Pesticide Biochemistry and Physiology 130 (2016) 65–70

Contents lists available at ScienceDirect

Pesticide Biochemistry and Physiology

journal homepage: www.elsevier.com/locate/pest

# Differential effects of glyphosate and aminomethylphosphonic acid (AMPA) on photosynthesis and chlorophyll metabolism in willow plants

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ARTICLE INFO

*Article history:*
Received 25 March 2015
Received in revised form 26 November 2015
Accepted 27 November 2015
Available online 28 November 2015

*Keywords:*
Aminolevulinic acid
Herbicide
Oxidative stress
Pigments
ROS
Photosynthesis

ABSTRACT

We used a willow species (*Salix miyabeana* cultivar SX64) to examine the differential secondary-effects of glyphosate and aminomethylphosphonic acid (AMPA), the principal glyphosate by-product, on chlorophyll metabolism and photosynthesis. Willow plants were treated with different concentrations of glyphosate (equivalent to 0, 1.4, 2.1 and 2.8 kg $ha^{-1}$) and AMPA (equivalent to 0, 0.28, 1.4 and 2.8 kg $ha^{-1}$) and evaluations of pigment contents, chlorophyll fluorescence, and oxidative stress markers (hydrogen peroxide content and antioxidant enzyme activities) in leaves were performed after 12 h of exposure. We observed that AMPA and glyphosate trigger different mechanisms leading to decreases in chlorophyll content and photosynthesis rates in willow plants. Both chemicals induced ROS accumulation in willow leaves although only glyphosate-induced oxidative damage through lipid peroxidation. By disturbing chlorophyll biosynthesis, AMPA induced decreases in chlorophyll contents, with consequent effects on photosynthesis. With glyphosate, ROS increases were higher than the ROS-sensitive threshold, provoking chlorophyll degradation (as seen by pheophytin accumulation) and invariable decreases in photosynthesis. Peroxide accumulation in both AMPA and glyphosate-treated plants was due to the inhibition of antioxidant enzyme activities. The different effects of glyphosate on chlorophyll contents and photosynthesis as described in the literature may be due to various glyphosate:AMPA ratios in those plants.

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## 1. Introduction

Since the introduction of glyphosate-resistant (GR) plants, glyphosate-based products have become the most widely used herbicides globally. Glyphosate has a short half-life and has been considered one of the least toxic herbicides [1,2]. However, following glyphosate application and its degradation by microorganisms, the aminomethylphosphonic acid (AMPA), the principal glyphosate by-product, was detected in soils and in water [1]. Similarly to soil and aquatic microorganisms, it was proposed that some plants can metabolize glyphosate to sarcosine through C-P lyase activity [3], or to AMPA through glyphosate oxidase (GOX) activity [4]. GOX pathway is usually the most expected one, since AMPA is found as the major glyphosate-metabolite in plants [5]. In addition to AMPA produced through glyphosate metabolism, plants can also be presumably exposed to AMPA through their contact with environmental matrices (i.e., soil and water) [1]. However, not so many studies have examined the effects of AMPA on plants [4,6,7] and to date, only high AMPA concentrations were described to induce detrimental effects in GR and non-GR plants [6].

Glyphosate effects on plant physiological processes have recently been reviewed [8], and include deleterious effect on photosystem II quantum efficiency as well as electron transport rate (ETR). It is known that decreased photochemical efficiency is associated with glyphosate-induced decreases in chlorophyll contents [9–11], although the exact mechanisms by which glyphosate affects chlorophyll contents are not well known. It was assumed that by disturbing plant mineral nutrient (i.e., Mg and Fe) uptake, glyphosate can affect chlorophyll biosynthesis [12]. However, glyphosate effects on mineral nutrition is contradictory, and a number of studies did not report any effect [13,14]. It was proposed that even at high glyphosate concentrations in the plant phloem, glyphosate was unable to compete with biological chelating agents for minerals implicated in chlorophyll biosynthesis [15]. Reddy et al. [4], alternatively argued that AMPA, and not glyphosate, was responsible for the deleterious effects observed on chlorophyll biosynthesis in GR soybeans. It is important to note, however, that decreased chlorophyll contents have been observed even in plants that do not degrade glyphosate to AMPA [9,10]. Increased reactive oxygen species (ROS) concentrations and oxidative stress have been also observed in glyphosate-exposed plants [16,17], as in many other herbicide-exposed plants [18], although according to Moldes et al. [19] the slight oxidative stress induced by glyphosate has no relevance to plant mortality. However, it is widely accepted that modulation in

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http://dx.doi.org/10.1016/j.pestbp.2015.11.010
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ROS metabolism can affect plant physiology [20], which can be related to some deleterious indirect effects of glyphosate on photosynthesis (even in GR plants [11,21]), i.e., by inducing chlorophyll degradation and chloroplast functional losses [22].

