UNIVERSITÉ DU QUÉBEC À
MONTRÉAL

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

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BY

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JUILLET 2015

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MONTRÉAL

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

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ABBREVIANTIONS

ABA	Abscisic acid
ALA	δ-aminolevulinic acid
AMPA	Aminomethylphosphonic acid
ANOVA	Analyse of variance
APX	Ascorbate peroxidase
ARA	Acetylene reduction activity
AsA	Ascorbate
ATP	Adenosine triphosphate
C-P lyase	Carbon-to-phosphorus lyase
CAT	Catalase
DAHP	3-deoxy-D-arabinoheptulosonic acid-7-phosphate
DCMU	3-(3, 4 dichlorophenyl)-1, 1-dimethyl urea
DHA	Dehydroascorbate
DHAR	Dehydroascorbate reductase
DNOC	4,6-dinotro-*o*-cresol
EPSPS	5-enolpyruvylshikimate-3-phosphate synthase
ETR	Electron transport rate
Fv/Fm	Maximal PSII photochemical efficien
g_s	Stomatal conductance

I_k	Minimum saturating irradiance
GSH	Gluthatione
GST	Glutathione-*S*-transferase
GPX	Glutathione peroxidase
GuPX	Guaiacol peroxidase
GAT	Glyphosate acyl transferase
GOX	Glyphosate oxidoreductase
GR	Glyphosate-resistant
GS	Glyphosate-sensitive
H_2O_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

qP	Photochemical quenching
Q_A	Plastoquinone
RBS	Riparian buffer strip
ROS	Reactive oxygen species
$^1O_2$	reactive singlet oxygen
Rubisco	Ribulose 1,5-biphosphate carboxylase oxygenase
SOD	Superoxide dismutase
SRC	Short-rotation coppice
hGSH	Thiol homoglutathione
Tyr	Tyrosine
UQF_{rel}	Relative unquenched fluorescence
Yield	PSII quantum yield

RÉSUMÉ GÉNERAL

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 entraine son accumulation dans les eaux souterraines ou les rivières. À ceci, il faut ajouter que l'acide aminomethylphosphonique – 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 PO_4^{-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 PO_4^{-3} dans l'assimilation du glyphosate. Considérant la fertilisation en PO_4^{-3} comme une pratique courante dans les grandes cultures, nous avons également étudié les effets du PO_4^{-3} sur l'absorption du glyphosate par les saules. La fertilisation en PO_4^{-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 PO_4^{-3} puisse être utilisé pour accentuer les capacités de phytoremédiation 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 différentiels 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 PO_4^{-3} et les interactions entre l'un et l'autre peuvent entrainer 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 PO_4^{3-} transporters in glyphosate uptake by willow roots. By using a PO_4^{3-} transporter inhibitor (phosphonophormic acid-PFA) we shown the involvement of PO_4^{3-} transporters in glyphosate uptake by roots. As phosphate fertilization (PO_4^{-3}) is a common practice in agricultural fields submitted to glyphosate applications, we evaluated the effects of PO_4^{-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, PO_4^{-3} fertilization increased glyphosate accumulation and assured photosynthesis rates. Therefore PO_4^{-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® 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 PO_4^{3-} 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-dinotro-*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, 1997*a*). 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.*, 2011*b*, 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, 2009*a*; 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 (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 PO_4^{3-} have a role in the phytoremediation capacity of willow plants?** As 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 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.

REFERENCES

Adegbidi HG, Briggs RD, Volk T a., White EH, Abrahamson LP. 2003. Effect of organic amendments and slow-release nitrogen fertilizer on willow biomass production and soil chemical characteristics. Biomass and Bioenergy **25**, 389–398.

Ahsan N, Lee D-G, Lee K-W, Alam I, Lee S-H, Bahk JD, Lee B-H. 2008. Glyphosate-induced oxidative stress in rice leaves revealed by proteomic approach. Plant physiology and biochemistry **46**, 1062–70.

Annett R, Habibi HR, Hontela A. 2014. Impact of glyphosate and glyphosate-based herbicides on the freshwater environment. Journal of Applied Toxicology **34**, 458–479.

Beltrano J, Ruscitti M, Arango C, Ronco M. 2013. Changes in the accumulation of shikimic acid in mycorrhized *Capsicum annum* grown with application of glyphosate and phosphorus. Theoretical and Experimental Plant Physiology **25**, 125–136.

Benbrook CM. 2012. Impacts of genetically engineered crops on pesticide use in the U.S. -- the first sixteen years. Environmental Sciences Europe **24**, 24.

Blanchoud H, Moreau-Guigon E, Farrugia F, Chevreuil M, Mouchel JM. 2007. Contribution by urban and agricultural pesticide uses to water contamination at the scale of the Marne watershed. The Science of the total environment **375**, 168–79.

Bott S, Tesfamariam T, Kania A, Eman B, Aslan N, Römheld V, Neumann G. 2011. Phytotoxicity of glyphosate soil residues re-mobilised by phosphate fertilisation. Plant and Soil **342**, 249–263.

Cakmak I, Yazici A, Tutus Y, Ozturk L. 2009. Glyphosate reduced seed and leaf concentrations of calcium, manganese, magnesium, and iron in non-glyphosate resistant soybean. European Journal of Agronomy **31**, 114–119.

Cavanagh A, Gasser MO, Labrecque M. 2011. Pig slurry as fertilizer on willow plantation. Biomass and Bioenergy **35**, 4165–4173.

Cerdeira AL, Duke SO. 2006. The current status and environmental impacts of glyphosate-resistant crops: a review. Journal of environmental quality **35**, 1633–58.

Cobb A. 1992. *Herbicides and plant physiology*. London: Chapman and Hall.

Cobb AH, Reade JP. 2010. The inhibition of amino acid byosynthesis. In: Cobb AH, Reade JP., eds. Herbicides and plant physiology. Wiley-Blackwell, 176–197.

Dagenais G. 2014. Analyse stratégique de la gouvernance de l'eau en milieu agricole : Normes, acteurs, enjeux, stratégies.

Denis M-H, Delrot S. 1993. Carrier-mediated uptake of glyphosate in broad bean (*Vicia faba*) via a phosphate transporter. Physiologia plantarum **87**, 569–575.

Ding W, Reddy KN, Zablotowicz RM, Bellaloui N, Arnold Bruns H. 2011. Physiological responses of glyphosate-resistant and glyphosate-sensitive soybean to aminomethylphosphonic acid, a metabolite of glyphosate. Chemosphere **83**, 593–8.

Duke SO. 2011. Glyphosate degradation in glyphosate-resistant and -susceptible crops and weeds. Journal of agricultural and food chemistry **59**, 5835–41.

Gavrilescu M, Demnerová K, Aamand J, Agathos S, Fava F. 2014. Emerging pollutants in the environment: present and future challenges in biomonitoring, ecological risks and bioremediation. New biotechnology **32**, 147–156.

Gomes MP, Carvalho M, Cristina T, Lanza L, Marques M, Duarte M, Oliveira C De, Nogueira G, Soares ÂM, Souza Q De. 2012. Arsenic-sensitivity in *Anadenanthera peregrina* due to arsenic-induced lipid peroxidation. International Journal of Applied Science and Technology **2**, 55–63.

Gomes MP, Soares AM, Garcia QS. 2014. Phosphorous and sulfur nutrition modulate antioxidant defenses in *Myracrodruom urundeuva* plants exposed to arsenic. Journal of hazardous materials **276**, 97–104.

Guidi W, Pitre FE, Labrecque M. 2013. Short-rotation coppice of willows for the production of biomass in Eastern Canada. In: Matovic MD, ed. Biomass Now - Sustainable Growth and Use. InTech Open Science, 421–448.

Hénault-Ethier L, Smedbol E, Dagenais G, Gomes M, Racine J-F, Juneau P, Lepage L, Labrecque M, Lucotte M. 2014. Les bandes riveraines en agriculture: Une approche pluridisciplinaire pour une application concrète. Vecteur Environnement, 52–57.

Keoleian GA, Volk TA. 2005. Renewable Energy from Willow Biomass Crops: Life Cycle Energy, Environmental and Economic Performance. Critical Reviews in Plant Sciences **24**, 385–406.

Kuzovkina Y, Volk TA. 2009. The characterization of willow (Salix L.) varieties for use in ecological engineering applications: Co-ordination of structure, function and autecology. Ecological Engineering **35**, 1178–1189.

Labrecque M, Teodorescu TI. 2003. High biomass yield achieved by Salix clones in SRIC following two 3-year coppice rotations on abandoned farmland in southern Quebec, Canada. Biomass and Bioenergy **25**, 135–146.

MDDEP. *Politique de protection des rives, du littoral et des plaines inondables, in Gazette officielle du Québec.* Quebec.

Mirck J, Isebrands JG, Verwijst T, Ledin S. 2005. Development of short-rotation willow coppice systems for environmental purposes in Sweden. Biomass and Bioenergy **28**, 219–228.

Miteva L-ELP-E, Ivanov S V, Alexieva VS. 2010. Alterations in glutathione pool and some related enzymes in leaves and roots of pea plants treated with the herbicide glyphosate. Russian Journal of Plant Physiology **57**, 131–136 LA – English.

Monsanto. 2005. *Backgrounder: Glyphosate Half-Life in Soil.*

Moreno NC, Sofia SH, Martinez CBR. 2014. Genotoxic effects of the herbicide Roundup Transorb and its active ingredient glyphosate on the fish *Prochilodus lineatus*. Environmental toxicology and pharmacology **37**, 448–54.

Morin F, Vera V, Nurit F, Tissut M, Marigo G. 1997. Glyphosate uptake in *Catharanthus roseus* cells : Role of a phosphate transporter. Pesticide Biochemistry and Physiology **58**, 13–22.

Neumann G, Kohls S, Landsberg E, Stock-Oliveira Souza K, Yamada T, Romheld V. 2006. Relevance of glyphosate transfer to non-target plants via the rhizosphere. Journal of plant diseases and protection **969**, 963–969.

Perttu KL. 1998. Environmental justification for short-rotation forestry in Sweden. Biomass and Bioenergy **15**, 1–6.

Reddy KN, Rimando AM, Duke SO. 2004. Aminomethylphosphonic acid, a metabolite of glyphosate, causes injury in glyphosate-treated, glyphosate-resistant soybean. Journal of agricultural and food chemistry **52**, 5139–43.

Reynolds HL, Smith a. a., Farmer JR. 2014. Think globally, research locally: Paradigms and place in agroecological research. American Journal of Botany **101**, 1631–1639.

Saroha MK, Sridhar P, Malik VS. 1998. Glyphosate-Tolerant Crops: Genes and Enzymes. Journal of Plant Biochemistry and Biotechnology **7**, 65–72.

Sergiev IG, Alexieva VS, Ivanov S, Moskova II, Karanov EN. 2006. The phenylurea cytokinin 4PU-30 protects maize plants against glyphosate action. Pesticide Biochemistry and Physiology **85**, 139–146.

Siehl D. 1997. Inhibitors of EPSPS synthase, glutamine synthetase and histidine synthesis. In: Roe R, Burton J, Kuhr R, eds. Herbicide activity: toxicology, biochemistry and molecular biology. Amsterdam: IOS Press, 37–67.

Stow CA, Dyble J, Kashian DR, *et al.* 2014. Phosphorus targets and eutrophication objectives in Saginaw Bay: A 35year assessment. Journal of Great Lakes Research **40**, 4–10.

Tesfamariam T, Bott S, Cakmak I, Römheld V, Neumann G. 2009. Glyphosate in the rhizosphere—Role of waiting times and different glyphosate binding forms in soils for phytotoxicity to non-target plants. European Journal of Agronomy **31**, 126–132.

Tilman D. 1999. Global environmental impacts of agricultural expansion: The need for sustainable and efficient practices. Proceedings of the National Academy of Sciences **96**, 5995–6000.

Vereecken H. 2005. Mobility and leaching of glyphosate: a review. Pest management science **61**, 1139–51.

Zan CS, Fyles JW, Girouard P, Samson R a. 2001. Carbon sequestration in perennial bioenergy, annual corn and uncultivated systems in southern Quebec. Agriculture, Ecosystems & Environment **86**, 135–144.

Zobiole LHSS, Kremer RJ, Oliveira Jr. RS, Constantin J, Oliveira RS. 2011. Glyphosate affects chlorophyll, nodulation and nutrient accumulation of 'second generation' glyphosate-resistant soybean (*Glycine max* L.). Pesticide Biochemistry and Physiology **99**, 53–60.

Zobiole LHS, Kremer RJ, de Oliveira Jr. RS, Constantin J. 2012. Glyphosate effects on photosynthesis, nutrient accumulation, and nodulation in glyphosate-resistant soybean. Journal of Plant Nutrition and Soil Science **175**, 319–330.

CHAPTER I

ALTERATION OF PLANT PHYSIOLOGY BY GLYPHOSATE AND ITS BY-PRODUCT AMINOMETHYLPHOSPHONIC ACID (AMPA), AN OVERVIEW[1]

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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, 1997*a*). 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.*, 2010*d*, 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.* (2004*a*) 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}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 exudated 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 this 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.*, 2004*a*), 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 Q_A and Q_B, by competing for Q_B 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; Yanniccari *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 pheophytin, 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.*, 2011*b*; 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.

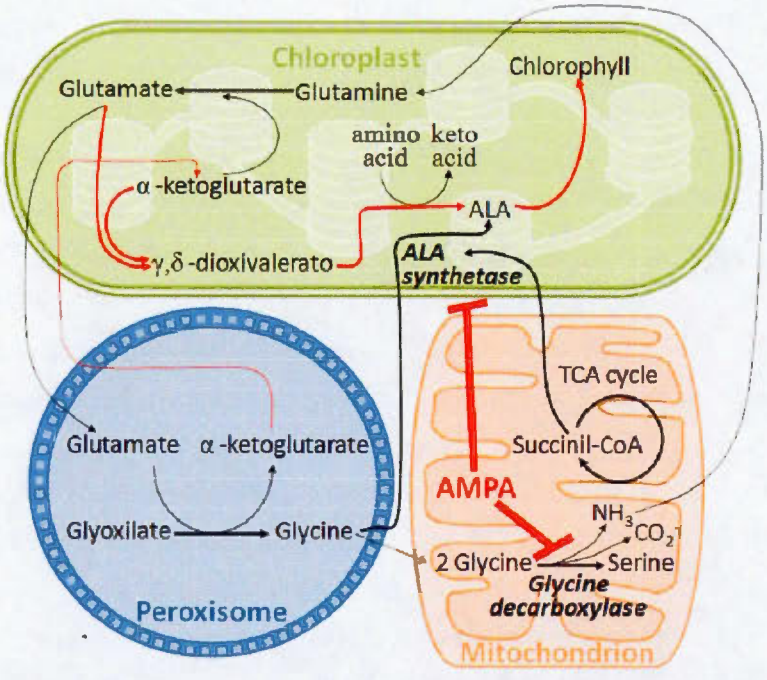

Fig.1.1: AMPA effects on chlorophyll biosynthesis. Due to its similarities, AMPA is suggested to compete with glycine metabolic pathways leading to decreased aminolevulinic acid (ALA) production (an intermediary of chlorophyll biosynthesis). By competing with glycine for the enzyme active sites, AMPA can decrease glutamate content (via a decreased NH_3 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-synthethase, 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.*, 2011*b*), 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), pheophytin*a* (Pheo) and plastoquinone (Q_A) as cofactors (Kern and Renger, 2007). The D1/D2 heterodimer possess two redox tyrosine subunits Tyr_Z (Y_Z) and Tyr_D (Y_D) (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. Tyr_Z 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 immerged 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

Effect	Parameters		References
↓[a]	F_O	Minimal fluorescence level	Zobiole *et al.* (2011a); Choi *et al.* (2012)
	F_m	Maximum fluorescence level	
↓	qP	Photochemical quenching	Mateos-Naranjo *et al.* (2009); Zobiole *et al.* (2011a); Vivancos *et al.* (2011); Huang *et al.* (2012); Yanniccari *et al.* (2012)
↓	F_v/F_m	Maximum quantum efficiency of PSII	Mateos-Naranjo *et al.* (2009); Vivancos *et al.* (2011); Yanniccari *et al.* (2012); Choi *et al.* (2012)
↑	NPQ or qN	Non-photochemical quenching	Mateos-Naranjo *et al.* (2009); Vivancos *et al.* (2011);
↓	NPQ or qN	Non-photochemical quenching	Huang *et al.* (2012); Zobiole *et al.* (2011a)
↓	ETR	Electron transport rate	Muñoz-Rueda *et al.* (1986); Zobiole *et al.* (2011a); Huang *et al.* (2012); Yanniccari *et al.* (2012)
↓	ΦPSII	Quantum efficiency of PSII	Mateos-Naranjo *et al.* (2009); Zobiole *et al.* (2011a)
↓	Fv'/Fm'	Intrinsic efficiency of PSII	Zobiole *et al.* (2011a); Huang *et al.* (2012); Yanniccari *et al.* (2012)

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.*, 2011*b*; Ding *et al.*, 2011). In these conditions, the CO_2 assimilation capacity is reduced, leading to an increased intracellular concentration of CO_2 (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, 1997*a*). 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 María *et al.* (2006) in *Lupinus albus* leaves, where a 26% reduction of Rubisco activity occurred after five days of exposure to10mM 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, 1997*a*; 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, 1997*a*). 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.*, 2010*c*). 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 María *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.*, 2011*a*) 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.*, 2010*c*). 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_2 fixation was due to inhibition of photosynthesis and carbon substrate availability (Zablotowicz 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_2 fixation (Zobiole *et al.*, 2012). Zobiole *et al.* (2010*b*) reported that nickel (Ni) was immobilized by glyphosate and could thereby compromise symbiotic N_2 fixation in GR soybean since Ni is required by N_2-fixing microorganisms for the hydrogenase that processes hydrogen gas generated during N_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_2 fixation process (Zobiole *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 *et al.*, 2011). Indeed, studying the AMPA effect on GR and GS sensitive soybean plants, Ding *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.

1.3.3 Glyphosate and plant mineral nutrition

The effect of glyphosate on plant mineral nutrition has not yet been extensively studied (Zobiole *et al.*, 2010*c*). Moreover, results published concerning to GR crops are contradictory. Some studies did not report any effect (Bailey *et al.*, 2002; Rosolem *et al.*, 2010), while others have shown that glyphosate disturbed plant nutritional status (Cakmak *et al.*, 2009; Su *et al.*, 2009; Zobiole *et al.*, 2010*c*, 2011*b*, 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.* (2012*b*) 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.*, 2011*b*).

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.*, 2011*b*). Significant decreases in all macro- and micronutrient contents have been observed in GR soybean plants exposed to glyphosate (Zobiole *et al.*, 2011*a*, 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.* (2011*a*), 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.*, 2011*b*). 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.*, 2012*a*).

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, gluthatione (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_2O_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 H_2O_2 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 gluthatione reductase and in the enhancement of the GST activities which, in addition to the increase in both total and oxidized gluthatione 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.

