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Photosynthetic Efficiency and Antioxidative Response of Soybean Exposed to Selective Herbicides: A Field Study

Lidija Begović [1], Nikola Jurišić [1,2], Martina Šrajer Gajdošik [3], Alma Mikuška [1] and Selma Mlinarić [1,*]

[1] Department of Biology, Josip Juraj Strossmayer University of Osijek, Cara Hadrijana 8/A, 31000 Osijek, Croatia; lbegovic@biologija.unios.hr (L.B.); n.jurisic96@gmail.com (N.J.); amikuska@biologija.unios.hr (A.M.)
[2] Elementary School Matija Antun Reljković Cerna, Šetalište Dr. Franje Tuđmana 3, 32272 Cerna, Croatia
[3] Department of Chemistry, Josip Juraj Strossmayer University of Osijek, Cara Hadrijana 8/A, 31000 Osijek, Croatia; msgajdosik@kemija.unios.hr
* Correspondence: smlinaric@biologija.unios.hr; Tel.: +385-31-399-936

Abstract: Soybean (*Glycine max* (L.) Merr.) is one of the most economically important crops in the world due to its nutritional value. To optimize soybean growth and yield, a wide range of commercial herbicides intended for weed control in crops are used. Although the herbicides used are selective, they can still cause oxidative stress and disturb photosynthetic reactions in soybean crops. In this work, the influence of commercial selective herbicides for weed control on the photosynthetic efficiency and antioxidative response of two soybean cultivars was investigated. The parameters describing the photosynthetic performance of soybean were derived by measuring in vivo chlorophyll *a* fluorescence while the antioxidative response was evaluated by determining activities of the selected antioxidative enzymes and parameters of oxidative stress at different development stages. The results showed a different response of the two soybean cultivars to herbicide treatment. Both investigated soybean cultivars showed that herbicide treatment did not cause oxidative damage. However, they revealed different adaptation mechanisms of photosynthetic apparatus. A negative impact of herbicide application was observed mainly on the electron transport chain in both varieties.

Keywords: *Glycine max* (L.) Merr.; bentazone; OJIP transients; driving forces; oxidative stress

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1. Introduction

Soybean (*Glycine max* (L.) Merr.) is an annual herbaceous plant from the legume family (Fabaceae), originating in Asia. It is one of the most cultivated legumes in tropical, subtropical and temperate regions and, due to its nutritional value, it represents economically one of the most important crops worldwide [1,2]. Soybean is the largest source of plant protein and has a high oil content, and is therefore of great importance for human food and livestock feed production [1,3,4]. In addition to being a source of valuable nutrients, soybean is an important plant in crop rotation due to the nitrogen fixation which occurs through the symbiotic relationship with rhizobia bacteria, enriching the soil and reducing the need for fertilization [1,3].

Development of soybean can be divided into vegetative and reproductive (generative) stages [5,6]. The initial stage (VE) represents the emergence of the plant from the ground. When the first simple leaves develop, the plant is in the second vegetative stage; the cotyledon stage (VC); and, with the development of the first trifoliate leaves, the strain moves to the V1 stage, i.e., the stage of the first true leaves. Stages from V1 to V(n) refer to the number of trefoils that can be found on a soybean stalk. After the last vegetative stage, the soybean plant goes into reproductive stages starting with the beginning of flowering (R1) [5,7].

A significant problem when growing soybeans are weeds, grass and broad-leaf, competing for water, light, nutrients and space, thus increasing production costs, causing

difficulties in harvesting and reducing the quality of the product. Therefore, use of herbicide has become an everyday practice [8,9]. Herbicides can be specific in regard to plant species and act either by destroying parts of plants that they come into direct contact with or after their absorption through leaves or roots [10–12]. There are numerous mechanisms by which herbicides inhibit various plant processes. Electron transport inhibitors remove or deactivate one or more electron carriers preventing further photosynthesis, which results in the plant's death [13]. Furthermore, "uncoupler" herbicides enable the transport of protons through the thylakoid membrane and degrade the existing transmembrane pH gradient, which ultimately leads to reduced ATP generation, without inhibition of electron transport [4,11]. They can also oxidize the water oxidation system ("oxygen evolving complex"; OEC) of photosystem II, reduce the amount of plastoquinone, and lead to the creation of artificial cyclic transport of electrons around PSII, acting as redox transmitters [14,15]. In contrast to the "uncoupler" mechanism, energy transfer inhibitors act directly on phosphorylation. Another mechanism is represented by compounds that compete with electron acceptors for electrons and prevent the further transfer of electrons between photosystems [10,16]. Although herbicide application is an established and efficient approach for weed control in crop cultivation, herbicides can also have negative effects on the growing crops, including damage and delayed crop growth, and reduced yield [10,17].

Photosynthesis is a fundamental process in plants where solar radiation is absorbed and converted into chemical energy vital for sustaining plant growth and metabolism. However, during this process, a portion of the absorbed energy is re-emitted as fluorescence or released as heat [12]. The release of fluorescence occurs due to various processes, including the recombination of excited electron and hole pairs within the photosystem II (PSII) reaction center and the subsequent relaxation of the excited states of chlorophyll molecules. In cases where the electron transport within the photosynthetic system is partially or completely obstructed due to environmental or physiological stress factors, the emission of fluorescence becomes more pronounced. This increased fluorescence intensity serves as an indicator of reduced utilization of photosynthetically active radiation by plants [12,18,19]. The stress−induced alterations in the electron transport chain disrupt the balance between the rate of light absorption and the rate of energy utilization, leading to an accumulation of excitation energy that cannot be effectively processed through the photochemical reactions [12,18,20].

The fluorescence of chlorophyll *a* is very small (1−2% of the absorbed light), but the fluorescence spectrum is different from the absorbed light. Chlorophyll *a* absorbs light with wavelengths of 430−490 nm (blue spectrum) and 630−670 nm (red spectrum), while re-emitted light is always in the red spectrum [21]. Monitoring and analyzing the changes in chlorophyll *a* fluorescence, can provide insights into the functionality and efficiency of the photosynthetic apparatus; as well as the impact of stressors; such as drought, temperature extremes and nutrient deficiency signals emitted by plants [22]. Additionally, chlorophyll *a* fluorescence (ChlF) has proven valuable in assessing the impact of herbicides on photosynthetic processes [12,23].

