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Research article

Glyphosate effects on gas exchange and chlorophyll fluorescence responses of two *Lolium perenne* L. biotypes with differential herbicide sensitivity

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ABSTRACT

Despite the extensive use of glyphosate, how it alters the physiology and metabolism of plants is still unclear. Photosynthesis is not regarded to be a primary inhibitory target of glyphosate, but it has been reported to be affected by this herbicide. The aim of the current research was to determine the effects of glyphosate on the light and dark reactions of photosynthesis by comparing glyphosate-susceptible and glyphosate-resistant *Lolium perenne* biotypes. After glyphosate treatment, accumulation of reduced carbohydrates occurred before a decrease in gas exchange. Stomatal conductance and CO_2 assimilation were reduced earlier than chlorophyll fluorescence and the amount of chlorophyll in susceptible plants. In the glyphosate-resistant biotype, stomatal conductance was the only parameter slightly affected only 5 days post-application. In susceptible plants, the initial glyphosate effects on gas exchange could be a response to a feedback regulation of photosynthesis. Since the herbicide affects actively growing tissues regardless of the inhibition of photosynthesis, the demand of assimilates decreased and consequently induced an accumulation of carbohydrates in leaves. We concluded that stomatal conductance could be a very sensitive parameter to assess both the susceptibility/resistance to glyphosate before the phytotoxic symptoms become evident.

1. Introduction

Glyphosate is the most extensively used herbicide worldwide [1]. The target enzyme of glyphosate is 5-enolpyruvylshikimate-3-phosphate synthase (EPSPs; EC. 2.5.1.19). This enzyme catalyzes the reaction of shikimate-3-phosphate and phosphoenolpyruvate to yield 5-enolpyruvylshikimate-3-phosphate [2]. Glyphosate applications elicit the inhibition of the enzyme and the accumulation of shikimic acid [3]. Consequently, the aromatic amino acid pools are depleted [2], and thus insufficient to maintain the necessary protein synthesis, which is consistent with the slow development of symptoms [4]. Supporting this, glyphosate-induced growth inhibition is reversed by the addition of aromatic amino acids in several plant cell cultures [5,6]. However, some researchers have found no reversal of glyphosate-induced phytotoxicity and no evidence of aromatic amino acid deficiencies after glyphosate application [7,8].

Photosynthesis is not regarded as a primary inhibitory target of glyphosate, but it has been reported to be affected by this herbicide [9]. Glyphosate causes a rapid and continuous inhibition of photosynthetic CO_2 assimilation in glyphosate-sensitive plants [10,11]. Furthermore, depletion of intermediates of the photosynthetic carbon reduction cycle has been observed promptly following the application of glyphosate [12]. These effects may be a consequence of an unregulated flux into the shikimate pathway due to depletion downstream from EPSPs of a feedback regulator of the first enzyme in the pathway [13]. Other authors argue that stomatal closure is an important factor contributing to a decrease in CO_2 assimilation [14].

Abbreviations: A, carbon assimilation rate; A_{max}, CO_2-saturated rate of CO_2 assimilation; A_{sat}, light-saturated carbon assimilation rate; Ca, external CO_2 concentration; Ci, partial pressure of CO_2 in the intercellular air space; DPA, days post-application; EPSPs, 5-enolpyruvylshikimate-3-phosphate synthase; ETR, photosynthetic electron transport rates; F_0, minimal fluorescence in dark-adapted leaves; Fm, maximal fluorescence in dark-adapted leaves; Fm′, maximal fluorescence in light-adapted leaves; Fs′, fluorescence of light-adapted leaves in steady state; Fv/Fm, maximum quantum yield of PSII in dark-adapted leaves; Fv′/Fm′, intrinsic efficiency of PSII; gs, stomatal conductance; J_{max}, maximum potential rate of electron transport contributing to ribulose 1,5-bisphosphate regeneration; PPFD, photosynthetically photon flux density; qP, photochemical quenching; Rubisco, ribulose 1,5-bisphosphate carboxylase/oxygenase; RuBP, ribulose 1,5-bisphosphate; V_{cmax}, ribulose 1,5-bisphosphate carboxylation.

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Recently, the effects of glyphosate have been studied using new molecular methods such as transcriptional comparison, proteomic approaches, and metabolomic profiling [15], but the full picture of the sequence of metabolic disturbances after EPSPs inhibition is not yet clear [16]. Despite the extensive use of this herbicide, there are still uncertainties concerning the precise mechanisms by which glyphosate kills susceptible plants and how it alters the physiology and metabolism in glyphosate-resistant plants [10].

The current research was aimed to determine the effects of glyphosate on the light and dark reactions of photosynthesis by comparing two *Lolium perenne* L. biotypes with differential sensitivity to the herbicide.

2. Materials and methods

Experiments were carried out in La Plata, Argentina (34° S, 58° W) under semi-controlled conditions (i.e. greenhouse) during 2010 and 2011. Each experiment was repeated twice.

