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Impact of phosphate on glyphosate uptake and toxicity in willow

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Highlights

Phosphate increased glyphosate uptake and decreased its toxicity in willows

PO_4^{3-} concentrations ≥ 200 mg l^{-1} doubled glyphosate uptake by willow roots

PO_4^{3-} concentrations ≥ 200 mg l^{-1} increased antioxidant system activity

PO_4^{3-} maintained photosynthesis rates by inducing reactive oxygen species scavenging

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Abstract

Phosphate (PO_4^{3-}) has been shown to increase glyphosate uptake by willow, a plant species known for its phytoremediation potential. However, it remains unclear if this stimulation of glyphosate uptake can result in an elevated glyphosate toxicity to plants (which could prevent the use of willows in glyphosate-remediation programs). Consequently, we studied the effects of PO_4^{3-} on glyphosate uptake and toxicity in a fast growing willow cultivar (*Salix miyabeana* SX64). Plants were grown in hydroponic solution with a combination of glyphosate (0, 0.001, 0.065 and 1 mg l^{-1}) and PO_4^{3-} (0, 200 and 400 mg l^{-1}). We demonstrated that PO_4^{3-} fertilization greatly increased glyphosate uptake by roots and its translocation to leaves, which resulted in increased shikimate concentration in leaves. In addition to its deleterious effects in photosynthesis, glyphosate induced oxidative stress through hydrogen peroxide accumulation. Although it has increased glyphosate accumulation, PO_4^{3-} fertilization attenuated the herbicide's deleterious effects by increasing the activity of antioxidant systems and alleviating glyphosate-induced oxidative stress. Our results indicate that in addition to the glyphosate uptake, PO_4^{3-} is involved in glyphosate toxicity in willow by preventing glyphosate induced oxidative stress.

Keywords: antioxidant enzymes; fertilization; herbicide; phosphorus; photosynthesis

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1. INTRODUCTION

Glyphosate [*N*-(phosphonomethyl)glycine] is a systemic, non-selective and broad-spectrum herbicide for controlling both annual and perennial weeds. Since the introduction of glyphosate-resistant (GR) plants, it has been the most widely used herbicide worldwide [1]. Its half-life under laboratory conditions can range from 30 to 40 days [2–4], but in the field can vary from 2 to 197 days [5]. Although it has been found to be quickly degraded by microbial activity [6], its combined ability to adsorb to soil particles and to disperse throughout the soil profile contribute to its accumulation in soils [7]. In this context, it is important to consider soil physicochemical characteristics when evaluating both glyphosate accumulation and mobility. Among soil proprieties, phosphorous (P) content has been considered one of the key factors controlling glyphosate availability [8]. Inorganic phosphate (PO_4^{3-}) and glyphosate's methylphosphonic group compete for similar adsorbing sites [9,10], and, as a result, glyphosate sorption and its availability in soil solution are determined by the soil's capacity to adsorb PO_4^{3-}.

Phosphorous is an essential nutrient, participating in crucial metabolic events, such as energy transfer and protein metabolism in plants [11], and PO_4^{3-} fertilization of soil is a common agricultural practice to assure plant growth and development [12]. PO_4^{3-} fertilization of agricultural fields submitted to glyphosate application may invariably influence the herbicide's bioavailability in soil solution, since the two compete for soil adsorbing sites [13]. Like PO_4^{3-}, glyphosate has high water solubility [6] and can easily be transferred to aquatic systems through runoff. Agriculture thus represents a potential source of both PO_4^{3-} and glyphosate for aquatic ecosystems. Glyphosate presence in the environment has been observed and its hazardous effects on non-target organisms have been described [14]. Such findings have led to the realization that techniques to reduce glyphosate leaching from agricultural soils and clean up glyphosate-enriched soils must be developed. In this context, the use of riparian buffer strips (RBS) composed of fast growing species may constitute an alternative approach for limiting the migration of such agricultural wastes into adjacent waterways. Willows in RBS have been shown to be highly effective at retaining water contaminants such as phosphorus [15], and their potential to attenuate glyphosate runoff specifically has been studied in agricultural sites in Quebec (Canada) [16]. Once desorbed and mobile in soil solution, glyphosate becomes available for root uptake [7]. By retaining the herbicide in their tissues, willows in RBS can then reduce glyphosate bioavailability and its runoff to aquatic ecosystems. Moreover, these plants can be

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harvested following short rotation cycles and used for bioenergy production [17,18], helping to remove the contaminant from the environment.

Situated at the interface of agricultural lands, plants in RBS can be submitted to both glyphosate and PO_4^{3-} runoff. Much as glyphosate and phosphate compete on soil adsorption sites, they vie for access to membrane carriers [19,20]. In some plant species, PO_4^{3-} regulates glyphosate uptake [20] and translocation [21]. Recently, we showed that addition of PO_4^{3-} increased glyphosate uptake by willow roots, a result that was related to the increased membrane cell stability of roots exposed to glyphosate under PO_4^{3-} treatment [22]. Consequently, we hypothesized that the ability of willow to phytoremediate glyphosate in RBS could be increased by PO_4^{-3}. However, as a glyphosate-sensitive plant, willow can suffer herbicidal effects, which can undermine their potential efficacy as components of a RBS. Therefore, the following question arises: can PO_4^{-3}-stimulated increase in glyphosate uptake result in increased glyphosate toxicity to willow?

Glyphosate's herbicidal effects are linked to the inhibition of the 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), which prevents the biosynthesis of aromatic amino acids in plants [23]. As a result of EPSPS inhibition, shikimate accumulation occurs, and its concentration has been used as a bioindicator of glyphosate exposure in plants [7]. As recently discussed by Gomes *et al.* (2014*a*), glyphosate can also indirectly affect other plant physiological processes such as photosynthesis, inducing oxidative stress. In addition to its role in glyphosate uptake, PO_4^{3-} may play a role in plant response to the herbicide, as it can help them avoid some of the contaminant's deleterious effects. For example, it was observed that by increasing PO_4^{3-} uptake, plant tolerance to arsenate (which, like glyphosate, is chemically similar to PO_4^{-3}) is increased through activation of antioxidant systems, preventing oxidative burst induced by the trace-element [25,26].

In this study, we evaluated the effects of addition of PO_4^{3-} on glyphosate toxicity and uptake capacity in a fast growing willow cultivar (*Salix miyabeana* SX64). This cultivar's rapid, voluminous biomass production, associated with its high tolerance to stress factors (i.e., soil contamination by metals and organic pollutants) [27], together constitute important features of plant species being considered as candidates for phytoremediation programs [28], particularly in the context of riparian buffer strips.

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2. MATERIAL AND METHODS

2.1 General plant growth conditions and harvesting

Cuttings of the *Salix miyabeana* cultivar SX64 approximately 20 cm long were used in this greenhouse experiment conducted from September to December 2013. The greenhouse was maintained at 25/22 °C (± 3 °C) day/night temperature with natural light supplemented by sodium vapor lamps to provide a 12 h photoperiod and an average photosynthetic active radiation of 619 µmol photons m^{-2} s^{-1}. Cuttings were grown in plastic boxes filled with distilled water amended with King Max© (Montreal, Canada) nutrient solution 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% K2O, 2% Ca, 0.05% Fe, 0.05% Mn) following the product's instructions. The solutions were continuously aerated, and renewed every 15 days. To minimize interference with unknown ingredients in the commercial formulation, analytical-grade glyphosate (Pestanal grade) obtained from Sigma-Aldrich (Oakville, Canada) was used in the experiments.

