>2</sub>, 0.81 µM ZnCl<sub>2</sub>, 0.37 µM CuCl<sub>2</sub>, 0.001 µM Na<sub>2</sub>MoO<sub>4</sub>) [36]. Growing conditions of plants were controlled. Illumination was 200 µmol m<sup>-2</sup> s<sup>-1</sup> for 12 h and occurred from 6:00 a.m. until 18:00 p.m. (White LED (5700 K) illumination with supplementary FAR LEDs; PSI, Drásov, Czech Republic). The day/night temperatures and the relative humidity were 24/22 °C and 55–60%, respectively [37]. For the experiments, 5- to 6-week-old intact plants in 3–4 developed leaf-level stages were used.

### 2.2. Treatments

Leaves of soybean and ragweed plants were sprayed with Basagran® herbicide solution (BASF, Ludwigshafen, Germany) diluted in double-distilled water to a final concentration of 720 or 1440 g ha<sup>-1</sup> of active ingredient bentazon (3-isopropyl-1H-2,1,3-benzothiadiazin-4(3H)-one 2,2-dioxide). To investigate the daytime- or night-time- and light-dependent effects of different concentrations of bentazon, plants were treated at several times, such as in the late afternoon (5:00 p.m.), in the evening (9:00 p.m.), at dawn (4:00 a.m.) and in the morning (8:00 a.m.) [37]. Plant responses were detected at 9:00 a.m. in the second light phase of the day after each treatment (Figure 1). As a control treatment, spraying with double-distilled water was used in all times, the results of which were averaged.

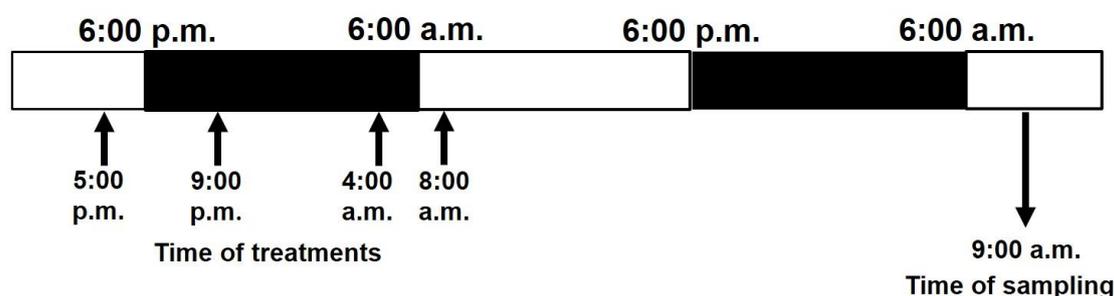


Figure 1. Experimental setup of bentazon treatments and time of samplings.

### 2.3. Measurement of Photosynthetic Activity

To detect the chlorophyll *a* fluorescence parameters in leaves of soybean and common ragweed plants, pulse amplitude modulation (PAM) chlorophyll fluorometer (PAM-2000; Heinz-Walz, Effeltrich, Germany) was used. Before the measurements of the minimal fluorescence yield of the dark-adapted state ( $F_0$ ), leaves were dark-adapted for 15 min. After dark-adaptation, firstly the maximal fluorescence in dark-adapted state ( $F_m$ ) was measured and the following parameters were calculated: The maximal

quantum efficiency of PSII photochemistry ( $F_v/F_m = (F_m - F_0)/F_m$ ), the actual quantum yield of PSII electron transport in the light-adapted state ( $\Phi_{PSII} = (F_m' - F_s)/F_m'$ ) and the photochemical quenching coefficient ( $qP = (F_m' - F_s)/(F_m' - F_0')$ ). Finally, the light-induced photoprotection through thermal dissipation of energy was determined  ${}^aNPQ = (F_m - F_m')/F_m'$  [38,39].

#### 2.4. Determination of Photosynthetic Pigment Content

Twenty-five mg of leaf tissues of soybean and common ragweed plants were homogenised in 1 mL of 100% (v/v) cold acetone, and extracted for one day at 4 °C in the dark. Samples were centrifuged (12,000× g for 15 min at 4 °C). The pellet was extracted again with 1 mL of cold acetone/Tris buffer solution (80:20 v/v, pH = 7.8) for another one day at 4 °C in the dark. Supernatants were collected after centrifugation (12,000× g, 15 min, 4 °C), and the absorbance was measured by spectrophotometer (KONTRON, Milano, Italy). Contents of photosynthetic pigments were calculated based on Lichtenthaler and Wellburn [40].

#### 2.5. Determination of H<sub>2</sub>O<sub>2</sub> Content

200 mg of leaf tissues of soybean and common ragweed plants were homogenised with 1 mL of 0.1% (w/v) ice-cold trichloroacetic acid (TCA), then the samples were centrifuged at 12,000× g for 10 min at 4 °C. Then 0.25 mL of 50 mM potassium phosphate buffer (pH 7.0) and 0.5 mL of 1 M potassium iodide (KI) were added to 0.25 mL of the supernatant. Samples were measured by spectrophotometer (KONTRON, Milano, Italy) after 10 min at 390 nm. The amount of H<sub>2</sub>O<sub>2</sub> was determined using a standard curve prepared from increasing concentration of H<sub>2</sub>O<sub>2</sub> [41]. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

#### 2.6. Determination of Activities of Key Antioxidant Enzymes

Two hundred and fifty mg of leaf tissue of soybean and common ragweed plants were homogenised with 1 mL of ice-cold extraction buffer (100 mM phosphate buffer (pH 7.0); 1 mM phenylmethylsulfonyl fluoride (PMSF) and 1% (w:v) polyvinyl-polypyrrolidone (PVPP)), then the homogenate was centrifuged (12,000× g for 20 min at 4 °C). This supernatant was used for superoxide dismutase (SOD) and catalase (CAT) enzyme activity assays. The extraction for ascorbate peroxidase activity (APX) was performed in the presence of 1 mM ascorbate (AsA). The absorbance of samples was detected by spectrophotometer (KONTRON, Milano, Italy). SOD (EC 1.15.1.1) activity was measured by determining the ability of the enzyme to inhibit the photochemical reduction of nitro blue tetrazolium (NBT) in the presence of riboflavin under light. One unit (U) means the amount of SOD activity causing 50% inhibition of NBT reduction under light. CAT activity (EC 1.11.1.6) was measured by detecting the consumption of H<sub>2</sub>O<sub>2</sub> at 240 nm for 3 min at 25 °C ( $\epsilon_{240} = 39.4 \text{ mM}^{-1} \text{ cm}^{-1}$ ). One unit of CAT activity means the amount of enzyme needed to decompose 1  $\mu\text{mol min}^{-1}$  H<sub>2</sub>O<sub>2</sub>. APX activity (EC 1.11.1.11) was calculated by measuring the decrease in AsA amount at 290 nm for 3 min at 25 °C ( $\epsilon_{290} = 2.8 \text{ mM}^{-1} \text{ cm}^{-1}$ ). One unit of APX activity means the amount of enzyme needed to oxidize 1  $\mu\text{mol min}^{-1}$  AsA [42]. All enzyme activities were expressed as U mg<sup>-1</sup> protein. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). Soluble protein concentration in samples was measured by the method of Bradford [43] using bovine serum albumin (BSA) as a standard.