2.1. Plant material and growing conditions

L. perenne L. seeds from a population from the south of Buenos Aires province [17] were grown for 8 weeks in order to obtain at least four tillers from each plant by tiller separation. When the propagules had three to four tillers, each clone was treated with 0.0, 0.7, 1.4, or 2.8 kg ae ha^{-1} formulated glyphosate (isopropylamine salt of glyphosate, Roundup®, 360 g ae L^{-1}, Monsanto Argentina) one week after transplanting, following Baerson et al. [18]. On the basis of these preliminary screens, one clone was characterized as 'susceptible' (no survivors at 0.7 kg ae ha^{-1} or higher doses) and the other one as 'glyphosate-resistant' (survivor at 2.8 kg ae ha^{-1} and lower doses). In pots of 500 cm^3 with sterile soil, plants of both biotypes were further subdivided into new propagules (replicates) and grown for additional four weeks before use in the following experiments.

The plants were grown in a greenhouse and pots were irrigated daily to field capacity. Fertilizer (12:10:20, Nitrofoska®, Compo Argentina) (1 g L^{-1}) was added to the irrigation water every 15 days.

Five replicates of each biotype were sprayed with glyphosate using a laboratory belt sprayer calibrated to deliver 200 L ha^{-1} at 1.08 kg ae ha^{-1}. Herbicide was applied at tillering, on plants with three to four tillers. The five replicates of controls of both biotypes were sprayed with twice distilled water.

2.2. Gas exchange measurements after glyphosate application

Gas exchanges of both the susceptible and the resistant biotypes were compared after glyphosate treatment. The light-saturated carbon assimilation rate (A_{sat}) and stomatal conductance (gs) were measured with an Infra Red Gas Analyzer (IRGA) using a CIRAS-2 portable photosynthesis system (PP Systems®, Hertfordshire, UK). Light-saturated irradiance intensity was previously deducted in control plants by means of CO_2 assimilation rate *versus* irradiance curves at different photosynthetically photon flux density (PPFD, 0 to 1500 μmol m^{-2} s^{-1}). Assimilation rates reached saturation around 1000 μmol m^{-2} s^{-1}, which is consistent with the fact that *L. perenne* is a species with C_3 metabolism.

The assessments were performed at 25 °C with 360 ppm of external CO_2 concentration (Ca) and at a saturated PPFD of 1000 μmol m^{-2} s^{-1}, between 10:00 h and 15:00 h. The recordings were conducted choosing the last fully expanded leaf of each plant, at 1, 2, 3, 5 and 7 days post-application (DPA). Measurements were alternated (between biotypes and treatments) in order to reduce the bias due to time.

2.3. Gas exchange response to CO_2 concentration (A/Ci curves)

In glyphosate-treated and control plants of both biotypes, the carbon assimilation rate (A) and gs were measured as a response to different Ca (50, 100, 250, 360, 500, 600, 700, 800 and 900 ppm) at a saturated PPFD of 1000 μmol m^{-2} s^{-1} and at 2 DPA. For every assessment, the partial pressure of CO_2 in the intercellular air space (Ci) was recorded, and the evolution of A and gs was then plotted as a function of Ci. The Ci/Ca ratio was calculated for each recording. Five replicates by treatment were used. The instrument and conditions used were similar to those described in the previous experiment.

The maximum rate of ribulose 1,5-bisphosphate (RuBP) carboxylation (V_{cmax}), maximum potential rate of electron transport contributing to RuBP regeneration (J_{max}), A_{sat} and CO_2-saturated rate of CO_2 assimilation (A_{max}) were calculated to analyze the A/Ci curves using an estimator utility provided by McMurtrie and Wang [19].

2.4. Chlorophyll fluorescence measurements and chlorophyll content after glyphosate application

The photochemical activity of the photosynthetic tissue was measured periodically on the last expanded leaves of glyphosate-susceptible and -resistant plants in five replicates of each treated clone, as described in the first experiment. Fluorescence measurements were determined with a modulated fluorometer (FMS 1, Hansatech®, Norfolk, UK) using the saturation pulse method [20].

In order to adapt plants to light, they were subjected to a photosynthetically active radiation of 1000 μmol m^{-2} s^{-1}, provided by an external halogen lamp, for at least 30 min prior to the measurement of maximal (Fm′) and steady-state (Fs′) fluorescences.

Similarly, to adapt plants to darkness, they were subjected to darkness for 30 min and then maximal (Fm) and minimal (F_0) fluorescence signals were. Based on these traits (light and/or dark adapted-state), the following variables were estimated according to Rosenqvist & van Kooten [21]: maximum quantum yield of PSII (Fv/Fm), photosynthetic electron transport rates (ETR, where 0.5 was assumed as the fraction of the excitation energy distributed to PSII and 0.84 as the fractional light absorption of the leaf), photochemical quenching (qP) and intrinsic efficiency of PSII (Fv′/Fm′).

Chlorophyll content was recorded with a portable chlorophyll meter (SPAD 502, Minolta®, Konica Minolta Sensing, Inc.). The value of chlorophyll per leaf section was the average of three measurements taken at the middle third of the last expanded leaf.