After an initial growth period (45 days), healthy (vigorous and without leaf chlorotic spots) and uniform (similar height) plants were transferred to containers (35 L), 15 plants per container. Each of these was filled with the desired treatment solutions and under continuous aeration. A randomized block design with three containers (corresponding to the replications) per treatment was used. Glyphosate was added to the solution at concentrations of 0, 0.001, 0.065 and 1 mg l^{-1}, while phosphate (as KH_2PO_4) was added at 0, 200 and 400 mg PO_4^{3-} l^{-1}. Glyphosate concentrations were chosen based on the range of environmental concentrations found in streams of agricultural areas in Canada and the United States [29–31]. These values are also in accordance with glyphosate-concentrations observed in different soils, i.e, in the USA (0.001-0.476 mg kg^{-1} [29]) and Argentina (0.5 – 5 mg kg^{-1} [32]). Phosphate doses were chosen according to common fertilization practices in agricultural fields [12,33,34]. Potassium was provided as KNO_3, nitrogen as both KNO_3 and NH_4NO_3, and pH was adjusted to 6.7 $\pm$ 0.1. Both physiological and glyphosate measurements were performed at two, three and seven days after treatment inductions. Until seventh day of treatment, the presence of aminomethylphosphonic acid (AMPA) was not detected in the growth solution for the low glyphosate concentrations ($\leq$ 0.065 mg l^{-1}; data not shown). However, the AMPA concentration increased from 1.1 to 9.8 µg l^{-1} in the growth solution of the highest glyphosate treatment (1 mg l^{-1}) from the seventh to tenth

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day of treatment (data not shown), respectively, probably due to microbial degradation. Therefore, evaluations were reported up to the seventh days (since the main goal here was to investigate PO_4^{3-} and glyphosate interaction, without AMPA effects).

After chlorophyll fluorescence measurements (see section 2.3), three plants form each container (a total of nine plants/treatment/day) were harvested and divided into root and shoot fractions. The fractions were washed thoroughly with distilled water and their fresh biomass was measured. Then, the samples of the seventh (first fully expanded leaf from apex) to ninth leaves and roots of plants from the same container were pooled (to assure sufficient biomass for evaluations), to constitute one replicate, for a total of three replicates/treatment/day. These samples were immediately frozen in liquid nitrogen and stored at -80 ºC

2.2 Chemical analyses

For phosphorus evaluation, 0.1 g of leaves or roots were digested in 5 ml of concentrated HNO_3 (GR) in a microwave oven (ETHOS 1, Milestone Italy), at 175 ºC for 10 min. Then, the solutions were cooled, filtered through Whatman nº40 filter paper and brought to a volume of 10 ml with ultra-pure water. The filtered extracts were preserved at 4 ºC until analysis. Phosphorus concentrations in solution were evaluated according to Sarruge and Haag [35].

Glyphosate and AMPA extraction-purification was performed following Goscinny *et al.* (2012), with modifications. Roots (0.2 g) or leaves (0.1 g) were placed in 50 ml Falcon tubes. After addition of 10 ml of acidified ultrapure water (pH 2.0), 10 ml of methanol and 5 ml of dichloromethane, samples were homogenized with a high-speed homogenizer (Ultra-Turrax® T8 Digital, IKA, Germany) for 1 min. Samples were subsequently centrifuged at 4,000 rpm for 20 min at 4 °C. Then, 40 or 200 µl of root and leaves supernatant extract, respectively, were transferred into a 1.5 mL vial and dried under nitrogen (N_2) flow. A derivatization procedure was carried out by adding 500 µl of trifluoroethanol (TFE) and 1 ml of trifluoroacetic anhydre (TFAA). To ensure complete dissolution of glyphosate and AMPA, vials were vortexed before being heated at 90°C for an hour. After being cooled down to room temperature, samples were evaporated to dryness under stream of N_2. Prior to GC-ECD injection, samples were dissolved in 800 µL of ethyl acetate and 200 µL of pyridine whereas a 1 µL of 1-bromopentadecane is finally

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added in order to monitor injection reproducibility. A Varian GC 3800 gas chromatograph equipped with a Restek RXI-5SIL MS capillary column (30 m x 0.25 mm ID, 0.25 µm) was used to analyze samples. The chromatographic conditions used for glyphosate 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.mn^{-1} to 250°C, hold 10.0 min, for a total run of 30.17 min. High purity hydrogen was used as carrier gas (at a flow of 1.4 mL.min^{-1}), and the injection volume was 2 µL. To minimize uncertainty of chromatographic measurements, 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 Mocack *et al.* [37]. 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.

Glyphosate total accumulation was calculated according to this formula: Glyphosate total accumulation = [(concentration of glyphosate + AMPA in roots)*roots fresh weight] + [(concentration of glyphosate + AMPA in leaves)*leaves fresh weight].

2.3 Physiological evaluations

For chlorophyll fluorescence measurements, samples from the first, second and third fully expanded leaves (seventh to ninth leaves from the apex) were first dark-acclimated for 20 min and chlorophyll fluorescence emission was assessed using a pulse-amplitude modulation (PAM) fluorometer (model PAM-2500, WALZ, Effeltrich, Germany). A rapid light curve (RLC) analysis was performed according to Juneau *et al.* [38]. Saturating pulses were triggered at 0.8 min intervals with varying actinic light intensity for each step (0, 32, 43, 61, 87, 131, 190, 284, 416, 619, 912 µmol photons m^{-2} s^{-1}). Using the RLC, the operational PSII quantum yield (Φ'_M), was calculated following Genty *et al.* [39]. To compare treatments, fluorescence results from 619 µmol photons m^{-2} s^{-1} (most similar irradiation in relation to light growth conditions) were used.

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Shikimate concentrations of leaves were evaluated following the methods of Bijay and Dale [40]. Hydrogen peroxide (H_2O_2) contents were measured following Velikova *et al*. [41]. To study antioxidant enzymes, 0.1 g of plant tissue (roots or leaves) was macerated in 1 ml of an extraction buffer solution containing 100 mM potassium buffer (pH 7.8), 100 mM EDTA, 1 mM L-ascorbic acid and 2% PVP (m/v). Protein content of samples was determined using the Bradford method. Activity of catalase (CAT; EC1.11.1.6 [42]), ascorbate peroxidase (APX; EC 1.11.1.11 [43]) and glutathione peroxidase (GPX; E.C. 1.11.1.9 [44]) were measured by standard procedures.

2.4 Statistical analyses

Results were expressed as the averages of three replicates. Statistical analyses were performed using JMP software 10.0 (SAS Institute Inc). Results were submitted to normality (Shapiro–Wilk) and homogeneity (Brown-Forsythe) tests and then were statistically evaluated. Univariate repeated measures ANOVA, with Time as the within-subject factor and Glyphosate and Phosphate addition as the main effects, was used to analyze differences in the studied variables throughout the time of exposure to the treatments. Phosphate, glyphosate and the interaction between phosphate and glyphosate were included within the model. The sphericity of the data was tested by the Mauchly's criteria to determine whether the univariate F tests for the within-subjects effects were valid. In cases of invalid F, the Greenhouse-Geisser test was used to estimate epsilon (ε). Contrast analysis was used when there were significant differences in the studied variables between treatments (Table 1S and 2S).