Various biotic and abiotic stressors induce overproduction and accumulation of reactive oxygen species (ROS), causing oxidative stress in plants. The cellular antioxidant machinery of plants consists of both enzymatic and non-enzymatic antioxidants, which play a crucial role in regulating levels of reactive oxygen species (ROS) and upholding cellular homeostasis [24]. Investigation of the antioxidant response to herbicide-induced stress in both crops and weeds has been a recent focus [25–29]. Herbicide treatment triggers the production of antioxidant compounds in plants as a protective response to stress. Changes in enzymatic antioxidants have been observed in crops and weeds following exposure to herbicides, and the generation of reactive oxygen species (ROS) resulting in lipid peroxidation has been reported [25,26,30]. Lipid peroxidation is a biochemical phenomenon characterized by the attacking of oxidizing agents, such as free radicals, on lipids that contain carbon−carbon double bonds, particularly polyunsaturated fatty acids (PUFAs) [31]. This process leads to various detrimental effects, including diminished membrane fluidity,

reduced membrane potential, heightened ion permeability and the potential rupture of cells, resulting in the release of cellular contents [32].

Based on Croatian legislation, a combination of commercial herbicides Harmony SX and Laguna 75 WG is commonly used to reduce the broad-leaf weeds competing with soybean crops. They are usually applied from emergence up to the development of fourth leaves. Harmony SX is translocation and residual herbicide that remains in the soil in an active form even after application and its active substance is thifensulfuron−methyl, while Laguna 75 WG is a translocation and partially residual herbicide with 75% oxasulfuron as an active substance. Both act as inhibitors of amino acid biosynthesis in young meristem parts of the plant, thus stop cell division and growth of plants. Herbicide Basagran 480 is a selective contact herbicide which is applied between the V1 and V3 stages of soybean development. The active substance is bentazone, which embeds into the chlorophyll molecule and disrupts the photosynthesis process [10,33–35]. Despite the fact that selective herbicides are solely intended to suppress the growth of weeds, foliar application might induce oxidative stress and disrupt electron transport of photosynthetic reactions to non-targeted crops. Therefore, the aim of this work was to investigate whether commercial herbicides intended to control broad-leaved weeds in agricultural crops cause oxidative stress in two cultivars of soybean (*Glycine max* (L.) Merr.): Ika and Zora, and to determine how these herbicides affect the fluxes of energy through active reaction centers of photosystem II.

2. Materials and Methods

2.1. Plant Meterial

Two soybean cultivars (*Glycine max* (L.) Merr.), Zora and Ika, were grown and sampled in fields in Cerna, Croatia (45°18′52.05″ N, 18°70′84.27″ E and 45°18′17.25″ N, 18°72′07.53″ E). The plants were treated with herbicides against weeds as standard, and a part of the plants was left untreated and served as a control. Three measurements and samplings were carried out from May to July, until the soybean was treated with selective herbicides used for weed control. Ten days after herbicide treatments, in the stages of cotyledons (VC), leaf development (V3) and the beginning of flowering (R1), photosynthetic performance measurement and biochemical analyses were undertaken (Figure 1). The developmental stages were determined according to Fehr and Caviness [5]. Both cultivars were measured and sampled on the same day and under the same conditions.

2.2. Herbicide Treatments

The soybeans were treated three times with three types of herbicides: Laguna 75 WG, Harmony SX and Basagran 480 according to the manufacturer's instructions. The first treatment took place at the cotyledon stage (VC) with a mixture of herbicides Laguna 75 WG and Harmony SX in a split application where the exact amount of herbicide is distributed and applied two or more times at certain time intervals. The second treatment took place at the leaf development stage (V3) also in a split application of the herbicides Laguna 75 WG and Harmony SX. The third herbicide application took place at the beginning of flowering stage (R1) and the herbicide Basagran 480 was added to the mixture of Laguna 75 WG and Harmony SX.

2.3. Biochemical Analyses

Soybean leaves were collected, stored in cool boxes and transported to the laboratory. Randomly selected soybean leaves from five plants of each cultivar and treatment were collected to prepare a composite sample, and five replicates made for each analysis. Plant tissue was ground into fine powder by using liquid nitrogen. Ground leaf tissue was used for determination of catalase (CAT, EC 1.11.1.6) and ascorbate peroxidase (APX, EC 1.11.1.11) activity as well as for the determination of lipid peroxidation level (LP), concentration of H_2O_2 and photosynthetic pigments.

