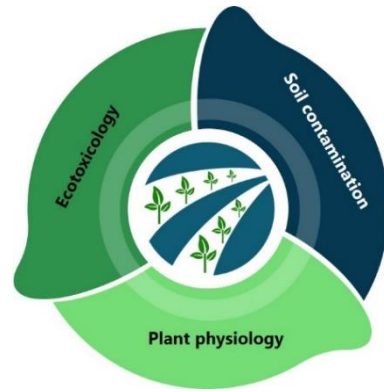




Mitigating glyphosate effects on crop plants and soil functions - strategies to minimise its potential toxicity

Cristiano Fortuna Soares

Doctoral Program in Biology

Biology Department

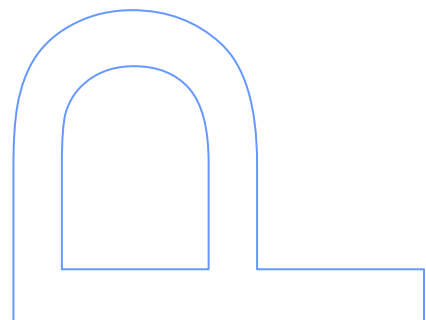
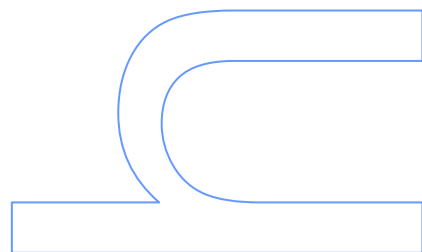
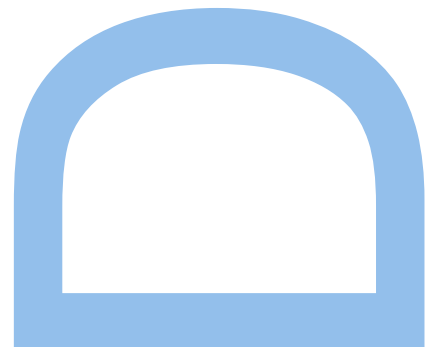
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Supervisor

Fernanda Fidalgo, Associate Professor with Habilitation, FCUP

Co-supervisor

Ruth Pereira, Assistant Professor with Habilitation, FCUP



Brief note

This thesis, presented to the Faculty of Sciences of University of Porto (FCUP) for the obtention of the PhD degree in Biology, was written on the basis of the point two of the Article 4 of the *Regulamento Geral dos Terceiros Ciclos de Estudos da Universidade do Porto*, which was elaborated in agreement with the Article 38 of the Portuguese Law Decree nº 74/2006. Summarising all the experimental work performed by the candidate, this thesis compiles the research articles listed below, which were prepared in collaboration with co-authors. The candidate hereby declares that himself is the main author of all publications, having played a major role in experimental conception and design, laboratorial work, data analysis and writing. The thesis was developed between Plant Stress lab (GreenUPorto/FCUP) and LabRisk (GreenUPorto/FCUP) and was financially supported by *Fundação para a Ciência e a Tecnologia* (FCT) through a doctoral scholarship (SFRH/BD/115643/2016).

List of publications:

1. **Soares, C.**, Carvalho, M. E., Azevedo, R. A., Fidalgo, F., 2019. Plants facing oxidative challenges — A little help from the antioxidant networks. *Environmental and Experimental Botany* 161, 4-25.
2. **Soares, C.**, Pereira, R., Spormann, S., Fidalgo, F., 2019. Is soil contamination by a glyphosate commercial formulation truly harmless to non-target plants? – Evaluation of oxidative damage and antioxidant responses in tomato. *Environmental Pollution* 247, 256-265.
3. Spormann, S., **Soares, C.**, Fidalgo, F., 2019. Salicylic acid alleviates glyphosate-induced oxidative stress in *Hordeum vulgare* L.. *Journal of Environmental Management* 241, 226-234.
4. **Soares, C.**, Pereira, R., Martins, M., Tamagnini, P., Serôdio, J., Moutinho-Pereira, J., Cunha, A., Fidalgo, F., 2020. Glyphosate-dependent effects on photosynthesis of *Solanum lycopersicum* L. — An ecophysiological, ultrastructural and molecular approach. *Journal of Hazardous Materials* 398, 122871.
5. Fernandes, B., **Soares, C.**, Braga, C., Rebotim, A., Ferreira, R., Ferreira, J., Fidalgo, F., Pereira, R., Cachada, A., 2020. Ecotoxicological assessment of a glyphosate-based herbicide in cover plants: *Medicago sativa* L. as a model species. *Applied Sciences* 10(15), 5098.

6. **Soares, C.**, Nadais, P., Sousa, B., Pinto, E., Ferreira, I. M., Pereira, R., Fidalgo, F., 2021. Silicon improves the redox homeostasis to alleviate glyphosate toxicity in tomato plants — Are nanomaterials relevant? *Antioxidants* 10(8), 1320.
7. **Soares, C.**, Rodrigues, F., Sousa, B., Pinto, E., Ferreira, I. M., Pereira, R., Fidalgo, F., 2021. Foliar application of sodium nitroprusside boosts *Solanum lycopersicum* L. tolerance to glyphosate by preventing redox disorders and stimulating herbicide detoxification pathways. *Plants* 10(9), 1862.
8. **Soares, C.**, Mateus, P., Pereira, R., Fidalgo, F., Modulation of the non-target phytotoxicity of glyphosate by soil organic matter in tomato (*Solanum lycopersicum* L.) plants. *Under Review in Environmental Pollution*.
9. **Soares, C.**, Nogueira, V., Fernandes, B., Paiva, C., Cachada, A., Fidalgo, F., Pereira, R., Ecotoxicological relevance of glyphosate and flazasulfuron to soil habitat and retention functions – single vs combined exposures. *Under Review in Science of the Total Environment*.

In all the above-mentioned publications, the host institution supporting the PhD studies of the candidate was listed as:

- GreenUPorto – Sustainable Agrifood Production Research Centre, Biology Department, Faculty of Sciences, University of Porto, Rua Campo Alegre s/n, 4169-007, Porto, Portugal



To my grandpa, the best one out there...

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This one is for you, vú...

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Resumo

Atualmente, o glifosato (GLY) continua a ocupar uma posição de destaque no mercado dos pesticidas, sendo o herbicida mais aplicado a nível mundial. Descrito como um composto de ação pós-emergente, sistémica e não-seletiva, a atividade herbicida do GLY centra-se na inibição da via do chiquimato, uma via metabólica presente exclusivamente em plantas e algumas espécies de microrganismos. Com base no seu modo-de-ação, sempre se assumiu que o GLY não afetaria, substancialmente, organismos não-alvo, com a exceção de espécies vegetais. Além disso, uma vez em contacto com o solo, esperase que o GLY seja rapidamente inativado, quer por adsorção a componentes do solo, quer por degradação microbiana, deixando de representar uma ameaça para o meio ambiente, incluindo plantas não-alvo. No entanto, especialmente no decorrer da última década, as preocupações acerca dos possíveis riscos ambientais do GLY têm aumentado, motivando e reforçando a necessidade de estudos científicos que explorem os impactos deste herbicida em espécies não-alvo. Neste sentido, o presente trabalho pretende avaliar os efeitos da contaminação ambiental por GLY em plantas não-alvo e nas funções do solo, bem como desenvolver estratégias sustentáveis que minimizem os riscos do GLY para espécies de interesse agronómico. De forma a atingir estes dois objetivos principais, seguiu-se uma abordagem multidisciplinar, com estudos que se estendem desde a fisiologia vegetal e bioquímica à ecotoxicologia do solo e análise de risco.

Embora os efeitos por detrás da atividade herbicida do GLY se encontrem bem descritos em plantas alvo (ervas-daninhas), bem como em variedades resistentes e sensíveis (por exemplo, a soja e o milho), os impactos deste agroquímico, enquanto contaminante do solo, no desenvolvimento e crescimento vegetal permanecem por caracterizar. Assim, numa primeira fase, foi realizada uma série de ensaios de exposição para avaliar os mecanismos de toxicidade do GLY em plantas não-alvo, focando não só na sua fitotoxicidade macroscópica, mas também na modulação do estado fisiológico da planta. Os resultados sugeriram que resíduos de GLY [testado a 10, 20 e 30 mg kg⁻¹ em *Solanum lycopersicum* L. (tomateiro) e a 8, 12, 18, 27 e 40 mg kg⁻¹ em *Medicago sativa* L. (alfafa)] no solo afetaram significativamente o crescimento vegetal, tanto ao nível das raízes como da parte aérea, num modo dependente da concentração. Além disso, dados moleculares, bioquímicos e ecofisiológicos demonstraram, inequivocamente, que a exposição de *S. lycopersicum* ao GLY resultou em marcadas alterações fisiológicas, especialmente relacionadas com desequilíbrios redox. De facto, verificou-se a ocorrência de stresse oxidativo em ambos os órgãos, com impactos na produção de espécies reativas de oxigénio (ROS) e na sua neutralização pelo sistema antioxidante (AOX). Estas

alterações surgiram associadas a perdas de viabilidade celular e a danos ultraestruturais no mesófilo foliar, sendo igualmente acompanhadas pela degradação de pigmentos fotossintéticos e pela redução dos níveis de transcritos de genes que codificam importantes proteínas envolvidas no metabolismo fotossintético (D1, CP47 e ribulose-1,5-bisfosfato carboxilase oxigenase – RuBisCO; EC 4.1.1.39). Contudo, em termos de fluxo de carbono (C), não se observaram efeitos significativos quando se analisou o rendimento fotossintético. Neste ponto, a primeira grande questão do presente trabalho foi respondida: a contaminação do solo por GLY, em níveis ecologicamente relevantes, representa um risco acrescido para plantas não-alvo, induzindo alterações bioquímicas e moleculares afetas ao metabolismo oxidativo e fotossintético que se traduzem numa inibição substancial do crescimento vegetal.

Uma vez confirmados e explorados os impactos de resíduos de GLY, procedeu-se ao desenvolvimento e avaliação de estratégias verdes para reduzir o stresse induzido por GLY (10 mg kg^{-1}) em culturas de interesse agronómico. Numa primeira fase, explorou-se, detalhadamente, o potencial de diferentes compostos [silício (Si) e nano-Si a 1 mM ; óxido nítrico (NO) a $200 \text{ }\mu\text{M}$; ácido salicílico (SA) a $100 \text{ }\mu\text{M}$] para reduzir a fitotoxicidade do herbicida. Os efeitos do Si, tanto na sua forma iónica como nanométrica (nano-SiO₂), e do NO foram estudados em *S. lycopersicum*, enquanto que os benefícios do SA foram testados em *Hordeum vulgare* L. (cevada). De uma forma geral, os resultados sugeriram que todos os compostos foram capazes de aliviar, pelo menos parcialmente, os efeitos fitotóxicos do GLY, promovendo o crescimento vegetal. Dado que a homeostasia redox foi substancialmente afetada pelo herbicida, foi dada particular relevância à dinâmica entre a sobreprodução de ROS e a ativação do sistema AOX. Com efeito, em resposta aos co-tratamentos (Si, nano-SiO₂, NO e SA), observou-se uma franca estimulação da resposta AOX, especialmente da componente enzimática, permitindo uma melhor gestão intracelular das ROS (peróxido de hidrogénio – H₂O₂ – e anião superóxido – O₂^{•-}), que assegurou a manutenção da homeostasia redox da célula. Em termos comparativos, de todas as abordagens estudadas, o co-tratamento com Si ou NO, via pulverização foliar, parece ser a estratégia mais promissora de ser implementada em contexto agrícola. Além disso, importa realçar que a aplicação exógena de NO permitiu também reduzir o impacto do GLY a nível da floração e frutificação, processos que se mostraram negativamente afetados pela exposição ao herbicida. Numa segunda fase, e de forma complementar, avaliou-se o papel da matéria orgânica (MO) na redução da biodisponibilidade do GLY no solo. De acordo com o observado, os impactos do GLY (10 mg kg^{-1}) em plantas de tomate ocorreram em menor escala em solos enriquecidos com MO [10 e 15% (m/m)], quando comparados com solos mais pobres [$2,5$ e $5,0\%$ (m/m)]. Com base nos parâmetros bioquímicos e fisiológicos estudados, os níveis mais altos de MO no solo, especialmente 10 e 15% (m/m), resultaram numa diminuição da toxicidade não-alvo do herbicida, quer

pela promoção da sua adsorção, quer pela prevenção de desequilíbrios oxidativos, sem impactos substanciais ao nível da nutrição azotada.

Reconhecendo que a dinâmica dos agroecossistemas em muito depende da interação de diferentes comunidades biológicas, que integram espécies de níveis tróficos distintos, a última componente deste trabalho pretendeu estudar a ecotoxicidade de um herbicida à base de GLY, focando particularmente nas funções de habitat e retenção do solo. No entanto, dado que os agricultores aplicam frequentemente misturas de diferentes herbicidas, os efeitos da co-exposição a resíduos de GLY e de flazassulfurão (FLA; um herbicida da classe das sulfonilureias, comumente aplicado em conjunto com o GLY) foram também avaliados em plantas não-alvo (*M. sativa*) e em oligoquetas (*Eisenia fetida* Savigny). De uma maneira geral, concentrações crescentes de GLY (6, 9, 13, 20 and 30 mg kg⁻¹), assim como elutriados preparados a partir de solos contaminados, não representaram um risco acrescido para organismos não-alvo [*E. fetida*, *Folsomia candida* Willem, *Lemna minor* L., *Raphidocelis subcapitata* (Korshikov) Nygaard et al.], inibindo apenas a capacidade reprodutiva de oligoquetas a níveis relativamente elevados (≥ 13 mg kg⁻¹). No que diz respeito aos ensaios de co-exposição, registou-se uma prevalência dos impactos individuais do FLA, onde se observaram efeitos a concentrações mais baixas (82, 122, 184, 275, 413 μ g kg⁻¹) para os organismos estudados. Tais observações reforçam que a análise de risco de compostos individuais pode subestimar os efeitos esperados em condições reais, onde aplicações sucessivas e cumulativas de vários ingredientes ativos (a.i.) são realizadas.

Sob uma perspetiva holística, a investigação que integra esta tese permitiu obter uma visão clara e robusta acerca das consequências da contaminação do solo por GLY para espécies de plantas não-alvo e para as funções do solo, seguindo metodologias ecologicamente relevantes. Mais ainda, para além de identificar os principais mecanismos responsáveis pela fitotoxicidade do GLY, conseguiu gerar-se conhecimento prático de como a ecotoxicidade deste herbicida pode ser reduzida. No futuro, de modo a validar as estratégias propostas em condições reais, deverão ser realizados ensaios de campo utilizando solos agrícolas contaminados.

Palavras-chave

Análise de risco; contaminação dos solos; ecotoxicologia; fisiologia vegetal; plantas não-alvo; stresse oxidativo; toxicidade por herbicidas.

Abstract

Nowadays, glyphosate (GLY) occupies a leading position in the pesticide market, being the most used herbicide worldwide. Described as a non-selective, systemic, and post-emergence herbicide, GLY primarily acts by inhibiting the shikimate pathway, a metabolic chain exclusively found in plants and some microorganisms. Based on its mode-of-action, GLY has always been considered to be narrowly toxic to non-target organisms other than plants. Moreover, it has also been claimed that, when in contact with the soil, GLY is promptly inactivated, either by adsorption or microbial degradation, not posing a threat to the surrounding environment, including non-target plants. However, especially over the last decade, further concerns about the possible environmental hazards of GLY have been raised, urging the need of additional studies dealing with soil contamination by GLY and its impacts in non-target biota. In this way, the present work aimed to assess the effects of GLY contamination on plants and soil quality, as well as to develop eco-friendly strategies to minimise its risks towards crops. In order to achieve these main goals, a multi-disciplinary approach was designed, with studies ranging from plant physiology and biochemistry to soil ecotoxicology and risk assessment.

Although the general effects behind GLY herbicidal activity are well described in target plants (*i.e.* weeds), and in sensitive and resistant varieties (such as soybean and maize), not much is known concerning the impacts of this agrochemical, as a soil contaminant, on non-target plant growth and development. Thus, at the beginning, a set of single exposure experiments was carried out to evaluate GLY's toxicity mechanisms in non-target plants, focusing not only on its macroscopic phytotoxicity, but also on the modulation of the plant's physiological status. Results suggested that soil residues of GLY [tested at 10, 20 and 30 mg kg⁻¹ in *Solanum lycopersicum* L. (tomato) and at 8, 12, 18, 27 and 40 mg kg⁻¹ in *Medicago sativa* L. (alfafa)] greatly hampered plant growth performance, in both shoots and roots, in a concentration-dependent manner. Moreover, molecular, biochemical and ecophysiological data clearly showed that *S. lycopersicum*'s exposure to GLY resulted in marked alterations in plant physiology, most of them related to redox imbalances. With effect, shoots and roots of GLY-exposed tomato plants underwent a state of oxidative stress, impacting reactive oxygen species (ROS) production and affecting their neutralization by the plant antioxidant (AOX) system. These alterations were linked to decreases of cell viability and leaf ultrastructure damage, this being followed by pigment losses and downregulation of genes encoding important photosynthetic proteins (D1, CP47 and ribulose-1,5-bisphosphate carboxylase-oxygenase – RuBisCO; EC 4.1.1.39). However, from a carbon (C) flux perspective, no major consequences were observed

when analysing the photosynthetic yield. At this moment, the first major question underlying this thesis was answered: soil contamination by GLY, at environmentally relevant levels, is a serious threat for non-target plants, inducing a series of biochemical and molecular disturbances related to the oxidative and photosynthetic metabolism, which further translates into a strong inhibition of plant growth.

Once the knowledge around GLY impacts towards non-target plants was obtained, focus was shifted to the development and implementation of green strategies to reduce GLY-induced (10 mg kg^{-1}) stress in crops. Here, two complementary approaches were followed. First, the potential of different compounds [1 mM silicon (Si) and nano-Si; $200 \text{ }\mu\text{M}$ nitric oxide (NO); $100 \text{ }\mu\text{M}$ salicylic acid (SA)] to alleviate GLY-mediated impacts on plant growth and physiology was tested. The effects of Si, either as bulk or nanomaterial (nano-SiO₂), and NO were investigated in *S. lycopersicum*, while the benefits of SA against GLY toxicity were studied in *Hordeum vulgare* L. (barley). Altogether, the results pointed towards the alleviation of GLY phytotoxic symptoms, at least partially, by all tested compounds, promoting a higher growth of both shoots and roots. Since the redox homeostasis was strongly affected by GLY, particular attention was drawn to the interplay between ROS and the AOX system. With effect, in response to the co-treatments (Si, nano-SiO₂, NO and SA), a much more prominent AOX response was observed, especially in what concerns the enzymatic component, which helped to keep ROS (hydrogen peroxide – H₂O₂ – and superoxide anion – O₂^{•-}) under control, thereby ensuring the maintenance of cellular redox homeostasis. When comparing the tested approaches, the co-treatment with bulk Si or NO, via foliar spraying, seemed to be the most promising strategy to be implemented in a real agricultural scenario. In addition, GLY-mediated impairment of flowering and fruit set was also partially counteracted by the foliar application of NO, reinforcing its effective potential. In complement, the role of organic matter (OM) in limiting GLY bioavailability in soils was also evaluated. From what could be observed, GLY-mediated impacts (10 mg kg^{-1}) in tomato plants were reduced in OM-enriched soils [10 and 15% (m/m)], when compared to soils with lower contents [2.5 and 5.0% (m/m)]. Based on the collected findings, the high levels of OM in the soil, especially 10 and 15% (m/m), were effective in limiting GLY phytotoxicity either by promoting its adsorption and/or by preventing redox disorders, with no major impacts in the nitrogen (N) metabolism.

Recognizing that agroecosystems' health and dynamics depend on the interaction between different biological communities, integrating species of different trophic levels, the last chapter of this thesis sought to evaluate the ecotoxicological relevance of GLY-based herbicides, mainly focusing on soil habitat and retention functions. However, as farmers often apply mixtures of different herbicides, the impacts of a co-exposure to residues of

GLY and flazasulfuron (FLA; a sulfonylurea herbicide commonly applied together with GLY) towards non-target plants (*M. sativa*) and soil oligochaetes (*Eisenia fetida* Savigny) were also assessed. In general, increased concentrations of GLY (6, 9, 13, 20 and 30 mg kg⁻¹), as well as soil elutriates prepared from contaminated soils, did not present a major risk towards non-target organisms [*E. fetida*, *Folsomia candida* Willem, *Lemna minor* L., *Raphidocelis subcapitata* (Korshikov) Nygaard et al.], only impairing earthworms' reproduction at relatively high levels (≥ 13 mg kg⁻¹). Regarding the co-exposure tests, plant growth and oligochaetes reproduction were majorly affected, with a prevalence of FLA single impacts, where significant effects were observed at low concentrations (82, 122, 184, 275, 413 μ g kg⁻¹) for all studied species. Such findings confirm that the risk assessment of individual compounds can be uninformative about the expected effects in real situations, in which successive and cumulative applications of several active ingredients (a.i.) are usually carried out.

From a holistic perspective, the research encompassing this thesis allowed to achieve a clear and robust insight into the main consequences of soil contamination by GLY for non-target plants and soil functions, using environmentally relevant methodologies. Also, besides unravelling the main mechanisms behind GLY toxicity, practical knowledge on how its ecotoxicity can be reduced was gathered. In the future, field-scaled studies, using natural contaminated soils, should be performed in order to validate the proposed strategies under real conditions.

Keywords

Ecotoxicology; herbicide toxicity; non-target plants; oxidative stress; plant physiology; risk assessment; soil contamination.

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Abbreviations, acronyms and symbols

$\cdot\text{OH}$ – hydroxyl radical	CaMV – cauliflower mosaic virus
$^1\text{O}_2$ – singlet oxygen	Car – carotenoids
2,4-D – 2,4-dichlorophenoxyacetic acid	CAT – catalase
a.i. – active ingredient	Cd – cadmium
ABA – abscisic acid	CDNB – 1-chloro,2,4-dinitrobenzene
Abs – absorbance	CH₃ – methyl
ADP – adenosine diphosphate	CH₃CO₂K – potassium acetate
AL – actinic light	Chl – chlorophyll
Al – aluminium	C_i – intercellular CO ₂ concentration
ALA – δ-aminolevulinic acid	CL – confidence limits
AlCl₃ – aluminium chloride	CN⁻ – cyanide
ALS – acetolactase synthase	CO₂ – carbon dioxide
AMPA – aminomethyl phosphonic acid	Cr – chromium
ANOVA – analysis of variance	CS – chorismate synthase
AOX – antioxidant	CTL – control
APAO – acetylated polyamine oxidase	Cu – copper
APX – ascorbate peroxidase	CYP450 – cytochrome P450
AsA – ascorbate	Cys – cysteine
AsA-GSH – ascorbate-glutathione	DAHPS – 3-deoxy-D-arabino-heptulosonate-7-phosphate-synthase
ATP – adenosine triphosphate	DAO – diamine oxidase
B – boron	DDT – dichlorodiphenyl trichloroethane
C – carbon	dH₂O – deionised water
Ca – calcium	DHA – dehydroascorbate
CaCO₃ – calcium carbonate	DHAR – dehydroascorbate reductase
Cad – cadaverine	

DHQ/SDH – dehydroquinate dehydratase-shikimate dehydrogenase

DHQS – 3- dehydroquinate synthase

dm (d.m.) – dry mass

DT₅₀ – half-life

DTNB – 5,5'-dithiobis-(2-nitrobenzoic acid)

DTT - dithiothreitol

dw (d.w.) – dry weight

E – transpiration rate

E4P – erythrose 4-phosphate

EC₅₀ – half maximal effective concentration

EDTA - ethylenediaminetetraacetic acid

EFSA – European Food Safety Authority

EPA – Environmental Protection Agency

EPSP – 5-enolpyruvylshikimate-3 phosphate

EPSPS – 5-enolpyruvylshikimate-3 phosphate synthase

ETC – electron transport chain

ETR – electron transport rate

EU – European Union

F₀ - minimal fluorescence

FAD – flavin adenine dinucleotide

FAO – Food and Agriculture Organization

Fe – iron

Fe³⁺ – ferric ion

FISH – fluorescence *in situ* hybridization

FLA – flazasulfuron

F_m - maximal fluorescence yield

fm (f.m.) – fresh mass

Fmoc - 9-Fluorenylmethoxycarbonyl

Fmoc-Cl - 9-Fluorenylmethoxycarbonyl chloride

F_v/F_m - maximum quantum yield of PSII

fw (f.w.) – fresh weight

GBH – glyphosate-based herbicides

GLY – glyphosate

GPOX – guaiacol peroxidase

GPX – glutathione peroxidase

GR – glutathione reductase

GRes – glyphosate-resistant

GS – glutamine synthetase

g_s – stomatal conductance

GSH – glutathione

GSSG – oxidised glutathione

GST – glutathione S-transferase

H – hydrogen

H₂O₂ – hydrogen peroxide

H₂S – hydrogen sulphide

H₂SO₄ – sulphuric acid

HCl – hydrochloric acid

Hg – mercury

HO₂[•] - hydroperoxyl radical

HS – Hoagland solution

HSM – halosulfuron-methyl

HSP – heat shock proteins

IAA – indole-3-acetic acid

IARC – International Agency for Research on Cancer

IC₅₀ – half maximal inhibitory concentration

IRGA – infrared-gas analyses

IUPAC – International Union of Pure and Applied Chemistry

K – potassium

KCN – potassium cyanide

KI – potassium iodide

LC-MS/MS – liquid chromatography with *tandem* mass spectrometry

LEA – late embryogenesis abundant

LOX – lipoxygenase

LP – lipid peroxidation

MDA – malondialdehyde

MDHA – monodehydroascorbate

MDHAR – monodehydroascorbate reductase

MeJa – methyl jasmonate

Met – methionine

Met-R-SO – methionine-R-sulfoxides

Met-S-SO – methionine-S-sulfoxides

Mg – magnesium

MgCl₂ – magnesium chloride

Mn – manganese

MS – Murashige and Skoog

N – nitrogen

N₂ – molecular nitrogen

Na – sodium

Na₂CO₃ – sodium carbonate

Na₂SiO₃·5H₂O – sodium metasilicate pentahydrate

NaCl – sodium chloride

NAD – nicotinamide adenine dinucleotide

NADPH – nicotinamide adenine dinucleotide phosphate

NaN₃ – sodium azide

NBT – nitroblue tetrazolium

NH₂ – amine

NH₄⁺ – ammonium

NH₄OH – ammonium hydroxide

Ni – nickel

NiO – nickel oxide

NM – nanomaterial

NO – nitric oxide

NOEC – No Observed Effect Concentration

NPQ – non-photochemical quenching

NR – nitrate reductase

O₂ – molecular oxygen

O₂⁻ – superoxide anion

OECD – Organization for Economic Co-operation and Development

OM – organic matter

ONOO⁻ – peroxynitrite

OsO₄ – osmium tetroxide

P – phosphorous

P5C – Δ¹-pyrroline-5-carboxylate

P5CR – Δ¹-pyrroline-5-carboxylate reductase

P5CS – Δ¹-pyrroline-5-carboxylate synthetase

PAM – pulse amplitude modulated fluorometry

PAO – polyamine oxidase	ROS – reactive oxygen species
PAR – photosynthetically active radiation	RT – room temperature
PAs – polyamines	RuBisCO – ribulose-1,5-bisphosphate carboxylase oxygenase
PCA – principal component analysis	S – sulphur
PCD – programmed cell death	S3P – shikimic acid-3-phosphate
PEP – phosphoenolpyruvate	SA – salicylic acid
PFA – paraformaldehyde	SD – standard deviation
PG – plastoglobuli	SDOS-PAGE - sodium dodecyl sulphate–polyacrylamide gel electrophoresis
PK – potassium phosphate	SDS – sodium dodecyl sulphate
PMSF – phenylmethylsulfonyl fluoride	SEM – standard error of the mean
P_N – net CO ₂ assimilation rate	Si – silicon
PO₄³⁻ – phosphate	SiO₂ – silicon dioxide
POD – peroxidases	SK – shikimate kinase
POEA - polyethoxylated amine	SLA – specific leaf area
PPFD – photosynthetic photon flux density	SN – supernatant
PPPs – plant protection products	SNP – sodium nitroprusside
Pro – proline	SOD – superoxide dismutase
PS – photosystem	Spd – spermidine
PsbO – oxygen evolution protein	Spm – spermine
PTFE – polytetrafluoroethylene	SpmO – spermine oxidase
PUFAs – polyunsaturated fatty acids	TAC – total antioxidant capacity
Put – putrescine	Tau – taurine
qPCR – quantitative polymerase chain reaction	TBA – thiobarbituric acid
QTL – quantitative trait loci	TCA – trichloroacetic acid
RBOH - NADPH oxidase	TEM – transmission electron microscopy
rETR – relative electron transport rate	TiSO₄ – titanium sulphate
RNS – reactive nitrogen species	TMV - tobacco mosaic virus

TNB – 2-nitrobenzoic acid

WUE_i – water use efficiency

TPC – total phenol content

XOD – xanthine oxidase

US – United States

Zn – zinc

UV – ultraviolet

ϵ – molar extinction coefficient

WHC – water holding capacity

Φ PSII – effective quantum yield of PSII

WT – wild-type

Thesis structure and layout

The present thesis was structured and organised into different chapters, each one targeting a specific objective. A systematic representation of the thesis layout can be observed in Figure 1. Aiming at providing a holistic contextualization of the main topics of this work, a General Introduction (Chapter I) was prepared, focusing on the state-of-the-art of GLY and its potential risks to agroecosystems. Furthermore, a literature review covering the main topics around the regulation of the redox homeostasis in plants under adverse growth conditions, such as the case of soil contamination, was carried out and is presented in Chapter II. The main objectives and biological questions of the thesis are detailed described in Chapter III. Afterwards, Chapter IV encompasses the experimental work performed to understand GLY-mediated risks to several non-target plant species, including the model crop *Solanum lycopersicum* L. and the cover plant, *Medicago sativa* L. Upon gathering this knowledge, focus was specifically paid to the development and implementation of eco-friendly tools to reduce GLY-induced stress, either by the application of different phytoprotective compounds [such as silicon (Si), nitric oxide (NO), and salicylic acid (SA)] or by modulating the organic matter (OM) content of the soil (Chapter V). For recognizing that, when evaluating herbicides' environmental safety, attention must be driven to the whole ecosystem, and not only to a single fraction, the last component of the experimental work underlying this thesis sought to unravel the ecotoxicological relevance of GLY-based herbicides, at environmental relevant concentrations, towards soil habitat and retention functions, by evaluating their effects on soil organisms and aquatic species (Chapter VI). Lastly, intending on summing up the main outputs of the various tasks and some exciting perspectives, a section dedicated to the General Conclusions (Chapter VII) was prepared.