3. RESULTS

3.1 Total phosphorus, Glyphosate and AMPA concentrations in plant tissues

Phosphorus concentrations in roots and leaves were increased by phosphate addition (hereafter referred simply as PO_4^{-3}), regardless of the glyphosate concentration and duration of exposure (Fig. 1). A significant interaction between time of exposure and glyphosate was

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observed for phosphorus concentrations in roots and leaves. Indeed, the reduction of phosphorus concentration in plant tissues was less reduced over time in glyphosate treated plants ($P < 0.05$).

Glyphosate concentration in roots and leaves was higher for the first time of evaluation (Table 1S, Figs. 2 and 3). A significant interaction between time of exposure, glyphosate and PO_4^{3-} addition was observed for glyphosate concentrations in roots and leaves (Table 1S, Figs. 2 and 3). For glyphosate concentrations ≥ 0.065 mg l^{-1}, PO_4^{3-} increased glyphosate concentration in roots and leaves ($P < 0.0001$) (Fig. 2 and 3). For the first time of evaluation, PO_4^{3-} fertilized plants treated with glyphosate concentration ≥ 0.065 mg l^{-1} showed greater glyphosate concentrations.

AMPA concentration in roots and leaves was higher for the first time of evaluation (Table 1S, Figs. 2 and 3). At that point, PO_4^{3-} increased AMPA concentration in the tissues of plants exposed to glyphosate doses ≥ 0.065 mg l^{-1}. We noticed also that glyphosate total accumulation was also higher for the first time of evaluation and (Tables 1 and 1S) and for concentrations ≥ 0.065 mg of glyphosate l^{-1}, total accumulation was increased by PO_4^{-3}.

3.2 Physiological evaluations

3.2.1 Photosynthesis

A significant interaction between time of exposure, glyphosate and PO_4^{3-} addition was observed for operational PSII quantum yield (Φ'_M) (Table 2S). In plants treated with PO_4^{3-}, Φ'_M was always greater after seven days of exposure regardless of the glyphosate and PO_4^{3-} addition (Fig. 4). After two days of treatment, PO_4^{3-} decreased the operational PSII quantum yield (Fig. 4) whereas glyphosate addition increased yield in plants not fertilized with PO_4^{-3} (Fig. 4). In contrast, yield decreased in plants treated with 400 mg PO_4^{3-} l^{-1} as glyphosate doses increased (Fig. 4). After three days of exposure, PO_4^{3-} increased Φ'_M of glyphosate-treated plants (Fig. 4). Similarly, after seven days of treatment induction, PO_4^{3-} increased Φ'_M of plants subjected to 1 mg glyphosate l^{-1} (Fig. 4).

3.2.2 Shikimate concentration in leaves

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Regardless of the duration of evaluation periods (2, 3 and 7 days), shikimate concentration in leaves was greater in glyphosate-treated plants (P > 0.01; Fig. 3). A significant interaction was observed between glyphosate and PO_4^{3-} addition (P = 0.0002*), and PO_4^{3-} increased shikimate concentration in leaves of plants treated with glyphosate (Fig. 4).

3.2.3 Hydrogen peroxide (H_2O_2) concentration in leaves

Hydrogen peroxide concentration was always greater in leaves of glyphosate-treated plants, regardless of the duration of evaluation (P > 0.01; Fig. 4). A significant interaction between time of exposure, glyphosate and PO_4^{3-} addition was observed (Table 2S). In plants without PO_4^{3-} fertilization, H_2O_2 concentration was greater after three days of exposure (P < 0.0001). On the other hand, for PO_4^{3-} fertilized plants treated with glyphosate doses ≥ 0.065 l^{-1}, H_2O_2 concentration in leaves were greater for the first time of evaluation (P = 0.0004). After two days of exposure, PO_4^{3-} increased H_2O_2 concentration in leaves of plants treated with 0.065 and 1 mg glyphosate l^{-1} (Fig. 4). In contrast, after three days of exposure, PO_4^{3-} significantly decreased H_2O_2 concentration in leaves of glyphosate-treated plants (Fig. 4). After seven days of exposure to the highest glyphosate treatment (1 mg l^{-1}), the addition of 400 mg PO_4^{3-} l^{-1} increased H_2O_2 concentration in leaves (Fig. 4).

3.2.4 Antioxidant enzymes

A significant interaction between glyphosate and PO_4^{3-} addition was observed for MDA content (lipid peroxidation) of roots (P < 0.0001). PO_4^{3-} decreased lipid peroxidation in roots of glyphosate-treated plants (Fig. 5). Glyphosate addition increased catalase (CAT), ascorbate peroxidase (APX), and glutathione peroxidase (GPX) activity in roots (P < 0.0001; Fig. 5). A significant interaction between time of exposure, glyphosate and PO_4^{3-} addition was observed for APX activity in roots (Table 2S), which was increased by PO_4^{3-}. Moreover, APX activity in roots was lower after two days of exposure in PO_4^{3-} fertilized plants treated with glyphosate(P = 0.0003). On the other hand, GPX activity in roots was increased by PO_4^{3-} for all evaluation times (Fig. 5).

While CAT activity of leaves (P < 0.05) was not affected by PO_4^{-3}, it was increased by glyphosate addition for all treatment durations (P < 0.0001; Fig. 6). A significant interaction

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between time of exposure, glyphosate and PO_4^{3-} addition was observed for APX activity (Table 2S). PO_4^{3-} fertilized plants exposed to glyphosate showed lower APX activity for the first time of evaluation. After two days of exposure, APX activity in leaves of glyphosate-treated plants was decreased by PO_4^{3-} (Fig. 6). However, after three or seven days, APX activity in plants treated with glyphosate was increased by PO_4^{3-} (Fig. 6). Glyphosate addition increased GPX activity in leaves (Fig. 6).

4. DISCUSSION

Agricultural soils are subjected to frequent fertilization with PO_4^{-3} and application of glyphosate-based herbicides [45]. The influence of soil P content on glyphosate uptake by plants has been investigated previously [20–22]. Here, we demonstrated that, in addition to glyphosate uptake (Figs. 2 and 3), PO_4^{3-} also has an impact on its toxicity, modulating physiological responses related to growth and biomass production, such as photosynthesis (Fig. 4).

As expected, PO_4^{3-} fertilization increased P concentration in roots and leaves of willows (Fig. 1). The P-phytoremediation capacity of willow has been already reported [46–48], and therefore, was not the focus of our study. However, we demonstrated here that the higher P concentration found in PO_4^{3-} fertilized plants may have a role in the plant's physiological responses to glyphosate. It is widely reported that increased P nutrition in plants under stress has beneficial effects on physiology [25,49], but to our knowledge, no information exists on about the effects of PO_4^{3-} fertilization on glyphosate toxicity. Interestingly, we noticed higher P nutrition in glyphosate treated plants. Recently we demonstrated that PO_4^{3-} fertilization increased glyphosate uptake by willow roots [22], but now, we see also increased P concentrations in the plant tissues in response to glyphosate. In this case, glyphosate could increase the P-requirement in plant tissues, as P was seen to alleviate glyphosate-deleterious effects, such as root injuries and lipid peroxidation, in willow plants [22]. By increasing the expression of P-transporters in root cell membranes, glyphosate could increase P nutrition in plants, however this hypothesis should be further investigated.