2.5. Glyphosate effects on free reducing sugar

Free reducing sugar levels in the last expanded leaf samples of five replicates were determined using the Somogyi–Nelson method with modifications [22,23]. Five hundred milligrams of leaf tissue were harvested at 1 and 2 DPA and 5 ml of ethanol (85% *v*/*v*) was added to each sample. Subsequently, these were heated for 5 min at 100 °C and the extract was removed and stored. Later, 5 ml of ethanol was added to each sample again and heated for 5 min at 100 °C. This procedure was repeated three times. The extracts were adjusted to 10 ml by evaporation at 80 °C to obtain the final extract. Then, 1 ml of low-alkalinity copper reagent was added to 250 μl of extract and heated for 10 min at 100 °C. Afterward, 0.5 ml of arsenomolybdate reagent was added with gentle stirring for 10 s. Finally, each sample was adjusted to 25 ml with bi-distilled water and quantified with a double-beam spectrophotometer (Shimadzu UV-160A, Shimadzu Corporation) at 520 nm. The determination of the concentration of reducing sugar was based on a standard curve generated using known quantities of sucrose (B.D.H.®) after hydrolysis with 0.03 M HCl (100 °C, 5 min).

2.6. Statistical analysis

All data were analyzed by multi-factorial ANOVA. Residual plots indicated that the variances were normally distributed and Bartlett's test was used to determine variance homogeneity. Means were compared using Fisher's LSD test ($p < 0.05$) when there were significant differences.

3. Results

3.1. Gas exchange measurements after glyphosate application

The saturated carbon assimilation rate of the susceptible biotype was not significantly affected until two days after glyphosate treatment. Treated plants had an A_{sat} 25% lower than their controls when measured at 2 DPA (Fig. 1a). Subsequently, the photosynthetic rate was reduced by 44% by glyphosate at 7 DPA (Fig. 1a). In contrast, A_{sat} was not significantly different from that of controls within 7 DPA in the glyphosate-treated resistant biotype (Fig. 1b).

Two days after glyphosate application, the gs of the susceptible biotype was significantly inhibited by the herbicide, around 30% less than the controls (Fig. 1c). Afterward, gs decreased over time and reached a value 55% lower at 7 DPA (Fig. 1c). In the glyphosate-resistant biotype, gs was inhibited as compared with control plants at 5–7 DPA, when the decrease was about 20% compared with their controls (Fig. 1d).

3.2. Gas exchange response to CO_2 concentration

Carbon assimilation rate as a function of Ci was assessed at 2 DPA, when the initial A_{sat} differences between glyphosate-treated and control plants were detected in the susceptible biotype at 360 ppm of external CO_2.

The evolution of A as a function of the partial pressure of CO_2 in the intercellular air space (Ci) was significantly different between susceptible plants treated with glyphosate and their controls. Thus, A was inhibited by about 30–50% due to the effect of glyphosate treatment when Ci was 75 ppm or higher (Fig. 2a). In contrast, no significant differences were found in the glyphosate-resistant biotype in A as a function of Ci between glyphosate-treated and control plants (Fig. 2b).

Maximum rate of RuBP carboxylation (V_{cmax}) decreased significantly with glyphosate treatment in the susceptible biotype (see the slopes of both curves in Fig. 2a). V_{cmax} showed a value 32% lower due to the herbicide effect. Instead, the glyphosate-resistant biotype showed no significant variation in this parameter (Table 1). Glyphosate treatment led to a J_{max} 31% lower in the susceptible biotype, but caused no significant changes in the glyphosate-resistant plants. A_{sat} and A_{max} were significantly affected by glyphosate treatment only in the susceptible biotype. In this sense, A_{sat} and A_{max} were reduced by 44 and 37%, respectively, comparing glyphosate-treated plants with controls in the susceptible biotype (Table 1).

In the susceptible biotype, the CO_2 compensation point increased significantly (from 51.0 to 71.9 ppm) due to the effect of glyphosate treatment. In contrast, the glyphosate-resistant biotype showed no significant differences in the CO_2 compensation point between treatments (Table 1).

In both biotypes, gs tended to decrease when Ci increased. The glyphosate treatment reduced gs in susceptible plants. Furthermore, when considering the same Ci, gs was inhibited by 50% (Fig. 2c). In contrast, the herbicide did not affect gs in the resistant biotype at any Ci (Fig. 2d).