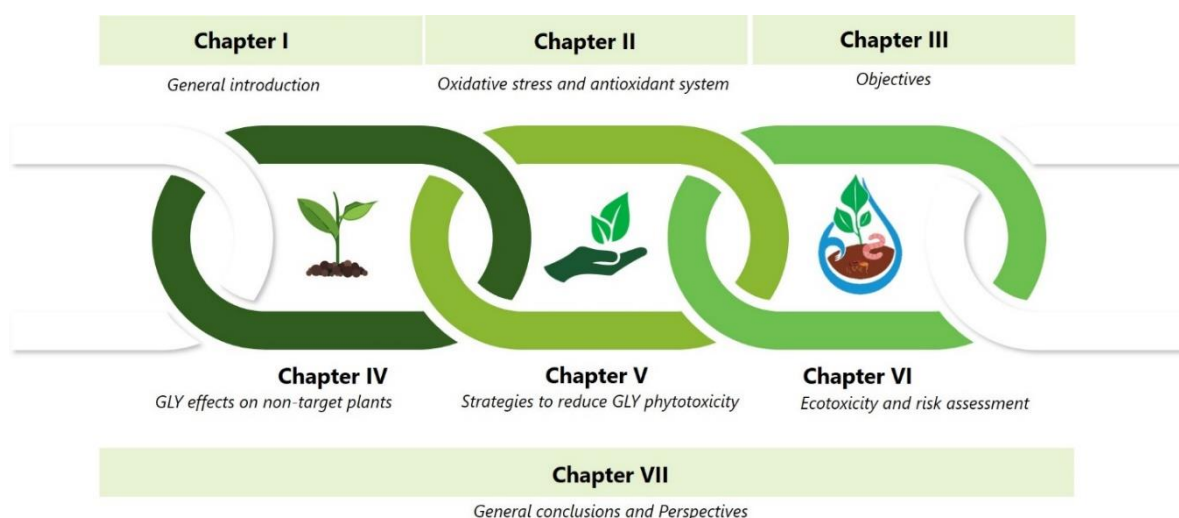


Figure 1. Graphic representation of the structure and layout of the present thesis.

CHAPTER I. GENERAL INTRODUCTION

General Introduction

“The development of agriculture about 12 000 years ago changed the way humans lived. They switched from nomadic hunter-gatherer lifestyles to permanent settlements and farming.” – the lines of this recent *National Geographic* publication (<https://www.nationalgeographic.org/article/development-agriculture/>) perfectly portray the importance that agriculture has always assumed in the development of humans and of human societies over time. Since millennia ago, agricultural products are the main source of food for both human and animal feeding, by the cultivation of cereal, horticultural and fruit crops (Tudi et al., 2021). Given the obvious dependence of agriculture towards the environment, especially in what concerns climatic conditions, water supply, and land quality, changes in abiotic and biotic factors can quickly affect crop growth and development, impacting the global food production (Del Buono, 2021). This aspect gains particular relevance nowadays, with the effects of climate change and environmental degradation becoming increasingly challenging and pushing agriculture outside its boundaries to achieve high productivity rates (Aguilera et al., 2020). Yet, in order to ensure a proper food supply for a growing population – which will, according to recent projections, reach the mark of 9.8 billion people by 2050 (<https://www.un.org/development/desa/en/news/population/world-population-prospects-2017.html>), – agricultural systems must be improved. Encompassing the perspective of the Food and Agriculture Organization (FAO) on *How to Feed the World by 2050* (http://www.fao.org/fileadmin/templates/wsfs/docs/Issues_papers/HLEF2050_Global_Agriculture.pdf), during the current century agriculture will endure unprecedented challenges, namely i) the rising global demand for food goods, ii) the ability to adapt and overcome climate change-related threats, iii) the decrease of soil quality and scarcity of natural resources (e.g. water availability), and iv) the need to respond to environmental policies and to demanding markets requesting more sustainable and healthier food products (Hasan et al., 2021; Hasanuzzaman et al., 2020; MacLaren et al., 2020). Indeed, especially since the last half of the 20th century, modern agricultural practices have become progressively dependent on the application of phytopharmaceutical products, where fertilisers and pesticides are included (Lykogianni et al., 2021). The importance of these agrochemicals towards agriculture should not – and cannot – be ignored. As a result of different pests and diseases, whose occurrence is expected to increase in frequency and intensity in the next decades (Das et al., 2016; Raza et al., 2019), crop productivity losses ascending to 70% could be anticipated if no pesticides were applied (reviewed by Tudi et al., 2021). In spite of that, a heated debate around the negative impacts of these chemicals on terrestrial and aquatic ecosystems, which has emerged in the 80s of the last

century, keeps in the loop, since the non-target toxicity of most of these compounds has not yet been fully unravelled.

1. Pesticides – history, market, and current trends

Pesticides are chemical substances, either natural or synthetic, designed to protect crops from pests and diseases (Mandal et al., 2020). According to the group of target species, they include a variety of classes, namely herbicides, insecticides, fungicides, rodenticides, bactericides, among others (Sharma et al., 2019b). Although historically the use of pesticides dates back several thousand years ago, before the development of synthetic agrochemicals, only natural tools were available to control the devastating effects of pests and disease vectors (Tudi et al., 2021). In those times, people relied on animal- and plant-based compounds to control several pests, especially insects and mites. For instance, pyrethrum – a natural compound extracted from dried flowers of *Chrysanthemum cinerariifolium* L. (chrysanthemum) – was used as a natural insecticide for more than 2000 years (Bernardes et al., 2015). Also, based on different reports, the use of inorganic compounds, such as copper sulphate and lime arsenic, on an industrial scale became particularly eminent during the 19th century, given their long-proved efficiency against fungi (reviewed by Tudi et al., 2021). Common examples, which are still in use today, are the *Bordeaux mixture* (copper sulphate and calcium hydroxide) (Lamichhane et al., 2018) and the *Paris Green* [copper (II) acetoarsenite] (Bencko and Foong, 2017). In spite of that, the pesticide industry started to arise only after the 40s of the 1900s, with the first synthetic pesticides being developed during *World War II* (Bernardes et al., 2015; Tudi et al., 2021). The first of its own was probably dichlorodiphenyltrichloroethane (DDT), a compound formulated in the 1800s, but only characterised as an insecticide in 1939 by Hermann Müller, the famous Nobel Prize winner in Physiology or Medicine in 1946. Initially, it was used for non-agricultural purposes, namely for eradicating the vectors of typhus, yellow fever, and malaria (Gomes et al., 2020), even being applied in clothes to prevent insect damage. In 1946, however, a report published in *Nature* by Shaw (1946) described for the first time “some uses” of this pesticide in agriculture, whose use lasted until the 70s/80s of the 20th century, when the Environmental Protection Agency (EPA) of the United States advised against its utilisation (<https://www.epa.gov/ingredients-used-pesticide-products/ddt-brief-history-and-status>). Within this aspect, the widely recognised book “*Silent Spring*” by Rachel Carson is worth mentioning, given its impact on public awareness of DDT’s potential hazards. Besides DDT, during the 40s of the 20th century, the synthetic auxin analogue 2,4-dichlorophenoxyacetic acid (2,4-D) was manufactured in the United Kingdom (UK) during *World War II*, with its commercialisation being quickly

spread all over the globe due to its easy and low-cost production (Hasanuzzaman et al., 2020).

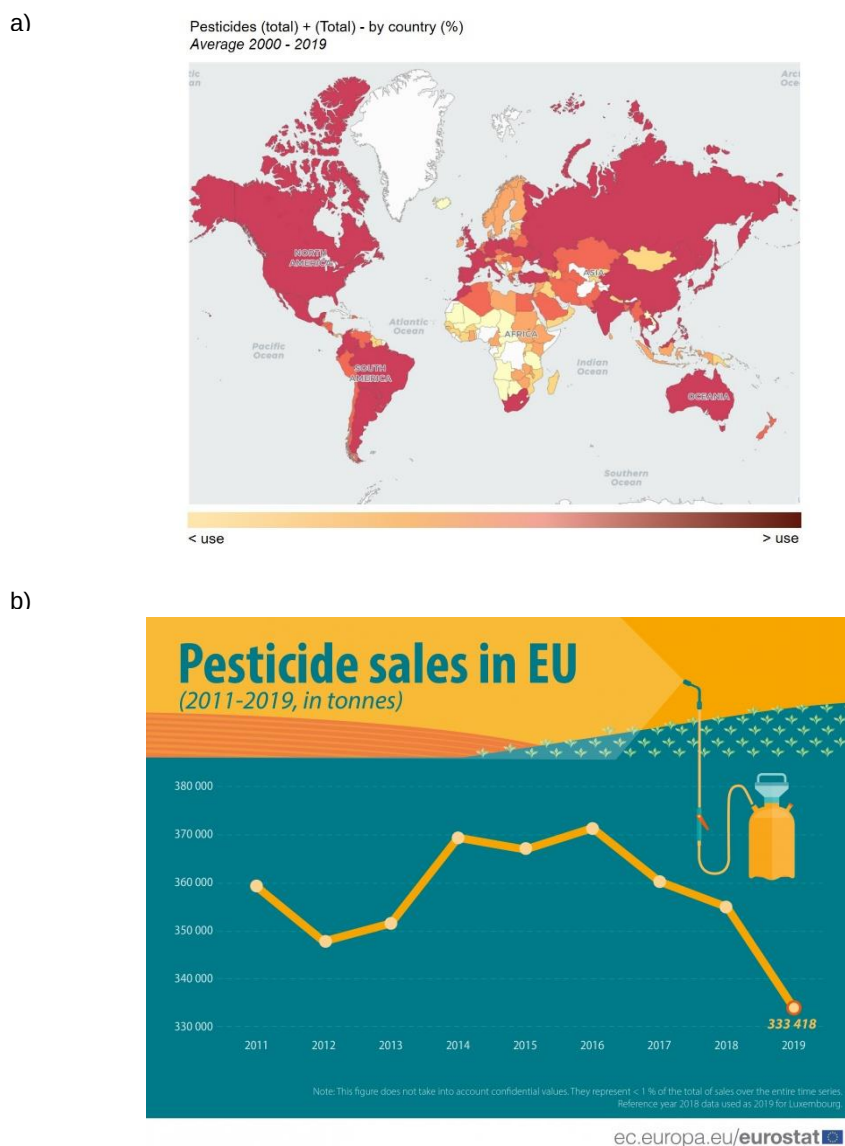


Figure 1. (a) Pesticide global use per area of cropland (kg ha^{-1}) between 2000 and 2019. Light and dark colours indicate lower and higher values, respectively. Data retrieved from FAO (<https://www.fao.org/faostat/en/#data/RP/visualize>). (b) Total pesticide sales, expressed in tonnes, of the European Union (EU) between 2011 and 2019. Retrieved from Eurostat (https://ec.europa.eu/eurostat/statisticsexplained/index.php?title=File:Pesticides_sales_2019data-01.jpg).

Since the development of the first synthetic pesticides, hundreds of formulations have been created and patented. Based on statistic data, in the 1960s, the pesticide market did not surpass the \$10 billion and less than 100 active ingredients were available to the farmers (Lykogianni et al., 2021). However, due to the “Green Revolution” – a period of

time where agricultural systems were maximised by the application of pesticides and fertilisers, allied to extensive farming systems (John and Babu, 2021) –, the pesticide industry has been steadily rising over the decades, reaching a market size value of almost \$90 billion in 2019, with a generalised worldwide representation (Figure 1a). Additionally, it is expected that this value will further increase at a 11.5% growth rate to more than \$130 billion by 2023 (<https://www.thebusinessresearchcompany.com/report/pesticides-market>). Nowadays, the main drivers of the uptrend in the pesticide industry are America and Asia, while the European Union (EU) has not been facing major fluctuations in the total volume of pesticide expenditures since 2011 (<https://www.fao.org/faostat/en/#data/RP/visualize>).

Moreover, as illustrated in Figure 1b, in 2019, the EU registered the lowest value for pesticide sales in the last ten years. In spite of this, not all European countries are decreasing their pesticide application rates. According to Eurostat (<https://ec.europa.eu/eurostat/web/products-eurostat-news/-/ddn-20200603-1>), the total volume of pesticide sales is still rising in Slovakia, France, Austria and Cyprus. On the contrary, other countries, including Portugal, have succeeded in significantly lowering their pesticide footprint in the last few years (Figure 2). In Europe, namely in Portugal (Figure 2), fungicides and bactericides are currently the most representative group (45%) of pesticides, being followed by herbicides (32%) and insecticides (11%) (<https://ec.europa.eu/eurostat/web/products-eurostat-news/-/ddn-20200603-1>). However, from a worldwide perspective, herbicides or weed killers are the ones accounting for the highest application volume, tailed by insecticides and fungicides.

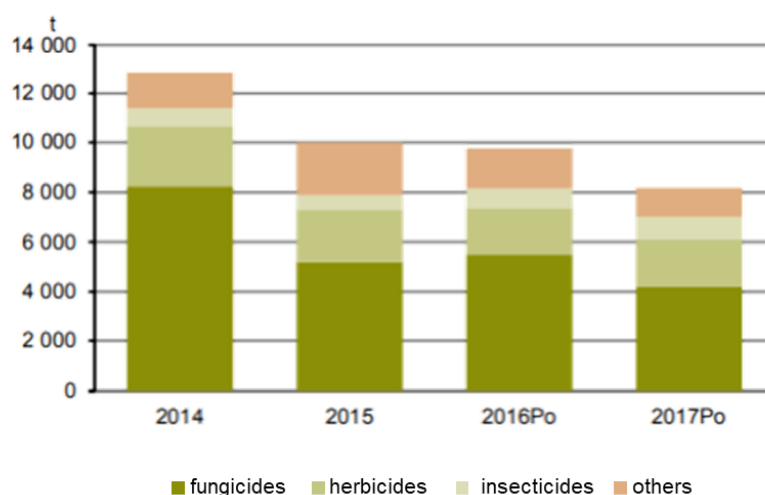


Figure 2. Sales of pesticides, in tonnes, according to the main classes (fungicides, herbicides, insecticides and others) in Portugal between 2014 and 2017. Retrieved from Instituto Nacional de Estatística (INE, 2018).

Since long ago, the growth of undesirable plants, commonly known as weeds, has been significantly hampering agricultural production, once these organisms compete directly with crops for light, water, mineral nutrients, and space (Hasanuzzaman et al., 2020), while also acting as natural reservoirs for disease vectors (e.g. insects) (Boydston et al., 2008; Swinton et al., 1994; Ziska, 2016). In the face of the unprecedented climate change, the increase of the atmospheric carbon dioxide (CO₂) is also expected to benefit weed growth, whose ability to adapt to new climatic and edaphic conditions can be higher than that of crops. In fact, these species display a lot more genetic diversity, since they have not been subjected to artificial selection for desirable growth traits (Korres et al., 2016). Therefore, and regardless of the current trends about conservation agriculture, the widespread use of herbicides is still expected to increase on the next years, as weed interference can be responsible for yield losses up to 70% (Hasan et al., 2021). Based on the projections of two Market Research and Advisory companies, the herbicide segment is expected to rise at a pace of around 5%, attaining a total market value of \$38 billion by 2025 (<https://www.alliedmarketresearch.com/herbicides-market>) and surpassing more than \$50 billion revenue by 2027 (<https://www.marketresearchfuture.com/reports/herbicides-market-4853>). On the European level, this ascending trend can also be found, with forecasts estimating a similar growth rate and a market size around \$9.4 billion, a 25% increase in relation to the value of 2017 (\$7.5 billion) (<https://www.marketdataforecast.com/market-reports/europe-herbicides-market>).

Herbicides can be classified according to different features, including their mode-of-action, selectivity, target crop, and active ingredient (a.i.). Some of the most globally applied herbicides are glyphosate (GLY), 2,4-D and atrazine, albeit the later is not allowed in Europe. Notwithstanding their role in weed management, there is a growing controversy around their possible non-target environmental and health effects (Hasanuzzaman et al., 2020).

2. Glyphosate (GLY) – from an agriculture ally to an emerging contaminant

2.1. Historical contextualization

From all ever-existing weed killers, GLY has rapidly become the best-selling herbicide of all time, being used for both agricultural and non-agricultural purposes (Benbrook, 2016; Duke, 2018). Before the introduction of GLY into the agri-food scenario during the 70s of the 20th century, this molecule was firstly described in 1950 as a metal chelator agent, with its original synthesis being attributed to a Swiss chemist, Dr Henri Martin, a worker of a small pharmaceutical company (Cilag) based in Switzerland (Franz et al., 1997). At the time, since no therapeutic properties could be attributed to the recently synthesised

compounds, GLY failed to reach the chemistry industry. In 1959, Johnson & Johnson – then owners of the former Cilag – decided to sell samples of several products, including GLY, to Aldrich Chemical to be tested for multiple applications; yet, no potential applicability was anticipated. Almost after a decade, in the late 60s, Monsanto started to seek promising molecules to be used as herbicides. Finally, in 1970, Dr John Franz tested the herbicidal activity of GLY in a greenhouse context (Baird et al., 1971). Quickly after, in 1974, GLY was patented for herbicide use under the trade name of RoundUp® (Duke and Powles, 2008), with Monsanto owning its rights until the start of the new millennium (Richmond, 2018). Following its commercialization, GLY became increasingly popular, though its potential applications were still limited, due to its non-selective action (*i.e.* it affects not only weeds but also crops). For this reason, at the beginning, GLY was only used for weed control in roads and streets, between rows in orchards and vineyards, and/or before crop instalment to prevent the growth of undesirable plants. Even so, when compared to other chemical options at the time (such as paraquat and diquat), GLY emerged as a very promising alternative, since it had a slower action and, once incorporated by plants, was quickly translocated to meristems, exerting its herbicidal action. Allied to these characteristics, GLY-based formulations were also considered safer than the other prevailing herbicides, with a low acute and chronic toxicity towards animals (Duke, 2018).

Over its pathway towards global recognition, the moment when transgenic GLY-resistant crops (GRes; branded as RoundUp® Ready™) were introduced in the market is, undoubtedly, the most important turning point, having contributed massively to the popularity and non-ending applications of GLY into the agri-food scenario (Duke and Powles, 2008). With this breakthrough technology, farmers could start using GLY for weed control before, during and after crop settlement, growth, and harvesting. The first genetically engineered varieties of soybean (*Glycine max* L.), maize (*Zea mays* L.) and cotton (*Gossypium hirsutum* L.) resistant to GLY were commercialised in 1996 (Benbrook, 2016, 2012). Since then, and especially in the United States of America (USA), almost 90% of the total land used for growing these crops is occupied by GLY-resistant genotypes (Benbrook, 2016). Presently, GLY continues to be the major chemical tool for weed control, but faces several drawbacks related not only to its possible non-target toxicity (which will be discussed below), but also to the emergence of resistant weed species. Accordingly, persistent and cumulative applications of GLY-based herbicides have been culminating in increasing weed resistance all over the World. Based on a recent report, more than 48 weed species were identified as GLY-resistant, some of them occurring in fields where GRes crops are sown (Baek et al., 2021). Although paradoxically, farmers often increase the application frequency to face this emerging issue, bringing a series of

subsequent disadvantages, such as higher costs and environmental problems. A comprehensive review of the environmental impacts of GLY will be discussed later on this chapter (topic 2.5.).

2.2. Global use and future projections

Paired with its growing popularity in terms of efficiency, the total volume of sales of GLY-based herbicides has also been steadily increasing since the start of its commercialization, but especially in the last three decades. For instance, while in 1994 a total of almost 57 000 tonnes of GLY were used for agricultural purposes, this value scaled up to more than 180 000 tonnes in 2000 and to almost 830 000 tonnes in 2014 (Figure 3). According to a recent review (Benbrook, 2016), besides the development of GRes crops, there are other factors explaining this quick and stable ascension of GLY over the recent years: i) a larger area of land is being used for agriculture, expanding the total area treated with GLY; ii) the implementation of no-till practises, often associated with an increased application of herbicides to control weeds; iii) the reduced price of GLY; iv) the setup of new application modes and novel applications for different crops.

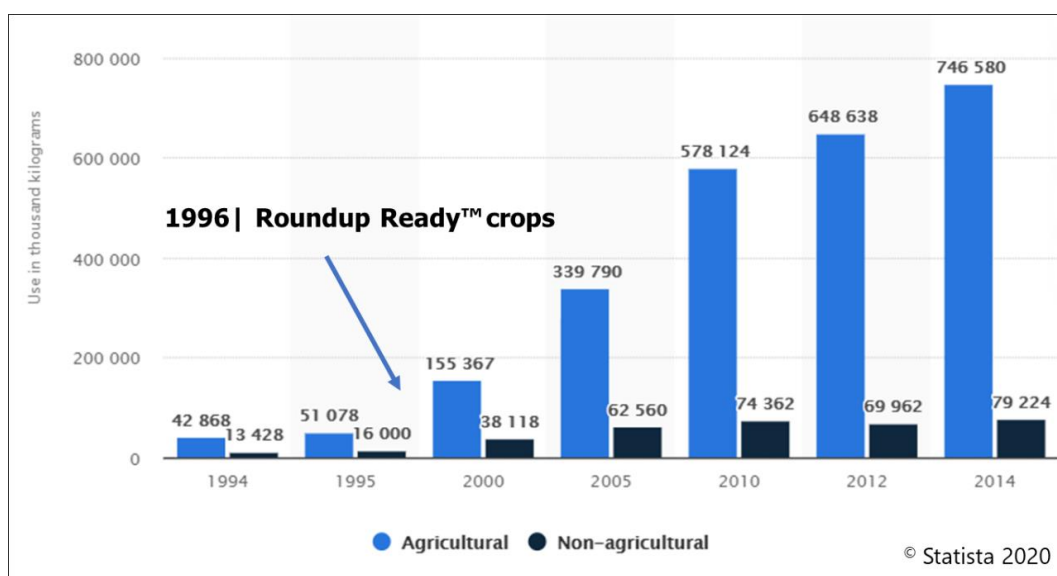


Figure 3. Total use of glyphosate (GLY), in thousand kg, for agricultural and non-agricultural purposes between 1994 and 2014. Adapted from Statista® (<https://www.statista.com/statistics/567250/glyphosate-use-worldwide/>).

In the EU, the sales of GLY in 2017 summed up to a total of 49 427 tonnes, representing 33% of the total European herbicide market and 7% of the total volume sold of GLY in the world (Antier et al., 2020). On a relative scale, it seems that the global ascension of GLY in terms of total sales is being mainly driven by other regions, such as Asia, rather than by Europe. The European countries with the highest volume of sales were France, Poland, Germany, Italy and Spain, accounting for more than 50% of the total GLY expenditures in

the EU. In terms of evolution, there has been no clear trend over the last decade, with the values of 2017 being identical to those recorded in 2013. Although Portugal sales represent only a fraction of 3% of the total value, data indicate that, from 2011 to 2017, Portugal has increased the sales of GLY by about 17%, accounting for almost 70% of the national herbicide market. Moreover, when considering the use of GLY for agricultural practices, Portugal ranks as the 4th EU country (0.35 kg a.i. ha⁻¹), placed higher than other countries such as France, Italy and Spain, and way above the mean value of the EU (0.24 kg a.i. ha⁻¹) (Antier et al., 2020).

2.3. Properties, mode-of-action and general effects

In terms of chemistry, GLY [*N*-(phosphonomethyl)glycine] is a phosphonomethyl derivate of glycine, being considered as a polyprotic molecule with three polar functional groups (phosphonate, carboxyl and amino group) (Figure 4) (Martinez et al., 2018; Mertens et al., 2018; Singh et al., 2020). It results from the oxidative linkage between the methyl group (CH₃) of methylphosphonic acid with the amino group (NH₂) of the amino acid glycine. It is characterised for being a weak acid, displaying an anionic behaviour (Tzanetou and Karasali, 2020). In general, herbicide formulations contain GLY in the form of a salt, mostly potassium, ammonium, trimethylsulphonium and isopropyl-ammonium, in order to increase GLY's solubility (Cuhra et al., 2016; Travlos et al., 2017). The first formulation reaching the market was the isopropylamine salt of GLY (Duke and Powles, 2008), but, nowadays, several options are available, and not all RoundUp[®] products have the same GLY salt: while RoundUp[®] Ultra Max II has GLY in the form of potassium salt, RoundUp[®] Original or RoundUp[®] Ultra make use of GLY combined with isopropylamine. Moreover, GLY-based formulations also contain diverse surfactant agents, since GLY salts *per se* do not have a great ability to interact with plants, due to the hydrophobicity of plant surfaces, such as cuticles (Hertel et al., 2021).

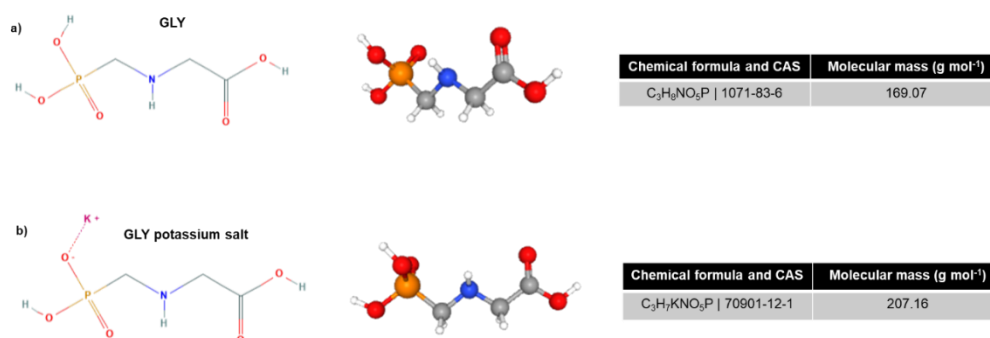


Figure 4. Molecular structure (2D and 3D), chemical formula, CAS number, and molecular mass (g mol⁻¹) of glyphosate (GLY) (a) and GLY potassium salt (b). Retrieved from PubChem[®].