The time of exposure had significant effect on some physiological parameters studied (biomass production, shikimate and H_2O_2 contents and in the activity of photosynthesis- Φ'_M -

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and some antioxidant enzymes), but this factor mainly have an effect on the glyphosate concentration in plant tissues. Indeed, glyphosate concentrations were higher after two days of exposure in plants treated with glyphosate doses ≥ 0.065 mg l^{-1} (Fig. 2 and 3). One can argue that this could be related to the increase in biomass production over the time. However, glyphosate total accumulation was greater after two days of exposure (Table 1). It is important to note that glyphosate accumulation takes into account the total biomass production of plants (Equation 1). Therefore, we can assume that the glyphosate uptake by roots was greater during the first two days of exposure due to high diffusional pressure leading to efficient transport of the herbicide [20] inside the root cells. This has important outcome as the plant physiology was directly responsible to the glyphosate uptake (as discussed below). For the lowest glyphosate concentration (0.001 mg l^{-1}), we did not observe a significant influence of PO_4^{3-} on glyphosate uptake by roots, as is evident in total accumulation levels ($P<0.05$; Table 1). Due to their chemical similarities, glyphosate and PO_4^{3-} share the same membrane transport carriers [19,20]. These costly energetic transporters have been shown to be involved in glyphosate uptake primarily at low glyphosate concentrations (< 0.032 mg l^{-1}) [20]. Therefore, the absence of PO_4^{3-} influence on glyphosate uptake by roots under lower glyphosate concentrations (0.001 mg l^{-1}) could be related to their competition for root-absorbing sites. However, under higher glyphosate concentrations (> 0.001 mg l^{-1}, in this study), a linear diffusional process is superimposed upon the active uptake of glyphosate [20]. In such a scenario, PO_4^{3-} can indeed influence glyphosate uptake, as observed in the present study (Figs. 2, 3 and Table 1), but, how can PO_4^{3-} increase glyphosate uptake by roots?

It was previously shown that P has a protective effect against oxidative damage induced by trace-elements, through a P-induced increase in antioxidant system activity [25,50]. In our study, plants submitted to glyphosate showed darker and thicker roots in relation to control (data not shown), recognized visual symptoms of stress [51–53]. Since glyphosate is known to induce oxidative damage in plants [54,55], we investigated oxidative stress markers to elucidate whether the process of glyphosate uptake by roots induces oxidative stress, and to evaluate the possible protective role of PO_4^{3-} in willow roots.

The increased lipid peroxidation and activation of enzymatic antioxidant systems in glyphosate-treated plants (Fig. 5) clearly indicate the presence of herbicide induced oxidative

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stress in roots. However, PO_4^{3-} was found to decrease glyphosate-induced oxidative damage by increasing the activity of enzymatic antioxidant systems, mainly ascorbate peroxidase (APX) and glutathione peroxidase (GPX) (Fig. 5). Plants showing high APX and GPX activity also showed decreased lipid peroxidation in roots (Fig. 5). As for catalase (CAT), APX and GPX are involved in scavenging hydrogen peroxide (H_2O_2), a reactive oxygen species (ROS) whose accumulation leads to oxidative damage, i.e., lipid peroxidation [56]. Lipid peroxidation is an irreversible burst process leading to cell membrane destruction, with direct impact on cell membrane constitution and stability [57]. Therefore, by stimulating antioxidant systems, PO_4^{3-} alleviated glyphosate-induced oxidative stress, ensuring the stability of cell membranes, and then allowing glyphosate diffusion and uptake through roots.

Interestingly, we observed the presence of AMPA (Fig. 2), the principal glyphosate by-product [58], in roots of plants exposed to glyphosate. We also detected the presence of AMPA in the growth solution at 2, 3 and 7 days after treatment induction (data not shown), which could be related to glyphosate degradation by microbial activity (as the systems used were not in axenic condition). The uptake of AMPA could explain its presence in root tissues, however, the concentration observed in the growing solution was very low ($\leq$ 1.1 µg l^{-1} for the highest glyphosate treatment) in relation to root AMPA concentrations (Fig. 2). This evidence suggests that willow plants were able to degrade glyphosate to AMPA through glyphosate oxidase enzyme activity, as already observed in several plant species [59]. The presence of AMPA in concentration in leaves indicates that, as for glyphosate, AMPA was translocated from roots to shoots and/or that glyphosate was also metabolized in leaves. In contrast to glyphosate, the addition of PO_4^{3-} had no obvious effect on AMPA concentrations in roots and leaves (Figs. 2 and 3). However, as a glyphosate metabolite, AMPA was considered in the calculation of total glyphosate accumulation, in which PO_4^{3-} had a significant effect, as noted previously.

Once in leaves, glyphosate interferes in with the shikimic acid pathway by inhibiting EPSPS activity, resulting in accumulation of shikimate [23]. We observed shikimate accumulation in glyphosate-treated plants (Fig. 3). Moreover in these plants, PO_4^{3-} increased shikimate concentration, which may be due to the greater glyphosate concentration in leaves of PO_4^{3-}-treated plants (Fig. 4). Therefore, we would expect that the increased glyphosate concentration in leaves associated with PO_4^{3-} would amplify the deleterious effects of glyphosate

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on leaf physiology. Indeed, after two days of exposure, we observed that, in the presence of glyphosate, PO_4^{-3}-fertilized plants showed decreased photosynthesis (as measured by the operational PSII quantum yield, Fig. 4). However, this pattern was inversed at the following measurement occasion (day 3) and, after 7 days, PO_4^{-3}-fertilized plants showed higher or no-significantly different rates of photosynthesis in relation to control (Fig. 4). We recently showed that glyphosate-induced ROS generation is implicated in decreased rates of photosynthesis [60]. Increased H_2O_2 concentration was observed in leaves of plants treated with glyphosate ($P<0.05$; Fig. 4), indicating herbicide-induced ROS generation in leaves. Moreover, similar to our findings in a previous study [60], increased H_2O_2 concentration higher than ~2 µg g FW^{-1} in leaves was related to decreased operational PSII quantum yield (Fig. 4). H_2O_2 is an important signaling molecule [61], however, once accumulated, it interferes with several plant metabolic pathways. For example, by suppressing the synthesis *de novo* of photosystem II D1 protein [62] or by inducing destruction of chloroplast membrane systems through lipid peroxidation [63], H_2O_2 accumulation decreases photosynthetic activity (for more examples, see Gomes *et al*., 2015*b*). However, how can PO_4^{3-} prevent H_2O_2 accumulation and its deleterious effects on photosynthesis in leaves of herbicide-treated plants? In an attempt to answer this question, we investigated the activity of antioxidant enzymes in leaves (Fig. 6).

This activity is central in determining the threshold between the role of ROS as signalling molecules and inducers of oxidative burst. In this context, plants have developed systems to control ROS accumulation, including important enzymatic H_2O_2 scavengers, such as CAT, APX and GPX [56]. CAT activity was found to have increased in glyphosate-exposed plants, however, it was not affected by PO_4^{3-} (Fig. 6). In contrast, APX and GPX activity were modulated by PO_4^{3-}, and an intrinsic implication of these enzymes (mainly APX) in the fine control of H_2O_2 concentration in leaves was noted (Fig. 6). After two days of exposure, APX activity was lower in those glyphosate-treated plants exposed to PO_4^{3-} in which H_2O_2 accumulation had been noted. However, after three days of exposure, these plants showed decreased H_2O_2 concentration and increased APX (as well as GPX) activity in leaves. APX activity remained higher in these plants after seven days of exposure, allowing controlled H_2O_2 concentrations in leaves and normal photosynthesis rates. Therefore, even as it increased glyphosate concentration in leaves, PO_4^{3-}

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induced the activity of enzymatic antioxidant systems, which controlled the ROS generation induced by the herbicide, ultimately ensuring normal photosynthesis rates.