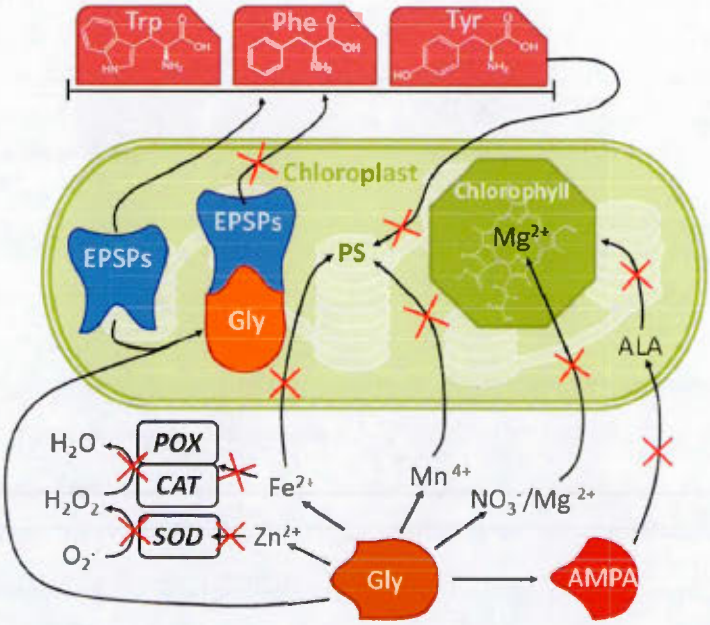

Fig. 1.2: Glyphosate and AMPA effects in some plant physiological process (red "X"). Glyphosate main effect is due the inhibition of aromatic amino acids (tryptophan –Trp, phenylalanine -Phe and tyrosine – Tyr) production by inhibiting 5-enolpyruvylshikimate-3-phosphate (EPSPS) synthase. Glyphosate can 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 - $O_2^{\cdot-}$ and hydrogen peroxide - H_2O_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.*, 2010*a*) 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.*, 2010*a*). 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) (Cakmak *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 (GA_3) 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*a*). 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 rhyzosphere 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.

1.5 REFERENCES

Ahsan N, Lee D-G, Lee K-W, Alam I, Lee S-H, Bahk JD, Lee B-H. 2008. Glyphosate-induced oxidative stress in rice leaves revealed by proteomic approach. Plant physiology and biochemistry **46**, 1062–70.

Bailey WA, Poston DH, Wilson HP, Hines TE. 2002. Glyphosate Interactions with Manganese 1. Weed Technology **16**, 792–799.

Baur JR. 1979. Effect of glyphosate on auxin transport in corn and cotton tissues. Plant physiology **63**, 882–886.

Bellaloui N, Reddy KN, Zablotowicz RM, Abbas HK, Abel CA. 2009. Effects of glyphosate application on seed iron and root ferric (III) reductase in soybean cultivars. Journal of agricultural and food chemistry **57**, 9569–9574.

Bellaloui N, Zablotowicz RM, Reddy KN, Abel CA. 2008. Nitrogen metabolism and seed composition as influenced by glyphosate application in glyphosate-resistant soybean. Journal of agricultural and food chemistry **56**, 2765–72.

Bolweel GP, Bozak K, Zimmerlin A. 1994. Plant cytochrome P450. Phytochemistry **37**, 1491–1506.

Borggaard OK, Gimsing AL. 2008. Fate of glyphosate in soil and the possibility of leaching to ground and surface waters: a review. Pest Management Science **456**, 441–456.

Bromilow RH, Chamberlain K, Tench AJ, Williams RH. 1993. Phloem translocation of strong acids—glyphosate, substituted phosphonic and sulfonic acids—in *Ricinus communis* L. Pesticide Science **37**, 39–47.

Burton JD, Balke NE. 2012. Glyphosate uptake by suspension-cultured potato (*Solanum tuberosum* and *S. brevidens*) cells. Weed Science **36**, 146–153.

Cakmak I, Yazici A, Tutus Y, Ozturk L. 2009. Glyphosate reduced seed and leaf concentrations of calcium, manganese, magnesium, and iron in non-glyphosate resistant soybean. European Journal of Agronomy **31**, 114–119.

Cañero AI, Cox L, Redondo-Gómez S, Mateos-Naranjo E, Hermosín MC, Cornejo J. 2011. Effect of the herbicides terbuthylazine and glyphosate on photosystem II photochemistry of young olive (*Olea europaea*) plants. Journal of Agricultural and Food Chemistry **59**, 5528–5534.

De Carvalho LB, Alves PLDCA, González-Torralva F, Cruz-Hipolito HE, Rojano-Delgado AM, De Prado R, Gil-Humanes J, Barro F, de Castro MDL. 2012. Pool of resistance mechanisms to glyphosate in *Digitaria insularis*. Journal of agricultural and food chemistry **60**, 615–22.

Choi CJ, Berges JA, Young EB. 2012. Rapid effects of diverse toxic water pollutants on chlorophyll *a* fluorescence: variable responses among freshwater microalgae. Water research **46**, 2615–26.

Cobb A. 1992. *Herbicides and plant physiology*. London: Chapman and Hall.

Coupland D, Caseley JC. 1979. Presence of 14C activity in root exudates and guttation fluid from Agropyron repens treated with 14C-labelled glyphosate. New Phytologist **83**, 17–22.

Cruz-Hipolito H, Rojano-Delgado A, Domínguez-Valenzuela J a., Heredia A, Castro MDL, Prado R. 2011. Glyphosate tolerance by *Clitoria ternatea* and *Neonotonia wightii* plants involves differential absorption and translocation of the herbicide. Plant and Soil **347**, 221–230.

Cuypers A, Vangronsveld J, Clijsters H. 2001. The redox status of plant cells (AsA and GSH) is sensitive to zinc imposed oxidative stress in roots and primary leaves of *Phaseolus vulgaris*. Plant Physiology and Biochemistry **39**, 657–664.

Descalzo RC, Punja ZK, Lévesque CA, Rahe JE. 1998. Glyphosate treatment of bean seedlings causes short-term increases in Pythium populations and damping off potential in soils. Applied Soil Ecology **8**, 25–33.

Dewick PM. 1998. The biosynthesis of shikimate metabolites. Natural product reports **15**, 17–58.

Ding W, Reddy KN, Zablotowicz RM, Bellaloui N, Arnold Bruns H. 2011. Physiological responses of glyphosate-resistant and glyphosate-sensitive soybean to aminomethylphosphonic acid, a metabolite of glyphosate. Chemosphere **83**, 593–8.

Duke SO. 2011. Glyphosate degradation in glyphosate-resistant and -susceptible crops and weeds. Journal of agricultural and food chemistry **59**, 5835–41.

Duke SO, Lydon J, Koskinen WC, Moorman TB, Chaney RL, Hammerschmidt R. 2012*a*. Glyphosate effects on plant mineral nutrition, crop rhizosphere microbiota, and plant disease in glyphosate-resistant crops. Journal of agricultural and food chemistry **60**, 10375–97.

Duke SO, Powles SB. 2008. Glyphosate: a once-in-a-century herbicide. Pest Management Science **64**, 319–325.

Duke SO, Reddy KN, Bu K, Cizdziel JV. 2012*b*. Effects of glyphosate on the mineral content of glyphosate resistant soybeans (*Glycine max*). Journal of agricultural and food chemistry **60**, 6764–6771.

Duke SO, Rimando AM, Pace PF, Reddy KN, Smeda RJ. 2003. Isoflavone, Glyphosate, and Aminomethylphosphonic Acid Levels in Seeds of Glyphosate-Treated, Glyphosate-Resistant Soybean. Journal of Agricultural and Food Chemistry **51**, 340–344.

Eker S, Ozturk L, Yazici A, Erenoglu B, Romheld V, Cakmak I. 2006. Foliar-applied glyphosate substantially reduced uptake and transport of iron and manganese in sunflower (*Helianthus annuus* L.) plants. Journal of agricultural and food chemistry **54**, 10019–25.

Fedtke K, Duke S. 2005. Herbicides. In: Hock B, Elstner E, eds. Plant Toxicology. New York: Marcel Dekker, 247–330.

Feng PCC, Chiu T, Douglas Sammons R. 2003. Glyphosate efficacy is contributed by its tissue concentration and sensitivity in velvetleaf (*Abutilon theophrasti*). Pesticide Biochemistry and Physiology **77**, 83–91.

Feng PCC, Tran M, Chiu T, Douglas Sammons R, Heck GR, CaJacob CA. 2004. Investigations into glyphosate-resistant horseweed (*Conyza canadensis*): retention, uptake, translocation, and metabolism. Weed Science **52**, 498–505.

Fischer RS, Berry A, Gaines CG, Jensen RA. 1986. Comparative action of glyphosate as a trigger of energy drain in eubacteria. Journal of bacteriology **168**, 1147–54.

Foyer CH, Noctor G. 2011. Ascorbate and glutathione: the heart of the redox hub. Plant physiology **155**, 2–18.

Franz J, Mao M, Sikorski J. 1997. *Glyphosate: A Unique and Global Herbcide.* Washington.

Gaines TA, Zhang W, Wang D, *et al.* 2010. Gene amplification confers glyphosate resistance in *Amaranthus palmeri.* Proceedings of the National Academy of Sciences of the United States of America **107**, 1029–34.

Gaspar T, Coumans M. 1987. Root formation. In: Ga JM, Durzan DJ, eds. Cell and Tissue Culture in Forestry. Martinus Nijhoff Publishers, 202–217.

Ge X, d'Avignon DA, Ackerman JJH, Sammons RD. 2010. Rapid vacuolar sequestration: the horseweed glyphosate resistance mechanism. Pest management science **66**, 345–8.

Gomes MP, Marques TCLLSM, Nogueira MOGN, Castro EM, Soares ÂM. 2011. Ecophysiological and anatomical changes due to uptake and accumulation of heavy metal in Brachiaria decumbens. Scientia Agricola **68**, 566–573.

Gosselink RJA, de Jong E, Guran B, Abächerli A. 2004. Co-ordination network for lignin—standardisation, production and applications adapted to market requirements (EUROLIGNIN). Industrial Crops and Products **20**, 121–129.

Gougler JA, Geiger DR. 1981. Uptake and distribution of N-phosphonomethylglycine in sugar beet plants. Plant Physiology **68**, 668–672.

Gressel J. 2002. *Molecular Biology of Weed Control.* London: Taylor & Francis.

Gunes A, Inal A, Bagci EG, Coban S, Pilbeam DJ. 2007. Silicon mediates changes to some physiological and enzymatic parameters symptomatic for oxidative stress in spinach (*Spinacia oleracea* L.) grown under B toxicity. Scientia Horticulturae **113**, 113–119.

Hernandez A, Garcia-Plazaola JI, Becerril JM. 1999. Glyphosate effects on phenolic metabolism of nodulated soybean (*Glycine max* L. merr.). Journal of agricultural and food chemistry **47**, 2920–5.

Hetherington PR, Reynolds TL, Marshall G, Kirkwood RC. 1999. The absorption, translocation and distribution of the herbicide glyphosate in maize expressing the CP-4 transgene. Journal of Experimental Botany **50**, 1567–1576.

Holt JS, Powles SB, Holtum JAM. 1993. Mechanisms and agronomic aspects of herbicide resistance. Annual Review of Plant Physiology and Plant Molecular Biology **44**, 203–229.

Hornby D, Bateman GL, Gutteridge RJ, Lucas P, Osbourn AE, Ward E, Yarham D. 1988. *Take-all disease of cereals: A regional perspective.* Wallingford, UK: CAB International.

Huang J, Silva EN, Shen Z, Jiang B, Lu H. 2012. Effects of glyphosate on photosynthesis, chlorophyll fluorescence and physicochemical properties of cogongrass (*Imperata cylindrical* L.). Plant Omics Journal **5**, 177–183.

Jiang L-X, Jin L-G, Guo Y, Tao B, Qiu L-J. 2013. Glyphosate effects on the gene expression of the apical bud in soybean (*Glycine max*). Biochemical and biophysical research communications **437**, 544–9.

Johal GS, Huber DM. 2009. Glyphosate effects on diseases of plants. European Journal of Agronomy **31**, 144–152.

Juneau P, Qiu B, Deblois CP. 2007. Use of chlorophyll fluorescence as a tool for determination of herbicide toxic effect: Review. Toxicological & Environmental Chemistry **89**, 609–625.

Kern J, Renger G. 2007. Photosystem II: structure and mechanism of the water:plastoquinone oxidoreductase. Photosynthesis research **94**, 183–202.

Kielak E, Sempruch C, Mioduszewska H, Klocek J, Leszczyński B. 2011. Phytotoxicity of Roundup Ultra 360 SL in aquatic ecosystems: Biochemical evaluation with duckweed (*Lemna minor* L.) as a model plant. Pesticide Biochemistry and Physiology **99**, 237–243.

King CA, Purcell LC, Vories ED. 2001. Plant growth and nitrogenase activity of glyphosate-tolerant soybean in response to foliar glyphosate applications. Agronomy Journal **93**, 179–186.

Kitchen LM. 1980. Inhibition of chlorophyll and delta -aminolevulinic acid synthesis by glyphosate. Dissertation Abstracts International, B **41**, 2006–2007.

Kremer R. 2003. Soil biological processes are influenced by roundup ready soybean production. Phytopathology **93**, S104.

Kremer RJ, Means NE. 2009. Glyphosate and glyphosate-resistant crop interactions with rhizosphere microorganisms. European Journal of Agronomy **31**, 153–161.

Kremer RJ, Means NE, Kim S. 2005. Glyphosate affects soybean root exudation and rhizosphere microorganisms. International Journal of Analytical Environmental Chemistry **85**, 1165–1174.

Laitinen P, Rämö S, Siimes K. 2007. Glyphosate translocation from plants to soil – does this constitute a significant proportion of residues in soil? Plant and Soil **300**, 51–60.

Lee TT, Dumas T. 1983. Effect of glyphosate on ethylene production in tobacco callus. Plant physiology **72**, 855–7.

Lee TT, Dumas T, Jevnikar JJ. 1983. Comparison of the effects of glyphosate and related compounds on indole-3-acetic acid metabolism and ethylene production in tobacco callus. Pesticide Biochemistry and Physiology **20**, 354–359.

Marchiosi R, Lucio Ferrarese MDL, Bonini EA, Fernandes NG, Ferro AP, Ferrarese-Filho O. 2009. Glyphosate-induced metabolic changes in susceptible and glyphosate-resistant soybean (*Glycine max* L.) roots. Pesticide Biochemistry and Physiology **93**, 28–33.

De María N, Becerril JM, García-Plazaola JI, Hernandez A, De Felipe MR, Fernandez-Pascual M. 2006. New insights on glyphosate mode of action in nodular metabolism: Role of shikimate accumulation. Journal of agricultural and food chemistry **54**, 2621–8.

Marsh HVJ, Evans HJ, Matrone G. 1963. Investigations of the Role of Iron in Chlorophyll Metabolism II. Effect of Iron Deficiency on Chlorophyll Synthesis. Plant Physiology **38**, 638–642.

Mateos-Naranjo E, Redondo-Gómez S, Cox L, Cornejo J, Figueroa ME. 2009. Effectiveness of glyphosate and imazamox on the control of the invasive cordgrass *Spartina densiflora*. Ecotoxicology and Environmental Safety **72**, 1694–1700.

Matschke J, Machácková I. 2002. Changes in the content of indole-3-acetic acid and cytokinins in spruce, fir and oak trees after herbicide treatment. Biologia Plantarum **45**, 375–382.

Miteva LP-EP-E, Ivanov S V., Alexieva VS. 2010. Alterations in glutathione pool and some related enzymes in leaves and roots of pea plants treated with the herbicide glyphosate. Russian Journal of Plant Physiology **57**, 131–136 LA – English.

Moldes CA, Medici LO, Abrahão OS, Tsai SM, Azevedo RA. 2008. Biochemical responses of glyphosate resistant and susceptible soybean plants exposed to glyphosate. Acta Physiologiae Plantarum **30**, 469–479.

Monquero PA, Christoffoleti PJ, Osuna MD, De Prado RA. 2004. Absorção, translocação e metabolismo do glyphosate por plantas tolerantes e suscetíveis a este herbicida. Planta Daninha **22**, 445–451.

Muñoz-Rueda A, Gonzalez-Murua C, Becerril JM, Sánchez-Díaz MF. 1986. Effects of glyphosate [N-(phosphonomethyl)glycine] on photosynthetic pigments, stomatal response and photosynthetic electron transport in *Medicago sativa* and *Trifolium pratense*. Physiologia Plantarum **66**, 63–68.

Neumann G, Kohls S, Landsberg E, Stock-Oliveira Souza K, Yamada T, Romheld V. 2006. Relevance of glyphosate transfer to non-target plants via the rhizosphere. Journal of plant diseases and protection **969**, 963–969.

Orcarav L, Zulet A, Zabalza A, Royela M. 2012. Impairment of carbon metabolism induced by the herbicide glyphosate. Journal of Plant Physiology **169**, 27–33.

Pline WA, Edmisten KL, Wilcut JW, Wells R, Thomas J. 2003. Glyphosate-induced reductions in pollen viability and seed set in glyphosate-resistant cotton and attempted remediation by gibberellic acid (GA3). Weed Science **51**, 19–27.

Powles SB, Preston C. 2010. Evolved glyphosate resistance in plants: Biochemical and genetic basis of resistance. Weed Technology **20**, 282–289.

Preston C, Wakelin AM, Dolman FC, Bostamam Y, Boutsalis P. 2009. A decade of glyphosate-resistant Lolium around the world: Mechanisms, genes, fitness, and agronomic management. Weed Science **57**, 435–441.

Ralph PJ. 2000. Herbicide toxicity of *Halophila ovalis* assessed by chlorophyll *a* fluorescence. Aquatic Botany **66**, 141–152.

Reddy KN, Rimando AM, Duke SO. 2004. Aminomethylphosphonic acid, a metabolite of glyphosate, causes injury in glyphosate-treated, glyphosate-resistant soybean. Journal of agricultural and food chemistry **52**, 5139–43.

Reddy KN, Zablotowicz RM. 2003. Glyphosate-resistant soybean response to various salts of glyphosate and glyphosate accumulation in soybean nodules. Weed Science **51**, 496–502.

Ricordi A, Tornisielo V, Almeida G. 2007. Translocação de 14C-glifosato entre *Brachiaria brizantha* e mudas de café (*Coffea arabia*) e citros (*Citrus limonia* Osbeck). Simpósio Internacional sobre Glyphosate. Botucatu, 307–310.

Rohacek K, Kloz M, Bina D, Batysta F, Vacha F. 2008. Investigation of Non-photochemical Processes in Photosynthetic Bacteria and Higher Plants Using Interference of Coherent Radiation – A Novel Approach., 1549–1552.

Rosolem CA, José G, Andrade M De, Lisboa P, Zoca SM, Glifosate TCOM. 2010. Manganese uptake and redistribution in soybean as affected by glyphosate., 1915–1922.

Samaranayaka AGP, Li-Chan ECY. 2011. Food-derived peptidic antioxidants: A review of their production, assessment, and potential applications. Journal of Functional Foods **3**, 229–254.

Satchivi NM, Wax LM, Stoller EW, Briskin DP. 2000. Absorption and translocation of glyphosate isopropylamine and trimethylsulfonium salts in *Abutilon theophrasti* and *Setaria faberi*. Weed Science **48**, 675–679.

Senem Su Y, Ozturk L, Cakmak I, Budak H. 2009. Turfgrass species response exposed to increasing rates of glyphosate application. European Journal of Agronomy **31**, 120–125.

Sergiev IG, Alexieva VS, Ivanov S, Moskova II, Karanov EN. 2006. The phenylurea cytokinin 4PU-30 protects maize plants against glyphosate action. Pesticide Biochemistry and Physiology **85**, 139–146.

Serra A-A, Nuttens A, Larvor V, Renault D, Couée I, Sulmon C, Gouesbet G. 2013. Low environmentally relevant levels of bioactive xenobiotics and associated degradation products cause cryptic perturbations of metabolism and molecular stress responses in *Arabidopsis thaliana*. Journal of experimental botany **64**, 2753–66.

Servaites JC, Tucci MA, Geiger DR. 1987. Glyphosate effects on carbon assimilation, ribulose bisphosphate carboxylase activity, and metabolite levels in sugar beet leaves. Plant physiology **85**, 370–4.

Sharma SD, Singh M. 2001. Environmental factors affecting absorption and bio-efficacy of glyphosate in Florida beggarweed (*Desmodium tortuosum*). Crop Protection **20**, 511–516.

Sharon A, Amsellem Z, Gressel J. 1992. Glyphosate suppression of an elicited defense response : Increased susceptibility of *Cassia obtusifolia* to a mycoherbicide. Plant physiology **98**, 654–9.

