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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).