#### 2.7. Measurements of Ascorbate (AsA) and Glutathione (GSH) Levels

Two hundred and fifty mg of leaves were homogenised with 1 mL of 5% (w/v) TCA. Samples were centrifuged at 12,000× g for 20 min at 4 °C. To assay total AsA, 10 mM dithiothreitol (DTT) was added to the mixture and the excess DTT was eliminated by using 0.5% (w/v) N-ethylmaleimide (NEM). AsA concentrations were measured in the mixture of 10% (w/v) TCA, 43% (w/v) H<sub>3</sub>PO<sub>4</sub>, 4% bipyridyl, 3% (w/v) FeCl<sub>3</sub> using spectrophotometer (KONTRON, Milano, Italy) at 525 nm [42].

GSH concentration was detected using an enzymatic assay containing 100 mM phosphate buffer (pH 7.5), 1 mM 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), 1 mM NADPH, 1 U of glutathione reductase

(baker's yeast, Sigma-Aldrich) and 20  $\mu\text{L}$  of the supernatant in 1 mL volume. GSH concentrations were measured spectrophotometrically at 412 nm [42]. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

### 2.8. Determination of Glutathione S-Transferase (GST) Activity

Two hundred and fifty mg of leaf tissues of soybean and common ragweed plants were homogenised in 1 mL of 100 mM phosphate buffer (pH 7.0) containing 1 mM phenylmethylsulfonyl fluoride (PMSF) and 1% (w:v) polyvinyl-polyrrolidone (PVPP) on ice, then the homogenate was centrifuged at  $12,000\times g$  for 20 min at 4 °C. The supernatant was used for the determination of GST (EC 2.5.1.18) activity by using 1-chloro-2,4-dinitrobenzene (CDNB) and reduced glutathione (GSH) as substrate [44]. GST activity was determined after the addition of CDNB to the mixture based on the increment at 340 nm for 3 min spectrophotometry (KONTRON, Milano, Italy). One unit of GST activity is the amount of the enzyme that produces 1  $\mu\text{mol}$  conjugated product in 1 min ( $\epsilon_{340} = 9.6 \text{ mM}^{-1} \text{ cm}^{-1}$ ). All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). Soluble protein concentration in samples was measured by the method of Bradford [43] using bovine serum albumin (BSA) as a standard.

### 2.9. Determination of Malondialdehyde (MDA) Content

One hundred mg of leaf tissues of soybean and common ragweed plants were homogenized with 1 mL of 0.1% TCA. Before the centrifugation ( $12,000\times g$ , 20 min, 4 °C) 0.1 mL of 4% butylated hydroxytoluene (BHT) was added to the samples; 0.5 mL of supernatant was added to 2 mL 0.5% thiobarbituric acid (TBA) dissolved in 20% TCA and incubated in boiling water for 30 min. The absorbance of the mixture was detected at 532 nm and the non-specific absorbance was measured at 600 nm using a spectrophotometer (KONTRON, Milano, Italy). Malondialdehyde (MDA) content was determined based on the extinction coefficient of  $155 \text{ mM}^{-1} \text{ cm}^{-1}$  [45]. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

### 2.10. Determination of Electrolyte Leakage (EL) and Leaf Fresh Mass (FM)

Firstly, leaf fresh mass per individual plant was determined using a laboratory scale (Mettler-Toledo; Greifensee, Switzerland).

For detection of electrolyte leakage (EL) from leaves, 50 mg of leaf tissues of soybean and common ragweed plants were incubated in 20 mL double-distilled water for 2 h at 25 °C in darkness. Then the conductivity of water was determined (C1) with a conductivity meter (HANNA Instruments, Woonsocket, RI, USA). To determine the total conductivity (C2), the samples were boiled for 40 min. EL was calculated based on the actual conductivity (C1) as a percentage of total conductivity (C2) ( $\text{EL} (\%) = (C_1/C_2) \times 100$ ) [46].