Shetty N, Jørgensen HL, Jensen J, Collinge D, Shetty HS. 2008. Roles of reactive oxygen species in interactions between plants and pathogens. In: Collinge D, Munk L, Cooke BM, eds. Sustainable disease management in a European context SE - 6. Springer Netherlands, 267–280 LA.

Siehl D. 1997. Inhibitors of EPSPS synthase, glutamine synthetase and histidine synthesis. In: Roe R, Burton J, Kuhr R, eds. Herbicide activity: toxicology, biochemistry and molecular biology. Amsterdam: IOS Press, 37–67.

Smiley RW. 1992. Influence of Glyphosate on *Rhizoctonia* Root Rot, Growth, and Yield of Barley. Plant Disease **76**, 937–942.

Tanaka R, Tanaka A. 2007. Tetrapyrrole biosynthesis in higher plants. Annual Review of Plant Biology **58**, 321–346.

Tesfamariam T, Bott S, Cakmak I, Römheld V, Neumann G. 2009. Glyphosate in the rhizosphere—Role of waiting times and different glyphosate binding forms in soils for phytotoxicity to non-target plants. European Journal of Agronomy **31**, 126–132.

Tóth SZ, Schansker G, Strasser RJ. 2005. In intact leaves, the maximum fluorescence level (FM) is independent of the redox state of the plastoquinone pool: A DCMU-inhibition study. Biochimica et Biophysica Acta (BBA) - Bioenergetics **1708**, 275–282.

Van Eerd LL, Hoagland RE, Zablotowicz RM, Hall JC. 2003. Pesticide metabolism in plants and microorganisms. Weed Science **51**, 472–495.

Vivancos PD, Driscoll SP, Bulman CA, Ying L, Emami K, Treumann A, Mauve C, Noctor G, Foyer CH. 2011. Perturbations of amino acid metabolism associated with glyphosate-dependent inhibition of shikimic acid metabolism affect cellular redox homeostasis and alter the abundance of proteins involved in photosynthesis and photorespiration. Plant physiology **157**, 256–68.

Wang C-Y. 2001. Effect of Glyphosate on Aromatic Amino Acid Metabolism in Purple Nutsedge (*Cyperus rotundus*). Weed Technology **15**, 628–635.

Xiang W-S, Wang X-J, Ren T-R, Ju X-L. 2005. Expression of a wheat cytochrome P450 monooxygenase in yeast and its inhibition by glyphosate. Pest management science **61**, 402–6.

Yanniccari M, Tambussi E, Istilart C, Castro AM. 2012. Glyphosate effects on gas exchange and chlorophyll fluorescence responses of two *Lolium perenne* L. biotypes with differential herbicide sensitivity. Plant Physiology and Biochemistry **57**, 210–217.

Yasuor H, Abu-abied M, Belausov E, Madmony A, Sadot E, Riov J. 2006. Glyphosate-Induced Anther Indehiscence in Cotton Is Partially Temperature Dependent and Involves Cytoskeleton and Secondary Wall Modifications and Auxin Accumulation 1. Plant physiology **141**, 1306–1315.

Zablotowicz RM, Reddy KN. 2004. Glyphosate-Resistant Transgenic Soybean: A Minireview. **831**, 825–831.

Zablotowicz RM, Reddy KN. 2007. Nitrogenase activity, nitrogen content, and yield responses to glyphosate in glyphosate-resistant soybean. Crop Protection **26**, 370–376.

Zobiole LHS, Bonini EA, Oliveira RS, Kremer RJ, Ferrarese-Filho O. 2010*a*. Glyphosate affects lignin content and amino acid production in glyphosate-resistant soybean. Acta Physiologiae Plantarum **32**, 831–837.

Zobiole LHS, Kremer RJ, Oliveira RS, Constantin J. 2011*a*. Glyphosate affects micro-organisms in rhizospheres of glyphosate-resistant soybeans. Journal of applied microbiology **110**, 118–27.

Zobiole LHSS, Kremer RJ, Oliveira Jr. RS, Constantin J, Oliveira RS. 2011*b*. Glyphosate affects chlorophyll, nodulation and nutrient accumulation of "second generation"

glyphosate-resistant soybean (*Glycine max* L.). Pesticide Biochemistry and Physiology **99**, 53–60.

Zobiole LHS, Kremer RJ, de Oliveira Jr. RS, Constantin J. 2012. Glyphosate effects on photosynthesis, nutrient accumulation, and nodulation in glyphosate-resistant soybean. Journal of Plant Nutrition and Soil Science **175**, 319–330.

Zobiole LHS, Oliveira RS, Kremer RJ, Constantin J, Yamada T, Castro C, Oliveira F a., Oliveira a. 2010*b*. Effect of glyphosate on symbiotic N_2 fixation and nickel concentration in glyphosate-resistant soybeans. Applied Soil Ecology **44**, 176–180.

Zobiole LHS, Oliveira RS, Morgan Huber D, Constantin J, Castro C, Oliveira FA, Oliveira A. 2009. Glyphosate reduces shoot concentrations of mineral nutrients in glyphosate-resistant soybeans. Plant and Soil **328**, 57–69.

Zobiole LHS, Oliveira RS, Visentainer J V, Kremer RJ, Bellaloui N, Yamada T. 2010*c*. Glyphosate affects seed composition in glyphosate-resistant soybean. Journal of agricultural and food chemistry **58**, 4517–4522.

Zouni A, Witt HT, Kern J, Fromme P, Krauss N, Saenger W, Orth P. 2001. Crystal structure of photosystem II from Synechococcus elongatus at 3.8 A resolution. Nature **409**, 739–43.

CHAPTER II

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

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ABSTRACT

Phosphate (PO_4^{3-}) fertilization is a common practice in agricultural fields also targets for glyphosate application. Due to their chemical similarities, PO_4^{3-} and glyphosate compete for soil adsorbing sites, with PO_4^{3-} fertilization increasing glyphosate bioavailability in the soil solution. After PO_4^{3-} fertilization, its concentration will be elevated in the soil solution and both PO_4^{3-} 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 PO_4^{3-} 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 PO_4^{3-}, 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 PO_4^{3-} transporters. We observed, however, that PO_4^{3-} increased glyphosate uptake by roots, an effect that was related to increased root cell membrane stability. Our results indicate that PO_4^{3-} has an important role in glyphosate physiological effects. Under agricultural conditions, PO_4^{3-} 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 (PO_4^{3-}) content appears to be one of the most important factors driving this glyphosate binding (Borggaard, 2011). As PO_4^{3-} 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 PO_4^{3-} determines glyphosate availability in soil solutions, and increases in soil PO_4^{3-} concentrations

may result in increased glyphosate availability (Bott *et al.*, 2011). Phosphorous is an essential element for plants, participating in crucial physiological events, and PO_4^{3-} fertilization is a common agricultural practice (Lopes, 2004). In agricultural fields where glyphosate has been applied, 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 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 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 PO_4^{3-} for root uptake? 2) Under situations of high availability of both compounds, how does 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 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 PO_4^{3-} transporters in glyphosate uptake by willow roots.

Using a PO_4^{3-} transporter inhibitor (phosphonophormic acid-PFA) we were able to demonstrate the involvement of 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 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 PO_4^{3-} additions (Fig 2.1). According to Denis and Delrot (1993), 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 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 PO_4^{3-} in glyphosate uptake by roots.

The addition of 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 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 PO_4^{3-} to soil matrices

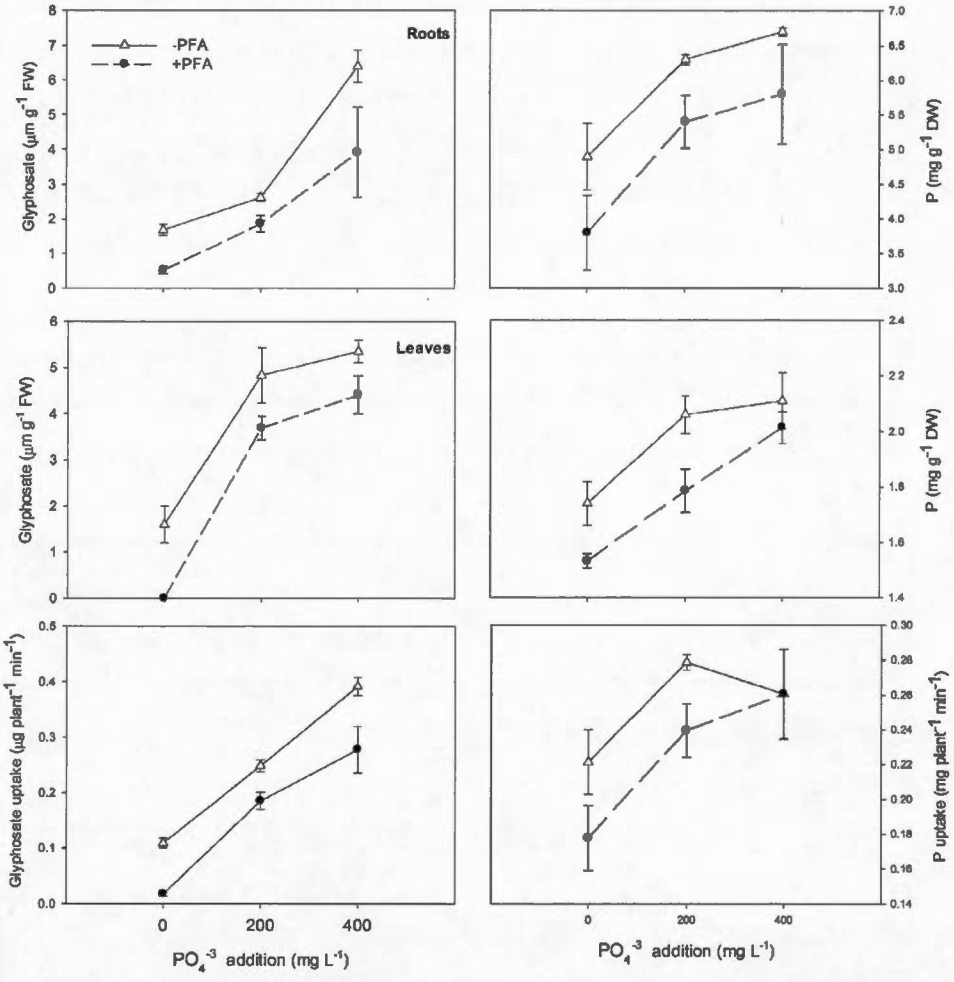

Fig 2.1. Glyphosate and phosphorus (P) concentration of roots and leaves and glyphosate uptake rates of willows grown under different phosphate (PO_4^{3-}) concentrations and treated with 0mM phosphonophormic acid (-PFA) or 5mM (+PFA).

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, 2014*b*). Like trace-elements (Gomes *et al.*, 2013, 2014*b*), glyphosate is known to induce oxidative stress in plants (Gomes *et al.*, 2014*a*). 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.

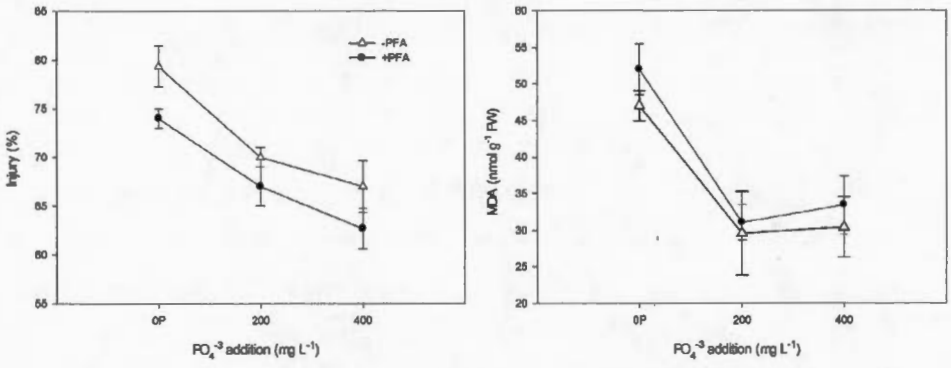

Fig 2.2. Root cellular injury and lipid peroxidation in roots of willow plants grown under different phosphate (PO_4^{3-}) concentrations and treated with 0mM phosphonophormic acid (-PFA) or 5mM (+PFA).

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 PO_4^{3-} 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 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 P, assuring 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.*, 2014*a*; 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

Salix 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 µmol photons m^{-2} s^{-1}. Cuttings were grown in plastic boxes filled with distilled water amended with King Max nutrient solutions (7% P_2O_5, 11% K_2O, 1.5% Mg, 1.27% S, 0.07% B, 0.002% Mo, 0.12% Zn) and B (4% N, 1% NH_4^+, 3% NO_3^{-2}, 10%

K2O, 2% Ca, 0.05% Fe, 0.05% Mn) (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 phosphonophormic 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 PO_4^{3-} (as KH_2PO_4). 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 PO_4^{3-}, -PFA + 200 ppm PO_4^{3-}, -PFA + 400 ppm PO_4^{3-}, +PFA + 0 ppm PO_4^{3-}, +PFA + 200 ppm PO_4^{3-}, and +PFA + 400 ppm PO_4^{3-}. 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:

$$Uptake = \frac{\text{root + leaf glyphosate concentrations}}{T_1 - T_0} \quad (1)$$

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:

$$\%CMS = \frac{1 - \left(\frac{T1}{T2}\right)}{1 - \left(\frac{C1}{C2}\right)} * 100$$

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 (N_2) 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 N_2 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).

2.3. REFERENCES

Annett R, Habibi HR, Hontela A. 2014. Impact of glyphosate and glyphosate-based herbicides on the freshwater environment. Journal of Applied Toxicology **34**, 458–479.

Beltrano J, Ruscitti M, Arango C, Ronco M. 2013. Changes in the accumulation of shikimic acid in mycorrhized *Capsicum annum* grown with application of glyphosate and phosphorus. Theoretical and Experimental Plant Physiology **25**, 125–136.

Borggaard O. 2011. Does phosphate affect soil sorption and degradation of glyphosate? - A Review. Trends in Soil Science and Plant Nutrition **2**, 16–27.

Bott S, Tesfamariam T, Kania A, Eman B, Aslan N, Römheld V, Neumann G. 2011. Phytotoxicity of glyphosate soil residues re-mobilised by phosphate fertilisation. Plant and Soil **342**, 249–263.

Chien SH, Prochnow LI, Tu S, Snyder CS. 2011. Agronomic and environmental aspects of phosphate fertilizers varying in source and solubility: An update review. Nutrient Cycling in Agroecosystems **89**, 229–255.

Clua A, Conti M, Beltrano J. 2012. The effects of glyphosate on the growth of birdsfoot trefoil (*Lotus corniculatus*) and its interaction with different phosphorus contents in soil. Journal of Agricultural Science **4**, 208–218.

Correia F V, Moreira JC. 2010. Effects of Glyphosate and 2, 4-D on earthworm (Eisenia foetida) in laboratory tests. Bulletin of environmental contamination and toxicology **85**, 264 – 268.

Correll DL. 1998. The role of phosphorus in the eutrophication of receiving waters: A review. , 261–266.

Coupe R, Kalkhoff S, Capel P, Gregoire C. 2012. Factors affecting the fate and transport of glyphosate and AMPA into surface waters of agricultural watersheds in the United States and Europe. Geophysical Research Abstracts **14**, 5877.

Denis M-H, Delrot S. 1993. Carrier-mediated uptake of glyphosate in broad bean (*Vicia faba*) via a phosphate transporter. Physiologia plantarum **87**, 569–575.

Gomes MP, Carvalho M, Carvalho GS, Garcia QS, Guilherme LRG, Soares AM. 2013. Phosphorus improves arsenic phytoremediation by *Anadenanthera*

peregrina by alleviating induced oxidative stress. International Journal of Phytoremediation **15**, 633–646.

Gomes MP, Smedbol E, Chalifour A, Hénault-Ethier L, Labrecque M, Lepage L, Lucotte M, Juneau P. 2014*a*. Alteration of plant physiology by glyphosate and its by-product aminomethylphosphonic acid (AMPA), an overview. Journal of Experimental Botany **65**, 4691–703.

Gomes MP, Soares AM, Garcia QS. 2014*b*. Phosphorous and sulfur nutrition modulate antioxidant defenses in *Myracrodruom urundeuva* plants exposed to arsenic. Journal of hazardous materials **276**, 97–104.

Goscinny S, Unterluggauer H, Aldrian J, Hanot V, Masselter S. 2012. Determination of glyphosate and its metabolite AMPA (Aminomethylphosphonic Acid) in cereals after derivatization by isotope dilution and UPLC-MS/MS". Food Analytical Methods **5**, 1177–1185.

Hénault-Ethier L, Smedbol E, Dagenais G, Gomes M, Racine J-F, Juneau P, Lepage L, Labrecque M, Lucotte M. 2014. Les bandes riveraines en agriculture: Une approche pluridisciplinaire pour une application concrète. Vecteur Environnement, 52–57.

Hodges DM, Delong JM, Forney CF, Prange RK. 1999. Improving the thiobarbituric acid-reactive-substances assay for estimating lipid peroxidation in plant tissues containing anthocyanin and other interfering compounds. Planta **1**, 604–611.

Krutz LJ, Senseman SA, Zablotowicz RM, Matocha MA. 2005. Reducing herbicide runoff from agricultural fi elds with vegetative fi lter strips: A review. Weed Science **53**, 353–367.

Laitinen P, Rämö S, Siimes K. 2007. Glyphosate translocation from plants to soil – does this constitute a significant proportion of residues in soil? Plant and Soil **300**, 51–60.

Lin C-H, Lerch RN, Goyne KW, Garrett HE. 2011. Reducing herbicides and veterinary antibiotics losses from agroecosystems using vegetative buffers. Journal of environmental quality **40**, 791–799.

Lopes AS. 2004. Fertilizer use by crop in Brazil. FAO, Rome.

Mirck J, Isebrands JG, Verwijst T, Ledin S. 2005. Development of short-rotation willow coppice systems for environmental purposes in Sweden. Biomass and Bioenergy **28**, 219–228.

Morin F, Vera V, Nurit F, Tissut M, Marigo G. 1997. Glyphosate uptake in *Catharanthus roseus* cells : Role of a phosphate transporter. Pesticide Biochemistry and Physiology **58**, 13–22.

Pham HT, Miyagawa S, Kosaka Y. 2015. Distribution patterns of trees in paddy field landscapes in relation to agro-ecological settings in northeast Thailand. Agriculture, Ecosystems & Environment **202**, 42–47.

Sanchez CA. 2006. Phosphorus. In: Barker AV, Pealbean DJ, eds. Handbook of plant nutrition. New York, NY: Taylor & Francis, 51–90.

Sarruge JR, Haag HP. 1974. *Plant chemical analysis.* Piracicaba: Escola Superior de Agricultura 'Luiz de Queiroz'.

Sharma RK, Agrawal M. 2006. Single and combined effects of cadmium and zinc on carrots: Uptake and bioaccumulation. Journal of Plant Nutrition **29**, 1791–1804.

Sullivan C, Ross W. 1979. Selecting for drought and heat resistance sorghum. In: Mussell H, Taples T, eds. Stress physiology in crops plants. USA: John Willey and Sons, 264–281.

Yajima D, Motani H, Hayakawa M, Sato Y, Sato K, Iwase H. 2009. The relationship between cell membrane damage and lipid peroxidation under the condition of hypoxia-reoxygenation: analysis of the mechanism using antioxidants and electrion transport inhibitors. Cell biochemistry and function **27**, 338–343.

CHAPTER III

IMPACT OF PHOSPHATE ON GLYPHOSATE UPTAKE AND TOXICITY IN WILLOW ‡

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ABSTRACT

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

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,

1998; Keoleian and Volk, 2005), helping to remove the contaminant from the environment.