5. CONCLUSION

In the present study, we clearly demonstrated the potential as a glyphosate uptake species of *S. miyabeana* cultivar SX64. We have also shown here that phosphate fertilization influenced glyphosate uptake and toxicity in willow. By increasing the activity of antioxidant systems and alleviating glyphosate-induced oxidative stress in both roots and leaves of willow plants, fertilization with PO_4^{3-} increased glyphosate accumulation and protected photosynthetic activity. It remains however unclear how PO_4^{3-} can induce increases in the activity of antioxidant systems. Extracellular PO_4^{3-} has been shown to be a signaling molecule affecting the expression of several genes in Murine osteoblasts [64]. Likewise, PO_4^{3-} could also modulate the expression of antioxidant enzymes genes in plants, but further studies are required.

As a result of their capacity to uptake glyphosate willows may be considered as a potential species to compose efficient riparian buffer strips. At the interface of agricultural lands, plants are submitted to agricultural discharge, such as herbicides (glyphosate) and phosphate runoff. As shown here, the positive interaction between phosphate and glyphosate increases the capacity of willows to uptake herbicides, which could therefore prevent or mitigate the effects of the discharge of this herbicide into aquatic ecosystems. Since in natural environment conditions are not controlled as it was here, we cannot confirm the glyphosate phytoremediation ability of willows. However, our results showing an influence of PO_4^{3-} on glyphosate uptake and toxicity in willow, pave the path for new studies addressing the influences of the soil proprieties (physical, chemical and biological characteristics) and environmental factors (i.e, temperature and light) on the willow's ability to uptake glyphosate.

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Figure Captions

Fig 1. Phosphorus concentration (mg g DW^{-1}) in roots and leaves of willow plants grown for two, three and seven days in nutrient solution amended with increasing doses of glyphosate (0, 0.001, 0.065 and 1.000 mg l^{-1}) and phosphate (0, 200 and 400 mg l^{-1}). Values are means ± SE of three replicates. The concentrations of added phosphate were 0 (filled circle), 200 (open circle) and 400 (filled inverted triangle) mg l^{-1}.

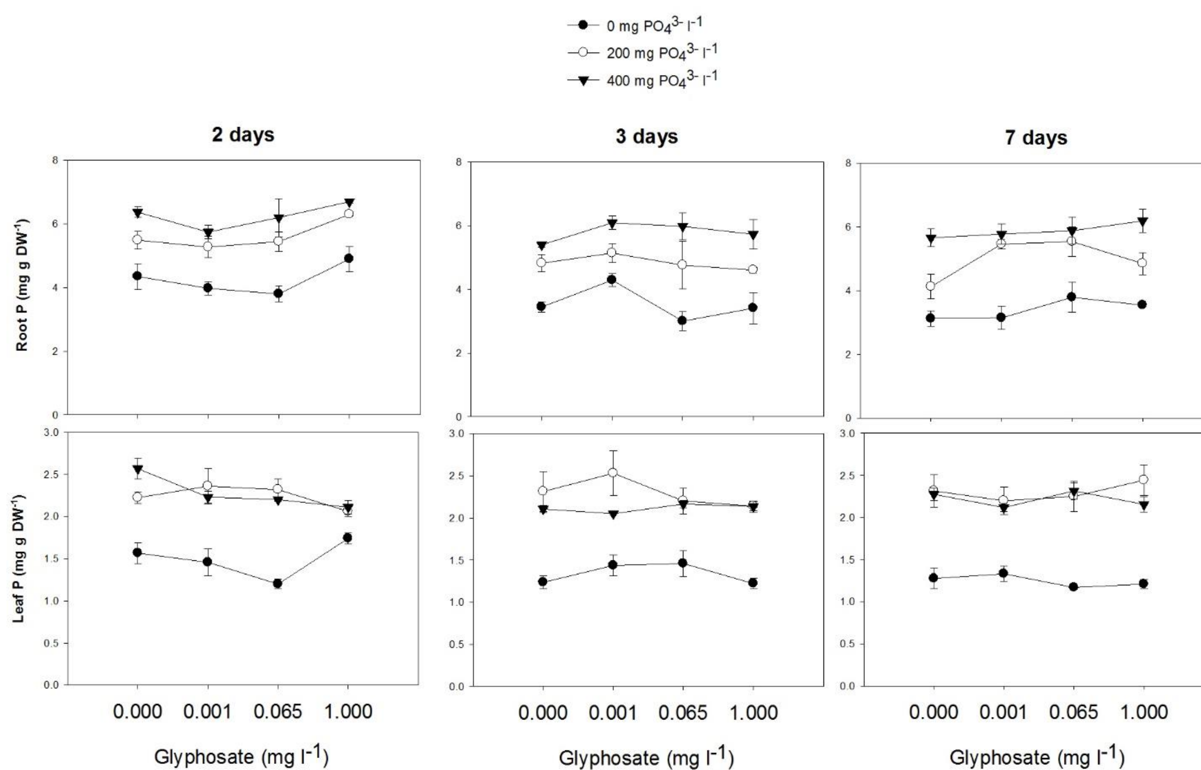

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Fig 2. Glyphosate and aminomethylphosphonic acid (AMPA) content (μg g FW^{-1}) in roots of willow plants grown for two, three and seven days in nutrient solution amended with increasing doses of glyphosate (0, 0.001, 0.065 and 1.000 mg l^{-1}) and phosphate (0, 200 and 400 mg l^{-1}). Values are means ± SE of three replicates. The concentrations of added phosphate were 0 (filled circle), 200 (open circle) and 400 (filled inverted triangle) mg l^{-1}. Values followed by * within the same glyphosate and phosphorus concentration, are significantly different (P>0.05) by the Scott-Knott multiple range test.