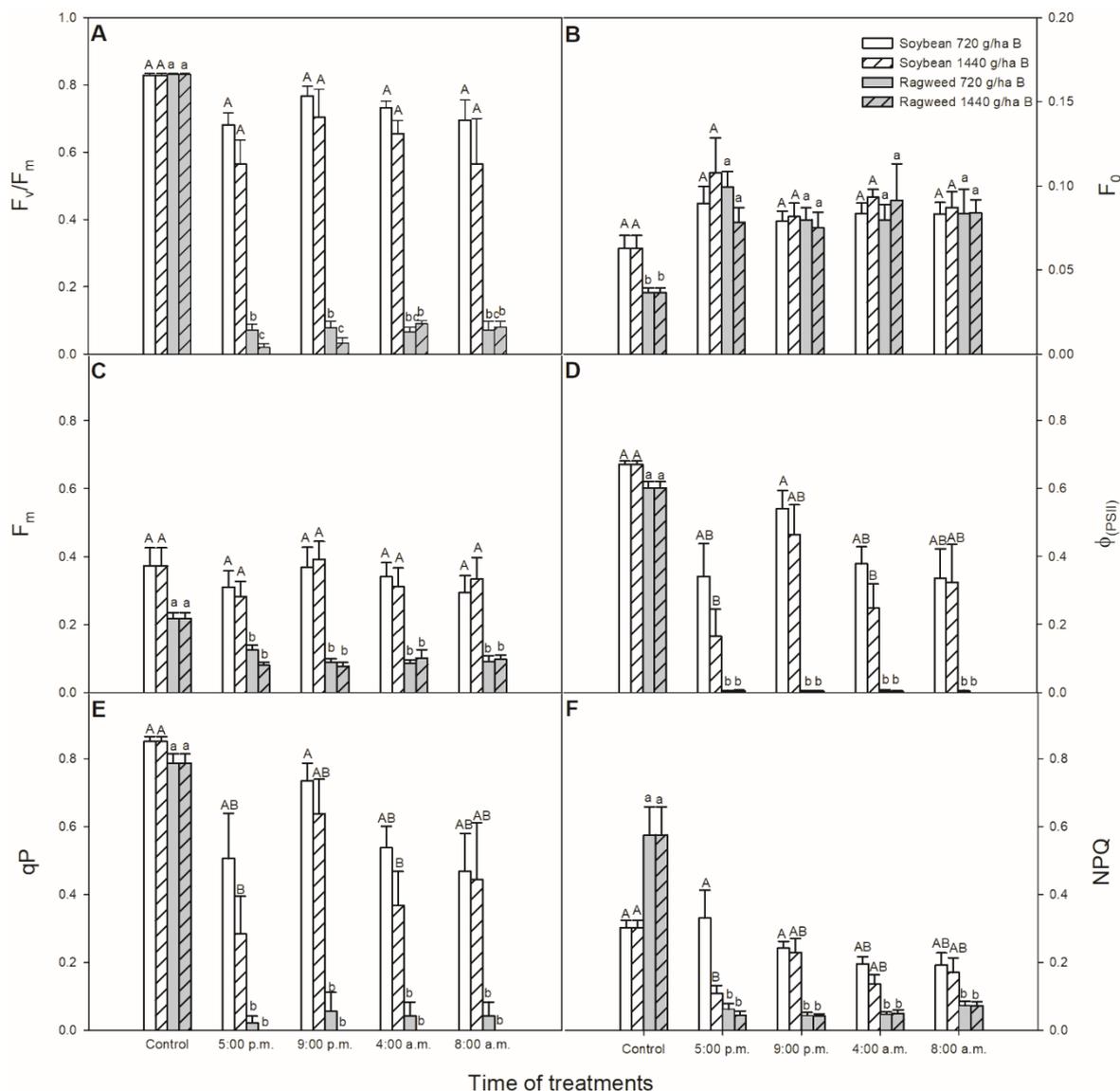
### 2.11. Statistical Analysis

Each experiment contains at least three biological replicates (at least three plants per treatment) and the entire experiment was conducted three times. Results are expressed as mean  $\pm$  SE calculated by Microsoft Excel 2016. Statistical analysis was accomplished using Sigma plot 11.0 software (SPSS Science Software, Erkrath, Germany). Differences between the treatments in the case of each plant species were statistically analyzed by one-way ANOVA using Tukey's test. The means of each treatment were significant if  $p \leq 0.05$ .

## 3. Results

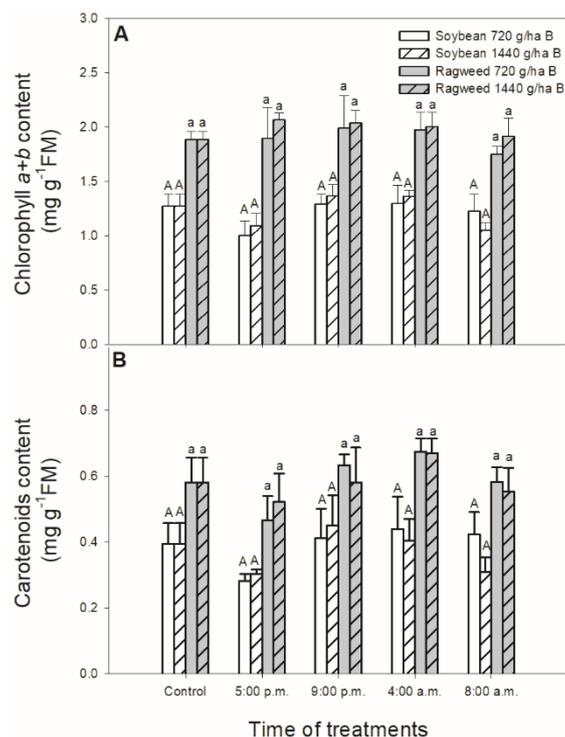
First of all, the daytime- and night-time-dependent effects of different bentazon concentrations were tested on the photosynthetic activity of both soybean and common ragweed plants based on the measurements of chlorophyll *a* fluorescence parameters. The maximal quantum yield of PSII

photochemistry ( $F_v/F_m$ ) did not change in soybean plants one light cycle later after bentazon treatments. In contrast to these results,  $F_v/F_m$  significantly decreased after bentazon concentrations were applied to both in common ragweed plants, independently of the time of the treatments (Figure 2A). Similar to changes in the  $F_v/F_m$  parameter, there were no significant changes in parameters of the minimal fluorescence yield of the dark-adapted state ( $F_0$ ; Figure 2B) and the maximal fluorescence in the dark-adapted state ( $F_m$ ; Figure 2C) after none of the bentazon treatments in leaves of soybean plants. In contrast to this observation,  $F_0$  significantly increased (Figure 2B) and  $F_m$  significantly decreased (Fig. 2C) in common ragweed plants after bentazon application at all times. In addition, the actual quantum yield of PSII electron transport in the light-adapted state ( $\Phi_{PSII}$ ) significantly declined in soybean plants after the application of  $1440 \text{ g ha}^{-1}$  bentazon at 5:00 p.m. and 4:00 a.m. Moreover, a significant decline in  $\Phi_{PSII}$  was measured in common ragweed plants exposed to both bentazon concentrations at every time point (Figure 2D). The photochemical quenching coefficient (qP) also decreased significantly in common ragweed plants after bentazon exposure in all cases. In soybean leaves, qP decreased significantly after the treatments with  $1440 \text{ g ha}^{-1}$  bentazon at 5:00 p.m., as well as at 4:00 a.m. (Figure 2E). The non-photochemical quenching (NPQ) parameter was basically higher in common ragweed leaves, which were reduced by the herbicide treatments in this plant species similarly in the case of  $\Phi_{PSII}$  and qP. However, bentazon did not influence significantly the NPQ in the concentration of  $720 \text{ g ha}^{-1}$  in soybean but it was decreased in the case of  $1440 \text{ g ha}^{-1}$  at 5:00 p.m. (Figure 2F).



