UNIVERSITÉ DU QUÉBEC À
MONTRÉAL

WILLOWS (Salix miyabeana SX64) AS A
POTENTIAL GLYPHOSATE-
PHYTOREMEDIATOR SPECIES:
UPTAKE AND PHYSIOLOGICAL
EFFECTS

THESIS SUBMITTED
IN PARTIAL FULFILLMENT OF THE
REQUIREMENTS FOR THE
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IN ENVIRONMENTAL SCIENCES
OF THE UNIVERSITÉ DU QUÉBEC À
MONTRÉAL

BY
MARCELO PEDROSA GOMES
JUILLET 2015
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UNIVERSITÉ DU QUÉBEC À MONTRÉAL

LES SAULES (Salix miyabeana SX64) POUR LA PHYTOREMÉDIATION DU GLYPHOSATE : ASSIMILATION ET EFFETS PHYSIOLOGIQUES

THÈSE PRÉSENTÉE COMME EXIGENCE PARTIELLE DU DOCTORAT EN SCIENCES DE L’ENVIRONNEMENT DE L’UNIVERSITÉ DU QUÉBEC À MONTRÉAL

PAR MARCELO PEDROSA GOMES JUILLET 2015
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It is better to try, albeit in vain, than to just sit doing nothing until the end. I prefer to walk in the rain than to hide at home. I prefer to be happy and crazy than to live a normal and ordinary life.
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Glyphosate and AMPA effects in some plant physiological process (red "X"). Glyphosate main effect is due the inhibition of aromatic amino acids (tryptophan -Trp, phenylalanine -Phe and tyrosine -Tyr) production by inhibiting 5-enolpyruvylshikimate-3-phosphate (EPSPS) synthase. Glyphosate can affect photosynthesis by depriving Tyr contents and chelating metal ions important to photosystem (PS) structure and chlorophyll biosynthesis. Moreover, metal chelation deprives cells of important co-factors needed for enzymatic antioxidant system activity (superoxide dismutase - SOD, catalase - CAT and peroxidase - POX), leading to oxidative stress by accumulation of reactive oxygen species (superoxide -O₂⁻ and hydrogen peroxide -H₂O₂). Aminomethylphosphonic acid (AMPA) can also inhibit photosynthesis by inhibiting the synthesis of aminolevulinic acid (ALA), an important precursor of chlorophylls (see Figure 1.1).

Glyphosate and phosphorus (P) concentration of roots and leaves and glyphosate uptake rates of willows grown under different phosphate (PO₄³⁻) concentrations and treated with 0mM phosphonophormic acid (-PFA) or 5mM (+PFA).

Root cellular injury and lipid peroxidation in roots of willow plants grown under different phosphate (PO₄³⁻) concentrations and treated with 0mM phosphonophormic acid (-PFA) or 5mM (+PFA).

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<td>Abbreviation</td>
<td>Description</td>
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</tr>
<tr>
<td>ABA</td>
<td>Abscisic acid</td>
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<tr>
<td>ALA</td>
<td>δ-aminolevulinic acid</td>
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<tr>
<td>AMPA</td>
<td>Aminomethylphosphonic acid</td>
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<td>ANOVA</td>
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<td>DCMU</td>
<td>3-(3, 4 dichlorophenyl)-1, 1-dimethyl urea</td>
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<td>Dehydroascorbate</td>
</tr>
<tr>
<td>DHAR</td>
<td>Dehydroascorbate reductase</td>
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<tr>
<td>DNOC</td>
<td>4,6-dinotro-o-cresol</td>
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<tr>
<td>EPSPS</td>
<td>5-enolpyruvyshikimate-3-phosphate synthase</td>
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<tr>
<td>ETR</td>
<td>Electron transport rate</td>
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<td>Fv/Fm</td>
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\( I_k \) Minimum saturating irradiance
GSH Glutathione
GST Glutathione-S-transferase
GPX Glutathione peroxidase
GuPX Guaiacol peroxidase
GAT Glyphosate acyl transferase
GOX Glyphosate oxidoreductase
GR Glyphosate-resistant
GS Glyphosate-sensitive
H\(_2\)O\(_2\) Hydrogen peroxide
MDA Malondialdehyde
NPQ Non-photochemical quenching
OPPP Oxidative pentose phosphate pathway
PEP Phosphoenolpyruvate
Pheo Pheophytin \( a \)
P Phosphorus
PO\(_4\)\(^{-3}\) Phosphate
PPRLPI *Politique de protection des rives, du littoral et des plaines inondables*
PSI and PSII Photosystems I and II
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<td>Photochemical quenching</td>
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<td>$Q_A$</td>
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<td>RBS</td>
<td>Riparian buffer strip</td>
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<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
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<tr>
<td>$^1O_2$</td>
<td>Reactive singlet oxygen</td>
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RÉSUMÉ GÉNÉRAL

LES SAULES (Salix miyabeana SX64) POUR LA PHYTOREMÉDIATION DU GLYPHOSATE : ASSIMILATION ET EFFETS PHYSIOLOGIQUES

Depuis l’introduction en agriculture d’organismes résistants au glyphosate (GR), l’utilisation de produits à base de glyphosate est répandue mondialement. L’usage de plus en plus courant et souvent mal contrôlé d’herbicides constitue une nouvelle problématique d’autant que le glyphosate est aujourd’hui reconnu comme un polluant potentiel. Le glyphosate peut être facilement adsorbé par les particules du sol et présente une grande mobilité, ce qui contribue à son accumulation dans les diverses couches du sol. Plus encore, le glyphosate se caractérise par une grande solubilité ce qui facilite sa circulation dans le sol et entraîne son accumulation dans les eaux souterraines ou les rivières. À ceci, il faut ajouter que l’acide aminométhylphosphonique – AMPA (qui est le principal composé de dégradation du glyphosate) peut également s’accumuler dans le sol et les eaux et constituer un problème non moins grave pour l’environnement. C’est pourquoi il est urgent de mieux comprendre les effets toxiques du glyphosate et de l’AMPA et de développer des méthodes de mitigation environnementales de ces composés.

Dans ce travail, les effets glyphosate et la possible tolérance à cet herbicide ont été étudiés chez un cultivar de saule à croissance rapide (Salix miyabeana SX64). Les cultures intensives sur courtes rotations (CICR) de saules peuvent en effet être envisagées comme une technique économique pour mitiger cette problématique. Ainsi, l’étude qui a été conduite, en milieu contrôlé et en utilisant des approches de culture hydroponique, a permis de tester le potentiel de ces saules comme technologie verte pour limiter la diffusion du glyphosate dans l’environnement. Le rôle des transporteurs de $P_{04}^{-3}$ dans l’absorption du glyphosate au niveau racinaire des saules a été étudié. En utilisant des inhibiteurs de ces transporteurs, en l’occurrence (l’acide phosphonophormique-PFA), nous avons pu montrer le rôle de ces transporteurs de $P_{04}^{-3}$ dans l’assimilation du glyphosate. Considérant la fertilisation en $P_{04}^{-3}$ comme une pratique courante dans les grandes cultures, nous avons également étudié les effets du $P_{04}^{-3}$ sur l’absorption du glyphosate par les saules. La fertilisation en $P_{04}^{-3}$ favorise l’accumulation de glyphosate ce qui a pour effet de stimuler l’activité des systèmes antioxydants et d’atténuer les stress oxydatifs induits par le glyphosate tant dans les racines que dans les feuilles des saules. On conçoit ainsi que le $P_{04}^{-3}$ puisse être utilisé pour accentuer les capacités de phytoémédiation du glyphosate par les saules. Outre les effets inhibiteurs du glyphosate sur la voie métabolique du shikimate, nous avons également observé des effets délétères du glyphosate sur la photosynthèse des saules. C’est pour cette raison que nous avons étudié de plus près les mécanismes physiologiques affectés par une exposition au glyphosate (Facteur ©...
540), notamment sur les processus photosynthétiques des saules. Il s’agit d’une première démonstration du mode d’action du glyphosate sur le métabolisme de plante. Le glyphosate agissant sur la voie métabolique du shikimate, les processus photosynthétiques et des événements oxydatifs. Finalement, comme les plantes sont également exposées à l’AMPA suivant la dégradation du glyphosate, nous avons aussi étudié les effets différenciels comparatifs de ces deux composés sur le métabolisme chlorophyllien et la photosynthèse. Dans ce cas, nous avons montré que l’AMPA et le glyphosate déclenchaient différents mécanismes affectant négativement le contenu en chlorophylle et la photosynthèse des saules. Alors que l’AMPA perturbe la biosynthèse de la chlorophylle, le glyphosate agit sur la dégradation de celle-ci en provoquant des dommages oxydatifs et conséquemment une diminution du taux de photosynthèse.

En somme, nous pouvons conclure que les saules peuvent être utilisés pour remédier des sols contaminés par le glyphosate. Nous avons également montré que les effets délétères sont principalement reliés au fait que le glyphosate induise des stress oxydatifs. L’absorption du glyphosate par les racines des saules est fortement influencée par la présence de $\text{PO}_4^{3-}$ et les interactions entre l’un et l’autre peuvent entraîner des effets positif ou négatif sur l’environnement. Enfin, et compte tenu des mécanismes d’action mentionnés plus haut, il nous semble important d’étudier les effets de cet herbicide sur les organismes non ciblés.

**Mots-clés:** agriculture, acide aminomethylphosphonique, contamination, glyphosate, herbicide
ABSTRACT

Since the introduction of glyphosate-resistant (GR) plants in agriculture, glyphosate-based products are now the most used herbicide worldwide. The widespread and uncontrolled use of the herbicide has become a new global concern since glyphosate has been recognized as a potential environmental pollutant. Glyphosate's ability to adsorb to soil particles, associated with its high mobility through soil layers, has contributed to its accumulation in the soil. Moreover, glyphosate's high solubility facilitates its transport and it can rapidly reach groundwater and rivers. Additionally, aminomethylphosphonic acid – AMPA (the principal glyphosate by-product, which accumulates in the soil and water following glyphosate degradation) has been reported as being phytotoxic. Therefore, studies regarding the toxic effects of glyphosate and AMPA and the development of techniques to mitigate environmental contamination by glyphosate are urgently claimed.

Here, we examined glyphosate tolerance and glyphosate herbicidal effects on a fast growing willow cultivar (Salix miyabeana cultivar SX64). Willows grown in short-rotation coppices (SRC) have been considered as a cost-effective system to mitigate environmental cues. Therefore, under greenhouse conditions and using a hydroponic system, we assessed the potential of willows as a green technology option to limit glyphosate diffusion into the environment. Firstly, we evaluated the involvement of \text{Pho\textsuperscript{+}} transporters in glyphosate uptake by willow roots. By using a \text{PO}_4\textsuperscript{3-} transporter inhibitor (phosphonophormic acid-PFA) we shown the involvement of \text{Pho\textsuperscript{+}} transporters in glyphosate uptake by roots. As phosphate fertilization (\text{PO}_4\textsuperscript{3-}) is a common practice in agricultural fields submitted to glyphosate applications, we evaluated the effects of \text{PO}_4\textsuperscript{3-} addition in glyphosate uptake and toxicity in willows. By increasing the activity of antioxidant systems and alleviating glyphosate-induced oxidative stress in both roots and leaves, \text{PO}_4\textsuperscript{3-} fertilization increased glyphosate accumulation and assured photosynthesis rates. Therefore \text{PO}_4\textsuperscript{3-} fertilization could be used to assure the success of glyphosate-phytoremediation programs using willows. Apart to be an inhibitory of shikimate pathway, we observed glyphosate-deleterious effects in photosynthesis of willows. Therefore, we studied the physiological mechanisms involved in the deleterious effects of a glyphosate-based herbicide (Factor\textsuperscript{®} 540) on photosynthesis and related physiological processes of willow. For the first time, a glyphosate-based herbicide mode of action interconnecting its effects on shikimate pathway, photosynthetic process and oxidative events in plants was presented. Finally, as plants are expected to be exposed to AMPA through glyphosate degradation or via environmental matrices, we investigated the differential effects of glyphosate and AMPA on the chlorophyll metabolism and photosynthesis. We demonstrated that AMPA and glyphosate trigger different mechanisms leading to decreases in chlorophyll content and photosynthesis in willow plants. While AMPA
disturbed chlorophyll biosynthesis, glyphosate induced chlorophyll degradation through oxidative damages, both resulting in decreased photosynthetic rates.

In summary, we conclude that willows can be used in programs to remediate glyphosate-contaminated soils and that its deleterious effects on plants are strongly linked to induced oxidative stress. Our results indicate that PO₄³⁻ and glyphosate have strong interactions concerning the herbicide uptake by roots which can result in positive or detrimental effects to the environment. In addition, as an oxidative-stress inductor, it is urgent to study the effects of herbicide in non-target organisms.

**Key-Words:** agriculture, aminomethylphosphonic acid, contamination, glyphosate, herbicide
GENERAL INTRODUCTION

The intensification of agriculture production as projected for the near future will have major detrimental impacts on the global environment, including both terrestrial and aquatic non-agricultural ecosystems (Tilman, 1999). The doubling of food production in recent years has been associated with tremendous increases in the use of agricultural supplements and the land areas under cultivation (Tilman, 1999), focusing attention on the risks of fertilizer inputs (i.e., nitrogen and phosphate) that can greatly impact freshwater and marine ecosystems through eutrophication (Stow et al., 2014) – although the scientific community has more recently directed its attention to another potential environmental contaminant: herbicides.

The chemical control of weeds is a relatively recent technology that was initiated at the turn of the twentieth century (Cobb and Reade, 2010). Dangerous and corrosive chemicals such as copper sulphate, calcium cyanamide, sodium chlorate, and sulphuric acid were initially used in weed control (Cobb and Reade, 2010), and the first synthetic herbicide, DNOC (4,6-dinitro-o-cresol), was produced in 1932 in France and offered the potential for selective control of annual weeds among cereal crops (Cobb and Reade, 2010). The production of synthetic herbicides has subsequently increased greatly, and is now a tremendously large business (Reynolds et al., 2014). Due to their indiscriminate use, however, herbicides are now considered among the most hazardous pollutants worldwide (Gavrilescu et al., 2014).

With the advance of biotechnology, the world is now confronting a new and revolutionary product: genetically engineered crops – with the promise that this new technology will ensure both food production and environmental gains through reductions in the use of herbicides. Since the introduction of glyphosate-resistant (GR) crops in 1996, glyphosate [N-(phosphonomethyl)glycine] has become the most widely used herbicide globally (Duke, 2011) for total vegetation control (Cobb,
1992). Glyphosate is as a broad-spectrum, non-selective systemic herbicide and its herbicidal effect is mainly due to the inhibition of 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), an enzyme in the shikimate pathway necessary for the biosynthesis of aromatic amino acids (Siehl, 1997a). Glyphosate-resistant crops were created by the stable integration of a transgene encoding for a glyphosate-insensitive EPSPS enzyme (Saroha et al., 1998). However, in contrast to often repeated claims by proponents, while glyphosate has been used in diverse situations, the volumes of herbicides applied to control weeds have continually increased (Benbrook, 2012).

Despite being described as quickly degraded by microbial activity (Monsanto, 2005), glyphosate's ability to adsorb to soil particles, associated with its high mobility through soil layers, has contributed to its accumulation in the soil (Beltrano et al., 2013). Moreover, glyphosate's high solubility facilitates its transport, it can rapidly reach the groundwater and rivers (Vereecken, 2005; Blanchoud et al., 2007). Attention has recently been directed to the presence of glyphosate in the environment and its hazardous effects on non-target organisms, such as algae (Annett et al., 2014), plants (Tesfamariam et al., 2009) and animals (Moreno et al., 2014). Increasing numbers of observations suggest a relationship between prolonged glyphosate application and negative effects on various non-target organisms in agro-ecosystems (Neumann et al., 2006). In this context, the development of techniques to mitigate glyphosate contamination of soil and water resources is of utmost importance.

Apart its effect shikimate pathway, secondary or indirect effects of glyphosate on plant physiology may be related to its mechanisms leading to plant death. This is also supported by the detrimental effects of glyphosate on animals, which do not possess the shikimate pathway, indicating that it can interact with metabolic pathways other than its primary target (EPSPS). As a metal chelator, glyphosate could deprive plants from important nutrients (Cakmak et al., 2009) which have important roles as enzymatic co-factors, biomolecular constituents, and antioxidative systems. Oxidative stress, more specifically lipid peroxidation, induced by glyphosate (Sergiev et al.,
2006; Ahsan et al., 2008), is known to severely damage the cell integrity which may lead to cell death. Moreover, increased ROS production can negatively interfere with photosynthetic processes, for example by decreasing the chlorophyll content, photochemical efficiency, and C metabolism, leading to reduction in plant growth. Indeed, deleterious effects of glyphosate in photosynthesis and chlorophyll contents (even in GR plants) have been reported (Zobiole et al., 2011b, 2012). Additionally, aminomethylphosphonic acid – AMPA (the principal glyphosate by-product, which accumulates in the soil and water following glyphosate degradation) has been reported as being phytotoxic (Cerdeira and Duke, 2006). Very few studies so far have focused on the effects of AMPA on plants (Reddy et al., 2004). A detailed overview of glyphosate and AMPA effects on plant physiology is given in Chapter 1. In summary, studies regarding the toxic effects of glyphosate and AMPA and the development of techniques to mitigate environmental contamination by glyphosate are claimed.

In 1996, the provincial government of Quebec adopted a waterway protection act (Politique de protection des rives, du littoral et des plaines inondables – PPRLPI) within a broader framework of urban development regulations grouped under the Loi sur l’aménagement et l’urbanisme. These regulations stipulate the creation and maintenance of riparian buffer strips (RBS) at least three meters wide (MDDEP). However, since the management practices and plant species to be maintained in these riparian strips have not yet been regulated, bare soil or soil covered by spontaneous vegetation are frequently considered as fulfilling these requirements – even if their efficiency in mitigating waterway contamination is questionable. Farmers also often consider the three meters reserved for RBS as an abusive loss of land that could better be used for agricultural production (Dagenais, 2014). Thus, a search for plant species that could both serve to reduce pollution and provide economic benefits to farmers would be welcome.
Willows (Salix miyabeana) grown in short-rotation coppices (SRC) – a management system in which fast growing woody crops are planted at high densities and harvested following short rotation cycles (Perttu, 1998) – are often used for bioenergy production in Northern Europe and North America. Experimental yields of 12 to 20 oven-dry tons (odt) ha⁻¹ y⁻¹ have been reported in SRC using willows (Labrecque and Teodorescu, 2003; Adegbidi et al., 2003), although typical yields and those of commercial plantations are often considerably lower (about two to six odt ha⁻¹) (Keoleian and Volk, 2005). Nevertheless, SRC using willows is still considered a cost-effective technology (Keoleian and Volk, 2005). This cropping system is also known for its effective remediation of many environmental issues, including soil decontamination and organic waste disposal (Mirck et al., 2005; Kuzovkina and Volk, 2009a; Cavanagh et al., 2011). Additionally, this woody crop cultivation practice can contribute to soil quality by improving soil organic matter contents (Zan et al., 2001), improving soil structure, minimizing disturbances, conserving soil and water, and enhancing nutrient cycling (Guidi et al., 2013). In this context, willow SRC could be indicated to compose RBS in Quebec’s agricultural lands, which may represent both a source of carbon-neutral fuel and a green technology for mitigating water contamination by agricultural wastes.

Two experimental RBS stands planted with the willow cultivar S. miyabeana SX64 at densities of either 33,000 or 55,000 stems/ha were established in 2009 at the interface of agricultural fields and the north bank of the St Lawrence River (Quebec, Canada). During the following years (from 2011 to 2013) farmers planted GR-maize and soy in rotation in the experimental fields and applied agrochemicals (including a glyphosate-based herbicide) in accordance with their usual agricultural practices. The growth and development of the willow plants were followed for three years, and it was observed that the willows adapted well to the stress caused by crop cultivation, producing high biomass yields (Hénault-Ethier et al., 2014).
Based on these findings, we examined glyphosate tolerance and glyphosate herbicidal effects on *S. miyabeana* SX64 to assess the potential of using these willows as a green technology option to limit glyphosate diffusion into the environment; we also investigated the underlying physiological actions of this chemical in willow plants. In this context, the following objectives (questions) and hypothesis were formulated:

a) **How is glyphosate taken up by roots?** It has been proposed that glyphosate competes with phosphate ($$\text{PO}_4^{3-}$$) for protoplast membrane carriers (Denis and Delrot, 1993; Morin *et al.*, 1997), and we tested to determine if this same energy intensive transport system is used in roots;

b) **Does $\text{PO}_4^{3-}$ have a role in the phytoremediation capacity of willow plants?** As $\text{PO}_4^{3-}$ has been shown to control glyphosate adsorption and its availability in the soil solution (Bott *et al.*, 2011) and to alleviates oxidative stress in plants (Gomes *et al.*, 2013, 2014), we investigated the effects of $\text{PO}_4^{3-}$ on glyphosate uptake and its toxicity to willow plants;

c) **How does glyphosate affect plant photosynthesis?** Despite its specific site of action, glyphosate can affect other physiological processes in plants, such as photosynthesis, and can induce oxidative stress (Sergiev *et al.*, 2006; Miteva *et al.*, 2010). It is not known, however, how the inhibition of the shikimate pathway is related to oxidative stress. In this context, we sought to determine if the deleterious effects of glyphosate on photosynthesis is related to glyphosate-induced oxidative stress in willow leaves;

d) **Do aminomethylphosphonic acid (AMPA) and glyphosate trigger different mechanisms leading to decreases in chlorophyll content and photosynthesis in willow plants?** It has been argued that AMPA (the main glyphosate by-product) is responsible (and not glyphosate itself) for the deleterious effects observed on chlorophyll biosynthesis in GR
soybeans (Reddy *et al.*, 2004; Ding *et al.*, 2011). We hypothesized that both chemicals could be responsible for the deleterious effects on the photosynthetic apparatus of willows, and we therefore examined the differential effects of glyphosate and AMPA on chlorophyll metabolism and photosynthesis.

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CHAPTER I

ALTERATION OF PLANT PHYSIOLOGY BY GLYPHOSATE AND ITS BY-PRODUCT AMINOMETHYLPHOSPHONIC ACID (AMPA), AN OVERVIEW¹


ABSTRACT

It is generally claimed that glyphosate kills undesired plants by affecting the 5-enolpyruvylshikimate-3-phosphate (EPSPS) synthase enzyme, disturbing the shikimate pathway. However, the mechanisms leading to plant death may also be related to glyphosate secondary or indirect effects on plant physiology. Moreover, some plants can metabolize glyphosate to aminomethylphosphonic acid (AMPA) or be exposed to AMPA from different environmental matrices. AMPA is a recognized phytotoxin, and its co-occurrence with glyphosate could modify glyphosate effects on plant physiology. The present review provides an overall picture of alterations of plant physiology caused by environmental exposure to glyphosate and its metabolite AMPA, and summarizes their effects on several physiological processes, with a special emphasis on photosynthesis, from photochemical events to carbon assimilation and translocation, and oxidative stress in plants. In an interconnected manner, the glyphosate and AMPA effects on several plant physiology processes were linked aiming to better understand their phytotoxicity and glyphosate herbicidal effects.