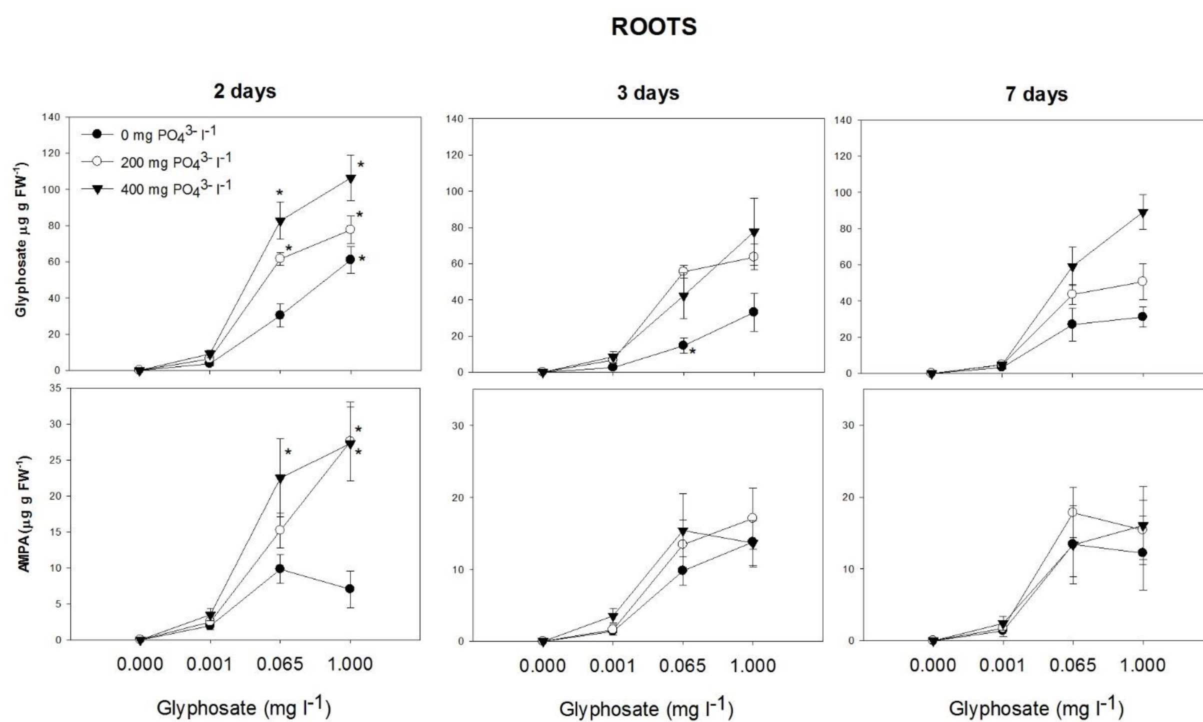

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Fig 3. Glyphosate and aminomethylphosphonic acid (AMPA) content (μg g FW^{-1}) in leaves of willow plants grown for two, three and seven days in a nutrient solution amended with increasing doses of glyphosate (0, 0.001, 0.065 and 1.000 mg l^{-1}) and phosphate (0, 200 and 400 mg l^{-1}). Values are means ± SE of three replicates. Values followed by *, within the same glyphosate and phosphorus concentration, are significantly different (P>0.05) by the Contrast test.

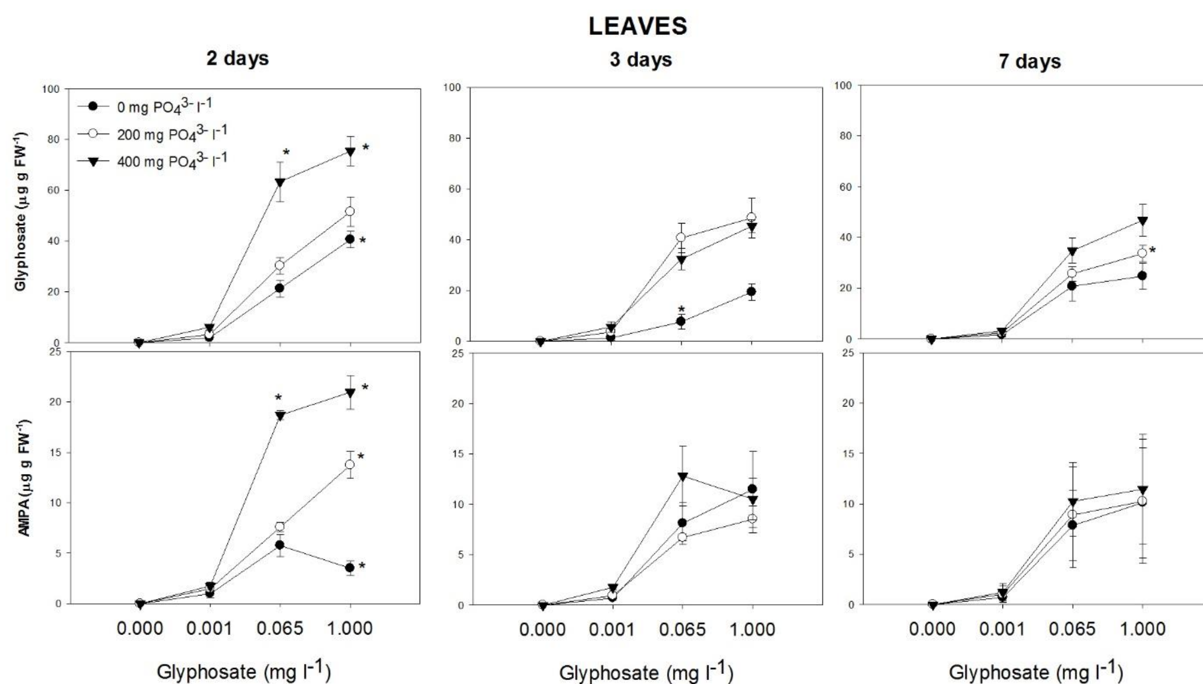

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Fig 4. PSII quantum yield (Φ'_M), shikimate and hydrogen peroxide (H_2O_2) (µg g FW^{-1}) concentrations in leaves of willow plants grown for two, three and seven days in a nutrient solution amended with increasing doses of glyphosate (0, 0.001, 0.065 and 1.000 mg l^{-1}) and phosphate (0, 200 and 400 mg l^{-1}). Values are means ± SE of three replicates. Values followed by *, within the same glyphosate and phosphorus concentration, are significantly different (P>0.05) by the Contrast test.