In terms of herbicidal action, GLY is considered as a broad-spectrum pesticide, with post-emergent and non-selective activity, being commonly applied as foliar spray on weeds foliage for growth control (Gomes et al., 2014). Regarding its mode-of-action, GLY was designed to specifically interfere with a biochemical route exclusively found in plants and some microorganisms, the shikimate pathway (Figure 5) (Duke and Powles, 2008; Steinrücken and Amrhein, 1980). Given their structural similarity, GLY competes directly with phosphoenolpyruvate (PEP), one of the substrates of 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS; EC 2.5.1.19). This enzyme assumes a leading role in the shikimate pathway, catalysing the reaction between the glycolytic intermediate PEP and shikimic acid-3-phosphate (S3P) to produce enolpyruvylshikimate-3-phosphate (EPSP). Thus, upon the presence of GLY, EPSPS activity is inhibited, leading to an overaccumulation of shikimate which results in lower levels of EPSP and chorismite, precursors of several aromatic amino acids (phenylalanine, tyrosine and tryptophan), which are also involved in the biosynthesis of important specialised metabolites in plant growth, such as indole-3-acetic acid (IAA), anthocyanins and flavonoids (Tzin and Galili, 2010).

Upon contact with the leaves, GLY experiences a rapid initial absorption through the cuticle, being then slowly transported via symplast. This foliar uptake and distribution is driven by distinct aspects, including the plant species, the development stage, the herbicide concentration and other environmental factors, especially those altering plant water relations (reviewed by Gomes et al., 2014). Once incorporated, the movement of GLY through the symplast route can either occur by passive diffusion, in a process independent from the pH, or by utilising specific phosphate (PO_4^{3-}) carriers located in the cell membranes (Denis and Delrot, 1993; Morin et al., 1997). Afterwards, GLY can reach the vascular bundles, being then translocated to other parts of the plant via phloem, similarly to photoassimilates (Dill et al., 2010). Finally, GLY will end up accumulating in tissues with a high metabolic index, namely root and shoot meristems, where it will exert its herbicidal action (Gomes et al., 2014; Singh et al., 2020). However, GLY accumulation in other structures, such as tubers, rhizomes and root nodules, is also commonly observed (Cakmak et al., 2009). Recently, studies conducted with several plant species also point towards the existence of a root absorption pathway, since residues of GLY in the soil can end up affecting plant growth. Although not as explored as the movement through the foliage, it is thought that, given the presence of a methylphosphonic group in the molecular skeleton of GLY, it can compete with PO_4^{3-} to be uptaken by roots (Gomes et al., 2016b). After exposure to GLY, plants start to develop a series of phytotoxicity symptoms, which includes foliar chlorosis and necrosis, this being accompanied by a deregulation of leaf morphology, inducing foliar wrinkling and apex malformations, especially at the shoot

apical meristem. In general, the development of these symptoms is gradual, ultimately leading to plant death over the course of some days or weeks (Singh et al., 2020).

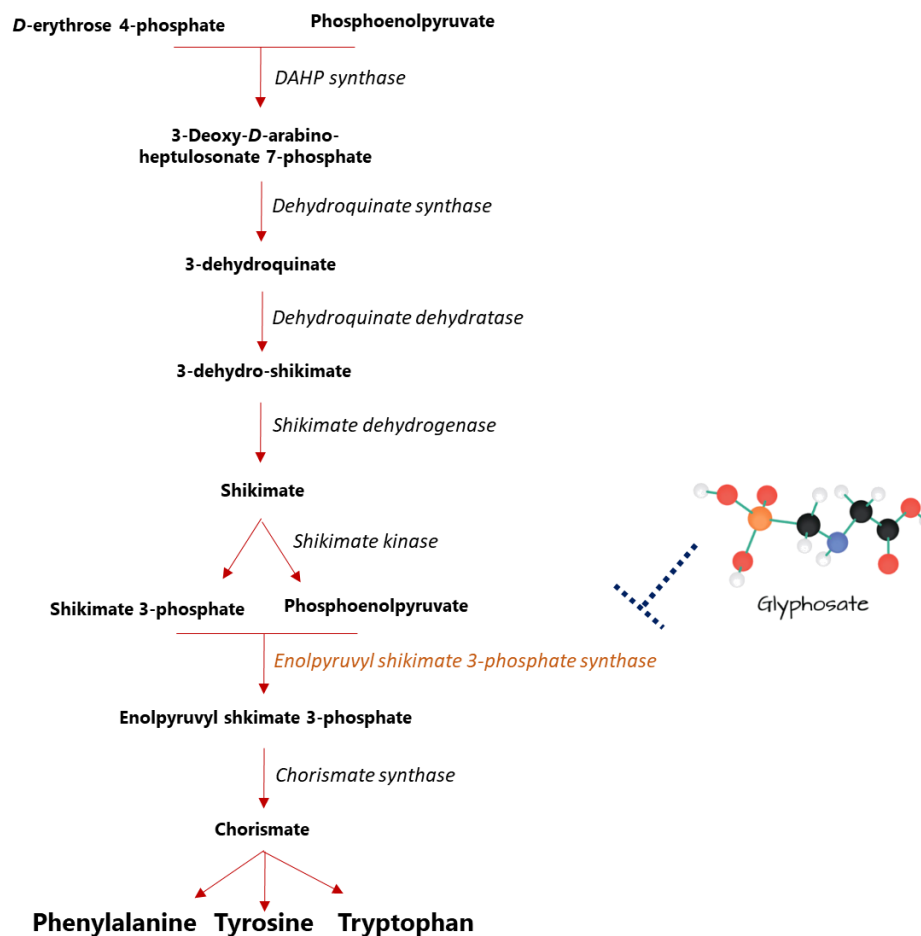


Figure 5. The shikimate pathway and glyphosate (GLY) interference with one of its biochemical steps. The shikimate pathway consists in a series of 7 steps catalysed by multiple enzymes in a sequential fashion to produce chorismate. The biosynthetic chain initiates with the interaction between phosphoenolpyruvate (PEP) and erythrose 4-phosphate (E-4P), both derived from the cellular carbon (C) metabolism, in a reaction mediated by 3-deoxy-d-arabino-heptulosonate-7-phosphate synthase (DAHPS; EC 2.5.1.54), producing 3-dehydroquaianate. Next in line is 3-dehydroquinate synthase (DHQS; EC 4.2.3.4), which is involved in the formation of 3-dehydroquinate from the previous intermediate. The next two steps are ensured by a bifunctional enzyme – 3-dehydroquinate dehydratase/shikimate 5-dehydrogenase (DHQ/SDH; EC 4.2.1.10 and EC 1.1.1.25) – which arises the biosynthesis of shikimate, this being posteriorly activated by the shikimate kinase (SK; EC 2.7.1.71) to shikimate 3-phosphate. Afterwards, the 5-enolpyruvylshikimate 3-phosphate synthase (EPSPS; EC 2.5.1.19) will catalyse the production of enolpyruvylshikimate 3-phosphate (EPSP). Whenever GLY is present, it has the ability to directly compete with one of the substrates (PEP) of this enzyme, leading to its inactivation. The last step is mediated by another enzymatic reaction, in which chorismate synthase (CS; EC 4.2.3.5) converts EPSP to chorismate, a key metabolite for the synthesis of the aromatic amino acids phenylalanine, tyrosine and tryptophan. *Adapted from Maeda and Dudareva (2012).*

2.4. Accumulation and fate of GLY in the soil

Due to the widespread use of GLY over the last decades, residues of this agrochemical are frequently found in several environmental matrices, such as surface waters and soils, especially in agricultural areas. GLY can enter the environment through different processes and mechanisms, potentially altering the ecosystem's dynamics and services, with possible impacts towards non-target biota (Figure 6). Upon foliar application of GLY, either by the action of wind, rain, gravity or spilling accidents, part of the applied formulations can be deposited in the soil, where they can be accumulated. Indeed, it is acknowledged that only a minor amount (about 0.1%) of the applied agrochemical will effectively reach the target organism, being the majority of it lost to the environment, especially soils (Hernández et al., 2013; Shahena et al., 2020). Alongside, it has been shown that GLY exudation from plant roots and its release from dead plant material can highly contribute to its persistence in the soil (Myers et al., 2016; Tesfamariam et al., 2009). Thus, it is not surprising that, nowadays, GLY and aminomethylphosphonic acid (AMPA) – its main degradation product – are two of the most recurrent residues found in agricultural soils. According to a very recent report (Silva et al., 2019), GLY and AMPA were found in 21 and 42%, respectively, of samples collected from agricultural soils of 11 European countries. Moreover, according to Geissen et al. (2021), both compounds were the most frequent and abundant ones in topsoils from Portugal, Spain and the Netherlands. In terms of contamination levels, based on several data, GLY residues can reach the mg kg^{-1} range, with studies reporting concentrations around or below 3 mg kg^{-1} in soils of agricultural areas in South America and Europe. Similarly, Primost et al. (2017) and Peruzzo et al. (2008) have found GLY concentrations up to 5 and 8 mg kg^{-1} , respectively. Worst-case scenarios, published recently, document GLY levels of about 40 mg kg^{-1} in olive groves in Greece and 608 mg kg^{-1} in crop fields in Mexico (Karanasios et al., 2018; Muñoz et al., 2019). Furthermore, although the levels found in water samples are often within the order of $\mu\text{g L}^{-1}$, there are already studies in China reporting concentrations as high as 15 mg L^{-1} (Wei et al., 2016), thus being expected that soil levels can exceed this value in response to the persistence of GLY.

Once accumulated in the soil, GLY can undergo different processes, namely mineralisation, immobilisation or leaching, whose dynamics are mostly dependent on soil composition and characteristics (reviewed by Bai and Ogbourne, 2016). In a similar manner, other aspects, including soil water content and temperature, are also important drivers of GLY persistence and degradation, whose half-life (DT_{50}) in the soil can vary from several weeks to months or even one year (Bento et al., 2016; Padilla and Selim, 2020). Thus, upon arrival to the soil surface, GLY can be degraded, adsorbed onto soil

particles (such as clays and organic matter – OM), and/or move to other soil horizons (Borggaard and Gimsing, 2008; Landry et al., 2005; Strange-Hansen et al., 2004). The mineralisation of GLY by the action of microorganisms can occur in two different ways, leading to the production of AMPA, or glycine and sarcosine (Bai and Ogbourne, 2016). According to several authors, the mineralisation of both GLY and AMPA is affected by different soil properties and by the soil microbiome. It is recognised that soils with a high pH, an increased concentration of PO_4^{3-} and low levels of copper (Cu) or iron (Fe) typically contribute for an accelerated mineralisation of GLY (Mamy and Barriuso, 2005; Morillo et al., 2000; Zhang et al., 2015). On a complementary perspective, the studies of Franz et al. (1997) and Von Wiren-Lehr et al. (1997) found a positive association between soil respiration rate and microbial biomass, and GLY degradation. Yet, opposite findings have been also reported, where no apparent relationship could be identified (reviewed by Borggaard and Gimsing, 2008). The dynamics underlying GLY mineralisation into AMPA are of particular interest, since this molecule – recognised as GLY's primary degradation product – is also considered to be a potent toxin (Gomes et al., 2014).

As reviewed by Bai and Ogbourne (2016), GLY has a strong adsorption coefficient, being rapidly immobilised upon the contact with the soil. As an example, previous studies have shown that 20% of the total GLY added to the soil can be adsorbed after only 3 h of application (Shushkova et al., 2009). In general, the mobility of organic compounds in soils is greatly dependent on their sorption features. Due to the nonpolar nature of most pesticides, they are mostly adsorbed by OM (Pérez-Lucas et al., 2021). In contrast, by possessing three polar functional groups (carboxyl, amino, and phosphonate) in its chemical structure, GLY preferentially interacts with inorganic elements such as aluminium (Al) and Fe (Kanissery, 2019), rather than with organic complexes. Given the presence of a methylphosphonic group in its structure, GLY can compete with PO_4^{3-} for the same adsorption sites, reason why the PO_4^{3-} content of the soil majorly influences GLY bioavailability (Padilla and Selim, 2020). In fact, both compounds can form stable complexes by ligand exchange with Al and Fe. Moreover, previous research has also suggested that, although they can compete for some sorption sites, there might be also an additive behaviour, with sorption sites being able to sorb both compounds (Borggaard and Gimsing, 2008). Besides the chemical affinity between GLY and the mineral group, the pH of the soil also plays a key role in the modulation of GLY adsorption to the soil components. Usually, as the soil pH increases, the sorption of GLY decreases (Bai and Ogbourne, 2016; Borggaard and Gimsing, 2008; Padilla and Selim, 2020). Despite being often assumed that soil OM does not play a role in the fate of GLY in the soil (De Jonge et al., 2001; Gerritse et al., 1996; Mamy and Barriuso, 2005), former research has identified interaction points between humic acids and GLY (Albers et al., 2009). Also, Piccolo et al.

(1996) observed that GLY was strongly adsorbed to samples of purified humus, by the existence of hydrogen (H) bonds. Additionally, it has also been postulated that soil OM can indirectly affect GLY adsorption dynamics, either by blocking surface sites or by promoting the stabilisation of metal oxides with high sorption features (Borggaard and Gimsing, 2008). Overall, it can be assumed that GLY persistence and accumulation in the soil are very dynamic processes, being generally favoured in soils with low pH and PO_4^{3-} content, high levels of Al and Fe, and rich in OM (Bai and Ogbourne, 2016). On the contrary, soils with low OM and high pH are more susceptible to GLY (and AMPA) leaching, contributing to their transfer to the aquatic compartment (Laitinen et al., 2009; Shushkova et al., 2009).

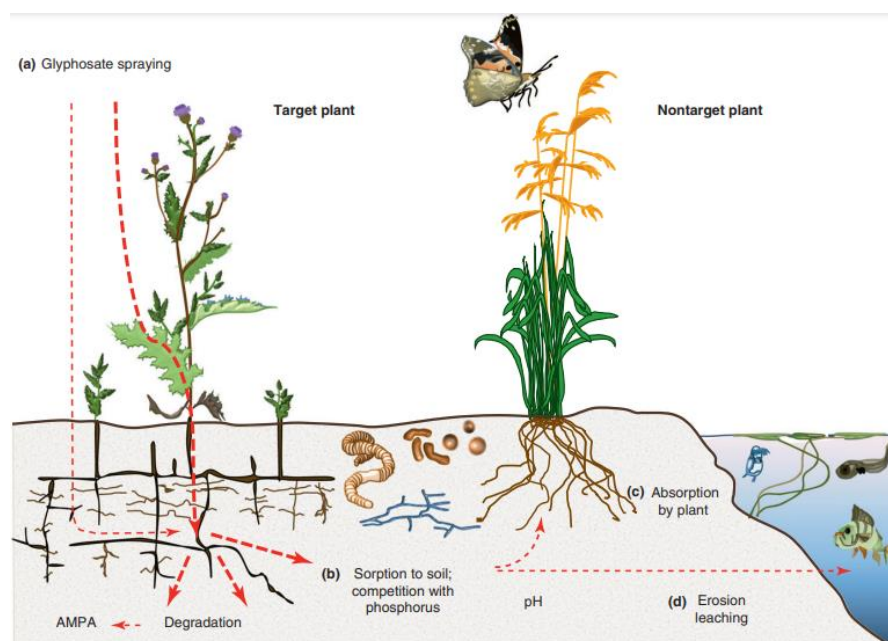


Figure 6. Mechanisms by which GLY can reach the soil and move to other environmental compartments. Upon treatment of the foliage of target plants (*i.e.* weeds) with GLY, a significant portion of the total applied volume can be lost by gravity or rainfall leaf washing, reaching the soil. Once there, and depending on the characteristics of the soil itself, GLY can remain adsorbed to soil particles (*e.g.* clays, metallic ions, organic compounds), be degraded by microbial action, consequently leading to the production of AMPA, and/or be remobilised. Afterwards, the resolubilised GLY can become available to soil biota, such as animals, microorganisms and plants, or move to other compartments, such as freshwater courses. Retrieved from: Helander et al. (2012).

2.5. Toxicity of glyphosate towards non-target species

Since their patent, GLY-based herbicides have been branded not only as a novel and efficient tool to prevent weed growth, but also as an environmentally safer option than other conventional counterparts (Duke, 2018). This assumption arises because GLY

interferes with a biochemical pathway absent in animals and, once in contact with the soil, it is readily metabolised and degraded by the microbial community, not posing a threat to non-target plants (Bai and Ogbourne, 2016; Kanissery et al., 2019). As highlighted in Bayer's website (<https://www.bayer.com/en/glyphosate/glyphosate-RoundUp>), the use of RoundUp® products actually has an active role in preserving biodiversity and soil quality, since the application of GLY-based herbicides reduces the need of tillage practices. However, especially in the last decade, a rising concern over the environmental and human health effects of GLY exposure has started to tackle the attention of the scientific community and the overall population (Bai and Ogbourne, 2016; Richmond, 2018; Singh et al., 2020; Tarazona et al., 2017), although with high controversy. From a wide perspective, while in 2015 and 2017 the International Agency for Research on Cancer (IARC) considered GLY to be a potential carcinogenic (Cressey, 2015; IARC, 2017, 2015), the European Food Safety Authority (EFSA) and the United States Environmental Protection Agency (US-EPA) rebutted any relation between GLY and cancer risk (EFSA, 2017; EPA, 2017). The most recent report, drawn this year by the European Commission (EC), states that GLY *"can be safely used for its intended applications when used in accordance with the label instructions"* (<https://www.glyphosate.eu/useful-information/>). Based on literature surveys conducted by Peillex and Pelletier (2020) and Klingelhöfer et al. (2021), more studies are needed to clearly understand the risks of GLY to human health. In spite of the ongoing controversy around GLY hazards to humans, attention must also be paid to its potential impacts in the ecosystems. With effect, and reinforcing what was previously discussed, GLY accumulation in agricultural soils is an emerging issue, with residues of this herbicide being capable of interacting with soil organisms (Bai and Ogbourne, 2016; Richmond, 2018) and plants (Gomes et al., 2014) (Figure 6). Up to now, research dealing with the possible toxicity of GLY (as well as AMPA), when present as an environmental contaminant, to terrestrial invertebrates, soil microorganisms and non-target plants is still inconclusive. Actually, the available studies do not always employ realistic approaches, in what regards the applied concentrations and exposure conditions, which hinders the obtention of a global picture on the real risks of GLY (and AMPA) towards non-target species.

Given the relevance of the current work, in the following subsections, a comprehensive and thorough sum-up of the most recent research exploring the non-target effects of GLY on soil invertebrates and plants will be provided. However, one should not underestimate the impacts that GLY can portray towards soil microflora. Soil microorganisms play several vital roles in soil health and functions, with direct consequences on crop health (Lehmann et al., 2020). By modulating nutrient acquisition and hormone balance, beneficial microorganisms are also a great aid in plant defence against pests and diseases (Arif et

al., 2020; Dubey et al., 2019). Since the shikimate pathway is also present in several species of bacteria and fungi, it is not surprising that GLY can modulate soil's microbiome, considered as the main route for GLY biodegradation (Van Bruggen et al., 2018), with potential effects on the microbial community and enzymatic activity of the rhizosphere and bulk soil (Arango et al., 2014; Banks et al., 2014; Cherni et al., 2015; Druille et al., 2016; Schafer et al., 2014). However, there is still a lot of divergent data concerning this issue, with studies pointing effects ranging from positive (Araújo et al., 2003; Haney et al., 2002), to neutral (Lane et al., 2012; Weaver et al., 2007; Zabaloy et al., 2012) and harmful (Kremer and Means, 2009; Newman et al., 2016a,b; Zobiole et al., 2011b). For instance, the beneficial bacteria from *Burkholderia*, *Rhizobium* and *Pseudomonas*, as well as arbuscular mycorrhizal fungi, were found to be negatively affected by GLY exposure (Arango et al., 2014; Druille et al., 2016; Schafer et al., 2014; Zobiole et al., 2011b). In opposition, the recent study of Schlatter et al. (2017) concluded that GLY did not majorly impact the structure and biodiversity of bacterial communities of an agricultural soil. In spite of that, considering the different sensitivity among microorganisms towards GLY, even minimal modifications in terms of microbiome composition and diversity can favour the abundance of pathogenic strains, such as *Fusarium* spp., with direct consequences to plant health (Fernandez et al., 2009; Johal and Huber, 2009; Rosenbaum et al., 2014).

2.5.1. Soil invertebrates

Covering a large spectrum of different types of organisms, soil invertebrates, such as worms (e.g. Oligochaeta and Enchytraeidae), nematodes (Nematoda), springtails (Collembola), and mites (Acari), play essential roles in the dynamics of the soil ecosystem. Altogether, they contribute, among others, for increasing soil porosity (due to their burrowing activity), ii) enhancing water infiltration and retention, preventing soil compaction, mineralising OM (Gunstone et al., 2021), nutrient cycling, soil formation, creating habitats and providing food to other organisms (Reed et al., 2016), aspects that assume particular relevance in the frame of agricultural sustainability. However, based on recent studies and a report from FAO, modern agriculture practices, namely the widespread use of pesticides, are currently a worrying trend, being considered as the main responsables for soil biodiversity losses (Gunstone et al., 2021). Although the risk assessment of hazardous compounds, including GLY, requires the evaluation of potential effects towards soil invertebrates, there it is still a lot of divergent opinions on whether residues of this herbicide, as well as its main degradation product AMPA, are able to negatively affect soil biota. When exploring the impacts of GLY towards soil invertebrates, Niemeyer et al. (2018) reported that the recommended dose of different commercial

formulations containing GLY (RoundUp® Original, Trop®, Zapp® Qi 620 and Crucial®) did not have any major effect on the avoidance and feeding activity of earthworms (*Eisenia andrei* Bouché) and collembola (*Folsomia candida* Willem). Also, previous research on the non-target effects of GLY (provided as Montana® at the recommended application dose) towards *F. candida* showed that this species did not avoid a GLY-contaminated soil (Santos et al., 2012). Similarly, by evaluating the short-term impact of GLY (as Rodeo® XL; 1080 g a.i. ha⁻¹) in the earthworm *Lumbricus terrestris* L., a recent study (Nuutinen et al., 2020) suggested the absence of toxicity of GLY to this species, with no effects on biomass, reproduction and survival. However, and opposing to these results, Pochron et al. (2020) concluded that GLY (26.3 mg kg⁻¹), but not two of its commercial formulations (RoundUp® Super Concentrate and RoundUp Ready-to-Use-III®, at 26.3 mg kg⁻¹), harms *E. fetida* individuals, impairing their biomass and survival up to 30%, in relation to unexposed organisms. Interestingly, the experiments conducted by Gaupp-Berghausen et al. (2015) contrastingly suggest that GLY-based herbicides (RoundUp® Alphée and RoundUp® Speed, at 50% of the recommended application dose) are capable of negatively affecting the activity and reproduction of earthworms. Indeed, several works have been finding substantial differences between the a.i. itself and different commercial formulations in what regards their toxicological profile (Bonnet et al., 2007; Maderthaner et al., 2020; Mesnage et al., 2015; Pereira et al., 2009; Piola et al., 2013; Pochron et al., 2020). A previous work aimed at comparing the ecotoxicological relevance of two GLY-based herbicides (RoundUp® FG and Mon 8750) towards *E. andrei* revealed that the former formulation (RoundUp® FG) was much more toxic than the latter, with effects being correlated with the presence of additives, such as surfactants, present in the commercial formulation (Piola et al., 2013). Besides being able to affect earthworm's behaviour, reproduction and survival, there are also reports suggesting that GLY-mediated effects can be related to the induction of morphological aberrations, such as body elevation, coiling and curling (Correia and Moreira, 2010). Although in this last study, authors tested concentrations of GLY up to 1000 mg kg⁻¹ (10, 50, 500 and 1000 mg kg⁻¹), visible effects were evident right from the lowest one (10 mg kg⁻¹) (Correia and Moreira, 2010). The occurrence of metabolic disorders, such as DNA damage, neurotoxicity and redox disorders/disbalances, has been the focus of several studies dealing with GLY toxicity in soil invertebrates (Contardo-Jara et al., 2009; Piola et al., 2013; Salvio et al., 2016; Zhou et al., 2013), though no apparent dose-response relationships could be drawn since a high variability among studies is found. Recently, Simões et al. (2018) conducted a thorough analysis of the non-target effects of a GLY-based herbicide (Montana®; tested concentrations up to 4 mg kg⁻¹) towards the model species *F. candida*, paying attention to different levels of organization – from behavioural responses to molecular endpoints.

According to their data, which included proteomics and transcriptomics, the recorded macroscopic impacts are likely related to a disruption of the lipid and oxidative metabolism, inducing oxidative stress and impairing the cellular respiration, with subsequent impacts on developmental and reproduction processes. From a different perspective, Owagboriaye et al. (2021) assessed how the gut microbiome of different earthworm species (*Alma millsoni* Beddard, *Eudrilus eugeniae* Kinberg and *Libyodrilus violaceus* Beddard) was affected by the presence of GLY (in the form of RoundUp® Alphée; sprayed at 115.49 mL m⁻²) in the soil. Indeed, serving as temporary habitats for different taxa of soil microorganisms, changes in gut's microflora can portray alterations in the earthworm physiological status, conditioning its behaviour, activity and ecological roles. In terms of total biodiversity, a decrease was observed in response to the herbicide. Moreover, based on the data obtained, upon an eight-week exposure to GLY, individuals showed a dominance of *Enterobacter*, *Pantoea* and *Pseudomonas* (80%), organisms barely found in the control group.

Overall, there is a high variability of results concerning the ecotoxicological relevance of GLY-based herbicides, which reinforces the need of future studies encompassing an integrative perspective on the impacts of GLY as well as of other herbicides (either as single a.i. and as commercial formulations) under realistic conditions, especially focusing on long-term exposures. Indeed, studies exploring the chronic effects of GLY on soil invertebrates – as well as in other species, such as non-target plants and microorganisms – is of utmost importance, since GLY is frequently applied each year.

2.5.2. Non-target plant species – how can GLY affect their growth?

Given the non-selective nature of GLY, the exposure of non-target plants to residues of this herbicide usually results in growth retardation and morphological alterations, significantly hampering plant development (Singh et al., 2020). Moreover, it is also accepted that even GRes crops can be negatively affected by GLY (Zobiole et al., 2012, 2011a, 2010a). The primary effects of GLY on the growth of non-target plant species arise from its herbicidal action, capable of inhibiting the production of essential amino acids, especially in meristematic zones (Gomes et al., 2014). As previously stated, when in the soil, both GLY and AMPA can be uptaken by the roots (Gomes et al., 2014), quickly reaching root and shoot apices through xylem movement. Indeed, works performed with sunflower (*Helianthus annuus* L.), Johnson grass (*Sorghum halepense* L.) and maize (*Z. mays*), revealed that this herbicide tends to accumulate in high active metabolic tissues (Eker et al., 2006; Hetherington et al., 1999; Vila-Aiub et al., 2012). In general, common GLY phytotoxic symptoms include the occurrence of chlorosis and necrosis, followed by a

marked reduction of biomass production and organ elongation (Gomes et al., 2014). GLY-mediated effects on growth have been documented for different plant species, such as willow (*Salix miyabeana* Seemen; Gomes et al., 2017, 2016a), tomato (*Solanum lycopersicum* L.; Khan et al., 2020; Singh et al., 2017b), pea (*Pisum sativum* L.; Singh et al., 2017a), tea plant (*Camelia sinensis* (L.) Kuntze; Tong et al., 2017), duckweed (*Lemna minor* L.) (Sikorski et al., 2019), *Hydrocharis dubia* (Bl.) Backer and *Vallisneria natans* (Lour.) H.Hara (Zhong et al., 2018). As reported for other contaminants, GLY effects are mostly dependent on the plant species and developmental stage, as well as the exposure conditions (e.g. concentration, application mode, frequency and intensity). For instance, Singh et al. (2017b) reported that the addition of GLY (40 mg L⁻¹) to a nutrient solution significantly reduced the growth of tomato plants after 30 d, with inhibitions up to 50% in relation to unexposed plants. Similarly, according to Kahn et al. (2020), the exposure of tomato seedlings to GLY, either supplemented to the nutrient solution (up to 30 mg L⁻¹) or added as a contaminant to the soil (up to 30 mg kg⁻¹), reduced plant growth upon exposure to the highest concentrations. Nowadays, it is universally recognised that GLY toxicity is not strictly related to its interference with the shikimate pathway, with it being also able to negatively affect other key biochemical, molecular and metabolic events, such as mineral nutrition, hormone balancing, photosynthesis and redox homeostasis (Gomes et al., 2014 and references therein). Thus, pinpointing the exact mechanisms by which GLY affects the overall physiological status of the plant, either by focusing on molecular, biochemical or cellular pathways, is essential to concretely unravel its risks towards plants, including non-target species and also resistant genotypes which are still injured by GLY application in a certain extent. In the last years, extensive research has been conducted with this purpose; however, given their practical relevance, most of the studies focus on the foliar effects of GLY – mimicking a situation of spray drift or direct application in the case of GRes crops – not concentrating on the impacts upon exposure to soil and water residues of this herbicide through the roots.