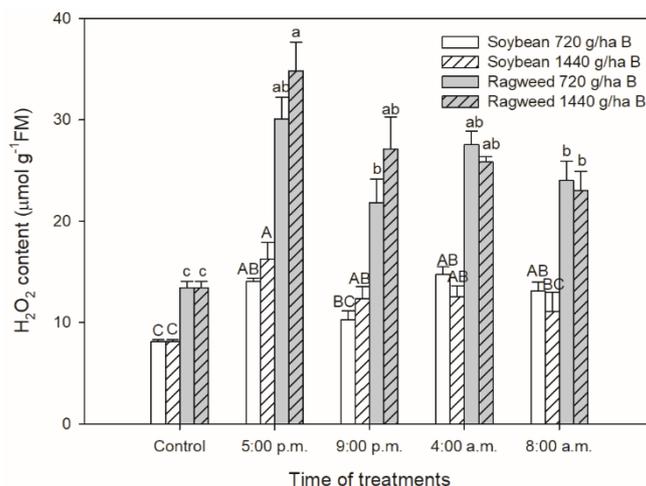
**Figure 2.** Changes in the chlorophyll *a* fluorescence parameters ( $F_v/F_m$  (A);  $F_0$  (B);  $F_m$  (C);  $\phi_{PSII}$  (D);  $qP$  (E); NPQ (F)) in leaves of soybean (white columns) and common ragweed plants (grey columns) treated foliar with 720 or 1440 g ha<sup>-1</sup> bentazon at different times (at the end of light cycle (5:00 p.m.), at night (9:00 p.m.), at dawn (4:00 a.m.) and in the morning (8:00 a.m.)). Measurements were disposed of in the morning at the second light phase of the day (9:00 a.m.) after the treatments. Means  $\pm$  SE,  $n = 3$ . Means marked with different letters are significantly different at  $p \leq 0.05$  as determined by Tukey's test (upper case letters show the different effects in soybean and lower case letters show the different effects in common ragweed plants).

In contrast to the significant changes in chlorophyll *a* fluorescence parameters of soybean and common ragweed plants, bentazon treatments did not change significantly either the content of chlorophyll *a + b* (Figure 3A) or the carotenoids content (Figure 3B) in soybean and common ragweed leaves during this time period.



**Figure 3.** Changes in the chlorophyll *a + b* (A) and carotenoids (B) content in leaves of soybean (white columns) and common ragweed plants (grey columns) treated foliar with 720 or 1440 g ha<sup>-1</sup> bentazon at different times (at the end of light cycle (5:00 p.m.), at night (9:00 p.m.), at dawn (4:00 a.m.) and in the morning (8:00 a.m.)). Measurements were disposed of in the morning at the second light phase of the day (9:00 a.m.) after the treatments. Means ± SE, n = 3. Means marked with different letters are significantly different at  $p \leq 0.05$  as determined by Tukey's test (upper case letters show the different effects in soybean and lower case letters show the different effects in common ragweed plants).

Effects of oxidative stress induced by bentazon treatments were detected by the measurements of H<sub>2</sub>O<sub>2</sub> content in leaves of soybean and common ragweed plants. H<sub>2</sub>O<sub>2</sub> levels were basically higher in common ragweed compared to soybean leaves but bentazon application caused much higher H<sub>2</sub>O<sub>2</sub> generation in common ragweed plants. Bentazon in 1440 g ha<sup>-1</sup> concentration induced significant H<sub>2</sub>O<sub>2</sub> production in soybean leaves treated at all times except at 8:00 a.m. In addition, the lower herbicide concentration did not elevate it significantly in the case of the treatment at night at 9:00 p.m. (Figure 4). The application of bentazon induced significant increase in H<sub>2</sub>O<sub>2</sub> levels in common ragweed leaves compared to the control, which was the highest in the late afternoon (5:00 p.m.)-treated leaves while H<sub>2</sub>O<sub>2</sub> content was the lowest in the case of 720 g ha<sup>-1</sup> bentazon application in the night (Figure 4).

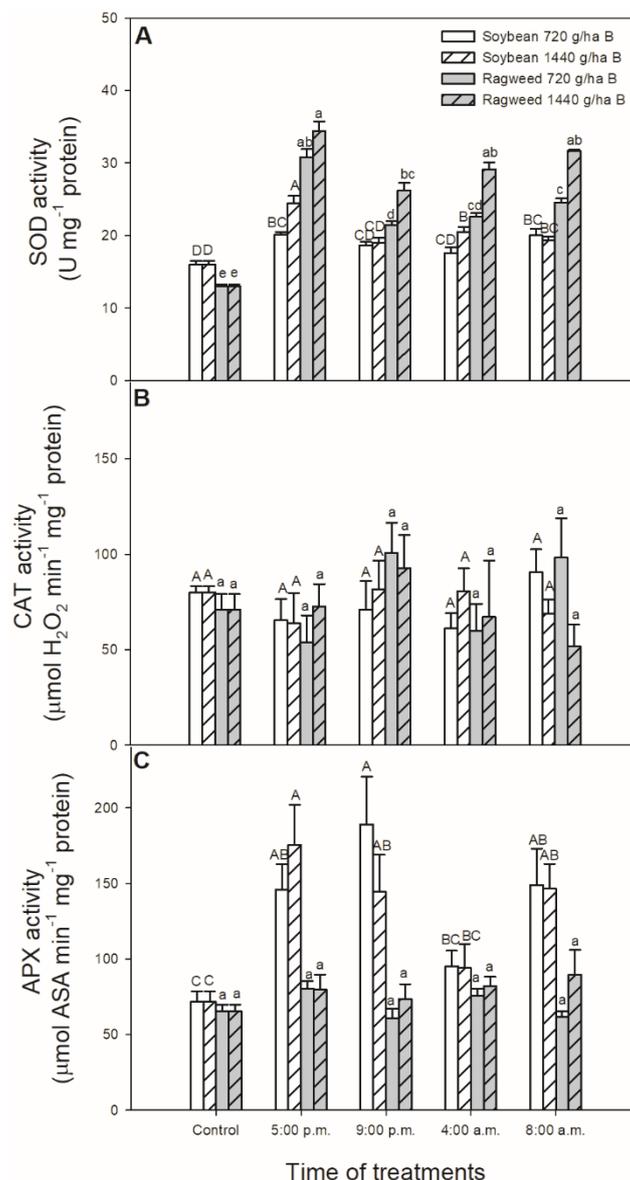


**Figure 4.** Changes in the H<sub>2</sub>O<sub>2</sub> content in leaves of soybean (white columns) and common ragweed plants (grey columns) treated foliar with 720 or 1440 g ha<sup>-1</sup> bentazon at different times (at the end of light cycle (5:00 p.m.), at night (9:00 p.m.), at dawn (4:00 a.m.) and in the morning (8:00 a.m.)). Measurements were disposed of in the morning at the second light phase of the day (9:00 a.m.) after the treatments. Means ± SE, n = 3. Means marked with different letters are significantly different at  $p \leq 0.05$  as determined by Tukey's test (upper case letters show the different effects in soybean and lower case letters show the different effects in common ragweed plants).