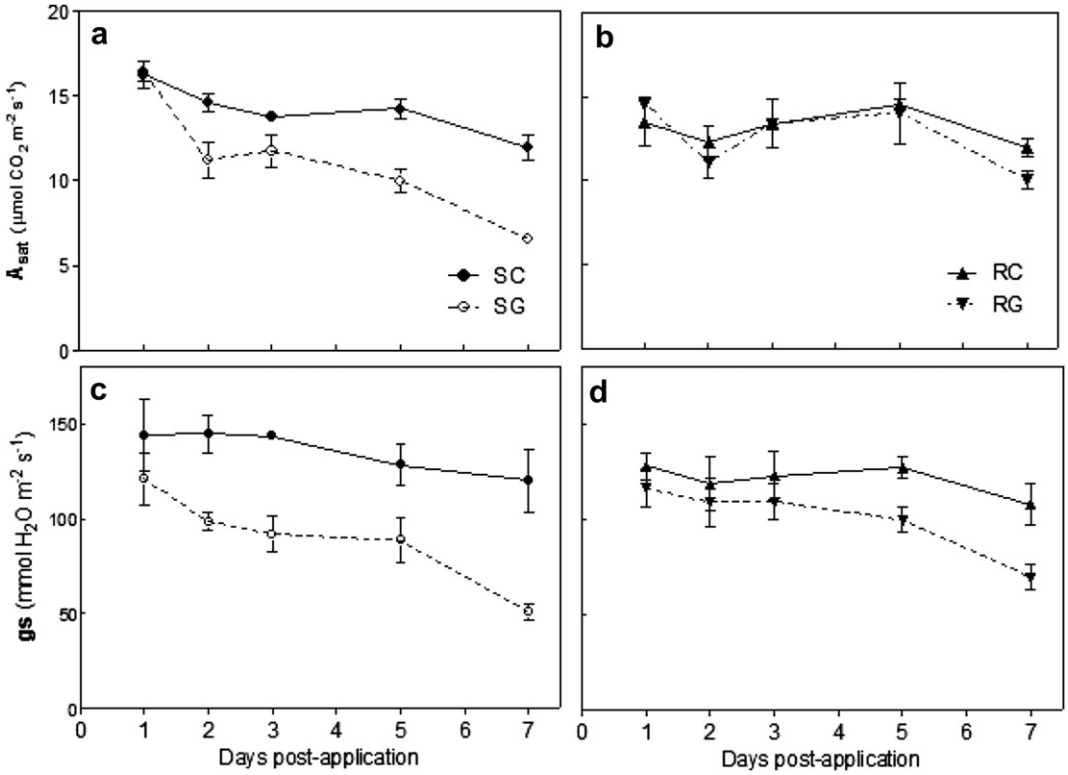

Fig. 1. Light-saturated rate of carbon assimilation (A_{sat}) and stomatal conductance (gs) during post-application in the susceptible *Lolium perenne* biotype (a, c) treated with glyphosate (SG) and controls (SC) and in the glyphosate-resistant biotype (b, d) treated with glyphosate (RG) and controls (RC). Values are means ($n = 5$) and vertical bars represent ±standard errors.

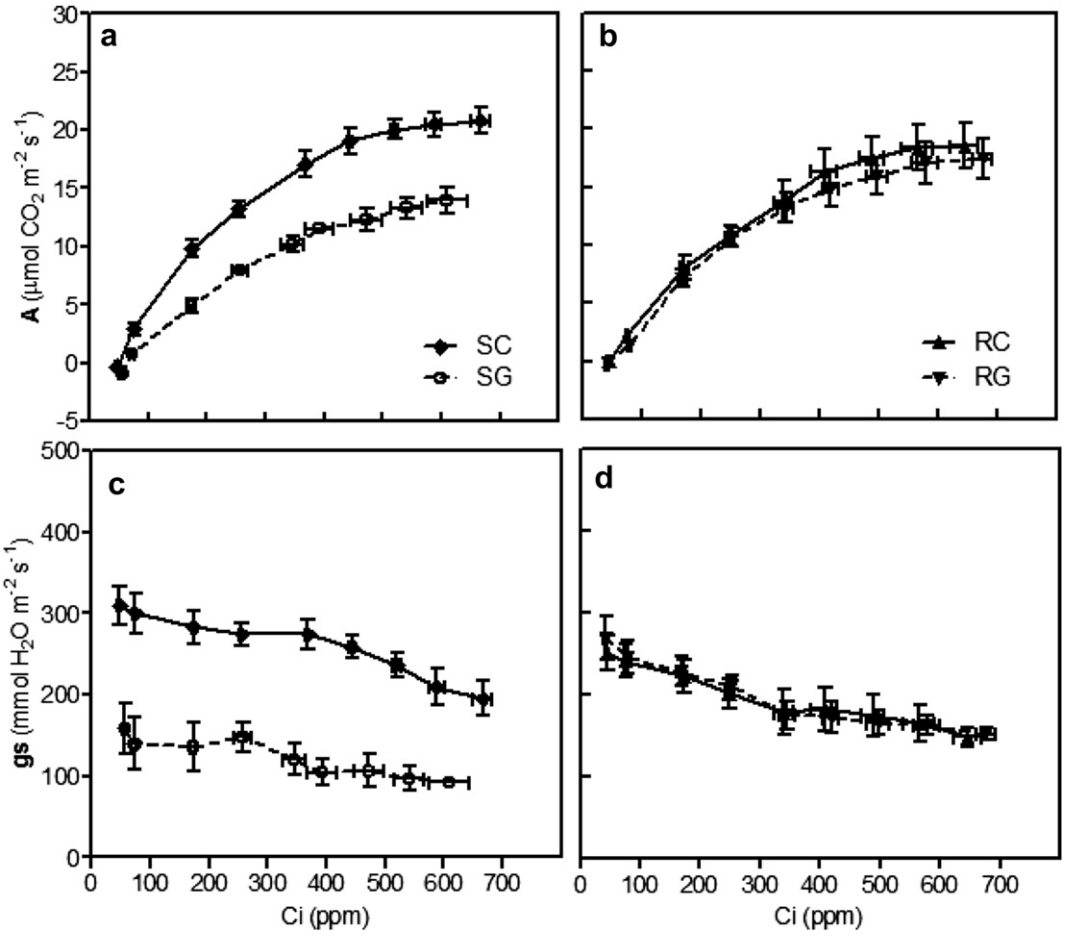

Fig. 2. Photosynthetic (*A*) and stomatal conductance (gs) responses to CO_2 concentration in the intercellular air space (Ci) at 2 days post-application in the susceptible *Lolium perenne* biotype (a, c) treated with glyphosate (SG) and controls (SC) and in glyphosate-resistant biotype (b, d) treated with glyphosate (RG) and controls (RC). Values are means ($n = 5$) and horizontal and vertical bars represent ±standard errors.