Keywords: AMPA, glyphosate, herbicide exposure, oxidative stress, photosynthesis, plant nutrition; phytotoxicity

1.1 INTRODUCTION

Glyphosate [N-(phosphonomethyl)glycine] is currently the most widely used herbicide in the world and its use in agriculture has tremendously increased since the introduction of glyphosate-resistant (GR) plants (Duke and Powles, 2008). The glyphosate molecule was first synthesized by Henri Martin from Cilag, a small Swiss pharmaceutical company, but was only tested as an herbicide in 1970 by John E. Franz of Monsanto Co. (Franz et al., 1997). In 1974, after patented for herbicide use, glyphosate (as isopropylamine salt of glyphosate) reached the market as a post-emergence, non-selective herbicide (Duke and Powles, 2008), and has been used for total control of vegetation (Cobb, 1992), since it is a broad-spectrum, non-selective systemic herbicide.

In agricultural fields, glyphosate is sprayed on plant foliage; however a portion of the chemical can be deposited directly to the soil surface or blown by the wind to neighboring soils and plants, leading to glyphosate exposure of non-target
Plants. Moreover, the waterway contamination is a source of glyphosate transfer to adjacent agricultural field, especially in fields irrigated through pumping into surface water bodies. Another important source of glyphosate exposure is the exudation from roots of sprayed plants (Coupland and Caseley, 1979) and its release from dead plants (Neumann et al., 2006). Recent studies suggested a risk of glyphosate toxicity to non-target plants due to rhizosphere transfer of glyphosate (Tesfamariam et al., 2009). Once in soil, glyphosate is adsorbed on soil particles, degraded by microbes or can migrate in deeper soil horizons via soil pores or root canals. However, some agricultural practices, such as phosphorous amendments may re-solubilize glyphosate in soils, making it available for leaching (Borggaard and Gimsing, 2008) and available to the rhizosphere of non-target plants.

 Nowadays, a number of studies have focused on the environmental presence of the major glyphosate metabolite, the aminomethylphosphonic acid (AMPA). Having a short half-life, glyphosate is quickly degraded to AMPA in soils by microorganism (Franz et al., 1997; Van Eerd et al., 2003). A similar mechanism of glyphosate degradation has been proposed in plants (Reddy et al., 2004). Therefore the co-occurrence of glyphosate and AMPA is expected in plant tissues due to glyphosate degradation and/or AMPA uptake from environmental matrices. Upon penetrating the plant tissues, glyphosate will reach active metabolic sites, such as root and shoot meristems after being translocated through vascular tissues (Satchivi et al., 2000), following the same pathway as photoassimilates (Monquero et al., 2004). Similarly, AMPA can also be translocated to diverse plant tissues (Reddy et al., 2004). Therefore, plant organs such as nodules, root tips and shoot apex, which show high metabolism and growth, represent important sinks for glyphosate/AMPA (Hetherington et al., 1999; Feng et al., 2003).

Glyphosate herbicidal effects are due the inhibition of the 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), an enzyme from the shikimate pathway, which leads to prevention of the biosynthesis of the amino acids
phenylalanine, tyrosine and tryptophan (Siehl, 1997a). Even though the changes in physiological plant processes induced by glyphosate and AMPA have not been considered as primary effects, they could contribute to its herbicidal effects. In addition, physiological processes of GR-plants may also be affected by glyphosate. Although at a lower extent compared to glyphosate-sensitive (GS) plants, glyphosate effects on photosynthesis, mineral nutrition and oxidative events in GR-plants have been reported (Henrique Saes Zobiole et al., 2009; Zobiole et al., 2010d, 2012). In addition, AMPA is a recognized phytotoxin (Reddy et al., 2004) whose exact mechanism of action has not been yet completely elucidated. Once present in plant tissues AMPA can contribute to glyphosate effects on physiological processes. Moreover, Reddy et al. (2004a) attested that glyphosate induced injuries in GR-plants is due to AMPA formed from glyphosate degradation. The glyphosate-sensitivity of GR-plants is widely variable depending on GR-plant species and cultivars. So, it is intrinsically important to elucidate the glyphosate and AMPA effects on physiological processes related to plant growth and production in order to better understand glyphosate-herbicidal mechanism and its possible effects on non-target plants.

Glyphosate has been shown to affect plant physiological mechanisms such as photosynthesis, carbon metabolism, mineral nutrition and oxidative events, and to disturb plant-microorganism interactions (Kremer and Means, 2009; Kielak et al., 2011; Zobiole et al., 2012). Despite the fact that AMPA has been less studied, it was shown to affect chlorophyll biosynthesis and to cause plant growth reductions (Reddy et al., 2004; Serra et al., 2013). In this review, we provide an overall picture of glyphosate and AMPA effects on plant primary physiological processes, with a special emphasis on photosynthesis, from photochemical events to carbon assimilation and translocation, and oxidative stress in plants.
1.2. UPTAKE AND TRANSLOCATION OF GLYPHOSATE AND AMPA IN PLANTS

In leaves, the uptake of glyphosate is a biphasic process which involves a rapid initial penetration through the cuticle, followed by a slow uptake through the symplast (Monquero et al., 2004). Entrance into the symplast could be either through a passive diffusion mechanism that is not affected by pH (Gougler and Geiger, 1981), or by an endogenous transport system, possibly a phosphate carrier within the cellular membrane (Burton and Balke, 2012). The duration of this process depends on several factors such as species and plant age, environmental conditions and herbicide concentration (Monquero et al., 2004). Environmental factors are also known to affect glyphosate absorption. For example, glyphosate absorption is modulated by any factors that alter the water potential of plants (such as soil moisture and relative humidity) (Sharma and Singh, 2001), the production of cuticular wax (such as low light intensity) (Franz et al., 1997) or its hydration and the mineral absorption through influencing transpiration rate (such as temperature) (Sharma and Singh, 2001).

After penetrating the leaves, glyphosate will reach active metabolic sites, such as root and shoot meristems after being translocated to vascular tissues (Satchivi et al., 2000). Glyphosate's movement through phloem follows the same pathway as other photoassimilates, which are produced in photosynthetically active tissues and migrate towards growth or storage plant tissues such as roots, tubers, rhizomes, young leaves and meristematic zones (Monquero et al., 2004). Plant organs with high metabolism and growth, such as nodules, root tips and shoot apex, represent important sinks for glyphosate (Cakmak et al., 2009). As for the photoassimilates, glyphosate translocation to these tissues changes during the plant life cycle (Monquero et al., 2004). Using $[^{14}\text{C}]$glyphosate, Feng et al. (Feng et al., 2003) demonstrated that in Abutilon theophrasti, 45 and 34% of the glyphosate absorbed after foliar application was translocated to roots and meristem, respectively. Low
inhibition thresholds (0.23 and 0.21 ppm glyphosate, respectively) were measured in these tissues indicating glyphosate phytotoxicity. In contrast, stem showed much higher inhibition threshold (8.4 ppm). Moreover, authors observed a linear relationship between glyphosate dose and tissue concentration, but, the tissue distribution pattern was independent of the dose used. Even at low foliar application rates, the sink tissues accumulate glyphosate at very high concentrations, and a single foliar application of glyphosate at a rate of 0.5 kg ha$^{-1}$ can lead to glyphosate accumulation up to 0.05 ppm in the sink organs (King et al., 2001).

A new topic of interest in glyphosate translocation in plants concerns the exudation from roots to soil which has been observed in some plant species (Neumann et al., 2006; Laitinen et al., 2007). It was shown that following exudation, glyphosate can inhibit growth of adjacent plants and seedlings (Kremer et al., 2005). For example, a glyphosate-resistant soybean plant may exude 1500 ng of glyphosate in the rhizosphere in 16 days (Kremer et al., 2005). Using $^{14}$C-glyphosate, Ricoldi et al. (2007) reported the translocation of glyphosate from target to non-target plants. However, the non-target plants showed little growth inhibition in relation to the decrease observed in the target plant, and so, the growth of non-target plants was considered not to be affected by the amount of glyphosate exuded through the rhizosphere of nearby plants (Ricordi et al., 2007). Laitinen et al. (2007) observed that after 8 days, 12% of the glyphosate applied on leaves of Chenopodium quinoa was detected in roots and about 4% in the surrounding soil. Two weeks later, 8 to 12% of the applied glyphosate was detected in soil samples. According to these authors, the same results were found in field studies, where the plant roots contribute to the glyphosate amounts detected in soil residues. Glyphosate exudation by plant roots can also be considered as an environmental source of glyphosate exposure for non-target plants and should be included into risks assessment models.

The results of Ricoldi et al. (2007) presented above also confirm glyphosate uptake through a root pathway. However, in soil, glyphosate has been proposed to be
quickly degraded to AMPA (Franz et al., 1997; Van Eerd et al., 2003). Due their chemical similarities, both glyphosate and AMPA are expected to be up taken from soil solution. Once in root tissues, glyphosate and AMPA can reach active metabolic sites such as the shoot meristems through xylem conduction. Despite root uptake of glyphosate and AMPA could also constitute an important source of these molecules to plants, information regarding glyphosate root uptake are scarce and to date, and no study has been performed with AMPA. As AMPA phytotoxic effects was found even in GR-plants (Reddy et al., 2004a), this studies are urgently needed.

1.3. ALTERATION OF PLANT PHYSIOLOGY BY GLYPHOSATE/AMPA

Glyphosate has been shown to affect several plant physiological processes which could also be linked to glyphosate-herbicidal effects. Reinforcing this statement, some studies have contested the glyphosate effects strictly due to EPSPS inhibition, as depletion of aromatic amino acids were not verified in glyphosate treated plants (Wang, 2001; Serra et al., 2013). As a metal chelator, glyphosate could deprive plants of important nutrients which have major roles as enzymatic co-factors and biomolecules constituents. Additionally, AMPA is known to be by itself a phytotoxin, which could, in addition to its own effects, amplify glyphosate indirect effects on physiological process. On the other hand, due its chemical similarity, AMPA can compete with glycine in biological sites and pathways, affecting among others chlorophyll biosynthesis and, thus, the photosynthetic process.

1.3.1 Photosynthesis

Photosynthesis is the major biochemical process occurring in photoautotroph organisms and is known to be affected by various anthropogenic factors. Some herbicides were found to interrupt directly photosynthetic electron transport. For example, 3-(3, 4 dichlorophenyl)-1, 1-dimethyl urea (DCMU) is known to block the electron flow between QA and QB, by competing for QB binding sites (Tóth et al., 2005). Other herbicides, such as glyphosate, will affect photosynthesis indirectly by
inhibiting the biosynthesis of carotenoids, chlorophylls, fatty acids or amino acids (Fedtke and Duke, 2005). As an EPSPS competitive inhibitor, glyphosate blocks the shikimate pathway inhibiting the biosynthesis of secondary metabolites in plants, including compounds related to photosynthesis, such as quinones (Dewick, 1998). However, it is not clear how glyphosate leads to plant death, and hypothesis such as depletion of protein stock and drainage of carbon from other vital pathways have been advanced (Duke and Powles, 2008). A closer look at glyphosate’s effects on photosynthetic processes may shed light on this hypothesis. Indeed, numerous field and greenhouse studies have indicated a decreased photosynthetic rate in plants following glyphosate exposure (Mateos-Naranjo et al., 2009; Zobiole et al., 2012; Yannicciar et al., 2012).

1.3.1.1 Chlorophyll biosynthesis

The first step in photosynthesis occurs in the thylakoid membranes of chloroplasts, by light excitation of photosynthetic pigments, including chlorophylls. This initiates the electron flow, where electrons are transferred from a special pair of chlorophyll $a$ to pheophitin, the primary electron acceptor (Rohacek et al., 2008). Some studies reported a decreased chlorophyll content in plants after glyphosate application due to the degradation or the inhibition of chlorophyll biosynthesis (Mateos-Naranjo et al., 2009; Zobiole et al., 2011b; Huang et al., 2012). Glyphosate may prevent chlorophyll synthesis indirectly by decreasing the magnesium content in leaves, as shown by Cakmak et al. (Cakmak et al., 2009), which leads to a decreased chlorophyll content and photosynthetic rate (Zobiole et al., 2012). Indeed, the incorporation of Mg by magnesium chelatase in the porphyrin structure is a necessary step leading to the synthesis of chlorophyll molecules (Tanaka and Tanaka, 2007). Cakmak et al. (Cakmak et al., 2009) demonstrated that foliar application of glyphosate will decrease concentrations of cations in shoots and seeds of glyphosate-sensitive soybeans. Similarly, by inducing iron (Fe) deficiency, glyphosate may prevent the biosynthesis of aminolevulic acid (ALA), a component of the chlorophyll
biosynthetic pathway (Marsh et al., 1963). Catalase and peroxidase, both enzymes implicated in ALA biosynthesis, are highly sensitive to Fe deprivation (Marsh et al., 1963). Glyphosate is a strong cation chelator, due to its carboxyl and phosphonate groups, forming complexes with nutrients in plant tissues, thus making them unavailable for biological processes, including photosynthesis (Cakmak et al., 2009). In addition, glyphosate was proposed to interfere on ALA biosynthesis by controlling the conversion of alpha-ketoglutarate to ALA and/or the condensation of glycine with succinyl-CoA to form ALA and carbon dioxide (Kitchen, 1980).

Interestingly, Reddy et al. (2004a) reported that glyphosate's deleterious effects on chlorophyll biosynthesis are primarily dependent on the degradation rate of glyphosate into AMPA. In other words, AMPA (not glyphosate) was proposed to be responsible for the hazardous effects on chlorophyll biosynthesis, but by an unknown mechanism. Recently, Serra et al. (2013) showed decreased amounts of glycine, serine and glutamate in plants treated with AMPA. The lack of glycine/glutamate in AMPA-treated plants could thus reduce ALA and chlorophyll contents, since these amino acids are required during ALA biosynthesis through ALA-synthetase and γ,δ-dioxivalerate (DOVA) cycles, respectively (Fig. 1.1). Furthermore, we may advance, based on the ALA metabolic pathway that, due to its similarity with glycine, AMPA can affect ALA production by competing: 1) with glycine in the photorespiration process (leading to deprivation of glutamate contents) and/or 2) with the substrate of ALA-synthetase active site. However, further studies are needed to provide evidence on how exactly AMPA may alter chlorophyll biosynthesis.
AMP A effects on chlorophyll biosynthesis. Due to its similarities, AMP A is suggested to compete with glycine metabolic pathways leading to decreased aminolevulinic acid (ALA) production (an intermediary of chlorophyll biosynthesis). By competing with glycine for the enzyme active sites, AMPA can decrease glutamate content (via a decreased NH₃ content needed for glutamate synthesis) and serine production. Decreased glutamate contents could then contribute to lower ALA production by reducing the content in γ,δ-dioxivalerato. Similarly, competing with glycine for the active site of the ALA-synthetase, AMPA can also affect ALA production.

Similarly, in the case of plants without contact with AMPA and devoided of glyphosate oxidoreductase (the enzyme responsible for glyphosate degradation into AMPA), glyphosate can induce a decrease in the chlorophyll content by depriving nitrogen assimilation (Zobiole et al., 2011b), which will lead to reduced glutamate production, affecting ALA and chlorophyll biosynthesis as shown previously by Kitchen (1980).
1.3.1.2 Photochemical reactions

After light excitation of chlorophyll molecules in the light harvesting complexes, the energy is transferred to the photosystems I (PSI) and II (PSII) reaction centers (Rohacek et al., 2008). PSII reaction center is formed by two monomers, D1 and D2 proteins, with chlorophyll \(a\) complex (P680), pheophytina (Pheo) and plastoquinone (QA) as cofactors (Kern and Renger, 2007). The D1/D2 heterodimer possess two redox tyrosine subunits TyrZ (YZ) and TyrD (YD) (Kern and Renger, 2007). Vivancos et al. (2011) have shown that glyphosate affects the abundance of proteins associated to PSII by disrupting the aromatic amino acid biosynthesis, including tyrosine, in GS soybeans. TyrZ is actively implicated in the electron transport chain as an electron donor from the manganese cluster (Mn-cluster) to the oxidized P680 complex. The Mn-cluster is the enzymatic complex involved in the water photolysis process. It oxidizes two water molecules, releasing \(O_2\), four protons (\(H^+\)), and four electrons entering the photosynthetic electron transport chain (Zouni et al., 2001). The released protons contribute to the proton gradient in the thylakoid membrane, leading to adenosine triphosphate (ATP) synthesis (Kern and Renger, 2007). Limited capacity of electron transport after glyphosate exposure was shown by Vivancos et al. (2011), but the implication of the amino acid tyrosine was not clear. Another factor that has to be considered is the Mn-cluster structure. This cluster contains four Mn ions, one calcium (Ca) ion, five oxygen atoms and water molecules (Zouni et al., 2001). There is no evidence that glyphosate will disturb the formation of the Mn-cluster associated to PSII, however one could reasonably expect an effect since glyphosate is known to be a strong metal chelator, forming stable complexes with Mn and Ca in plants, which become unavailable for biological processes (Cakmak et al., 2009). Reducing the availability of PSII associated metals and amino acids could reduce its capacity to transfer light energy into the electron transport chain, and may explain the reduced photosynthetic activity observed when plants are exposed to glyphosate.
It was demonstrated that chlorophyll a fluorescence measurements can be used to evaluate plant photosynthetic performance and therefore is useful to study direct and indirect effects of herbicides on photosynthesis (Juneau et al., 2007). The effects of glyphosate on photosynthesis (evaluated by chlorophyll fluorescence kinetics) have been investigated by some authors, using in vitro and in vivo studies (Table 1.1). In these studies, glyphosate was shown to inhibit PSII activity, electron transport rate, and non-photochemical energy dissipation processes. In addition to these effects, glyphosate can also alter the activity of PSI (Muñoz-Rueda et al., 1986) and decrease NADH and NADPH pools (Vivancos et al., 2011). On the other hand, some studies revealed no effects of glyphosate on photosynthetic activity using chlorophyll a fluorescence measurements (Cañero et al., 2011). Differences observed among these studies may be due to methodological differences. For example, Cañero et al. (Cañero et al., 2011) treated young olive trees (Olea europaea) by adding glyphosate to their soil medium, while other fluorescence studies on superior plants (Vivancos et al., 2011; Zobiole et al., 2012) used foliar applications. Ralph (Ralph, 2000), meanwhile, did his experiment using an immersed seagrass, Halophila ovalis, planted in sandy loam sediments, in a media with liquid IPA glyphosate salts diluted in filtered seawater, using a recirculating flow-through system, with chlorophyll a measurements being done underwater. This methodology is quite different and this may explain the differences among the effects of glyphosate on the measured photosynthetic parameters.
Table 1.1 Glyphosate effects on plants, measured by chlorophyll a fluorescence

<table>
<thead>
<tr>
<th>Effect</th>
<th>Parameters</th>
<th>Description</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>↑</td>
<td>$F_0$</td>
<td>Minimal fluorescence level</td>
<td>Zobiole et al. (2011a); Choi et al. (2012)</td>
</tr>
<tr>
<td>↓</td>
<td>$F_m$</td>
<td>Maximum fluorescence level</td>
<td>Mateos-Naranjo et al. (2009); Zobiole et al. (2011a); Vivancos et al. (2011); Huang et al. (2012); Yannicciari et al. (2012)</td>
</tr>
<tr>
<td>↓</td>
<td>$Q_P$</td>
<td>Photochemical quenching</td>
<td>Mateos-Naranjo et al. (2009); Vivancos et al. (2011); Yannicciari et al. (2012); Choi et al. (2012)</td>
</tr>
<tr>
<td>↓</td>
<td>$F_r/F_m$</td>
<td>Maximum quantum efficiency of PSII</td>
<td>Mateos-Naranjo et al. (2009); Vivancos et al. (2011); Huang et al. (2012); Zobiole et al. (2011a)</td>
</tr>
<tr>
<td>↑</td>
<td>NPQ or qN</td>
<td>Non-photochemical quenching</td>
<td>Huanco-Rueda et al. (1986); Zobiole et al. (2011a); Huang et al. (2012); Yannicciari et al. (2012)</td>
</tr>
<tr>
<td>↓</td>
<td>NPQ or qN</td>
<td>Non-photochemical quenching</td>
<td>Mateos-Naranjo et al. (2009); Zobiole et al. (2011a)</td>
</tr>
<tr>
<td>↓</td>
<td>ETR</td>
<td>Electron transport rate</td>
<td>Mateos-Naranjo et al. (2009); Zobiole et al. (2011a); Huang et al. (2012); Yannicciari et al. (2012)</td>
</tr>
<tr>
<td>↓</td>
<td>$\phi_{PSII}$</td>
<td>Quantum efficiency of PSII</td>
<td>Zobiole et al. (2011a); Huang et al. (2012); Yannicciari et al. (2012)</td>
</tr>
<tr>
<td>↓</td>
<td>$F_v/F_m'$</td>
<td>Intrinsic efficiency of PSII</td>
<td>Zobiole et al. (2011a); Huang et al. (2012); Yannicciari et al. (2012)</td>
</tr>
</tbody>
</table>

Upward- and downward-pointing arrows indicate increased or decreased of the parameter, respectively.
1.3.1.3 Carbon metabolism

Glyphosate and AMPA also affect photosynthesis by modifying carbon metabolism in plants. It was reported that net carbon exchange and stomatal conductance was decreased after foliar application of glyphosate and AMPA (Mateos-Naranjo et al., 2009; Zobiole et al., 2011b; Ding et al., 2011). In these conditions, the CO₂ assimilation capacity is reduced, leading to an increased intracellular concentration of CO₂ (Mateos-Naranjo et al., 2009; Ding et al., 2011).

In addition to these effects on gas exchange, the levels of ribulose-1,5-biphosphate (RuBP) and 3-phosphoglyceric acid (PGA) are reduced after glyphosate exposure (Servaites et al., 1987; Siehl, 1997a). It seems that glyphosate may also reduce ribulose 1,5-biphosphate carboxylase oxygenase activity (Rubisco) in sugar beet (Servaites et al., 1987). This result was also reported by de Maria et al. (2006) in Lupinus albus leaves, where a 26% reduction of Rubisco activity occurred after five days of exposure to 10mM of glyphosate. All these effects impact the plant efficiency to fix atmospheric carbon and reduce it into sugars. Another hypothesis that was advanced to explain glyphosate’s perturbation on the carbon metabolism is related to a carbon flow into the shikimic pathway caused by a lack of regulation. Indeed, glyphosate’s inhibition of the shikimate pathway, leads to an accumulation of shikimate-3-phosphate in the chloroplasts, which becomes a carbon sink (Siehl, 1997a; Duke and Powles, 2008). Arogenate, a by-product of chorismate, was shown to inhibit the enzyme 3-deoxy-D-arabinoheptulosonic acid-7-phosphate (DAHP) synthase, the first enzyme involved in the shikimic pathway catalyzing the condensation of phosphoenolpyruvate (PEP) with D-erythrose-4-phosphate (Siehl, 1997a). Siehl (1997) suggested that the inhibition of DAHP synthase by arogenate is the key regulation process for the shikimate pathway. In case of glyphosate exposure, this important regulation pathway cannot occur since chorismate and all its by-products are not synthesized, resulting in a carbon flow towards this pathway and the accumulation of shikimate-3-phosphate.
Glyphosate can also impair carbon metabolism by interfering with sugar metabolism and translocation. Studying the effects of glyphosate on *Pisum sativum* plants, Orcaray *et al.* (Orcarav *et al.*, 2012) found a carbohydrate accumulation in both leaves and roots of glyphosate-treated plants. As growth was stopped, the carbohydrate accumulation in roots was attributed to a lack of utilization of available sugars, which further caused soluble carbohydrate accumulation in leaves. Servaites *et al.* (Servaites *et al.*, 1987) reported that glyphosate had no effect on sucrose synthesis and transport, but did reduce starch synthesis, four hours after foliar application on sugar beet leaves. They also found that glucose-6-phosphate levels increased during day-time, but there was no difference observed in the levels of fructose-6-phosphate or triose-phosphate.