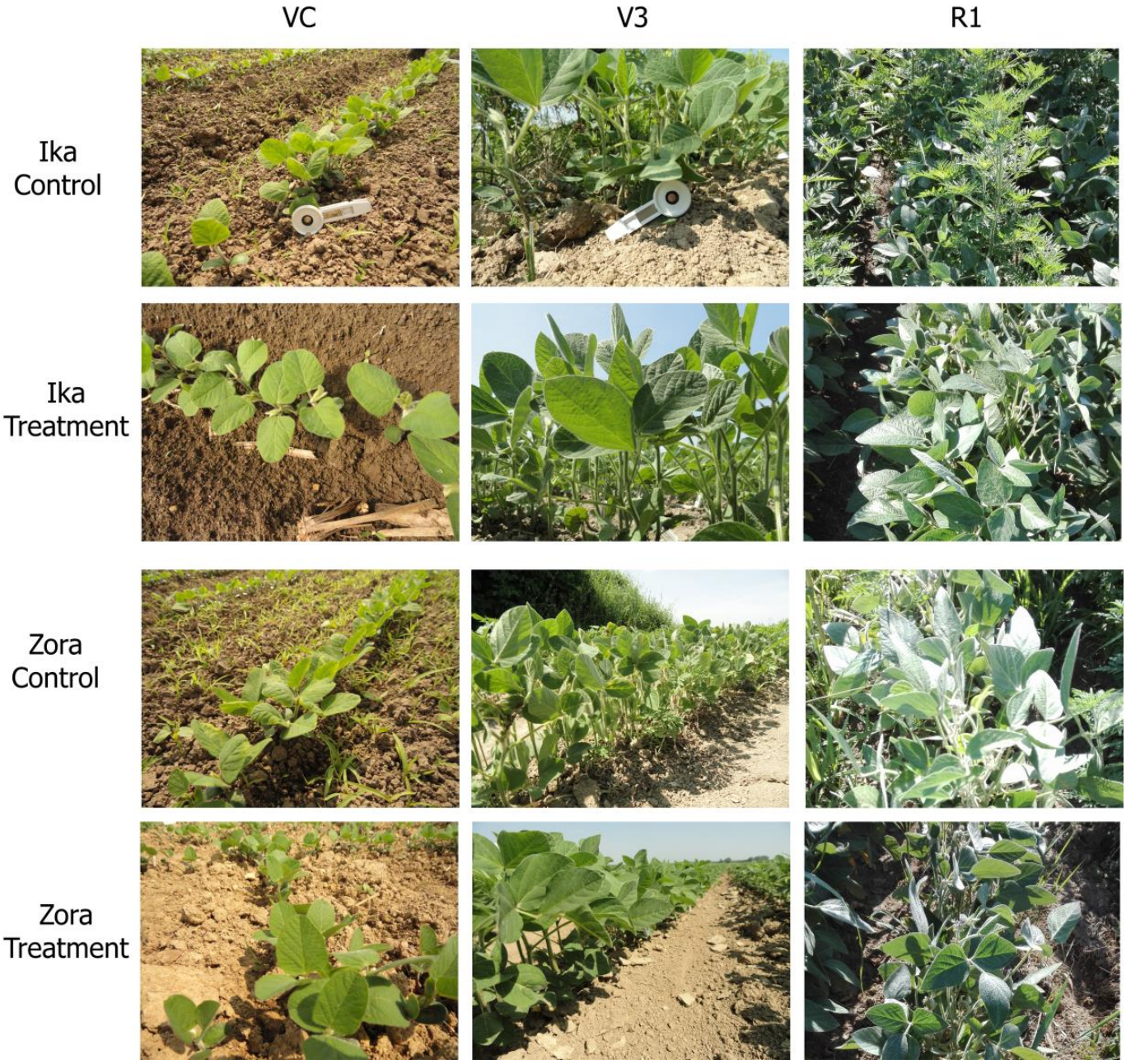

Figure 1. Soybean cultivars (*Glycine max* (L.) Merr.), Zora and Ika; grown in fields in Cerna; at the developmental stages of cotyledons (VC), leaf development (V3) and the beginning of flowering (R1) in control (untreated), and after treatment with selective herbicides.

Catalase enzyme activity was determined by monitoring the decrease in absorbance due to the decomposition of H_2O_2 at 240 nm spectrophotometrically (Specord 40, Analytik Jena, Jena, Germany). For the extraction, ice cold 100 mM potassium phosphate (K−P) buffer (pH = 7.5) was used and polyvinylpyrrolidone (PVP) was added. Reaction mixture contained 100 mM K−P buffer (pH = 7.0), 30% H_2O_2 and enzyme extract. The change in absorbance per minute per gram of fresh tissue weight (FW) was used to calculate total catalase enzyme activity [36].

Ascorbate peroxidase (APX) enzyme activity is based on monitoring the decrease in absorbance due to the decomposition of H_2O_2 at 290 nm spectrophotometrically [37]. Ice cold 100 mM K−P extraction buffer (pH = 7.0) with the addition of 5 mM Na−ascorbate was used with the addition of PVP. Reaction mixture (pH = 7.0) contained 50 mM K−P

buffer (pH 7.0), 0.1 mM EDTA, 50 mM ascorbic acid, 12 mM H_2O_2 and enzyme extract. The change in absorbance per minute per gram of FW was a measure of the activity of the APX enzyme.

The level of lipid peroxidation was determined as the amount of substances that react with thiobarbituric acid (TBA) and form colored thiobarbituric acid reactive substances (TBARS) [38]. The absorbance was measured spectrophotometrically at 532 while the value for non-specific absorption was measured at 600 nm. The concentration of TBARS products was calculated using the molar extinction factor of 155 $mM^{-1}cm^{-1}$.

The concentration of hydrogen peroxide (H_2O_2) is based on the formation of a complex between H_2O_2 and titanium (IV) ions that precipitates and dissolves in H_2SO_4 [39]. After the precipitate was completely dissolved, the absorbance was measured at 415 nm spectrophotometrically and the concentration of H_2O_2 was calculated by using the molar extinction factor of 1.878 $mM^{-1}cm^{-1}$.

2.4. Photosynthetic Pigments Determination

Powdered soybean tissue was extracted with 1 mL of 100% acetone for 24 h at −20 °C. The samples were diluted after the extraction, and the absorbance was measured spectrophotometrically at 470 nm, 645 nm and 662 nm, using pure acetone as a blank. Total chlorophylls (Chla *a*+*b*) and carotenoid (Car) content was calculated using the coefficients according to Lichtenthaler [40].

2.5. Chlorophyll a Fluorescence Measurements

The measurement of chlorophyll *a* fluorescence was conducted in vivo in the field during the early morning (between 7 and 9 a.m.) to avoid possible photoinhibition. Chl measurements were performed by using Plant Efficiency Analyzer (PEA, Hansatech, Norfolk, UK) on 15 (n = 15) dark-adapted plants (30 min) per group of each cultivar and at each developmental stage (10 days after the herbicide treatments). Induction of Chl transients (OJIP) were made by applying a pulse of saturating red light (maximum intensity at 650 nm, photon flux of 3200 µmol m^{-2} s^{-1}). One-second measurements induced changes in fluorescence and the measured data were used to calculate JIP-test parameters (Table 1) [41].

Table 1. Definitions of selected JIP-test parameter [18,42–44].

Quantum Efficiencies and Flux Ratios	
ABS/RC	Effective antenna size of an active reaction center (RC). Expresses the total number of photons absorbed by Chl molecules of all RC divided by the total number of active RCs
ET_0/RC	Electron transport per active RC
TR_0/RC	Maximal trapping rate of PSII. Describes the maximal rate by which an excitation is trapped by the RC
DI_0/RC	Effective dissipation per active RC
RE_0/RC	Electron flux reducing end electron acceptors at the PSI acceptor side per RC
TR_0/DI_0	Flux ration trapping per dissipation
$\varphi_{P0} = TR_0/ABS$	Maximum quantum yield of primary photochemistry, the probability that an absorbed photon will be trapped by the PSII RC and will reduce one Q_A
$\psi_{E0} = ET_0/TR_0$	Probability that an absorbed photon will enter the electron transport chain, electron transport efficiency
$\varphi_{E0} = ET_0/ABS$	Quantum yield for electron transport
$\delta_{R0} = RE_0 - ET_0$	Probability that an electron is transported from reduced PQ to the electron acceptor side of PSI
$\varphi_{R0} = RE_0/ABS$	Quantum yield of electron transport from Q_A^- to the PSI end electron acceptors
Performance Index and Driving Forces	
SFI	Structure–function index on absorption basis; $(RC/ABS) \times \varphi_{P0} \times \psi_{E0}$
PI_{ABS}	Performance index (potential) for energy conservation from photons absorbed by PSII to the reduction in intersystem electron acceptors; $[\gamma_{RC}/(1-\gamma_{RC})][\varphi_{P0}/(1-\varphi_{P0})][\psi_{E0}/(1-\psi_{E0})]$
$DF_{ABS} = \log PI_{ABS}$	Total driving forces for photosynthesis of the observed system, created by summing up the partial driving forces for each of the several bifurcations

The mean values of 15 measurements were used to draw OJIP transient treated and untreated plants of each cultivar at three developmental stages after herbicide treatment. Every treatment was normalized to its corresponding control at the specific developmental stage. Specific events of OJIP transient in the OK, OJ, JI and PI phases were presented as differences in the variable fluorescence shown as ΔV_{OP}, ΔV_{OK}, ΔV_{OJ}, ΔV_{JI} and ΔV_{IP} normalized to corresponding controls [44]. Driving forces (DF_{ABS}) for photosynthesis of the observed system electron transport were shown as log PI_{ABS}. Corresponding partial driving forces: $\log \gamma_{RC}/(1-\gamma_{RC})$, $\log \varphi_{P0}/(1-\varphi_{P0})$, and $\log \psi_{E0}/(1-\psi_{E0})$ were summed and the difference for the control and treated plants, respectively, ΔDF_{ABS} was calculated as $\Delta DF = DF_{treatment} - DF_{control}$ [43].