3.3. Chlorophyll fluorescence variations along the time and chlorophyll content after glyphosate application

Periodic recordings of fluorescence parameters showed that the maximum quantum yield of PSII in dark-adapted leaves (Fv/Fm) was not significantly affected by glyphosate treatment before 5 DPA in susceptible plants. After 5 DPA, the herbicide caused a gradual inhibition of Fv/Fm, which was about 30% at 7 DPA (Fig. 3a). Conversely, the Fv/Fm ratio did not reveal changes in the maximum quantum yield of PSII after glyphosate treatment in resistant plants whose average value of Fv/Fm was 0.81 (Fig. 3b).

In the susceptible biotype, ETR showed an average value of 145 μmol m^{-2} s^{-1} without any significant difference between treatments until 5 DPA, when glyphosate treatment drastically inhibited ETR and reached an average value of 61 μmol m^{-2} s^{-1}, which remained stable at 7 DPA (Fig. 3c). In the glyphosate-resistant biotype, herbicide treatment did not induce significant disturbances in ETR compared with controls. This parameter was around 141 μmol m^{-2} s^{-1} within 7 DPA (Fig. 3d).

Glyphosate treatment changed the photochemical quenching (qP, an estimator of PSII centers in open state) in the susceptible biotype after 5 DPA. In this sense, the proportion of PSII open reaction centers decreased by 42 and 52% at 5 and 7 DPA, respectively (Fig. 3e). In contrast, there were no significant differences in the qP of glyphosate-treated plants compared with their controls in the resistant biotype. Photochemical quenching average value was about 0.50 in both treatments for this biotype (Fig. 3f).

In the susceptible biotype, the intrinsic efficiency of PSII (Fv′/Fm′) was inhibited by glyphosate treatment at 5 DPA and stabilized onwards. In this case, Fv′/Fm′ decreased around 30% compared with controls (Fig. 3g). The glyphosate-resistant biotype showed no significant differences in Fv′/Fm′ (Fig. 3h).

Initially, the glyphosate treatment did not alter the chlorophyll content in leaves of both biotypes. A significant chlorophyll

Table 1
Parameters of photosynthetic CO_2 response curves: Light- and CO_2-saturated rate of carbon assimilation (A_{sat} and A_{max}, respectively), maximum carboxylation velocity of Rubisco (V_{cmax}), maximum potential rate of electron transport contributing to ribulose 1,5-bisphosphate regeneration (J_{max}) and CO_2 compensation point (ΓCO_2) in the susceptible and resistant *Lolium perenne* biotypes under glyphosate treatment and controls. Values are means ($n = 5$) ± standard error. * indicates statistical significance ($p < 0.05$).

	Susceptible biotype		Glyphosate-resistant biotype	
	Control	Glyphosate	Control	Glyphosate
A_{sat} (μmol m^{-2} s^{-1})	14.00 ± 0.69	7.73 ± 0.35*	9.94 ± 0.80	10.47 ± 1.08
A_{max} (μmol m^{-2} s^{-1})	21.78 ± 0.63	13.60 ± 0.80*	18.16 ± 1.60	17.92 ± 1.85
V_{cmax} (μmol m^{-2} s^{-1})	67.07 ± 1.68	45.47 ± 2.69*	58.13 ± 6.92	52.16 ± 3.53
J_{max} (μmol m^{-2} s^{-1})	131.03 ± 2.68	89.73 ± 5.93*	112.58 ± 11.27	104.46 ± 7.87
ΓCO_2 (ppm)	51.02 ± 7.01	71.90 ± 6.97*	50.80 ± 5.14	61.83 ± 7.08