The main antioxidant enzymes playing a crucial role in H<sub>2</sub>O<sub>2</sub> metabolism were further analysed upon herbicide exposure. First of all, the activity of SOD, which catalyzes the dismutation of •O<sub>2</sub><sup>-</sup> into molecular oxygen and H<sub>2</sub>O<sub>2</sub>, was measured in the leaf tissues. Both bentazon treatments increased the SOD activity in soybean plants and the highest activity was measured in leaves treated at 5:00 p.m. with 1440 g ha<sup>-1</sup> bentazon (Figure 5A). In contrast to soybean plants, SOD activity was significantly higher for 1440 g ha<sup>-1</sup> compared to 720 g ha<sup>-1</sup> bentazon treatments in common ragweed. Application of bentazon in 1440 g ha<sup>-1</sup> in the late and early light phase of the day (at 5:00 p.m. and 8:00 a.m.) caused two maxima in SOD activities in common ragweed leaves (Figure 5A).

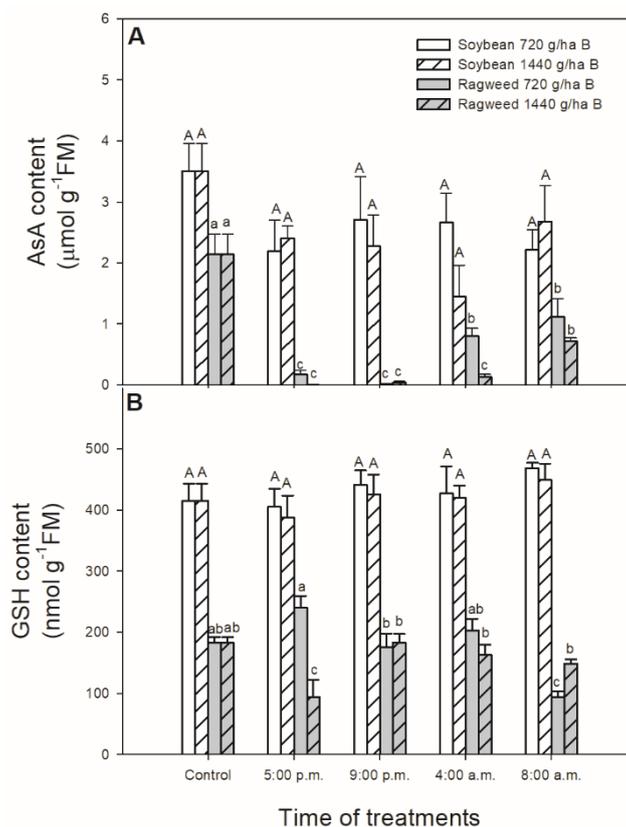
The activity of CAT enzyme catalyzing the decomposition of H<sub>2</sub>O<sub>2</sub> to H<sub>2</sub>O and O<sub>2</sub>, did not display any significant changes in either soybean or common ragweed leaves but it was elevated slightly in the night (at 9:00 p.m.)-treated common ragweed plants (Figure 5B).

In contrast to CAT, the activity of APX, the other H<sub>2</sub>O<sub>2</sub>-detoxifying enzyme using AsA as co-substrate, significantly increased in soybean leaves in case of treatments in all times except at dawn (Figure 5C). At the same time, significant concentration-dependent effects of bentazon were not detected on APX in this plant species. Surprisingly, bentazon did not cause any significant changes in APX activity in common ragweed leaves (Figure 5C).



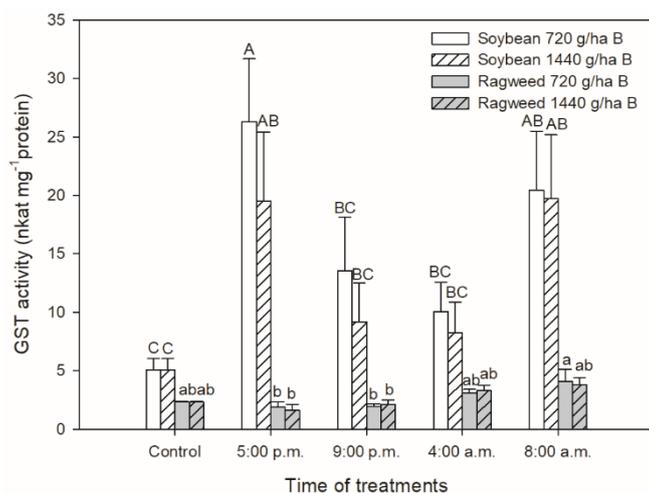
**Figure 5.** Changes in the activity of superoxide dismutase (SOD; **A**), catalase (CAT; **B**) and ascorbate peroxidase (APX; **C**) in leaves of soybean (white columns) and common ragweed plants (grey columns) treated foliar with 720 or 1440 g ha<sup>-1</sup> bentazon at different times (at the end of light cycle (5:00 p.m.), at night (9:00 p.m.), at dawn (4:00 a.m.) and in the morning (8:00 a.m.)). Measurements were disposed of in the morning at the second light phase of the day (9:00 a.m.) after the treatments. Means  $\pm$  SE, n = 3. Means marked with different letters are significantly different at  $p \leq 0.05$  as determined by Tukey's test (upper case letters show the different effects in soybean and lower case letters show the different effects in common ragweed plants).

As shown in Figure 6 neither 720 nor 1440 g ha<sup>-1</sup> bentazon treatment caused any significant changes in total AsA content in the leaves of soybean plants (Figure 6A). However, in common ragweed plants treated with any bentazon concentrations, AsA contents decreased significantly, especially in the case of the herbicide application at 5:00 p.m. and 9:00 p.m. (Figure 6A). Both AsA and GSH contents were lower in leaves of common ragweed compared to soybean plants (Figure 6). Levels of GSH also did not change in soybean leaves (Figure 6B). In contrast to this, GSH levels decreased with 1440 g ha<sup>-1</sup> bentazon treatment at 5:00 p.m. and with 720 g ha<sup>-1</sup> bentazon application at 8:00 a.m. in leaves of common ragweed plants (Figure 6B).