Due to their rapid and voluminous biomass production, in association with their high tolerance to stress factors, willows are potential candidates for phytoremediation programs [23] and in Quebec (Canada), willows have been used to compose riparian buffer strips (RBS). In the interface of agricultural lands, plants in RBS are submitted to agricultural wastes, including herbicide contamination during the herbicide application or its runoff. Therefore, in this study, we used a glyphosate-sensitive willow species (*Salix miyabeana* cultivar SX64) to examine the differential effects of glyphosate and AMPA on chlorophyll metabolism and photosynthesis.

## 2. Materials and methods

### 2.1. Greenhouse experiments

Eighteen *S. miyabeana* cultivar SX64 cuttings (corresponding to the replicates) approximately 20 cm long (acquired from the Institut de recherche en biologie végétale (the Research Center of the Montreal Botanical Garden, Canada)) were planted in plastic containers (35 l) containing distilled water amended with 20 ml of King Max nutrient solutions A (7% $P_2O_5$, 11% $K_2O$, 1.5% Mg, 1.27% S, 0.07% B, 0.002% Mo, 0.12% Zn) and B (4% N, 1% $NH_4^+$, 3% $NO_3^{-2}$, 10% $K_2O$, 2% Ca, 0.05% Fe, 0.05% Mn) (Montreal, Canada). The growth solutions were continuously aerated and renewed every 15 days. The greenhouse was maintained at 25/22 °C (±3 °C) day/night temperature under natural light conditions and supplemented by sodium vapor lamps to provide a 12 h photoperiod and an average photosynthetic active radiation level of 619 μmol photons $m^{-2}$ $s^{-1}$. Forty-five day old plants were used in all treatments. 100 μl of a freshly prepared herbicide solutions were hand-sprayed uniformly on each of the first three fully expanded leaves (corresponding to seventh to ninth leaves counting down from the shoot apex). This spray volume did not result in any runoff from the leaves. Glyphosate (56.15, 84.21 and 112.30 mM) and AMPA (17.10, 85.50 and 171.01 mM) applied concentrations corresponded to field applications of 1.4, 2.1 and 2.8 kg glyphosate $ha^{-1}$ and 0.28, 1.4 and 2.8 kg AMPA $ha^{-1}$, respectively. Moreover, glyphosate rates represent scenarios of 50, 75 and 100% of the standard field herbicide application rate normally used in the agricultural areas in Quebec, Canada. Both the glyphosate and AMPA solutions were prepared with Tween 20 (0.5% *v/v*). To minimize interference by unknown ingredients in commercial formulations, analytical-grade glyphosate (Pestanal grade) and AMPA (≥99%) obtained from Sigma-Aldrich (Oakville, Canada) were used. Tween 20 (0.5% *v/v*) treated plants were included as controls.

Photosynthetic evaluations (using chlorophyll fluorescence kinetic measurements) were performed 12 h after the initiation of the treatments, when the first to third fully expanded leaves (corresponding to seventh to ninth leaves counting down from the shoot apex) were collected, washed thoroughly with distilled water and subsequently wrapped in foil and stored at −80 °C until analyzed (glyphosate/AMPA concentrations and pigments and oxidative responses).