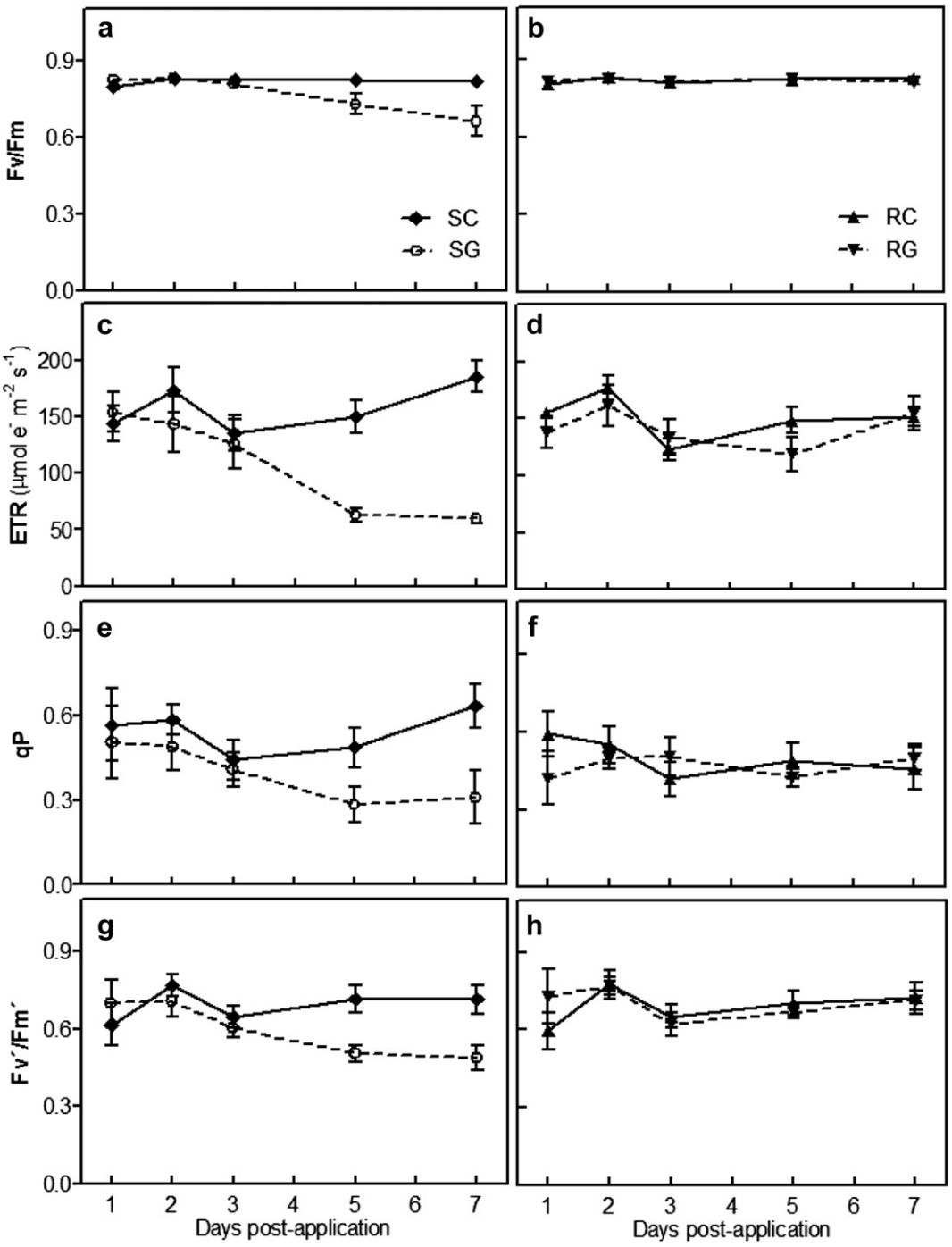

Fig. 3. Maximum quantum yield of PSII in dark-adapted leaves (Fv/Fm), photosynthetic electron transport rates (ETR, estimated from chlorophyll fluorescence measurements), photochemical quenching (qP) and intrinsic efficiency of PSII (Fv′/Fm′) during post-application in the susceptible *Lolium perenne* biotype (a, c, e, g) treated with glyphosate (SG) and controls (SC) and in glyphosate-resistant biotype (b, d, f, h) treated with glyphosate (RG) and controls (RC). Values are means ($n = 5$) and vertical bars represent ±standard errors.

reduction was observed within 7 DPA in susceptible plants as a first response to glyphosate treatment. However, no significant effects were found on chlorophyll content in glyphosate-resistant biotype leaves (Fig. 4).

3.4. Glyphosate effects on free reducing sugar

The glyphosate treatment led to free reducing sugar accumulation in leaves of the susceptible biotype, which was significantly higher 24 h after glyphosate application. Leaves of susceptible treated plants showed 45% more free reducing sugar than their controls, while the resistant biotype showed no significant variations in the level of free reducing sugar in response to glyphosate treatment (Fig. 5).

4. Discussion

The inhibition of CO_2 assimilation on leaves after glyphosate application has been widely documented in several species [11,14,24]. However, the causes of this effect attributed to the

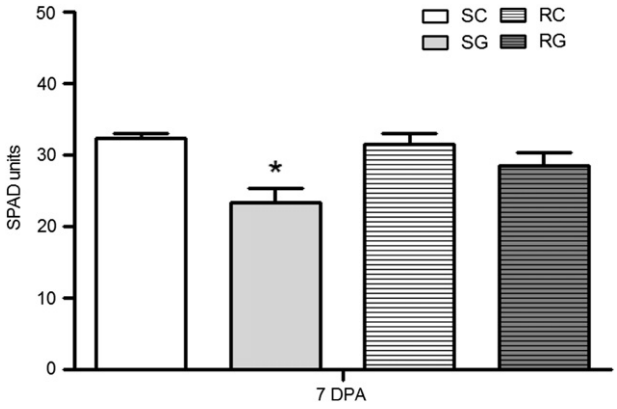

Fig. 4. Chlorophyll content (SPAD units) in leaves at 7 days post-application (DPA) in the susceptible *Lolium perenne* biotype under glyphosate treatment (SG) and controls (SC) and in the glyphosate-resistant biotype under glyphosate treatment (RG) and controls (RC). Values are means ($n = 5$) and vertical bars represent ±standard errors; * indicates statistical significance ($p < 0.05$).

herbicide are still unclear. A simultaneous decrease in stomatal conductance and CO_2 assimilation has been previously observed by Geiger et al. [11] and Olesen and Cedergreen [25]. The latter suggested that this could be the result of a cessation of carbon fixation based on a decrease in RuBP regeneration rather than a direct effect on stomatal conductance. Servaites et al. [12] proposed that glyphosate induces a depletion of carbon or phosphate or both from the photosynthetic carbon reduction cycle, reducing the rate of regeneration of RuBP and photosynthesis. Glyphosate treatment causes the depletion of intermediates of the Calvin cycle and the accumulation of shikimate, which may be related through the regulation of the shikimate pathway via arogenate [13].