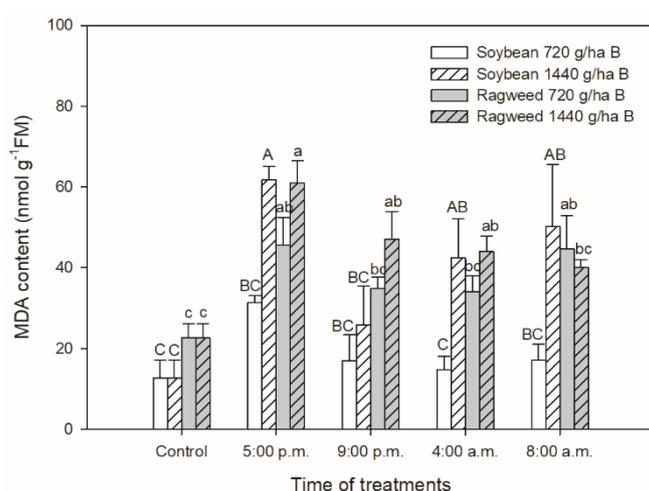
**Figure 6.** Changes in the ascorbate (AsA; **A**) and glutathione (GSH; **B**) content in leaves of soybean (white columns) and common ragweed plants (grey columns) treated foliar with 720 or 1440 g ha<sup>-1</sup> bentazon at different times [at the end of light cycle (5:00 p.m.), at night (9:00 p.m.), at dawn (4:00 a.m.) and in the morning (8:00 a.m.)]. Measurements were disposed of in the morning at the second light phase of the day (9:00 a.m.) after the treatments. Means  $\pm$  SE,  $n = 3$ . Means marked with different letters are significantly different at  $p \leq 0.05$  as determined by Tukey's test (upper case letters show the different effects in soybean and lower case letters show the different effects in common ragweed plants).

Not only the key antioxidant enzymes but also the activity of the key detoxifying enzyme, GST was determined in the leaves of these plant species. GST activity was basically higher in soybean compared to common ragweed leaves (Figure 7). Enzyme activity of GST was elevated by bentazon application in both concentrations and the significantly highest levels were measured in the soybean leaves treated in the light phase of the day (at 5:00 p.m. and 8:00 a.m.). GST activity also increased significantly in common ragweed leaves but only in the case of treatment at 8:00 a.m. for 720 g ha<sup>-1</sup> bentazon (Figure 7).



**Figure 7.** Changes in the glutathione S-transferase (GST) activity in leaves of soybean (white columns) and common ragweed plants (grey columns) treated foliar with 720 or 1440 g ha<sup>-1</sup> bentazon at different times (at the end of light cycle (5:00 p.m.), at night (9:00 p.m.), at dawn (4:00 a.m.) and in the morning (8:00 a.m.)). Measurements were disposed of in the morning at the second light phase of the day (9:00 a.m.) after the treatments. Means  $\pm$  SE, n = 3. Means marked with different letters are significantly different at  $p \leq 0.05$  as determined by Tukey's test (upper case letters show the different effects in soybean and lower case letters show the different effects in common ragweed plants).

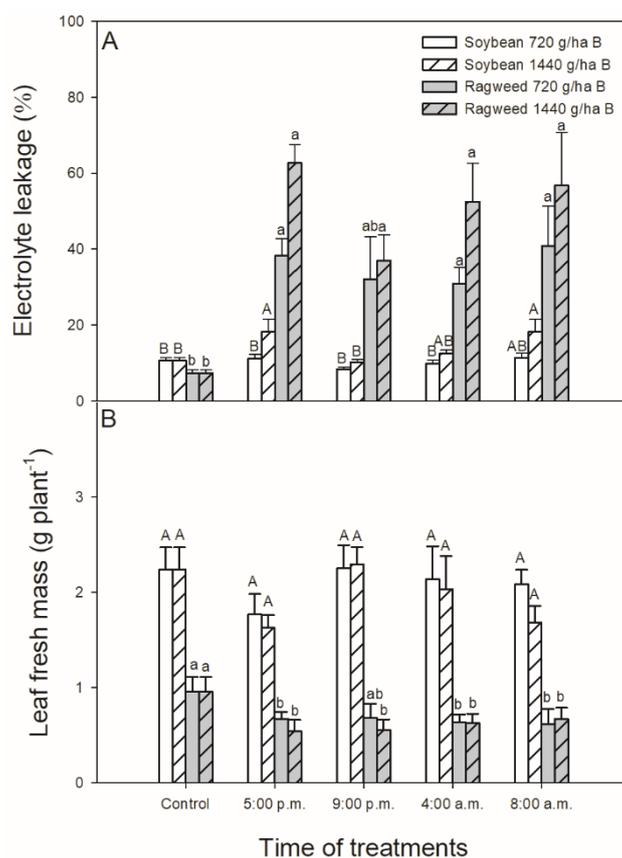
The effects of oxidative stress on lipids were determined based on the measurements of MDA content in the leaves. MDA content increased significantly after 1440 g ha<sup>-1</sup> bentazon treatment in soybean plants, especially in the case of treatments in the light phase (at 5:00 p.m. and 8:00 a.m.) (Figure 8). Similar tendencies were found in the case of common ragweed, but in the case of this plant species, the 720 g ha<sup>-1</sup> bentazon was able to increase the rate of the lipid peroxidation in the light phase too, based on the changes in MDA content (Figure 8). The lowest levels in MDA content were measured in case of bentazon treatments in the dark (at 9:00 p.m. and 4:00 a.m.).



**Figure 8.** Changes in the malondialdehyde (MDA) content in leaves of soybean (white columns) and common ragweed plants (grey columns) treated foliar with 720 or 1440 g ha<sup>-1</sup> bentazon at different times (at the end of light cycle (5:00 p.m.), at night (9:00 p.m.), at dawn (4:00 a.m.) and in the morning (8:00 a.m.)). Measurements were disposed of in the morning at the second light phase of the day (9:00 a.m.) after the treatments. Means  $\pm$  SE, n = 3. Means marked with different letters are significantly different at  $p \leq 0.05$  as determined by Tukey's test (upper case letters show the different effects in soybean and lower case letters show the different effects in common ragweed plants).

The cell viability based on the measurement of electrolyte leakage (EL) changed significantly upon 1440 g ha<sup>-1</sup> bentazon treatment in soybean plants treated in the light phase of the day (at 5:00 p.m. and 8:00 a.m.) (Figure 9A). In parallel, the same concentration of bentazon caused the highest EL from common ragweed leaves at the same day times but significant differences between the two applied herbicide concentrations were not detected (Figure 9A).