### 2.2. Glyphosate and AMPA concentrations

For glyphosate/AMPA evaluations, the first to third fully expanded leaves from each plant were pooled and ground in liquid nitrogen with a mortar and pestle. The extraction-purification steps were performed according to Goscinny et al. [24] with the following modifications: 0.01 g of leaves were placed in 50 ml Falcon tubes and 10 ml of ultrapure water (pH 2), 10 ml of methanol, and 5 ml of dichloromethane were added, and the samples homogenized in a high-speed homogenizer (Ultra-Turrax® T8 Digital, IKA, Germany) for 1 min. The samples were then centrifuged at 4000 rpm for 20 min at 4 °C and 40 μl of the supernatant extract was transferred to a 1.5 ml vial and dried under nitrogen ($N_2$) flow. The derivatization procedure was carried out following Börjesson and Torstensson's method [25] by adding 500 μl of trifluoroethanol (TFE) and 1 ml of trifluoroacetic anhydride (TFAA). Both chemicals were acquired from Sigma-Aldrich (Oakville, Canada). To assure complete dissolution of the glyphosate and AMPA, the vials were vortexed before being heated to 90 °C for 1 h. After returning to room temperature, the samples were evaporated to dryness under $N_2$ flow. Prior to GC-ECD injection, the samples were dissolved in 800 μl of ethyl acetate and 200 μl of pyridine. A Varian GC 3800 gas chromatograph equipped with a Restek RXI-5SIL MS capillary column (30 m × 0.25 mm ID, 0.25 μm) was used to analyze the samples. The chromatographic conditions used for glyphosate/AMPA detection were as follows: injector temperature, 250 °C; detector temperature, 300 °C; oven temperature program, 60 °C, hold for 0.50 min, 6 °C $min^{-1}$ to 170, 60 °C $min^{-1}$ to 250 °C, hold 10.0 min, for a total run of 30.17 min. High purity hydrogen was used as the carrier gas, with a 1.4 ml $min^{-1}$ constant flow; the injection volume was 2 μl. GC-ECD performance parameters were checked on a daily basis to verify their suitability for the purpose of glyphosate/AMPA analysis. Limit of detection (LOD) and limit of quantification (LOQ) were determined based on the method described in Mocak et al. [26]. The calculated LOD and LOQ were 0.02 μg $l^{-1}$ and 0.06 μg $l^{-1}$ and 0.03 μg $l^{-1}$ and 0.09 μg $l^{-1}$ for glyphosate and AMPA, respectively. Calibration curves of six points showed good linearity for both analytes ($r^2 = 0.96$; $P < 0.0001$ and $r^2 = 0.99$; $P < 0.0001$ for glyphosate and AMPA, respectively) in the domain of expected samples concentration. Each batch of samples included three blanks, five standards and five spiked control samples (roots or leaves for the corresponding plant tissue in evaluation).

### 2.3. Chlorophyll fluorescence kinetics and pigment contents

Chlorophyll fluorescence kinetics were assayed using a pulse-amplitude modulated (PAM) fluorometer (model PAM-2500, WALZ, Effeltrich, Germany) with dark-adapted (20 min) leaves. An 11-step rapid light curve (RLC) was performed, with saturating pulses triggered at 0.8 min intervals with actinic light intensities varying for each step (0, 32, 43, 61, 87, 131, 190, 284, 416, 619, 912 μmol photons $m^{-2}$ $s^{-1}$). The maximal photochemical efficiency of PSII (Fv/Fm), the relative electron transport rate through PSII (ETR), and non-photochemical quenching (NPQ) were calculated according to Kitajima and Butler [27], Krall and Edwards [28], and Bilger and Björkman [29] respectively. To compare the effects of the different treatments, ETR and NPQ from the 619 μmol photons $m^{-2}$ $s^{-1}$ step (irradiation levels similar to the original growth conditions) were used.

Three leaf disks (approximately 5 mm in diameter) were taken from each leaf for pigment evaluations. After determining the fresh weighs of the samples, the disks were macerated and chlorophyll, carotenoid [30], and pheophytin [31] were extracted in 80% acetone. The spectral absorption of the extracts (from 300 to 800 nm) was measured using a Varian Cary® 300 Bio UV–vis spectrophotometer (Varian, USA). The concentrations (μg/g fresh leaf weight) of the chlorophylls and total carotenoids were calculated using the equations described by Lichtenthaler and Wellburn [30], while pheophytin *a* and *b* concentrations were calculated according to Vernon [31].

### 2.4. Oxidative responses

Oxidative responses, hydrogen peroxide ($H_2O_2$), lipid peroxidation (MMA contents), and antioxidant enzyme activities were examined following the methods described by Gomes et al. [32]. To assess antioxidant enzyme activities, the seventh to ninth fully expanded leaves from each plant were pooled and grind with a mortar and pestle in liquid nitrogen. Then, 0.1 g of leaves was macerated in 1 ml of an extraction buffer containing the following: 100 mM potassium buffer (pH 7.8), 100 mM EDTA, 1 mM L-ascorbic acid, and 2% PVP (m/v). The protein contents of the samples were determined using the Bradford

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