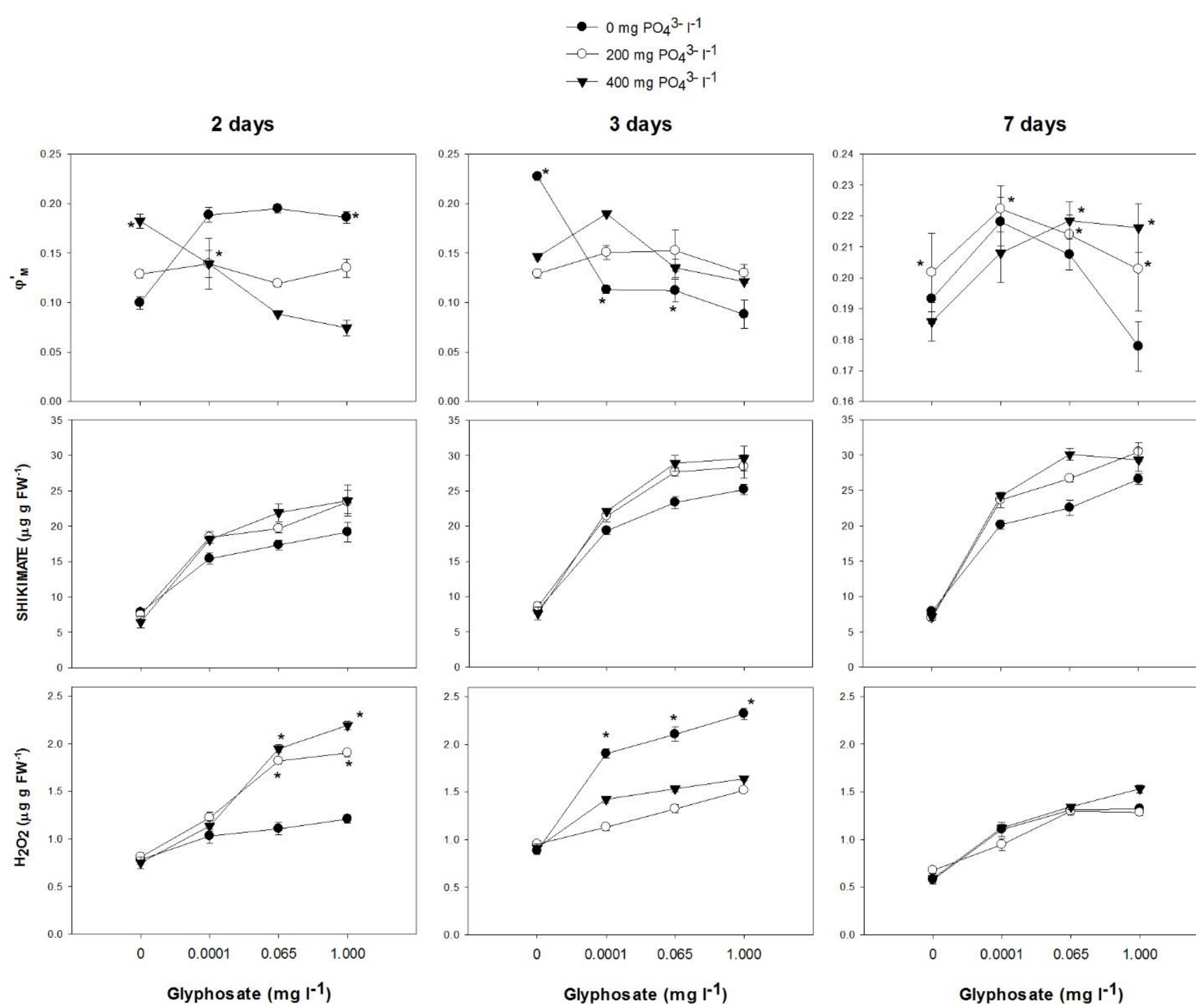

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Fig 5. Lipid peroxidation (MDA content, nmol g^{-1} FW), and activity of catalase (CAT, µmol H_2O_2 min^{-1} mg^{-1} protein), ascorbate peroxidase (APX µmol ascorbate min^{-1} mg^{-1} protein) and glutathione peroxidase (GPX, µmol glutathione min^{-1} mg^{-1} protein) in roots of willow plants grown for two, three and seven days in a nutrient solution amended with increasing doses of glyphosate (0, 0.001, 0.065 and 1.000 mg l^{-1}) and phosphate (0, 200 and 400 mg l^{-1}). Values are means ± SE of three replicates.