Mineral nutrition

Based on several reports, GLY-mediated inhibition of plant growth can be linked to its interference with the nutritional status. For being a metal chelator, GLY may affect the normal uptake of mineral nutrients by plant roots (Gomes et al., 2014). Yet, most of the work performed so far evaluated the relationship between GLY and mineral nutrition upon foliar exposure, not paying attention to the root pathway. Even so, according to Cakmak (2009), GLY-mediated deregulation of plant nutritional status can be the result of two different process: the immobilization of several cations *in planta* and/or the interference

with nutrient absorption at the root level. In fact, due to its carboxyl and phosphonate polar groups, GLY can establish strong complexes with divalent cations, promoting their immobilization (Gomes et al., 2014). When in solution, GLY can form complexes with different macro and micronutrients, which, on one hand, decrease the herbicide bioavailability, but, on the other hand, can also end up reducing the uptake of essential elements (reviewed by Mertens et al., 2018). However, regarding this point, contrasting evidence has been collected so far – while some reports observed a negative relationship between GLY and the accumulation pattern of several macro and micronutrients (Cakmak et al., 2009; Eker et al., 2006; Su et al., 2009), others did not find major alterations in terms of mineral nutrition (Duke et al., 2012). Yet, based on studies conducted with GRes soybean genotypes, the foliar application of GLY was found to negatively affect the cellular levels of different macro and micronutrients (Zobiolo et al., 2011, 2010b). Significant reductions in the levels of all macronutrients (nitrogen – N, phosphorous – P, sulphur – S, calcium – Ca and magnesium – Mg) were likewise reported in leaves of common bean plants upon treatment with increasing doses of GLY (Rabello et al., 2014). In a similar manner, Eker et al. (2006) has also documented that GLY application decreased the cellular levels of several essential nutrients in sunflower leaves, pointing that the influence of GLY on plant's mineral nutrition should not be ignored. Additionally, it was previously shown that GLY can interfere with the activity of ferric (Fe^{3+}) chelate reductase (EC 1.16.1.10), one of the key enzymes involved in root uptake of Fe (Eker et al., 2006; Ozturk et al., 2008). Actually, results obtained with soybean plants, either GLY-resistant or susceptible, strengthens the idea that Fe metabolism can be majorly affected by GLY, since Fe reductase activity was majorly inhibited in both genotypes upon herbicide exposure (Bellaloui et al., 2009). Given that Fe is a vital co-factor of many enzymes involved in several aspects of the plant physiology, this aspect needs to be adequately addressed, especially in non-target plants exposed to residues of this herbicide through the soil. Due to their chemical similarities, one of the mineral nutrients whose uptake and assimilation can be particularly affected by GLY is PO_4^{3-} . As commented above, GLY and PO_4^{3-} compete for the same absorption channels in root cells (Gomes et al., 2016b). It was previously reported that PO_4^{3-} soil supplementation may even contribute for a higher GLY uptake, promoting its re-solubilization, and exacerbating its effects on plant growth (Bott et al., 2011). Interestingly, when studying the interaction dynamics between GLY and PO_4^{3-} in a hydroponic system (with no soil particles for adsorption processes), Gomes et al. (2016b) observed that GLY uptake was enhanced as the PO_4^{3-} levels increased. Overall, more studies are needed to concretely unravel the influence of GLY on the mineral status of non-target plants, being now hypothesized that GLY can affect the uptake of essential

macro and micronutrients, this being mostly dependent on the soil's physical-chemical characteristics (Bott et al., 2011).

Hormone balance

As previously commented, GLY and AMPA are mainly accumulated in highly active metabolic tissues, such as root and shoot meristems (Gomes et al., 2014), which are also preferential sites for the biosynthesis of different plant growth regulators (Taiz et al., 2015). Once accumulated in plant cells, GLY can affect hormone metabolism by influencing their biosynthesis, not only by blocking the production of specific precursors but also by inhibiting key enzymes of the metabolic cascade. From all phytohormones, indole-3-acetic acid (IAA) – considered as the “growth hormone” – is the most abundant and important auxin, being actively involved, among other processes, in cell elongation, root growth and nodulation, phototropism and gravitropism, while also regulating the apical dominance (Taiz et al., 2015). Although there are multiple biosynthetic pathways responsible for IAA production, most of them depend on the presence of tryptophan, one of the three amino acids derived from the shikimate pathway (Morffy and Strader, 2020). By reducing the intracellular levels of IAA, the indirect effects of GLY on plants can be even greater, not only in terms of growth, but also in the establishment of symbiotic relations with microorganisms (Kremer and Means, 2009). Moreover, although not so explored as IAA, it has been suggested that GLY can hinder the biosynthesis of other phytohormones, such as gibberellins, brassinosteroids, and jasmonic acid, probably as a result of its negative effect of on cytochrome P450 (EC; 1.14.-.-) enzymes (reviewed by Gomes et al., 2014).

Photosynthesis and carbon metabolism

Considered as one of the central aspects of plant metabolism, changes in photosynthesis can be translated into several cellular, physiological and developmental disorders (Sharma et al., 2019a; Taiz et al., 2015), severely impacting crop growth. Although frequently viewed as an independent process, the photosynthetic hub is strongly connected to other metabolic events. One of those is the shikimate pathway, since chorismate is involved in the biosynthesis of quinones, a widely-known class of electron carriers (Nowicka and Kruk, 2010). Therefore, it is not surprising that the exposure of plants to GLY – at the foliar or root levels – can induce significant alterations in the photosynthetic metabolism, both in susceptible and resistant plants (Gomes et al., 2017; Khan et al., 2020; Yanniccari et al., 2012; Zobiole et al., 2012, 2010a). These effects can be the result of a direct consequence of the herbicide, since GLY is known to promote chlorophyll degradation, but can also arise due to the action of AMPA, which is capable of inhibiting chlorophyll biosynthesis, by

reducing the intracellular levels of an important intermediate, δ -aminolevulinic acid (Gomes et al., 2014). Losses in total chlorophylls were previously reported in different models, such as *S. miyabeana* (Gomes et al., 2017), *P. sativum* (Singh et al., 2017a), *S. lycopersicum* (Singh et al., 2017b), *G. max* (Moldes et al., 2008), *Salvinia natans* L. (Liu et al., 2019), and *H. dubia* (Zhong et al., 2018). Furthermore, deleterious effects of GLY on the biosynthesis of carotenoids, amino acids, and fatty acids (Fedtke and Duke, 2005), as well as on the abundance of proteins associated with photosystem (PS) II (Vivancos et al., 2011), have already been suggested by several authors, revealing its indirect effect on the dysregulation of the photosynthetic metabolism. This GLY-mediated inhibition of different photosynthetic players can also arise from its interference with the plant mineral status, inducing deficiency of elements required as metallic co-factors of pigments and enzymes (Gomes et al., 2014). A strong downregulation of processes involved in the photochemical phase of photosynthesis has been largely documented (Mateos-Naranjo and Perez-Martin, 2013; Zobiole et al., 2011). Actually, not only the direct light-dependent reactions of photosynthesis appear to be inhibited by GLY, but also the Calvin cycle, due to poor gas exchange capacity and inhibition of its main enzyme, ribulose-1,5-bisphosphate carboxylase oxygenase (RuBisCO; EC 4.1.1.39) (De María et al., 2005; Mateos-Naranjo et al., 2009). In addition, the intracellular overaccumulation of shikimate, especially in chloroplasts, can shift the carbon flux, since the dysregulation of the shikimate pathway ends up affecting the negative feedback control process (Duke, 1988), resulting in a decreased photosynthetic potential.

Redox homeostasis

Since most of the processes occurring in plant cells are dependent on redox reactions, recognised as one of the most conserved responses across all types of organisms, a proper balance between oxidised and reduced compounds is pivotal for the cellular homeostasis (Dietz, 2003). The tightly and elegant regulation of these dynamic reactions, known as the redox control, ensures the maintenance of the redox homeostasis, essential for cellular growth and development (Das et al., 2015; Kapoor et al., 2015). However, as a consequence of different environmental fluctuations, including xenobiotic (*e.g.* herbicide) exposure, this balance can be jeopardised, resulting in redox disorders (Gill and Tuteja, 2010). In this way, besides directly affecting the normal functioning of particular and specific cellular events, similarly to other stressors, GLY-associated impacts in plant physiology are strongly linked to its ability to induce oxidative damage, by an overproduction of reactive oxygen species (ROS) and/or an inhibition of the antioxidant (AOX) defences (Gomes et al., 2014; Hasanuzzaman et al., 2020). From the available

literature, it appears that GLY can indeed affect the redox homeostasis of plant cells, although the specific ways by which ROS and the AOX players are modulated by the herbicide are not always identical. As shown by different authors, the exposure of plants to soil residues of GLY is capable of inducing great redox disorders, elevating the levels of lipid and protein oxidation. For instance, the exposure of willow plants to sublethal doses of GLY (0, 56.15, 84.21, and 112.30 mM) evoked an overproduction of ROS, accompanied by an upsurge of several AOX metabolites (Gomes et al., 2017). The same pattern was also reported by Singh et al. (2017a), in which pea plants exposed to GLY (40 mg L⁻¹) experienced a marked reduction of growth, this being accompanied by an excessive accumulation of ROS. By applying a proteomic-based approach, Ahsan et al. (2008) found that several AOX enzymes were upregulated in response to both GLY and paraquat in rice (*Oryza sativa* L.) leaves. Similarly, Singh et al. (2017b) reported that GLY exposure resulted in enhanced activities of several AOX enzymes. Due to GLY's unique mode-of-action and particular chelator features, GLY-mediated action on the cellular redox homeostasis emerges as a cascade-like phenomenon, since the induced oxidative damage can then compromise enzyme activity and stability, gene integrity and organelle functions (Sharma et al., 2012). For instance, by depriving plants from essential metals, GLY can decrease the activity of defence enzymes, boosting ROS overaccumulation, downstream hampering other aspects of plant physiology, such as photosynthesis. Actually, it is known that excessive levels of hydrogen peroxide (H₂O₂) can affect thylakoids membrane integrity and inhibit gene expression (Sharma et al., 2019a). Given the relevance of this topic for the present thesis, the following section is entirely dedicated to the plant redox homeostasis, describing: i) the characteristics, generation processes and cellular effects of the main ROS; ii) the antioxidant battery, with regard to its non-enzymatic and enzymatic components; iii) the oxidative challenges faced by plants; and iv) the transgenerational effects of ROS.

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CHAPTER II. AN OVERVIEW OF THE MULTIFACETED PLANT ANTIOXIDANT SYSTEM – KEEPING ROS UNDER CONTROL

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Plants facing oxidative challenges—A little help from the antioxidant networks[☆]



Cristiano Soares^a, Marcia E.A. Carvalho^b, Ricardo A. Azevedo^b, Fernanda Fidalgo^{a,*}

^a GreenUPorto – Centro de investigação em Produção Agroalimentar Sustentável, Biology Department, Faculty of Sciences, University of Porto, Rua Campo Alegre s/n, 4169-007, Porto, Portugal

^b Departamento de Genética, Escola Superior de Agricultura “Luiz de Queiroz”, Universidade de São Paulo, Av. Pádua Dias, 11, CP. 83, CEP 13418-900, Piracicaba, Brazil

Plants facing oxidative challenges - a little help from the antioxidant networks

Abstract

A large number of reviews have discussed many aspects of oxidative burst due to plant exposure to biotic and abiotic stresses, including the dual role of reactive oxygen species (ROS) as both signaling and toxic compounds, and the strategies developed by plants to cope with this oxidative imbalance. In this review, we have concentrated on fresh new information and other promising and emerging topics of oxidative stress and antioxidant (AOX) mechanisms in plants, giving particular attention to genotoxicity, transgenerational alterations and quantitative trait loci (QTL) associated with enhancements in the plant tolerance to stresses. Furthermore, besides the discussion of the “classical” enzymatic and non-enzymatic components of plant defense, novel aspects about the components of the AOX machinery, which now includes sugars, annexins and dehydrins, are also presented, along with a final section on future directions in this field.

Keywords

Antioxidant machinery; cytogenotoxicity; enzymatic antioxidant; non-enzymatic antioxidant; oxidative stress; quantitative trait loci; reactive oxygen species; redox homeostasis; signaling compounds; transgenerational effects

Forward

As plant physiologists, it is amazing to see how this research topic developed over the years and how many efforts have been made for a better understanding of plant metabolism and physiology. Thus, our exercise in this review is not only to present some key aspects on the topics based on the literature, but also to provide the readers with our thoughts about how this field of research is shaping up for the next few years. We obviously do not intend to cover all aspects and all types of stress, but we can point in the direction of aspects that we believe should be the main focus of attention and where we would most likely concentrate efforts so that we continue to make major advances on this and related topics.

What a headache!

The first real problem, which anyone who has been working on this topic will most likely agree with, is that it became literally impossible to track and follow what has been published in the last 5-10 years and there is no light at the end of the tunnel that it will be easier in the coming years. Even if your work is centred on one specific type of stress factor (drought, for instance), the number of papers being published is astonishing. So, how on earth we, or anybody working on these topics, would be able to track these papers and try to select the key findings that have shaken the field? That is hard work and one can easily get a headache by trying to do it.

1. INTRODUCTION

Oxygen-derived atmosphere allowed the appearance of aerobic organisms and several energy generation systems that use molecular oxygen (O_2) as the final electron acceptor, and although molecular oxygen is relatively unreactive, its reduction leads to the production of reactive oxygen species - ROS - which are naturally and continuously produced (about 1-2% of the total consumed O_2) as a consequence of the aerobic cell metabolism (Bhattacharjee, 2005; Mittler 2017). ROS play a dual role depending on their concentration in plant cells: at low levels, they can act as intracellular signaling agents, inducing a positive response in the antioxidant (AOX) system; however, at high levels, all forms of ROS become toxic and capable of interacting with all kinds of organic molecules, such as nucleic acids and lipids (Sharma et al., 2012; Foyer, 2018). Thus, oxidative stress arises from a disproportion between ROS production and elimination, being a complex biochemical and physiological phenomenon (Mittler, 2017). Both biotic and abiotic adverse conditions enhance ROS generation, which requires from plants a rapid and efficient mechanism to manage ROS homeostasis according to the environmental challenges (Mittler 2017). On one hand, the oxidative burst may act as an effective bactericidal mechanism in plants (Dröge et al., 2002). On the other hand, excessive ROS generation can trigger oxidative-induced damages, such as protein oxidation, cytotoxicity, and even DNA abandonment, hence threatening the cellular viability (Sharma et al., 2012; Oldenburg and Bendich, 2015; Carvalho et al, 2018a). In order to maintain the cell redox homeostasis, plants possess a powerful and multifaceted AOX system that is composed by enzymatic and non-enzymatic mechanisms (Figure 1), which are involved in sensing, detoxification, elimination and/or neutralization of ROS (Gratão et al., 2005; Liebthal et al., 2018). The following sections will highlight the chemistry and production sites of the main ROS in plant cells, as well as describe the AOX system in plants with special focus on the latest advances in oxidative stress studies.

2. ROS: TYPES, SOURCES AND FEATURES

The production of ROS is mainly related to two chemical phenomena: i) the transference of energy to the O_2 , arising the production of singlet oxygen ($^1O_2$), and/or ii) the transference of 1, 2 or 3 electrons to oxygen, giving rise to the superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($\cdot OH$), respectively (Mittler, 2017). Some of the reactions that naturally produce ROS, as well as the plant AOX components responsible for their scavenging, are shown in the Table 1.

Table 1. Biological and chemical sources of reactive oxygen species (ROS) and their enzymatic and non-enzymatic scavenger mechanisms

ROS types	Sources		Scavengers	
	Non-enzymatic	Enzymatic	Non-enzymatic	Enzymatic
$^1O_2$	Energy transference from triplet chlorophyll (Chl)	Indirectly dependent on lipoxygenases	β -carotene, α -tocopherol, glutathione (GSH), Flavonoids, Proline, Polyamines, Plastoquinone	
	Energy transference from triplet excited P680			
	Energy transference from triplet carbonyls			
$O_2^{\cdot-}$	Reaction with reduced ferredoxin	Peroxisomal membranes dependent on NAD(P)H	Ascorbate (AsA), GSH, cysteine (Cys), Sugars, Dehydrins, Cytochrome <i>b559</i> , Spontaneous dismutation	Superoxide dismutase (SOD)
	Electron transport chain (ETC) of photosystem (PS) I and PSII ETC of mitochondrial complexes I and III	Xanthine oxidase		
$\cdot OH$	Haber-Weiss reaction		Sugars, AsA, Flavonoids, Proline, Polyamines, GSH	
	Photo-fenton reaction			
	Inner-sphere electron transfer			
H_2O_2	Spontaneous dismutation of $O_2^{\cdot-}$	NADPH oxidase, Polyamine oxidases (PAO), Acetylated polyamine oxidase (APAO), Diamine oxidase (DAO), Xanthine oxidase (XOD), Glycolate oxidase (GOX), Acyl-CoA oxidase, Sulphite oxidase, Glutathione or ascorbate oxidase, Class III peroxidases, pH dependent cell-wall peroxidases, Urate oxidase, Sarcosine oxidase, SOD, Oxalate oxidase	Flavonoids, AsA, Cys, Met	Catalase (CAT), ascorbate peroxidase (APX), GSH peroxidase (GPX), guaiacol peroxidase (GPOX), glutathione S-transferase (GST)

2.1. Singlet oxygen

Singlet oxygen ($^1\text{O}_2$) production is linked to the energy dissipation from chlorophyll triplet state to O_2 , being induced under strong light conditions and/or low carbon dioxide (CO_2) assimilation rate, situations when this ROS can damage photosystem I and II (PSI and PSII, respectively) (Gill and Tuteja, 2010). It has been recently shown an extra pathway for $^1\text{O}_2$ production, which is catalysed by lipoxygenase that subsequently leads to the formation of triplet carbonyls ($^3\text{L}=\text{O}^*$), another electronically excited species that transfers energy to the O_2 resulting in $^1\text{O}_2$ generation (Prasad et al., 2017). Regarding its chemistry, $^1\text{O}_2$ is a highly reactive radical, with a short life-time between 4-100 μs , able to react with different biological molecules, triggering lipid peroxidation and oxidising proteins, fatty acids and nucleic acids (Mittler 2017; Singh et al., 2018). Cellular metabolites, such as β -carotene, tocopherol or plastoquinone, are able to quench $^1\text{O}_2$ that, when in excess, also triggers the up-regulation of several defense genes (Krieger-Liszkay et al., 2008).

2.2. Superoxide anion

The superoxide radical ($\text{O}_2^{\cdot-}$) is usually the first ROS to be produced, and its generation is mainly associated with electron transport chains (ETC), whereby the major sources of $\text{O}_2^{\cdot-}$ within plant cells are mitochondria and chloroplast in complexes I and III, and PSI and PSII, respectively (Noctor et al., 2006; Sharma et al., 2012). However, its production in other organelles, such as peroxisomes, can also take place (Gill and Tuteja, 2010). When compared to other ROS, $\text{O}_2^{\cdot-}$ is classified as a moderate reactive radical with a short half-life and low mobility, due to its negative charge and consequent inability to cross biological membranes (Demidchik, 2015). The superoxide radical cannot directly interfere with organic macromolecules and its toxicity is associated with its powerful reducing ability, hence changing Fe^{3+} to Fe^{2+} that can later interact with H_2O_2 and give rise to the production of $\cdot\text{OH}$, which is one of the most toxic ROS (Ahmad et al., 2008; Demidchik, 2015; Mittler 2017). This reaction is globally known as the Haber-Weiss reaction, being its last step, where Fe^{2+} interacts with H_2O_2 , referred to as Fenton's reaction (Cuypers et al., 2016). Moreover, $\text{O}_2^{\cdot-}$ can suffer a process of protonation, inducing the production of hydroperoxyl radical ($\text{HO}_2^{\cdot-}$), a more reactive and stable molecule, permeable through biological membranes (Bielski et al., 1983).

2.3. Hydrogen peroxide

Hydrogen peroxide (H_2O_2) and $\text{O}_2^{\cdot-}$ are considered primary ROS, but the first one can impose a more severe oxidative stress condition due to its higher stability when compared to $\text{O}_2^{\cdot-}$ (Sharma et al., 2012). H_2O_2 production is coupled to the electron transport in ETCs of different organelles (e.g. mitochondria, chloroplast, endoplasmic reticulum and plasma membrane), photorespiration metabolism and β -oxidation of fatty acids (Sharma et al., 2012; Mittler, 2017). The polyamine oxidases (PAOs; EC 1.5.3.17), enzymes from polyamine metabolism, also release H_2O_2 as one of their byproducts in the apoplast (Pottosin and Shabala, 2014; Handa et al., 2018a) and probably in the peroxisomes (Hao et al., 2018). The high toxicity of H_2O_2 can be easily explained by its chemical nature: it has no unpaired electrons and possesses a relatively long half-life (1 ms), so it is able to cross biological membranes and to diffuse across long distances, increasing the number of potential sites of action (Gupta et al., 2015). Calvin cycle-related enzymes are extremely sensitive to H_2O_2 and high levels of this ROS can directly reduce CO_2 assimilation (Scandalios, 1993).

2.4. Hydroxyl radical

Hydroxyl radical ($\cdot\text{OH}$) is the most dangerous and reactive ROS, which is produced as a result of the Haber-Weiss reaction, due to the interaction between $\text{O}_2^{\cdot-}$ and H_2O_2 in the presence of redox-active metals such as copper (Cu) and iron (Fe) (Cuyper et al., 2016). Interestingly, *in vitro* assays provided clues about the possibility of a direct role of cadmium (Cd) and zinc (Zn), which are usually considered as physiologically non-redox-active metals, on $\cdot\text{OH}$ generation through Fenton-like reactions (Kuznetsov et al., 2014). $\cdot\text{OH}$ radical has a very short half-life of around 1 ns (Mittler 2017); therefore, its major targets and sites of action are closely located to its production site (Sharma et al., 2012). In addition to its chemical features that lead to a high reactivity and, consequently, a high toxicity, $\cdot\text{OH}$ can cause serious damage to all organic molecules despite its extremely short life-time, and such potential damages can be enhanced because there is no enzymatic mechanism responsible for its degradation and metabolism. Not by coincidence, high levels of $\cdot\text{OH}$ are involved in programmed cell death (PCD) (Gill and Tuteja, 2010; Sharma et al., 2012; Demidchik, 2015).

3. ANTIOXIDANT MACHINERY

3.1. Non-enzymatic components

The non-enzymatic AOX system comprises diverse molecules, especially low mass metabolites like glutathione (GSH), ascorbate (AsA), flavonoids and proline (Pro) (Figure 1), that are able to neutralise, remove and/or transform ROS, allowing the management and sensing of ROS homeostasis in order to achieve the cellular redox balance in plants under stress (Gratão et al., 2005; Mittler, 2017; Carvalho et al., 2018d). The efficiency and behaviour of non-enzymatic AOX machinery to stress depend on diverse factors, such as type of stress, time-length of exposure and its intensity, plant species and their genotypes, organ or tissue, among others (Carvalho et al., 2018c, Borges et al., 2018; Piotto et al., 2018). Therefore, both positive and negative responses and outcomes from the non-enzymatic AOX have been largely reported in plants under exposure to different stressors like salinity (Ahanger and Agarwal, 2017; Farhangi-Abriz and Torabian, 2017; Gadelha et al., 2017;), drought (Çelik et al., 2017; Lima et al., 2018), extreme temperatures and high light (Szymańska et al., 2017 and references therein), metal/metalloid toxicity (Soares et al., 2016a; López-Orenes et al., 2017; Handa et al., 2018), nanoparticles (Arruda et al., 2015; Doğaroğlu and Köleli, 2017; Salehi et al., 2018; Soares et al., 2018a) and xenobiotics (Sharma et al., 2016a,b,c; Soares et al., 2018b; Shahzad et al., 2018).

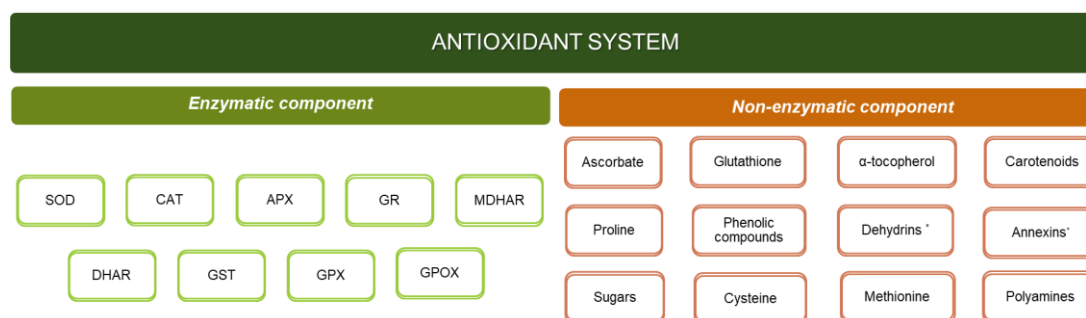


Figure 1. Enzymatic and non-enzymatic antioxidant (AOX) players in a typical plant cell. Words marked with a * represent new emerging components of the plant AOX system.

3.1.1. Proline

Proline (Pro) acts by (i) avoiding ROS production and (ii) scavenging them (Sharma et al., 2006; Signorelli et al., 2014). Pro may scavenge $\cdot\text{OH}$ through a reaction that converts this amino acid to γ -aminobutyric acid (Signorelli et al., 2014); however, Pro is not directly involved in the protection against $\text{O}_2^{\cdot-}$, nitric oxide, nitrogen dioxide and peroxynitrite (Signorelli et al., 2016a). Since NADPH consumption for Pro synthesis is necessary, this

amino acid acts as an electron sink and prevents ROS generation (Sharma et al., 2006). In this context, a new Pro role in green tissues of plants under stress was recently proposed: acting as NAD⁺ regenerator, Pro accumulation could be a mechanism to avoid photo-inhibition in a manner analogous to fermentation (Signorelli et al., 2016b).

Pro biosynthesis can be achieved by two different pathways: via glutamic acid or via ornithine, being observed that the first is the most frequent, when Pro is synthesised from glutamate through the intermediate Δ^1 -pyrroline-5-carboxylate (P5C), a reaction that involves the catalytic activity of two different enzymes, Δ^1 -pyrroline-5-carboxylate synthetase (P5CS; EC 2.7.2.11) and Δ^1 -pyrroline-5-carboxylate reductase (P5CR; EC 1.5.1.2) (Gill and Tuteja, 2010). Current studies have shown that, in addition to transcriptional control, Pro production is modulated at post-transcriptional level, which may depend on pyridine nucleotide pools (NADP vs NAD), as well as the concentrations of Pro, chloride (Gilberti et al., 2014), and metabolites from lysine catabolism (Azevedo and Arruda, 2010; Kiyota et al., 2015). Yet, it was recently suggested that Pro synthesis in plants under Cd exposure is stimulated by decreases in Fe concentration (Sharmila et al., 2017), so Fe starvation, which is commonly reported as a Cd-induced effect coupled to negative outcomes in leaves, can be a possible mechanism actively modulated to increase Pro concentration and improve plant tolerance to Cd toxicity.

3.1.2 Cystein

Cysteine (Cys) and methionine (Met) are the principal sulfur (S)-containing amino acids because they are two of the canonical 20 amino acids that are incorporated into proteins (Brosnan and Brosnan, 2006). Due to the presence of a thiol group, Cys exhibits a reducing power that enables it to participate in redox reactions (Kim et al., 2018). Thiol groups and S-containing amino acids are very susceptible sites for attack by ROS, so that activated oxygen can use an H atom from Cys residues to form a thiyl radical that will cross-link to a second thiyl radical to form a disulphide bridge (Sharma and Dietz, 2006). A high AOX action of Cys has been confirmed in assays using different oxidant compounds, with Cys exhibiting the highest scavenging activity for O₂^{•-}, but the lowest activity (~12.6%) for H₂O₂ when compared to others S-containing amino acids such as Met (~20.7%) and taurine (Tau – an amino sulfonic acid) (~52%) (Kim et al., 2018). The role of Cys in plant response to stress also involves sensing of ROS by key peptides/proteins through oxidation of conserved Cys residues (Sharma and Dietz, 2006). Furthermore, Cys is a substrate for the production of hydrogen sulfide (H₂S), an emerging gasotransmitter that, by inducing alternative respiration capacity, AOX activity and metallothionein genes expression, has been shown to enhance plant tolerance to Cd exposure (Jia et al., 2016).