Although only very few studies were conducted on the effect of AMPA on carbon metabolism, it was shown that soluble sugar content was decreased in AMPA-treated plants (Serra *et al.*, 2013). However, we found no study demonstrating direct AMPA effects on photochemical and carbon assimilatory pathways, as reported for glyphosate. AMPA effects on photosynthesis were obtained in plants few days after exposure to AMPA and were related to the effects on chlorophyll contents (Reddy *et al.*, 2004; Ding *et al.*, 2011). GR and GS soybeans had decreased photosynthetic activity in presence of AMPA up to 10 days after exposure, and the recovery of photosynthesis was slowed down and occurred 28 days after exposure (Ding *et al.*, 2011). The nitrogen nutrition and the activity of nitrate reductase of these plants were not affected. Thus, in contrast to glyphosate, AMPA does not appear to affect nitrogen assimilation in plants. Reduced photosynthesis could thus be linked to AMPA-induced alterations in glutamate, glycine and serine contents (Serra *et al.*, 2013), which could lead to a decreased chlorophyll content by inhibition of ALA biosynthesis (Fig. 1.1). As plants are not deprived of nitrogen, the production of amino acids can be recovered leading to normalization of chlorophyll contents and photosynthesis, as demonstrated by Ding *et al.* (2011).
1.3.2 Nitrogen metabolism

Glyphosate effects on nitrogen metabolism have mostly been studied in soybean (*Glycine max*), a Leguminosae species in which symbiotic nitrogen fixation represents about 40 to 70% of the plant’s total nitrogen requirement (Zablotowicz and Reddy, 2007). Maintaining this significant nitrogen input is important for profitable soybean yields, and for sustaining long-term soil productivity, especially in soils with low concentrations of available nitrogen where cultural rotations are done with high nitrogen consuming crops such as maize (Zablotowicz and Reddy, 2007). Glyphosate can influence nitrogen metabolism through direct effects on the rhizobial symbiont or indirectly by affecting the physiology of the host plants (Zobiole et al., 2010c). Aside from plants, microorganisms also possess EPSPS enzymes and are therefore susceptible to glyphosate (Fischer et al., 1986). For example, the soybean nitrogen fixing symbiont *Bradyrhizobium japonicum*, possesses a glyphosate-sensitive EPSPS and accumulates shikimate and hydroxybenzoic acids, such as protocatechuic and/or gallic acids, upon exposure to glyphosate. This leads to growth inhibition and death induction at high glyphosate concentration (de Maria et al., 2006). The accumulation of protocatechuic acid in soybean nodules of glyphosate-treated plants suggested a possible translocation of the herbicide to the nodules. This hypothesis was reinforced by the reduced nitrogenase activity showed in the *B. japonicum* bacteroids (Hernandez et al., 1999). Moreover, glyphosate residues were also found in nodules of GR soybean from plants under routine herbicide application in field conditions (Reddy and Zablotowicz, 2003). The toxic effects of glyphosate in prokaryote constituents of bacteroids may be attributed to: 1) its negative effect on the synthesis of aromatic amino acids; 2) the accumulation of potential toxic intermediates of the shikimic acid pathway; or 3) the exacerbate chemical energy (ATP and PEP) spent in the shikimate pathway (Fischer et al., 1986).
The introduction of GR plants may have unforeseen consequences for symbiotic microorganisms associated with soybeans (Zobiole et al., 2011a) since glyphosate can be transferred to root nodules (Reddy and Zablotowicz, 2003) and excreted into the rhizosphere (Kremer et al., 2005). Several studies report decreased nitrogen nutrition in plants exposed to glyphosate which has been related to glyphosate effects on N fixation/assimilation (Zablotowicz and Reddy, 2007; Bellaloui et al., 2008; Zobiole et al., 2012). Zablotowicz and Reddy (2004) demonstrated direct effect of glyphosate on soybean symbiotic bacteria *B. japonicum* by monitoring nodule parameters and acetylene reduction activity (ARA). However, until now, results mentioned in the literature are still inconsistent regarding the effects of glyphosate on the nodulation and nitrogen metabolism of GR plants. King et al. (King et al., 2001) reported decreased nodulation and nitrogen fixation activity (by evaluating ARA) in 21-days-old soybeans plants. Similarly, Zobiole et al. (2012) reported decreased nodulation in GR plants at different growth stages. ARA inhibition has been associated to sensitivity of *B. japonicum* strain to glyphosate (Hernandez et al., 1999). In a 3 year field study, Zablotowicz and Reddy (2007) reported only a slight effect on nitrogen fixation and/or assimilation in GR soybean at glyphosate recommended application rate, but a consistent reduction for higher concentrations. On the other hand, Bellaloui et al. (2008) have not observed glyphosate effect on nitrogen fixation in a 2 year field study. However, these authors reported decreased nitrate assimilation, probably due to glyphosate-induced effects on carbon metabolism. Although decreased nodulation may vary among soybean cultivars and plant growth stages, it was previously shown that glyphosate may affect nodulation of approximately 45% of GR soybean cultivated in Brazil (Zobiole et al., 2010c). The loss of energy and fixed N\(_2\) provided by *B. japonicum* may be significant factors responsible for reduced growth and production observed in some GR soybean fields (Hernandez et al., 1999).
It was reported that the effect of glyphosate on symbiotic N\textsubscript{2} fixation was due to inhibition of photosynthesis and carbon substrate availability (Zabloutowicz and Reddy, 2007). Kremer and Means (2009) reported that glyphosate can affect rhizospheric interactions between plants and microorganisms, for example, interfering in the balance of plant indole-3-acetic acid (IAA), which leads to lower root nodulation. Moreover, the reduction of nutrient accumulation in plants exposed to glyphosate can also affect symbiotic N\textsubscript{2} fixation (Zobiole \textit{et al.}, 2012). Zobiole \textit{et al.} (2010b) reported that nickel (Ni) was immobilized by glyphosate and could thereby compromise symbiotic N\textsubscript{2} fixation in GR soybean since Ni is required by N\textsubscript{2}-fixing microorganisms for the hydrogenase that processes hydrogen gas generated during N\textsubscript{2} fixation. In addition, glyphosate may interfere with the availability of other minerals which are metal cofactors required for the activity of many enzymes involved in the N\textsubscript{2} fixation process (Zobiole \textit{et al.}, 2012).

Concerning AMPA, its effects on nodulation, nitrogen fixation and nitrogen assimilation need further investigation. However, AMPA was proposed not to interfere on nitrogen nutrition (Ding \textit{et al.}, 2011). Indeed, studying the AMPA effect on GR and GS sensitive soybean plants, Ding \textit{et al.} (2011) did not observe any AMPA effect on nodulation parameters (nodule number and dry weight) and on nitrogen fixation through acetylene reduction. These results suggest that even if AMPA is being translocated to nodules, it does not appear toxic to the fixing symbionts.

\textbf{1.3.3 Glyphosate and plant mineral nutrition}

The effect of glyphosate on plant mineral nutrition has not yet been extensively studied (Zobiole \textit{et al.}, 2010c). Moreover, results published concerning to GR crops are contradictory. Some studies did not report any effect (Bailey \textit{et al.}, 2002; Rosolem \textit{et al.}, 2010), while others have shown that glyphosate disturbed plant nutritional status (Cakmak \textit{et al.}, 2009; Su \textit{et al.}, 2009; Zobiole \textit{et al.}, 2010c, 2011b, 2012).
Studying the effects of one or two glyphosate applications (at the recommended rate of 0.86 kg ha\(^{-1}\)) on the mineral content in leaves and seeds of GR soybeans grown in greenhouse and field, Duke et al. (2012b) found no change in the concentrations of Ca, Mg, Mn, Zn, Fe, Cu, Sr, Ba, Al, Cd, Cr, Co or Ni. Hence, glyphosate did not appear to influence mineral nutrition of GR soybean while used for weed management in the field. Accordingly, Rosolem et al. (Rosolem et al., 2010) did not find any effect of glyphosate on soybean Mn nutrition, and concluded that the effect of glyphosate on soybean nutrition depends on the cultivar, the plant development stage, the physiological condition, and finally on the growth conditions. However, the glyphosate molecule was first patented as a metal chelator (Bromilow et al., 1993), and, this primarily reported mode of action could support the hypothesis that its application can exert some effects on plant nutrition and, therefore affect growth, even in resistant plants (Zobiole et al., 2011b).

According to Cakmak et al. (2009), glyphosate may induce nutritional disturbances by immobilizing certain nutrients in plants and/or interfering with their uptake and translocation. Moreover, the effects of glyphosate on the mineral content of GR crops were associated with the greater susceptibility of these crops to plant diseases (Johal and Huber, 2009; Kremer and Means, 2009). The accumulation of glyphosate in plant tissues may, in association with its chelating property, reduce the free activity of cationic mineral nutrient, leading to their deficiency in cells (Cakmak et al., 2009). Glyphosate is easily bound to divalent cations by its carboxyl and phosphonate groups, forming insoluble or very stable complexes, leading to immobilization of several divalent cations in plant tissues (Bellaloui et al., 2009; Cakmak et al., 2009; Su et al., 2009; Zobiole et al., 2011b). Significant decreases in all macro- and micronutrient contents have been observed in GR soybean plants exposed to glyphosate (Zobiole et al., 2011a, 2012). According to the aforementioned authors, these plants were rendered less efficient in nutrient uptake and translocation and suffered from potential chelating effects of glyphosate. The decreased mineral
concentrations in glyphosate exposed soybean leaves (Zobiole et al., 2012) is in agreement with the possible inhibition of uptake and/or transport of nutrients due to the formation of glyphosate-metal complexes within plant tissues (Eker et al., 2006). Moreover, reduced nutrient uptake and accumulation may be related to glyphosate reducing root growth (Zobiole et al., 2012). According to Zobiole et al. (2011a), glyphosate doses affect plant macronutrient accumulation in the following order: Ca > Mg > N > S > K > P whether glyphosate was applied as a single dose or as multi doses. Meanwhile, micronutrient accumulation is affected in the following order: Fe > Mn > Co > Zn > Cu > B > Mo, and Fe > Co > Zn > Mn > Cu > Mo > B, for single and sequential glyphosate applications, respectively. Moreover, the negative effects of glyphosate on plant mineral nutrition varied depending on their growth stages, with younger plants being more sensitive than plants receiving glyphosate at a later growth stage (Zobiole et al., 2011b). Nutritional disturbances in GR plants may be related to the frequently observed plant injury known as “yellow flashing” following glyphosate application (Zablotowicz and Reddy, 2007). This yellow flashing occurring shortly after glyphosate application was also proposed to be attributed to AMPA toxic effect and not to mineral deficiencies (Duke et al., 2012a).

In a short term uptake study using radiolabeled elements, Eker et al. (2006) reported that root-to-shoot translocation of micronutrients were severely inhibited in sunflower plants in the following order Mn > Fe > Zn. Studying the mineral nutrition of some turf grass species, Su et al. (2009) observed that glyphosate applications reduced shoot concentration of mineral nutrients, especially Ca, Mg, Fe and Mn. Although research in this field is still limited, it is important to know that the glyphosate interactions with plant mineral nutrition could amplify the toxicity of glyphosate for GS-plants and lead to some glyphosate toxicity in GR-plants by interfering with mineral nutrient availability (Su et al., 2009).
1.3.4 Oxidative stress

Along with the inhibition of specific target sites, the glyphosate action also leads to oxidative stress in plants which is most probably a secondary effect of the blocked shikimate pathway (Ahsan et al., 2008). Plants have developed mechanisms to cope with oxidative stress induced by reactive oxygen species (ROS) accumulation by synthesizing enzymatic and non-enzymatic antioxidants (Gunes et al., 2007). Among the enzymatic systems, activities of ROS-scavenging enzymes and content of malondialdehyde (MDA), a product of membrane lipid peroxidation, are frequently used as indicators of oxidative stress in plants (Gunes et al., 2007). Although changes in these oxidative stress markers were reported under various stress conditions, little information is currently available concerning the effects of glyphosate on oxidative stress.

Maize (Zea mays) leaves exposed to glyphosate showed an increased level of lipid peroxidation, glutathione (GSH), free proline content and ion flux (Sergiev et al., 2006). In a gene expression analysis, Ahsan et al. (Ahsan et al., 2008) found that glyphosate application generates hydrogen peroxide (H$_2$O$_2$) resulting in peroxidation and destruction of lipids in rice (Oryza sativa) leaves. Moreover, these authors also observed a decrease of the Rubisco large subunit content and an increase in the accumulation of antioxidant enzymes, including ascorbate peroxidase (APX), glutathione-S-transferase (GST), thioredox h-type, nucleoside diphosphate kinase 1 (NDPK1), peroxiredoxin and chloroplast precursor of superoxide dismutase [Cu-Zn] (SOD) within leaves treated with glyphosate.

Testing GR and susceptible (GS) soybean plants exposed to glyphosate, Moldes et al. (2008) did not observe a significant impact of glyphosate on lipid peroxidation. However, there was a significant increase in the levels of soluble amino acids in roots and leaves, greater in GS than in GR soybean cultivars. Soluble amino acids have antioxidant action (Samaranayaka and Li-Chan, 2011), which may prevent the lipid peroxidation. Furthermore, the oxidative stress generated by glyphosate in
these plants was noted by the modulation in catalase (CAT) and guaiacol peroxidase (GuPX) activities. Studying the phytotoxicity of glyphosate in duckweed (Lemma minor), Kielak et al. (2011) observed that glyphosate causes damages similar to abiotic and biotic stressors, leading to an over-accumulation of putrescine, spermidine and total polyamines, which were connected to oxidative burst induced by glyphosate. Duckweed tissues treated with glyphosate also showed higher CAT and APX activities, demonstrating that oxidative stress can be induced by glyphosate.

Oxidative stress in pea (Pisum sativum), wheat and maize were observed in plants exposed to glyphosate. Indeed, increased MDA (lipid peroxidation) and H₂O₂ contents and activation of antioxidant enzymes (SOD, CAT and glutathione peroxidase - GPX) were found (Sergiev et al., 2006; Miteva et al., 2010). Moreover, glyphosate application on both leaves and roots of pea plants resulted in the activation of glutathione reductase and in the enhancement of the GST activities which, in addition to the increase in both total and oxidized glutathione contents, highlight the oxidative stress induced by glyphosate in these plant tissues (Miteva et al., 2010).

Studying herbicidal effects on Arabidopsis thaliana plants, Serra et al. (2013) observed increased accumulation of inositol, ascorbate and serine. Inositol and ascorbate are oxidative-stress markers and their increased contents is related to increased oxidative stress in plants (Foyer and Noctor, 2011). In addition, serine (as a cysteine precursor) is involved in glutathione metabolism (Foyer and Noctor, 2011), and its accumulation could also be an indicator of oxidative-stress. Interestingly, A. thaliana treated with AMPA did not shown an increase in these oxidative-stress markers (Serra et al., 2013). While ascorbate and inositol contents did not statistically differ from control plants, the serine content was decreased, due to a decreased glycine content (as these two amino acid are intrinsically linked to metabolic pathways) (Serra et al., 2013). Thus, in contrast to glyphosate, AMPA was not suggested to induce oxidative stress in A. thaliana plants (Serra et al., 2013).
The oxidative damage due to glyphosate exposure could be associated with glyphosate effects on plant nutrition. For instance, metal deficiency could increase oxidative stress in plants because redox-active metals such as copper are known to perform antioxidative protection in plant cells (Cuypers et al., 2001). Moreover, metal deficiency such as Zn and Fe, could impair activities of antioxidant enzyme systems (Fig. 1.2). On the other hand, AMPA may not induce oxidative damage since this glyphosate by-product was not proven to induce nutritional disturbance (as glyphosate does). However, no study has confirmed this hypothesis. Apart from acting as hazardous molecules carrying on oxidative burst, ROS can also act as plant signaling and are involved in several plant physiological processes. Specifically, ROS are intrinsically related to plant hormone action, thus, it is important to study glyphosate-induced oxidative events as they can directly interfere with plant growth and development.
Fig. 1.2: Glyphosate and AMPA effects in some plant physiological process (red "X"). Glyphosate main effect is due the inhibition of aromatic amino acids (tryptophan –Trp, phenylalanine -Phe and tyrosine – Tyr) production by inhibiting 5-enolpyruvylshikimate-3-phosphate (EPSPS) synthase. Glyphosate can affect photosynthesis by depriving Tyr contents and chelating metal ions important to photosystem (PS) structure and chlorophyll biosynthesis. Moreover, metal chelation deprives cells of important co-factors needed for enzymatic antioxidant system activity (superoxide dismutase – SOD, catalase – CAT and peroxidase – POX), leading to oxidative stress by accumulation of reactive oxygen species (superoxide -O$_2^-$ and hydrogen peroxide - H$_2$O$_2$). Aminomethylphosphonic acid (AMPA) can also inhibit photosynthesis by inhibiting the synthesis of aminolevelinic acid (ALA), an important precursor of chlorophylls (see Figure 1.1).

1.3.5 Glyphosate and lignin

Lignin represents a significant proportion of all the fixed carbon by plants, accounting for approximately 30% of the organic carbon in the biosphere (Gosselink et al., 2004). The lignification of cellulose microfibrils was described as an adaptive mechanism that helps to maintain plant stability and tolerance to biotic and abiotic stresses (Gomes et al., 2011). Lignin production is controlled by phenylalanine, a key product of the shikimate pathway (Zobiole et al., 2010a) and thus, can be largely affected by glyphosate. Indeed, even GR soybean plants treated with glyphosate have shown to produce less lignin when compared to non-treated plants (Zobiole et al., 2010a). According to Marchiosi et al. (2009), glyphosate may reduce lignin synthesis due to the inhibition of EPSPS followed by the reduced supply of cinnamate precursors. Reduction in lignin content is an important physiological aspect of glyphosate’s herbicidal effect since the lignin content is associated to the morphological and functional quality of plant organs (Gaspar and Coumans, 1987). Depriving plants of suitable lignin content may make them vulnerable to diseases as well as to nutrition and water balance disturbances. The role of lignin in plant-pathogen defense is widely discussed and the lignin deposition in root cortical cell walls is recognized to help maintain root turgescence (Gomes et al., 2011), leading to adequate water and mineral uptake. Despite its importance, however, studies of glyphosate affecting lignin content in plants remain scarce.
1.3.6 Glyphosate/AMPA and plant hormones

The glyphosate and AMPA accumulation in active metabolic tissues (i.e., growing and metabolic active sites, shoot and root apical meristems) (Cakrnak et al., 2009) are identical to the main production sites of plant hormones, and thus, glyphosate/AMPA detrimental effects in plants can induce hormonal disturbance. Thus, glyphosate exposed plants could have both growth and development affected.

Auxin is a critical hormone related to plant growth and development processes. Indole-3-acetic acid (IAA), the key auxin, is synthesized from tryptophan and indolic tryptophan precursor (products from shikimic acid pathway). Therefore, by inhibiting shikimate pathway, glyphosate may prevent auxin biosynthesis. Evaluating the gene expression of the apical bud in soybean, Jiang et al. (2013) observed different expression of genes involved in auxin metabolism pathways (genes related to auxin response factor, genes encoding to proteins in the auxin-responsive family and IAA genes). The results indicated that the effects of glyphosate on these genes may perturb cell enlargement and plant growth (Jiang et al., 2013). Moreover, by interfering in the balance of IAA, glyphosate can affect rhizospheric interactions between plants and microorganisms, leading, for example, to lower root nodulation (Kremer and Means, 2009).

Sublethal doses of glyphosate was also shown to reduce the velocity of indolacetic acid (IAA) basipetal transport in cotton seedlings (Baur, 1979). Application of 1.44 kg acid equivalent ha\(^{-1}\) at the beginning of flower bud appearance (eight-leaf stage) in glyphosate-resistant cotton (Gossypium hirsutum) resulted in an increase in IAA level in anthers, resulting in inhibition of anther dehiscence (Yasuor et al., 2006). This IAA accumulation was related to glyphosate inhibition of auxin transport (Yasuor et al., 2006). Pretreatment of tobacco callus (Nicotiana tabacum) with glyphosate and AMPA perturbed IAA metabolism by increasing both conjugation and oxidation, and consequently lowered the level of free IAA leading to
growth inhibition (Lee et al., 1983). However, at identical concentrations, AMPA were less active then glyphosate (Lee et al., 1983).

Sergiev et al. (2006) observed alleviation of glyphosate detrimental effects in maize plants following treatment with the phenylurea cytokinin 4PU-30. Three- and four-year-old Picea pungens treated with a mixture of glyphosate and haxazinone showed decreased cytokinins (Cyt) contents (Matschke and Machácková, 2002). The strongest decrease was observed in the root upper and middle part (Matschke and Machácková, 2002), which coincides with the Cyt producing and glyphosate accumulating sites. These results indicate that glyphosate can also affect Cyt metabolism in plants. Similarly, gibberellic acid (GA₃) application to GR cotton has some remedial effects on pollen viability, which can also indicate glyphosate effects in GA metabolism (Pline et al., 2003). In higher plants, in addition to participate in the biosynthesis of lignins, fatty acids, flavonoids, etc., the cytochrome P450 monooxygenases also participate in gibberellic acid, brassinosteroids and jasmonic acid biosynthetic pathway (Bolweel et al., 1994). Glyphosate inhibition of P450 activity was seen in yeast (Xiang et al., 2005), which could also be expected in plants, corroborating to the glyphosate effects in plant hormones. Glyphosate was also shown to interfere with other hormones such as ethylene (Lee and Dumas, 1983) and abscisic acid (ABA) (Jiang et al., 2013). Although extremely important, the effects of glyphosate and AMPA in plant hormones metabolism are still unclear, and little attention has been done to AMPA effects in plant hormones metabolism and biosynthetic pathways.

1.3.7 Glyphosate and plant diseases

Herbicides are known to increase specific plant diseases (Hornby et al., 1988). Due to its interaction with rhizosphere microorganisms and plant physiological features, glyphosate is also expected to modulate diseases in plants. Glyphosate effects on plants diseases were extensively reviewed (Johal and Huber, 2009; Duke et al., 2012). Due to the induction of mineral nutrient disturbances, glyphosate can
greatly affect plant growth and resistance to diseases and pests (Johal and Huber, 2009). Moreover, increase in root infection may be due to the shutdown of plant protection compounds production, such as phytoalexins (Kremer et al., 2005), which are synthesized by the plants via the shikimate pathway (Sharon et al., 1992).