Photosynthetic responses to CO_2 concentration (i.e. A/Ci curves) can provide support to several important parameters related to leaf physiology [26]. In this sense, the results of the present work show that the RuBP regeneration-limited photosynthesis induced by glyphosate is evidenced by a low J_{max} compared with controls in the susceptible biotype. Under RuBP regeneration-limited conditions, RuBP carboxylase/oxygenase (Rubisco) uses RuBP faster than it is synthesized and, thus, A does not respond to increasing Ci [26]. Accordingly, the current results show evidence that the lower A of glyphosate-treated susceptible plants compared with controls is based on a limitation by Rubisco reflected in a lower V_{cmax}. The change in the slope of CO_2 assimilation response at low Ci indicates that Rubisco activity *in vivo* changed. This is consistent with the results by Servaites et al. [12], who found that RuBP level and Rubisco activation level decrease after glyphosate application in sugar beet. More recently, Ahsan et al. [15] showed that proteins corresponding to the large and small subunits of Rubisco are down-regulated in response to glyphosate in rice. Modulation of Rubisco protein content is considered to be a major means by which the rate of A is adjusted to match sink activity in plants grown at high CO_2 [27]. In this way, sugars are a part of a repertoire of signals that coordinate the source–sink interaction [27].

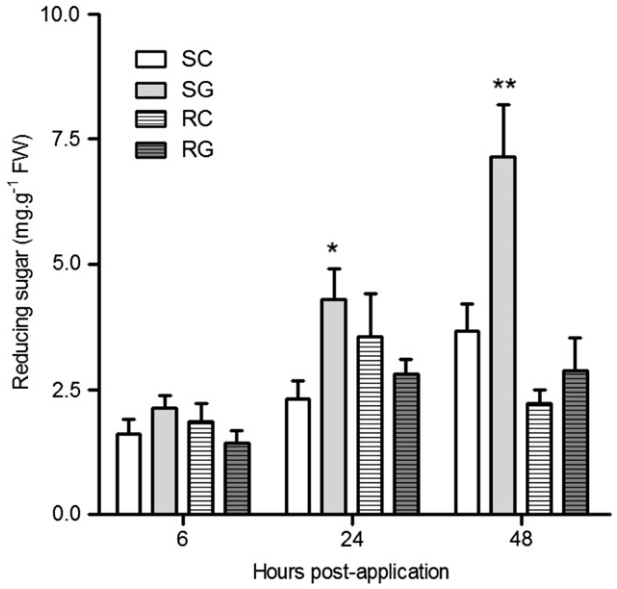

Fig. 5. Free reducing sugar content in leaves at 6, 24 and 48 h post-application in the susceptible *Lolium perenne* biotype under glyphosate treatment (SG) and controls (SC) and in the glyphosate-resistant biotype under glyphosate treatment (RG) and controls (RC). Values are means ($n = 5$) and vertical bars represent ±standard errors; * and ** indicate statistical significance ($p < 0.05$ and $p < 0.01$ respectively).

The CO_2 compensation point increased markedly in leaves of susceptible plants treated with glyphosate. These findings are consistent with previous reports, where the decrease in net carbon exchange was accompanied by an increase in the CO_2 compensation point. Photorespiration seems not to be involved because no glyphosate effects were detected in this physiological process [28].

Glyphosate-induced stomatal closure, assessed in the susceptible biotype, could be an important factor contributing to the decreased CO_2 assimilation [14]. The herbicide led to a decrease in stomatal conductance, a situation that was not reversed at low CO_2 concentrations. Given the above, our results indicate that the initial glyphosate effects on stomatal conductance and gas exchange could be a response to a feedback regulation of photosynthesis. Since the herbicide affects actively growing tissues regardless of the inhibition of photosynthesis [29], the assimilate demand decreased, inducing carbohydrate accumulation in leaves. The growth arrest detected after the inhibition of the biosynthesis of amino acids suggests an impaired metabolism that does not allow the utilization of the available carbohydrates at the expected rate [16]. Subsequently, the inhibition of photosynthesis and stomatal conductance may be caused by the accumulation of end-products of photosynthesis. The current results show that the reducing sugar accumulation had occurred before gas exchange decreased. This is consistent with the results by Oracray et al. [16], who detected an accumulation of glucose in leaves and roots of *Pisum sativum* as an early response to glyphosate treatment when measured before photosynthesis decline.