Yet, Cys metabolism is also required for Met, GSH, phytochelatin and metallothionein biosynthesis, so it is a central metabolite in AOX defense and metal sequestration (Sharma and Dietz, 2006). In comparison to Met, Cys exhibited the highest chelating activities against Cu^{2+} and Zn^{2+} during *in vitro* assay, although it was not able to chelate Fe^{2+} (Kim et al., 2018).

3.1.3. Methionine

A fraction of the Met residues is surface exposed in some proteins, being susceptible to oxidation to Met sulfoxide residues (Brosnan and Brosnan, 2006). This amino acid can play direct and indirect roles in the reduction of ROS generation and/or alleviation of their potential damaging effects due to the presence of S in its structure (Brosnan and Brosnan, 2006; Sharma and Dietz, 2006). Accordingly, it was shown that Met intramembrane accumulation, which was achieving in certain organisms through an evolutionary strategy involving deviant genetic code, increases AOX and cytoprotective properties in living cells (Bender et al., 2008). A recent study revealed that Met exhibited, under *in vitro* conditions, higher H_2O_2 scavenging capacity than Cys (Kim et al., 2018). According to these authors, Met was also able to chelate heavy metals such as Cu^{2+} and Zn^{2+} , although with a lower efficiency in comparison to Cys and ethylenediamine tetraacetic acid (EDTA) (natural and artificial chelators, respectively), indicating its possible role in limiting ROS generation at the source. In addition, Met is the precursor of several compounds with different roles in plants, such as nicotinamide (management in metal homeostasis), ethylene (cell signaling) and polyamines (AOX defense) (Sharma and Dietz, 2006), reinforcing the idea that this amino acid has a number of functions in plant response to oxidative stress.

3.1.4. Glutathione

The tripeptide glutathione (GSH) is a non-protein thiol that is able to chemically react with $\text{O}_2^{\cdot-}$, $\cdot\text{OH}$ and H_2O_2 , functioning as an efficient radical scavenger (Sharma et al., 2012). GSH also acts as a cellular buffer, contributing to the maintenance of the reduced state of several cell components during both normal and stressful conditions (Foyer and Noctor, 2005); for instance, GSH is needed for ascorbate (AsA) regeneration because it is the substrate for dehydroascorbate reductase (DHAR; EC 1.8.5.1). GSH is synthesised in the cytosol and chloroplasts by specific enzymes - glutamylcysteine ligase (EC 6.3.2.2) and glutathione synthetase (EC 6.3.2.3) (Gill and Tuteja, 2010), but it was also detected in vacuoles, endoplasmic reticulum and mitochondria (Mittler and Zilinskas, 1993; Jiménez et al., 1998).

Due to its reducing power, GSH is also coupled to protein synthesis, enzymatic regulation and expression of stress-responsive genes (Gill and Tuteja, 2010; Sharma et al., 2012). In this context, the ratio between reduced GSH and oxidised glutathione (GSSG) is a valuable information about the redox state of the cell. The increased amount of GSH relative to GSSG is accomplished by the action of glutathione reductase (GR; EC 1.6.4.2), by increasing GSH biosynthesis and/or GSSG degradation or, alternatively, due to their long-distance transport (Gill and Tuteja, 2010). Yet, such mobility is also important for the distribution of S among plant parts (Noctor et al., 2002).

3.1.5. Ascorbic acid

Ascorbic acid (AsA), commonly known as vitamin C, is the most abundant AOX metabolite in plant cells (Smirnoff, 2008). It is capable of directly interacting with different ROS, neutralising the toxic effects of $^1\text{O}_2$, $\text{O}_2^{\cdot-}$ and $\cdot\text{OH}$, as well as acting as an electron donor in enzymatic reactions leading to reductions in the content of H_2O_2 through ascorbate peroxidase (APX; EC 1.11.1.11) activity (Smirnoff, 2008; Gill and Tuteja, 2010; Sharma et al., 2012). This water soluble AOX, which can reach up to 300 mM in plant cells (Smirnoff, 2008), is found in distinct subcellular compartments, with chloroplasts representing 30-40% of cell's total AsA content (Gill and Tuteja, 2010). A recent study, however, showed that elevated AsA concentrations might act as a pro-oxidant in the presence of high H_2O_2 concentrations, stimulating the Fenton reaction and contributing to the enhancement of oxidative stress in rice leaves when subjected to intense light radiation (Castro et al., 2018).

Regarding its biosynthesis, AsA is produced in the mitochondria by L-galactono- γ -lactone dehydrogenase (EC 1.3.2.3), being posteriorly transported to other organelles via active transport or facilitated diffusion (Sharma et al., 2012). Upon normal conditions, the major content of AsA corresponds to its reduced form, whose pool is maintained due to monodehydroascorbate reductase (MDHAR; EC 1.6.5.4) and DHAR activities (Gill and Tuteja, 2010), as shown in this article's sub-section named "ascorbate-glutathione (AsA-GSH) cycle enzymes". In addition, AsA actively participates in the control of mitosis, cellular elongation, senescence and cell death, also acting as stabiliser of enzymes with prosthetic metallic ions (Ahmad et al. 2008; Gill and Tuteja, 2010; Queirós, 2012).

3.1.6. Carotenoids

Carotenoids, a class of lipophilic compounds that has more than 700 species, are one of the most abundant naturally occurring pigments produced by both photosynthetic (cyanobacteria, plants and algae) and non-photosynthetic organisms (some bacteria, fungi

and invertebrates) (Nisar et al., 2015). They can be distinguished in two classes: the oxygen-free carotenoids, called carotenes (*e.g.* β -carotene and lycopene), and their oxygen-containing derivatives, called xanthophylls (*e.g.* lutein and zeaxanthin) (Havaux, 2014). In photosynthetic organs, these low-molecular-weight metabolites can inhibit the production of $^1\text{O}_2$ by quenching both triplet sensitiser and excited chlorophyll, protecting the photosynthetic machinery (Li et al., 2008) and reducing lipid peroxidation (Gill and Tuteja, 2010). According to Havaux (2014), since β -carotene is close to the primary site of $^1\text{O}_2$ production in chloroplasts (*i.e.* the PSII reaction center), its oxidation can be considered as an early event during photostress, so that β -carotene oxidation metabolites may constitute primary sensors of light stress in plants. In addition to their role as accessory pigments responsible for absorbing light at 400 and 550 nm in the antenna complexes, carotenoids and their sub-products are also crucial for the assembly of PS, as well as for developmental regulation by directly modulating the production of two plant hormones, strigolactones and abscisic acid (ABA), since carotenoids serve as their precursors (Cazzonelli, 2011; Ruiz-Sola and Rodríguez-Concepción, 2012; Havaux, 2014; Nisar et al., 2015).

3.1.7. Flavonoids

Flavonoids, a class of secondary metabolites which comprises more than 10000 substances, are a group of phenolic compounds exclusively produced by plant organisms (Agati and Tattini, 2010; Pollastri and Tattini, 2011). Based on their chemical structure, they are classified as anthoxanthins (where flavones and flavonols are included), flavanones, flavanonols, flavans and anthocyanidins (*e.g.* anthocyanins) (Gill and Tuteja, 2010). The AOX capacity of flavonoids is due to their capacity to directly interacting with ROS (*e.g.* $^1\text{O}_2$ and H_2O_2), but also due to their ability to serve as substrate for different peroxidases (Pourcel et al., 2007; Hernández et al., 2009). Accumulation of flavonoids takes place in different plant parts (from leaves to pollen), in exudates on leaf surface and in external appendices such as trichomes. In terms of organelles, these metabolites can be found in the cell wall, chloroplast, vacuole, endoplasmic reticulum, and nucleus (Gill and Tuteja, 2010; Agati et al., 2012).

In vacuoles, the modulation of peroxidase activity may depend on flavonoids, since ascorbate has low affinity to vacuolar peroxidases; thus, it appears that flavonoids have a major role in the vacuolar H_2O_2 detoxification, being oxidised by peroxidases and then regenerated by AsA (peroxidase-flavonoid-ascorbate system) (Gill and Tuteja, 2010 and references therein; Agati et al., 2012). It is proposed that flavonoids can act synergistically with other $^1\text{O}_2$ neutralising AOX like carotenoids, reducing the exit of this ROS from the

chloroplast and, consequently, limiting its oxidative damage on the nuclear DNA (Agati et al., 2007; 2012). Moreover, since flavonoids are able to interact with the polar region of phospholipids, these AOX molecules contribute to the membrane lipid homeostasis, thus preventing envelope membrane disruption and oxidative injury (Erlejman et al., 2004). In addition, derivatives of quercetin found in nuclei can inhibit the production of $\cdot\text{OH}$, even at high H_2O_2 concentrations, thus preventing ROS-induced stress on the DNA (Brown et al., 1998).

3.1.8. α -Tocopherol

Tocopherols, which include four isomers (α -, β -, γ -, δ -tocopherol), are characterised by a specific number and position of methyl (CH_3) groups in the 2-methyl-6-cromanol ring. Tocopherols are found in the plastids, associated with the envelope and thylakoid membranes, and in plastoglobuli. The most abundant isomer is α -tocopherol (90% of the foliar total tocopherol content), which possesses the highest AOX activity due to the presence of three CH_3 groups in its molecular structure (Blokhina et al., 2003; Foyer and Noctor, 2003). α -Tocopherol is particularly active in the thylakoid membranes, where it can directly interact with $^1\text{O}_2$, $\cdot\text{OH}$, and also with some lipid radicals derived from the oxidation of the polyunsaturated fatty acids (PUFAs), thus preventing lipid peroxidation. α -Tocopherol can neutralise $^1\text{O}_2$ through an energy transference mechanism, leading to the production of different quinones and epoxides. α -Tocopherol quinone, one of these products, exhibits AOX properties identical to α -tocopherol and seems to be involved in the PSII energy dissipation (Munné-Bosch and Alegre, 2002a).

Considering the impossibility of regenerating α -tocopherol from sub-reaction products like quinones and other oxidised derivatives, AOX properties may be impaired. By contrast, the reaction of α -tocopherol with lipid peroxidation (alkoxy, peroxy radicals) produces tocopheroxy radicals that allow the regeneration of α -tocopherol through intervention of AsA, GSH and co-enzyme Q (Munné-Bosch and Alegre, 2002b). In chloroplasts, α -tocopherol preserves the integrity of the membranes and increases the stiffness of these structures, influencing fluidity and the permeability for small molecules and ions (Munné-Bosch and Alegre, 2002a). The role of α -tocopherol in membrane stability associated with its contribution to the redox homeostasis in chloroplasts (Munné-Bosch and Alegre, 2003; Lin et al., 2004; Munné-Bosch, 2005; Shao et al., 2008), as well as to the regulation of the concentration of some phytohormones, such as jasmonic acid (Souza et al., 2017), leads to the assumption that α -tocopherol may interact with the main components of the signal transduction pathways, suggesting that tocopherol's functions exceed the AOX activity (Munné-Bosch and Alegre, 2002a; Hyun et al., 2011).

3.1.9. Polyamines

Polyamines (PAs) are small aliphatic organic compounds widely distributed in nature and their ubiquity across all kingdoms of life implies that these compounds play seminal roles in cells, as corroborated by modifications from gene expression to cell proliferation when the cellular PAs content is modified (Ioannidis and Kotzabasis, 2014; Miller-Fleming et al., 2015; Pál et al., 2017). The critical role of PAs in stress tolerance is indicated by a number of evidence: (i) the transcript levels of PAs biosynthetic genes and the activities of their encoded enzymes are induced by stresses; (ii) the elevation of endogenous PAs levels by exogenous supply, or overexpression of PA biosynthetic genes, results in enhanced stress tolerance; and (iii) the reduction of endogenous PAs is accompanied by compromised stress tolerance (Liu et al., 2015). Putrescine (Put), spermidine (Spd) and spermine (Spm) are three major PAs in plants, in which some of others types of PAs, like cadaverine (Cad), can be found (Miller-Fleming et al., 2015).

These low-molecular-weight compounds change ROS homeostasis by modifying AOX systems and modulating ROS generation (Das and Misra, 2004; Liu et al., 2015). For instance, *in vitro* studies showed that Put, Spd, Spm and Cad are powerful $\cdot\text{OH}$ scavengers, whilst Spd or Spm may also quench $^1\text{O}_2$ at higher concentrations (Das and Misra, 2004). Indirectly, PAs may potentially protect the genetic material and enzymes from oxidative-induced damages due to their capacity, as polycations, of binding to distinct anionic macromolecules, such as DNA, RNA, chromatin and proteins (Alcázar et al., 2010). However, PA metabolism also generates H_2O_2 during reactions catalysed by enzymes collectively named PA oxidases (PAOs; EC 1.5.3.17), such as acetylated polyamine oxidase (APAO; EC 1.5.3.13), the spermine oxidase (SpmO; EC 1.5.3.16), and diamine oxidase (DAO; EC 1.4.3.22) (Miller-Fleming et al., 2015). Usually presented as organic polycations, PAs are bases that can be found in a charged or uncharged form; the latter represents less than 0.1% of the total PAs pool but it may exert a crucial role in cell chemiosmosis (Ioannidis and Kotzabasis, 2014).

3.1.10. Sugars

Water-soluble sugars such as glucose and sucrose, and water-soluble carbohydrates derived from sucrose (sucrosyl oligosaccharides, which includes the raffinose family oligosaccharides and fructans), are recognised as compounds necessary for the coordination of plant responses to oxidative stresses (Van den Ende and Valluru, 2009), possibly by a direct reaction with ROS and by inducing the expression of genes related to the production of other AOX compounds, for instance, Pro (Sami et al., 2016). Several studies have demonstrated that plant-derived sugars show $\cdot\text{OH}$ scavenging capabilities

during Fenton reactions with Fe^{2+} and H_2O_2 , triggering the formation of less detrimental sugar radicals that may undergo regeneration to non-radical carbohydrates (Nishizawa et al., 2008; Peshev et al., 2013). Fructans are probably involved in ROS-scavenging systems of vacuoles (Peshev et al., 2013), while raffinose may play a role in chloroplastidic ROS detoxification (Nishizawa et al., 2008; Schneider and Keller, 2009). Van den Ende and Valluru (2009) have hypothesized that prevention of lipid peroxidation is potentially mediated by membrane-associated fructans that, due to their ideal positions in membranes, may react with some ROS, such as $\text{O}_2^{\cdot-}$ and $\cdot\text{OH}$, retrieving H to form water and generating oxidised fructan free radicals, which might be rapidly reduced again to fructans by AsA or by other vacuolar AOXs (phenolic compounds and anthocyanins). According to these authors, NADPH oxidase/peroxidase / fructan/phenolic compound's system within the tonoplast, which can be associated with the inner side of the tonoplast (peroxidase /fructan/ phenolic compounds) and be present in the vacuolar lumen (fructan/ phenolic compounds), may be linked with the cytoplasmic redox systems.

Further investigations provided evidence for the occurrence of these reactions with sugars in tissues of control and stressed *Arabidopsis* plants, in which the expected sugar recombination and degradation products were observed (Matros et al., 2015). In this same work, oxidation products of endogenous sugars were also assessed in barley, which exhibited increased abundance in comparison to the non-oxidised precursor during oxidative stress conditions, indicating that such non-enzymatic reactions with the $\cdot\text{OH}$ are included into plant AOX mechanisms. In line with these evidence, it has been recently shown that the overexpression of alkaline/neutral invertase gene, which produces an enzyme that hydrolyses sucrose irreversibly into glucose and fructose, provided a greater reducing sugar content, concurrently conferring an enhanced tolerance to multiple stresses (cold, high salinity and drought), due to lower ROS levels, reduced oxidative damages, decreased water loss rate, and increased photosynthesis (Dahro et al., 2016).

3.1.11. Emerging components

3.1.11.1. Dehydrins

Dehydrins, a class of LEA (*late embryogenesis abundant*) proteins, accumulate abundantly in plants under diverse abiotic stresses, such as water, salt and temperature stress, when they can act in sequestering ions, stabilising membranes, or as chaperones (Tunnacliffe and Wise, 2007). Currently, their role as radical scavengers has been reported (Halder et al., 2016), and a recent work provided direct evidence for the protection granted by dehydrins to isolated chloroplasts when added externally during oxidative stress conditions, and also when synthesised *in planta* (Halder et al., 2018). These authors

showed that, by overexpressing dehydrin genes in transgenic tobacco, two *Sorghum bicolor* L. dehydrins (*SbDHN1* and *SbDHN2*) could protect plants by scavenging $O_2^{\cdot-}$ or by conferring an overall protective effect to the enzymes responsible for dismutation of this free radical. Accordingly, the heterologous expression of *ZmDHN13*, a maize dehydrin gene that is induced by H_2O_2 , led to decreases in $O_2^{\cdot-}$ generation and reductions in the malondialdehyde (MDA) content in tobacco subjected to oxidative stress (Liu et al., 2017).

In another report, overexpression of the *Hevea brasiliense* dehydrins, *HbDHNs*, in *Arabidopsis thaliana* L. Heynh. led to increases in tolerance to salt, drought and osmotic stresses in transformed plants, which exhibited higher activity levels of AOX enzymes, and lower accumulation of H_2O_2 and $O_2^{\cdot-}$ (Cao et al., 2017). By contrast, the silencing of *CaDHN3*, which is a dehydrin gene induced by the hormones ABA and methyl jasmonate (MeJA), resulted in decreases in the tolerance to abiotic stresses (cold, salt and mannitol) in transformed plants when compared to the control ones (Jing et al., 2016). According to Liu et al. (2017), dehydrins ability to mitigate oxidative stress is due to: (i) their capacity to bind metal ions, inhibiting ROS production at the source; (ii) their high content of AOX amino acids such as lysine, histidine and glycine; (iii) their skill to non-specifically bind proteins and membranes, protecting their function and structure; and (iv) their capability to bind DNA, which may repair or protect the DNA from damage caused by environmental stresses. According to Hanin et al. (2011), oxidation of the amino acid residues occurs when dehydrin reacts with ROS, whereas covalent bonds are formed during metal ion binding.

3.1.11.2. Annexins

Annexins, an evolutionarily conserved family of proteins, are involved in membrane trafficking, cytoskeletal organization, cellular homeostasis and ion transport (Yadav et al., 2018). They also have been associated to distinct protective mechanisms for the mitigation of oxidative stress in plants, including peroxidase activity (Gorecka et al., 2005; Mortimer et al., 2009). They are abundant cytosolic proteins (up to 2% of the total soluble protein pool) that possess redox-sensitive Cys, which confer them the ability to participate in the cellular protein thiol pool (Szalonek et al., 2015). According to these authors, the overexpression of an endogenous annexin (STANN1) in potato (*Solanum tuberosum* L.) provided an increased plant tolerance to drought by enhancing the capacity of cytosolic AOX buffer. STANN1 (which contains two Cys residues) may probably prevent ROS overaccumulation by either direct ROS neutralization and further regeneration through NADPH-dependent thioredoxin/glutaredoxin systems, so functioning as acceptor of

excess electrons leaking from over-reduced photosynthetic ETC. Alternatively, STANN1 may be used as an acceptor for ROS diminishing GSSG formation (Szalonek et al., 2015).

Recent studies have confirmed and provided evidence that annexins are especially relevant for plant tolerance against salt stress (Ijaz et al., 2017; Ahmed et al., 2018). It was shown that overexpression of the annexin gene *AnnSp2* enhanced drought and salt tolerance through modulation of ABA synthesis and ROS scavenging in *AnnSp2*-transgenic plants, which exhibited higher total chlorophyll content, lower lipid peroxidation levels, increased AOX enzyme activities and higher levels of Pro in comparison to non-transgenic plants (Ijaz et al., 2017). Accordingly, heterologous expression of *Brassica juncea* L. annexin, *AnnBj2*, also conferred salt tolerance and ABA insensitivity in transgenic tobacco seedlings (Ahmed et al., 2018). It was proposed that annexins may not only mediate increases in the cytosolic Ca^{2+} level, but also sense it and interact with distinct Ca^{2+} -dependent protein kinases (CDPK, CBL-CIPK), hence forming a complex with Ca^{2+} that can regulate downstream components, which include the phosphorylation of different transcription factors like *NAC*, *MYB*, *AP2/ERF*, *WRKY*, *bZIP* and *bHLH*. All of this leads to the transcriptional activation, either ABA-dependent or independent, of different salt and osmotic stress-responsive genes (Yadav et al., 2018).

3.2. Enzymatic components

Together with the non-enzymatic components of the AOX machinery, the enzymatic players provide a complex and multifaceted protective mechanism to maintain ROS homeostasis in order to avoid oxidative-induced damages in plant cells and support plant development (Gratão et al., 2015; Mittler, 2017). Superoxide dismutase (SOD; EC 1.15.1.1), several peroxidases like catalase (CAT; EC 1.11.1.6) and guaiacol peroxidase (GPX; EC 1.11.1.9), glutathione S-transferase (GST; EC 2.5.1.18), and a set of enzymes from the ascorbate-glutathione (AsA-GSH) cycle, which includes ascorbate peroxidase (APX), glutathione reductase (GR), monodehydroascorbate reductase (MDHAR) and dehydroascorbate reductase (DHAR), take part of the enzymatic AOX components (Figure 1). Considering their role as ROS scavengers, variations in their activity and/or transcript accumulation are a common feature in plants under biotic and abiotic stresses, as those induced by water deprivation (Ali et al., 2017; Antoniou et al., 2017; Zhou et al., 2017; Yildizli et al., 2018), salinity (Ali et al., 2017; Sarabi et al., 2017; Siddiqui et al., 2017), and exposure to nanoparticles (Soares et al., 2016b; 2018a; Da Costa and Sharma, 2016; Tripathi et al., 2016; Salehi et al., 2018) and organic compounds (Sharma et al., 2017a; de Sousa et al., 2017; Soares et al., 2018b; Zhong et al., 2018).

3.2.1. Superoxide dismutase

Superoxide dismutase (SOD), an ubiquitous protein found in diverse aerobic organisms such as bacteria, animals and plants, is considered the first enzymatic defence line against oxidative stress (Gratão et al., 2005). It catalyses the $O_2^{\cdot-}$ dismutation in H_2O_2 and molecular oxygen, thus having a pivotal role in ROS detoxification by affecting the levels of both $O_2^{\cdot-}$ and H_2O_2 and preventing the toxicity associated to $O_2^{\cdot-}$ (Abouzari and Fakheri, 2015). Additionally, SOD is able to tightly coordinate the production of $\cdot OH$ generated from Waber-Weiss reaction as consequence of $O_2^{\cdot-}$ removal (Gupta et al., 2018). This metalloenzyme, whose intracellular levels can reach up to 10 μM (Fink and Scandalios, 2002), can be classified in three classes within higher plants, depending on the ion present in its active center: Cu/Zn-SOD, manganese (Mn)-SOD and Fe-SOD (Perry et al., 2010; Sharma et al., 2012). Structurally, Fe-SOD and Mn-SOD are closely related, although Fe cannot replace the Mn ion in the active center; Cu/Zn-SOD, by possessing two metallic ions in its structure, has distinct chemical and physical properties, which result in differences at the structural level (Scandalios, 1997).

The identification of SOD isoforms can be experimentally performed by negative staining in accordance to their sensitivity to potassium cyanide (KCN) and H_2O_2 , being Cu/Zn-SOD sensitive to both inhibitors, Fe-SOD sensitive to H_2O_2 and Mn-SOD resistant to KCN and H_2O_2 (Azevedo et al., 1998). Based on previous phylogenetic studies, it is supposed that the evolution of SODs isoenzymes is related to changes in the availability of the metallic ion. Thus, it appears that Fe-SOD is the oldest group of SODs, since Fe^{2+} was initially more abundant than Cu^{2+} and Mn^{2+} (Alscher et al., 2002; Mittler, 2017). Nevertheless, all SOD isoenzymes are encoded by nuclear genes, being, after translation, transported to other cellular compartments due to a NH_2 -terminal target sequence (Pan et al., 2006). Although the number of isoenzymes, as well as their relative abundance, are dependent on plant species and environmental circumstances, Cu/Zn-SOD is the most abundant form (Gill and Tuteja, 2010; Singh et al., 2018).

SOD isoenzymes can be differentially found in several subcellular compartments: Cu/Zn-SOD is generally present in the cytosol, chloroplasts, peroxisomes and apoplast; Mn-SOD is fundamentally associated with the mitochondrial matrix, despite its reported occurrence also in the peroxisome; Fe-SOD, a specific plant SOD, is found in the chloroplasts, coupled with thylakoid membranes (Gill and Tuteja, 2010). The regulation of SOD expression and activity depends on both development and environmental aspects (Bowler et al., 1992), including exposure to ozone (Azevedo et al., 1998) and heavy metals such as Cd (Carvalho et al., 2018a,c,d) and Cu (Fidalgo et al., 2013; Branco-Neves et al.,

2017). Indeed, given the high AOX activity of SOD, its overexpression in plant species can be an efficient tool to increase abiotic and biotic stress tolerance.

3.2.2. Catalase

Catalase (CAT), which is a tetrameric heme-containing protein with enzymatic function found in different taxa of aerobic organisms, was the first AOX enzyme to be discovered and functionally characterised (Sharma et al., 2012). Catalase is responsible for the intracellular detoxification of H_2O_2 by converting it into H_2O and O_2 (Gill and Tuteja, 2010; Weydert and Cullen, 2010). Although there are several enzymes involved in H_2O_2 degradation, CAT occupies a central role in this scavenging process because it does not require any reducing power (Gill and Tuteja, 2010). Moreover, CAT exhibits one of the highest turn-over rates among AOX enzymes, in which one CAT molecule is able to reduce 6 million H_2O_2 molecules per minute (Gill and Tuteja, 2010). Despite the high specificity of CAT to H_2O_2 , its activity is only efficient when high levels of H_2O_2 are present because its affinity for H_2O_2 is relatively lower than other enzymes, such as APX and other peroxidases (Mittler, 2002).

The localization of CAT in plant cells is intrinsically related to the sources of H_2O_2 . Given the aerobic metabolism of peroxisomes, CAT is commonly found in this organelle, although several authors have already reported its occurrence in other subcellular compartments, such as mitochondria, chloroplasts and cytosol (Corpas et al., 2001). Thus, though displaying a more restrict location than SOD, CAT is also very important for limiting H_2O_2 diffusion across plant cells (Bowler et al., 1992). Plant organisms have three main classes of CATs that are classified according to their expression profiles: class I is present in photosynthetic tissues and are light-dependent; class II is majorly found in vascular tissues; and class III is detected in seeds and early stages of seedling's development (Gill and Tuteja, 2010).

In *Arabidopsis*, the CAT gene family comprises three genes (*cat 1-3*), whose expression differs from control and stress conditions, thus reinforcing the involvement of CAT in plant stress responses. Moreover, gene-silencing studies revealed that the knockdown of *cat2* had a much stronger negative effect on total CAT activity than the knockdown of the other two genes (Mhamdi et al., 2010). In a similar manner to SOD, changes in CAT activity are often correlated to the establishment of oxidative stress conditions (see articles reviewed by Gill and Tuteja, 2010 and Gupta et al., 2015). Based on different reports, it is supposed that CAT behaviour is highly dependent on plant species and environmental context. Furthermore, there is a certain disparity between published data. Indeed, several authors defended CAT's importance in the AOX defence machinery, whilst others do not value its

role against stress (Queirós, 2012). Since CAT activity can be inhibited by $O_2^{\cdot-}$ (Kono and Fridovich, 1982), the overproduction of this ROS can partially explain several reports about reductions in CAT activity in plants under oxidative stress (Iannone et al., 2015; Borges et al., 2018; Soares et al., 2018a; 2018b).

3.2.3. AsA-GSH cycle enzymes

3.2.3.1. Ascorbate peroxidase

Ascorbate peroxidase (APX) is an enzyme present in different organisms, such as plants and algae, that catalyses the H_2O_2 disproportion into water and monodehydroascorbate (MDHA) by using reducing power from AsA (Mittler et al., 2004; Sharma et al., 2012). In this way, APX activity is greatly dependent on AsA availability, reason why its regeneration is a fundamental process (Foyer and Noctor, 2003; 2005). Differently from CAT, APX possesses a high affinity for H_2O_2 and can exert its functions even with low levels of this ROS, indicating that APX is primarily responsible for modulation of H_2O_2 levels necessary for signaling events, whilst CAT is mainly involved in preventing H_2O_2 -induced cellular damage by removing its excess (Mittler, 2002).