The leaf fresh mass per plant significantly decreased in common ragweed plants upon all bentazon treatments except at 9:00 p.m. with 720 g ha<sup>-1</sup> bentazon treatment but it did not change in soybean (Figure 9B).



**Figure 9.** Changes in the electrolyte leakage (EL; **A**) and leaf fresh mass (**B**) of soybean (white columns) and common ragweed plants (grey columns) treated foliar with 720 or 1440 g ha<sup>-1</sup> bentazon at different times (at the end of light cycle (5:00 p.m.), at night (9:00 p.m.), at dawn (4:00 a.m.) and in the morning (8:00 a.m.)) after one week. Measurements were disposed of in the morning at the second light phase of the day (9:00 a.m.) after the treatments. Means  $\pm$  SE, n = 3. Means marked with different letters are significantly different at  $p \leq 0.05$  as determined by Tukey's test (upper case letters show the different effects in soybean and lower case letters show the different effects in common ragweed plants).

#### 4. Discussion

Weed control costs billions of US dollars worldwide; thus, optimization of the herbicide application is one of the key questions in agriculture and other related industries [47]. Use of herbicides is essential for limiting and eliminating various weed species but at the same time it possesses many problems and risks; thus, their application must be minimized to account for the desired economic and environmental impacts [48]. However, there are strict regulations worldwide for herbicide usage [49]. In order to ensure sustainable and successful weed control and effective elimination of weeds, new and innovative strategies, technologies and methods must be developed. Results of basic and applied sciences can significantly serve these aims and provide information for a practical purpose.

Herbicides, which belong to photosynthesis inhibitors based on their mode of action (Groups 5–7), can provide the possibility to optimize their efficiency on plants by the selection of the daytime or night-time of their application. These herbicides, such as bentazon, inhibit the photosynthetic electron transport by binding to the  $Q_B$ -binding site of the D1 protein complex located in the chloroplast thylakoid membrane, which results in plants being unable to reoxidize  $Q_A$ , resulting in triplet–chlorophyll ( $^3\text{Chl}$ ) generation, which forms singlet oxygen ( $^1\text{O}_2$ ) upon reacting with molecular oxygen [13]. Therefore, high ROS production generated by inhibited electron transport in chloroplasts requires the presence of light. The herbicide treatment-caused ROS production and oxidative stress result in the damage of lipids, carbohydrates, proteins and nucleic acids inducing cell death in weeds [20,21]. The antioxidant system of plants plays a crucial role in the elimination and detoxification of high ROS levels protecting plants from lethal damage [50,51]. However, the activity of antioxidant enzymes and levels of non-enzymatic antioxidants are also highly dependent on light and dark conditions and on the circadian rhythm [18]. It is also known that phytotoxicity is less prevalent under low light conditions than under strong sunlight in the case of some herbicides [52,53]. Therefore, a well-chosen time of the herbicide application, especially at dawn, where the basic antioxidant enzyme activities are low, can be essential in optimizing the use and effect of herbicides on crops and weeds. In contrast, the herbicide application late in the light period, where the basic antioxidant enzyme activities are high, can delay the toxic effects of several herbicides on weeds.

Bentazon treatments generally decreased all of the chlorophyll *a* fluorescence parameters independently of the time of the application in common ragweed plants, confirming the selective photosynthesis inhibitory effects of this chemical on this weed species. Both the increase in  $F_0$  and the decrease in  $F_m$  contributed to the decrease in  $F_v/F_m$  in common ragweed plants. This significant decrease in  $F_m$ , as well as in  $F_v/F_m$  parameters indicates that PSII cooperativity is inhibited and a large proportion of the PSII reaction centre is damaged [54]. It can be concluded that multiple effects of bentazon treatments (decrease in  $F_v/F_m$  and  $\Phi_{\text{PSII}}$ , as well as the inhibition of the increase of  $F_0$  and NPQ) resulted in chronic photoinhibition in leaves of common ragweed plants [55]. Interestingly, others found that 50 nmol l<sup>-1</sup> bentazon reduced  $F_v/F_m$  and  $\Phi_{\text{PSII}}$  after 18 days but increased  $F_0$  only after 25 days in the non-target pea plants suggesting that this herbicide has a long and time-dependent effect on the photosynthetic activity [56]. It is well known that the uptake of bentazon through the cell membranes is a rapid process [57]; thus, it can act on photosynthesis despite the metabolization to less phytotoxic glucosyl conjugates [58]. In contrast to common ragweed,  $F_v/F_m$  did not change but  $\Phi_{\text{PSII}}$  decreased after bentazon treatments in the light phase of the day and at dawn in soybean, suggesting that bentazon reduced the photosynthetic activity in soybean but did not cause significant photoinhibition at this time point in soybean [59]. The effect of bentazon on the photosynthesis of soybean plants is dependent on the concentration of the treatments based on the changes in qP and NPQ, which decreased with the higher bentazon concentration. Surprisingly, the chlorophyll *a* fluorescence parameters did not change significantly after 720 g ha<sup>-1</sup> bentazon treatment at night (9:00 p.m.) in soybean leaves, suggesting a higher degree of basic defence processes in the early dark phase of the day in this plant species and underlining the significance of night-time in herbicide application to crops.

Excess energy in PSII under light condition can lead to the production of ROS, which can destroy the electron transport components and PSII structure, causing more ROS production and initiation of cell death [60,61]. In accordance, photoinhibition in common ragweed plants and the significant decrease in NPQ after all bentazon treatments cannot dissipate excessive pressure accumulated in PSII reaction centres and cannot protect PSII under light conditions, which could result in high ROS production in chloroplasts of herbicide-treated leaves [62]. Interestingly, the carotenoid content did not change significantly during this time period after herbicide application, the pigments of which can scavenge singlet oxygen under stress condition [63]. Earlier, it was found that bentazon treatments induced a rapid production of ROS within 24 hours in cyanobacterium (*Synechococcus elongatus* PCC7942) [14], peanut leaves (*Arachis hypogaea* L.) [16] and common sage (*Salvia officinalis*

L.) [15]. Based on our results, application of bentazon caused significantly higher  $H_2O_2$  generation, which was lower in case of  $720\text{ g L ha}^{-1}$  bentazon treatment at night in common ragweed similarly to in soybean plants. These results can confirm the significant role of the presence of light and the active photosynthesis in ROS production [64] at the time of the application of this herbicide. Moreover, these results confirm also the significance of basic activities of antioxidant enzymes depending on the circadian rhythm in the detoxification of ROS in this process [65].