Situated at the interface of agricultural lands, plants in RBS can be submitted to both glyphosate and PO_4^{3-} runoff. Much as glyphosate and phosphate compete on soil adsorption sites, they vie for access to membrane carriers (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.*, 2015*a*). 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, 1997*b*). 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.* (2014*a*), glyphosate can also indirectly affect other plant physiological processes such as photosynthesis, inducing oxidative stress. In addition to its role in glyphosate uptake, PO_4^{3-} may play a role in plant response to the herbicide, as it can help them avoid some of the contaminant's deleterious effects. For example, it was observed that by increasing PO_4^{3-} uptake, plant tolerance to arsenate (which, like glyphosate, is chemically similar to PO_4^{-3}) is increased through activation of antioxidant systems, preventing oxidative burst induced by the trace-element (Gomes *et al.*, 2012, 2014*c*).

In this study, we evaluated the effects of addition of PO_4^{3-} on glyphosate toxicity and uptake capacity in a fast-growing willow cultivar (*Salix miyabeana* SX64). This cultivar's rapid, voluminous biomass production, associated with its high tolerance to stress factors (i.e., soil contamination by metals and organic pollutants) (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% P_2O_5, 11% K_2O, 1.5% Mg, 1.27% S, 0.07% B, 0.002% Mo, 0.12% Zn) and B (4% N, 1% NH_4^+, 3% NO_3^{-2}, 10% K2O, 2% Ca, 0.05% Fe, 0.05% Mn) following the product's instructions. The solutions were continuously aerated, and renewed every 15 days. To minimize interference with unknown ingredients in the commercial formulation, analytical-grade glyphosate (Pestanal grade) obtained from Sigma-Aldrich (Oakville, Canada) was used in the experiments.

After an initial growth period (45 days), healthy (vigorous and without leaf chlorotic spots) and uniform (similar height) plants were transferred to containers (35 L), 15 plants per container. Each of these was filled with the desired treatment solutions and under continuous aeration. A randomized block design with three containers (corresponding to the replications) per treatment was used. Glyphosate was

added to the solution at concentrations of 0, 0.001, 0.065 and 1 mg l^{-1}, while phosphate (as KH_2PO_4) was added at 0, 200 and 400 mg PO_4^{3-} l^{-1}. Glyphosate concentrations were chosen based on the range of environmental concentrations found in streams of agricultural areas in Canada and the United States (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 KNO_3, nitrogen as both KNO_3 and NH_4NO_3, and pH was adjusted to 6.7 $\pm$ 0.1. Both physiological and glyphosate measurements were performed at two, three and seven days after treatment inductions. Until seventh day of treatment, the presence of aminomethylphosphonic acid (AMPA) was not detected in the growth solution for the low glyphosate concentrations ($\leq$ 0.065 mg l^{-1}; data not shown). However, the AMPA concentration increased from 1.1 to 9.8 μg l^{-1} in the growth solution of the highest glyphosate treatment (1 mg l^{-1}) from the seventh to tenth day of treatment (data not shown), respectively, probably due to microbial degradation. Therefore, evaluations were reported up to the seventh days (since the main goal here was to investigate PO_4^{3-} and glyphosate interaction, without AMPA effects).

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

3.2.2 Chemical analyses

For phosphorus evaluation, 0.1 g of leaves or roots were digested in 5 ml of concentrated HNO_3 (GR) in a microwave oven (ETHOS 1, Milestone Italy), at 175 °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 (N_2) flow. A derivatization procedure was carried out by adding 500 µl of trifluoroethanol (TFE) and 1 ml of trifluoroacetic anhydre (TFAA). To ensure complete dissolution of glyphosate and AMPA, vials were vortexed before being heated at 90°C for an hour. After being cooled down to room temperature, samples were evaporated to dryness under stream of N_2. Prior to GC-ECD injection, samples were dissolved in 800 µL of ethyl acetate and 200 µL of pyridine whereas a 1 µL of 1-bromopentadecane is finally added in order to monitor injection reproducibility. A Varian GC 3800 gas chromatograph equipped with a Restek RXI-5SIL MS capillary column (30 m x 0.25 mm ID, 0.25 µm) was used to analyze samples. The chromatographic conditions used for glyphosate detection were as follows: injector temperature, 250°C; detector temperature, 300°C; oven temperature program, 60°C, hold for 0.50 min, 6°C.min^{-1} to 170, 60°C.mn^{-1} to 250°C, hold 10.0 min, for a total run of 30.17 min. High purity hydrogen was used as carrier

gas (at a flow of 1.4 $mL.min^{-1}$), and the injection volume was 2 µL. To minimize uncertainty of chromatographic measurements, GC-ECD performance parameters were checked on a daily basis to verify their suitability for the purpose of glyphosate/AMPA analysis. Limit of detection (LOD) and limit of quantification (LOQ) were determined based on the method described in Mocack *et al.* (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 ($r^2 = 0.96$; $p < 0.0001$ and $r^2 = 0.99$; $p < 0.0001$ for glyphosate and AMPA, respectively) in the domain of expected samples concentration. Each batch of samples included three blanks, five standards and five spiked control samples (roots or leaves for the corresponding 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_2O_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 (ε). 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 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 ($P < 0.05$).

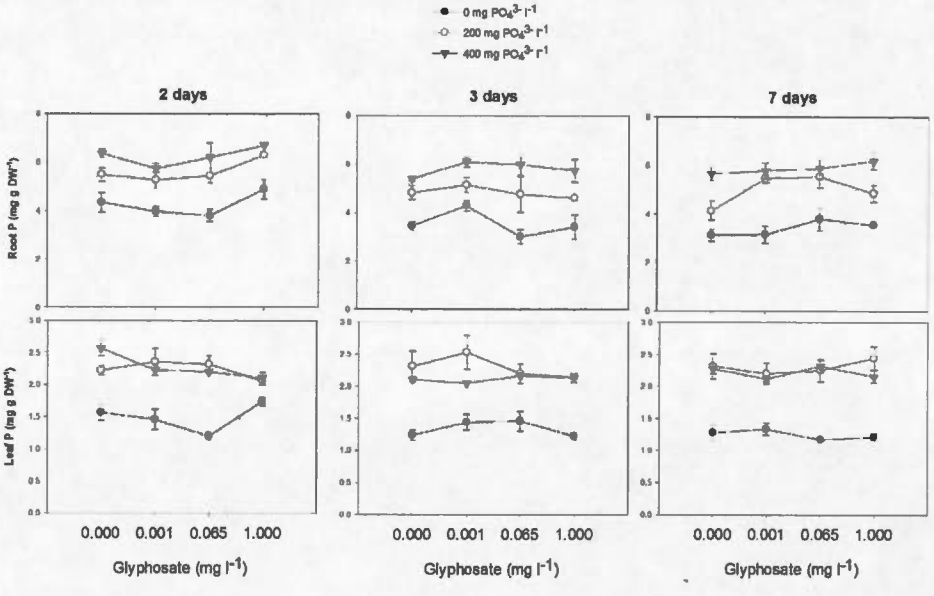

Fig 3.1. Phosphorus concentration (mg g DW^{-1}) in roots and leaves of willow plants grown for two, three and seven days in nutrient solution amended with increasing doses of glyphosate (0, 0.001, 0.065 and 1.000 mg l^{-1}) and phosphate (0, 200 and 400 mg l^{-1}). Values are means ± SE of three replicates. 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 PO_4^{3-} addition was observed for glyphosate concentrations in roots and leaves (Table 1S, Figs. 2 and 3). For glyphosate concentrations ≥ 0.065 mg l^{-1}, PO_4^{3-} increased glyphosate concentration in roots and leaves ($P < 0.0001$) (Fig. 3.2 and 3.3). For the first time of evaluation, PO_4^{3-} fertilized plants treated with glyphosate concentration ≥ 0.065 mg l^{-1} showed greater glyphosate concentrations.

AMPA concentration in roots and leaves was higher for the first time of evaluation (Table 3.1S, Figs. 3.2 and 3.3). At that point, PO_4^{3-} increased AMPA concentration in the tissues of plants exposed to glyphosate doses ≥ 0.065 mg l^{-1}. We 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 ≥ 0.065 mg of glyphosate l^{-1}, total accumulation was increased by PO_4^{3-}.

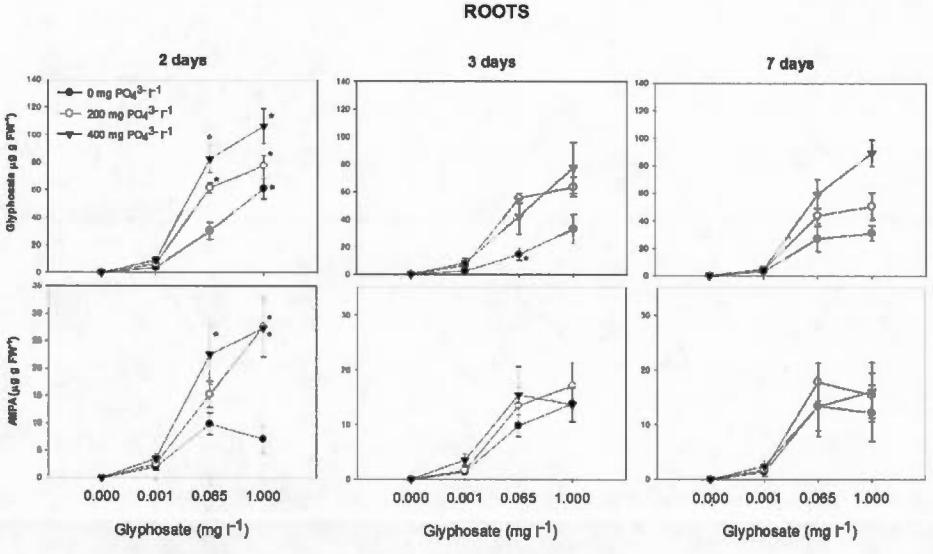

Fig 3.2. Glyphosate and aminomethylphosphonic acid (AMPA) content (µg g FW^{-1}) in roots of willow plants grown for two, three and seven days in nutrient solution amended with increasing doses of glyphosate (0, 0.001, 0.065 and 1.000 mg l^{-1}) and phosphate (0, 200 and 400 mg l^{-1}). Values are means ± SE of three replicates. The concentrations of added phosphate were 0 (filled circle), 200 (open circle) and 400

(filled inverted triangle) mg l^{-1}. Values followed by *, within the same glyphosate and phosphorus concentration, are significantly different (P>0.05) by the Contrast test.

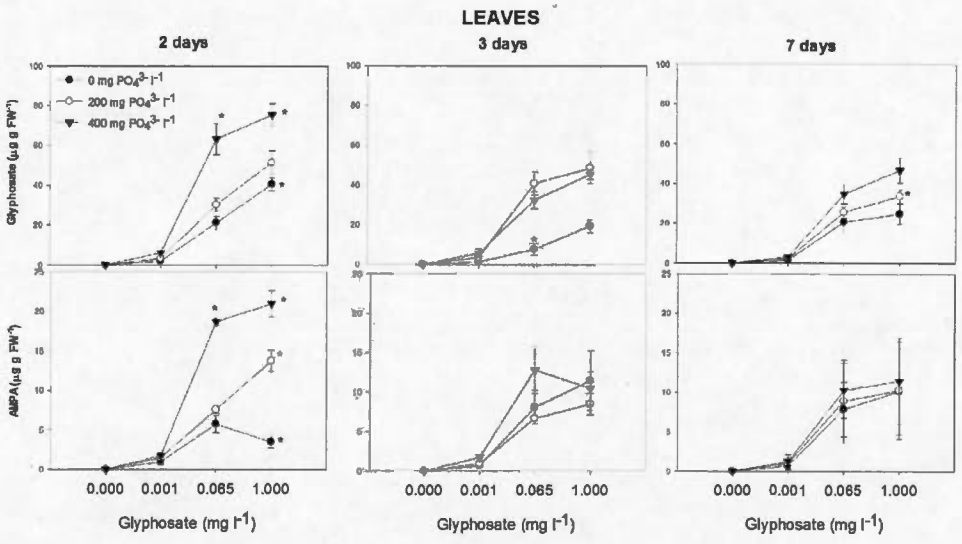

Fig 3.3. Glyphosate and aminomethylphosphonic acid (AMPA) content (µg g FW^{-1}) in leaves of willow plants grown for two, three and seven days in a nutrient solution amended with increasing doses of glyphosate (0, 0.001, 0.065 and 1.000 mg l^{-1}) and phosphate (0, 200 and 400 mg l^{-1}). Values are 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}).

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

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

3.2 Physiological evaluations

3.1.1 Photosynthesis

A significant interaction between time of exposure, glyphosate and PO_4^{3-} addition was observed for operational PSII quantum yield (Φ'_M) (Table 3.2S). In plants treated with PO_4^{3-}, Φ'_M was always greater after seven days of exposure, regardless of the glyphosate and PO_4^{3-} addition (Fig. 3.4). After two days of treatment, PO_4^{3-} decreased the operational PSII quantum yield (Fig. 3.4) whereas glyphosate addition increased yield in plants not fertilized with PO_4^{-3} (Fig. 3.4). In contrast, yield decreased in plants treated with 400 mg PO_4^{3-} l^{-1} as glyphosate doses increased (Fig. 3.4). After three days of exposure, PO_4^{3-} increased Φ'_M of glyphosate-treated plants (Fig. 3.4). Similarly, after seven days of treatment induction, PO_4^{3-} increased Φ'_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 PO_4^{3-} addition ($P = 0.0002^*$), and PO_4^{3-} increased shikimate concentration in leaves of plants treated with glyphosate (Fig. 3.4).

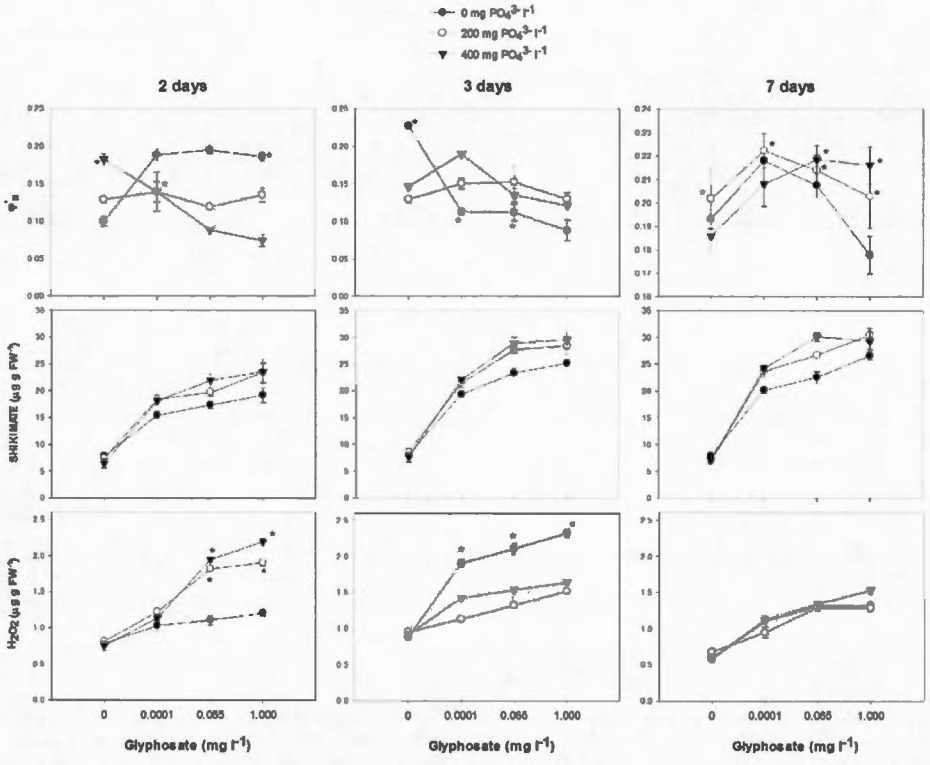

Fig 3.4. PSII quantum yield (Φ'_M), shikimate and hydrogen peroxide (H_2O_2) (µg g FW^{-1}) concentrations in leaves of willow plants grown for two, three and seven days in a nutrient solution amended with increasing doses of glyphosate (0, 0.001, 0.065 and 1.000 mg l^{-1}) and phosphate (0, 200 and 400 mg l^{-1}). Values are means ± SE of three replicates. Values followed by *, within the same glyphosate and phosphorus concentration, are significantly different (P>0.05) by the Contrast test.

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 ≥ 0.065 l^{-1}, H_2O_2 concentration in leaves were greater for the first time of evaluation ($P = 0.0004$) . After two days of exposure, PO_4^{3-} increased H_2O_2 concentration in leaves of plants treated with 0.065 and 1 mg glyphosate l^{-1} (Fig. 3.4). In contrast, after three days of exposure, PO_4^{3-} significantly decreased H_2O_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_2O_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).

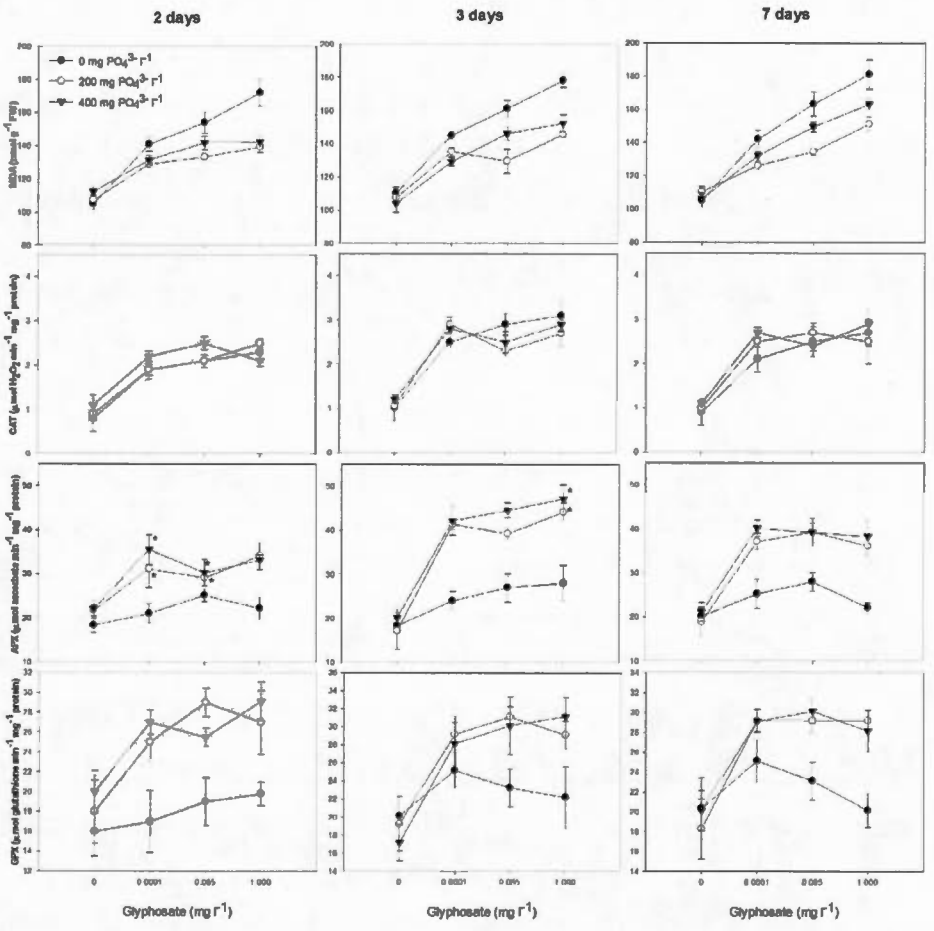

Fig 3.5. Lipid peroxidation (MDA content, nmol g^{-1} FW), and activity of catalase (CAT, µmol H_2O_2 min^{-1} mg^{-1} protein), ascorbate peroxidase (APX µmol ascorbate min^{-1} mg^{-1} protein) and glutathione peroxidase (GPX, µmol glutathione min^{-1} mg^{-1} protein) in roots of willow plants grown for two, three and seven days in a nutrient solution amended with increasing doses of glyphosate (0, 0.001, 0.065 and 1.000 mg l^{-1}) and phosphate (0, 200 and 400 mg l^{-1}). Values are means ± SE of three replicates. 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 PO_4^{3-}, 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 PO_4^{3-} addition was observed for APX activity (Table 3.2S). PO_4^{3-} fertilized plants exposed to glyphosate showed lower APX activity for the first time of evaluation. After two days of exposure, APX activity in leaves of glyphosate-treated plants was decreased PO_4^{3-} (Fig. 3.6). However, after three or seven days, APX activity in plants treated with glyphosate was increased by PO_4^{3-} (Fig. 3.6). Glyphosate addition increased GPX activity in leaves (Fig. 3.6).