APXs are encoded by a small multigenic family, whose transcription rates are regulated by different stimuli, such as H_2O_2 concentration and redox signals (Shigeoka et al., 2002). To date, based on amino acid sequences, 5 distinct classes of APX were identified in plants and classified according to their subcellular location. These classes include isoenzymes present in the cytosol (cAPX), in the chloroplast (at the stroma – sAPX – and bound to thylakoid's membrane – tAPX) and in the mitochondria and peroxisomal membranes, mitAPX and pAPX, respectively (Gill and Tuteja, 2010; Sharma et al., 2012; Gupta et al., 2015). The expression of cAPX enzymes – encoded by *APX1-2* genes – can be regulated at both transcriptional and post-translational levels. In fact, it is known that several molecules, such as ABA, GSH, ROS and 3'-phosphoadenosine 5'-phosphate (PAP) can be transported to the nucleus and interact with APX-coding genes, modifying their expression patterns; also, Cys-32 has an active role in shaping redox changes in cAPX protein structure, thus contributing to the regulation of the enzymatic activity of this enzyme (Gupta et al., 2018).

Regarding chloroplastidic APX, different species show differential molecular mechanisms underlying APX expression. Accordingly, *Arabidopsis* have distinct genes for the expression of sAPX and tAPX. However, chloroplastidic APX isoenzymes of species such as tobacco, spinach and pumpkin, are coded by only one gene, and the differential expression between sAPX and tAPX is achieved by alternative splicing (Maruta and Ishikawa, 2018). Although future research is required to completely unveil the biological

functions of organelle-specific APX, it is currently accepted that cytosolic forms of APX are more related to stress defence responses, whilst APX from chloroplasts have a more important role in H₂O₂ signaling pathways (Gupta et al., 2018).

3.2.3.2. Monodehydroascorbate, dehydroascorbate and glutathione reductases

As a consequence of APX catalytic activity, AsA is oxidised to monodehydroascorbate (MDHA), a very unstable radical that can be spontaneously converted into AsA and dehydroascorbate (DHA) (Ushimaru et al., 1997). Yet, MDHA can also be enzymatically reduced by dehydroascorbate reductase (MDHAR), a flavin adenine dinucleotide (FAD) enzyme that is present in all plant species, using reducing power from NAD(P)H (Park et al., 2016). As APX, different forms of MDHAR can be found in distinct organelles, such as chloroplasts, mitochondria, peroxisomes and cytosol (Roychoudhury and Basu, 2012; Singh et al., 2018). Due to the non-enzymatic disproportion of MDHA to AsA and DHA, dehydroascorbate reductase (DHAR) is a key element of the AsA-GSH cycle, allowing the regeneration of AsA from its oxidised form – DHA (Taiz et al., 2015). In fact, DHAR requires GSH as reducing agent and has a great specificity for GSH as substrate, not being able to use other reducing compounds (Hossain et al., 1984). This enzyme, classified as a monomeric thiol protein, is essentially found in seed tissues, roots and green organs (Eltayeb et al., 2007). It is widely accepted that DHAR contributes to the cellular redox balance, with a fundamental role in plant tolerance to abiotic stress (Sharma et al., 2012). Last, glutathione reductase (GR) is also a relevant component of the AsA-GSH cycle, since it catalyses the reduction of GSSG to GSH, allowing the maintenance of GSH/GSSG ratio (Yannarelli et al., 2007). GR is regarded as a flavoenzyme with a disulfide group and can be found in different taxa of photosynthetic organisms, both prokaryotes and eukaryotes (Sharma et al., 2012). Like other flavin-containing proteins, GR presents a well-conserved sequence (Rossman fold), characterised by alternating series of β -strand α -helix, allowing the binding of adenosine diphosphate (ADP) portions of dinucleotide molecules (e.g. FAD) (Hanukoglu, 2015). Indeed, GR has three functional domains, two of them for FAD and NADH binding, and the other one involved in dimerization events (Berkholz et al., 2008).

As reviewed by Gill and Tuteja (2010), GR is mainly present in the chloroplasts, but it can also be found in mitochondria, cytosol and peroxisomes. In higher plants, there are, at least, two isoforms of GR (GR1 and GR2) encoded by two distinct genes. However, recent molecular advances revealed that, structurally, these genes are highly conserved across all plant kingdom (Tahmasebi et al., 2012). Comparatively, GR1 is shorter and

mainly found in the cytosol and peroxisomes, while GR2 has a longer N-terminal chain and is specific of chloroplasts and mitochondria (Kataya and Reumann, 2010). Globally, GR importance is intrinsically linked to the maintenance of cell's GSH content and several reverse-genetic studies have highlighted its involvement in plant abiotic stress tolerance (Gill and Tuteja, 2010; Sharma et al., 2012). Nevertheless, not much is available regarding the molecular regulatory mechanisms of GR under stress conditions and future research efforts must be pointed to this issue.

3.2.4. Peroxidases

Plant cells possess a diverse set of enzymes, globally known as peroxidases, involved in the intracellular detoxification of H_2O_2 by the oxidation of distinct chemical substrates (Bela et al., 2015). Peroxidases are also implicated in other important metabolic reactions, controlling cell growth, inducing defence mechanisms against pathogen infection and playing an active role in auxin and ethylene metabolism (Welinder et al., 2002; De Gara, 2004; Cosio and Dunand, 2009). Generally, peroxidases can be subdivided into two main groups – heme-containing co-factor peroxidases, which include APX (already described in this review) and guaiacol peroxidase (GPOX), and non-heme-containing peroxidases, the so-called thiol peroxidases, where glutathione peroxidases (GPX; EC 1.11.1.9) and thioredoxin peroxidases are featured (Bela et al., 2015; Dietz, 2016). According to some authors, enzymes like GSTs and annexins can also be considered as thiol-based peroxidases; however, strictly, only thioredoxin peroxidases and GPX are thiol peroxidases, due to their high affinity to peroxides (Dietz, 2016).

3.2.4.1. Thiol-based peroxidases

Glutathione peroxidases (GPX) can be found in different plant tissues and cellular organelles during distinct development stages (Mullineaux et al., 1998; Yang et al., 2005; 2006). Among other functions, like hormone-control of root growth, shoot organogenesis and inhibition of cell death, GPX particularly stand out for their role in the prevention of oxidative stress. Indeed, the upregulation of *GPX* genes under stress conditions is commonly reported, with an increased accumulation of GPX-related transcripts. GPX-encoding cDNA were found to be positively affected by different abiotic fluctuations, such as salinity, drought, low temperatures and metal toxicity, but also by biotic stresses, like pathogen attack (Li et al., 2000; Rodriguez Milla et al., 2003; Kang et al., 2004; Navrot et al., 2006; Gao et al., 2014). Moreover, besides ROS detoxification, GPX are also a key element in preserving the cellular redox state, by the maintenance of thiol/disulfide and NADPH/NADP⁺ balance, inducing redox changes in different nuclear signaling proteins

and mediating the crosstalk among different cellular pathways (see review by Bela et al., 2015). However, according to several authors, future studies are required to concretely unravel the biological functions and action mechanisms of GPX (Bela et al., 2015; Dietz, 2016).

3.2.4.2. Guaiacol peroxidase

Guaiacol peroxidase is an ubiquitous protein throughout all living organisms, being present in animals, microbes and plants, where it can be specifically found in distinct plant organs and organelles, especially in vacuoles, cytosol and cell wall (Sharma et al., 2012). As other peroxidases, GPOX can regulate H_2O_2 intracellular levels using different organic compounds as substrates, such as guaiacol or pyrogallol (Gill and Tuteja, 2010). Furthermore, due to its both intracellular and extracellular enzymatic activity, different reports suggest that GPOX is the key enzyme in H_2O_2 detoxification. Structurally, this heme-containing enzyme is composed by monomers of around 40–50 kDa and possess four conserved disulfide bridges and two structural Ca^{2+} ions (Gill and Tuteja, 2010; Das and Roychoudhury, 2014). Besides its role in plant oxidative stress tolerance, GPOX also participates in other essential biosynthetic pathways, contributing to cell wall's lignification, indole-3-acetic acid (IAA) catabolism and biosynthesis of ethylene (Sharma et al., 2012).

Given its great significance in cellular redox homeostasis, the activation and enhanced activity of GPOX in response to different kinds of adverse conditions have been largely reported (Gill and Tuteja, 2010; Sharma et al., 2012). Indeed, increases in GPOX activity in *Helianthus annuus* L. (sunflower) and *Vicia sativa* L. plants exposed to cadmium (Cd) was previously observed (Saidi et al., 2014; Rui et al., 2016). Likewise, the treatment of rice seedlings with boron (B) in a saline soil also resulted in enhanced activity of GPOX (Farooq et al., 2015), as well as in *Poa pratensis* L. plants under salt stress (Puyang et al., 2015). Besides, an augmented activity of this enzyme was further observed in plants growing under different stresses, such as heavy metal, drought, cold and ultraviolet (UV) radiation (Devi et al., 2012; Janmohammadi et al., 2012; Ibrahim et al., 2013; Caverzan et al., 2016; Eskandari et al., 2017).

3.2.5. Glutathione S-transferase

Glutathione S-transferase (GST) represents a class of enzymes present in different types of organisms, including animals and plants (Basantani and Srivastava, 2007; Ghelfi et al., 2011). The first report about their occurrence in plant species was published in 1970 in a study conducted with maize (Frear and Swanson, 1970). GSTs are responsible for the conjugation of GSH with different types of xenobiotics, particularly electrophilic substrates

(Gill and Tuteja, 2010). Besides their function as important AOX enzymes, GST are also involved in other metabolic events and physiological phenomena, acting as peroxidases under certain conditions and mediating nucleophilic aromatic substitution and isomerization reactions (Basantani and Srivastava, 2007). GSTs show a wide distribution in plant organisms, being present in different development stages and tissues (McGonigle et al., 2000). The superfamily of plant GSTs is grouped into distinct classes that, generally, are regarded as cytoplasmic proteins, though some isoenzymes were found to be located in chloroplasts, microsomes and apoplast (Gill and Tuteja, 2010). In parallel to what is described for other AOX enzymes, different studies have been exploring the potential of GSTs in increasing plant tolerance to different adverse circumstances. Indeed, it has been suggested that overexpression of GSTs in tobacco plants positively affect the seedling's growth under stressful conditions (Gill and Tuteja, 2010).

Cloning, expression, molecular modeling and docking analysis of sugarcane GST have also been performed in order to obtain a better understanding of the catalytic specificity; two mutants were designed and the tertiary structure models and the same docking procedure were performed to explain the interactions between sugarcane GSTs with GSH and 1-chloro-2,4-dinitrobenzene (CDNB) (Ghelfi et al., 2011). Such a detailed study was the first to carry out site-directed mutagenesis and docking analysis of sugarcane GST, in which the roles of selected residues at the H-site have been investigated.

It is important to bear in mind that the enzymes selected in this section, and which exhibit distinct isoenzymes, must be thoroughly analysed since as we have already shown, they may be located in distinct cell compartments and naturally respond differentially to the ROS produced as a result of an oxidative stress condition. Enzyme stimulation or inhibition may be the result of changes in the activity of specific isoenzymes, being, therefore, essential to assay the specific activity of these AOX enzymes, rather than evaluate their total activity.

4. OXIDATIVE CHALLENGES

At high levels every ROS become toxic and capable of interacting with all organic molecules, such as proteins, nucleic acids, lipids, and carbohydrates; thus, if the defence response is not enough to counteract and cope with the enhanced production of ROS, cell viability is threatened by the consequences from oxidative stress that involves lipid and protein oxidation, enzyme inhibition, cytogenotoxicity, and ultimately, activation of programmed cell death (PCD) (Gill and Tuteja, 2010; Sharma et al., 2012, Mittler 2017).

4.1. Lipid peroxidation

Lipid peroxidation (LP) can be described as a cascade of biochemical events resulting from ROS action on unsaturated fatty acids of cellular and subcellular membranes, compromising membrane integrity, fluidity, and selectivity, as well as originating several lipid radicals that can further enhance oxidative damage (Gill and Tuteja, 2010; Sharma et al., 2012; Anjum et al., 2015; Halliwell and Gutteridge, 2015; Singh et al., 2018). The occurrence of LP is tightly related to the establishment of oxidative stress, since the produced lipid-derived free radicals are also able to interact with other macromolecules, like proteins and DNA (Anjum et al., 2015). Regarding the process itself, LP involves three distinct phases – initiation, progression and termination. Usually, in the initiation (Phase I) of LP, distinct ROS, especially $\cdot\text{OH}$, but also $\text{O}_2^{\cdot-}$, capture an H atom from the unsaturated chains of polyunsaturated fatty acids (PUFAs), producing the lipid alkyl radical (Anjum et al., 2015 and references therein). Afterwards, this radical can react with O_2 , resulting in the production of another radical - lipid peroxy radical -, with the ability to propagate throughout the lipid chain, by sequentially removing H atoms of lateral chains of adjacent PUFAs (Progression – Phase II). Finally, the termination phase comprises the elimination of the produced radicals, with the subsequent generation of more stable molecules. For example, the resulting lipid hydroperoxide can be transformed into several chemical highly reactive compounds, such as malondialdehyde (MDA), alkanes, lipid epoxides and alcohols (Gill and Tuteja, 2010; Sharma et al., 2012).

Given the chemical phenomena involved, it is widely accepted that LP is mainly limited by the two initial phases (initiation and progression) and that, besides being induced by ROS interaction with PUFAs, LP can also be enzymatically catalysed, by the action of several peroxidases and lipoxygenases (EC 1.13.11.) (Gupta et al., 2015; Singh et al., 2018). Over the years, with the expansion of molecular and biochemical studies on plant stress responses, LP has been extensively used as a widely accepted warning signal of the occurrence of oxidative damage. In most cases, LP is assessed by the estimation of membrane ion leakage and/or by the quantification of one of LP sub-products, MDA - a molecule that, when in contact with thiobarbituric acid, originates a color product, whose intensity can be measured at 532 nm (Heath and Packer, 1968). Increases in LP have been reported for numerous plant species exposed to a wide range of adverse environmental conditions, including water stress (salinity, drought and flooding), heavy metal exposure (both in bulk and nano-sized forms), nutrient imbalances, UV radiation and xenobiotic exposure (Gill and Tuteja, 2010; Queirós et al., 2011; de Sousa et al., 2013; Fidalgo et al., 2013; Anjum et al., 2015; Soares et al., 2016a,b,c; 2018a; Branco-Neves et al., 2017).

4.2. Protein oxidation

Protein oxidation is defined as the covalent modification, which is an important class of posttranslational modifications, of a protein induced either by direct reactions with ROS or indirectly by conjugation with breakdown products of fatty acid peroxidation. Direct modification involves modulation of protein's activity through nitrosylation, carbonylation, disulphide bond formation, and glutathionylation (Sharma et al., 2012). Indirect modifications of proteins are specifically targeted to residues such as the amino acids arginine, histidine, lysine, Pro, threonine and tryptophan resulting in an increased susceptibility of proteins towards proteolytic degradation (Gill and Tuteja, 2010). Thiol groups and S-containing amino acids, such as Met and Cys, are more likely to be attacked by ROS (Cys and Met are quite reactive towards $^1\text{O}_2$ and $\cdot\text{OH}$), and particularly prone to oxidation, being consequently the most commonly modified ones (Sharma et al., 2012). Active oxygen can remove a H atom from Cys residues to form a thiyl radical, which cross-links to another thiyl radical to form a disulfide bridge (Hancock et al., 2006). In addition, the oxidation of protein Cys thiol groups can also generate sulfenic acid, sulfinic acid, and sulfonic acid derivatives (Costa et al., 2007). On the other hand, similarly to Cys, Met can undergo ROS-mediated oxidation. Protein Met residues are oxidised into methionine-S-sulfoxides (Met-S-SO) and methionine-R-sulfoxides (Met-R-SO) (Costa et al., 2007). The oxidised Met residues are readily reduced back to Met by methionine sulfoxide reductase (EC 1.8.4.13), a class of cytosolic and plastidic enzymes that are involved in ameliorating oxidative damage (Cabreiro et al., 2006). On the other hand, enzymes containing Fe-S centers are irreversibly inactivated by $\text{O}_2^{\cdot-}$, leading to enzyme inactivation (Sharma et al., 2012; Banerjee and Roychoudhury, 2018).

Proteins irreversibly inactivated cannot be repaired and have to be recognised and degraded by cellular proteolytic processes (Costa et al., 2007; Sharma et al., 2012), being extremely important its efficient degradation and removal for the maintenance of the cellular metabolism. It has been suggested that oxidised proteins are better substrates for proteolytic digestion, by getting ready for ubiquitination and then a target for degradation by the proteasome (Anjum et al., 2015). Carbonylation is an irreversible modification of proteins and the most commonly occurring oxidative protein modification, being usually used as marker for evaluating the intensity of protein oxidation that negatively affect the structure and function of different proteins, as channels, enzymes and receptors (Gill and Tuteja, 2010; Banerjee and Roychoudhury, 2018). Carbonylated proteins have been found in all plant cellular compartments: cytosol, chloroplast, peroxisome, nucleus and mitochondrion. In wheat leaves, the concentration of carbonylated proteins per mg protein was higher in the mitochondria than in chloroplasts and peroxisomes (Bartoli et al., 2004),

which may suggest that mitochondrial proteins are more susceptible to oxidative damage. Several abiotic stresses including drought, heat, salinity and heavy metals as well as biotic stresses, lead to the carbonylation of proteins. Nevertheless, the extent of carbonylation correlates to the stress characteristics as exposure time and the intensity of the stressor factor (Song et al., 2009; Lounifi et al., 2013).

4.3. Cytogenotoxicity

Cytogenotoxicity can be directly or indirectly triggered by several stressors that change the cell cycle and provoke chromosomal aberrations, such as sticky and lost chromosomes, chromosomal bridges, DNA breaks, and formation of micronucleus, hence frequently causing mutations, aneuploidy and polyploidy events (Leme and Marin-Morales, 2009; Oldenburg and Bendich, 2015; Carvalho et al., 2018a). These cytogenetic changes may impact cell expansion and metabolism, so impairing the development at cell, organ and plant levels (Shi et al., 2016). Since cytogenotoxicity can reach germline cell genome, some side-effects are also transmitted to the progeny. The main point is that, in several situations, cytogenotoxicity has been coupled to ROS overproduction, causing strand breakage, removal of nucleotides, and even the loss of the complete genetic material in certain organelles (Bandyopadhyay and Mukherjee, 2011; Sharma et al., 2012; Oldenburg and Bendich, 2015). For instance, ROS overproduced in response to drought and herbivory stresses have been associated to the phenomenon called “DNA abandonment”, which was observed in plastids of mature leaves from certain grasses (Oldenburg and Bendich, 2015). Depending on the type of ROS and interaction, DNA can suffer a wide range of problems, including sugar oxidation, strand disruption, depletion of nucleotides, changes in nitrogen bases and crosslinks between DNA and histones (Gill and Tuteja, 2010).

Among all ROS, $\cdot\text{OH}$ accounts for the majority of DNA damages, being capable of reacting with both purines and pyrimidines bases, as well as the deoxyribose sugar (Sharma et al., 2012; Halliwell and Gutteridge, 2015; Singh et al., 2018). For instance, $\cdot\text{OH}$ is responsible for the hydroxylation of guanine, stimulating the production of 8-oxo-7,8-dehydro-2'-deoxyguanosine, but also leads to the generation of hydroxymethyl urea, urea, thymine glycol and opened-rings of thymine and adenine (Tsuboi et al., 1998). Additionally, $\cdot\text{OH}$ -induced DNA-protein crosslinks cannot be automatically repaired, thus aggravating the consequences if transcription or replication occur before healing. Besides $\cdot\text{OH}$, $^1\text{O}_2$ only reacts with guanine, whilst $\text{O}_2^{\cdot-}$ and H_2O_2 do not react with any purine nor pyrimidine bases (Dizdaroglu, 1993; Halliwell and Gutteridge, 2015). Indeed, it is currently accepted that $\text{O}_2^{\cdot-}$ and H_2O_2 toxicity is mainly attributed to their involvement in the Fenton reaction,

where $\cdot\text{OH}$ is produced (Sharma et al., 2012). By affecting DNA replication and transcription, ROS-induced damage on nucleic acids may result in abnormalities in protein synthesis, signal transduction pathways, membrane stability, thus contributing to a lower metabolic efficiency and genomic instability, and compromising cell homeostasis (Gill and Tuteja, 2010; Sharma et al., 2012; Gupta et al., 2015). The diversity of ROS-mediated cytogenotoxicity effects, which also include epigenetic events, is illustrated in the Figure 2.

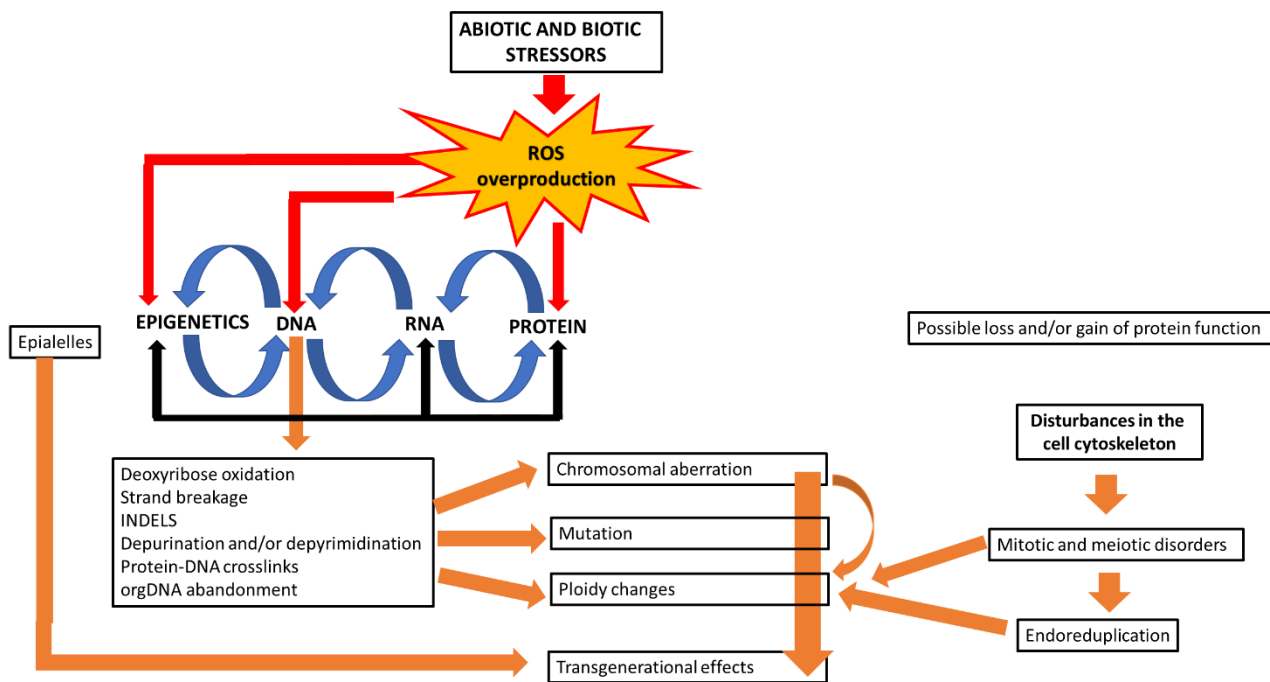


Figure 2. Potential direct and indirect ROS-induced cytogenotoxicity, resulting in cell cycle alterations, chromosomal abnormalities, ploidy modifications, mutation and also transgenerational effects.

5. TRANSGENERATIONAL EFFECTS

Embryo malformation and reductions in the seed provisioning can be triggered by environmental stresses to which the mother plant is submitted, potentially decreasing seed germination and seedling establishment (Marcos-Filho, 2016). Accordingly, progeny from *Pinus pinaster* plants grown in favorable conditions presented higher tolerance to the pathogen *Fusarium circinatum* than the offspring from plants grown in unfavourable conditions, and such ability was related to transgenerational modifications associated to enhancements in the AOX activity (Vivas et al., 2013, 2014). However, some plants are able to remember past incidents and to use this stored knowledge — the so-called memory — to enhance the progeny tolerance to continuous or upcoming stresses, not only those which challenged their parents, but also other types of adverse conditions – cross-tolerance (Herman and Sultan, 2011). Although several cues indicate the involvement of

AOX machinery in both triggering transgenerational changes (Locato et al., 2018) and improving offspring tolerance to stressors (Tabassum et al., 2017), only few studies showed modifications in gene expression and/or activity of enzymatic and non-enzymatic components, or ROS generation and their side-effects like lipid and protein peroxidation (gray lines, Table 2).

6. QUANTITATIVE TRAIT-LOCI FOR TOLERANCE TO OXIDATIVE STRESS

The objective of quantitative trait-loci (QTL) studies is to find QTLs that can be implemented into breeding programs via marker-assisted selection. In general, the major objective of crop breeding is high yield, combined with insensitivity to biotic and abiotic stresses. So far, QTL studies have been successful for introgressing and pyramiding major-effect genes (Zhang et al., 2017). There are several studies using QTLs, the large majority in recent years, but only few of them directly investigated enzymatic and non-enzymatic components of the AOX machinery, ROS generation and oxidative stress indicators (*i.e.* MDA content). For instance, Jiang et al. (2009) mapped QTLs for leaf MDA content associated with stress tolerance in rice. Another example was the identification of QTLs for Pro accumulation related to barley tolerance against drought and salinity tolerance (Fan et al., 2015). A list of QTLs related to tolerance to oxidative stress is compiled in Table 3.

Table 2. Beneficial transgenerational effects on progeny of different plant species due to the exposure of parental generation to biotic or abiotic stressors, in comparison to the offspring from plants grown in control, non-stressful conditions.