2.6. Statistical Analysis

Statistical analysis of two treated (T) and untreated (C) soybean cultivars (Ika and Zora) at three developmental stages (VC, V3 and R1) was performed by using Statistica software (ver. 14, TIBCO Software Inc., Palo Alto, CA, USA). Differences between all measured groups were compared by analysis of variance (ANOVA), after which the Tukey HSD *post hoc* test was performed at a significance level of $p < 0.05$. The results are presented as mean ± standard deviation (SD) of 15 replicates ($n = 15$) for the ChlF measurements, and 5 replicates ($n = 5$) for biochemical analyses and pigment determination normalized to their corresponding controls (Control = 1). The raw data are shown in the supplementary tables.

3. Results

3.1. Antioxidative Response after Herbicide Treatments

The relative level of lipid peroxidation, expressed as TBARS content (Figure 2a), was shown to be significantly lower after herbicide treatment at V3 and R1 stages in Ika cultivar and VC and R1 stage in Zora cultivar compared to corresponding untreated (control) plants in the same stage. The level of produced H_2O_2 (Figure 2b) was shown to be unchanged after the herbicide treatment in both cultivars and all developmental stages. Relative catalase (CAT) activity (Figure 2c) measured in cultivar Ika showed significantly higher activities at VC and V3 stages, while cultivar Zora showed higher activities at V3 and R1 stages compared to their controls. Both cultivars revealed the highest activities at V3 stage. Relative ascorbate peroxidase The Chl *a+b* (Figure 2e) and Car (Figure 2f) significantly increased after herbicide treatment at V3 stage in cultivar Ika, while in cultivar Zora, there was a significant increase in Chl *a+b* in V3 and R1 stages, and in Car in V3 stage compared to control.

3.2. Effect of Herbicide Treatment on PSII Functioning

The OP normalized prompt ChlF transients (Figure 3a,f) revealed obvious differences in the shapes of the OJIP curves. Early stages of development; VC and V3, in both cultivars, regardless of treatment; showed somewhat distorted shapes. After a fast increase in ChlF intensity at J step, an apparent decline of I step occurred. However, the later stage, R1, revealed characteristic shapes of transient in both cultivars and treatments.

Herbicide treatment in Ika cultivar induced positive ΔV_{OK} (L band, Figure 3b) amplitude at V3 stage, and at VC and R1 the amplitudes are negative. However; in cultivar Zora (Figure 3g), at stage VC, herbicide treatment induced a positive amplitude; but at later stages, there were no major changes compared to their corresponding controls. While in Ika cultivar, herbicide treatment induced positive amplitudes of ΔV_{OJ} (K band, Figure 3c) only at V3 and R1 stages, in cultivar Zora (Figure 3h), positive K band amplitudes were observed in all three stages compared to control.

Similar responses in both cultivars were observed for ΔV_{IJ} (H band, Figure 3d,i) and ΔV_{IP} (G band, Figure 3e,j) after herbicide treatment. There were positive amplitudes at V3 stage, and negative ones at VC and R1 stages compared to their controls.