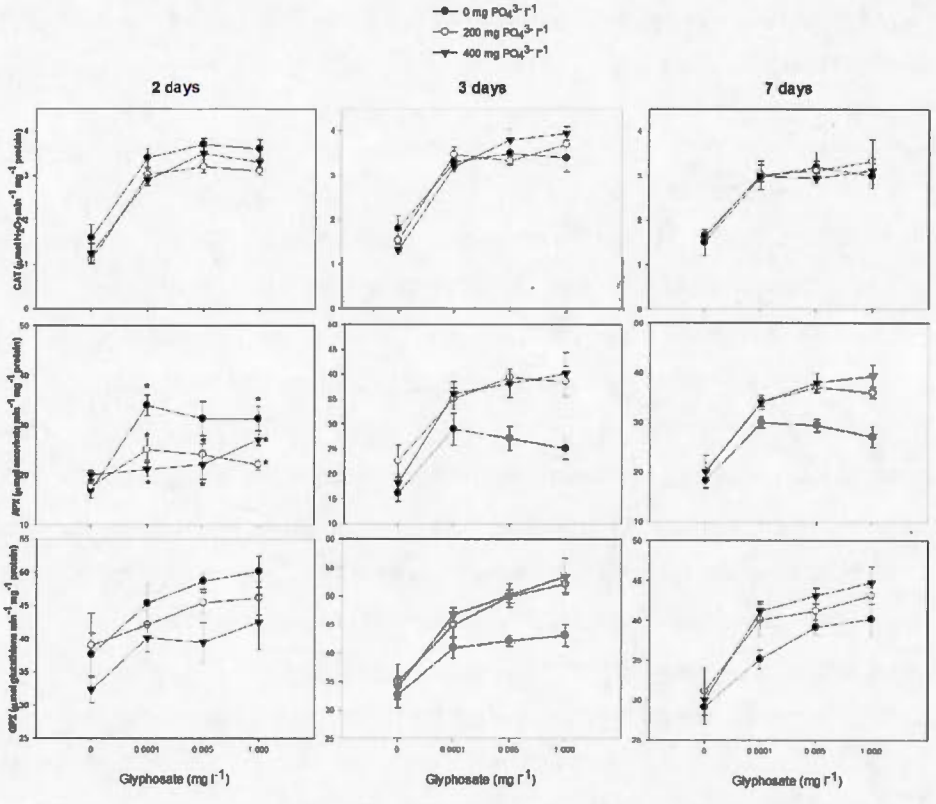

Fig 3.6. Activity of catalase (CAT, μmol H_2O_2 min^{-1} mg^{-1} protein), ascorbate peroxidase (APX μmol ascorbate min^{-1} mg^{-1} protein) and glutathione peroxidase (GPX, μmol glutathione min^{-1} mg^{-1} protein) in leaves of willow plants grown for two, three and seven days in a nutrient solution amended with increasing doses of

glyphosate (0, 0.001, 0.065 and 1.000 mg l^{-1}) and phosphate (0, 200 and 400 mg l^{-1}). Values are means ± SE of three replicates.

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.*, 2015*a*). 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, 2009*b*; 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.*, 2014*c*), 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.*, 2015*a*), 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.*, 2015*a*). By increasing the expression of P-transporters in root cell membranes, glyphosate could increase P nutrition in plants, however this hypothesis should be further investigated. The time of exposure had significant effect

on some physiological parameters studied (biomass production, shikimate and H_2O_2 contents and in the activity of photosynthesis- Φ'_M - 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.*, 2013*a*, 2014*c*). 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 PO_4^{3-} 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, PO_4^{3-} was found to decrease glyphosate-induced oxidative damage by increasing the activity of enzymatic antioxidant systems, mainly ascorbate peroxidase (APX) and glutathione peroxidase (GPX) (Fig. 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 (H_2O_2), 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, PO_4^{3-} alleviated glyphosate-induced oxidative stress, ensuring the stability of cell membranes, and then allowing glyphosate diffusion and uptake through roots.

Interestingly, we observed the presence of AMPA (Fig. 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 $\mu g\ l^{-1}$ 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 PO_4^{3-} 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 PO_4^{3-} 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, 1997*b*). We observed shikimate accumulation in glyphosate-treated plants (Fig. 3.3). Moreover in these plants, PO_4^{3-} increased shikimate concentration, which may be due to the greater glyphosate concentration in leaves of PO_4^{3-}-treated plants (Fig. 3.4). Therefore, we would expect that the increased glyphosate concentration in leaves associated with PO_4^{3-} would amplify the deleterious effects of glyphosate on leaf physiology. Indeed, after two days of exposure, we observed that, in the presence of glyphosate, PO_4^{-3}-fertilized plants showed decreased photosynthesis (as measured by the operational PSII quantum yield, Fig. 3.4). However, this pattern was inversed at the following measurement occasion (day 3) and, after 7 days, PO_4^{3-}-fertilized plants showed higher or no-significantly different rates of photosynthesis in relation to control (Fig. 3.4). We recently showed that glyphosate-induced ROS generation is implicated in decreased rates of photosynthesis (Gomes *et al.*, 2015*b*). Increased H_2O_2 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.*, 2015*b*), increased H_2O_2 concentration

higher than ~2 μg g FW^{-1} in leaves was related to decreased operational PSII quantum yield (Fig. 3.4). H_2O_2 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), H_2O_2 accumulation decreases photosynthetic activity (for more examples, see Gomes *et al.*, 2015*b*). However, how can PO_4^{3-} prevent H_2O_2 accumulation and its deleterious effects on photosynthesis in leaves of herbicide-treated plants? In an attempt to answer this question, we investigated the activity of antioxidant enzymes in leaves (Fig. 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 H_2O_2 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 PO_4^{3-} (Fig. 3.6). In contrast, APX and GPX activity were modulated by PO_4^{3-}, and an intrinsic implication of these enzymes (mainly APX) in the fine control of H_2O_2 concentration in leaves was noted (Fig. 3.6). After two days of exposure, APX activity was lower in those glyphosate-treated plants exposed to PO_4^{3-} in which H_2O_2 accumulation had been noted. However, after three days of exposure, these plants showed decreased H_2O_2 concentration and increased APX (as well as GPX) activity in leaves. APX activity remained higher in these plants after seven days of exposure, allowing controlled H_2O_2 concentrations in leaves and normal photosynthesis rates. Therefore, even as it increased glyphosate concentration in leaves, PO_4^{3-} 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 PO_4^{3-} increased glyphosate accumulation and protected photosynthetic activity. It remains, however, unclear how PO_4^{3-} can induce increases in the activity of antioxidant systems. Extracellular PO_4^{3-} has been shown to be a signaling molecule affecting the expression of several genes in Murine osteoblasts (Rendenbach *et al.*, 2014). Likewise, PO_4^{3-} could also modulate the expression of antioxidant enzymes genes in plants, but further studies are required.

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

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REFERENCES

Ahsan N, Lee D-G, Lee K-W, Alam I, Lee S-H, Bahk JD, Lee B-H. 2008. Glyphosate-induced oxidative stress in rice leaves revealed by proteomic approach. Plant physiology and biochemistry **46**, 1062–70.

Arduini I, Godbold DL, Onnis A. 1994. Cadmium and copper change root growth and morphology of Pinus pinea and Pinus pinaster seedlings. Physiologia Plantarum **92**, 675–680.

Arduini I, Godbold DL, Onnis a. 1995. Influence of copper on root growth and morphology of Pinus pinea L. and Pinus pinaster Ait. seedlings. Tree physiology **15**, 411–5.

Azevedo R, Alas R, Smith R, Lea P. 1998. Response of antioxidant enzymes to transfer from elevated carbon dioxide to air and ozone fumigation, in the leaves and roots of wild-type and a catalase-deficient mutant of barley. Physiologia Plantarum **104**, 280–292.

Beltrano J, Ruscitti M, Arango C, Ronco M. 2013. Changes in the accumulation of shikimic acid in mycorrhized *Capsicum annum* grown with application of glyphosate and phosphorus. Theoretical and Experimental Plant Physiology **25**, 125–136.

Benbrook CM. 2012. Impacts of genetically engineered crops on pesticide use in the U.S. -- the first sixteen years. Environmental Sciences Europe **24**, 24.

Bijay KS, Dale LS. 1998. Rapid determination of glyphosate injury to plants and identification of glyphosate-resistant plants. Weed Science Society of America **12**, 527–530.

Borggaard O. 2011. Does phosphate affect soil sorption and degradation of glyphosate? - A Review. Trends in Soil Science and Plant Nutrition **2**, 16–27.

Bott S, Tesfamariam T, Kania A, Eman B, Aslan N, Römheld V, Neumann G. 2011. Phytotoxicity of glyphosate soil residues re-mobilised by phosphate fertilisation. Plant and Soil **342**, 249–263.

Cerdeira AL, Duke SO. 2006. The current status and environmental impacts of glyphosate-resistant crops: a review. Journal of environmental quality **35**, 1633–58.

Clua A, Conti M, Beltrano J. 2012. The effects of glyphosate on the growth of birdsfoot trefoil (*Lotus corniculatus*) and its interaction with different phosphorus contents in soil. Journal of Agricultural Science **4**, 208–218.

Coupe R, Kalkhoff S, Capel P, Gregoire C. 2012. Factors affecting the fate and transport of glyphosate and AMPA into surface waters of agricultural watersheds in the United States and Europe. Geophysical Research Abstracts **14**, 5877.

Denis M-H, Delrot S. 1993. Carrier-mediated uptake of glyphosate in broad bean (*Vicia faba*) via a phosphate transporter. Physiologia plantarum **87**, 569–575.

Duke SO, Powles SB. 2008. Glyphosate: a once-in-a-century herbicide. Pest Management Science **64**, 319–325.

Fillion M, Brisson J, Guidi W, Labrecque M. 2011. Increasing phosphorus removal in willow and poplar vegetation filters using arbuscular mycorrhizal fungi. Ecological Engineering **37**, 199–205.

Foyer CH, Noctor G. 2011. Ascorbate and glutathione: the heart of the redox hub. Plant physiology **155**, 2–18.

Genty B, Wonders J, Baker N. 1990. Non-photochemical quenching of F0 in leaves is emission wavelength dependent: Consequences for quenching analysis and its interpretation. Photosynthesis research **26**, 133–139.

Giesy J, Dobson S, Solomon K. 2000. Ecotoxicological Risk Assessment for Roundup® Herbicide. In: Ware G, ed. Reviews of Environmental Contamination and Toxicology. Reviews of Environmental Contamination and Toxicology SE - 2. Springer New York, 35–120.

Giroux I, Roy N, Lamontagne C. 2010. Présence de Pesticides dans l'Eau Souterraine en Milieu Agricole : Étude Pilote du Bassin Versant de la Rivière Châteauguay. Canadian Water Resources Journal **35**, 527–542.

Gomes MP, Carvalho M, Carvalho GS, Garcia QS, Guilherme LRG, Soares AM. 2013. Phosphorus improves arsenic phytoremediation by *Anadenanthera peregrina* by alleviating induced oxidative stress. International Journal of Phytoremediation **15**, 633–646.

Gomes MP, Carvalho M, Cristina T, Lanza L, Marques M, Duarte M, Oliveira C De, Nogueira G, Soares ÂM, Souza Q De. 2012. Arsenic-sensitivity in *Anadenanthera peregrina* due to arsenic-induced lipid peroxidation. International Journal of Applied Science and Technology **2**, 55–63.

Gomes M, Maccario S, Lucotte M, Labrecque M, Juneau P. 2015*a*. Consequences of phosphate application on glyphosate uptake by roots: impacts for environmental management practices. Science of The Total Environment **In press**.

Gomes M, Le Manac'h S, Labrecque M, Hénault-Ethier L, Lucotte M, Juneau P. 2015*b*. Understanding the interconnected effects of a glyphosate-based herbicide on photosynthesis and related physiological processes in willow plants. The Plant journal **submitted**.

Gomes MP, Marques TCLLSM, Nogueira MOGN, Castro EM, Soares ÂM. 2011. Ecophysiological and anatomical changes due to uptake and accumulation of heavy metal in *Brachiaria decumbens*. Scientia Agricola **68**, 566–573.

Gomes MP, Smedbol E, Chalifour A, Hénault-Ethier L, Labrecque M, Lepage L, Lucotte M, Juneau P. 2014*a*. Alteration of plant physiology by glyphosate and its by-product aminomethylphosphonic acid (AMPA), an overview. Journal of Experimental Botany **65**, 4691–703.

Gomes MP, Soares AM, Garcia QS. 2014*b*. Phosphorous and sulfur nutrition modulate antioxidant defenses in *Myracrodruom urundeuva* plants exposed to arsenic. Journal of hazardous materials **276**, 97–104.

Goscinny S, Unterluggauer H, Aldrian J, Hanot V, Masselter S. 2012. Determination of glyphosate and its metabolite AMPA (Aminomethylphosphonic Acid) in cereals after derivatization by isotope dilution and UPLC-MS/MS". Food Analytical Methods **5**, 1177–1185.

Guidi W, Pitre FE, Labrecque M. 2013. Short-rotation coppice of willows for the production of biomass in Eastern Canada. In: Matovic MD, ed. Biomass Now - Sustainable Growth and Use. InTech Open Science, 421–448.

Gunes A, Pilbeam D, Inal A. 2009. Effect of arsenic–phosphorus interaction on arsenic-induced oxidative stress in chickpea plants. Plant and Soil **314**, 211–220.

Hénault-Ethier L, Smedbol E, Dagenais G, Gomes M, Racine J-F, Juneau P, Lepage L, Labrecque M, Lucotte M. 2014. Les bandes riveraines en agriculture:

Une approche pluridisciplinaire pour une application concrète. Vecteur Environnement, 52–57.

Juneau P, Barnett A, Méléder V, Dupuy C, Lavaud J. 2015. Combined effect of high light and high salinity on the regulation of photosynthesis in three diatom species belonging to the main growth forms of intertidal flat inhabiting microphytobenthos. Journal of Experimental Marine Biology and Ecology **463**, 95–104.

Keoleian GA, Volk TA. 2005. Renewable Energy from Willow Biomass Crops: Life Cycle Energy, Environmental and Economic Performance. Critical Reviews in Plant Sciences **24**, 385–406.

Kuzovkina Y a. YA, Volk T a. 2009. The characterization of willow (*Salix* L.) varieties for use in ecological engineering applications: coordination of structure, function and autecology. Ecological Engineering **35**, 1178–1189.

Labrecque M, Teodorescu TI. 2005. Field performance and biomass production of 12 willow and poplar clones in short-rotation coppice in southern Quebec (Canada). Biomass and Bioenergy **29**, 1–9.

Laitinen P, Rämö S, Siimes K. 2007. Glyphosate translocation from plants to soil – does this constitute a significant proportion of residues in soil? Plant and Soil **300**, 51–60.

Li W-X, Chen T-B, Huang Z-C, Lei M, Liao X-Y. 2006. Effect of arsenic on chloroplast ultrastructure and calcium distribution in arsenic hyperaccumulator *Pteris vittata* L. Chemosphere **62**, 803–9.

Lopes AS. 2004. Fertilizer use by crop in Brazil. FAO, Rome.

Marques TCLLM, Moreira FMS, Siqueira JO. 2000. Crescimento e teor de metais de mudas de espécues arbóreas. Pesquisa Agropecuária Brasileira **35**, 121–132.

Marschner H. 1995. *Mineral nutrition of higher plants*. San Diego: Academic Press.

McWhorter CG, Jordan TN, Wills GD. 1980. Translocation of 14C-glyphosate in soyhean (*Glycine max*) and Johnsongrass (Sorghum halepense). Weed Science **28**, 113–118.

Mocak J, Bonde AM, Mitchell S, Scollary G. 1997. A statistical overview of standard (IUPAC and ACS) and new procedures for determining the limits of

detection and quantification: Application to voltammetric and stripping techniques. Pure and Applied Chemistry **69**, 297–328.

Morin F, Vera V, Nurit F, Tissut M, Marigo G. 1997. Glyphosate uptake in *Catharanthus roseus* cells : Role of a phosphate transporter. Pesticide Biochemistry and Physiology **58**, 13–22.

Mullins G. 2009. *Phosphorus Agriculture & the Environment.*

Nakano Y, Asada K. 1981. Hydrogen Peroxide is Scavenged by Ascorbate-specific Peroxidase in Spinach Chloroplasts. Plant and Cell Physiology **22**, 867–880.

Nomura NS, Hilton HW. 1977. The adsorption and degradation of glyphosate in five Hawaiian sugarcane soils. Weed Research **17**, 113–121.

Perttu KL. 1998. Environmental justification for short-rotation forestry in Sweden. Biomass and Bioenergy **15**, 1–6.

Peruzzo PJ, Porta A a, Ronco AE. 2008. Levels of glyphosate in surface waters, sediments and soils associated with direct sowing soybean cultivation in north pampasic region of Argentina. Environmental pollution **156**, 61–6.

Reddy KN, Rimando AM, Duke SO, Nandula VK. 2008. Aminomethylphosphonic acid accumulation in plant species treated with glyphosate. Journal of agricultural and food chemistry **56**, 2125–30.

Rendenbach C, Yorgan TA, Heckt T, Otto B, Baldauf C, Jeschke A, Streichert T, David JP, Amling M, Schinke T. 2014. Effects of extracellular phosphate on gene expression in Murine osteoblasts. Calcified Tissue International **94**, 474–483.

Rueppel ML, Brightwell BB, Schaefer J, Marvel JT, Agri- M. 1977. Metabolism and degradation of glyphosate in soil and water. Journal of agricultural and food chemistry **25**, 517–528.

Sarruge JR, Haag HP. 1974. *Plant chemical analysis.* Piracicaba: Escola Superior de Agricultura 'Luiz de Queiroz'.

Scribner E, Battaglin W, Gillion R, Meyer M. 2007. *Concentrations of glyphosate, its degradation product, aminomethylphosphonic acid, and glufosinate in ground- and surface-water, rainfall, and soil samples collected in the United States, 2001-06: U.S.*

Sergiev IG, Alexieva VS, Ivanov S, Moskova II, Karanov EN. 2006. The phenylurea cytokinin 4PU-30 protects maize plants against glyphosate action. Pesticide Biochemistry and Physiology **85**, 139–146.

Shetty N, Jørgensen HL, Jensen J, Collinge D, Shetty HS. 2008. Roles of reactive oxygen species in interactions between plants and pathogens. In: Collinge D, Munk L, Cooke BM, eds. Sustainable disease management in a European context SE - 6. Springer Netherlands, 267–280 LA – English.

Siehl DL. 1997. Inhibitors of EPSP synthase, gluthamine synthetase and histidine synthesis. In: Roe RM, Burton JD, Kuhr RJ, eds. Herbicide activity: toxicology, biochemistry, and molecular biology. Amsterdam: IOS, 37–67.

Singh BR, Lal R. 2005. Phosphorus Management in Low-Input Agricultural Systems. In: Sims JT, Sharpley AN, eds. Phosphorus: Agriculture and the Environment. Madison, WI: American Society of Agronomy, Crop Science Society of America, and Soil Science Society of America, 729–759.

Skłodowski M, Kiedrzyńska E, Kiedrzyński M, Urbaniak M, Zielińska KM, Kurowski JK, Zalewski M. 2014. The role of riparian willows in phosphorus accumulation and PCB control for lotic water quality improvement. Ecological Engineering **70**, 1–10.