Studying GR-soybean plants, Kremer (Kremer, 2003) reported that fungal colonization of GR soybean roots increased significantly after application of glyphosate but not when conventional post-emergence herbicides were applied. Gressel (Gressel, 2002) argued that transgenic EPSPS of GR soybean could be less efficient than the wild-type genotype and could produce insufficient amount of phytoalexins to prevent fungal infection. However, in the sole study on phytoalexin production in GR-soybeans until now, Duke et al. (Duke et al., 2003) showed no effect on phytoalexins content in plants following glyphosate exposure. Thus, the susceptibility of GR-plants to pathogenic infections should not be associated with decreased phytoalexin contents (due ineffective EPSPS) but to overall changes in plant physiology which could reduce plant resilience.

Descalzo et al. (1998) observed an increased population of the fungus-like pathogen *Pythium sp.* in the rhizosphere and in soils amended with heat-killed roots from glyphosate treated beans. This population increase was not observed in control treatments (without glyphosate). Authors concluded that herbicide treatment of plants increased the pathogen population damping off soil potential. Similarly, Smiley (1992) observed a relationship between glyphosate application and plant diseases in a *Rhizoctonia*-infected soil. As the interval between glyphosate application and planting spring barley was shortened, severity of *Rhizoctonia* root rot increased. Thus, glyphosate treatment can induce a massive release of organic compounds from dying roots of target plants which can induce proliferation of a wide range of soil-pathogenic fungi.
GR soybeans can also release glyphosate into the rhizosphere which could influence the microbial community, enhancing communities of microorganisms pathogenic to GR soybean and causing a buildup of detrimental species that may affect subsequent crops (Kremer et al., 2005). Indeed, glyphosate can be used as a nutrient source by specific fungal species. Moreover, Kremer et al. (Kremer et al., 2005) demonstrated that glyphosate exudates from soybean root tips and that glyphosate application also increases carbohydrate and amino acid contents in root exudates. Together, both glyphosate and high levels of soluble carbohydrates and amino acids associated with glyphosate treatment of the soybean plants may provide a selective C and N source that stimulates growth of selected rhizospheric fungi.

Glyphosate effects on photosynthesis and on oxidative stress may also be linked to glyphosate increasing plant susceptibility to diseases. For example, ROS have crucial roles in pathogenesis, and are reported to be involved in processes such as the hypersensitive response of plant-pathogen incompatibility interactions; limiting pathogen infection by reinforcing plant cell walls and/or killing pathogens directly; programmed cell death (PCD); and in signaling acquired resistance (Shetty et al., 2008). Modulations of the system coordinating ROS content may lead to loss of ROS beneficial functions, thereby causing oxidative stress and subsequent effects and favouring pathogenic infections.

1.3.8 Glyphosate resistance in weeds

Until 1993, the natural development of weed tolerance or resistance to glyphosate was a topic virtually absent from the literature (Holt et al., 1993). However, the glyphosate tolerant weed populations have nowadays increased substantially, as reported by the International Survey of Herbicide Resistant Weeds (www.weedscience.org). The morphological or physiological reasons of such wide genetic variation in resistance to glyphosate are not well understood (Su et al., 2009). Glyphosate resistance in crops and weeds develops via two primary mechanisms referred to “non-target site” (reduced absorption and/or translocation) and “target site
resistance" (mutation and gene amplification) (Cruz-Hipolito et al., 2011). Sequestration of glyphosate into vacuoles (Ge et al., 2010), EPSPS gene amplification (Gaines et al., 2010), EPSPS mutation (Preston et al., 2009; Powles and Preston, 2010), reduced translocation of glyphosate (Powles and Preston, 2010) and poor penetration and translocation of the herbicide to apical growing points (Cruz-Hipolito et al., 2011) have been described to confer plant glyphosate resistance. Mechanisms involving plant metabolism of glyphosate have not been found to contribute to resistance of any weeds (Feng et al., 2004). However, Duke (Duke, 2011) recently suggested that some plants possess a glyphosate oxidoreductase (GOX) enzyme involved in degradation of glyphosate into AMPA. This enzyme may be related in part to the observed natural tolerance of some species. However, as AMPA could be also toxic, these plants might be able to degrade AMPA by carbon-to-phosphorus (C-P) lyases, enzymes responsible for direct cleavage of organophosphonate C-P, avoiding AMPA accumulation (Van Eerd et al., 2003). Studying the weed species Digitaria insularis, de Carvalho et al. (de Carvalho et al., 2012) observed that more than 90% of glyphosate was degraded into AMPA, glyoxylate, and sarcosine in resistant biotypes, whereas only a small amount of herbicide (up to 11%) was degraded by the susceptible biotype. In resistant biotypes, the substitution of a proline with threonine and of a tyrosine with a cysteine at the positions 182 and 310 of the EPSPS, respectively, were observed. Therefore, the authors concluded that absorption, translocation, metabolism, and gene mutation play an important role in the D. insularis glyphosate resistance. Studying glyphosate-resistant Amaranthus palmeri populations, Gaines et al. (Gaines et al., 2010) verified that the EPSPS gene amplification was heritable in pseudo-F2 populations and conferred glyphosate resistance. Both resistant and susceptible A. palmeri plants had EPSPS activity inhibited by glyphosate, however, resistant plants contained in their genome 5- to 160-fold more copies of the EPSPS gene.
Mineral nutrition could also lead to glyphosate resistance, as plants showing high concentrations of some cationic nutrients may significantly reduce glyphosate phytotoxic effects through formation of poorly soluble glyphosate complexes (Su et al., 2009). Moreover, the horizontal transference of GOX or C-P lyase genes from soil microbes to plants may also be possible (Duke, 2011). The glyphosate tolerant weed populations have increased vertiginously, as reported by the International Survey of Herbicide Resistant Weeds (www.weedscience.org). Consequently, further studies in relation to glyphosate resistance are needed as the population of glyphosate-tolerant weeds has considerably increased.

1.4 CONCLUSION

It is generally claimed that glyphosate kills undesired plants by affecting the EPSPS synthase enzyme, disturbing the synthesis of aromatic amino acids. However, glyphosate has several secondary or indirect effects on plant physiology which may also explain glyphosate herbicidal effects. The toxicity of glyphosate could be related to its effects on other physiological processes such as mineral nutrition and photosynthesis and to the plant’s hormone and oxidative status. The alteration of these cellular processes could be directly linked to the deleterious effects of glyphosate observed on plant growth and production. As a metal chelator, glyphosate could deprive plants from important nutrients which have important roles as enzymatic co-factors, biomolecules constituents and anti-oxidative systems. Oxidative stress, more specifically lipid peroxidation, induced by glyphosate is known to severely damage the cell integrity which may lead to cell death. Moreover, increased ROS production can negatively interfere with photosynthetic processes, for example, by decreasing the chlorophyll content, photochemical efficiency and carbon metabolism, leading to reduction in plant growth.

It is also important to note that some plants can metabolize glyphosate to AMPA or be exposed to AMPA via different environmental matrices, which could
amplify glyphosate effects on plant physiology. AMPA by itself has been considered phytotoxic, although at not to the same extent than glyphosate. AMPA has been proposed to be the responsible for injuries in GR-plants exposed to glyphosate (Reddy et al., 2004). In fact, AMPA could interfere in chlorophyll biosynthesis by inhibiting ALA formation. However, some studies showed rapid recovery of plant chlorophyll content and photosynthetic activity after AMPA exposure (Ding et al., 2011). Therefore, further studies on AMPA effects on plant physiology are clearly needed. These studies will provide evidence for the development of more efficient GR plants. Since some GR plants are able to degrade glyphosate to AMPA, it is important to study if this metabolite is responsible of the observed deleterious effects found in some plants treated with glyphosate or if these symptoms are related to the indirect effects of glyphosate on plant physiology.

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CHAPTER II

CONSEQUENCES OF PHOSPHATE APPLICATION ON GLYPHOSATE UPTAKE BY ROOTS: IMPACTS FOR ENVIRONMENTAL MANAGEMENT PRACTICES

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ABSTRACT

Phosphate (PO₄³⁻) fertilization is a common practice in agricultural fields also targets for glyphosate application. Due to their chemical similarities, PO₄³⁻ and glyphosate compete for soil adsorbing sites, with PO₄³⁻ fertilization increasing glyphosate bioavailability in the soil solution. After PO₄³⁻ fertilization, its concentration will be elevated in the soil solution and both PO₄³⁻ and glyphosate will be readily available for runoff into aquatic ecosystems. In this context, man-made riparian buffer strips (RBS) at the interface of agricultural lands and waterways can be used as a green technology to mitigate water contamination. The plants used in RBS form a barrier to agricultural wastes that can limit runoff, and the ability of these plants to take up these compounds through their roots plays an important role in RBS efficacy. However, the implications of PO₄³⁻ for glyphosate uptake by roots are not yet clearly demonstrated. Here, we addressed this problem by hydroponically cultivating willow plants in nutrient solutions amended with glyphosate and different concentrations of PO₄³⁻, assuring full availability of both chemicals to the roots. Using a phosphate carrier inhibitor (phosphonophormic acid-PFA), we found that part of the glyphosate uptake is mediated by PO₄³⁻ transporters. We observed, however, that PO₄³⁻ increased glyphosate uptake by roots, an effect that was related to increased root cell membrane stability. Our results indicate that PO₄³⁻ has an important role in glyphosate physiological effects. Under agricultural conditions, PO₄³⁻ fertilization can amplify glyphosate efficiency by increasing its uptake by the roots of undesired plants. On the other hand, since simultaneous phosphate and glyphosate runoffs are common, non-target species found near agricultural fields can be affected.

Keywords: Contamination; herbicide; phosphate carriers; phytoremediation; riparian buffer strips; transport.

2.1 MAIN

Since the introduction of glyphosate-resistant (GR) plants, glyphosate has become the most widely used herbicide globally (Coupe et al., 2012). Once applied in the field, glyphosate invariably penetrates into the soil, where it can be adsorbed onto soil particles, and phosphate (PO₄³⁻) content appears to be one of the most important factors driving this glyphosate binding (Borggaard, 2011). As PO₄³⁻ and the methylphosphonic group of glyphosate compete for the same adsorbing sites (Bott et al., 2011; Clua et al., 2012), the capacity of soils to adsorb PO₄³⁻ determines glyphosate availability in soil solutions, and increases in soil PO₄³⁻ concentrations...
may result in increased glyphosate availability (Bott et al., 2011). Phosphorous is an essential element for plants, participating in crucial physiological events, and $\text{PO}_4^{3-}$ fertilization is a common agricultural practice (Lopes, 2004). In agricultural fields where glyphosate has been applied, $\text{PO}_4^{3-}$ fertilization may influence soil herbicide bioavailability (Laitinen et al., 2007). Furthermore, these two compounds may be readily available for runoff, and be leached in adjacent waterways.

The detrimental effects of agricultural $\text{PO}_4^{3-}$ inputs to aquatic systems have been widely discussed (Correll, 1998; Chien et al., 2011), and attention has more recently been focused on the presence of glyphosate and its detrimental effects on aquatic ecosystems (Annett et al., 2014). In this context, implantation of man-made riparian buffer strips (RBS) at the boundary of the agricultural lands may limit runoff and decrease water contamination. The ability of these plants to perform efficient root uptake of these agricultural wastes plays an important role in RBS efficacy (Krutz et al., 2005; Lin et al., 2011). It was advance that glyphosate may compete with $\text{PO}_4^{3-}$ for membrane carriers (Denis and Delrot, 1993; Morin et al., 1997), as it occurs with soil absorption sites. Therefore, the following questions arise: 1) Does glyphosate compete with $\text{PO}_4^{3-}$ for root uptake? 2) Under situations of high availability of both compounds, how does $\text{PO}_4^{3-}$ influence glyphosate uptake by plants?

To address these questions, plants were grown in a hydroponic system to assure high availabilities of both glyphosate and $\text{PO}_4^{3-}$ to the root systems of a fast growing willow cultivar (Salix miyabeana cultivar SX64). Willow species have been successfully used in various phytoremediation programs, from soil decontamination to organic waste disposal (Mirck et al., 2005). In Quebec, willow plants have been used to establish riparian buffer strips to control the runoff of agricultural wastes (Hénault-Ethier et al., 2014) and, in that context, it becomes important to evaluate the ability of willows to take up glyphosate, as well as the implications of different agricultural practices on glyphosate-uptake efficacy by this species. First, we evaluated the involvement of $\text{PO}_4^{3-}$ transporters in glyphosate uptake by willow roots.
Using a $\text{PO}_4^{3-}$ transporter inhibitor (phosphonophoric acid-PFA) we were able to demonstrate the involvement of $\text{PO}_4^{3-}$ transporters in glyphosate uptake by roots as glyphosate concentrations in plant tissues (roots and leaves) since glyphosate uptake decreased in the presence of PFA (Fig 2.1). The involvement of phosphate carriers in glyphosate uptake has been observed in protoplasts (Bott et al., 2011; Clua et al., 2012), improving our understanding of the glyphosate’s membrane transport, and the differential distribution of this herbicide in different plant tissues (Denis and Delrot, 1993). However, to our knowledge, the importance of $\text{PO}_4^{3-}$ carriers in primary glyphosate uptake by roots has not been described before.

Interestingly, we observed glyphosate in the tissues of plants exposed to both PFA treatments and $\text{PO}_4^{3-}$ additions (Fig 2.1). According to Denis and Delrot (1993), $\text{PO}_4^{3-}$ carriers are mostly involved in glyphosate uptake at low herbicide concentrations in protoplasts. This energy-dependent uptake was superimposed, however, on a linear diffusional process under increased xenobiotic concentrations (Denis and Delrot, 1993). Similarly, we demonstrated here that high $\text{PO}_4^{3-}$ concentrations in nutrient solutions increase glyphosate diffusion into roots (Fig. 2.1). These results lead us to our second question regarding the involvement of $\text{PO}_4^{3-}$ in glyphosate uptake by roots.

The addition of $\text{PO}_4^{3-}$ significantly increased glyphosate uptake by roots and its concentration in plant tissues (roots and leaves) (Fig 2.1). Recent studies have shown that as $\text{PO}_4^{3-}$ fertilization increases glyphosate remobilization from soil matrices, which then becomes more available for root uptake (Bott et al., 2011; Beltrano et al., 2013). In these studies, however, the adsorption of $\text{PO}_4^{3-}$ to soil matrices
Fig 2.1. Glyphosate and phosphorus (P) concentration of roots and leaves and glyphosate uptake rates of willows grown under different phosphate (PO$_4^{3-}$) concentrations and treated with 0mM phosphonophormic acid (-PFA) or 5mM (+PFA).

reduced PO$_4^{3-}$ availability in the soil solution, but neither the potential competition between PO$_4^{3-}$ and glyphosate for carrier sites, nor PO$_4^{3-}$ effects on glyphosate uptake
under situations of high availability of both chemicals were investigated. In the present study, we observed that despite their competition for the same membrane carriers, PO$_4^{3-}$ fertilization clearly increased plant glyphosate’s uptake. To better understand this stimulatory effect of PO$_4^{3-}$, we examined root cell injury by measuring cell membrane stability (CMS) (Fig. 2.2), and found that PO$_4^{3-}$-fertilization decreased root cell injuries induced by glyphosate (Fig. 2.2). Additionally, a negative correlation was observed between root cell injury and root PO$_4^{3-}$ concentrations ($r = -0.824$ and $r = -0.950$ for PFA treated and untreated plants respectively).

Increased phosphorous nutrition is known to protect plants from environmental stress (i.e., trace-elements contamination) through phosphorous stimulation of antioxidant systems, thus preventing oxidative tissue damage (Gomes et al., 2013, 2014b). Like trace-elements (Gomes et al., 2013, 2014b), glyphosate is known to induce oxidative stress in plants (Gomes et al., 2014a). Here, we demonstrated that glyphosate induced lipid peroxidation (malondialdehyde – MDA contents, from 29 to 52%) in willow roots (Fig. 2.2). PO$_4^{3-}$-protective effects in glyphosate-treated roots were observed, as lipid peroxidation decreased with increasing PO$_4^{3-}$ concentration (Fig. 2.2). Lipid peroxidation in biological membranes is the most obvious symptom of oxidative stress in plants, and has been directly linked to cell membrane constitution and stability (Yajima et al., 2009). By protecting cell membranes against oxidative bursts, PO$_4^{3-}$ may favor root absorption, and explain the greater glyphosate concentrations observed in PO$_4^{3-}$ fertilized plants.

Raising PO$_4^{3-}$ fertilization levels in glyphosate treated soils leads to increased glyphosate availability for root uptake and increases the deleterious effects of glyphosate in plants (Bott et al., 2011). However, it is important to note that, in addition to releasing glyphosate into the soil solution, PO$_4^{3-}$ adsorption onto soil binding sites can create situations of low PO$_4^{3-}$ availability for plants impairing their growth. In this context, PO$_4^{3-}$ fertilization should be considered in studies of glyphosate toxicity since we observed that despite PO$_4^{3-}$-competition with glyphosate
for root absorbing sites (PO$_4^{3-}$ transporters), PO$_4^{3-}$ fertilization assured high phosphorous contents (which normally range from 0.1 to 1% (Sanchez, 2006)) and also increased glyphosate uptake by willow roots.

![Fig 2.2](image-url)  
**Fig 2.2.** Root cellular injury and lipid peroxidation in roots of willow plants grown under different phosphate (PO$_4^{3-}$) concentrations and treated with 0mM phosphonophormic acid (-PFA) or 5mM (+PFA).

These findings have crucial impacts on environmental management strategies at different levels: 1) Glyphosate efficiency — if we assume that PO$_4^{3-}$ increases glyphosate uptake by roots in weed species (as demonstrated here for willow), the co-application of P and glyphosate should lead to higher assimilation of glyphosate by these undesirable plants, thus contributing to herbicide efficiency; 2) Glyphosate toxicity — it is known, on the other hand, that both glyphosate and phosphate readily runoff agricultural fields to surrounding areas, resulting in glyphosate exposure of non-target plants. In these cases, PO$_4^{3-}$ fertilization and/or the resuspension of PO$_4^{3-}$ present in soils and sediments would have detrimental environmental effects by increasing glyphosate uptake by non-target plants; 3) Glyphosate phytoremediation — PO$_4^{3-}$ could be used as an auxiliary tool in phytoremediation programs directed towards glyphosate contaminated soils, as increasing glyphosate uptake by tolerant...
plants established in riparian buffer strips would help to prevent its runoff into surrounding waters.

The identification of potential \( \text{PO}_4^{2-} \) carriers, as well as investigations of glyphosate metabolism in roots could contribute to the selection of plant species suitable for phytoremediation programs. Additionally, studies of \( \text{P} \) carriers involved in glyphosate uptake by roots could contribute to the development of better GR plants. In this context, new GR plants could be developed by selecting those with membrane carriers having high specific affinities for \( \text{P} \), assuring \( \text{P} \) nutrition with less glyphosate uptake (thus decreasing secondary glyphosate toxic effects on GR plants). However, in this case, the expected increased free glyphosate concentration might have detrimental effects on soil biological community (Correia and Moreira, 2010; Gomes et al., 2014a; Pham et al., 2015), and therefore, strategies to accelerate glyphosate's dissipation or biodegradation (e.g. by increasing soils microbial glyphosate degrader community) should be investigated for their efficiency and impacts on the environment.

2.2 MATERIAL AND METHODS

2.2.1 General plant growth conditions

*S. miyabeana* cultivar SX64 cuttings approximately 20 cm long were acquired from the “Institut de recherche en biologie végétal” (the Research Center of the Montreal Botanical Garden, Canada). Greenhouse experiments were conducted from September to December/2013 at the Université du Québec à Montréal (Montreal, Canada). The greenhouse was maintained at 25/22 °C (± 3 °C) day/night temperatures, with natural light being supplemented by sodium vapor lamps to provide a 12 h photoperiod and an average photosynthetic active radiation level of 619 \( \mu \text{mol photons m}^{-2} \text{s}^{-1} \). Cuttings were grown in plastic boxes filled with distilled water amended with King Max nutrient solutions (7% \( \text{P}_2\text{O}_5 \), 11% \( \text{K}_2\text{O} \), 1.5% Mg, 1.27% S, 0.07% B, 0.002% Mo, 0.12% Zn) and B (4% N, 1% \( \text{NH}_4^+ \), 3% \( \text{NO}_3^- \), 10%
K2O, 2% Ca, 0.05% Fe, 0.05% Mn) (Montreal, Canada) following product instructions. The solutions were continuously aerated and renewed every 15 days. Healthy 45 day-old plants (with sufficient biomass) were used in the experiments. To minimize interference by unknown ingredients in commercial formulations, analytical-grade glyphosate acid (Pestanal grade) obtained from Sigma-Aldrich (Oakville, Canada) was used in the experiments.

After plant harvesting, the roots were thoroughly washed with distilled water and then held for 15 min in distilled water (-PFA) or in 5 mM phosphonophosphonic acid (+PFA) (Denis and Delrot, 1993). The plants were then transferred to plastic beakers containing freshly prepared glyphosate solutions (1 mg L⁻¹) amended with 0, 200 or 400 ppm of PO₄³⁻ (as KH₂PO₄). Phosphate doses were chosen following common fertilization practices in agricultural soils (Lopes, 2004). The solution pH was checked and adjusted (by manual addition of 1 M HCl or 1 M NaOH) to 6.7 ± 0.1, the pH observed in agricultural soils to be treated with glyphosate in Quebec (Canada) (data not shown). The experimental design was randomized, with six treatments using five plants as follows: -PFA + 0 ppm PO₄³⁻, -PFA + 200 ppm PO₄³⁻, -PFA + 400 ppm PO₄³⁻, +PFA + 0 ppm PO₄³⁻, +PFA + 200 ppm PO₄³⁻, and +PFA + 400 ppm PO₄³⁻. After 30 min of exposure to their respective treatments, all roots and samples of the seventh to ninth nodes counting from the apex of the plant (first fully expanded leaves) were collected, washed thoroughly in distilled water, and stored at -80 °C until analyzed. Glyphosate measurements were performed as described in the "Phosphorous and Glyphosate concentrations" section below.

Glyphosate uptake (Sharma and Agrawal, 2006) was calculated using the following formula:

\[
Uptake = \frac{\text{root + leaf glyphosate concentrations}}{T_1 - T_0}
\]

where \(T_1\) is the experimental period (30 min).
2.2.2 Cell membrane stability and Lipid Peroxidation

Cell membrane stability was measured in 500 mg of roots using a conductivity meter (YSI Model 345, Yellow Springs, Ohio) according to Sullivan and Ross (1979), and calculated using the following formula:

\[
\text{%CMS} = \frac{1 - \left(\frac{T_1}{T_2}\right)}{1 - \left(\frac{C_1}{C_2}\right)} \times 100
\]

where T and C are the conductivities of the treated and control (without glyphosate treatment) samples respectively. T1 and C1 represent electrolyte leakage (dS m\(^{-1}\)) after submerging the samples in de-ionized water at 25 °C for 4 hours. T2 and C2 represent the total electrolyte concentrations as measured in samples heated in boiling water for 1 hour and then cooled to room temperature. T and C are the conductivities of the treated and control samples respectively. T1 and T2 correspond to the first and second solution conductivity determinations of treated samples, and C1 and C2 are the respective values for the control.