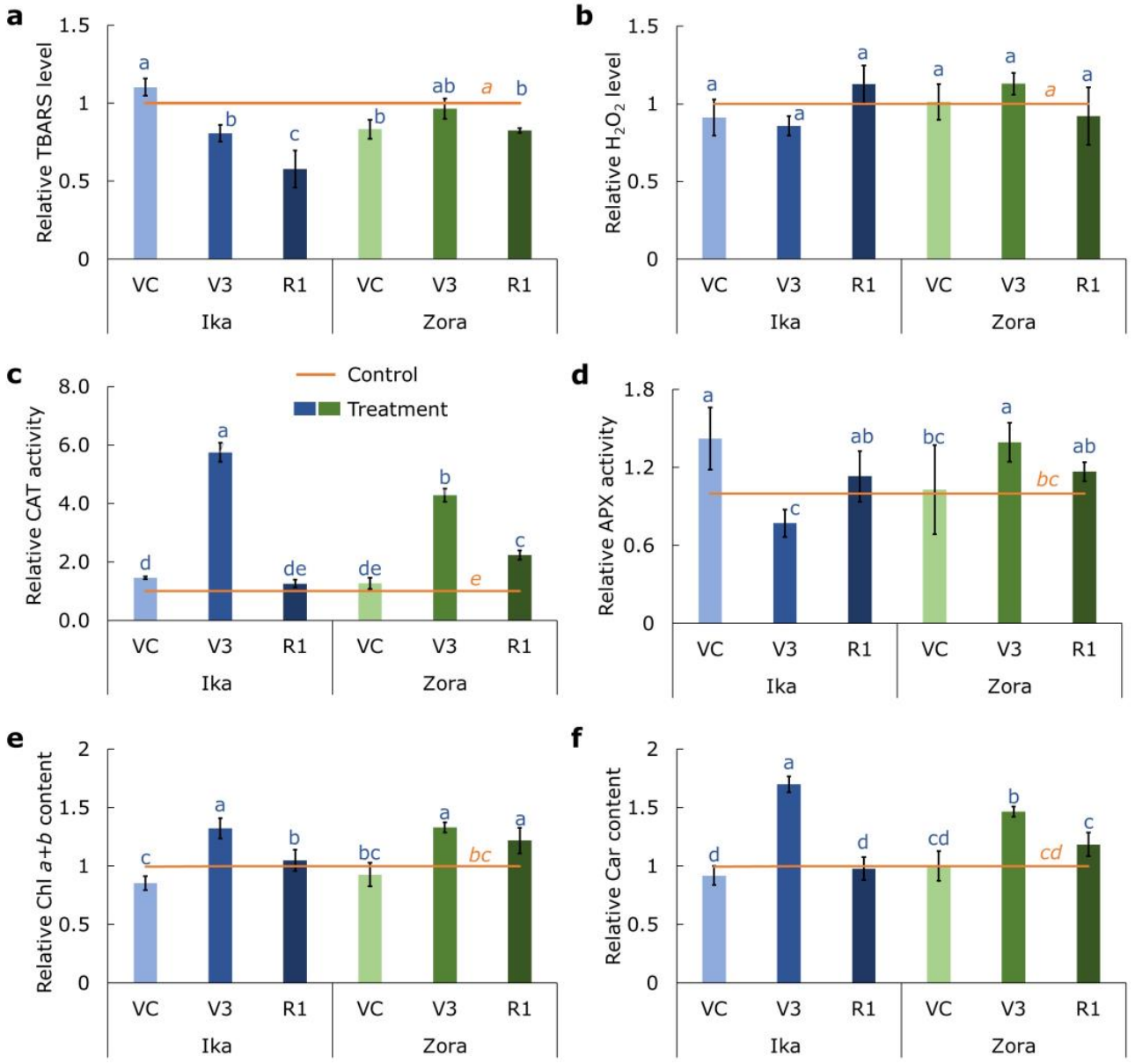

Figure 2. Changes in relative level of thiobarbituric acid reactive substances (TBARS, nmol g^{-1} FW; (**a**)) and hydrogen peroxide (H_2O_2, nmol g^{-1} FW; (**b**)), relative catalase activity (CAT, ΔA min^{-1} mg^{-1} FW; (**c**)), relative peroxidase activity (APX, ΔA min^{-1} mg^{-1} FW; (**d**)), relative content of total chlorophylls (Chl *a*+*b*, mg g^{-1} FW; (**e**)) and carotenoids (Car, mg g^{-1} FW; (**f**)) in untreated (control) and herbicide treated soybean leaves at three developmental stages. Bars represent means ± SD (n = 5) of treated plants relative to control (orange line = 1). Different letters determine significant difference at $p < 0.05$ (ANOVA, Tukey HSD). Original data of biochemical parameters are shown in Supplementary Table S1.

Spider plots (Figure 4) show normalised values of calculated biophysical parameters after herbicide treatments of two soybean cultivars; specific energy fluxes per Q_A^- reducing PSII RC (ABS/RC, DI_0/RC, TR_0/RC, ET_0/RC, and RE_0/RC), quantum yields (φ_{P0}, ψ_{E0}, φ_{E0}, δ_{R0} and φ_{R0}), flux ratio of trapping and dissipation (TR_0/DI_0), performance index on absorption basis (PI_{ABS}) and structure−function index (SFI_{ABS}). Values are normalised to their respective controls. Radar plots revealed a differential response of two soybean cultivars after herbicide treatments. Significant changes in almost all parameters were observed at V3 and R1 stages after herbicide treatments in cultivar Ika, while, in cultivar Zora, such changes were observed at VC stage compared to their controls.

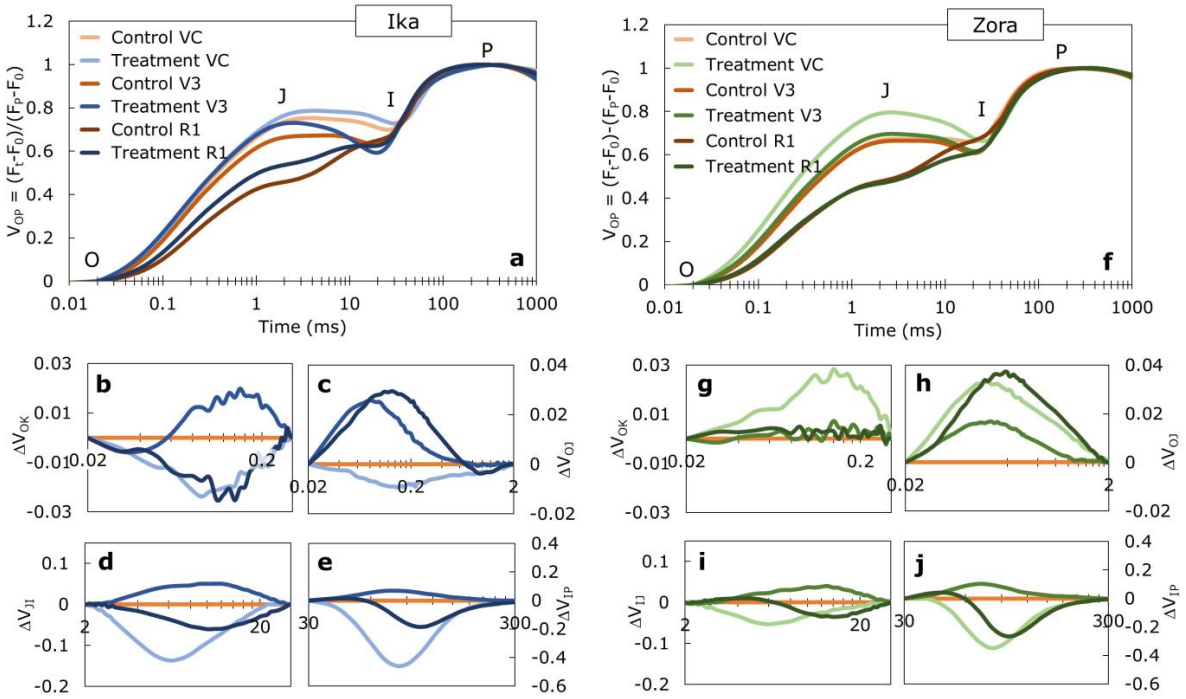

Figure 3. Changes in the shapes of ChlF transients measured in untreated and after herbicide-treated soybean leaves at three developmental stages (**a**,**f**). Curves represent average kinetics of 15 measurements (n = 15) per variety, treatment and developmental stage. Averaged ChlF data are normalized between OP (**a**,**f**), OK (**b**,**g**), OJ (**c**,**h**), JI (**d**,**i**) and IP (**e**,**j**) steps of OJIP transient. Difference kinetics ΔV_t are plotted at different time ranges to reveal L (ΔV_{OK}), K (ΔV_{OJ}), H (ΔV_{IJ}) and G (ΔV_{IP}) bands. Average values of corresponding controls in each developmental stage after herbicide treatment are used as referent values.