Plants evolved antioxidant systems that keep the generation of ROS below a harmful level [50,51]. SOD means the first element of this system, which can dismutate  $\bullet O_2^-$  into  $O_2$  and  $H_2O_2$  [66]. Earlier it was found that SOD activity was the highest late in the light phase and decreased during the night in tomato (*Solanum lycopersicum* L.) leaves [27,31]. SOD activity was significantly induced by bentazon treatment in both plant species. It was the highest in leaves of soybean after the treatment at 5:00 p.m. with bentazon and in common ragweed plants after the treatment in the late and early light phase of the day (at 5:00 p.m. and 8:00 a.m.). These changes in SOD activity are in good accordance with  $H_2O_2$  results, especially in the case of  $720\text{ g ha}^{-1}$  bentazon treatment. It shows also strong correspondence with the results of Radwan et al. [16], who measured significant increase in SOD activity of peanut leaves after Basagran<sup>®</sup> treatments. These results also confirm that both oxidative stress and antioxidant defence responses are induced by bentazon in herbicide-tolerant plant species, such as soybean. Thus the effective defence of soybean, in parallel with other detoxification pathways, can depend on the antioxidant defence system, which is regulated by the circadian clock and the light or dark conditions [26], providing a practical way to maximize the herbicide application.  $H_2O_2$  can be mostly degraded by the two key antioxidant enzymes, CAT and APX. APX has a higher affinity to hydrogen peroxide and can detoxify it in low concentrations, whereas CAT shows a high reaction rate but a low affinity to hydrogen peroxide [18]. In addition, the regulation of these antioxidant enzymes also shows circadian and/or diurnal rhythm, as well as a light dependency [28,29]. Surprisingly, the activity of CAT did not change significantly upon bentazon treatments in either soybean or common ragweed leaves but it was slightly elevated in the night-treated common ragweed plants. Interestingly, CAT activity neither changed in *Synechococcus elongatus* [14] but it increased in peanut [16] and common sage plants [15]. In contrast to CAT, APX activity significantly rose in soybean leaves, except in dawn-treated plants, independently of the applied bentazon concentrations. APX activity also increased in peanut [16] and common sage plants [15]. Surprisingly, bentazon treatments did not change APX activity in common ragweed plants contributing to the high  $H_2O_2$  levels in these herbicide-treated weeds.

In parallel with antioxidant enzymes, AsA and GSH levels show also diurnal changes in plants [28,33]. Interestingly bentazon treatments did not cause a significant decrease in total AsA and GSH contents in leaves of soybean plants, which can effectively contribute to the detoxification of toxic ROS in this plant species [50], where these antioxidant contents were basically higher compared to common ragweed plants. In contrast to soybean, AsA content decreased after herbicide application, especially in the case of the treatments at 5:00 p.m. and at 9:00 p.m. in common ragweed leaves. GSH also decreased after  $1440\text{ g ha}^{-1}$  bentazon treatment at 5:00 p.m. in this plant species. These significant changes with bentazon can negatively influence the ROS-mediated toxic effects in tissues of common ragweed.

GST also plays a key role in plant detoxification system, which enzyme shows circadian- and light-dependent activity with a maximum late in the light and early dark period [34]. GST also contributes to the detoxification of various herbicides, depending on their chemical structure [67,68]. Earlier it was found that GST activity by time- and concentration-dependent manner increased in cyanobacterium (*Anabaena cylindrica*) after bentazon exposure [12]. Based on our investigations, the activity of GST was basically higher in soybean plants and it was significantly elevated by bentazon treatments, especially in the light phase-treated leaves (at 5:00 p.m. and 8:00 a.m.). Surprisingly, GST activity was increased only in 8:00 a.m.-treated common ragweed leaves. These results suggest that GST could not moderate cell death induction in common ragweed leaves.

Changes in MDA content show the herbicide-induced effects of oxidative stress on lipids. Bentazon induced high degree of lipid peroxidation in various crops and weeds [12,14–16]. MDA contents increased significantly after 1440 g ha<sup>-1</sup> bentazon treatments in the leaves of soybean, especially in case of the light phase treatments similarly to the common ragweed plants. The lowest levels in MDA content were measured after bentazon treatments in the dark. Based on our result, herbicide-induced lipid peroxidation is not only dependent on the herbicide's concentration rate and type, and plant species as earlier described [15,69–71], but also on the time of its application.

Oxidative damage of lipids can contribute to loss of cell membrane integrity and cell death in plants [20]. EL, which is a cell viability test based on the integrity of cell membranes, increased after bentazon treatments at high concentration in the light phase of the day (at 5:00 p.m. and 8:00 a.m.) in both investigated plant species but leaf fresh mass per plants decreased significantly only in common ragweed plants confirming the lethal effects of herbicide application on this species. In contrast to changes in EL in soybean leaves, this plant species outgrows the harmful effects of bentazon one week later (data not shown). Earlier same effects of bentazon on EL were detected in *Anabaena cylindrical* [12]. At the same time, lower concentration of bentazon exposure also significantly elevated EL in common ragweed plants and significant differences between the two applied herbicide concentrations were not detected, suggesting that bentazon in lower concentration at well-chosen times can effectively kill common ragweed plants.

## 5. Conclusions

This work provides firstly a comparative biochemical and physiological analysis and comprehensive study of photosynthetic activity, antioxidants and GST activity in soybean and common ragweed plants exposed to different concentrations of the herbicide bentazon. The daytime- or night-time-dependent effects of bentazon application on the photosynthetic activity and defence responses in the weed-crop model system were specially analysed. According to our results it can be concluded that changes in photosynthetic activity, as well as plant defence responses and/or cell death induced by bentazon are not only dependent on the applied herbicide concentration and plant species but also on the time of the application. Here we firstly demonstrated that treatments with bentazon in the early dark period of the day can delay the lethal effects of this herbicide based on the measurements in H<sub>2</sub>O<sub>2</sub> content, SOD activity, MDA content and EL. Our results indicate that bentazon in lower concentration is also effective for cell death induction in the late dark phase (at dawn) and early morning in common ragweed plants where the EL and MDA content significantly increased and leaf fresh mass per plants decreased, while defence responses of soybean plants were highly activated at this time point based on the changes in APX and GST activity; moreover, EL and leaf fresh mass per plant did not change. Furthermore, these results indicate that the antioxidant defence system contributes to cell survival by limiting the oxidative stress-triggering effects of bentazon besides other xenobiotic detoxification pathways in both sensitive and tolerant plant species; thus, these result can provide relevant information in the aspect of the time- and concentration-dependent practical application of photosynthesis inhibitor herbicides.

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