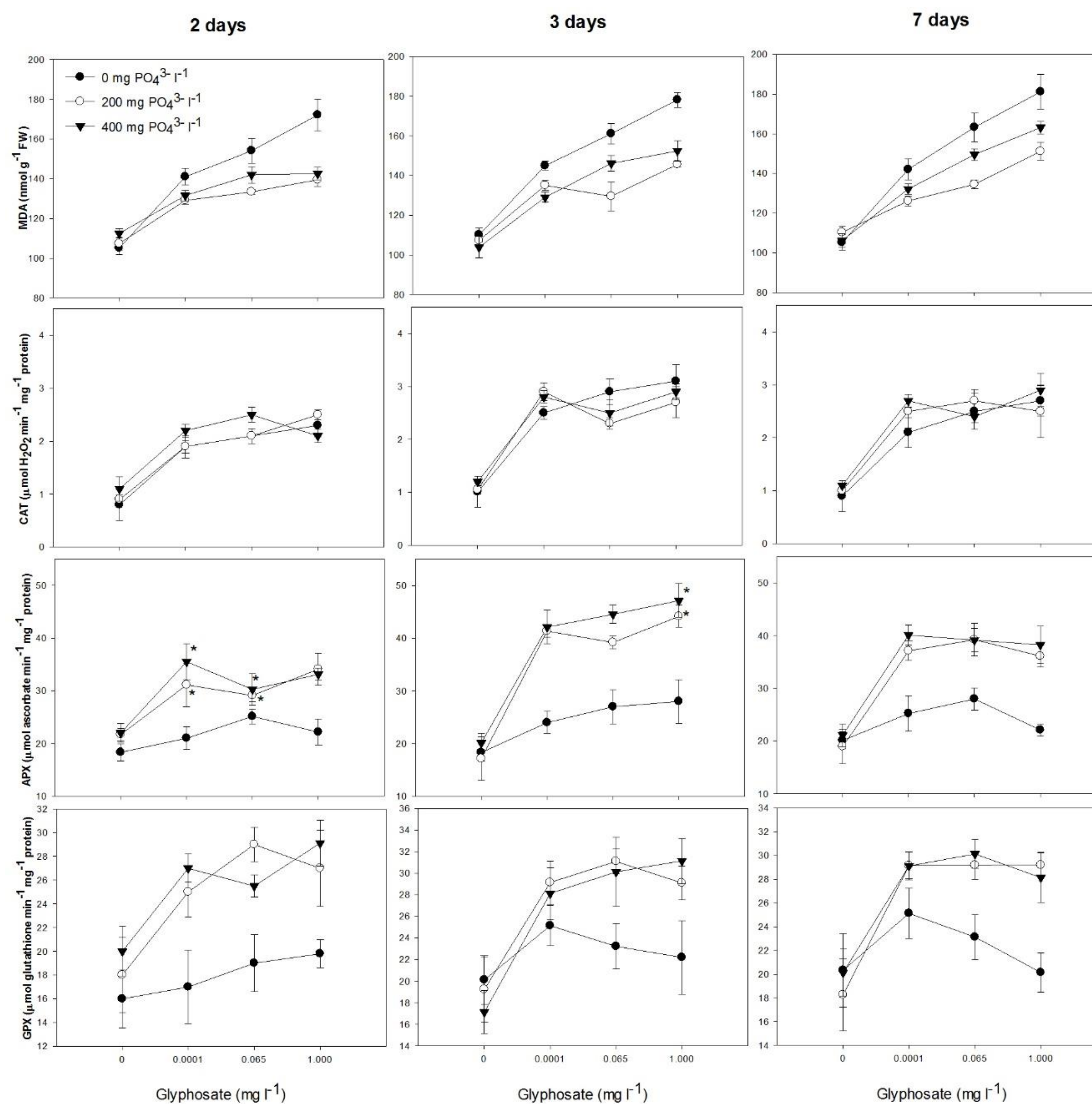

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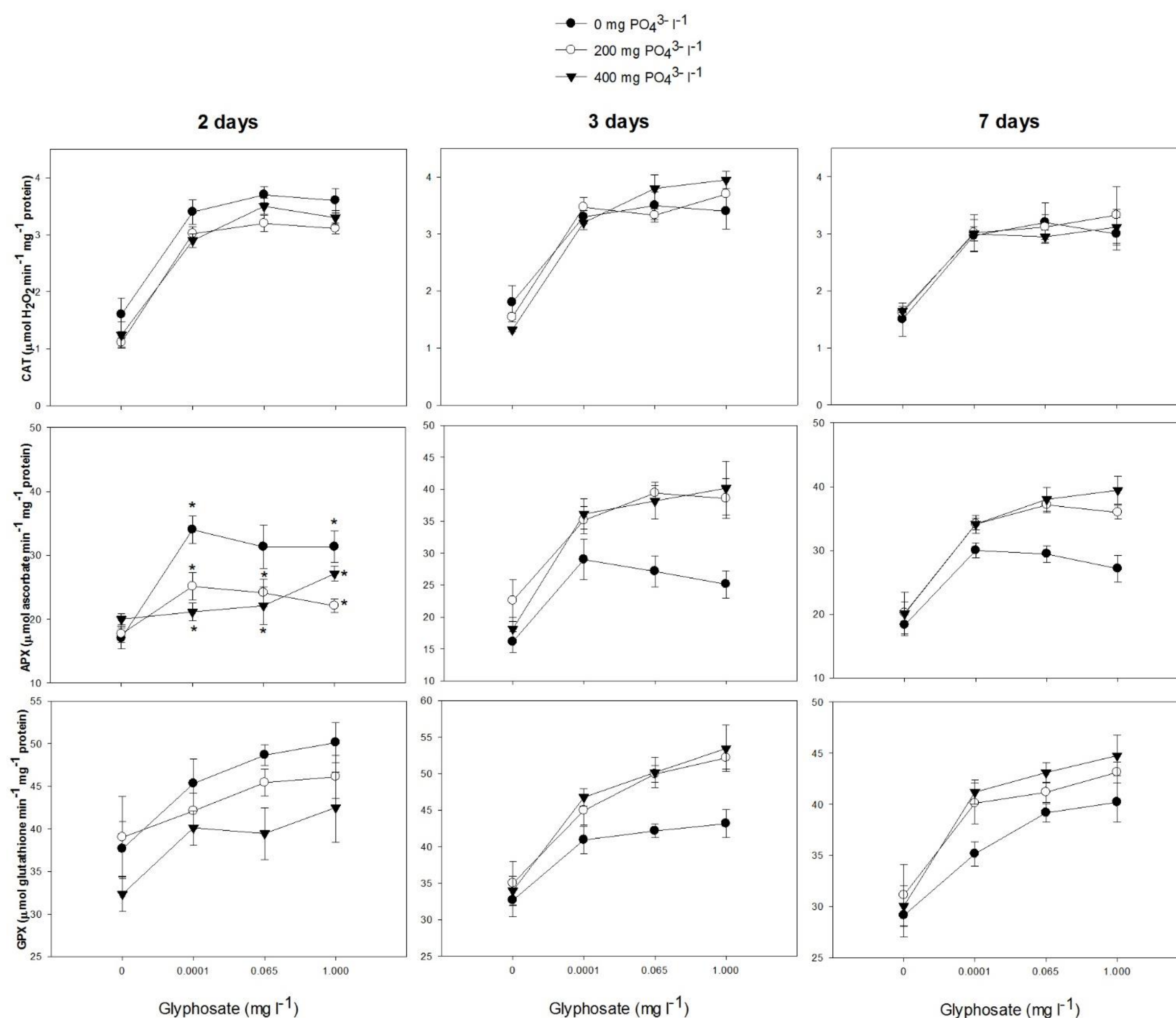
Fig 6. Activity of catalase (CAT, µmol H_2O_2 min^{-1} mg^{-1} protein), ascorbate peroxidase (APX µmol ascorbate min^{-1} mg^{-1} protein) and glutathione peroxidase (GPX, µmol glutathione min^{-1} mg^{-1} protein) in leaves of willow plants grown for two, three and seven days in a nutrient solution amended with increasing doses of glyphosate (0, 0.001, 0.065 and 1.000 mg l^{-1}) and phosphate (0, 200 and 400 mg l^{-1}). Values are means ± SE of three replicates. Values followed by *, within the same glyphosate and phosphorus concentration, are significantly different (P>0.05) by the Contrast test.



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Tables

Table 1. Total accumulation of glyphosate in willow plants grown for two, three and seven days in a nutrient solution amended with increasing doses of glyphosate (0, 0.001, 0.065 and 1.000 mg l^{-1}) and phosphate (0, 200 and 400 mg l^{-1}).

Glyphosate (mg l^{-1})	Phosphate (mg l^{-1})	Total accumulation (µg DW $plant^{-1}$)		
		2 days	3 days	7 days
0	0	-	-	-
	200	-	-	-
	400	-	-	-
0.001	0	17.48	12.38	16.89
	200	31.57 (180.58)	31.18 (251.77)	27.82 (164.72)
	400	40.94 (232,48)	40.55 (327.39)	27.66 (163.75)
0.065	0	130.98*	78.97*	154.30*
	200	229.28 (175.04)	299.94 (379.80)	253.28 (164.14)
	400	402.84 (307.55)	232.00 (293.77)	306.14 (198.40)
1.000	0	186.93*	146.84*	160.90*
	200	378.46 (20245)	314.81 (214.38)	288.12 (179.07)
	400	533.26 (285.26)	358.50 (244.14)	455.42 (283.04)

Treatment means (n = 3). Values followed by * within the same glyphosate concentration, are significantly different (P>0.05) by the contrast test. Values in the brackets represent the % of increase in total accumulation by PO_4^{3-} treatment in relation to their respective PO_4^{3-} unfertilized controls.

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Table 1S: Repeated-measures ANOVA for the effects of addition of phosphate (mg l^{-1}) and glyphosate (mg l^{-1}) and time of exposure (days) on total phosphorus (P; mg g DW^{-1}), glyphosate and AMPA concentrations (µg g FW^{-1}) in roots and leaves and on total glyphosate accumulation (µg DW $plant^{-1}$) in willow.

Source of Variation	D.F	Roots			Leaves			Total accum.
		P	Gly	AMPA	P	Gly	AMPA	
PO_4^{3-}	2	<0.0001*	<0.0001*	0.0088*	<0.0001*	<0.0001*	<0.0001*	<0.0001*
Gly	3	0.0297*	<0.0001*	<0.0001*	0.3110	<0.0001*	<0.0001*	<0.0001*
PO_4^{3-} x Gly	6	0.4615	0.0005*	0.2926	0.1414	0.0004	0.1292	<0.0001*
Time	2	<0.0001*	<0.0001*	<0.0001*	0.0461*	<0.0001*	0.0218*	<0.0001*
Time x PO_4^{3-}	4	0.2850	<0.0001*	<0.0001*	0.0047*	<0.0001*	<0.001*	<0.0001*
Time x Gly	6	<0.0001*	<0.0001*	<0.0001*	0.0319*	<0.0001*	0.3907	<0.0001*
Time x PO_4^{3-} x Gly	12	0.0701	<0.0001*	<0.0001*	0.0006*	<0.0001*	0.0005*	<0.0001*

D.F. Degrees of freedom

*Significant

ACCEPTED MANUSCRIPT

Table 2S: Repeated-measures ANOVA for the effects of addition of phosphate (mg l^{-1}) and glyphosate (mg l^{-1}) and time of exposure (days) on PSII quantum yield (Φ'_M), hydrogen peroxide concentrations in leaves (H_2O_2) (µg g FW^{-1}) and ascorbate peroxidase (APX) activity (µmol ascorbate min^{-1} mg^{-1} protein) in roots and leaves.

Source of Variation	D.F	Φ'_M	H_2O_2.	APX	
				Roots	Leaves
PO_4^{3-}	2	0.0184*	0.0005*	<0.0001*	<0.0001*
Gly	3	<0.0001*	<0.0001*	<0.0001*	<0.0001*
PO_4^{3-} x Gly	6	0.0006*	0.0270*	0.0006*	0.0002*
Time	2	<0.0001*	<0.0001*	<0.0001*	<0.0001*
Time x PO_4^{3-}	4	<0.0001*	0.0002*	<0.0001*	<0.0001*
Time x Gly	6	<0.0001*	0.3354	<0.0001*	<0.0001*
Time x PO_4^{3-} x Gly	12	<0.0001*	0.0255*	<0.0001*	<0.0001*

D.F. Degrees of freedom

* significant