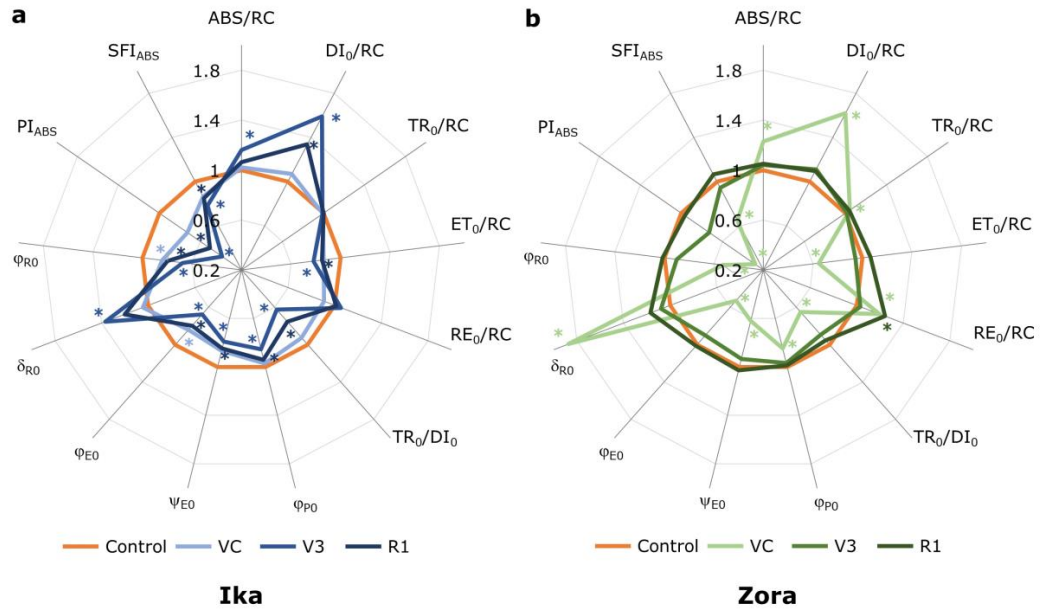

Figure 4. Normalized values relative to corresponding control of selected ChlF parameters characterizing the functioning of PSII are measured in untreated and herbicide-treated soybean leaves at three developmental stages in two cultivars: Ika (**a**) and Zora (**b**). For description of parameters, see Table 1. Values of each cultivar are shown relative to corresponding control (Control = 1). Each curve represents average kinetics of 15 replicates (n = 15) per cultivar, treatment and developmental stage. Asterisks (*) represent a significant difference compared to corresponding control at $p < 0.05$ (ANOVA, Tukey HSD). Original data of spider plots are shown in Supplementary Table S2.

Driving forces in PSII with respect to absorption (Figure 5) are summed up by the individual partial driving forces log $\gamma_{RC}/(1-\gamma_{RC})$, log $\varphi_{P0}/(1-\varphi_{P0})$, and log $\psi_{E0}/(1-\psi_{E0})$. The difference in DF_{ABS} is calculated by subtracting the value of individual control DF from the treated ones. PI_{ABS} significantly decreased at V3 and R1 stages after herbicide treatment in Ika cultivar. Such a decrease was achieved due to more negative values of all three partial DFs; however, the decrease in log $\varphi_{P0}/(1-\varphi_{P0})$ contributed the most. In cultivar Zora, PI_{ABS} was shown to be significantly lower at VC stage compared to control. A significant ΔDF_{ABS} decrease was also the result of more negative values of all three partial DFs, but log $\psi_{E0}/(1-\psi_{E0})$ contributed the most.

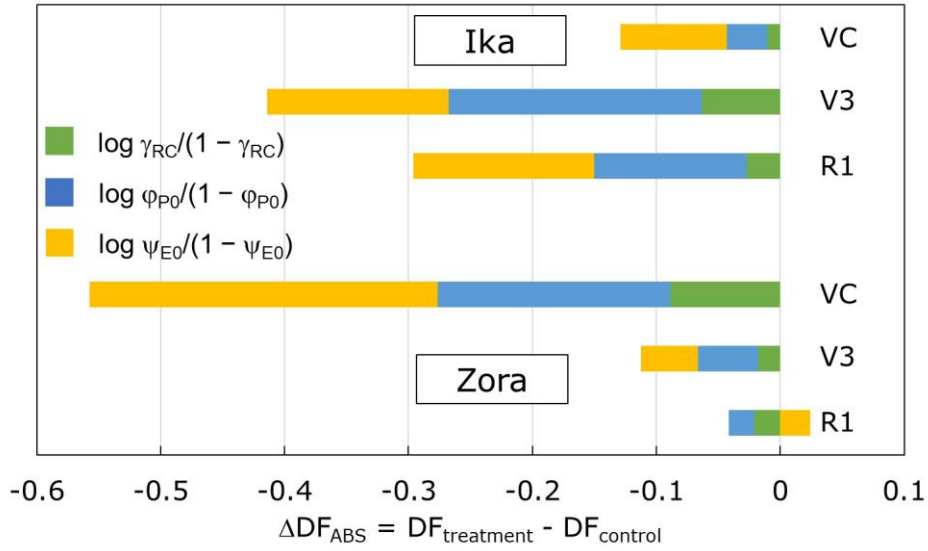

Figure 5. Changes in difference of driving forces (ΔDF_{ABS}) in untreated and herbicide-treated soybean leaves at three developmental stages are calculated as the difference between herbicide-treated and untreated samples. Each DF_{ABS} is calculated by adding up their partial driving forces: log $\gamma_{RC}/(1-\gamma_{RC})$, log $\varphi_{P0}/(1-\varphi_{P0})$, and log $\psi_{E0}/(1-\psi_{E0})$. Each parameter presents average of 15 replicates (n = 15) per cultivar, treatment and developmental stage. Original data of DFs are shown in Supplementary Table S3.

4. Discussion

Commercial post-emergence herbicides for the protection of agricultural crops from weeds are nowadays an essential way to ensure optimal growth and yield of soybean crops [28]. Although weed control management uses methods and active substances that usually do not influence the growth of non-target species, herbicide application is reported to have an influence on agricultural crops [28,45–47]. Based on Croatian legislation, the last treatment with the recommended combination of herbicides should be by the end of the emergence of the third trefoils (V3) and before the emergence of the flowers (R1). The rate of herbicide action on targeted, as well as non-targeted species, could vary from a very slow up to a very fast response after the application [48]. Non-targeted species usually have the ability to quickly metabolize the herbicide. Thus, the herbicides have a rather short half-life of only 4–6 hours compared to slower degradation in targeted weeds [49]. However, herbicide residuals usually remain in the environment for longer periods of time, causing harmful effects on humans, animals and plants. Despite that, non-targeted species usually have tolerance to specific herbicides due to their fast-metabolic degradation [50–52]. Moreover, several recent investigations confirmed that herbicide treatments have little or no effect on final yield production of soybeans [47,53–56].

However, this work was intended to detect the effect of foliar application of selective herbicides on photosynthetic and antioxidative response of two soybean cultivars. Results revealed opposite responses of two selected soybean cultivars. Our results suggested that neither cultivar was under oxidative stress after herbicide application. Namely, an

accumulation of TBARS and increased levels of H_2O_2 are recognized as good indicators of oxidative stress [24,57,58]. However, herbicide application induced a differential photosynthetic response. While the Ika cultivar showed higher susceptibility in the stages of emergence of leaves (V3) and at the beginning of flowering (R1), cultivar Zora was shown to be more sensitive at the stage of cotyledon emergence (VC).