Lipid peroxidation (MDA content) was evaluated in 0.2 g of roots according to Hodges et al. (1999). The results are expressed in % in relation to control plants (without glyphosate treatment).

2.2.3 Phosphorous and Glyphosate concentrations

Phosphorus concentrations were measured in plant samples (~1 g of fully expanded leaves or roots) according to Sarruge and Haag (1974). The glyphosate extraction-purification steps were performed according to Goscinny et al. (2012), with the following modifications: 0.2 g of roots or 0.1 g of leaves were weighted in 50 ml Falcon tubes, with the subsequent addition of 10 ml of ultrapure acidified water (pH 2), 10 ml of methanol, and 5 ml of dichloromethane. The samples were then
homogenized using ultrasound (Ultra-Turrax®) for 1 min. The extracts were then centrifuged at 4,000 rpm for 20 min at 4 °C. Forty µl of the root, or 200 µl of the leaf supernatant, were transferred to a 1.5 mL vial and dried under nitrogen (N₂) flow. Derivatization was carried out by adding 500 µl of trifluoroethanol (TFE) and 1 ml of trifluoroacetic anhydride (TFAA). To assure complete dissolution of the glyphosate, the vials were vortexed before being heated at 90°C for one hour. After returning to room temperature, the samples were evaporated to dryness under N₂ flow. Prior to GC-ECD injection, the samples were dissolved in 800 µL of ethyl acetate and 200 µL of pyridine. Finally, 1 µL of 1-bromopentadecane was 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 the 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.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.

2.2.4 Statistical analyses

The results are expressed as the average of five replicates. Statistical analyses were performed using JMP 10.0 software (SAS Institute Ins.). The data were submitted to tests of normality (Shapiro–Wilk) and homogeneity (Brown-Forsythe) and then statistically evaluated using two-way analysis of variance. The means were compared using the Tukey test at a 5% level of probability. To assure the reproducibility of the obtained data, we repeated twice the entire experiment, with no significant difference (data not shown).
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CHAPTER III
IMPACT OF PHOSPHATE ON GLYPHOSATE UPTAKE AND TOXICITY IN WILLOW

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ABSTRACT

Phosphate (PO₄³⁻) 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 PO₄³⁻ 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 PO₄³⁻ (0, 200 and 400 mg l⁻¹). We demonstrated that PO₄³⁻ 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, PO₄³⁻ 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, PO₄³⁻ is involved in glyphosate toxicity in willow by preventing glyphosate induced oxidative stress.

Key-words: antioxidant enzymes, fertilization, herbicide, phosphorus, photosynthesis

3.1. 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 (Coupe et al., 2012). Its half-life under laboratory conditions can range from 30 to 40 days (Nomura and Hilton, 1977; Rueppel et al., 1977; Smith and Aubin, 1993), but in the field can vary from 2 to 197 days (Giesy et al., 2000). Although it has been found to be quickly degraded by microbial activity (Duke and Powles, 2008), its combined ability to adsorb to soil particles and to disperse throughout the soil profile contribute to its accumulation in soils (Beltrano et al., 2013). 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 (Borggaard, 2011). Inorganic phosphate (PO$_4^{3-}$) and glyphosate's methylphosphonic group compete for similar adsorbing sites (Bott et al., 2011; Clua et al., 2012), 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 (Marschner, 1995), and PO$_4^{3-}$ fertilization of soil is a common agricultural practice to assure plant growth and development (Lopes, 2004). 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 (Laitinen et al., 2007). Like PO$_4^{3-}$, glyphosate has high water solubility (Duke and Powles, 2008) 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 (Tesfamariam et al., 2009). 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 (Skłodowski et al., 2014), and their potential to attenuate glyphosate runoff specifically has been studied in agricultural sites in Quebec (Canada) (Hénault-Ethier et al., 2014). Once desorbed and mobile in soil solution, glyphosate becomes available for root uptake (Beltrano et al., 2013). 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 harvested following short rotation cycles and used for bioenergy production (Perttu,
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 (Denis and Delrot, 1993; Morin et al., 1997). In some plant species, PO$_4^{3-}$ regulates glyphosate uptake (Denis and Delrot, 1993) and translocation (McWhorter et al., 1980). 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 (Gomes et al., 2015a). 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 (Siehl, 1997b). As a result of EPSPS inhibition, shikimate accumulation occurs, and its concentration has been used as a bioindicator of glyphosate exposure in plants (Beltrano et al., 2013). As recently discussed by Gomes et al. (2014a), 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 (Gomes et al., 2012, 2014c).
In this study, we evaluated the effects of addition of $\text{PO}_4^{3-}$ 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) (Labrecque and Teodorescu, 2005), together constitute important features of plant species being considered as candidates for phytoremediation programs (Marques et al., 2000), particularly in the context of riparian buffer strips.

3.2. MATERIAL AND METHODS

3.2.1 General plant growth conditions

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% $\text{P}_2\text{O}_5$, 11% $\text{K}_2\text{O}$, 1.5% Mg, 1.27% S, 0.07% B, 0.002% Mo, 0.12% Zn) and B (4% N, 1% $\text{NH}_4^+$, 3% $\text{NO}_3^-$, 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 KH₂PO₄) was added at 0, 200 and 400 mg PO₄³⁻ 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 (Scribner et al., 2007; Struger et al., 2008; Giroux et al., 2010). 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 (Scribner et al., 2007)) and Argentina (0.5 - 5 mg kg⁻¹ (Peruzzo et al., 2008)). Phosphate doses were chosen according to common fertilization practices in agricultural fields (Lopes, 2004; Singh and Lal, 2005; Mullins, 2009). Potassium was provided as KNO₃, nitrogen as both KNO₃ and NH₄NO₃, and pH was adjusted to 6.7 ± 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 (≤ 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 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 PO₄³⁻ 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 °C.
3.2.2 Chemical analyses

For phosphorus evaluation, 0.1 g of leaves or roots were digested in 5 ml of concentrated HNO₃ (GR) in a microwave oven (ETHOS 1, Milestone Italy), at 175 °C for 10 min. Then, the solutions were cooled, filtered through Whatman n°40 filter paper and brought to a volume of 10 ml with ultra-pure water. The filtered extracts were preserved at 4 °C until analysis. Phosphorus concentrations in solution were evaluated according to Sarruge and Haag (Sarruge and Haag, 1974).

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 (N₂) flow. A derivatization procedure was carried out by adding 500 µl of trifluoroethanol (TFE) and 1 ml of trifluoroacetic anhydride (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 N₂. 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 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.min⁻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. (Mocak et al., 1997). 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 (r² = 0.96; p < 0.0001 and r² = 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 analysed plant tissue).

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].

3.2.3 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. (Juneau et al., 2015). 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. (Genty et al., 1990). To
compare treatments, fluorescence results from 619 µmol photons m$^{-2}$ s$^{-1}$ (most similar irradiation in relation to light growth conditions) were used.

Shikimate concentrations of leaves were evaluated following the methods of Bijay and Dale (Bijay and Dale, 1998). Hydrogen peroxide (H$_2$O$_2$) contents were measured following Velikova et al. (Velikova et al., 2000). 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 (Azevedo et al., 1998)), ascorbate peroxidase (APX; EC 1.11.1.11 (Nakano and Asada, 1981)) and glutathione peroxidase (GPX; E.C. 1.11.1.9 (Takeda et al., 2003)) were measured by standard procedures.

3.2.4 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 ($\epsilon$). Contrast analysis was used when there were significant differences in the studied variables between treatments (Table 3.1S and 3.2S).
3.3. RESULTS

3.3.1 Total phosphorus, Glyphosate and AMPA concentrations in plant tissues

Phosphorus concentrations in roots and leaves were increased by phosphate addition (hereafter referred simply as $\text{PO}_4^{3-}$), regardless of the glyphosate concentration and duration of exposure (Fig. 3.1). A significant interaction between time of exposure and glyphosate was 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 ($P < 0.05$).

Fig 3.1. 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$^{-1}$.

Glyphosate concentration in roots and leaves was higher for the first time of evaluation (Table 3.1S, Figs. 3.2 and 3.3). A significant interaction between time of
exposure, glyphosate and \( \text{PO}_4^{3-} \) addition was observed for glyphosate concentrations in roots and leaves (Table 1S, Figs. 2 and 3). For glyphosate concentrations \( \geq 0.065 \) mg l\(^{-1}\), \( \text{PO}_4^{3-} \) increased glyphosate concentration in roots and leaves \( (P < 0.0001) \) (Fig. 3.2 and 3.3). For the first time of evaluation, \( \text{PO}_4^{3-} \) fertilized plants treated with glyphosate concentration \( \geq 0.065 \) mg l\(^{-1}\) showed greater glyphosate concentrations.

AMPA concentration in roots and leaves was higher for the first time of evaluation (Table 3.1S, Figs. 3.2 and 3.3). At that point, \( \text{PO}_4^{3-} \) increased AMPA concentration in the tissues of plants exposed to glyphosate doses \( \geq 0.065 \) mg l\(^{-1}\). We also noticed that glyphosate total accumulation was also higher for the first time of evaluation and (Tables 3.1 and 3.1S) and for concentrations \( \geq 0.065 \) mg of glyphosate l\(^{-1}\), total accumulation was increased by \( \text{PO}_4^{3-} \).

**ROOTS**

![Graphs showing glyphosate and AMPA content](image)

Fig 3.2. Glyphosate and aminomethylphosphonic acid (AMPA) content \( (\mu g \text{ g FW}^{-1}) \) in roots of willow plants grown for two, three and seven days in nutrient solution amended with increasing doses of glyphosate \( (0, 0.001, 0.065 \text{ and } 1.000 \text{ mg l}^{-1}) \) and phosphate \( (0, 200 \text{ and } 400 \text{ mg l}^{-1}) \). Values are means ± SE of three replicates. The concentrations of added phosphate were 0 (filled circle), 200 (open circle) and 400
Values followed by *, within the same glyphosate and phosphorus concentration, are significantly different (P>0.05) by the Contrast test.

**Fig 3.3.** 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.
Table 3.1. 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\(^{-1}\)).

<table>
<thead>
<tr>
<th>Glyphosate (μg l(^{-1}))</th>
<th>Phosphate (mg l(^{-1}))</th>
<th>2 days</th>
<th>3 days</th>
<th>7 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>0</td>
<td>200</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>0</td>
<td>400</td>
<td>17.48</td>
<td>12.38</td>
<td>16.89</td>
</tr>
<tr>
<td>0.001</td>
<td>200</td>
<td>31.57</td>
<td>31.18</td>
<td>27.82</td>
</tr>
<tr>
<td>0</td>
<td>400</td>
<td>(180.58)</td>
<td>(251.77)</td>
<td>(164.72)</td>
</tr>
<tr>
<td>0</td>
<td>200</td>
<td>130.98*</td>
<td>78.97*</td>
<td>154.30*</td>
</tr>
<tr>
<td>0.065</td>
<td>400</td>
<td>229.28</td>
<td>299.94</td>
<td>253.28</td>
</tr>
<tr>
<td>0</td>
<td>200</td>
<td>(232.48)</td>
<td>(327.39)</td>
<td>(163.75)</td>
</tr>
<tr>
<td>0</td>
<td>400</td>
<td>(175.04)</td>
<td>(379.80)</td>
<td>(164.14)</td>
</tr>
<tr>
<td>0.100</td>
<td>200</td>
<td>(186.93*)</td>
<td>146.84*</td>
<td>160.90*</td>
</tr>
<tr>
<td>1.000</td>
<td>400</td>
<td>(307.55)</td>
<td>(293.77)</td>
<td>(198.40)</td>
</tr>
<tr>
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<td>200</td>
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<tr>
<td>1.000</td>
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<td></td>
<td></td>
<td>533.26</td>
<td>358.50</td>
<td>455.42</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(285.26)</td>
<td>(244.14)</td>
<td>(283.04)</td>
</tr>
</tbody>
</table>

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.
3.2 Physiological evaluations

3.1.1 Photosynthesis

A significant interaction between time of exposure, glyphosate and \( \text{PO}_4^{3-} \) addition was observed for operational PSII quantum yield (\( \Phi_M' \)) (Table 3.2S). In plants treated with \( \text{PO}_4^{3-} \), \( \Phi_M' \) was always greater after seven days of exposure, regardless of the glyphosate and \( \text{PO}_4^{3-} \) addition (Fig. 3.4). After two days of treatment, \( \text{PO}_4^{3-} \) decreased the operational PSII quantum yield (Fig. 3.4) whereas glyphosate addition increased yield in plants not fertilized with \( \text{PO}_4^{3-} \) (Fig. 3.4). In contrast, yield decreased in plants treated with 400 mg \( \text{PO}_4^{3-} \) l\(^{-1}\) as glyphosate doses increased (Fig. 3.4). After three days of exposure, \( \text{PO}_4^{3-} \) increased \( \Phi_M' \) of glyphosate-treated plants (Fig. 3.4). Similarly, after seven days of treatment induction, \( \text{PO}_4^{3-} \) increased \( \Phi_M' \) of plants subjected to 1 mg glyphosate l\(^{-1}\) (Fig. 3.4).

3.1.2 Shikimate concentration in leaves

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. 3.4). A significant interaction was observed between glyphosate and \( \text{PO}_4^{3-} \) addition (\( P = 0.0002 \)), and \( \text{PO}_4^{3-} \) increased shikimate concentration in leaves of plants treated with glyphosate (Fig. 3.4).
3.1.3 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. 3.4). A significant interaction between time of exposure, glyphosate and $PO_4^{3-}$ addition was observed (Table 3.2S). 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 $\geq 0.065$ l$^{-1}$, H$_2$O$_2$ concentration in leaves were greater for the first time of evaluation ($P = 0.0004$). After two days of exposure, PO$_4^{3-}$ increased H$_2$O$_2$ concentration in leaves of plants treated with 0.065 and 1 mg glyphosate l$^{-1}$ (Fig. 3.4). In contrast, after three days of exposure, PO$_4^{3-}$ significantly decreased H$_2$O$_2$ concentration in leaves of glyphosate-treated plants (Fig. 3.4). 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$_2$O$_2$ concentration in leaves (Fig. 3.4).

3.1.4 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. 3.5). Glyphosate addition increased catalase (CAT), ascorbate peroxidase (APX), and glutathione peroxidase (GPX) activity in roots ($P < 0.0001$; Fig. 3.5). A significant interaction between time of exposure, glyphosate and PO$_4^{3-}$ addition was observed for APX activity in roots (Table 3.2S), 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. 3.5).
Fig 3.5. Lipid peroxidation (MDA content, nmol g⁻¹ FW), and activity of catalase (CAT, μmol H₂O₂ 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. Values followed by *, within the same glyphosate and phosphorus concentration, are significantly different (P>0.05) by the Contrast test.

While CAT activity of leaves (P < 0.05) was not affected by PO₄³⁻, it was increased by glyphosate addition for all treatment duration (P < 0.0001; Fig. 3.6). A
significant interaction between time of exposure, glyphosate and PO₄³⁻ addition was observed for APX activity (Table 3.2S). PO₄³⁻ 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 PO₄³⁻ (Fig. 3.6). However, after three or seven days, APX activity in plants treated with glyphosate was increased by PO₄³⁻ (Fig. 3.6). Glyphosate addition increased GPX activity in leaves (Fig. 3.6).

Fig 3.6. Activity of catalase (CAT, μmol H₂O₂ 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.

3.4. DISCUSSION

Agricultural soils are subjected to frequent fertilization with PO\(_4\)^{3-} and application of glyphosate-based herbicides (Benbrook, 2012). The influence of soil P content on glyphosate uptake by plants has been investigated previously (McWhorter et al., 1980; Denis and Delrot, 1993; Gomes et al., 2015a). Here, we demonstrated that, in addition to glyphosate uptake (Figs. 3.2 and 3.3), PO\(_4\)^{3-} also has an impact on its toxicity, modulating physiological responses related to growth and biomass production, such as photosynthesis (Fig. 3.4).

As expected, PO\(_4\)^{3-} fertilization increased P concentration in roots and leaves of willows (Fig. 3.1). The P-phytoremediation capacity of willow has been already reported (Kuzovkina and Volk, 2009b; Fillion et al., 2011; Guidi et al., 2013), 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 (Gunes et al., 2009; Gomes et al., 2014c), 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 (Gomes et al., 2015a), but now, we also see 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 (Gomes et al., 2015a). 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$_2$O$_2$ contents and in the activity of photosynthesis- $\Phi'_M$ - 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 $\geq 0.065$ mg l$^{-1}$ (Fig. 3.2 and 3.3). One can argue that this could be related to the increase in biomass production over time. However, glyphosate total accumulation was greater after two days of exposure (Table 3.1). 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 (Denis and Delrot, 1993) inside the root cells. This has important outcome as the plant physiology was directly responsible for 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 3.1). Due to their chemical similarities, glyphosate and PO$_4^{3-}$ share the same membrane transport carriers (Denis and Delrot, 1993; Morin et al., 1997). These costly energetic transporters have been shown to be involved in glyphosate uptake primarily at low glyphosate concentrations ($< 0.032$ mg l$^{-1}$) (Denis and Delrot, 1993). 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 (Denis and Delrot, 1993). In such a scenario, PO$_4^{3-}$ can indeed influence glyphosate uptake, as observed in the present study (Figs. 3.2, 3.3 and Table 3.1), 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 (Gomes et al., 2013a, 2014c). In our study, plants submitted to glyphosate showed darker and thicker roots in relation to control (data not shown), recognized visual symptoms of stress (Arduini et al., 1994, 1995; Gomes et al., 2011). Since glyphosate is known to induce oxidative damage in plants (Sergiev et al., 2006; Ahsan et al., 2008), 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 PO₄³⁻ in willow roots.

The increased lipid peroxidation and activation of enzymatic antioxidant systems in glyphosate-treated plants (Fig. 3.5) clearly indicate the presence of herbicide induced oxidative stress in roots. However, PO₄³⁻ 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. 3.5). Plants showing high APX and GPX activity also showed decreased lipid peroxidation in roots (Fig. 3.5). As for catalase (CAT), APX and GPX are involved in scavenging hydrogen peroxide (H₂O₂), a reactive oxygen species (ROS) whose accumulation leads to oxidative damage, i.e., lipid peroxidation (Foyer and Noctor, 2011). Lipid peroxidation is an irreversible burst process leading to cell membrane destruction, with direct impact on cell membrane constitution and stability (Yajima et al., 2009). Therefore, by stimulating antioxidant systems, PO₄³⁻ 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. 3.2), the principal glyphosate by-product (Cerdeira and Duke, 2006), 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 (≤ 1.1 μg l⁻¹ for the highest glyphosate treatment) in relation to root AMPA concentrations (Fig. 3.2). 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 (Reddy et al., 2008). 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 PO₄³⁻ had no obvious effect on AMPA concentrations in roots and leaves (Figs. 3.2 and 3.3). However, as a glyphosate metabolite, AMPA was considered in the calculation of total glyphosate accumulation, in which PO₄³⁻ had a significant effect, as noted previously.

Once in leaves, glyphosate interferes with the shikimic acid pathway by inhibiting EPSPS activity, resulting in accumulation of shikimate (Siehl, 1997b). We observed shikimate accumulation in glyphosate-treated plants (Fig. 3.3). Moreover in these plants, PO₄³⁻ increased shikimate concentration, which may be due to the greater glyphosate concentration in leaves of PO₄³⁻-treated plants (Fig. 3.4). Therefore, we would expect that the increased glyphosate concentration in leaves associated with PO₄³⁻ would amplify the deleterious effects of glyphosate on leaf physiology. Indeed, after two days of exposure, we observed that, in the presence of glyphosate, PO₄³⁻-fertilized plants showed decreased photosynthesis (as measured by the operational PSII quantum yield, Fig. 3.4). However, this pattern was inversed at the following measurement occasion (day 3) and, after 7 days, PO₄³⁻-fertilized plants showed higher or no-significantly different rates of photosynthesis in relation to control (Fig. 3.4). We recently showed that glyphosate-induced ROS generation is implicated in decreased rates of photosynthesis (Gomes et al., 2015b). Increased H₂O₂ concentration was observed in leaves of plants treated with glyphosate (P<0.05; Fig. 3.4), indicating herbicide-induced ROS generation in leaves. Moreover, similar to our findings in a previous study (Gomes et al., 2015b), increased H₂O₂ concentration
higher than ~2 μg g FW⁻¹ in leaves was related to decreased operational PSII quantum yield (Fig. 3.4). H₂O₂ is an important signaling molecule (Shetty et al., 2008), however, once accumulated, it interferes with several plant metabolic pathways. For example, by suppressing the synthesis de novo of photosystem II D1 protein (Takahashi and Murata, 2008) or by inducing destruction of chloroplast membrane systems through lipid peroxidation (Li et al., 2006), H₂O₂ accumulation decreases photosynthetic activity (for more examples, see Gomes et al., 2015b). However, how can PO₄³⁻ prevent H₂O₂ 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. 3.6).

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 H₂O₂ scavengers, such as CAT, APX and GPX (Foyer and Noctor, 2011). CAT activity was found to have increased in glyphosate-exposed plants, however, it was not affected by PO₄³⁻ (Fig. 3.6). In contrast, APX and GPX activity were modulated by PO₄³⁻, and an intrinsic implication of these enzymes (mainly APX) in the fine control of H₂O₂ concentration in leaves was noted (Fig. 3.6). After two days of exposure, APX activity was lower in those glyphosate-treated plants exposed to PO₄³⁻ in which H₂O₂ accumulation had been noted. However, after three days of exposure, these plants showed decreased H₂O₂ 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 H₂O₂ concentrations in leaves and normal photosynthesis rates. Therefore, even as it increased glyphosate concentration in leaves, PO₄³⁻ induced the activity of enzymatic antioxidant systems, which controlled the ROS generation induced by the herbicide, ultimately ensuring normal photosynthesis rates.
3.5. 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 $\text{PO}_4^{3-}$ increased glyphosate accumulation and protected photosynthetic activity. It remains, however, unclear how $\text{PO}_4^{3-}$ can induce increases in the activity of antioxidant systems. Extracellular $\text{PO}_4^{3-}$ has been shown to be a signaling molecule affecting the expression of several genes in Murine osteoblasts (Rendenbach *et al.*, 2014). Likewise, $\text{PO}_4^{3-}$ 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 $\text{PO}_4^{3-}$ 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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Table 3.18: 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.