Parental generation under exposure to	Species	Alterations probably coupled to the best performance of progenies from stressed than non-stressed parental plants		References
		↑	↓	
UV-C	<i>Arabidopsis thaliana</i> (L.) Heynh.	CAT activity	APX and POD activities	Čuk et al. (2010)
Salt	<i>Arabidopsis thaliana</i> (L.) Heynh.	Root length	PER, GST, lipoxigenase (LOX), MDHAR, CYP450, and dehydrin gene expression	Boyko et al. (2010)
Tobacco mosaic virus (TMV)	<i>Nicotiana tabacum</i> L.	Total phenolic concentration, PR gene expression, and callose deposition		Kathiria et al. (2010)
Nutrient-poor soil patches	<i>Cyperus esculentus</i> L.	Number of propagules		Dyer et al. (2010)
N deficiency	<i>Oryza sativa</i> L.	Plant height and dry weight		Kou et al. (2011)
Nickel (Ni), Cu, and Cd	<i>Arabidopsis thaliana</i> (L.) Heynh.	Root length		Rahavi et al. (2011)
Herbivory (simulated damage)	<i>Mimulus guttatus</i> Fisch. ex DC.	Trichome density		Scoville et al. (2011)
Salt	<i>Oryza sativa</i> L.	Plant height		Feng et al. (2012)
<i>Pseudomonas syringae</i> pv tomato (avirulent)	<i>Arabidopsis thaliana</i> (L.) Heynh.	PR gene expression		Slaughter et al. (2012)
Mercury (Hg)	<i>Oryza sativa</i> L.	Plant height and chlorophyll content		Ou et al. (2012)
<i>Pseudomonas syringae</i> pv toma to DC3000	<i>Arabidopsis thaliana</i> (L.) Heynh.	PR-1, WRKY6, WRKY53, and WRKY70 gene expression	Seed weight, pathogen-mediated lesions	Luna et al. (2012)
Low humidity	<i>Arabidopsis thaliana</i> (L.) Heynh.		Leaf stomatal frequency	Tricker et al. (2012, 2013)
Drought	<i>Polygonum persicaria</i> L.	Seedling size and biomass, and root length		Herman et al. (2012)
Salt	<i>Arabidopsis thaliana</i> (L.) Heynh.	Length of the main stem, total siliques		Suter and Widmer (2013)

Cd	<i>Arabidopsis thaliana</i> (L.) Heynh.	Shoot lenght		Truyens et al. (2013)
UV-C	<i>Arabidopsis thaliana</i> (L.) Heynh.		Leaf number	Migicovsky and Kovalchuk (2014)
Heat	<i>Arabidopsis thaliana</i> (L.) Heynh.	Leaf number	Leaf length	Migicovsky et al. (2014)
Heat	<i>Arabidopsis thaliana</i> (L.) Heynh.	Polyphenols and ascorbate concentrations	Lipid peroxidation, electrolyte leakage, protein oxidation	Zinta et al. (2014)
Herbivory (florivory)	<i>Pastinaca sativa</i> L.	Flower size		Jogesh et al. (2014)
Poor nutrient soil	<i>Plantago lanceolata</i> L.	Plant biomass, and root carbohydrate storage		Latzel et al. (2014)
Warm and dry conditions (relative)	<i>Silene vulgaris</i> (Moench) Garcke	Seed longevity, and heat shock protein (HSP) mRNA content		Mondoni et al. (2014)
Cd and Ni	<i>Agrostis capillaris</i> L.	Shoot and root length and biomass, Cd content		Truyens et al. (2014)
Wounding	<i>Mimulus guttatus</i> Fisch. ex DC.	Trichome density		Colicchio (2014) and Colicchio et al. (2017)
Ozone	<i>Lolium multiflorum</i> Lam.	GSH, and γ-tocopherol concentrations	GSSG/GSH ratio, seed production	Gundel et al. (2015)
Fe deficiency	<i>Arabidopsis thaliana</i> (L.) Heynh.	Seed dormancy and longevity		Murgia et al. (2015,2017)
Warm	<i>Cardamine alpina</i> Willd.	Seed viability		Bernareggi et al. (2015)
Cold	<i>Arabidopsis thaliana</i> (L.) Heynh.		Leaf number	Migicovsky and Kovalchuk (2015)
Cauliflower mosaic virus (CaMV)	<i>Arabidopsis thaliana</i> (L.) Heynh.	Larger seeds	SOD gene expression	Kalischuk et al. (2015)
Competition (increased plant community diversity)	<i>Biscutella didyma</i> L.			Metz et al. (2015)
Drought (relative)	<i>Impatiens capensis</i> Meerb.	Seed germination	ABA content	Maruyana et al. (2016)

Floral and root herbivore, and detritivore interaction	<i>Moricandia moricandioides</i> (Boiss.) Heywood	Seed quality (C:N ratio)		González-Megías (2016)
Heat	<i>Triticum aestivum</i> L.	APX, SOD and POD activities, and expression of genes related to APX, GPX, POX, CYP450, and alternative oxidase	MDA concentration	Wang et al. (2016)
Wounding (mechanical damages)	<i>Mimulus guttatus</i> Fisch. ex DC.	Trichome density		Akkerman et al. (2016)
Drought	<i>Genista tinctoria</i> L.	Germination		Walter et al. (2016)
Drought	<i>Avena sativa</i> L.	Thin roots, and seed Mg, Fe, Zn content	P content	Nosalewicz et al. (2016)
Grazing	<i>Larrea cuneifolia</i> Cav., <i>Larrea divaricate</i> Cav., <i>Monttea aphylla</i> (Miers) Hauman, <i>Atriplex lampa</i> (Moq.) Gillies, <i>Gutierrezia solbrigii</i> Cabrera, and <i>Grindelia chiloensis</i> (Cornel.) Cabrera	Seedling vigor	Seed germination	Tadey and Souto (2016)
Unfavourable edaphoclimatic features	<i>Pinus pinaster</i> Aiton	POX, SOD and CAT activities, and Cu/Zn SOD expression		Arencibia et al. (2016)
NaCl	<i>Arabidopsis thaliana</i> (L.) Heynh.	Seedling survivor		Wibowo et al. (2016)
<i>Phytophthora infestans</i> (Mont.) de Bary	<i>Solanum physalifolium</i> Rusby	Resistance to <i>P. infestans</i>	Lesions	Lankinen et al. (2016)
Herbivory	<i>Phaseolus lunatus</i> L.	β -glucosidase activity, and cyanide releasing	Mortality	Ballhorn et al. (2016)
Drought	<i>Oriza sativa</i> L.	SOD, CAT, and peroxidase (POD) activities		Zheng et al. (2017)
Drought	<i>Polygonum persicaria</i> L.	Seedling biomass, leaf area, and root length		Herman and Sultan (2016)
Herbivory	<i>Alternanthera philoxeroides</i> (Mart.) Griseb.	Internode elongation, N concentration in stolon	Soluble sugar in stolon, and starch in fine roots	Dong et al. (2017)
Drought	<i>Triticum aestivum</i> L.	Leaf osmotic potential, proline and glycine betaine contents, number of grains per spike, 100-grain dry weight, grain yield	Specific leaf area, Na content, MDA concentration	Tabassum et al. (2017)
Drought	<i>Lupinus angustifolius</i> L.	Plant height	ABA	Kalandyk et al. (2017)
Fire	<i>Pinus halepensis</i> Miller	Germination		Saracino et al. (2017)

Low temperature	<i>Vicia sativa</i> L.	Seed mass and viability	Electrical conductivity	Li et al. (2017)
N excess	<i>Stipa krylovii</i> Roshev.	Seed weight and production, and offspring biomass		Li et al. (2017)
Competition (increased plant community diversity)	<i>Knautia arvensis</i> (L.) Coult.	Seed production	Seed germination	Rottstock et al. (2017)
Herbivory	<i>Arabidopsis thaliana</i> (L.) Heynh.	Jasmonic acid (JA), and gibberellin, and isoleucine content	Seed dormancy, ABA	Singh et al. (2017)
Grazing	<i>Leymus chinensis</i> (Trin.) Tzvelev		Leaf area and width, stem length and diameter, and plant height	Ren et al. (2017)
Herbivory	<i>Brassica rapa</i> L.	Silique number		Kellenberger et al. (2018)
Cd	<i>Solanum lycopersicum</i> L.	Cd concentration	Mn concentration	Carvalho et al. (2018)
Drought	<i>Triticum aestivum</i> L.	SOD, CAT, APX, GPX, GR, MDHAR, and DHAR activities, and AsA, GSH, proline and glycine betaine concentrations	MDA concentration, and O ₂ ⁻ and H ₂ O ₂ generation	Wang et al. (2018)
Cu	<i>Silene vulgaris</i> (Moench) Garcke	Plant biomass, and number of flowers in offspring under N-deficiency stress		Sandner et al. (2018)
Drought	<i>Achnatherum inebrians</i> (Hance) Keng	C, N and P contents, water use efficiency and root dry weight when endophytic <i>Epichloë gansuensis</i> (C.J. Li & Nan) Schardl was present in the maternal generation		Xia et al. (2018)
Salt	<i>Suaeda vermiculata</i> Forssk. ex. J.F.Gmel.	Germination recovery	Germination rate	El-Keblawy et al. (2018)
Herbivory	<i>Raphanus sativus</i> L.		Palatability	Neylan et al. (2018)
Mepiquat clorid (growth retardant)	<i>Gossypium hirsutum</i> L.	Seed biomass and vigor, seedling root and shoot biomass, and P, K, boron (B) and Zn contents	Mn concentration	Zohaib et al. (2018)

Table 3. Quantitative trait loci (QTL) associated to enzymatic and non-enzymatic components of the AOX machinery, as well as to ROS generation and stress indicators (*i.e.* reactive oxygen species – ROS, and malondialdehyde – MDA content).

Species	Stressor	Trait	Putative gene / encoded enzyme	Chromosomal location (QTL name)	Reference
<i>Oryza sativa</i> L.	Cold		GST	12 (qCTS12a)	Andaya and Tai (2006)
<i>Solanum lycopersicum</i> L. x <i>Solanum penelli</i> (Correll)	Chilling	AsA concentration	MDHAR	9 (AA9.1+, AA9.2–, and AA9.3+, PW9.2.5 and Brix9.2.5)	Stevens et al. (2007,2008)
<i>Oryza sativa</i> L.	Cold		MDHAR	8 (qCTB8)	Kuroki et al. (2007)
<i>Oryza sativa</i> L.	No cited	MDA concentration		1 (qMDA-1a), 1 (qMDA-1b)	Jiang et al. (2009)
<i>Pisum sativum</i> L.	Frost	Glucose concentration		5 (GlcT2.b)	Dumont et al. (2009)
<i>Solanum penelli</i> (Correll) D'Arcy	Salt	Water-soluble AOX activity		2 (aox-s2.1), 3 (aox-s3.2), 3 (aox-s3.1), 7 (aox-s7.1), 8 (aox-s8.1), 12 (aox-s12.1)	Frery et al. (2010)
		Total phenolic concentration		1 (phe-s1.1), 2 (phe-s2.1), 4 (phe-s4.1), 5 (phe-s5.1)	
		Flavonoid concentration		5 (phe-s5.2), 6 (phe-s6.1), 7 (phe-s7.1), 11 (phe-s11.1), 12 (phe-s12.1)	
		POX activity		1 (fla-s1.1), 1 (fla-s1.2), 2 (fla-s2.1), 2 (fla-s2.2), 3 (fla-s3.1), 4 (fla-s4.1), 5 (fla-s5.1), 5 (fla-s5.2), 6 (fla-s6.1), 7 (fla-s7.1), 9 (fla-s9.1), 10 (fla-s10.1), 11 (fla-s11.1), 12 (fla-s12.1)	
				1 (pox-s1.1), 2 (pox-s2.1), 2 (pox-s2.2), 3 (pox-s3.1), 4 (pox-s4.1), 5 (pox-s5.1), 6 (pox-s6.1), 7 (pox-s7.1), 8 (pox-s8.1), 8 (pox-s8.2), 12 (pox-s12.1), 12 (pox-s12.2)	
<i>Oryza sativa</i> L.	Ozone	AsA concentration, and ascorbate oxidase expression		9 (OzT9)	Frei et al. (2010)
<i>Helianthus annuus</i> L.	Water deficit	Tocopherol concentration	POD, GST, and CAT	2.TTC.11, and 6.TTC.8	Haddadi (2010)
<i>Zea mays</i> L.	Water deficit		GST	4.TPC.1	
				5 (mQTL_GY_5), 7(mQTL_GY_7), 0(mQTL_GY_10a)	Almeida (2012)

<i>Oryza sativa</i> L.	Water submergence	SOD, CAT, APX, GPX, GR, and DHAR activities		9 (SUB1A)	Panda et al. (2012)
<i>Helianthus annuus</i> L.	Water deficit		CAT	8	Abdi et al. (2012)
			POD	17	
<i>Oryza rufipogon</i> Griff.	Salt		CYP450	2 (qCST2)	Liu et al. (2013)
			GST	10 (qCST10)	
<i>Glycine max</i> (L.) Merr.	Water deficit	MDA concentration		qMDA-G-2	Yang et al. (2014)
<i>Solanum penelli</i> (Correll) D'Arcy	None		Dehydrin, glutaredoxin	2	Bolger et al. (2014)
			CYP450	7	
			Cu/Zn SOD	8	
			MDHAR	9	
<i>Zea mays</i> L.	Water deficit		Cytochrome c oxidase	3 (qkw 15)	Wu et al. (2014)
			Tocopherol cyclase	5 (qgyld 17)	
			Anthocyanidin 3-o-glucosyltransferase	6 (qgyld 23)	
			Spermidine synthase	8 (qkw 24)	
<i>Hordeum vulgare</i> L.	Water deficit	Proline concentration		3 (QPC-D.TxFr.3H)	Fan et al. (2015)
	Salt	Proline concentration		3 (QPC-S.TxFr.3H)	
<i>Triticosecale</i> Wittm. ex A. Camus.	Cold	AOX activity		4A (QRASm4A-1)	Krzewska et al. (2015)
		AOX activity		5R (QARSsm5R-1 r)	
<i>Gossypium</i> spp.	Water deficit		Cu/Zn SOD	13	Kebede et al. (2015)
<i>Oryza sativa</i> L.	Salt	MDA concentration		6 (qSNC-6)	Deng et al. (2015)
<i>Helianthus annuus</i> L.	<i>Sclerotinia sclerotiorum</i> (Lib.) de Bary		GST	BsrSSKH41.1.1	Amoozadeh et al. (2015)
			POD	BsrSSKH41.17.1	
			GST	BsrSSU107.1.1	
			POD	BsrSSU107.17.1	
<i>Gossypium hirsutum</i> L.	Salt	SOD activity		A1, A7, A11, A13, D1, D6 and D7	Du et al. (2016)
		POD activity		A10, A13, D1, D6, D11, D12	
		CAT activity		A13	
		MDA concentration		A1, A2, A4, A5, A6, A9, A10, A11, A12, D1, D2, D6, D9	
<i>Oryza sativa</i> L.	Water deficit	POD activity		11	Zhou et al. (2016)

<i>Sorghum bicolor</i> (L.) Moench	Low-N		GST CYP450	1 (qMC2-1b) 6 (qGY-6b)	Gelli et al. (2016)
<i>Hordeum vulgare</i> L.	<i>Puccinia striiformis</i> Westend var hordei		CAT, NADPH oxidase (RBOH), peptide-methionine (R)-S-oxide reductase	4H (Qpsh4Hb)	Klos et al. (2016)
<i>Glycine max</i> (L.) Merr.	Different edaphoclimatic growing conditions	α -tocopherol concentration γ -tocopherol concentration δ -tocopherol concentration vitamin E concentration		14 (QaB2_1), 6(QaC2_1), 2(QaD1b_1), and 20 (Qal_1) 5 (QcA2_1), 4(QcC1_1), 6(QcC2_1), 18(QcG_1), (QcD1b-1), 10(QcO-1), 16(QcJ_1) 1(QdD1a_1), 13(QdF_1), and 20(QdI_1) 6 (QTVEC2_1), 6(QTVEC2_2), 2(QTVED1b_1), and 10(QTVEO_1)	Li et al. (2016)
<i>Triticum aestivum</i> L.	Heat		POD	1D (QHst.cph-1D)	Sharma et al. (2017)
<i>Oryza sativa</i> L.	Heat	MDA concentration MDA concentration		1 (qHTSF1.1) 4 (qHTSF4.1)	Vivitha et al. (2017)
<i>Hordeum vulgare</i> L.	Water deficit		Cu/Zn SOD Thioredoxin reductase POD Glutaredoxin Peroxisredoxin GPX APX Polyamine oxidase Peptide methionine sulfoxide reductase CAT	2H (MQTL2H.2) 2H (MQTL2H.2) 2H (MQTL2H.3) 2H (MQTL2H.3) 3H (MQTL3H.2) 4H (MQTL4H.2) 4H (MQTL4H.2) 7H (MQTL7H.3) 7H (MQTL7H.3) 4H (MQTL4H.4)	Zhang et al. (2017)
<i>Hordeum vulgare</i> L.	Water deficit/Heat	Glutathione concentration α -tocopherol, and γ - tocopherol concentrations MDA concentration		5H 7H	Templer et al. (2017)
<i>Gossypium hirsutum</i> L.	Salt			A2 and D9	Cai et al. (2017)
<i>Brachypodium distachyon</i> (L.) P.Beauv.	Water deficit		GST, and Fe/Mn SOD	2 (D-LWC2.2, D-Fv/Fm2.1, and D-WT2.1)	Jiang et al. (2017)
	Low-N		POD	9 (qAD-9, and qMC2-9)	

<i>Sorghum bicolor</i> (L.) Moench			CYP450 GST	3 (qTGW-3), 8 (qBY-8, and qGY-8) 1 (qTW-1, qAD-1b, and qMC1-1) D13 (qMDA-D13)	Gelli et al. (2017)
<i>Gossypium hirsutum</i> L.	Salt	MDA concentration			Diouf et al. (2017)
<i>Triticum aestivum</i> L.	Salt		GST POD Flavonoid 3 -monooxygenase Peroxidase precursor	AX-94408415 AX-94777280 AX-95684819 7HS	Hussain et al. (2017)
<i>Hordeum vulgare</i> L.	Salt				Xue et al. (2017)
<i>Zea mays</i> L.	Water deficit		CAT (cat2) CAT (cat1) APX SOD	1 (mQTL1-2) 5 (mQTL5-3) 6 (mQTL6-3) 9 (mQTL9-3)	Zhao et al. (2018)
<i>Hordeum vulgare</i> L.	Water deficit	α -tocopherol concentration γ -tocotrienol, and proline and sucrose concentrations γ -tocopherol concentration Lycopene and carotenoids concentration		6H (QdATf.6H) 3H (QdsiGTt.3H_2) 7H (QdsiGTf.7H_3)	Gudys et al. (2018)
<i>Oryza sativa</i> L.	Water deficit			9 (SUB1A)	Saha et al. (2018)
<i>Solanum lycopersicum</i> L.	Water deficit and salt		GPX Ferredoxin	8 (Firm8.1) 11 (FW11.2)	Diouf et al. (2018)

7. PERSPECTIVES

The study of plant stress responses is vast and has to involve groups from a number of fields. Any quick analysis of the available literature will show that the number of papers published on biotic and abiotic stresses in plants focusing on the AOX response is huge and increased dramatically over the last 15 years. For instance, back in the 1990`s only a few reports were available for heavy metal-induced stress. The number grew so much that many are published on a weekly basis. The main problem is that the large majority of these publications is repetitive and shows very little novel information. Most of the results are confirming previous findings and, while they may not actually cover novel aspects, a good share of them do bring information sometimes new for one plant species that is worth publishing despite the lack of a brand-new insight on this subject.

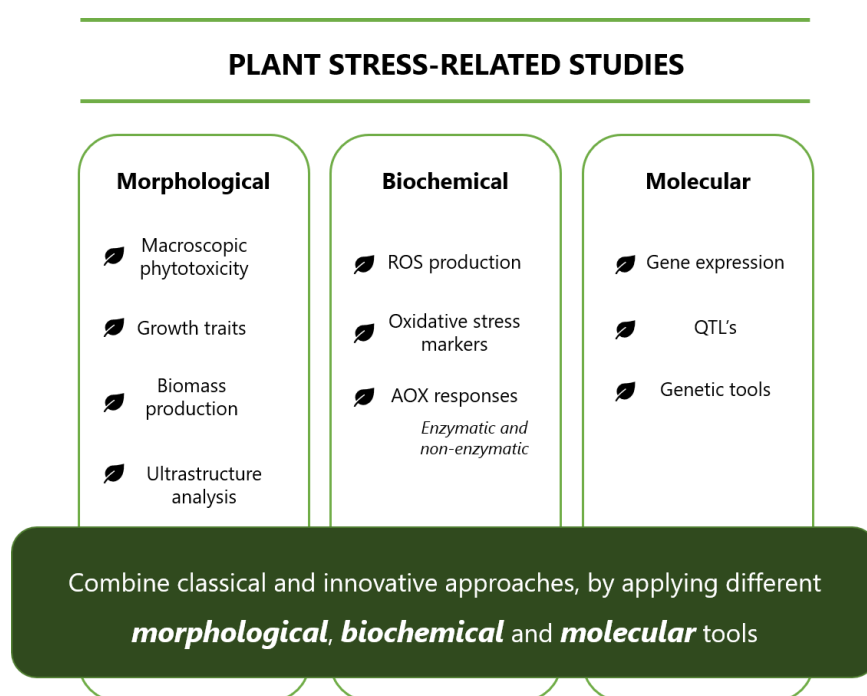


Figure 3. Comprehensive diagram integrating the available complementary approaches to study plant abiotic stress tolerance

So, currently, it is more important than ever to address and attempt new strategies and approaches. For instance, if on one hand we have a lot of work investigating AOX enzymes, on the other hand there is plenty to be done when cytogenotoxicity is concerned. Moreover, if we take the example of heavy metals, it would be interesting to deeply investigate their direct effects on protein and DNA structures. The Omics techniques are available and should be used more. The proteomics studies should also go a step forward to avoid being a typical descriptive study of protein ups and downs, in a similar manner to those involving the modulation of the AOX enzymes. The use of more refined techniques

such as fluorescence *in situ* hybridization (FISH) has to be employed, since its potential is immense, although it is seldomly used.

Transgenerational effects studies are also lagging behind but we feel they have the potential to better understand a number of processes that go beyond the typical study of plant responses to stress. Other approaches using QTLs, for instance, need to have more groups involved. Plant breeding research changed significantly over the last 15 years due to new approaches and we can advance a lot in producing better genotypes by combining the use of QTLs with stress response.

One other aspect that deserves more attention and, in recent years appears to have attracted more researchers, is the use of well-known techniques such as grafting to study stress signaling in plants. Grafting is widely used and possible for many important crops such as citrus and tomato (Gratão et al., 2015; Hippler et al., 2016). The possibility of combining scions and rootstocks from distinct genotypes with different degrees of tolerance/sensitivity to a certain type of stress is another tool that has not really been used as it could and should.

We feel that major advances can be gained with more intensive research using such approaches and techniques, independently of the type of environmental stress. Yet, it does not mean that what is being done is not useful, but on the contrary, the combination of all that was described in this review plus other aspects that were not the main focus of it, can be a very positive attitude towards helping plants to deal with stressful situations (Figure 3).

We have suggested some aspects that we feel deserve a great deal of attention and perhaps with the potential to more effectively contribute with real major advances to this topic. We would be pleased to see these and other ideas receiving more attention over the next few years. Plants do not really need a shrink, but a good AOX system can be very handy as both direct and indirect protective mechanisms to overcome the environmental challenges (Figure 4). That is why we need to understand it well.

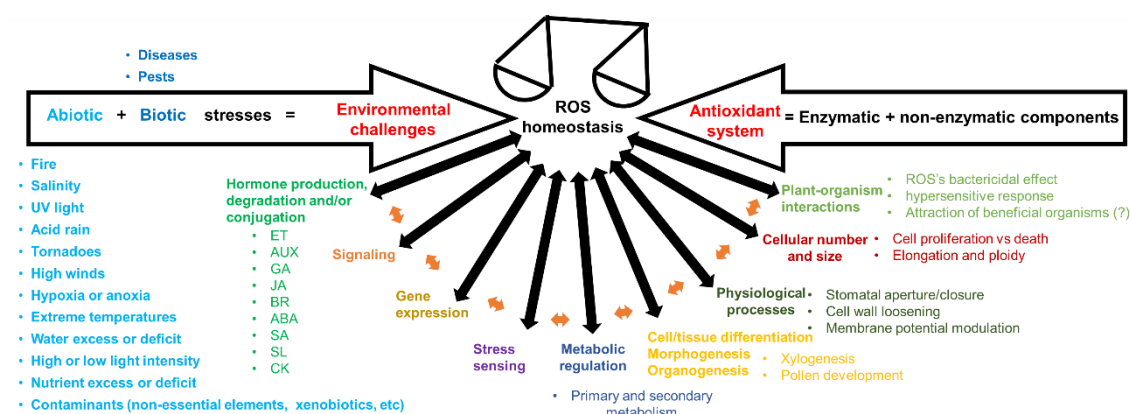


Figure 4. Integrated overview of the redox homeostasis in plant cells, focusing on the interplaying between the generation of ROS, as a result of both biotic and abiotic stresses, and their tightly control by the plant AOX system.

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CHAPTER III. MAIN OBJECTIVES

Biological questions and main goals

Since the authorization of GLY and its placement in the market is still approved in the USA (<https://www.epa.gov/ingredients-used-pesticide-products/glyphosate>) and in the EU (<https://www.efsa.europa.eu/en/topics/topic/glyphosate>), besides understanding its non-target phytotoxicity, it is essential the development of new eco-friendly tools to increase crop's tolerance to GLY, as well as to reduce its ecotoxicity towards soil organisms and functions. For this purpose, several questions underlying this PhD thesis have arise:

1. Do GLY residues in soils affect non-target plants growth and development?
2. What are the main biochemical and molecular mechanisms underlying this toxicity?
3. How can GLY uptake and ecotoxicity to non-target plants be reduced?
4. Does contamination of soils with residues of GLY and other herbicides result in the loss of soil habitat and production functions?

In order to achieve the main objectives, different experimental trials were designed focusing on a set of specific goals:

- Characterise the physiological effects of GLY on several non-target plants, giving particular attention to growth traits, photosynthetic endpoints and nitrogen nutrition;
- Understand the toxicity patterns of GLY in what regards the crosstalk between ROS generation and the performance of the plant AOX system;
- Assess if the application of different biostimulants [e.g. silicon (Si), nitric oxide (NO) and salicylic acid (SA)] results in a higher tolerance of crops to GLY, with a reduced bioaccumulation factor;
- Unravel the role of soil organic matter (OM) in limiting GLY bioavailability, thus decreasing its ecotoxicity towards non-target plants;
- Evaluate if GLY residues, at environmentally relevant concentrations, negatively affect soil's habitat and production functions, by studying different ecotoxicological endpoints on different trophic levels, through the employment of international standardised protocols.

CHAPTER IV. GLYPHOSATE-INDUCED TOXICITY IN NON-TARGET PLANTS



Is soil contamination by a glyphosate commercial formulation truly harmless to non-target plants? — Evaluation of oxidative damage and antioxidant responses in tomato[☆]

Cristiano Soares^{a,*}, Ruth Pereira^a, Sofia Spormann, Fernanda Fidalgo

GreenUPorto - Sustainable Agrifood Production Research Centre, Biology Department, Faculty of Sciences of University of Porto, Rua do Campo Alegre s/n, 4169-007, Porto, Portugal



Glyphosate-dependent effects on photosynthesis of *Solanum lycopersicum* L.—An ecophysiological, ultrastructural and molecular approach

Cristiano Soares^{a,*}, Ruth Pereira^a, Maria Martins^a, Paula Tamagnini^{b,c,d}, João Seródio^e, José Moutinho-Pereira^f, Ana Cunha^{g,h}, Fernanda Fidalgo^a

^a GreenUPorto - Sustainable Agrifood Production Research Centre, Department of Biology, Faculty of Sciences of University of Porto, Rua do Campo Alegre s/n, 4169-007 Porto, Portugal

^b Bioengineering and Synthetic Microbiology Group, ICS - Instituto de Investigação e Inovação em Saúde, University of Porto, Porto, Portugal

^c IBMC - Instituto de Biologia Celular e Molecular, University of Porto, Porto, Portugal

^d Biology Department, Faculty of Sciences of University of Porto, Rua do Campo Alegre s/n, 4169-007 Porto, Portugal

^e Biology Department and CESAM - Centre for Environmental and Marine Studies, University of Aveiro, Campus de Santiago, 3810-193 Aveiro, Portugal

^f CITAB - Centre for the Research and Technology of Agro-Environmental and Biological Sciences, Universidade de Trás-os-Montes e Alto Douro, 5000-801 Vila Real, Portugal

^g Biology Department & CITAB - Centre for the Research and Technology of Agro-Environmental and Biological Sciences, School of Sciences, University of Minho, Campus de Gualtar, 4710-057 Braga, Portugal

^h CEB - Centre of Biological Engineering, University of Minho, 4710-057 Braga, Portugal



Article

Ecotoxicological Assessment of a Glyphosate-Based Herbicide in Cover Plants: *Medicago sativa* L. as a Model Species

Beatriz Fernandes^{1,2,†}, Cristiano Soares^{1,3,*,†}, Cláudia Braga¹, Ana Rebotim¹, Rafael Ferreira¹, Joana Ferreira¹, Fernanda Fidalgo^{1,3}, Ruth Pereira^{1,3,†} and Anabela Cachada^{1,4}

¹ Department of Biology, Faculty of Sciences of the University of Porto, Rua do Campo Alegre, s/n, 4169-007 Porto, Portugal; up201503352@fc.up.pt (B.F.); 10180059@ess.ipp.pt (C.B.); up201603230@fc.up.pt (A.R.); up201604298@fc.up.pt (R.F.); up201604044@fc.up.pt (J.F.); ffidalgo@fc.up.pt (F.F.); acachada@fc.up.pt (A.C.)

² Department of Geosciences, Environment and Spatial Plannings, Faculty of Sciences of the University of Porto, Rua do Campo Alegre, s/n, 4169-007 Porto, Portugal

³ GreenUPORTO—Sustainable Agrifood Production Research Centre, Campus de Vairão, Rua da Agrária 747, 4485-646 Vairão, Portugal

⁴ CIIMAR, Novo Edifício do Terminal de Cruzeiros do Porto de Leixões, Avenida General Norton de Matos, S/N, 4450-208 Matosinhos, Portugal

* Correspondence: csoares@fc.up.pt (C.S.); ruth.pereira@fc.up.pt (R.P.)

† These authors contributed equally to this work and, therefore, both should be considered as first co-authors.