Prompt ChlF transients (Figure 3) are also good indicators of various environmental influences on the functioning of photosynthetic apparatus. Normalizations and differences between specific points in the OJIP curve reveal specific inflections that correspond to L, K, H and G bands used to describe specific events of primary photochemistry. Differential shapes of specific bands could be used to describe sensitive or resistant genotypes/cultivars to specific type of stress [41,59]. The L and K bands were shown recently to be reliable stress indicators [60–64]. The values of L and K bands (Figure 3b,g and Figure 3c,h) are derived from the OJIP curve to describe more closely the influence of herbicides on the energy connection of the PSII reaction centers and their antennae, and the state of the oxygen-evolving complex (OEC) [44]. Therefore, a positive L band indicates a low energetic connectivity between PSII units that is usually connected with a lower ability to utilize absorbed light energy efficiently for photosynthetic processes. A positive K band suggests inactivation of the OEC and/or increased size of functional antennae [44,65] as a result of increased susceptibility to specific stressful conditions of the investigated cultivar. Negative values of L and K bands, however, are indicators that parts of the PSII have a high connectivity and increased stability of the system [60,64,66]. Therefore, both positive L and K bands in Zora cultivar at VC stage suggested increased susceptibility of this cultivar at early stages. On the other side, positive L and K bands at V3 stages in Ika cultivar indicated a higher susceptibility in later developmental stages. However, both cultivars revealed positive H and G bands at V3 stage (Figure 3d,i and Figure 3e,j). The H band describes the reduction and oxidation rate of plastoquinone (PQ) pool, while the G band explains the reduction rate of the PSI acceptor side from PQ pool which depends on the availability of $NADP^+$ molecules. Both bands are considered as a thermal phase of induction curve [67–69]; which suggested that, at this stage, the oxidation capacity of PQ pool decreased which resulted in a higher accumulation of reduced electron carriers; thus, positive H and G-bands occurred. A similar decrease in PQ capacity was reported in stressful conditions such as drought [60], parasitic infestation [70] or low temperature [63].

In order to further investigate the effect of herbicide application on two soybean cultivars, the parameters of energy transfer by active reaction center (Figure 4a), absorption (ABS/RC), exciton capture (TR_0/RC), electronic transport (ET_0/RC), excess energy release (DI_0/RC) and electron flux reducing end electron acceptors at the PSI acceptor side (RE_0/RC) were calculated. Ika cultivar showed at V3 stage an increased ABS/RC; however, TR_0/RC and RE_0/RC remained unchanged. Since the ABS/RC increase was followed by a decrease in φ_{P0}, or TR_0/ABS, it suggested that certain parts of RCs were inactivated and transformed into the non-Q_A-reducing RCs after herbicide application [44]. The fact that TR_0/RC remained unchanged suggested that part of the functional antennae did not utilize energy into the functional RCs for Q_A reduction (lower ET_0/RC), but rather dissipated excess energy as heat, seen as increased DI_0/RC. Such reaction centers are called "silenced" or "heat sinks" because they cannot effectively use the absorbed energy in photochemical reactions, but release it harmlessly as heat [41,44,71]. Such results, in combination with a positive K band, suggest that the transformation of active reaction centers into silent ones was the result of inactive OEC [44]. Moreover, all quantum yields (φ_{P0}, φ_{E0} and φ_{R0}) decreased, suggesting that herbicide application downregulated the electron transport [59,72,73]. Such results suggested that Ika cultivar had an effective mechanism to inactivate RCs to avoid damage to the photosynthetic apparatus. As a result of the lower functionality of OEC and slower electron transfer further than plastoquinone Q_A, the level of H_2O_2 (Figure 1b) remained unchanged after herbicide treatment. Moreover, CAT activity (Figure 2c) and Car content (Figure 2f) significantly increased, leading to less damaged membranes seen as lower TBARS levels (Figure 2a). H_2O_2 molecules in smaller concentra-

tions often serve as signaling molecules for the activation of the antioxidant system [74], which could be the case in herbicide treated Ika cultivar at this stage. Moreover, such results suggested that increased CAT activity and increased Car content managed to protect the membrane integrity from the herbicide action [26,75].

A similar response after herbicide treatment was shown by cultivar Zora at VC stage. However, its mechanism was somewhat different. Even though herbicide treatment induced increased ABS/RC (Figure 4b), increased TR_0/RC suggested that inactivation of a certain fraction of RC was the result of transformation into non-Q_A-reducing RCs, but also of the increase in functional antennae size [44]. Despite this, the lower amount of absorbed energy was utilized, seen as lower ET_0/RC; however, a larger portion was dissipated as heat, represented as increased DI_0/RC. This was corroborated by the fact that both L and K bands showed positive inflections at this stage after herbicide treatment (Figure 3b,g). In addition, a decrease in all quantum yields (φ_{P0}, φ_{E0} and φ_{R0}) suggested the down-regulation of the electron transport [59,72,73]. At the same time, herbicide application on Zora cultivar at VC stage showed a lower TBARS level (Figure 1a) and unchanged H_2O_2 level (Figure 2b), as well as unchanged CAT and APX activities (Figure 2c,d). It has been shown that the extent of APX expression is directly correlated with the intensity and duration of the imposed stress as well as with the variety of imposed stress [75,76]. Accordingly, it can be assumed that the influence of herbicides on the Zora cultivar was not of high intensity in the cotyledon stage. However, a significant increase in excess energy released as heat (DI_0/RC) might be an efficient enough mechanism for protection from herbicide application.

In the flowering (R1) stage, except with herbicides based on the active substance oxasulfuron (Laguna 75 WG) and thifensulforon−methyl (Harmony SX), plants were also treated with a herbicide based on active substance bentazone (Basagran 480) that initially disrupts the chlorophyll molecule and blocks photosynthetic reactions [12]. However, after the application, only the cultivar Ika showed a certain response; but at lower intensity compared to previous treatment at V3 stage; while Zora cultivar revealed almost no response. It was found that herbicides based on the active substance bentazone, such as Basagran 480 used in this study, have no effect on the parameters of ABS/RC and TR_0/RC [72]. This was corroborated also in our study. However, the same study reported a negative influence of bentazone on electron transport. Our study confirmed this report, since Ika cultivar showed increased DI_0/RC followed by a decrease in electron transport, evident as a decrease in all measured parameters: ET_0/RC, φ_{P0}, φ_{E0} and φ_{R0}, ψ_{E0}, TR_0/DI_0. Both cultivars, however, revealed positive K bands at this stage after herbicide treatment. Nevertheless, H_2O_2 level and APX activity remained unchanged, while TBARS level decreased. A slight increase in CAT activity, even though nonsignificant, in Ika cultivar, was obviously efficient enough to prevent oxidative damage from herbicide action. It has been shown that treating soybeans with herbicides based on atrazine and bentazone may lead to a decrease in the activity of ascorbate peroxidase [35], but no such influence was observed in this study.