Smith AE, Aubin AJ. 1993. Degradation of 14C-glyphosate in Saskatchewan soils. Bulletin of environmental contamination and toxicology **50**, 499–505.

Struger J, Thompson D, Staznik B, Martin P, McDaniel T, Marvin C. 2008. Occurrence of glyphosate in surface waters of Southern Ontario. Bulletin of environmental contamination and toxicology **80**, 378–84.

Takahashi S, Murata N. 2008. How do environmental stresses accelerate photoinhibition? Trends in plant science **13**, 178–82.

Takeda T, Miyao K, Tamoi M, Kanaboshi H, Miyasaka H, Shigeoka S. 2003. Molecular characterization of glutathione peroxidase-like protein in halotolerant <i>Chlamydomonas<i/> sp. W80. Physiologia Plantarum **117**, 467–475.

Tesfamariam T, Bott S, Cakmak I, Römheld V, Neumann G. 2009. Glyphosate in the rhizosphere—Role of waiting times and different glyphosate binding forms in soils for phytotoxicity to non-target plants. European Journal of Agronomy **31**, 126–132.

Velikova V, Yordanov I, Edreva A. 2000. Oxidative stress and some antioxidant systems in acid rain-treated bean plants: Protective role of exogenous polyamines. Plant Science **151**, 59–66.

Yajima D, Motani H, Hayakawa M, Sato Y, Sato K, Iwase H. 2009. The relationship between cell membrane damage and lipid peroxidation under the condition of hypoxia-reoxygenation: analysis of the mechanism using antioxidants and electrion transport inhibitors. Cell biochemistry and function **27**, 338–343.

Table 3.1S: Repeated-measures ANOVA for the effects of addition of phosphate (mg l^{-1}) and glyphosate (mg l^{-1}) and time of exposure (days) on total phosphorus (P; mg g DW^{-1}), glyphosate and AMPA concentrations (µg g FW^{-1}) in roots and leaves and on total glyphosate accumulation (µg DW $plant^{-1}$) in willow.

Source of Variation	D.F	Roots	Leaves	Total accum.

D.F. Degrees od freedon * Significant

		P	Gly	AMPA	P	Gly	AMPA	
PO_4^{3-}	2	<0.0001*	<0.0001*	0.0088*	<0.0001*	<0.0001*	<0.0001*	<0.0001*
Gly	3	0.0297*	<0.0001*	<0.0001*	0.3110	<0.0001*	<0.0001*	<0.0001*
PO_4^{3-} x Gly	6	0.4615	0.0005*	0.2926	0.1414	0.0004	0.1292	<0.0001*
Time	2	<0.0001*	<0.0001*	<0.0001*	0.0461*	<0.0001*	0.0218*	<0.0001*
Time x PO_4^{3-}	4	0.2850	<0.0001*	<0.0001*	0.0047*	<0.0001*	<0.001*	<0.0001*
Time x Gly	6	<0.0001*	<0.0001*	<0.0001*	0.0319*	<0.0001*	0.3907	<0.0001*
Time x PO_4^{3-} x Gly	12	0.0701	<0.0001*	<0.0001*	0.0006*	<0.0001*	0.0005*	<0.0001*

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 (Φ'_M), hydrogen peroxide concentrations in leaves (H_2O_2) (µg g FW^{-1}) and ascorbate peroxidase (APX) activity (µmol ascorbate min^{-1} mg^{-1} protein) in roots and leaves.

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

D.F. Degrees of freedom

* Significant

CHAPTER IV

UNDERSTANDING THE INTERCONNECTED EFFECTS OF A GLYPHOSATE-BASED HERBICIDE ON PHOTOSYNTHESIS AND RELATED PHYSIOLOGICAL PROCESSES IN WILLOW PLANTS[4]

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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 (H_2O_2) accumulation. H_2O_2 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 H_2O_2 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*-(phosphonomethyl)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, 1997*a*) 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, 1997*a*). 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; Yanniccari *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, 1997*a*). Moreover, glyphosate can indirectly affect photosynthesis by inhibiting chlorophyll biosynthesis (Fedtke and Duke, 2005), decreasing stomatal conductance (Yanniccari *et al.*, 2012) and inducing nutritional disturbances (Cakmak *et al.*, 2009; Su *et al.*,

2009). 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 parameter[1] in leaves of *Salix miyabeana* (cultivar SX64).

Herbicide	Time	Chl	Carot	PQ	gs	ETR_{max}	I_k	qP	UOF_{rel}	NPQ	Fv/Fm
0	6	204.27	9.06bD	51.33	221.30cA	49.96bA	181.98bA	0.22cA	0.10	1.77bNS	0.77nsA
	24	153.55	8.07bA	48.78	344.00aA	64.23aA	243.58aA	0.35aA	0.08	1.32bA	0.78nsA
	48	152.49	10.51aA	44.57	296.13bA	40.79cA	142.40cA	0.24bA	0.08	2.49aA	0.79nsA
	72	179.11	11.78aA	37.24	354.00aA	59.72aA	263.34aA	0.25bA	0.10	1.58bA	0.79nsA
1.4	6	175.40	12.08aC	42.66	152.41cB	50.98aA	198.98aA	0.23aA	0.18	1.78aNS	0.74aA
	24	115.14	3.96cB	36.56	170.73cB	47.56aB	182.95aB	0.25aB	0.15	0.95bB	0.79aA
	48	79.10	7.50bB	35.47	321.11aB	27.82bB	104.67bB	0.12bB	0.14	1.37bB	0.76aA
	72	118.37	8.62bB	28.42	262.20bB	25.23bC	99.44bB	0.13bB	0.16	1.15bB	0.65bB
2.1	6	134.31	19.63aB	37.81	118.10aC	33.69bB	141.38aB	0.10bB	0.23	1.68aNS	0.76aA
	24	112.60	3.57cB	29.12	159.61bB	40.74aB	161.42aB	0.18aC	0.17	0.97bB	0.78aA
	48	67.20	8.12bB	28.78	173.70bC	19.50cB	70.49bC	0.07cC	0.20	1.15bB	0.72bB
	72	113.72	9.41bB	29.45	167.40bC	20.28cB	74.13bB	0.05cC	0.27	1.25bB	0.59cC
2.7	6	125.22	22.73aA	37.10	151.63bB	32.99aB	104.65bC	0.11bB	0.23	1.36aNS	0.70aB
	24	119.45	5.02bB	29.30	179.73aB	36.76aC	146.64aB	0.17aC	0.25	1.09aB	0.70aB
	48	45.38	6.64bB	29.75	189.63aC	16.76bC	70.75cC	0.05cC	0.26	1.09aB	0.65bC
	72	74.70	8.35bB	25.30	164.66bC	7.80cC	36.19dC	0.01dD	0.31	0.67bB	0.48cD
0		172.35a	9.85a	45.48a	303.35a	53.67a	207.82a	0.26a	0.09d	1.79a	0.78a
1.4		122.00c	8.04b	36.05b	226.61b	37.90b	146.51b	0.18c	0.15c	1.31b	0.73b
2.1		106.96c	10.18a	31.29c	154.70d	28.55c	111.85c	0.10d	0.21b	1.16c	0.71b
2.8		91.19d	10.68a	30.25c	171.41c	23.58d	89.56d	0.09e	0.27a	1.05c	0.63c
	6	159.80a	15.87a	42.23a	160.86c	41.90b	156.74b	0.18b	0.18a	1.65a	0.74b
	24	125.19b	5.16d	35.94b	213.52b	47.32a	183.75a	0.25a	0.16b	1.08b	0.77a
	48	86.04c	8.19bc	34.80b	222.60a	26.22c	97.08d	0.14c	0.17b	1.52a	0.73b
	72	121.47b	9.54b	30.10c	259.61b	28.26c	118.27c	0.12d	0.21a	1.65b	0.63c
F values	Herbicide	40.48***	7.19**	35.07***	218.00***	127.11***	88.35***	487.87***	76.18***	20.99***	50.54***
	Time	29.78***	110.16***	18.10***	80.01***	77.03***	49.80***	341.75***	6.15**	17.26***	54.56***
	Herbicide x Time	2.17ns	20.16***	0.957ns	16.92***	9.23***	11.56***	12.92***	1.87ns	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.

[1]Total chlorophyll and carotenoid contends (μg g^{-1} FW), stomatal conductance (g_s - mmol $m^{-2}s^{-1}$), maximum electron transport rate (ETR_{max} - μmol electrons m^{-2} s^{-1}), minimum saturating irradiance (I_k - μmol photons m^{-2} s^{-1}), photochemical quenching (qP), the relative unquenched fluorescence (UQF_{rel}), 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 H_2O_2 contents and lipid peroxidation

Hydrogen peroxide (H_2O_2) contents were always higher in plants exposed to the herbicide compared to control (Table 4.2; Figure 4.2). Remarkable increase in H_2O_2 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 ($P < 0.001$).

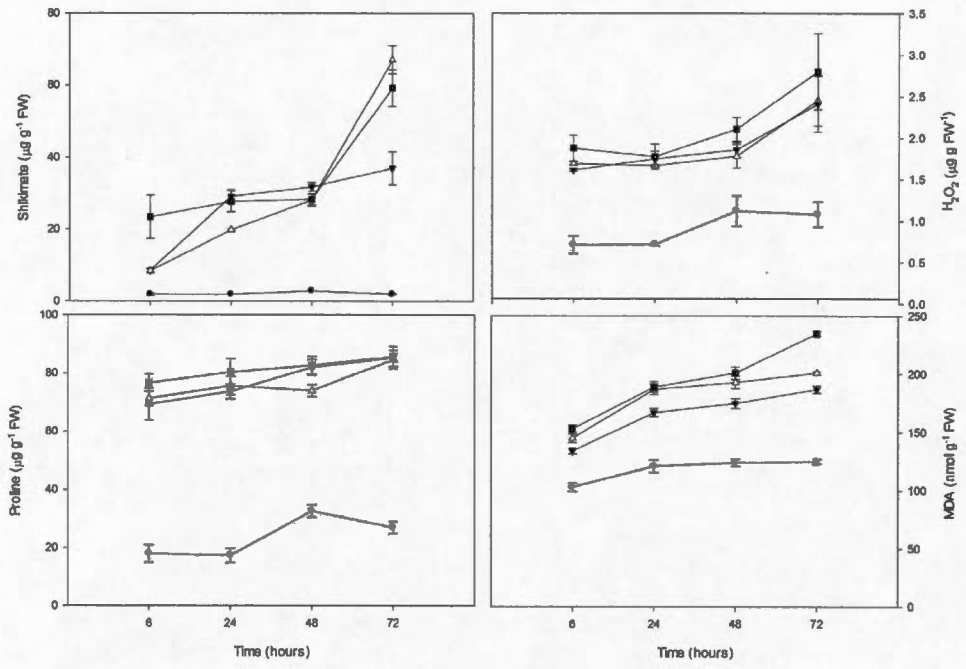

Figure 4.1. Shikimate, proline and hydrogen peroxide contents and lipid peroxidation (MDA contents) in leaves of *Salix miyabeana* (cultivar SX64) plants spread with doses of 0 (filled circle), 1.4 (filled inverted triangle), 2.1 (open triangle) and 2.8 (filled square) kg a.e ha^{-1} of the glyphosate-based 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_2O_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 miyabeana* (cultivar SX64).

Source of variation	Shikimate	Proline	H_2O_2	MDA	SOD	CAT	APX	GPX	GR
Herbicide	368.96*	937.89**	97.42**	1174.59**	2.69ns	93.25**	1.21ns	27.63***	52.02**
Time	277.00**	36.78***	36.22**	616.84***	1527.94**	13.63**	40.60**	228.75**	4.74**
Herbicide x Time	54.24***	4.04**	2.02*	100.92***	204.27***	1.25ns	20.11**	37.58***	4.59***
Scott-Knott multiple range test									
Herbicide (kg a.e ha^{-1})									
0	1.38e	23.75d	0.90c	118.25d	356.58	2.00b	24.50	41.45a	39.66b
1.4	26.58c	76.41b	1.90b	165.75c	363.08	4.47a	26.75	35.16b	55.25a
2.1	30.84b	77.58b	1.89b	181.50b	367.33	4.49a	26.44	35.08b	54.08a
2.8	34.67a	81.33a	2.14a	207.00a	365.33ns	4.41a	25.97ns	32.50c	53.16a
Time (hours)									
6	10.32d	58.83d	1.47b	133.75d	465.58a	3.31c	29.50a	47.66a	53.75a
24	19.36c	61.75c	1.47b	166.00c	452.91b	3.71b	31.75a	41.91b	50.16b
48	22.50b	67.83b	1.71b	173.25b	270.66c	3.90b	23.26c	31.70c	49.41b
72	41.29a	70.66a	2.18a	199.50a	263.58c	4.45a	19.15d	22.91d	48.84b

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 48h 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, 1997*a*) 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 UQFrel 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

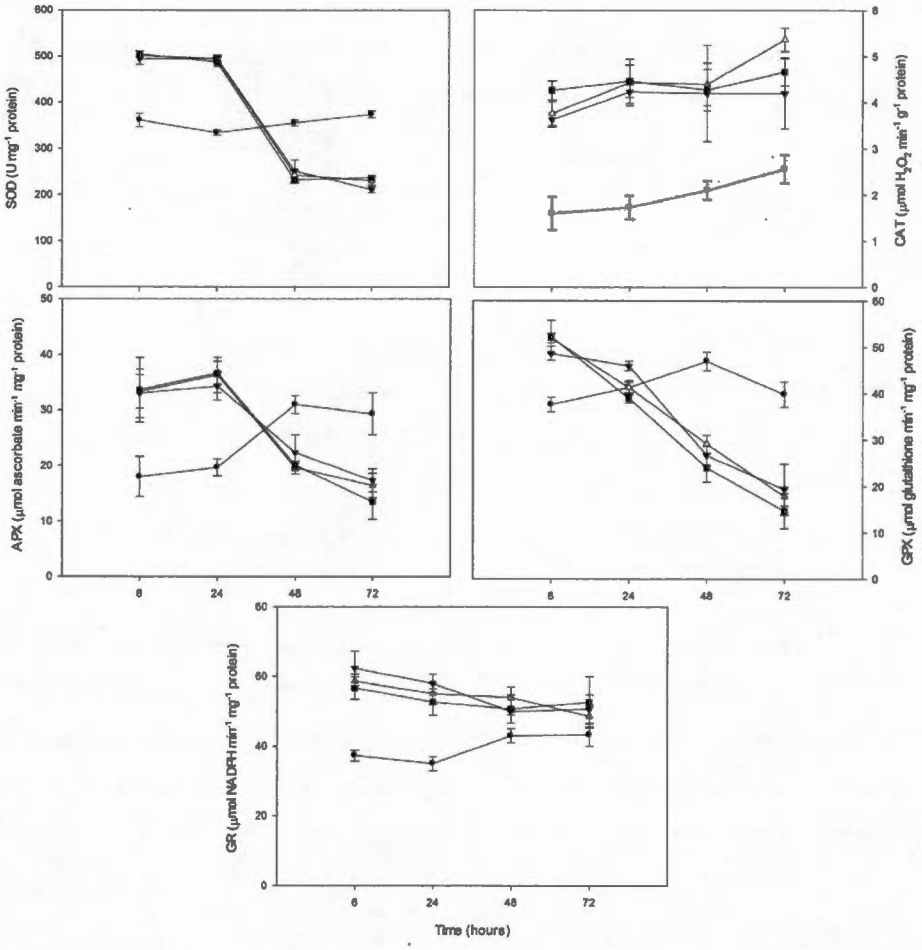

Figure 4.2. Superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), glutathione peroxidase (GPX) and glutathione reductase (GR) activities in leaves of *Salix miyabeana* (cultivar SX64) plants spread with doses of 0 (filled circle), 1.4 (filled inverted triangle), 2.1 (open triangle) 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 $\geq$ 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 H_2O_2 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 (F_V/F_M) 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 ($^1O_2$) 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 F_V/F_M 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 miyabeana* (cultivar SX64).

Herbicide	Time	AsA + DHA	AsA	DHA	Asa/DHA
0	6	975.00dB	524.00bA	451.00cB	1.17aA
	24	1077.00cB	448.00cA	629.00bB	0.71bA
	48	1221.33bA	614.00aNS	607.33bA	1.01aB
	72	1316.66aA	434.66cB	882.00aA	0.49cB
1.4	6	1157.00bA	373.66cB	783.33bA	0.47cB
	24	1221.33aA	282.00dB	939.33aA	0.30cB
	48	949.00cB	590.33aNS	358.66cB	1.65aA
	72	869.00dB	503.66bA	365.33cB	1.38bB
2.1	6	1159.33aA	380.00cB	779.33bA	0.48bB
	24	1198.66aA	315.33dB	883.33aA	0.35bB
	48	945.33bB	593.66aNS	351.66cB	1.70aA
	72	850.66cB	517.33bA	333.33cB	1.56aA
2.7	6	1175.33aA	349.00bC	826.33bA	0.42bB
	24	1201.66aA	298.66cB	903.00aA	0.33bB
	48	931.66bB	573.33aNS	358.33cB	1.73aA
	72	847.33cB	537.66aA	309.66cB	1.61aA
0		1147.50c	505.16a	642.33a	0.84b
1.4		1049.08c	437.41b	611.66b	0.95a
2.1		1038.50b	451.58b	586.91b	1.02a
2.8		1039.00a	439.66b	599.33b	1.02a
	6	1116.66b	406.66c	720.00b	0.64c
	24	1174.66a	336.00d	838.66a	0.42d
	48	1011.83c	592.83a	419.00d	1.49a
	72	970.91d	498.33b	472.58c	1.29b
F values	Herbicide	27.62^{***}	20.75^{***}	5.17^{**}	6.22^{**}
	Time	86.69^{***}	256.16^{***}	359.10^{***}	227.05^{***}
	Herbicide x Time	61.69^{***}	17.67^{***}	105.97^{**}	39.58^{***}

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 ζ–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 β-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_2O_2 content in pea and soybean plants (Moldes *et al.*, 2008; Miteva *et al.*, 2010). Similarly, we observed increased lipid peroxidation and increased H_2O_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_2O_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_2O_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_2O_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 *Loliumm perenne* plants (Olesen and Cedergreen, 2010; Yanniccari *et al.*, 2012). This reduced g_s can negatively affect carbon assimilation by limiting gas exchange (Zobiole *et al.*, 2010*b*), 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_2O_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

proline 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 proline 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 proline can be synthesized from ornithine, metabolic labeling studies indicate that, under stressing conditions, proline is mainly produced from glutamate (as reviewed by Hare & Cress, 1997). Therefore, the proline accumulation found in our study indicates that this pathway is highly activated (Figure 4.3#12). A special function of proline 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-aravino-heptulosonate-7-phosphate synthase) (Crowley, 2006), the herbicide (glyphosate) led to an unregulated flux of carbon into the shikimate pathway (Siehl, 1997*a*). 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).