<table>
<thead>
<tr>
<th>Source of Variation</th>
<th>D.F.</th>
<th>Roots</th>
<th>Leaves</th>
<th>Total accum.</th>
</tr>
</thead>
</table>

D.F. Degrees of freedom * Significant
<table>
<thead>
<tr>
<th></th>
<th>P</th>
<th>Gly</th>
<th>AMPA</th>
<th>P</th>
<th>Gly</th>
<th>AMPA</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\text{PO}_4^{3-}$</td>
<td>2</td>
<td>$&lt;0.0001^*$</td>
<td>$&lt;0.0001^*$</td>
<td>$0.0088^*$</td>
<td>$&lt;0.0001^*$</td>
<td>$&lt;0.0001^*$</td>
</tr>
<tr>
<td>Gly</td>
<td>3</td>
<td>0.0297$^*$</td>
<td>$&lt;0.0001^*$</td>
<td>$&lt;0.0001^*$</td>
<td>0.3110</td>
<td>$&lt;0.0001^*$</td>
</tr>
<tr>
<td>$\text{PO}_4^{3-} \times \text{Gly}$</td>
<td>6</td>
<td>0.4615</td>
<td>0.0005$^*$</td>
<td>0.2926</td>
<td>0.1414</td>
<td>0.0004</td>
</tr>
<tr>
<td>Time</td>
<td>2</td>
<td>$&lt;0.0001^*$</td>
<td>$&lt;0.0001^*$</td>
<td>$&lt;0.0001^*$</td>
<td>0.0461$^*$</td>
<td>$&lt;0.0001^*$</td>
</tr>
<tr>
<td>Time $\times$ $\text{PO}_4^{3-}$</td>
<td>4</td>
<td>0.2850</td>
<td>$&lt;0.0001^*$</td>
<td>$&lt;0.0001^*$</td>
<td>0.0047$^*$</td>
<td>$&lt;0.0001^*$</td>
</tr>
<tr>
<td>Time $\times$ Gly</td>
<td>6</td>
<td>$&lt;0.0001^*$</td>
<td>$&lt;0.0001^*$</td>
<td>$&lt;0.0001^*$</td>
<td>0.0319$^*$</td>
<td>$&lt;0.0001^*$</td>
</tr>
<tr>
<td>Time $\times$ $\text{PO}_4^{3-} \times$ Gly</td>
<td>12</td>
<td>0.0701</td>
<td>$&lt;0.0001^*$</td>
<td>$&lt;0.0001^*$</td>
<td>0.0006$^*$</td>
<td>$&lt;0.0001^*$</td>
</tr>
</tbody>
</table>
Table 3.2S: 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 (\(\Phi'M\)), hydrogen peroxide concentrations in leaves (H\(_2\)O\(_2\)) (µg g FW\(^{-1}\)) and ascorbate peroxidase (APX) activity (µmol ascorbate min\(^{-1}\) mg\(^{-1}\) protein) in roots and leaves.

<table>
<thead>
<tr>
<th>Source of Variation</th>
<th>D.F.</th>
<th>(\Phi'M)</th>
<th>(H_2O_2)</th>
<th>APX Roots</th>
<th>APX Leaves</th>
</tr>
</thead>
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<tr>
<td>PO(_4^{3-})</td>
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<td>0.0184*</td>
<td>0.0005*</td>
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<td>&lt;0.0001*</td>
</tr>
<tr>
<td>Gly</td>
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<td>&lt;0.0001*</td>
<td>&lt;0.0001*</td>
<td>&lt;0.0001*</td>
<td>&lt;0.0001*</td>
</tr>
<tr>
<td>PO(_4^{3-}) x Gly</td>
<td>6</td>
<td>0.0006*</td>
<td>0.0270*</td>
<td>0.0006*</td>
<td>0.0002*</td>
</tr>
<tr>
<td>Time</td>
<td>2</td>
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<td>&lt;0.0001*</td>
<td>&lt;0.0001*</td>
<td>&lt;0.0001*</td>
</tr>
<tr>
<td>Time x PO(_4^{3-})</td>
<td>4</td>
<td>&lt;0.0001*</td>
<td>0.0002*</td>
<td>&lt;0.0001*</td>
<td>&lt;0.0001*</td>
</tr>
<tr>
<td>Time x Gly</td>
<td>6</td>
<td>&lt;0.0001*</td>
<td>0.3354</td>
<td>&lt;0.0001*</td>
<td>&lt;0.0001*</td>
</tr>
<tr>
<td>Time x PO(_4^{3-}) x Gly</td>
<td>12</td>
<td>&lt;0.0001*</td>
<td>0.0255*</td>
<td>&lt;0.0001*</td>
<td>&lt;0.0001*</td>
</tr>
</tbody>
</table>

D.F. Degrees of freedom

* Significant
CHAPTER IV

UNDERSTANDING THE INTERCONNECTED EFFECTS OF A
GLYPHOSATE-BASED HERBICIDE ON PHOTOSYNTHESIS AND
RELATED PHYSIOLOGICAL PROCESSES IN WILLOW PLANTS

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Labrecque, Marc Lucotte, Philippe Juneau

4 Article submitted to J. Exp. Botany.
SUMMARY

Apart from its recognized inhibitory effect on the shikimate pathway, it is still not clear how glyphosate-based herbicides can affect photosynthesis. We studied the physiological mechanisms involved in the deleterious effects of a glyphosate-based herbicide (Factor® 540) on photosynthesis and related physiological processes of willow (Salix miyabeana cultivar SX64) plants. Sixty-days-old plants grown under greenhouse conditions were sprayed with different rates (0, 1.4, 2.1 and 2.8 kg a.e ha⁻¹) of the commercial glyphosate formulated salt Factor® 540. Evaluations were performed at 6, 24, 48 and 72 h after herbicide exposure. We established that the herbicide decreases chlorophyll, carotenoid and plastoquinone contents, promoting changes in the photosynthetic apparatus leading to decreased photochemistry which results in peroxide (H₂O₂) accumulation. H₂O₂ accumulation triggers proline production which can be associated with oxidative protection, NADP⁺ recovery and shikimate pathway stimulation. Ascorbate peroxidase (APX) and glutathione peroxidase (GPX) appeared to be the main peroxidases involved in the H₂O₂ scavenging. In addition to promoting decreases of the activity of the antioxidant enzymes, the herbicide induced decreases in ascorbate pool. For the first time, a glyphosate-based herbicide mode of action interconnecting its effects on shikimate pathway, photosynthetic process and oxidative events in plants is presented.

SIGNIFICANCE STATEMENT

We demonstrated, for the first time, that on top of the alteration of its primary target site (shikimate pathway), the glyphosate-based herbicide Factor 540® induces a series of interconnected events that leads to a decrease in photosynthetic activity of treated plants. Furthermore, we showed that the herbicide-deleterious effects on photosynthesis of willow plants are strongly related to herbicide-induced oxidative stress. Our findings permit to obtain a better understanding of the glyphosate-based herbicide phytotoxicity.

Key-Words: Herbicide, oxidative stress, photosynthesis, shikimate, willow
4.1 INTRODUCTION

Glyphosate \([N-(\text{phosphonomethyl})\text{glycine}]\) is the most broadly used herbicide worldwide since the introduction of glyphosate-resistant (GR) plants (Coupe et al., 2012). Although it has been suggested as one of the least toxic pesticides to animals and humans (Williams et al., 2000; Cerdeira and Duke, 2006), the widespread use of glyphosate together with its great solubility trigger some concerns about its possible effects on the environment.

Glyphosate negative effects on non-target plants (Gomes et al., 2014b; Bott et al., 2011) and aquatic organisms (Vendrell et al., 2009; Inderjit and Kaushik, 2010) have been largely described. By inhibiting the 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase (EC 2.5.1.19), glyphosate-based herbicides prevent biosynthesis of aromatic amino acids (Siehl, 1997a) leading to shikimic acid accumulation (Duke and Powles, 2008). The depletion of the aromatic amino acid pool leads to a reduction of protein synthesis necessary to growth maintenance (Siehl, 1997a). On the other hand, some studies showed that in some plants, aromatic amino acid deficiencies upon glyphosate application have not been found (Lee, 1981; Wang, 2001; Serra et al., 2013), although deleterious effects of glyphosate have been observed, indicating that glyphosate can affect other plant physiological processes (Gomes et al., 2014b). Numerous studies demonstrated a decrease in the photosynthetic rate of plants following treatment with glyphosate (Mateos-Naranjo et al., 2009; Zobiole et al., 2012; Yanniccarri et al., 2012). In glyphosate-sensitive plants, glyphosate causes inhibition of CO\(_2\) assimilation (Vivancos et al., 2011) and depletion of intermediates of the photosynthetic carbon reduction cycle (Servaites et al., 1987) which could be linked to the unregulated flux of carbon into the shikimate pathway (Siehl, 1997a). Moreover, glyphosate can indirectly affect photosynthesis by inhibiting chlorophyll biosynthesis (Fedtke and Duke, 2005), decreasing stomatal conductance (Yanniccarri et al., 2012) and inducing nutritional disturbances (Cakmak et al., 2009; Su et al.,
Nowadays, a special attention has been given to understand glyphosate-induced oxidative stress in plants (Gomes et al., 2014b).

Reactive oxygen species are quite toxic and their accumulation could lead to cell death and then, to plant growth reduction (Gomes et al., 2014b). Although being a major site of ROS production, photosynthetic apparatus is highly sensitive to ROS accumulation (Foyer and Noctor, 2011). By interacting with biological molecules, ROS can induce destruction of DNA, lipids and proteins (Foyer and Noctor, 2011). To avoid oxidative damage due to ROS accumulation, plants have developed enzymatic (e.g. superoxide dismutase [SOD], catalase [CAT], ascorbate peroxidase [APX], glutathione peroxidase [GPX] and glutathione reductase [GR]) and non-enzymatic (e.g., ascorbate and glutathione) systems (Foyer and Noctor, 2011). The activity of antioxidant systems as well as the lipid peroxidation extent are oxidative stress markers which were shown to be modulated by glyphosate exposure (Moldes et al., 2008; Ahsan et al., 2008; Miteva et al., 2010).

Photosynthesis-targeting herbicides, such as atrazine, are known to induce oxidative stress by inhibiting Hill’s reactions (Fedtke and Duke, 2005). Plants exposed to these kinds of herbicides are not able to cope with the mass of triplet chlorophyll molecules produced due the blockage of the electron transport flow, resulting in cell oxidative bursts due to ROS accumulation. On the other hand, although glyphosate induces oxidative bursts (Moldes et al., 2008; Ahsan et al., 2008; Miteva et al., 2010), it is not known how the inhibition of the shikimate pathway can be related to the observed oxidative stress. In this study we accessed the physiological mechanisms involved in the deleterious effects of a glyphosate-based herbicide (Factor® 540) on photosynthesis of willow (Salix miyabeana cultivar SX64) plants. For the first time, a glyphosate-based herbicide mode of action interconnecting glyphosate effects on shikimate pathway, plant photosynthetic process and oxidative events is described.
4.2 RESULTS

4.2.1 Pigment content, gas exchange and chlorophyll fluorescence

Total chlorophyll and plastoquinone contents were decreased in plants by herbicide exposure and by treatment time (Table 4.1; P > 0.001). Carotenoid contents were greater in herbicide-treated plants at 6 h for all applied doses (Table 4.1); then, carotenoid contents were decreased in plants exposed for at least 24 h to herbicide concentrations (P < 0.001). The stomatal conductance was decreased in herbicide-exposed plants for all the treatment times (P < 0.001; Table 4.1). Similar effects were observed on the maximum electron transport rate (ETR_{max}), the minimum saturating irradiance (I_{k}) and the photochemical quenching (qP), which were significantly reduced in treated plants (P < 0.001). However, for the first evaluation (6 h), ETR_{max}, I_{k} and qP were not decreased in plants treated with 1.4 kg a.e ha^{-1} (Table 4.1). The relative unquenched fluorescence (UQF_{rel}) increased in all treated plants (Table 4.1). Concomitantly, the non-photochemical quenching (NPQ) decreased in plants exposed for more than 24 h to the herbicide (P < 0.001; Table 4.1). The maximal PSII photochemical efficiency (F_{v}/F_{m}) was decreased in herbicide-treated plants (P < 0.001). Decreased F_{v}/F_{m} was seen in plants treated with 1.4 kg a.e ha^{-1} only after 72 h of herbicide exposure. Plants exposed to 2.1 kg a.e ha^{-1} showed decreases in F_{v}/F_{m} at 48 and 72 h of exposure. In contrast, in all the evaluations, plants exposed to 2.8 kg a.e ha^{-1} showed decreased F_{v}/F_{m} (Table 4.1).
Table 4.1. Interactive effects of herbicide (Factor® 540) doses (kg a.e ha⁻¹) and time (hours) on photosynthesis-related parameter¹ in leaves of *Salix miyabeana* (cultivar SX64).

<table>
<thead>
<tr>
<th>Herbicide</th>
<th>Time</th>
<th>Chl</th>
<th>Carot</th>
<th>PQ</th>
<th>gs</th>
<th>ETRleaf</th>
<th>Ig</th>
<th>qP</th>
<th>UQFrst</th>
<th>NPQ</th>
<th>Fv/Fm</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>6</td>
<td>204.27</td>
<td>9.06bD</td>
<td>51.33</td>
<td>221.30cA</td>
<td>49.96bA</td>
<td>181.98bA</td>
<td>0.22cA</td>
<td>0.10</td>
<td>1.77bNS</td>
<td>0.77nsA</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>153.55</td>
<td>8.07bA</td>
<td>48.78</td>
<td>344.00bA</td>
<td>64.23aA</td>
<td>243.58aA</td>
<td>0.35A</td>
<td>0.08</td>
<td>1.32bA</td>
<td>0.78mnsA</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>152.49</td>
<td>10.51aA</td>
<td>44.57</td>
<td>296.13bA</td>
<td>40.79cA</td>
<td>142.40cA</td>
<td>0.24bA</td>
<td>0.08</td>
<td>2.49aA</td>
<td>0.79msA</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>179.11</td>
<td>11.78aA</td>
<td>37.24</td>
<td>354.00aA</td>
<td>59.72aA</td>
<td>263.34aA</td>
<td>0.23bA</td>
<td>0.10</td>
<td>1.58bA</td>
<td>0.79msA</td>
</tr>
<tr>
<td>1.4</td>
<td>6</td>
<td>175.40</td>
<td>12.08aC</td>
<td>42.66</td>
<td>152.41cB</td>
<td>50.98aA</td>
<td>198.98aA</td>
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<tr>
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<td>3.90cB</td>
<td>36.56</td>
<td>170.73cB</td>
<td>47.56aB</td>
<td>182.95aB</td>
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<td>0.21a</td>
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<td>0.63c</td>
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F-values: Herbicide 40.48*** 7.19*** 35.07*** 218.00*** 127.11*** 88.35*** 487.87*** 76.18*** 20.99*** 50.54***

F-values: Time 29.78*** 110.16*** 18.10*** 80.01*** 77.03*** 49.80*** 341.75*** 6.15*** 17.26*** 54.56***

F-values: Herbicide x Time 2.17= 20.16*** 0.957= 16.92*** 9.23*** 11.56*** 12.92*** 1.87= 4.02= 9.42***

Significance of ANOVA: NS—not significant F ratio (P < 0.05); ** and ***—significant at P < 0.05, 0.01 and 0.001 respectively.
Total chlorophyll and carotenoid contents (μg g⁻¹ FW), stomatal conductance (gₛ - mmol m⁻² s⁻¹), maximum electron transport rate (ETR max - μmol electrons m⁻² s⁻¹), minimum saturating irradiance (Iₙₖ - μmol photons m⁻² s⁻¹), photochemical quenching (qP), the relative unquenched fluorescence (UQFₘₜₐₜ), non-photochemical quenching (NPQ) and the maximal PSII photochemical efficiency (Fv / Fm). Values represent the means of 3 replicates. Different lowercase letters in each column for herbicide doses and time represent significant differences at the P<0.05 level, based on the Scott-Knott multiple range tests. Different lowercase letters in the column for herbicide x time interactions represent significant differences of time for each herbicide dose at the P<0.05 level based on the Scott-Knott test. Different capital letters in the column for herbicide x time interactions represent significant differences of herbicide dose for each time of evaluation at the P<0.05 level based on the Scott-Knott test. Significance of ANOVA: NS - not significant F ratio (P < 0.05); *, ** and *** significant at P < 0.05, 0.01 and 0.001 respectively.

4.2.2 Shikimate and proline contents

The shikimate and proline contents of herbicide-treated plants were always higher than the control (Table 4.2; Figure 4.1). An important shikimate and proline accumulation was found in herbicide-treated plants after 72 h of treatment (P < 0.001) (Figure 4.1).

4.2.3 H₂O₂ contents and lipid peroxidation

Hydrogen peroxide (H₂O₂) contents were always higher in plants exposed to the herbicide compared to control (Table 4.2; Figure 4.2). Remarkable increase in H₂O₂ contents was noticed in herbicide-treated plants after 72 h (P < 0.01). Lipid peroxidation (MDA content) was always higher in plants exposed to the herbicide (Table 4.2; Figure 4.1). In control plants, MDA content slightly increased at 24 h (P > 0.001). Plants treated with herbicide showed increasing MDA content after 24 h of exposure with a pronounced increase being observed at 72 h (P < 0.001).
Figure 4.1. Shikimate, proline and hydrogen peroxide contents and lipid peroxidation (MDA contents) in leaves of *Salix miyabeana* (cultivar SX64) plants spread with doses of 0 (filled circle), 1.4 (filled inverted triangle), 2.1 (open triangle) and 2.8 (filled square) kg a.e ha⁻¹ of the glyphosate-based herbicide (Factor® 540). Values are means ± SE of three replicates.

4.2.4 Antioxidant responses

Plants treated with herbicide showed higher activity of all evaluated antioxidant enzymes after 6 h in relation to control (Table 4.2; Figure 4.2). We found that: 1) superoxide dismutase (SOD) and ascorbate peroxidase (APX) activities were higher in herbicide treated plants up to 24 h, and then were reduced for the following exposure times (P < 0.001); 2) catalase (CAT) activity was always higher in plants treated with the herbicide (P < 0.001); 3) similar to SOD and APX, glutathione
reductase (GPX) activity was also reduced in herbicide treated plants at 48 and 72 h of exposure; 4) glutathione reductase (GR) activity was higher in herbicide treated plants up to 48 h of exposure and did not differ between treatments at 72 h (P > 0.05).

**Table 4.2.** Results of the two-way ANOVA and Skott-Knott tests for the effects of herbicide (Factor 540) doses (kg a.e ha\(^{-1}\)) and time (hours) on shikimate (μmol g\(^{-1}\) FW), proline (μmol g\(^{-1}\) FW), \(H_2O_2\) (mmol g\(^{-1}\) FW), MDA (nmol g\(^{-1}\) FW), SOD (U mg\(^{-1}\) protein), CAT (μmol H\(_2\)O\(_2\) min\(^{-1}\) g\(^{-1}\) protein), APX (μmol ascorbate m\(^{-1}\) mg\(^{-1}\) protein), GPX (μmol glutathione m\(^{-1}\) mg\(^{-1}\) protein) and GR (μmol NADPH min\(^{-1}\) mg\(^{-1}\) protein) activity in leaves of *Salix miyabeanasa* (cultivar SX64).

<table>
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<th>Source of variation</th>
<th>Shikimate</th>
<th>Proline</th>
<th>(H_2O_2)</th>
<th>MDA</th>
<th>SOD</th>
<th>CAT</th>
<th>APX</th>
<th>GPX</th>
<th>GR</th>
</tr>
</thead>
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<td>937.89**</td>
<td>97.42***</td>
<td>1174.59***</td>
<td>2.69**</td>
<td>93.25**</td>
<td>1.21**</td>
<td>27.63**</td>
<td>52.02**</td>
</tr>
<tr>
<td>Time</td>
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<td>36.78***</td>
<td>36.22**</td>
<td>616.84***</td>
<td>1527.94***</td>
<td>13.63**</td>
<td>40.60**</td>
<td>228.75***</td>
<td>4.74**</td>
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<tr>
<td>Herbicide x Time</td>
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<td>4.04**</td>
<td>2.02*</td>
<td>100.92***</td>
<td>204.27***</td>
<td>1.25ns</td>
<td>20.11**</td>
<td>37.58***</td>
<td>4.59***</td>
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Scott-Knott multiple range test

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<td>199.50a</td>
<td>263.58c</td>
<td>4.45a</td>
<td>19.15d</td>
<td>22.91d</td>
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ns – not significant F ratio (P < 0.05), *, ** and *** significant at P < 0.05, 0.01 and 0.001, respectively. Treatment means from ANOVA. Values followed by the same letter, within the same source of variation, are not significantly different (P < 0.05) by the Scott-Knott multiple range test.

Regarding ascorbate pool (Table 4.3) we found that, in relation to control: 1) total ascorbate concentrations (AsA + DHA) were higher in herbicide-treated plants up to 24 h of exposure, and then were reduced for the following exposure times; 2)
the concentrations of the ascorbate reduced form (AsA) were greater in control plants up to 24 h, did not differ between treatments at 48 h and was increased in herbicide treated plants for 72 h; 3) the concentrations of oxidized form of ascorbate (DHA) were greater in herbicide treated plants up to 24 h and were reduced for the following exposure durations; 4) the AsA/DHA ratio was lower in 6 and 24 h treated plants compared to control, but was higher for the following treatment times.

4.3 DISCUSSION

We demonstrated in this study that a glyphosate-based herbicide affected not only the shikimate pathway, but several physiological processes in willow plants. Figure 4.3 represents an integrative model interconnecting the studied physiological parameters (in particular, shikimate pathway, photosynthetic processes and oxidative events) affected by long-term (48 and 72 h) exposure to a glyphosate-based herbicide. The various steps of this model are identified throughout the text as Figure 4.3, #1-19.

As expected, the glyphosate-based herbicide clearly inhibited the shikimate pathway in willow plants, as demonstrated by the shikimate accumulation (Figure 4.1). We can advance that by inhibiting the shikimate pathway, the glyphosate-based herbicide prevents the biosynthesis of several secondary plant compounds (such as plastoquinones and aromatic amino acids) (Siehl, 1997a) including plastoquinones as seen in Table 4.1 (Figure 4.3, #1). It is known that UQF_rel is an indicator of closed PSII reaction centers present under continuous illumination (Juneau et al., 2005) and qP represents the proportion of open PSII reaction centers (RCs) (Maxwell and Johnson, 2000). Therefore, the higher UQF_rel and reduced qP in treated plants indicate that the plastoquinone pool, and thus the PSII RCs were in a more reduced state than in control plants, a consequence of a lower PQ content (Figure 4.3#2 and 3) and/or less effective PSI. This may, together with the decrease in the minimum saturating irradiance (I_k) (for
Figure 4.2. Superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), glutathione peroxidase (GPX) and glutathione reductase (GR) activities in leaves of *Salix miyabeana* (cultivar SX64) plants spread with doses of 0 (filled circle), 1.4 (filled inverted triangle), 2.1 (open triangle) and 2.8 (filled square) kg a.e ha⁻¹ of the glyphosate-based herbicide (Factor® 540). Values are means ± SE of three replicates.
doses ≥ 1.4 kg a.e ha⁻¹), have contributed to the observed lower electron transport rate (ETR) in treated plants (Table 4.1; Figure 4.3#4) due to a lower ability for PSII to deliver electrons to the electron transport chain, leading to PSII saturation at low irradiance. However, we demonstrated that other effects of the glyphosate-based herbicide have also caused the decrease in the ETR (see below).