Regarding carbon metabolism, glyphosate promotes large changes in chlorophyll fluorescence [9,24,30]. Thus, chlorophyll fluorescence has been proposed as a tool for rapid screening of glyphosate effects [31,32]. However, our data show that stomatal conductance and CO_2 assimilation are both affected earlier than chlorophyll fluorescence in susceptible plants treated with glyphosate, suggesting that the decrease in ETR is a down-regulation process and not the initial cause of photosynthesis decrease. Later, at 5 and 7 DPA, there were damages to PSII (see Fig. 3c). In susceptible plants treated with glyphosate, values for Fv/Fm, ETR, qP, and Fv′/Fm′ were lower than those of their controls. Therefore, changes in ETR can be explained by changes in the proportion of PSII centers in open state (qP) and the intrinsic quantum yield of these centers (Fv′/Fm′). Thus, as from 5 DPA, the decrease in V_{cmax} and J_{max} suggests that the effect of glyphosate could be attributed to a lower activity of certain enzymes of the carbon cycle. Consequently, there was a decrease in the rate of electron transport, resulting, in turn, in an increase in the reduction state of the primary electron acceptor of PSII, pointed out by a decrease in qP, as argued by Fracheboud and Leipner [33]. The assessment of Fv/Fm

provides a measure of the intactness of the PSII and the light-harvesting complex [21]. Also, as from 5 DPA, glyphosate affected this parameter, indicating damage in PSII. However, this damage to PSII (suggested by Fv/Fm) seems to be a late event (Fig. 3a) and not the primary cause of the decrease in CO_2 assimilation rate.

The reducing sugar accumulation and the decrease of CO_2 assimilation in leaves were independent of the marked changes in chlorophyll fluorescence parameters. In other words, the photochemical activity of leaves was not affected until 5 DPA by glyphosate treatment. The photosynthetic electron transport and light harvesting are not directly affected by the herbicide and this would be triggered by alterations in carbon metabolism, as discussed above. This is in agreement with the results by Olesen and Cedergreen [25], who concluded that a cessation of CO_2 assimilation is a much more rapid and sensitive biomarker of glyphosate effect than the changes in chlorophyll fluorescence.

In susceptible plants, glyphosate inhibited CO_2 fixation as from 2 DPA, while the photosynthetic electron transport rate was unaffected at this time post-application. Therefore, the increase in the relation between ETR and CO_2 fixation after glyphosate application (9 μmol electron μmol CO_2^{-1} at 1 DPA *versus* near 13 μmol electron μmol CO_2^{-1} at 2 DPA; data not shown) indicates that a significant redirection of electrons to alternative electron sinks could have occurred [33]. The latter may explain previous reports which involved the generation of reactive oxygen species after glyphosate application [15], as a result of lipid peroxidation and the subsequent membrane damage in leaves. In our work, chlorophyll content decreased at 7 DPA in the susceptible biotype. However, whether oxidative damage is implicated in glyphosate-treated plants, it was a relatively later event and it seems not to be the primary cause of photosynthetic decrease.

Absolute fluorescence values are dependent on both the photochemical activities and the optical properties of the leaf, which could be markedly modified by differences in chlorophyll content [31]. In the current research, the chlorophyll content was not changed within 7 days after the application of herbicide, although variations in chlorophyll fluorescence parameters were previously detected. The decrease in chlorophyll contents might be explained by the direct damage on the chloroplast, and by degradation or perturbations in chlorophyll biosynthesis [34,35].

In the glyphosate-resistant biotype, a small inhibition of stomatal conductance was observed at 5 DPA and 7 DPA. Regarding the gas exchange and the photochemical activity, gs was the only parameter significantly affected by glyphosate, and these reductions appear not to be mediated by the Ci variations, thus, a direct effect of the herbicide on guard cells may be involved because these cells are more exposed to the herbicide application than mesophyll cells. Moreover, previous studies on sugar accumulations during longer post-application periods in the glyphosate-resistant biotype have shown a little accumulation of sugar in leaves after 5 DPA [29]. The current results suggest gs as a very sensitive parameter to assess both the susceptibility/resistance to glyphosate, in an early lapse before the phytotoxic symptoms become evident. This is interesting, since gs could be easily monitored (even more than IRGA measurements) using modern porometers under field conditions. The absence of alterations in carbon metabolism may explain why there were no changes in the chlorophyll fluorescence parameters of glyphosate-resistant plants. Intactness of PSII was evidenced by no changes in the value of Fv/Fm in glyphosate-treated plants compared with controls in the resistant biotype. In that sense, no changes were found in ETR after glyphosate application. Therefore, alterations in qP pointed out variations in the reduction state of the primary electron acceptor of PSII, thus changes in Fv′/Fm′ were not expected.

In the glyphosate-resistant biotype, we recorded a slight but not lethal growth inhibition which might be caused by the stomatal conductance reduction as a regulated metabolic processes in order to optimize the carbon fixation according to assimilate demands [36]. The lack of changes in the relation between ETR and CO_2 assimilation supports the absence of stress symptoms in the glyphosate-resistant biotype.

The current study thus provides evidences in the phytotoxic effects that are triggered in response to glyphosate application. In addition, it specifies the mechanisms by which the herbicide affects susceptible plants and it characterizes the glyphosate-resistant *L. perenne* plants physiologically. Finally, we concluded that stomatal conductance seems to be a sensitive biomarker for plant responses to glyphosate and it could be evaluated for screening tests in future studies.

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