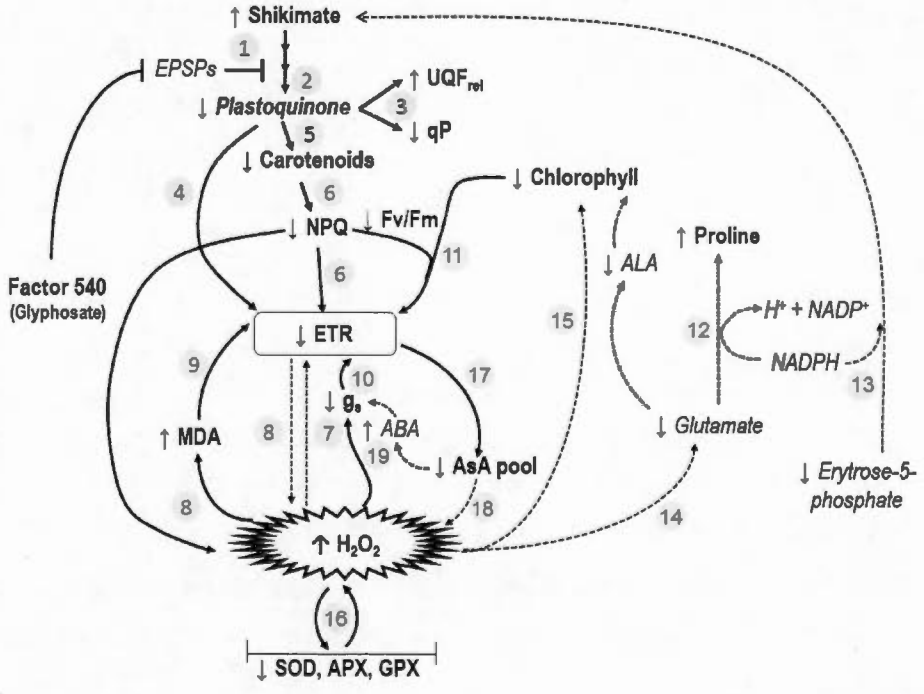

Figure 4.3. Interconnected model of the effects of the glyphosate-based-herbicide (Factor® 540) on photosynthesis and oxidative markers of willow plants. Numbers refer to the ones mentioned in the discussion. ABA, abscisic acid (ABA); ALA, δ-aminolevulinic acid; APX, ascorbate peroxidase; AsA, ascorbate; EPSPS, 5-enolpyruvylshikimate-3-phosphate synthase; ETR, electron transport rate; Fv/Fm, maximal PSII photochemical efficiency; GPX, glutathione peroxidase; g_s, stomatal conductance; H_2O_2, peroxide; I_k, 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-dioxivalerate cycles (Beale, 1978). Therefore, if glumatate 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 H_2O_2 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 photoinibitory 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 H_2O_2 accumulation in willow plants since: 1) decreased activities of both enzymes were related to increased H_2O_2 content in leaves; 2) even if in treated plants we observed higher catalase (CAT) activity, it was not able to prevent H_2O_2 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 H_2O_2 contents and oxidative damages to plants (Gomes *et al.*, 2013b). When H_2O_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 NADP(H)-dependent-GR activity can favor OPPP and contribute as a source of $NADP^+$ 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 g_s (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.*, 2010*b*, 2011*b*, 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, Decadon 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 (qP) (van Kooten and Snel, 1990), the relative unquenched fluorescence (UQF_{rel}) (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 (α), 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 (µg/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 ($NaBH_4$) 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 $NaBH_4$ 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, absoption 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

Ahsan N, Lee D-G, Lee K-W, Alam I, Lee S-H, Bahk JD, Lee B-H. 2008. Glyphosate-induced oxidative stress in rice leaves revealed by proteomic approach. Plant physiology and biochemistry **46**, 1062–70.

Aniya Y, Anders MW. 1992. Activation of rat liver microsomal glutathione *S*-transferase by hydrogen peroxide: Role for protein-dimer formation. Archives of Biochemistry and Biophysics **296**, 611–616.

Arisi A, Cornic G, Jouanin L, Foyer C. 1998. Overexpression of iron superoxide dismutase in transformed poplar modifies the regulation of photosynthesis at low CO2 partional pressures or following exposure to prooxidant methyl viologen. Plant physiology **117**, 565–574.

Bates LS, Waldren RP, Teare I. 1973. Rapid determination of free proline for water stress studies. Plant and Soil **39**, 205–208.

Beale S. 1978. *S*-aminolevulinic acid in plants: Its biosynthesis, regulation, and role in plastid development. Annual Review of Plant Physiology **29**, 95–120.

Bijay KS, Dale LS. 1998. Rapid determination of glyphosate injury to plants and identification of glyphosate-resistant plants. Weed Science Society of America **12**, 527–530.

Bott S, Tesfamariam T, Kania A, Eman B, Aslan N, Römheld V, Neumann G. 2011. Phytotoxicity of glyphosate soil residues re-mobilised by phosphate fertilisation. Plant and Soil **342**, 249–263.

Boussiba S. 2000. Carotenogenesis in the green alga *Haematococcus pluvialis*: Cellular physiology and stress response. Physiologia Plantarum **108**, 111–117.

Bouvier F, Backhaus RA, Camara B, Chem JB. 1998. Induction and control of chromoplast-specific carotenoid genes by oxidative stress induction and control of chromoplast-specific carotenoid genes by oxidative stress. The Journal of biological chemistry **273**, 30651–30659.

Bouvier F, D'Harlingues A, Hugueney P, Marin E, Marion-Poll A, Camara B. 1996. Xanthophyll biosynthesis. Cloning, expression, functional reconstitution, and regulation of beta -cyclohexenyl carotenoid epoxidase from pepper (*Capsicum annuum*). Journal of Biological Chemistry **271**, 28861–28867.

Cakmak I, Yazici A, Tutus Y, Ozturk L. 2009. Glyphosate reduced seed and leaf concentrations of calcium, manganese, magnesium, and iron in non-glyphosate resistant soybean. European Journal of Agronomy **31**, 114–119.

Cerdeira AL, Duke SO. 2006. The current status and environmental impacts of glyphosate-resistant crops: a review. Journal of environmental quality **35**, 1633–58.

Chaoui A, Habib Ghorbal M, El Ferjani E. 1997. Effects of cadmium-zinc interactions on hydroponically grown bean (Phaseolus vulgaris L.). Plant Science **126**, 21–28.

Coupe R, Kalkhoff S, Capel P, Gregoire C. 2012. Factors affecting the fate and transport of glyphosate and AMPA into surface waters of agricultural watersheds in the United States and Europe. Geophysical Research Abstracts **14**, 5877.

Crowley V. 2006. *The isozymes of 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase from Arabidopsis perform differential and overlapping roles in vivo and may be regulated by tyrosine* (V. Crowley, Ed.). Toronto: University of Toronto.

Demming-Adams B, Adams W. 2000. Photosynthesis: Harvesting sunlight safety. Nature **403**, 371–374.

Duke SO, Powles SB. 2008. Glyphosate: a once-in-a-century herbicide. Pest Management Science **64**, 319–325.

Eilers PHC, Peeters JCH. 1988. A model for the relationship between light intensity and the rate of photosynthesis in phytoplankton. Ecological Modelling **42**, 199–215.

Fan L, Vonshak A, Zarka A, Boussiba S. 1998. Does astaxanthin protect Haematococcus against light damage? Zeitschrift fur Naturforschung. C, Journal of biosciences **53**, 93–100.

Fedtke K, Duke S. 2005. Herbicides. In: Hock B, Elstner E, eds. Plant Toxicology. New York: Marcel Dekker, 247–330.

Ferreira D. 2011. Sisvar: a computer statistical analysis system. Ciência e Agrotecnologia **35**, 1039–1042.

Foyer CH, Leiandais M, Kunert KJ. 1994. Photooxidative stress in plants. Physiologia Plantarum **92**, 696–717.

Foyer CH, Noctor G. 2011. Ascorbate and glutathione: the heart of the redox hub. Plant physiology **155**, 2–18.

Gomes MP, Carvalho M, Carvalho GS, Garcia QS, Guilherme LRG, Soares AM. 2013*a*. Phosphorus improves arsenic phytoremediation by *Anadenanthera peregrina* by alleviating induced oxidative stress. International Journal of Phytoremediation **15**, 633–646.

Gomes MP, Duarte DM, Carneiro MMLC, Barreto LC, Carvalho M, Soares AM, Guilherme LRG, Garcia QS. 2013*b*. Zinc tolerance modulation in *Myracrodruon urundeuva* plants. Plant Physiology and Biochemistry **67**, 1–6.

Gomes MMP, Maccario S, Lucotte M, Labrecque M, Juneau P. 2015. Consequences of phosphate application on glyphosate uptake by roots: Impacts for environmental management practices. Science of The Total Environment **537**, 115–119.

Gomes MP, Smedbol E, Carneiro MMLC, Garcia QS, Juneau P. 2014*a*. Reactive Oxygen Species and Plant Hormones. In: Ahmad P, ed. Oxidative Damage to Plants: Antioxidant Networks and Signaling. New York: Academic Press, 65–81.

Gomes MP, Smedbol E, Chalifour A, Hénault-Ethier L, Labrecque M, Lepage L, Lucotte M, Juneau P. 2014*b*. Alteration of plant physiology by glyphosate and its by-product aminomethylphosphonic acid (AMPA), an overview. Journal of Experimental Botany **65**, 4691–703.

Gomes MP, Soares AM, Garcia QS. 2014*c*. Phosphorous and sulfur nutrition modulate antioxidant defenses in *Myracrodruom urundeuva* plants exposed to arsenic. Journal of hazardous materials **276**, 97–104.

Hare PD, Cress WA. 1997. Metabolic implications of stress-induced proline accumulation in plants. Plant Growth Regulation **21**, 79–102.

Hayat S, Hayat Q, Alyemeni MN, Wani AS, Pichtel J, Ahmad A. 2012. Role of proline under changing environments: a review. Plant signaling & behavior **7**, 1456–66.

Huang J, Silva EN, Shen Z, Jiang B, Lu H. 2012. Effects of glyphosate on photosynthesis, chlorophyll fluorescence and physicochemical properties of cogongrass (*Imperata cylindrical* L.). Plant Omics Journal **5**, 177–183.

Inderjit, Kaushik S. 2010. Effect of herbicides with different modes of action on physiological and cellular traits of Anabaena fertilissima. Paddy and Water Environment **8**, 277–282.

Juneau P, Green BR, Harrison PJ. 2005. Simulation of Pulse-Amplitude-Modulated (PAM) fluorescence: Limitations of some PAM-parameters in studying environmental stress effects. Photosynthetica **43**, 75–83.

Kitajima M, Butler WLW. 1975. Quenching of chlorophyll fluorescence and primary photochemistry in chloroplasts by dibromothymoquinone. Biochemica et biophysica acta **376**, 105–115.

Van Kooten O, Snel J. 1990. The use of chlorophyll fluorescence nomenclature in plant stress physiology. Photosynthesis research **25**, 147–150.

Krall JP, Edwards GE. 1992. Relationship between photosystem II activity and CO2 fixation in leaves. Physiologia Plantarum **86**, 180–187.

Kruk J, Karpinski S. 2006. An HPLC-based method of estimation of the total redox state of plastoquinone in chloroplasts, the size of the photochemically active plastoquinone-pool and its redox state in thylakoids of Arabidopsis. Biochimica et Biophysica Acta - Bioenergetics **1757**, 1669–1675.

Labrecque M, Teodorescu TI. 2005. Field performance and biomass production of 12 willow and poplar clones in short-rotation coppice in southern Quebec (Canada). Biomass and Bioenergy **29**, 1–9.

Lee TT. 1981. Effects of glyphosate on synthesis and degradation of chlorophyll in soybean and tobacco cells. Weed Research **21**, 161–164.

Lichtenthaler HK, Wellburn AR. 1983. Determinations of total carotenoids and chlorophylls a and b of leaf extracts in different solvents. Biochemical Society Transactions **11**, 591–592.

Mateos-Naranjo E, Redondo-Gómez S, Cox L, Cornejo J, Figueroa ME. 2009. Effectiveness of glyphosate and imazamox on the control of the invasive cordgrass *Spartina densiflora*. Ecotoxicology and Environmental Safety **72**, 1694–1700.

Maxwell K, Johnson GN. 2000. Chlorophyll fluorescence--a practical guide. Journal of experimental botany **51**, 659–668.

Miteva L-ELP-E, Ivanov S V, Alexieva VS. 2010. Alterations in glutathione pool and some related enzymes in leaves and roots of pea plants treated with the herbicide glyphosate. Russian Journal of Plant Physiology **57**, 131–136 LA – English.

Moldes CA, Medici LO, Abrahão OS, Tsai SM, Azevedo RA. 2008. Biochemical responses of glyphosate resistant and susceptible soybean plants exposed to glyphosate. Acta Physiologiae Plantarum **30**, 469–479.

Olesen CF, Cedergreen N. 2010. Glyphosate uncouples gas exchange and chlorophyll fluorescence. Pest management science **66**, 536–42.

Pompeu GB, Gratão PL, Vitorello VA, Azevedo RA. 2008. Antioxidant isoenzyme responses to nickel-induced stress in tobacco cell suspension culture. Scientia Agricola Agricola **65**, 548–552.

Redondo-Gómez S, Mateos-Naranjo E, Cambrollé J, Luque T, Figueroa ME, Davy AJ. 2008. Carry-over of differential salt tolerance in plants grown from dimorphic seeds of Suaeda splendens. Annals of botany **102**, 103–12.

Richter C. 1992. Reactive oxygen and DNA damage in mitochondria. Mutation Research/DNAging **275**, 249–255.

Sandmann G, Römer S, Fraser PD. 2006. Understanding carotenoid metabolism as a necessity for genetic engineering of crop plants. Metabolic engineering **8**, 291–302.

Serra A-A, Nuttens A, Larvor V, Renault D, Couée I, Sulmon C, Gouesbet G. 2013. Low environmentally relevant levels of bioactive xenobiotics and associated degradation products cause cryptic perturbations of metabolism and molecular stress responses in *Arabidopsis thaliana*. Journal of experimental botany **64**, 2753–66.

Servaites JC, Tucci M a, Geiger DR. 1987. Glyphosate effects on carbon assimilation, ribulose bisphosphate carboxylase activity, and metabolite levels in sugar beet leaves. Plant physiology **85**, 370–4.

Siehl D. 1997. Inhibitors of EPSPS synthase, glutamine synthetase and histidine synthesis. In: Roe R, Burton J, Kuhr R, eds. Herbicide activity: toxicology, biochemistry and molecular biology. Amsterdam: IOS Press, 37–67.

Sohal RS, Mockett RJ, Orr WC. 2002. Mechanisms of aging: an appraisal of the oxidative stress hypothesis. Free Radical Biology & Medicine **33**, 575–586.

Song FN, Yang CP, Liu XM, Liu GB. 2006. Effect of salt stress on activity of superoxide dismutase (SOD) in Ulmus primula L. Journal of Forestry Research **17**, 13–16.

Soshinkova TN, Radyukina NL, Korolkova D V, Nosov A V. 2013. Proline and functioning of the antioxidant system in Thellungiella salsuginea plants and cultured cells subjected to oxidative stress. Russian Journal of Plant Physiology **60**, 41–54.

Su YS, Ozturk L, Cakmak I, Budak H. 2009. Turfgrass species response exposed to increasing rates of glyphosate application. European Journal of Agronomy **31**, 120–125.

Vendrell E, Gómez de Barreda Ferraz D, Sabater C, Carrasco JM. 2009. Glyphosate on growth of four freshwater species of phytoplankton: A microplate bioassay. Bulletin of Environmental Contamination and Toxicology **82**, 538–542.

Vivancos PD, Driscoll SP, Bulman C a, Ying L, Emami K, Treumann A, Mauve C, Noctor G, Foyer CH. 2011. Perturbations of amino acid metabolism associated with glyphosate-dependent inhibition of shikimic acid metabolism affect cellular redox homeostasis and alter the abundance of proteins involved in photosynthesis and photorespiration. Plant physiology **157**, 256–68.

Walter a, Rascher U, Osmond B. 2003. Transitions in photosynthetic parameters of midvein and interveinal regions of leaves and their importance during leaf growth and development. Plant biology (Stuttgart, Germany) **6**, 184–91.

Wang C-Y. 2001. Effect of glyphosate on aromatic amino acid metabolism in purple nutsedge (*Cyperus rotundus*). Weed Technology **15**, 628–635.

Williams GM, Kroes R, Munro IC. 2000. Safety evaluation and risk assessment of the herbicide Roundup and its active ingredient, glyphosate, for humans. Regulatory toxicology and pharmacology: RTP **31**, 117–65.

Yabuta Y, Mieda T, Rapolu M, Nakamura A, Motoki T, Maruta T, Yoshimura K, Ishikawa T, Shigeoka S. 2007. Light regulation of ascorbate biosynthesis is dependent on the photosynthetic electron transport chain but independent of sugars in Arabidopsis. Journal of experimental botany **58**, 2661–71.

Yanniccari M, Tambussi E, Istilart C, Castro AM. 2012. Glyphosate effects on gas exchange and chlorophyll fluorescence responses of two Lolium perenne L. biotypes with differential herbicide sensitivity. Plant Physiology and Biochemistry **57**, 210–217.

Yoshida K, Shibata M, Terashima I, Noguchi K. 2010. Simultaneous determination of in vivo plastoquinone and ubiquinone redox states by HPLC-based analysis. Plant and Cell Physiology **51**, 836–841.

Zobiole LHSS, Kremer RJ, Oliveira Jr. RS, Constantin J, Oliveira RS. 2011. Glyphosate affects chlorophyll, nodulation and nutrient accumulation of 'second generation' glyphosate-resistant soybean (*Glycine max* L.). Pesticide Biochemistry and Physiology **99**, 53–60.

Zobiole LHS, Kremer RJ, de Oliveira Jr. RS, Constantin J. 2012. Glyphosate effects on photosynthesis, nutrient accumulation, and nodulation in glyphosate-resistant soybean. Journal of Plant Nutrition and Soil Science **175**, 319–330.

Zobiole LHS, de Oliveira RS, Kremer RJ, Constantin J, Bonato CM, Muniz AS. 2010. Water use efficiency and photosynthesis of glyphosate-resistant soybean as affected by glyphosate. Pesticide Biochemistry and Physiology **97**, 182–193.

CHAPTER V

DIFFERENTIAL EFFECTS OF GLYPHOSATE AND AMINOMETHYLPHOSPHONIC ACID (AMPA) ON PHOTOSYNTHESIS AND CHLOROPHYLL METABOISM OF WILLOW PLANTS[5]

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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.*, 2003*a*). 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.*, 2003*b*; 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.*, 2014*a*), 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% P_2O_5, 11% K_2O, 1.5% Mg, 1.27% S, 0.07% B, 0.002% Mo, 0.12% Zn) and B (4% N, 1% NH_4^+, 3% NO_3^{-2}, 10% K2O, 2% Ca, 0.05% Fe, 0.05% Mn) (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 (N_2) 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 N_2 flow. Prior to GC-ECD injection, the samples were dissolved in 800 µL of ethyl acetate and 200 µL of pyridine. A Varian GC 3800 gas chromatograph equipped with a Restek RXI-5SIL MS capillary column (30 m 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 ug L^{-1} and 0.06 µg L^{-1} and 0.03 µg L^{-1} and 0.09 µg L^{-1} for glyphosate and AMPA, respectively. Calibration curves of six points showed good linearity for both analytes ($r^2 = 0.96$; $p < 0.0001$ and $r^2 = 0.99$; $p < 0.0001$ for glyphosate and AMPA, respectively) in the domain of expected samples concentration. Each batch of samples included three blanks, five standards and five spiked control samples (roots or leaves for the corresponding plant tissue in evaluation).

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 PSII (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 (H_2O_2), 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.*, 2014*a*). 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 H_2O_2 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 H_2O_2 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.

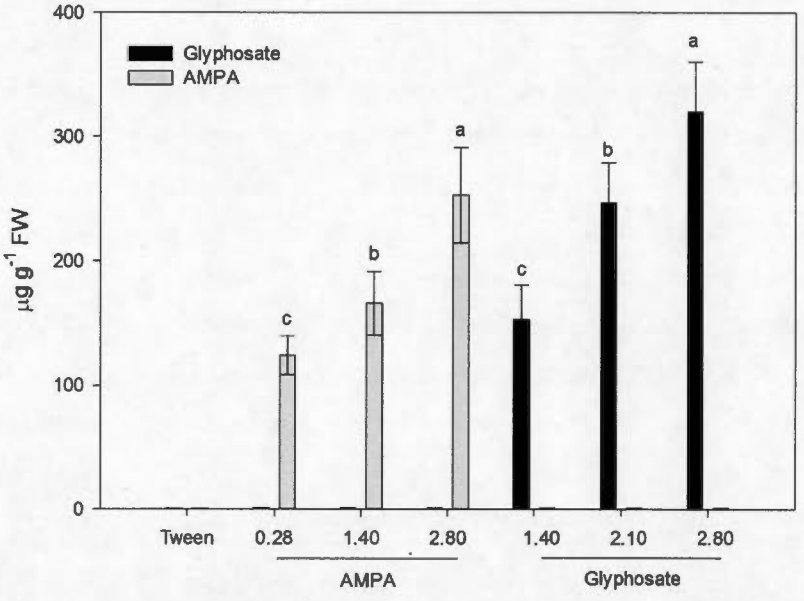

Fig.5.1. Glyphosate and AMPA concentrations in the leaves of willow plants treated with different concentrations of AMPA or glyphosate. Treatment means from ANOVA. Bars indicate the means ± standard errors of three replicates. Means followed by the same letter within the same column are not significantly 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.