The observed increase in the carotenoid content after 6 h in the herbicide-treated plants (Table 4.1) could be related to the concomitant increase in H₂O₂ concentration, since it is known that ROS presence can induce carotenogenic responses (Fan et al., 1998). Indeed, by the activation of latent biosynthetic enzymes (such as glutathione transferase and glutathione reductase) or by the expression of genes coding for carotenogenic enzymes, ROS may regulate carotenoid content (Aniya and Anders, 1992; Bouvier et al., 1998). Since the maximal PSII photochemical yield (Fᵥ/Fₘ) is a proxy of the PSII integrity (Walter et al., 2003), the undisturbed maximal PSII photochemical yield observed up to 24 h in treated plants (with the exception of the highest dose; Table 4.1) indicates that the glyphosate-based herbicide had no effect on the PSII integrity following short term exposure. This may be the consequence of the increased carotenoid content helping to prevent ROS-damages to PSII (Gomes et al., 2013b). Carotenoids are usually involved in the protection of the oxidative damage by the detoxification of oxygen singlets (¹O₂) produced by photosynthesis or by enzymatic conversion of other ROS to oxygen singlets (Boussiba, 2000). Although plants exposed to the highest herbicide concentration contain high carotenoid content, this was not sufficient to prevent oxidative damages to PSII (since we observed lower Fᵥ/Fₘ value).
### Table 4.3. Interactive effects of herbicide (Factor® 540) doses (kg a.e ha$^{-1}$) and time (hours) on total (AsA + DHA – nmol g$^{-1}$ FW), reduced (AsA – nmol g$^{-1}$ FW) and oxidized (DHA – nmol g$^{-1}$ FW) form of ascorbate in leaves of *Salix myabeanad* (cultivar SX64).

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<th>DHA</th>
<th>Asa/DHA</th>
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<td>358.33cB</td>
<td>1.73aA</td>
</tr>
<tr>
<td></td>
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<td>847.33cB</td>
<td>537.66aA</td>
<td>309.66cB</td>
<td>1.61aA</td>
</tr>
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<td>2.8</td>
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<td>1.02a</td>
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</tr>
</tbody>
</table>

**F values**

<table>
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<th>Herbicide</th>
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<th>20.75***</th>
<th>5.17***</th>
<th>6.22***</th>
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<tr>
<td>Time</td>
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<td>256.16***</td>
<td>359.10***</td>
<td>227.05***</td>
</tr>
<tr>
<td>Herbicide x Time</td>
<td>61.69***</td>
<td>17.67***</td>
<td>105.97***</td>
<td>39.58***</td>
</tr>
</tbody>
</table>
Values represent the means of 3 replicates. Different lowercase letters in each column for herbicide doses and time represent significant differences at the $P<0.05$ level, based on the Scott-Knott multiple range tests. Different lowercase letters in the column for herbicide x time interactions represent significant differences of time for each herbicide dose at the $P<0.05$ level based on the Scott-Knott test. Different capital letters in the column for herbicide x time interactions represent significant differences of herbicide dose for each time of evaluation at the $P<0.05$ level based on the Scott-Knott test. ns - not significant $F$ ratio ($P > 0.05$), *, ** and *** significant at $P < 0.05$, 0.01 and 0.001, respectively. Treatment means from ANOVA. Values followed by the same letter, within the same source of variation, are not significantly different ($P < 0.05$) by the Scott-Knott multiple range test.

These plants also showed higher lipid peroxidation (Figure 4.1) indicating higher oxidative damages. However, for exposure times longer than 24 h, plants treated with glyphosate-based herbicide showed reduced carotenoid content (Table 4.1). This can be a consequence of the inhibition of the shikimate pathway, since plastoquinone, is a co-factor of the phytoene desaturase and $\zeta$-carotene desaturase involved in the carotenoid biosynthesis pathway (Sandmann et al., 2006). Therefore, decreased plastoquinone content will affect directly carotenoid biosynthesis (Figure 4.3#5). We also observed that the non-photochemical energy dissipation (NPQ), one of the mechanisms by which plants can dissipate excess light energy absorbed by PSII light-harvesting complexes in order to minimize the generation of the highly reactive singlet oxygen ($^1O_2$) responsible of oxidative damages (Demming-Adams and Adams, 2000), decreased in presence of the glyphosate-based herbicide (Table 4.1). Since $\beta$-carotene is known to be the precursor of zeaxanthin, the first compound of xanthophyll cycle (Bouvier et al., 1996), reduced carotenoid content could lead to inhibition of the xanthophyll cycle reducing plant capacity for photoprotection and thus, leading to increased PSII damages (as shown by reduced $F_V/F_M$) (Figure 4.3#6). These decreases in the photosynthetic activity (shown by the decrease in ETR) and in the NPQ may also have contributed to a higher production of ROS due over-excitation of chlorophylls (Figure 4.3#7). Increased lipid peroxidation has been previously observed in glyphosate-exposed plants and was related to increased H$_2$O$_2$ content in pea and soybean plants (Moldes et al., 2008; Miteva et al., 2010). Similarly, we observed increased lipid peroxidation and increased H$_2$O$_2$ contents in
our study (Figure 4.1 and Figure 4.3#8). Lipid peroxidation resulting from an increased level of ROS (such as H$_2$O$_2$) has been shown to affect the integrity of the thylakoid membranes (Richter, 1992), contributing to the noted decrease in ETR (Figure 4.3#9). In addition, it has long been recognized that H$_2$O$_2$ is a potent inhibitor of photosynthesis, since even at low concentrations it can inhibit CO$_2$ fixation by oxidizing the thiol groups of some essential enzymes of the Calvin cycle (Foyer and Noctor, 2011). We can therefore advance that the observed decrease in photosynthesis (ETR) in presence of glyphosate-based herbicide may also be directly linked to the higher H$_2$O$_2$ content (Figure 4.3#8). Herbicide treated plants decreased their stomatal conductance ($g_s$) (Table 4.1), as it was previously reported in Hordeum vulgare (barley) and Lolium perenne plants (Olesen and Cedergreen, 2010; Yanniccare et al., 2012). This reduced $g_s$ can negatively affect carbon assimilation by limiting gas exchange (Zobiole et al., 2010b), which can limit photochemistry, and therefore can lead to a decreased ETR (Figure 4.3#10). The observed ETR reduction could also be due to the alteration of the integrity of PSII (lower F$_{v}$/F$_{M}$) (Table 4.1; Figure 4.3#11). In addition, the decrease in total chlorophyll content in presence of the glyphosate-based herbicide is responsible for a lower light interception and thus, the noted lower electron transport rate (ETR, Figure 4.3#11). Decreased chlorophyll contents when plants are exposed to herbicide application have been demonstrated previously and have been attributed to an increase chlorophyll degradation or to a decrease in chlorophyll synthesis.(Mateos-Naranjo et al., 2009; Cakmak et al., 2009; Huang et al., 2012).

In order to better understand the processes involved in H$_2$O$_2$ accumulation (and herbicide-induced oxidative damage), we investigated the activity of antioxidant system in treated plants. Increases in proline synthesis is a common response of plants to stress conditions (Hayat et al., 2012), and its accumulation in plants exposed to glyphosate was also documented (Huang et al., 2012), and has been interpreted as a proline-protective effect in response to stress. However, it is important to note that
Praline can also act as a significant signaling molecule in plant physiological processes, mainly under stress conditions (Hare and Cress, 1997). In the present study, we suggest that the observed praline accumulation in treated plants is associated to oxidative protection, NADP⁺ recovery and shikimate pathway stimulation (Figure 4.3#12 and #13). As we also observed, proline biosynthesis is commonly stimulated by increased cellular-ROS concentrations conditions (Soshinkova et al., 2013). Although praline can be synthesized from ornithine, metabolic labeling studies indicate that, under stressing conditions, praline is mainly produced from glutamate (as reviewed by Hare & Cress, 1997). Therefore, the praline accumulation found in our study indicates that this pathway is highly activated (Figure 4.3#12). A special function of praline in preventing oxidative damage and enhancing tolerance from abiotic oxidative stress has been proposed recently (Soshinkova et al., 2013). Proline accumulation in plants in response to glyphosate exposure was also documented (Huang et al., 2012), and has been interpreted as a proline-protective effect in response to stress. Moreover, due the loss of feedback control of the shikimate pathway by tyrosine (that regulates the activity of 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase) (Crowley, 2006), the herbicide (glyphosate) led to an unregulated flux of carbon into the shikimate pathway (Siehl, 1997a). As a result, there is an increased demand of erythrose-5-phosphate, the substrate of the first reaction of the shikimate pathway. Erythrose-5-phosphate is produced in the oxidative pentose phosphate pathway (OPPP), which is dependent on NAD(P)⁺ availability and inhibited by NADPH (Hare and Cress, 1997). During proline synthesis, NADPH is oxidized, therefore stimulating OPPP. Even a small change in the NAD(P)⁺/NADPH ratio may have a large effect on this redox-sensitive pathway (Hare and Cress, 1997). The oxidation of NADPH during proline synthesis, coupled to the reduction of NADP⁺ during the two oxidative steps of the OPPP, promotes a cycle of changes in NAD(P)⁺/NADPH ratio which stimulates proline biosynthesis, justifying its accumulation during stress (Hare and Cress, 1997). Therefore, upon the glyphosate-based herbicide exposure, the proline accumulation in
willow plants could also be linked to the OPPP stimulation for the production of the erythrose-5-phosphate which will be used in shikimate pathway (Figure 4.3#13).

Figure 4.3. Interconnected model of the effects of the glyphosate-based-herbicide (Factor® 540) on photosynthesis and oxidative markers of willow plants. Numbers refer to the ones mentioned in the discussion. ABA, abscisic acid (ABA); ALA, δ-aminolevulinic acid; APX, ascorbate peroxidase; AsA, ascorbate; EPSPS, 5-enolpyruvylshikimate-3-phosphate synthase; ETR, electron transport rate; Fv/Fm, maximal PSII photochemical efficiency; GPX, glutathione peroxidase; gs, stomatal conductance; H$_2$O$_2$, peroxide; I$_{s}$, minimum saturating irradiance; MDA, lipid peroxidation; NPQ, non-photochemical quenching; qP, photochemical quenching; SOD, superoxide dismutase; UQF$_{rel}$, the relative unquenched fluorescence. Literature-based information in the model are expressed in italic words and in dotted arrows. While, observed data obtained in the present study are introduced in the model as bold words and non-dotted arrows.
As mentioned previously, under stress conditions, proline is mainly produced from glutamate (Hare and Cress, 1997). Glutamate is also required during δ-aminolevulinic acid (ALA; a chlorophyll precursor) biosynthesis through ALA-synthetase and γ,δ-dioxovalerate cycles (Beale, 1978). Therefore, if glutamate was preferentially used for proline biosynthesis (as suggested by proline greater accumulation in treated plants in relation to control; Figure 4.1), a decrease in ALA biosynthesis may be obtained, therefore contributing to the decreased chlorophyll content observed in treated plants (Figure 4.3#14). The decrease in chlorophyll content may also be due to its degradation by increased ROS content (Figure 4.3#15).

Even though treated plants showed increased activities of antioxidant enzymes after 6 h exposure, they were not able to prevent both peroxide accumulation and lipid peroxidation indicating a clear deleterious effect of the glyphosate-based herbicide through oxidative burst. A strong inhibition of SOD, APX and GPX activities were observed in plants exposed to herbicide after 48 h. Simultaneously, increased H₂O₂ and decreased ETR were observed in these plants. SOD is the first defense enzyme against oxidative stress (Pompeu et al., 2008) and is closely related to stress resistance in plants (Song et al., 2006). Indeed, this enzyme was involved in the PSII protection against the effects of prooxidant herbicide, limiting carbon dioxide and photoinhibitory conditions (Foyer et al., 1994; Arisi et al., 1998). The observed decrease in SOD activity (Figure 4.2) can therefore contribute to the herbicide-deleterious effects on photosynthesis in willow plants. We also demonstrated the key role of ascorbate peroxidase (APX) and glutathione peroxidase (GPX) to prevent H₂O₂ accumulation in willow plants since: 1) decreased activities of both enzymes were related to increased H₂O₂ content in leaves; 2) even if in treated plants we observed higher catalase (CAT) activity, it was not able to prevent H₂O₂ accumulation. The importance of APX and GR in avoiding oxidative stress has also been observed in metal(loid) treated plants (Gomes et al., 2013a,b; Chaoui et al., 1997) and the inactivation/degeneration of these enzymes has been related to
increased \( \text{H}_2\text{O}_2 \) contents and oxidative damages to plants (Gomes et al., 2013b). When \( \text{H}_2\text{O}_2 \) accumulation exceeded the tolerance limit of plants, enzyme systems are prone to protein carbonilation – an irreversible oxidative process in which the side chains of Lys, Arg, Pro, and Thr are converted to aldehyde or keto groups (Sohal et al., 2002), which may have been occurring in willow plants exposed to the studied herbicide (Figure 4.3#16).

We also observed an interesting response for glutathione reductase (GR) activity for the longer time glyphosate-based herbicide exposure (48h and 72h). Indeed, its activity was not significantly decreased by the glyphosate-based herbicide exposure. GR is linked to APX and GPX activity by the glutathione-ascorbate cycle (Foyer and Noctor, 2011). However, as mentioned, the GR activity did not follow APX and GPX patterns. The maintenance of GR activity in treated plants indicates that APX and GPX activities were not limited by substrate availability, reinforcing that the proposed oxidative damage (protein carbonilation) of the enzymes could be responsible for their degeneration. Until regarding GR, we may hypothesize that, similarly to the proline production, the higher \( \text{NADP(H)} \)-dependent-GR activity can favor OPPP and contribute as a source of \( \text{NADP}^+ \) for photochemistry.

In addition to being the substrate for APX, ascorbate is an important antioxidant component of the cellular redox potential and has its activity linked to ascorbate-glutathione metabolic cycle (Foyer and Noctor, 2011). In the present study, we found a link between the reduced form of ascorbate (AsA) and the APX activity. Up to 24 h, treated plants showed higher APX activity that we can relate to the reduced AsA concentration in their leaves; similarly, decreased APX activity for the following treatment periods was related to the increased AsA content. On the other hand, the contrary was observed for the oxidized form of ascorbate (DHA). The accumulation of AsA, as shown by the increased AsA/DHA ratio in treated plants, shows that the DHA has been effectively recycled to AsA by ascorbate-glutathione cycle. We also observed that total ascorbate concentrations (AsA + DHA) were
reduced in herbicide treated plants (Table 4.3). It is known that ascorbate concentrations and ETR are closely linked as the light-dependent stimulation of ascorbate biosynthesis requires photosynthetic electron transport activity (Yabuta et al., 2007). Thus, reduced ETR in treated plants could explain the observed reduction in ascorbate pool (Figure 4.3#17). It is known that low ascorbate pool favors the increase in both ROS (Figure 4.3#18) and abscisic acid (ABA), leading to an increase in signal transduction through ROS-mediated and ABA-dependent signaling cascades (Foyer and Noctor, 2011). Among others, the interactive effect of ROS and ABA in stomatal movement is well studied, with increased ROS and ABA content inducing stomatal closure (Gomes et al., 2014a). This mechanism can also be related to the observed herbicide-induced decreases in gs (Figure 4.3#19).

As expected, the primary target site of the studied glyphosate-based-herbicide (Factor® 540) on willow plants is the shikimate pathway. We demonstrated, for the first time, that on top of the alteration of this primary target site, the herbicide induces a series of interconnected events that leads to a decrease in photosynthetic activity of the treated plants. Furthermore, we showed that the herbicide-deleterious effects on photosynthesis of willow plants are strongly related to herbicide-induced oxidative stress, and that reduction of photosynthesis may amplify the observed effect by inducing ROS production. Since photosynthetic processes of glyphosate-resistant plants have been shown to be affected by glyphosate-based herbicides (Zobiole et al., 2010b, 2011b, 2012), further studies are needed to establish if the presented model fits also to these plants.

4.4 EXPERIMENTAL PROCEDURES

4.4.1 Greenhouse experiments

Salix miyabeana cultivar SX64 was chosen for this study due to its high tolerance to stress factors, fast growth and great biomass production (Labrecque and Teodorescu, 2005). Twenty cm long cuttings were grown in plastic boxes (35 litters)
filled up with distilled water and 20 ml of King Max nutrient solution A and B (Montreal, Canada). The solutions were continuously aerated and renewed every 15 days. The pH of the medium was checked and adjusted on a weekly basis to 6.5 ± 0.1. 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 825 μmol photons m⁻² s⁻¹.

A randomized block design with 3 replicates in a 5 x 4 factorial scheme was used. Treatments consisted of five herbicide rates (0, 1.4, 2.1 and 2.8 kg a.e ha⁻¹) and four times of exposure (6, 24, 48, 72 h).

The herbicide tested in this study was a commercial formulation of the herbicide glyphosate (Factor 540°, IPCO, Winnipeg, Manitoba, Canada), which is normally used in the agricultural areas in Quebec. We selected doses of 0, 50, 75 and 100% of the herbicide application rate used by the farmers (i.e., 5 l of Factor° 540 to be applied with 140 l of water per ha: 114.20 mM glyphosate as active ingredient) which were equivalent to 0, 1.4, 2.1 and 2.8 kg a.e ha⁻¹, respectively. Freshly prepared herbicide solution was sprayed on foliage in a volume of nearly 5 mL per plant (75 ml per box). This sprayed volume of the solution did not cause any runoff from leaves.

Photosynthetic and biochemical evaluations were performed at 6, 24, 48, 72 h after the beginning of the treatments and were stopped after 72 h of exposure as plants from the highest glyphosate treatment showed pronounced intoxication symptoms, including several necrotic spots and loss of leaves (data not shown). After photosynthetic and stomatal conductance evaluations, plants were harvested and thoroughly washed with distilled water. Samples of the seventh to ninth fully expanded leaves were stored in aluminum foil paper at -80 °C until biochemical and oxidative damage evaluations.
4.4.2 Gas exchange, chlorophyll fluorescence and pigment contents

Gas exchange, chlorophyll fluorescence and pigment contents were measured on samples of the seventh (first fully expanded leaf from apex) to ninth leaves, for a total of three measurements per plant. Measurements of stomatal conductance ($g_s$) were performed using a leaf porometer (model SC-1, Decagon Devices Inc., Washington, USA). Then, these leaves were dark-acclimated for 20 min and the chlorophyll fluorescence emission was assessed using a pulse-amplitude modulation (PAM) fluorometer (model PAM-2500, WALZ, Effeltrich, Germany). An 11 step rapid light curve (RLC) was performed. Saturating pulses were triggered at 0.8 min intervals with actinic light intensity varying for each step (0, 31, 48, 76, 117, 179, 253, 405, 586, 874, 1326 µmol photons m$^{-2}$ s$^{-1}$). Using the RLC, the evaluation of the following parameters was performed: the relative rate of electron transport through PSII (ETR) (Krall and Edwards, 1992), the photochemical quenching ($q_P$) (van Kooten and Sneel, 1990), the relative unquenched fluorescence ($U_{QF}$) (Juneau et al., 2005), the non-photochemical quenching (NPQ) (Redondo-Gómez et al., 2008) and the maximal photochemical efficiency of PSII ($F_v/F_M$) (Kitajima and Butler, 1975).

To compare treatments, NPQ from the 874 µmol photons m$^{-2}$ s$^{-1}$ (most similar irradiation in relation to light growth conditions) were used. A ETR versus irradiance curves was also plotted and the initial slope of RLC ($a$), the maximum electron transport rate (ETR$_{max}$) and the minimum saturating irradiance ($I_k$) were calculated according to Eilers and Peeters (Eilers and Peeters, 1988).

For pigments evaluations, three foliar discs of approximately 5 mm in diameter were cut from each leaf, and after determining the fresh weight of the samples, their chlorophyll and carotenoid pigments were extracted in 80% acetone after macerating the discs with a mortar and pestle. The absorption of the extracts at 470 nm, 646 nm, and 663 nm was measured using a Varian Cary® 300 Bio UV-Vis spectrophotometer (USA). The concentrations ($\mu$g/g fresh leaf weight) of total
chlorophylls and carotenoids were then calculated using the equations described by Lichtenthaler & Wellburn (1983).

4.4.3 Biochemical evaluations

Shikimate and proline contents were evaluated following the methods of Bijay & Dale (1998) and Bates et al. (1973), respectively. To evaluate the pool of quinones in leaves, 0.1 g of fresh plant tissue was ground in liquid nitrogen, homogenized in 1000 μl of freeze-cold ethyl acetate and then centrifuged for 1 min at 6,590 x g (Kruk and Karpinski, 2006). The supernatant was then transferred to a collecting tube and the procedure was repeated twice (by adding 1000 μl of freeze-cold ethyl acetate to the pellet) to assure high extraction efficiency. Ten μl of cold 1 M sodium borohydride (NaBH₄) was added to the combined supernatant to convert quinone to its reduced form and then, samples were centrifuged for 2 min at 10,000 x g to remove the impurities (Yoshida et al., 2010). The standard of plastoquinone (PQ-9, 1 mM) was acquired from the laboratory of J. Kruk (Jagiellonian University, Poland). After dilution in ethanol, the amount of 20 μl of cold 1 M NaBH₄ were added to assure complete reduction of plastoquinone pool. The UHPLC (Agilent 1290 Infinity II LC, Wilmington, USA) measurements were performed according to (Yoshida et al., 2010), using UV-VIS detector, fluorescence detector, column (50 x 2.1 mm, ) isocratic solvent system (methanol/hexane, 340/20 vol/vol), flow rate of 0.31 ml/min, absorption detection wavelength at 255 nm, fluorescence excitation/emission detection at 290/330 nm, and injection volume of 1 μl.

4.4.4 Statistical analyses

Results were expressed as the averages of three replicates. Statistical analyses were performed using JMP 10.0 software (SAS Institute Ins.). The results were submitted to normality (Shapiro–Wilk) and homogeneity (Brown-Forsythe) tests and then, they were statistically evaluated using two-way analysis of variance. The means were compared using the Scott-Knott test at a 5% level of probability.
4.5 REFERENCES


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CHAPTER V

DIFFERENTIAL EFFECTS OF GLYPHOSATE AND AMINOMETHYLPHOSPHONIC ACID (AMPA) ON PHOTOSYNTHESIS AND CHLOROPHYLL METABOLISM OF WILLOW PLANTS

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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 (0, 1.4, 2.1 and 2.8 kg ha\(^{-1}\)) and AMPA (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 hs 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.

Key-words: aminolevulinic acid, herbicide, oxidative stress, pigment, ROS, photosynthesis, glyphosate, AMPA

5.1 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 (Williams et al., 2000; Cerdeira and Duke, 2006). 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 (Cerdeira and Duke, 2006). Similarly to soil and aquatic microorganisms, it was proposed that some plants can metabolize glyphosate to sarcosine through C-P lyase activity (RongFang et al., 2009), or to AMPA through glyphosate oxidase (GOX) activity (Reddy et al., 2004). GOX pathway is usually the most expected one, since AMPA is
found as the major glyphosate-metabolite in plants (Duke et al., 2003a). 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) (Cerdeira and Duke, 2006). However, not so many studies have examined the effects of AMPA on plants (Duke et al., 2003b; Reddy et al., 2004; Ding et al., 2011) and to date, only high AMPA concentrations were described to induce detrimental effects in GR and non-GR plants (Ding et al., 2011).