By measuring the structural−functional index, SFI_{ABS}, we can obtain structural and functional information about the strength of the influence of internal factors that trigger reactions within PSII [18]. A significant decrease in this parameter was observed in Zora cultivar at VC stage (Figure 4b), and at V3 and R1 stages in Ika cultivar (Figure 4a). Significantly lower values after herbicide treatment, compared to their corresponding controls, suggested a higher sensitivity of the Ika cultivar to commercial herbicide treatment compared to Zora in later stages of development. Zora cultivar, however, was more susceptible in the early developmental stage. Since this parameter includes several ratios describing specific events of primary photochemistry, it could be used as a reliable parameter to measure the susceptibility of the plant to specific conditions. Active substance bentazone in Basagran® 480 blocks the transfer of electrons by binding to the Q_B binding site on the D1 protein [12,72]. Since both bentazone and electrons compete for the same binding site on the D1 protein, it can be suggested that this herbicide had a negative effect on the efficiency

of photosynthesis at R1 phase of the Ika cultivar. On the other hand, the lower values in the earlier stages are the result of the action of two other commercial herbicides used, since both herbicides use foliar application and thus can interfere with photosynthetic processes.

Furthermore, one of the most sensitive parameters of the JIP test that also includes several events of the primary photochemistry is PI_{ABS}, the performance index for energy conservation from photons absorbed by PSII to the reduction in intersystem electron acceptors [12,77]. It can also be defined as the total amount of driving forces for photosynthesis, which includes all individual reactions within the observed system [42]. Each component or partial driving force of DF_{ABS} enables an estimate of how much an individual parameter contributes to the overall change in PI_{ABS} with respect to the concentration of active reaction centers in PSII, primary photochemical reactions and reoxidation of reduced plastoquinone Q_A^- [18]. Herbicide treatment showed a significant decrease in PI_{ABS} in Ika cultivar at V3 and R1 stages (Figures 4a and 5) and in Zora cultivar at VC stage (Figures 4b and 5). Such responses were, at the highest extent, the result of the negative influence of herbicide treatment on the functionality of the electron transport chain due to the more negative value of the partial driving force $\log \psi_{E0}/(1 - \psi_{E0})$. It suggested that herbicides caused a disturbed balance of redox reactions of electronic transport between PSII and PSI, which affected the overall photosynthetic efficiency of the herbicide-treated plants. Such results indicate that, despite the known site of action of active substances, selective herbicides also negatively affect photosynthetic electrons transport. In addition, a negative $\log \varphi_{P0}/(1 - \varphi_{P0})$ suggested that there was also an inhibition of the reaction centers of chlorophyll and direct obstruction of the primary acceptor Q_A. By interfering with the function of chlorophyll, the possibility of absorption of light is reduced, which leads to a smaller number of excited reaction centers, and thus ultimately to a decrease in photosynthetic efficiency [65].

In the VC and V3 stages, soybean plants were treated with the combination of two commercially available herbicides: Harmony SX and Laguna 75 WG, based on active substances thifensulforon−methyl and oxasulfuron, respectively. Both thifensulforon−methyl and oxasulfuron are sulfonylurea types of herbicides, used for post-emergence weed control. They are applied through the leaf surface and act as inhibitors of acetolactase synthase (ALS), a crucial enzyme included in the biosynthesis of amino acids with branched chains such as leucine, isoleucine and valine, which consequently might disrupt protein metabolism [49,52]. Except for direct inhibition of ALS, some vital metabolic pathways, such as photosynthetic transport and the respiration system, could be also depleted [52]. In that context, the photosynthetic response of two soybean cultivars described here could be also the result of thifensulforon−methyl and oxasulfuron influence on photosynthetic electron transport. Especially, since in Zora cultivar, there was a significant response of primary photosynthetic reactions at VC stage (Figures 3 and 4) compared to Ika cultivar. Soybean tolerance to this specific herbicide is based on its fast metabolic degradation, in contrast with the slower metabolism of targeted weed species [50–52]. Moreover, as a result of gene mutations involved in resistance response, the number of weed species resistant to this type of herbicide is growing [51]. Therefore, thifensulforon−methyl and/or oxasulfuron are usually combined with bentazone. Since the absorption and translocation of bentazone is slower in tolerant plants than in susceptible ones [78], it can be assumed that both the investigated cultivars, Ika and Zora, are somewhat tolerant to active substance bentazone. Previous reports on the bentazone effect found the absorbed energy cannot be fully used for photochemical processes [12,72] and there is an increased formation of triplet chlorophylls [76]. The lifespan of triplet chlorophyll is relatively long and it has the potential to react with oxygen to form singlet oxygen, one of the reactive oxygen individuals, which damages the proteins and membranes of the plant [58,72,79]. Due to such damage caused by the herbicide's application, plants can develop various defense mechanisms, such as increased gene expression for the activation of various enzymes that are associated with tolerance to herbicides [35]. This enables plants to develop defense mechanisms and thus more successfully detoxify and metabolize harmful substances [35,80,81]. Her-

bicide metabolism involves reductions, oxidations and hydrolysis of molecules by which molecules become more soluble in water and thus become less toxic [35,81]. It has also been shown that the response of plants differs if treated with only one herbicide or a combination of several herbicides [35]. In our case, despite the fact active substances in commercial herbicides Harmony SX and Laguna 75 WG do not affect the photosynthetic process directly, their application definitely induced the activation of defense mechanisms. By the time the bentazone was applied, both cultivars established efficient mechanisms to effectively reduce possible oxidative stress and to perform efficient photosynthetic reactions.

5. Conclusions

Considering the obtained results, it can be concluded that the application of selective herbicides for protection of soybean crops from broad-leaf weeds did cause specific responses in two investigated soybean cultivars, Ika and Zora. Both investigated cultivars showed that treatment with herbicides did not cause oxidative damage, but they showed different adaptation mechanisms of photosynthetic reactions. Cultivar Ika showed a response at later stages of development, namely at leaf development and at the beginning of the flowering stage, while cultivar Zora showed a response earlier, at the cotyledon development stage. Herbicide application caused inactivation of the oxygen-evolving complex and reaction centers which transformed into dissipative ones. In that way, absorbed energy was not utilized efficiently in primary photochemistry but rather dissipated harmlessly. Moreover, herbicide action mostly blocked electron transport, causing a decrease in overall photosynthetic functionality. However, despite the fact that selective application of herbicide caused a decrease in photosynthetic performance, both cultivars showed an increased resistance to applied herbicides in later stages of development.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agriculture13071385/s1, Table S1: Antioxidative response, Table S2: Spider plot data, Table S3: Driving forces data.

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