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

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.*, 2014*a*) 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 H_2O_2 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 H_2O_2 (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; Rodríguez *et al.*, 2007).

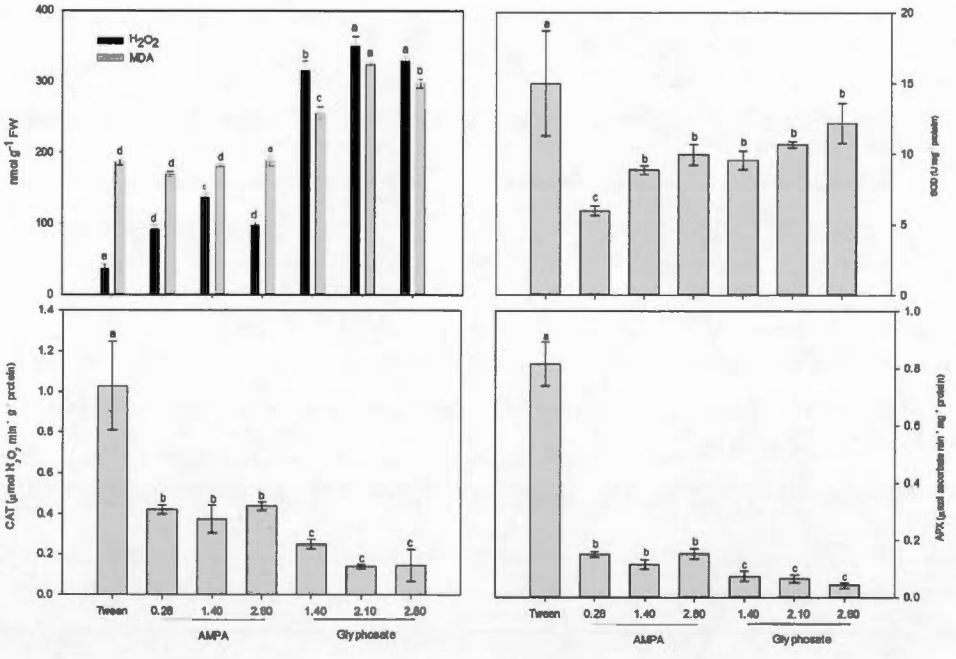

Fig. 5.2. Lipid peroxidation (MDA content), hydrogen peroxide content, and the activities of superoxide dismutase (SOD), catalase (CAT), and ascorbate peroxidase.

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.

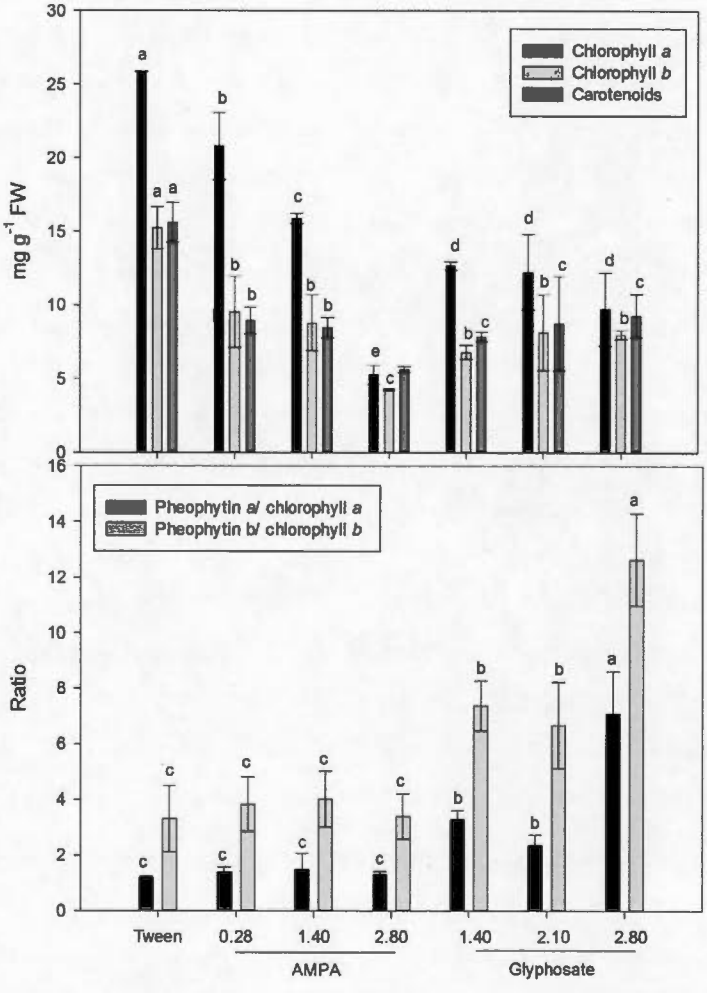

Fig. 5.3. Pigment (Chlorophyll *a*, *b* and carotenoids) contents and the pheophytin/chlorophyll ratios of leaves of willow plants treated with different concentrations of AMPA or glyphosate. Treatment means from ANOVA. Bars indicate the means ± standard errors of three replicates. Means followed by 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_2O_2 contents in leaves treated with glyphosate, with consequent increases in lipid peroxidation ($P < 0.05$; Fig. 5.2). Increased H_2O_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_2O_2 will react with subcellular components which can cause accentuated oxidative damage (Gomes *et al.*, 2014*b*). H_2O_2 is also an important cellular signaling compound, however, and plants should therefore be able to deal with certain H_2O_2 accumulation levels (Gomes *et al.*, 2014*b*). In this context, when the H_2O_2 content is below the threshold of H_2O_2 sensitivity, ROS do not cause oxidative bursts (as observed in AMPA-treated plants); H_2O_2 contents above the H_2O_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_2O_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 ($O_2^{\bullet-}$), 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-sacavenging 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.*, 2014*a*), 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 tetrapyrrole 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

Ahmad P. 2014. *Oxidative damages to plants: Antioxidant networks and signaling* (P Ahmad, Ed.). San Diego: Academic Press.

Ahsan N, Lee D-G, Lee K-W, Alam I, Lee S-H, Bahk JD, Lee B-H. 2008. Glyphosate-induced oxidative stress in rice leaves revealed by proteomic approach. Plant physiology and biochemistry : PPB / Société française de physiologie végétale **46**, 1062–70.

Bailey WA, Poston DH, Wilson HP, Hines TE. 2002. Glyphosate Interactions with Manganese 1. Weed Technology **16**, 792–799.

Bilger W, Björkman O. 1990. Role of the xanthophyll cycle in photoprotection elucidated by measurements of light-induced absorbance changes, fluorescence and photosynthesis in leaves of *Hedera canariensis*. Photosynthesis research **25**, 173–185.

Björkman O, Demmig B. 1987. Characteristics at 77 K among vascular plants of diverse origins *. Planta **170**, 489–504.

Bohne F, Linden H. 2002. Regulation of carotenoid biosynthesis genes in response to light in *Chlamydomonas reinhardtii*. Biochimica et Biophysica Acta **1579**, 26–34.

Börjesson E, Torstensson L. 2000. New methods for determination of glyphosate and (aminomethyl)phosphonic acid in water and soil. Journal of chromatography. A **886**, 207–16.

Boussiba S. 2000. Carotenogenesis in the green alga *Haematococcus pluvialis*: Cellular physiology and stress response. Physiologia Plantarum **108**, 111–117.

Cakmak I, Yazici A, Tutus Y, Ozturk L. 2009. Glyphosate reduced seed and leaf concentrations of calcium, manganese, magnesium, and iron in non-glyphosate resistant soybean. European Journal of Agronomy **31**, 114–119.

Cerdeira AL, Duke SO. 2006. The current status and environmental impacts of glyphosate-resistant crops: a review. Journal of environmental quality **35**, 1633–58.

Cobb AH, Reade JP. 2010. The inhibition of amino acid byosynthesis. In: Cobb AH,, In: Reade JP., eds. Herbicides and plant physiology. Wiley-Blackwell, 176–197.

Corniani N, Velini ED, Silva FML, Nanayakkara NPD, Witschel M, Dayan FE. 2014. Novel bioassay for the discovery of inhibitors of the 2-C-Methyl-D-erythritol 4-Phosphate (MEP) and terpenoid pathways leading to carotenoid biosynthesis. PLoS ONE **9**, e103704.

Demming-Adams B, Adams W. 2000. Photosynthesis: Harvesting sunlight safety. Nature **403**, 371–374.

Dhindsa RS, Plumb-Dhindsa P, Thorpe TA. 1981. Leaf senescence: correlated with increased levels of membrane permeability and lipid peroxidation, and decreased levels of superoxide dismutase and catalase. Journal of Experimental Botany **32**, 93–101.

Ding W, Reddy KN, Zablotowicz RM, Bellaloui N, Arnold Bruns H. 2011. Physiological responses of glyphosate-resistant and glyphosate-sensitive soybean to aminomethylphosphonic acid, a metabolite of glyphosate. Chemosphere **83**, 593–8.

Duke SO, Rimando AM, Pace PF, Reddy KN, Smeda JR. 2003*a*. Isoflavone, glyphosate, and aminomethylphosphonic acid levels in seeds of glyphosate-treated, glyphosate-resistant soybean. Journal of Agricultural and Food Chemistry **51**, 340–344.

Duke SO, Rimando AM, Pace PF, Reddy KN, Smeda JR, Smeda RJ. 2003*b*. Isoflavone, glyphosate, and aminomethylphosphonic acid levels in seeds of glyphosate-treated, glyphosate-resistant soybean. Journal of Agricultural and Food Chemistry **51**, 340–344.

Fedtke K, Duke S. 2005. Herbicides. In: Hock B,, In: Elstner E, eds. Plant Toxicology. New York: Marcel Dekker, 247–330.

Gilmore AM. 1997. Mechanistic aspects of xanthophyll cycle-dependent photoprotection in higher plant chloroplasts and leaves. Physiologia Plantarum **99**.

Gomes MP, Duarte DM, Carneiro MMLC, Barreto LC, Carvalho M, Soares

AM, Guilherme LRG, Garcia QS. 2013. Zinc tolerance modulation in *Myracrodruon urundeuva* plants. Plant Physiology and Biochemistry **67**, 1–6.

Gomes MP, Smedbol E, Chalifour A, Hénault-Ethier L, Labrecque M, Lepage L, Lucotte M, Juneau P. 2014*a*. Alteration of plant physiology by glyphosate and its by-product aminomethylphosphonic acid (AMPA), an overview. Journal of Experimental Botany **65**, 4691–703.

Gomes MP, Soares AM, Garcia QS. 2014*b*. Phosphorous and sulfur nutrition modulate antioxidant defenses in *Myracrodruom urundeuva* plants exposed to arsenic. Journal of hazardous materials **276**, 97–104.

Gonçalves J, Becker A, Cargnelutti D, Tabaldi L, Pereira L, Battisti V, Spanevello R, Morsch V, Nicoloso F, Schetinger M. 2007. Cadmium toxicity causes oxidative stress and induces response of the antioxidant system in cucumber seedlings. Brazilian Journal of Plant Physiology **19**, 223–232.

Goscinny S, Unterluggauer H, Aldrian J, Hanot V, Masselter S. 2012. Determination of glyphosate and its metabolite AMPA (Aminomethylphosphonic Acid) in cereals after derivatization by isotope dilution and UPLC-MS/MS". Food Analytical Methods **5**, 1177–1185.

Gunes A, Inal A, Bagci EG, Coban S, Pilbeam DJ. 2007. Silicon mediates changes to some physiological and enzymatic parameters symptomatic for oxidative stress in spinach (*Spinacia oleracea* L.) grown under B toxicity. Scientia Horticulturae **113**, 113–119.

Harris WR, Sammons RD, Grabiak RC, Mehrsheikh A, Bleeke MS. 2012. Computer simulation of the interactions of glyphosate with metal ions in phloem. Journal of agricultural and food chemistry **60**, 6077–6087.

Huang J, Silva EN, Shen Z, Jiang B, Lu H. 2012. Effects of glyphosate on photosynthesis, chlorophyll fluorescence and physicochemical properties of cogongrass (*Imperata cylindrical* L.). Plant Omics Journal **5**, 177–183.

Kitajima M, Butler WLW. 1975. Quenching of chlorophyll fluorescence and primary photochemistry in chloroplasts by dibromothymoquinone. Biochemica et biophysica acta **376**, 105–115.

Krall JP, Edwards GE. 1992. Relationship between photosystem II activity and CO2 fixation in leaves. Physiologia Plantarum **86**, 180–187.

Krause G, Weis E. 1991. Chlorophyll fluorescence and photosynthesis: The basics. Annual review of plant molecular biology **42**, 313–349.

Labrecque M, Teodorescu TI. 2005. Field performance and biomass production of 12 willow and poplar clones in short-rotation coppice in southern Quebec (Canada). Biomass and Bioenergy **29**, 1–9.

Lichtenthaler HK, Wellburn AR. 1983. Determinations of total carotenoids and chlorophylls a and b of leaf extracts in different solvents. Biochemical Society Transactions **11**, 591–592.

Maroli AS, Nandula VK, Dayan FE, Duke SO, Gerard P, Tharayil N. 2015. Metabolic profiling and enzyme analyses indicate a potential role of antioxidant systems in complementing glyphosate resistance in an Amaranthus palmeri biotype. Journal of Agricultural and Food Chemistry **63**, 9199–9209.

Mateos-Naranjo E, Redondo-Gómez S, Cox L, Cornejo J, Figueroa ME. 2009. Effectiveness of glyphosate and imazamox on the control of the invasive cordgrass *Spartina densiflora*. Ecotoxicology and Environmental Safety **72**, 1694–1700.

Matile P, Stefan H, Thomas H. 1999. Chlorophyll degradation. Annual review of plant molecular biology, 67–95.

Memon SA, Hou X, Wang L, Li Y. 2009. Promotive effect of 5-aminolevulinic acid on chlorophyll, antioxidative enzymes and photosynthesis of pakchoi (*Brassica campestris* ssp. chinensis var. communis Tsen et Lee). Acta Physiologiae Plantarum **31**, 51–57.

Miteva L.-E, Ivanov SV, Alexieva VS. 2010. Alterations in glutathione pool and some related enzymes in leaves and roots of pea plants treated with the herbicide glyphosate. Russian Journal of Plant Physiology **57**, 131–136 LA – English.

Mobin M, Khan NA. 2007. Photosynthetic activity, pigment composition and antioxidative response of two mustard (*Brassica juncea*) cultivars differing in photosynthetic capacity subjected to cadmium stress. Journal of plant physiology **164**, 601–10.

Mocak J, Bonde AM, Mitchell S, Scollary G. 1997. A statistical overview of standard (IUPAC and ACS) and new procedures for determining the limits of detection and quantification: Application to voltammetric and stripping techniques. Pure and Applied Chemistry **69**, 297–328.

Moldes CA, Medici LO, Abrahão OS, Tsai SM, Azevedo RA. 2008. Biochemical responses of glyphosate resistant and susceptible soybean plants exposed to glyphosate. Acta Physiologiae Plantarum **30**, 469–479.

Pitzschke A, Forzani C, Hirt H. 2006. Reactive oxygen species signaling in plants. Antioxidants & Redox Signaling **8**, 1757–1764.

Reddy K, Hoagland R, Zablotowicz R. 2000. Effect of glyphosate on growth, chlorophyll, and nodulation in glyphosate-resistant and susceptible soybean (*Glycine max*) varieties. Journal of New Seeds **2**, 37–52.

Reddy KN, Rimando AM, Duke SO. 2004. Aminomethylphosphonic acid, a metabolite of glyphosate, causes injury in glyphosate-treated, glyphosate-resistant soybean. Journal of agricultural and food chemistry **52**, 5139–43.

Rodríguez MC, Barsanti L, Passarelli V, Evangelista V, Conforti V, Gualtieri P. 2007. Effects of chromium on photosynthetic and photoreceptive apparatus of the alga *Chlamydomonas reinhardtii*. Environmental research **105**, 234–9.

RongFang C, HsiangHua W, ChingYuh W. 2009. Translocation and metabolism of injected glyphosate in lead tree (*Leucaena leucocephala*). Weed Science **57**, 229–234.

Rosolem CA, José G, Andrade M De, Lisboa P, Zoca SM. 2010. Manganese uptake and redistribution in soybean as affected by glyphosate. Revista Brasileira de Ciências do Solo **34**, 1915–1922.

Sandmann G, Römer S, Fraser PD. 2006. Understanding carotenoid metabolism as a necessity for genetic engineering of crop plants. Metabolic engineering **8**, 291–302.

Sergiev IG, Alexieva VS, Ivanov S, Moskova II, Karanov EN. 2006. The phenylurea cytokinin 4PU-30 protects maize plants against glyphosate action. Pesticide Biochemistry and Physiology **85**, 139–146.

Serra A-A, Nuttens A, Larvor V, Renault D, Couée I, Sulmon C, Gouesbet G. 2013. Low environmentally relevant levels of bioactive xenobiotics and associated degradation products cause cryptic perturbations of metabolism and molecular stress responses in *Arabidopsis thaliana*. Journal of experimental botany **64**, 2753–66.

Sharkey TD, Berry JA, Sage RF. 1988. Planta by room-temperature chlorophyll a fluorescence. Planta **176**, 415–424.

Somashekaraiah B V, Padmaja K, Prasad ARK. 1992. Phytotoxicity of cadmium ions on germinating seedlings of mung bean (Phaseolus vulgaris): Involvement of lipid peroxides in chlorphyll degradation. Physiologia Plantarum **85**, 85–89.

Takahashi S, Murata N. 2008. How do environmental stresses accelerate photoinhibition? Trends in plant science **13**, 178–82.

Vernon LEOP. 1960. Spectrophotometric determination of chlorophylls and pheophytins in plant extracts. Analytical Chemistry **32**, 1144–1150.

Welsch R, Beyer P, Hugueney P, Kleinig H, Lintig J Von. 2000. Regulation and activation of phytoene synthase, a key enzyme in carotenoid biosynthesis, during photomorphogenesis. Planta **211**, 846–854.

Williams GM, Kroes R, Munro IC. 2000. Safety evaluation and risk assessment of the herbicide Roundup and its active ingredient, glyphosate, for humans. Regulatory toxicology and pharmacology : RTP **31**, 117–65.

Xu F, Chang J, Cheng S., Zhu J, Li L., Wang Y, Cheng H. 2009. Promotive effect of 5-aminolevulinic acid on the antioxidant system in *Ginkgo biloba* leaves. African Journal of Biotechnology **8**, 3769–3776.

Zobiole LHSS, Kremer RJ, Oliveira Jr. RS, Constantin J, Oliveira RS. 2011. Glyphosate affects chlorophyll, nodulation and nutrient accumulation of 'second generation' glyphosate-resistant soybean (*Glycine max* L.). Pesticide Biochemistry and Physiology **99**, 53–60.

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_2O_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 PO_4^{3-} on glyphosate uptake by roots, and hypothesize that 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 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, 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 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 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 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

Denis M-H, Delrot S. 1993. Carrier-mediated uptake of glyphosate in broad bean (*Vicia faba*) via a phosphate transporter. Physiologia plantarum **87**, 569–575.