Glyphosate effects on plant physiological processes have recently been reviewed (Gomes et al., 2014a), and include deleterious effect on photosystem II quantum efficiency as well as electron transport rates (ETR). It is known that decreased photochemical efficiency is associated with glyphosate-induced decreases in chlorophyll contents (Mateos-Naranjo et al., 2009; Zobiole et al., 2011; Huang et al., 2012), 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 (Cakmak et al., 2009). However, glyphosate effects on mineral nutrition is contradictory, and a number of studies did not report any effect (Bailey et al., 2002; Rosolem et al., 2010). 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 (Harris et al., 2012). Reddy et al. (Reddy et al., 2004), 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 (Mateos-Naranjo et al., 2009; Huang et al., 2012). Increased reactive oxygen species (ROS) concentrations and oxidative stress have been observed in glyphosate-exposed plants (Sergiev et al., 2006; Ahsan et al., 2008), as in many other herbicide-exposed plants (Fedtke and Duke, 2005), although according to Moldes et al. (Moldes et al., 2008) the slight
oxidative stress induced by glyphosate has no relevance to plant mortality. However, it is widely accepted that modulation in ROS metabolism can affect plant physiology (Ahmad, 2014), which can be related to some deleterious indirect effects of glyphosate on photosynthesis (even in GR plants (Reddy et al., 2000; Zobiole et al., 2011)), i.e., by inducing chlorophyll degradation and chloroplast functional losses (Pitzschke et al., 2006).

Due to their rapid and voluminous biomass production, in association with their high tolerance to stress factors, willows are potential candidates for phytoremediation programs (Labrecque and Teodorescu, 2005) 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.

5.2 MATERIAL AND METHODS

5.2.1 Greenhouse experiments

Eighteen Salix 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 litters) containing distilled water amended with 20 ml of King Max nutrient solutions A (7% P₂O₅, 11% K₂O, 1.5% Mg, 1.27% S, 0.07% B, 0.002% Mo, 0.12% Zn) and B (4% N, 1% NH₄⁺, 3% NO₃⁻, 10% K₂O, 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).

5.2.2 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. (Goscinny et al., 2012) 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 4,000 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 (N₂) flow. The derivatization procedure was carried out following Börjesson and Torstensson’s method (Börjesson and Torstensson, 2000) 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 one hour. After returning to room temperature, the samples were evaporated to dryness under N₂ 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 x 0.25 mm ID, 0.25 μm) was used to analyze the 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.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 Mocack et al. (Mocak et al., 1997)). 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 (r² = 0.96; p < 0.0001 and r² = 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).
5.2.3 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 PSI (Fv/Fm), the relative electron transport rate through PSII (ETR), and non-photochemical quenching (NPQ) were calculated according to Kitajima and Butler (Kitajima and Butler, 1975), Krall and Edwards (Krall and Edwards, 1992), and Bilger and Björkman (Bilger and Björkman, 1990) 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 discs (approximately 5 mm in diameter) were taken from each leaf for pigment evaluations. After determining the fresh weighs of the samples, the discs were macerated and chlorophyll, carotenoid (Lichtenthaler and Wellburn, 1983), and pheophytin (Vernon, 1960) 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 (1983), while pheophytin a and b concentrations were calculated according to Vernon (1960).

5.2.4 Oxidative responses

Oxidative responses, hydrogen peroxide (H₂O₂), lipid peroxidation (MMA contents), and antioxidant enzyme activities were examined following the methods described by Gomes et al. (Gomes et al., 2013). 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: 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 method. The activities of the following enzymes were determined: superoxide dismutase (SOD; EC 1.15.1.1), catalase (CAT; EC 1.11.1.6), and ascorbate peroxidase (APX; EC 1.11.1.11).

5.2.5 Statistical analyses

All results were expressed as the average of three replicates. Statistical analyses were performed using JMP 10.0 software (SAS Institute Ins.). The data were submitted to tests of normality (Shapiro-Wilk) and homogeneity (Brown-Forsythe) and statistically evaluated using one-way analysis of variance. The means were compared using the Scott-Knott test at a 5% level of probability.

5.3 RESULTS AND DISCUSSION

As expected, leaf glyphosate and AMPA concentrations increased as glyphosate and AMPA treatment doses increased (P < 0.05; Fig. 5.1). AMPA concentrations were undetectable in the leaves of control and glyphosate-treated plants. Similarly, glyphosate concentrations were undetectable in leaves of control plants and AMPA-treated plants (Fig. 5.1). The absence of AMPA in glyphosate-treated plants indicated that hydroponically grown willow plants did not metabolize glyphosate to AMPA after three days of exposure. Glyphosate degradation in the leaves of GR soybean initiates after only one day of glyphosate exposure, and AMPA was suggested to be responsible for the observed decrease in chlorophyll content in that species (Reddy et al., 2004). The AMPA and glyphosate phytotoxic effects in plants were recently reviewed (Gomes et al., 2014a). Since glyphosate was not metabolized to AMPA (as AMPA was not detected in glyphosate-treated plants) and
AMPA-treated plants were not contaminated by glyphosate (as glyphosate was not detected in AMPA-treated plants) (Fig. 5.1), we were able to investigate the differential effects of these compounds on the leaf physiology (pigment contents, photosynthesis, and antioxidant systems) of willow plants.

The results of the present study demonstrated that both AMPA and glyphosate had phytotoxic effects on willow plants and caused decreases in photosynthesis, with the maximal photosystem II quantum yields (Fv/Fm) and the relative electron transport rates (ETR) being reduced by exposure to either compound at all applied rates (P < 0.05; Table 5.1). Decrease in the maximum PSII quantum yield (Fv/Fm) can be caused by the inhibition of the oxygen evolving complex (Björkman and Demmig, 1987), the inactivation of PSII reaction centers, or low photosynthetic pigment contents (Krause and Weis, 1991). AMPA is a very poor chelator, and it is not reported to affect Mn clusters which are involved in the oxygen evolving complex, therefore, the decrease in Fv/Fm in the presence of AMPA is probably related to changes in, or damage to, the PSII structure. This could also be expected in glyphosate-treated plants, as plants showing decreased Fv/Fm also demonstrated increased H2O2 contents in their leaves (Fig. 5.2). Peroxide accumulation is known to suppress the de novo synthesis of PSII proteins (especially the D1 protein) required for PSII repair and assembly (Takahashi and Murata, 2008). Therefore, by inducing H2O2 accumulation, as shown also previously for glyphosate in various species (Sergiev et al., 2006; Ahsan et al., 2008; Miteva et al., 2010; Maroli et al., 2015) AMPA and glyphosate can disturb plant photochemistry, as seen by the decreased ETR of treated plants (Table 5.1). Similar to the decrease in Fv/Fm, a decrease in ETR may be related to low chlorophyll contents – and decreased pigment contents were, in fact, observed in AMPA and glyphosate-treated plants (P < 0.05; Fig. 5.3). Since chlorophylls are essential constituents of active PS reaction centers and are responsible for light harvesting, decreased chlorophyll contents can contribute to reducing the number of functional PS and reduced light harvesting – being, therefore,
directly related to the observed decreases in Fv/Fm and ETR. One could hypothesize that ETR decreases could also be related to the destruction of other photosynthetic apparatus components, not associated with PSII, causing a decrease in the ability of PSI and/or the Calvin cycle to use energy from PSII (Sharkey et al., 1988) but, as will be discussed below, this possibility is unlikely.

**Fig.5.1.** Glyphosate and AMPA concentrations in the leaves of willow plants treated with different concentrations of AMPA or glyphosate. Treatment means from ANOVA. Bars indicate the means ± standard errors of three replicates. Means followed by the same letter within the same column are not significantly different (P < 0.05) by the Scott-Knott multiple range test.

Concomitant to decreases in photosynthetic activity, non-photochemical quenching (NPQ) also became reduced in treated plants (Table 5.1; P < 0.05). When electron transport between PSII and PSI is altered by environmental stress, the NPQ of the affected photosynthetic organisms will rapidly increase to protect the photosynthetic
apparatus against photoinhibition – and the xanthophyll cycle is the main component of this NPQ energy dissipation (Bilger and Björkman, 1990). When electron transport is reduced due to limited electron input from PSII, however, the trans-thylakoid ΔpH will diminish and the induction of the xanthophyll cycle consequently reduced (Gilmore, 1997). Therefore, in presence of glyphosate or AMPA, decreased maximal PSII quantum yield and relative electron transport will contribute to decreases in NPQ, leading to less protection for PSII, which will then amplify the negative effects on the PSII reaction centers of treated plants. We also noted lower carotenoid contents in plants showing decreased NPQ processes (P < 0.05; Fig. 5.3). Since, carotenoids (i.e., β-carotene) are precursors of xanthophylls (Demming-Adams and Adams, 2000), this may also contribute to the observed decreases in NPQ. Decreased carotenoid contents could also contribute to the observed decreases in Fv/Fm in treated plants, as carotenoids are known to protect against oxidative damages by quenching ROS produced by photosynthesis or by chloroplast enzyme reactions (Boussiba, 2000). Based on the effects of AMPA and glyphosate on NPQ and Fv/Fm, we can reasonably assume that the decrease in ETR is mainly related to impacts at the PSII level by ROS, and to a less extent (if it occurs) to a decrease in energy consumption at the PSI or the Calvin cycle level.

By inhibiting the shikimate pathway, glyphosate can directly induce decreases in carotenoid content by preventing plastoquinone biosynthesis (Cobb and Reade, 2010), as plastoquinone is a co-factor of the enzymes phytoene desaturase and ζ-carotene desaturase (Sandmann et al., 2006) – although how AMPA can induce decreases in carotenoid contents is not currently clear. We hypothesize that by inducing decreases in chlorophyll biosynthesis AMPA can reduce carotenoid levels, as the synthesis of both pigments is quantitatively and qualitatively coordinated by a shared common biosynthetic precursor, geranylgeranyldiphosphate (GGPP) (Welsch et al., 2000). In addition to the expression of their corresponding genes, it has been suggested that carotenoid accumulation is dependent on chlorophyll biosynthesis, the
development of the photosynthetic apparatus, and the photooxidation of pigments (Bohne and Linden, 2002). It has been proposed that AMPA affects chlorophyll biosynthesis (Reddy et al., 2004), and this is consistent with the observed effect of AMPA on carotenoid accumulation (Fig. 5.3).

**Table 5.1.** Photochemical efficiency of PSII (Fv/Fm), electron transport through PSII (ETR) and the non-photochemical quenching (NPQ) in leaves of *Salix miyabeana* (cultivar SX64) spiked with tween 0.5% (v/v) and glyphosate and AMPA in different rates.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Fv/Fm</th>
<th>ETR</th>
<th>NPQ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tween 0.5%</td>
<td>0.75a</td>
<td>46.58a</td>
<td>2.15a</td>
</tr>
<tr>
<td>0.28</td>
<td>0.59b</td>
<td>31.76b</td>
<td>1.28b</td>
</tr>
<tr>
<td>1.4</td>
<td>0.62b</td>
<td>32.46b</td>
<td>1.45b</td>
</tr>
<tr>
<td>2.8</td>
<td>0.48c</td>
<td>20.87c</td>
<td>0.92b</td>
</tr>
<tr>
<td>1.4</td>
<td>0.55b</td>
<td>26.19b</td>
<td>1.48b</td>
</tr>
<tr>
<td>2.1</td>
<td>0.55b</td>
<td>21.52c</td>
<td>1.45b</td>
</tr>
<tr>
<td>2.8</td>
<td>0.42c</td>
<td>13.84d</td>
<td>1.24b</td>
</tr>
</tbody>
</table>

Treatment means from ANOVA. Values followed by the same letter within the same column are not significantly different (P < 0.05) by the Scott-Knott multiple range test.

Chlorophyll *a* and *b* contents were drastically decreased by exposure to either AMPA or glyphosate (Fig. 5.3). To determine if these decreases in chlorophyll contents were due to decreased biosynthesis and/or increased degradation of chlorophyll, we investigated the pheophytin contents of AMPA and glyphosate-treated plants, as pheophytin is one of the degradation products of chlorophyll (Matile *et al.*, 1999). We observed that the pheophytin (*a,b*)/chlorophyll (*a,b*) ratios were not altered in AMPA-treated plants (P > 0.05; Fig. 5.3), indicating an effect on chlorophyll biosynthesis and not on its degradation. Although there are other possible action sites (Corniani *et al.*, 2014), it is known that AMPA decreases the amounts of glycine, serine, and glutamate in plants (Serra *et al.*, 2013), so that the observed decrease in chlorophyll biosynthesis may be explained by the hypothesis (Gomes *et al.*, 2014a) that AMPA directly affects chlorophyll biosynthesis by competing with
glycine in photorespiration processes and/or in the active site of δ-aminolevulinic acid (ALA) synthetase, depriving plants of the substrates needed in the chlorophyll biosynthetic pathway. In contrast, glyphosate-treated plants showed increases in their pheophytin/chlorophyll ratios (P < 0.05; Fig. 3), indicating that glyphosate induces chlorophyll degradation. We also only observed increased lipid peroxidation and greater H₂O₂ accumulations in glyphosate-treated plants (P < 0.05; Fig. 5.2). It is widely accepted that chlorophyll degradation involves hydroxyl radicals produced by reactions between superoxide anion and H₂O₂ (Dhindsa et al., 1981) as well as lipid peroxides (Somashekaraiah et al., 1992). Likewise, concomitant pheophytin accumulation and oxidative stress have been observed in plants exposed to toxic concentrations of trace elements (Mobin and Khan, 2007; Rodriguez et al., 2007).

![Fig. 5.2. Lipid peroxidation (MDA content), hydrogen peroxide content, and the activities of superoxide dismutase (SOD), catalase (CAT), and ascorbate peroxidase.](image-url)
Bars indicate the means ± standard errors of three replicates. Means followed by the same letter within the same column are not significantly different (P < 0.05) by the Scott-Knott multiple range test.

Fig. 5.3. Pigment (Chlorophyll a, b and carotenoids) contents and the pheophytin/chlorophyll ratios of leaves of willow plants treated with different concentrations of AMPA or glyphosate. Treatment means from ANOVA. Bars indicate the means ± standard errors of three replicates. Means followed by the same
letter within the same column are not significantly different (P < 0.05) by the Scott-Knott multiple range test.

The induction of peroxide accumulation following treatment with AMPA (P < 0.05; Fig. 5.2) does not appear to be sufficient to induce oxidative burst in terms of increased lipid peroxidation (P > 0.05; Fig. 5.2). These results are in agreement with a recent study demonstrating that AMPA did not induce oxidative stress in A. thaliana, indicating that AMPA effects are not due to oxidative damage (Serra et al., 2013). On the other hand, we observed the highest H$_2$O$_2$ contents in leaves treated with glyphosate, with consequent increases in lipid peroxidation (P < 0.05; Fig. 5.2). Increased H$_2$O$_2$ accumulation and lipid peroxidation after glyphosate exposure has likewise been observed in rice (Ahsan et al., 2008), maize (Sergiev et al., 2006), and pea (Miteva et al., 2010) plants. Once accumulated, H$_2$O$_2$ will react with subcellular components which can cause accentuated oxidative damage (Gomes et al., 2014b). H$_2$O$_2$ is also an important cellular signaling compound, however, and plants should therefore be able to deal with certain H$_2$O$_2$ accumulation levels (Gomes et al., 2014b). In this context, when the H$_2$O$_2$ content is below the threshold of H$_2$O$_2$ sensitivity, ROS do not cause oxidative bursts (as observed in AMPA-treated plants); H$_2$O$_2$ contents above the H$_2$O$_2$ sensitivity threshold, however, would be expected to cause oxidative damage (as in glyphosate-treated plants). Therefore, to better understand the processes involved in H$_2$O$_2$ metabolism and accumulation, we investigated the activities of the major antioxidant enzymes (SOD, CAT, and APX).

Increased enzymatic antioxidant system activities have been reported in plants exposed to glyphosate (Ahsan et al., 2008; Miteva et al., 2010), and the ability to avoid oxidative stress induced by glyphosate has been proposed to complement glyphosate resistance in plants (Maroli et al., 2015). However, little is known about the antioxidant responses of plants exposed to AMPA (Serra et al., 2013). We observed decreased SOD, CAT, and APX activities following both AMPA and glyphosate treatments. SOD is the first line of defense against ROS, being the major
scavenger of superoxide radicals ($O_2^-$), which are converted to $H_2O_2$, which is, in turn, scavenged by CAT and peroxidases (Gunes et al., 2007). CAT and APX activities were greatly reduced in treated-plants in comparison to SOD (Fig. 5.2). It means that although reduced in glyphosate and AMPA treated plants, the $H_2O_2$ generated by SOD activity was not scavenged by the $H_2O_2$-scavenging enzyme system (CAT and APX), which justify the observed $H_2O_2$ accumulation in those plants. We hypothesize that, by inhibiting ALA production (Gomes et al., 2014a), AMPA can induce decreases in SOD and $H_2O_2$-scavenging enzyme activities. It was shown that upon ALA application, plants reduced their $H_2O_2$ accumulation and increased the activities of enzymatic antioxidant systems, i.e., SOD, CAT, APX, glutathione reductase – GR, and dehydroascorbate reductase – DHAR (Memon et al., 2009; Xu et al., 2009). Additionally, ALA is a precursor in the tetrapyrrrole metabolic pathway in plants and important in catalase and peroxidases biosynthesis (Cobb and Reade, 2010). We noted, however, that antioxidant system inhibition was greater in glyphosate-treated plants, leading to increased lipid peroxidation through $H_2O_2$ accumulation. High accumulations of ROS provoke modifications in the side-chains of proteins, with Lys, Arg, Pro, and Thr being converted to aldehyde or keto-groups (Gonçalves et al., 2007). The observed decreases in antioxidant enzyme activities in glyphosate-treated plants could therefore be related to irreversible protein carbonylation and the inactivation of those enzymes.

5.4 CONCLUSION

We demonstrated that AMPA and glyphosate have different indirect effects in reducing chlorophyll content and photosynthesis in willow plants. While AMPA might interrupt chlorophyll biosynthesis, glyphosate seems to induce chlorophyll degradation through oxidative damage – both with resulting decreases in photosynthetic rates. Further studies should therefore investigate the combined effects of these two chemicals on plant physiology, and we note that the different effects of
glyphosate on chlorophyll contents and photosynthesis as described in the literature may be due to varying glyphosate:AMPA ratios in those plants – and therefore related to different ROS levels and to changes in the ratios of chlorophyll synthesis inhibition and chlorophyll degradation.

5.5 REFERENCES


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CONCLUSIONS AND PERSPECTIVES

We examined the herbicidal effects of glyphosate on willow (Salix miyabeana SX64) in terms of its impact on photosynthesis and oxidative stress and the phytoremediation capacity of the species.

Willows were observed to take up glyphosate through their roots, and their fast growth and high biomass production was indicative of their phytoremediation abilities. Phosphate (PO$_4^{3-}$) was found to modulate the glyphosate-uptake capacity of willows. While glyphosate competes for phosphate carriers in the roots, the addition of PO$_4^{3-}$ to the growth substrate actually increased glyphosate uptake and accumulation while at the same time alleviating glyphosate-induced damage to the root system. PO$_4^{3-}$ fertilization also had a protective effect in the leaves, as it stimulated antioxidant systems (mainly ascorbate peroxidase) and avoided the detrimental effects of glyphosate on photosynthesis.

We confirmed that the primary target of glyphosate (in the glyphosate-based-herbicide used [Factor 540]) is the shikimate pathway, although this herbicide induced a series of physiologically interconnected events that led to decreases in the photosynthetic activities of treated plants (which was strongly related to herbicide-induced oxidative stress). The ability of glyphosate to induce reactive oxygen species (ROS) accumulation (such as hydrogen peroxide – H$_2$O$_2$) and related oxidative bursts (lipid peroxidation, protein carbonylation) could help explain the diverse detrimental effects of this herbicide observed in glyphosate-resistant (GR) plants.

Finally, we investigated the effects of the main glyphosate by-product, aminomethylphosphonic acid (AMPA), on leaf physiological characteristics of willows, as it is known to induce decreases in chlorophyll content and photosynthesis. We observed, however, that both glyphosate and AMPA provoked decreases in the chlorophyll contents of willows through different mechanisms. While AMPA
disturbed chlorophyll biosynthesis, glyphosate induced chlorophyll degradation through oxidative damage.

In summary, we conclude that willows can be used in programs to remediate glyphosate-contaminated soils and that glyphosate's deleterious effects on plants are strongly linked to induced oxidative stress. Our results also have important environmental considerations:

1) We noted the influence of $\text{PO}_4^{3-}$ on glyphosate uptake by roots, and hypothesize that $\text{PO}_4^{3-}$ fertilization in agricultural fields can contribute to the herbicidal-efficacy of glyphosate by increasing glyphosate uptake through weed roots. However, if GR plants also increase glyphosate uptake with added $\text{PO}_4^{3-}$, it will presumably contribute to the deleterious effects of that herbicide in those crops. To our knowledge, however, these two observations have not yet been closely examined;

2) In this same context, $\text{PO}_4^{3-}$ fertilization can contribute to glyphosate remediation programs by increasing herbicide uptake by willow roots;

3) We observed that glyphosate strongly induces oxidative stress in plants that can lead to significant physiological damage, such as decreased photosynthetic abilities.

4) We observed that both glyphosate and AMPA can decrease plant chlorophyll contents, although in different ways. As such, the different effects of glyphosate on chlorophyll contents and photosynthesis reported in the literature might be due to different glyphosate/AMPA ratios in those plants – and therefore related to ROS levels and to changes in the rates and ratios of chlorophyll synthesis inhibition and chlorophyll degradation.

Although we have greatly contributed to understanding the glyphosate assimilation and physiological effects in plants, a number of questions remain. It would be of great importance investigating the processes involved in glyphosate
uptake by roots, such as the identification of $\text{PO}_4^{3-}$ carriers mediating glyphosate uptake. These studies could contribute to the development of better GR and phytoremediator plant species, with membrane carriers having high specific affinities for $\text{P}$ or increased capacity for glyphosate uptake, respectively. In the case of GR, this could assure P nutrition with less glyphosate uptake, thus decreasing secondary glyphosate toxic effects on that plants. Moreover, although already studied (Denis and Delrot, 1993) one can argue that the polarity of glyphosate molecule could difficult its diffusion through cell membranes and, therefore, this mechanism merits some attention.

Concerning plant physiology, it was not investigated yet the effects of $\text{PO}_4^{3-}$ on EPSP activity (the primary site of action of glyphosate), which could be related to the nutrient-alleviation of glyphosate herbicidal effects. Moreover, more studies will be needed on glyphosate's ability to induce oxidative stress, including the identification of ROS-generating sites resulting from glyphosate exposure. This information will contribute to our understanding of the sites of action of glyphosate, especially in species without the shikimate pathway (i.e., animals) that nonetheless show toxic responses to glyphosate. Accordingly, further studies of AMPA effects on plant physiology are clearly needed. These studies will provide evidence for the development of more-efficient GR plants in addition to contributing to understanding the glyphosate fate and the herbicide detrimental effects on the environment. In another hand, since some GR plants are able to degrade glyphosate to AMPA, it is important to study whether this metabolite is responsible for the observed deleterious effects found in some plants treated with glyphosate or if these symptoms are related to the indirect effects of glyphosate on plant physiology.

REFERENCES