Is soil contamination by a glyphosate commercial formulation truly harmless to non-target plants? – Evaluation of oxidative damage and antioxidant responses in tomato

Abstract

Using a realistic and environmentally relevant approach, the present study aimed at understanding the biochemical and physiological basis of glyphosate (GLY)-induced stress in non-target plant species, using tomato (*Solanum lycopersicum* L.) as a model. For this purpose, plants were grown for 28 d under different concentrations of a commercial formulation of GLY (RoundUp® UltraMax) - 0, 10, 20 and 30 mg kg⁻¹ soil. The exposure of plants to increasing concentrations of GLY caused a severe inhibition of growth (root and shoot elongation and fresh weight), especially in the highest treatments. In what regards the levels of reactive oxygen species (ROS), both hydrogen peroxide (H₂O₂) and superoxide anion (O₂^{•-}) remained unchanged in shoots, but significantly increased in roots. Moreover, a concentration-dependent decrease in lipid peroxidation (LP) was found in shoots, though in roots differences were only found for the highest concentration of GLY. The evaluation of the antioxidant (AOX) system showed that GLY interfered with several AOX metabolites (proline, ascorbate and glutathione) and enzyme activities (superoxide dismutase – SOD; catalase – CAT; ascorbate peroxidase – APX), generally inducing a positive response of the defence mechanisms. Overall, data obtained in this study unequivocally demonstrated that soil contamination by GLY, applied as part of its commercial formulation RoundUp® UltraMax, impairs the growth and physiological performance of tomato plants, and likely of other non-target plant species, after 28 d of exposure by clearly affecting the cellular redox homeostasis.

Keywords

Antioxidant system; glyphosate contamination; herbicides; non-target plants; oxidative stress; reactive oxygen species.

1. INTRODUCTION

Glyphosate [N-(phosphonomethyl)glycine; GLY], developed by Monsanto Company (S.A., Belgium, Europe) in the 70s, is the most widely used herbicide worldwide and acts as a post-emergent, non-selective systemic herbicide, often sprayed on leaves of undesired weeds for growth control (Duke and Powles, 2008). Over the years, the development of different GLY-resistant crops has been contributing for increasing GLY applications (Duke and Powles, 2008). Consequently, and as a result of leaching, runoffs, and wind after or even during application, a significant part of GLY can reach the soil and/or surface waters, affecting agroecosystems and non-target plant species, which are not intentionally treated/sprayed with the herbicide. Allied to this, since GLY can be released from dead plants (Neumann et al., 2006), the common tillage and non-tillage practices can potentiate its accumulation in soils at different depths. Alongside, GLY can also be exuded from roots of sprayed plants, in a process known as rhizosphere transfer (Coupland and Caseley, 1979; Tesfamariam et al., 2009). Altogether, these aspects can boost the risks of GLY contamination, since plants also have a root-to-shoot transport pathway for this herbicide (Ricordi et al., 2007). Thus, although different authors and entities, including the European Union (EU) (https://ec.europa.eu/food/plant/pesticides/glyphosate_en), consider GLY a low-risk herbicide, due to its rapid degradation in soil (DT_{50} in the field = 23.79 d, <http://sitem.herts.ac.uk/aeru/iupac/Reports/373.htm>), recent evidence suggest that even residual amounts of GLY can affect non-target plant species, like crops (Gomes et al., 2016b; Gomes et al., 2017a,b; Singh et al., 2017a,b; Soares et al., 2018d). Indeed, despite GLY can be metabolised by different microorganisms or be adsorbed to soil components, its re-solubilization in soil was already reported (Borggaard and Gimsing, 2008). Furthermore, the by-product of GLY's degradation, aminomethylphosphonic acid (AMPA) (Franz et al., 1997; Van Eerd et al., 2003), is also known to be a potent phytotoxin, inducing great negative impacts on plant growth. Although GLY and AMPA are frequently detected in the environment, studies reporting their levels in soils are less common than those for water resources. Nevertheless, GLY has been found in soils within the range of $\mu\text{g kg}^{-1}$ and mg kg^{-1} (Busse et al., 2001; Peruzzo et al., 2008). Recent works found GLY concentrations up to 5 and 8 mg kg^{-1} in agricultural soils (Primost et al., 2017; Peruzzo et al., 2008). Moreover, since maximum GLY levels in water samples reached 15 mg L^{-1} (Wei et al., 2016), it is expected that soil levels can exceed this value due to cumulative applications.

Upon contact with roots, both GLY and AMPA can be absorbed and transported through xylem and phloem, reaching highly active metabolic tissues, like shoot and root meristems (Gomes et al., 2014). Once inside the cell, GLY primarily exerts its effect by blocking the

activity of the 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) enzyme, disturbing the shikimate pathway, which is involved in the biosynthesis of several amino acids, like phenylalanine, tyrosine, and tryptophan (Siehl and Roe, 1997). However, it is currently recognised that GLY and AMPA effects on plant's physiology highly exceed the shikimate pathway, adversely affecting photosynthesis, carbon metabolism and mineral nutrition, and inducing the occurrence of oxidative damage (see review by Gomes et al., 2014). Accordingly, recent studies exploring the interaction between GLY and oxidative stress, suggested that GLY, even in GLY-resistant species, disrupts the redox homeostasis of the cell, favoring the production of reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2), superoxide anion ($\text{O}_2^{\cdot-}$) and hydroxyl radical ($\cdot\text{OH}$), inducing oxidative stress.

In order to counteract the detrimental effects of ROS, plants are equipped with a powerful and complex antioxidant (AOX) system, comprising several low-molecular-weight metabolites, such as glutathione (GSH), ascorbate (AsA) and proline (Pro) (non-enzymatic component), and a substantial number of enzymes involved in ROS detoxification and neutralization (enzymatic component), like superoxide dismutase (SOD; EC 1.15.1.1), catalase (CAT; EC 1.11.1.6) and ascorbate peroxidase (APX; EC 1.11.1.11) (Sharma et al., 2012; Soares et al., 2019). However, under different abiotic stresses, these defence pathways can be inhibited and/or not be enough to neutralise ROS, hence leading to oxidative injuries in lipids, DNA, and proteins and, ultimately, cell apoptosis (Gill and Tuteja, 2010).

As previously stated, the detrimental effects of GLY, along with the biochemical and molecular basis of its mode-of-action, are well studied in target plants, as well as in sensitive and resistant plant species (see works reviewed by Gomes et al., 2014). However, the ecotoxicological relevance of residual GLY concentrations to agroecosystems, in particular to non-target plants like crops, remains to be elucidated. In this way, this work firstly aims to unravel the impacts of soil contamination by GLY in the growth and development of the non-target species *Solanum lycopersicum* L., commonly known as tomato plant. To meet these goals, several biological questions will be answered: 1) What are the main macroscopic and morphological effects of soil contamination by GLY on tomato shoots and roots? 2) Does GLY evoke a severe oxidative stress condition? 3) How will the herbicide affect the performance of the AOX system? 4) Are the responses of tomato plants to GLY dependent on the concentration provided?

2. MATERIALS AND METHODS

2.1. Chemicals and test substrate

The herbicide RoundUp®UltraMax (Monsanto Europe, S.A., Belgium), a glyphosate-based (360 g GLY L⁻¹, potassium salt) herbicide, was acquired from a local supplier. A stock solution of 1 g GLY L⁻¹ was prepared by diluting the commercial formulation of the herbicide in deionised water (dH₂O), and used for obtaining the desired amount of GLY to be added to the soil. The substrate used in this study was an artificial soil (pH 6.0 ± 0.5, organic matter 5%), composed by sphagnum peat, quartz sand (< 2 mm) and kaolin clay (OECD, 2006).

2.2. Experimental design and plant growth conditions

Seeds of *Solanum lycopersicum* cv. Micro-Tom, were surface-sterilised with 70% (v/v) ethanol and 20% (v/v) commercial bleach (5% chlorine) for 5 min each, followed by a series of washing with dH₂O. Afterwards, seeds were placed in Petri dishes (10 cm diameter), containing half-strength Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) solidified with 0.625% (m/v) agar, transferred for a growth chamber [temperature: 25 °C; photoperiod: 16 h/8 h light/dark; photosynthetically active radiation (PAR): 150 µmol m⁻² s⁻¹] and left for germination for 7 d. After germination, plantlets were randomly selected and transferred for pots with 200 g_{dry} of the artificial soil supplemented with 0, 10, 20 and 30 mg kg⁻¹ GLY. The maximum soil water holding capacity (WHC_{max}), determined according to ISO (2005), was adjusted to 40% and the exact volume of deionised and distilled water required for this procedure was used as a carrier to prepare GLY solutions (from the 1 g L⁻¹ stock solution) to obtain the set of concentrations in soil above described. The selection of these concentrations was based on i) previous scientific works, ii) data concerning GLY contamination levels in soils, and iii) the recommended applied doses for agricultural practices (Primost et al., 2017; Gomes et al., 2016a; Mertens et al., 2018; Oliveira et al., 2016; Peruzzo et al., 2008; Singh et al., 2017a,b; Tesfamariam et al., 2009; Zhong et al., 2018). In fact, the first concentration (10 mg kg⁻¹) tested can be classified as environmentally realistic, while the other two (20 and 30 mg kg⁻¹) intend to simulate the effects of cumulative herbicide applications and/or overuse practices (Nguyen et al., 2016). After spiking, to ensure a proper homogenisation of the substrate, the soil was thoroughly and manually mixed. Five plantlets were placed in each pot, which was defined as the biological replicate. For each experimental condition, including the control (CTL), a total of eight biological replicates were prepared. After seedling, plants were immediately watered with 0.5 x Hoagland solution (HS; pH 5.8) (Taiz et al., 2015) and

grown for 28 d in a chamber, under the same conditions above described. At the end of the experiment, plants randomly selected from four biological replicates were collected, separated into shoots and roots and processed for measuring biometric (root and shoot length) and growth-related (fresh mass) parameters. Part of the plant material (from four biological replicates of each experimental condition) was immediately used for some biochemical assays, while the remaining was frozen in liquid N₂ and stored at -80 °C until analyses.

2.3. Oxidative stress biomarkers

2.3.1. ROS (O₂^{•-} and H₂O₂)

The levels of O₂^{•-} were quantified in samples of fresh material (around 250 mg) based on the method described by Gajewska and Sklodowska (2007). Briefly, after 2 h-incubation of plant material in dark conditions in a reaction mixture (2 mL), the resulting solution was heated (85 °C; 15 min) and further centrifuged (15 s; maximum speed). Finally, the absorbance (Abs) of the solution was recorded at 580 nm and O₂^{•-} levels were expressed as the Abs_{580 nm} h⁻¹ g⁻¹ fresh weight (fw). Regarding H₂O₂, the protocol of Jana and Choudhuri (1982) was employed. After homogenization of the plant material (ca 250 mg in 1.5 mL of extraction buffer) and centrifugation (25 min; 6 000 g; 4 °C), the supernatant (SN) reacted with 0.1% (m/v) TiSO₄ in 20% (v/v) H₂SO₄. Lastly, the Abs was read at 410 nm and the H₂O₂ concentration calculated, using the extinction coefficient (ε) of 0.28 μM⁻¹ cm⁻¹, and expressed in nmol g⁻¹ fw.

2.3.2. Lipid peroxidation (LP) and thiols

The analysis of LP was performed by the quantification of malondialdehyde (MDA) according to Heath and Packer (1968). Briefly, after the extraction of the plant material (ca 200 mg) with 0.1% (m/v) trichloroacetic acid (TCA), samples were incubated for 30 min, at 95 °C with 0.5% (m/v) thiobarbituric acid in 20% (m/v) TCA. Then, the Abs of the samples was recorded at 532 and 600 nm and the values obtained at 600 nm were subtracted to the ones at 532 nm to minimise unspecific turbidity effects. The content of MDA was calculated using a ε of 155 mM⁻¹ cm⁻¹ and expressed as nmol g⁻¹ fw. To analyse the levels of thiols, frozen aliquots of shoots (around 250 mg) were homogenised in an extraction solution [20 mM ethylenediaminetetraacetic acid (EDTA) and 20 mM AsA] and, then, used for the quantification of both, total and non-protein thiols according to Zhang et al. (2009). After centrifugation, the SN reacted with 10 mM Ellman's Reagent and was further incubated for colour development. For quantifying the non-protein thiols, proteins were precipitated with 10% (m/v) sulfosalicylic acid. Finally, the Abs of each sample was

read at 412 nm and the thiol levels estimated using a ϵ of $13\,600\text{ M}^{-1}\text{ cm}^{-1}$ and expressed in $\text{nmol g}^{-1}\text{ fw}$.

2.4. Quantification of AsA, GSH and Pro

The levels of total, reduced (AsA) and oxidised ascorbate (DHA) were quantified spectrophotometrically following the protocol of Gillespie and Ainsworth (2007). Aliquots of frozen shoots material (ca 250 mg) were extracted in 1.5 mL 6% (m/v) TCA on ice and centrifuged (10 min; 6 000 g ; 4 °C). The SN was mixed with a reaction mixture containing α - α' -dipyridyl and incubated for 1 h at 37 °C. For the determination of the total ascorbate content, prior to the reaction, the SN was treated with dithiothreitol (10 mM). Afterwards, the Abs of each sample was recorded at 525 nm.

GSH quantification was accomplished by following the method of Glutathione Assay Kit (CS0260; Sigma-Aldrich®). Briefly, frozen samples of shoots and roots (around 200 mg) were homogenised, on ice, in 3% (m/v) sulfosalicylic acid and centrifuged for 10 min at 10 000 g . Then, the SN was mixed with a reaction solution containing Ellman's Reagent (1.5 mg mL^{-1}) and incubated for 10 min in dark conditions. Finally, the Abs at 412 nm was recorded and used for quantifying the levels of GSH.

The content of proline in tissues was determined by the ninhydrin-based colorimetric assay (Bates et al., 1973). After extraction of the plant material (200 mg in 1.5 mL) in 3% (m/v) sulfosalicylic acid, the SN was mixed with glacial acetic acid and ninhydrin and incubated for 1 h at 96 °C. Finally, the complex proline-ninhydrin formed was extracted with toluene and the Abs of this complex was recorded at 520 nm.

For each AOX, a standard curve was prepared with solutions of known-concentrations and all the results were expressed in a fw basis.

2.5. Extraction of the antioxidant enzymes

The extraction of the main AOX enzymes (SOD, CAT and APX) was performed according to de Sousa et al. (2013). Frozen samples of shoot and root tissues were homogenised, on ice, in an extraction buffer, composed of 100 mM potassium phosphate (PK) (pH 7.3), 8% (m/v) glycerol, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1mM EDTA and 5 mM AsA, and centrifuged (4 °C) at 16 000 g for 25 min. Afterwards, the SN was used to measure enzyme's activity and total protein content (Bradford, 1976).

2.6. Activity quantification of SOD, CAT and APX

The total activity of SOD was quantified by spectrophotometry, based on the inhibition of the photochemical reduction of nitroblue tetrazolium (NBT), following the protocol of

Donahue et al. (1997). For each SN, a volume of each extract containing 15 µg of proteins was added to a reaction solution, containing 50 mM PK (pH 7.8), 0.1 mM EDTA, 13 mM L-methionine, 75 µM NBT and 0.0067 µM riboflavin. Samples were incubated for 10 min under 6 fluorescent 8 W lamps and, then, the Abs of each mixture was read at 560 nm. SOD activity was expressed as units of SOD mg⁻¹ of protein, in which one SOD unit is defined as the amount of enzyme that inhibits the photochemical reduction of NBT by 50%.

The activity of CAT was measured according to Aebi (1984), with slight modifications in a protocol adapted for UV-microplates. Briefly, 160 µL of PK buffer (pH 7.0) were added to 20 µL of sample extract and 20 µL of 100 mM H₂O₂. Then, the degradation of H₂O₂ was monitored at 240 nm for 30 s, in 5-s intervals. CAT activity was expressed in nmol H₂O₂ min⁻¹ mg⁻¹ of protein, using a ϵ of 39.4 M⁻¹ cm⁻¹.

The total activity of APX was spectrophotometrically quantified based on the method described by Nakano and Asada (1981), also adapted for UV-microplates. In this case, 170 µL of 50 mM PK buffer (pH 7.0) containing 0.6 mM AsA were combined with 20 µL of protein extract and 10 µL of 254 mM H₂O₂. Afterwards, AsA oxidation was followed over 30 s, in 5-s intervals, at 290 nm. APX activity was expressed as µmol AsA min⁻¹ mg⁻¹ of protein, using the AsA extinction coefficient of 2.8 mM⁻¹ cm⁻¹.

2.7. Statistics

All results were expressed as the mean of four biological replicates $\pm$ the standard deviation (SD). After checking the normality and homogeneity of data, an one-way ANOVA was performed, assuming a significance level of 0.05, in order to test the hypothesis of no significant differences between each GLY treatment (0, 10, 20 and 30 mg kg⁻¹) for all the parameters assessed in exposed plants. When differences were recorded, Tukey's *post-hoc* test was applied to discriminate differences between treatments. All statistical procedures were performed in GraphPad® Prism 7 (GraphPad Software Inc., USA).

3. RESULTS

3.1. Biometrics and growth-related parameters

The contamination of soil by GLY negatively affected plant growth, inducing several phytotoxic symptoms, like chlorosis and inhibition of shoot apical growth (Figure 1), followed by a decrease in the biometric parameters (Tables 1 and 2). As can be seen, the fresh biomass [shoots: $F(3, 14) = 81.93$; $p < 0.001$; roots: $F(3, 14) = 164$; $p < 0.001$] of tomato plants was significantly reduced up to 86 and 95% in shoots and roots, respectively, in relation to the CTL, in a dose-dependent manner. The same pattern was also observed

for shoots' height [$F(3, 87) = 71.33$; $p < 0.001$] and roots' length [$F(3, 91) = 65.51$; $p < 0.001$], once GLY induced marked declines in these parameters, especially in the two highest concentrations (Tables 1 and 2).

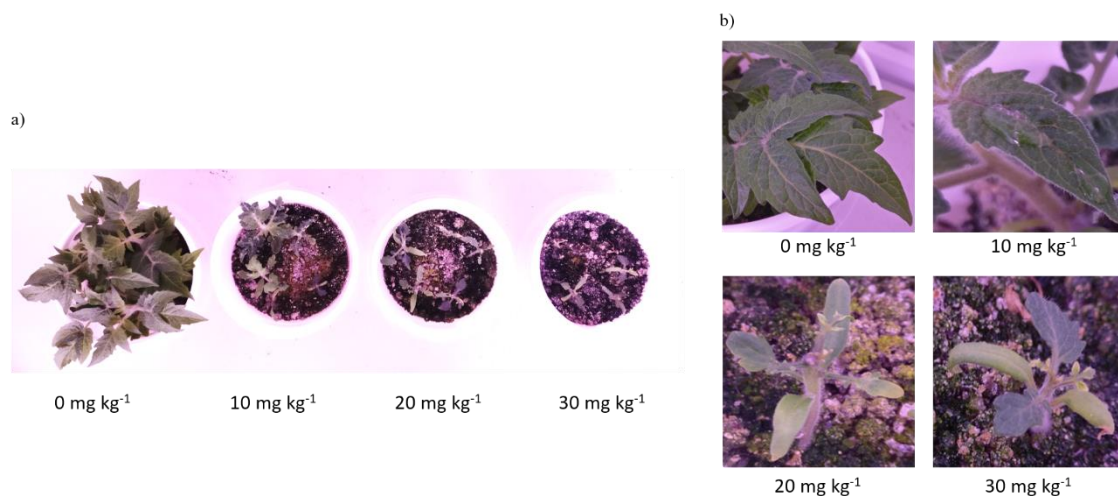


Figure 1. Effects of different concentrations (0, 10, 20 and 30 mg kg⁻¹) of GLY on *S. lycopersicum* plants, after 28 d of growth (a). Leaf chlorosis and shoot apex dysfunction induced by GLY, especially in the highest applied concentrations (b).

3.2. Oxidative stress markers

3.2.1. O₂^{•-} and H₂O₂ levels

The levels of O₂^{•-} and H₂O₂ showed different responses between shoots and roots of GLY-exposed plants (Tables 1 and 2). As it can be observed, in shoots, no significant differences were found among treatments for both analysed ROS [H₂O₂: $F(3, 8) = 3.05$; $p > 0.05$; O₂^{•-}: $F(3, 9) = 2.13$; $p > 0.05$] (Table 1). In roots, O₂^{•-} production [$F(3, 9) = 21.76$; $p < 0.001$] was strongly induced by GLY even in the lowest tested concentration, reaching an increase of 100% in the treatment of 30 mg kg⁻¹, in comparison with the CTL (Table 2). Regarding H₂O₂ [$F(3, 8) = 8.81$; $p < 0.01$], only plants exposed to 30 mg kg⁻¹ manifested a significant rise in its content (40% in relation to the CTL) (Table 2).

Table 1. Fresh weight, height, H₂O₂, O₂^{•-}, MDA, thiols, AsA, GSH and Pro contents in shoots of *S. lycopersicum* after 28 d of growth in OECD soil contaminated by increasing concentrations (0, 10, 20 and 30 mg kg⁻¹) of GLY.

Endpoint	[Glyphosate] mg kg ⁻¹			
	0	10	20	30
Fresh weight (g)	8.3 ± 0.3 a	6.8 ± 0.4 b	3.6 ± 0.5 c	1.1 ± 0.2 d
Shoot height (cm)	7.1 ± 0.2 a	5.9 ± 0.3 b	3.8 ± 0.3 c	2.6 ± 0.2 d

H ₂ O ₂ (nmol g ⁻¹ fw)	856 ± 61	1158 ± 128	768 ± 96	859 ± 94
O ₂ ^{•-} (Abs g ⁻¹ fw)	3.2 ± 0.2	4.2 ± 0.3	4.3 ± 0.4	4.2 ± 0.3
MDA (nmol g ⁻¹ fw)	25.9 ± 1.0 a	23.4 ± 0.9 a	18.1 ± 0.4 b	14.2 ± 1.7 b
Total thiols (μmol g ⁻¹ fw)	0.71 ± 0.03 a	0.80 ± 0.04 a	0.48 ± 0.05 b	0.44 ± 0.08 b
Protein-bond thiols (μmol g ⁻¹ fw)	0.50 ± 0.02 a	0.46 ± 0.02 a	0.27 ± 0.02 b	0.21 ± 0.03 b
Proline (μg g ⁻¹ fw)	78 ± 4 c	144 ± 26 c	279 ± 12 b	775 ± 123 a
Total ascorbate (nmol g ⁻¹ fw)	1.47 ± 0.20	1.14 ± 0.07	2.01 ± 0.46	1.97 ± 0.15
AsA (nmol g ⁻¹ fw)	1.27 ± 0.16	0.69 ± 0.09	1.06 ± 0.39	1.24 ± 0.11
DHA (nmol g ⁻¹ fw)	0.16 ± 0.04 b	0.39 ± 0.10 ab	0.56 ± 0.07 a	0.73 ± 0.16 a
AsA/DHA	6.8 ± 0.5 a	1.9 ± 0.8 b	1.8 ± 0.6 b	2.0 ± 0.6 b
GSH (nmol g ⁻¹ fw)	334 ± 35 a	243 ± 4 b	126 ± 4 c	287 ± 36 ab

Results are expressed as mean ± standard deviation (SD). Different letters after number indicate statistic differences at $p \leq 0.05$, according to Tukey test.

Table 2. Fresh weight, root length, H₂O₂, O₂^{•-}, MDA, GSH and Pro content in roots of *S. lycopersicum* after 28 d of growth in OECD soil contaminated by increasing concentrations (0, 10, 20 and 30 mg kg⁻¹) of GLY.

Endpoint	[Glyphosate] mg kg ⁻¹			
	0	10	20	30
Fresh weight (g)	2.60 ± 0.11 a	1.56 ± 0.02 b	0.43 ± 0.08 c	0.12 ± 0.02 c
Root length (cm)	27.5 ± 1.5 a	12.1 ± 1.4 b	5.8 ± 0.5 c	4.6 ± 0.6 c
H ₂ O ₂ (nmol g ⁻¹ fw)	1011 ± 25 b	1108 ± 123 b	981 ± 24 b	1411 ± 36 a
O ₂ ^{•-} (Abs g ⁻¹ fw)	2.55 ± 0.17 c	4.50 ± 0.22 ab	3.95 ± 0.21 b	5.09 ± 0.37 a
MDA (nmol g ⁻¹ fw)	10.8 ± 0.9 b	11.6 ± 0.7 b	11.6 ± 0.4 b	16.5 ± 2.1 a
Proline (μg g ⁻¹ fw)	46 ± 4 c	53 ± 1 bc	391 ± 99 a	284 ± 35 ab
GSH (nmol g ⁻¹ fw)	27 ± 2 c	52 ± 6 bc	95 ± 10 ab	140 ± 23 a

Results are expressed as mean ± standard deviation (SD). Different letters after number indicate statistic differences at $p \leq 0.05$, according to Tukey test.

3.2.2. MDA and thiols content

Shoots and roots of tomato plants showed different responses regarding MDA levels: in shoots, MDA content was reduced in a dose-dependent manner [F (3, 10) = 25.5; $p < 0.001$], with the highest GLY concentration causing a decline of about 50%, relatively to the CTL (Table 1); in roots [F (3, 10) = 5.30; $p < 0.05$], only plants exposed to 30 mg kg⁻¹ GLY were affected, with a 53% increase in MDA levels comparatively to the CTL (Table 2).

In what concerns the thiol levels, only shoot samples were analysed. Results regarding their total content, as well as their protein and non-protein fractions, are represented in Table 1. In this case, both total [$F(3, 11) = 11.55$; $p < 0.01$] and protein [$F(3, 11) = 33.83$; $p < 0.001$] thiols were significantly reduced by GLY exposure, especially in the two highest applied doses. Actually, plants exposed to 20 mg kg^{-1} GLY presented a decline of 32 and 45% in total and protein thiols, respectively, when compared to the CTL, while 30 mg kg^{-1} GLY induced an even more marked reduction (total thiols – 39%; protein thiols – 59%), although no significant differences were found between these treatments (20 and 30 mg kg^{-1}).

3.3. Antioxidant system performance

3.3.1. Non-enzymatic component – AsA, GSH and Pro

Ascorbate results are presented in Table 1. When considering total ascorbate levels [$F(3, 9) = 1.74$; $p > 0.05$], no significant differences among treatments were recorded. However, the ratio between AsA and DHA [$F(3, 9) = 16.56$; $p < 0.001$], which is a good indicator of the cellular redox status, was reduced in response to all GLY concentrations, by around 70% (Table 1). This reduction was likely caused by the observed rise in the oxidised form [DHA; $F(3, 11) = 6.09$; $p < 0.05$], whose levels were higher in all the treatments in comparison to the CTL.

Regarding GSH content, a contrasting pattern was detected among organs, being this AOX generally reduced in shoots [$F(3, 8) = 20$; $p < 0.001$] with the increase of GLY (declines of 24 and 63 in 10 and 20 mg kg^{-1} groups, respectively). Regarding roots, GSH content was positively affected throughout all treatments, with increases up to 4-fold [$F(3, 8) = 15.63$; $p < 0.01$] (Tables 1 and 2).

The levels of Pro were strongly affected by GLY in both plant organs [shoots: $F(3, 11) = 59.11$; $p < 0.001$; roots: $F(3, 7) = 9.88$; $p < 0.01$], being concentration-dependent in shoots, but not in roots. As it can be observed, shoots and roots of GLY-treated plants increased Pro levels up to 9- and 4-fold, respectively, in comparison with the CTL group.

3.3.2. Enzymatic component – SOD, CAT and APX

In order to assess the effects of increased doses of GLY on the performance of the plant enzymatic AOX system, the activity of three of the main AOX enzymes was studied in both shoots and roots of tomato plants (Figure 2). In shoots, the herbicide positively affected all of the three studied enzymes [SOD: $F(3, 8) = 25.64$; $p < 0.001$; CAT: $F(3, 8) = 21.76$; $p < 0.001$; APX: $F(3, 8) = 105.8$; $p < 0.001$], in a dose-dependent manner, with the highest concentration inducing the most evident increase in the activities of SOD (2.3-fold), CAT

(3-fold) and APX (4.4-fold), relatively to the control. Regarding roots, as can be seen in Figure 2, SOD activity [$F(3, 8) = 11.15$; $p < 0.01$] was repressed along with the increase of GLY concentrations in soil, with reductions of 61 and 41% in the two highest treatments. Contrastingly, CAT [$F(3, 8) = 34.53$; $p < 0.001$] and APX [$F(3, 8) = 11.91$; $p < 0.01$] activities in roots followed the same pattern observed in shoots, with increases dependent of the concentration of GLY in soil, reaching rises of 4-fold and 0.87-fold compared to the CTL, respectively (Figure 2).

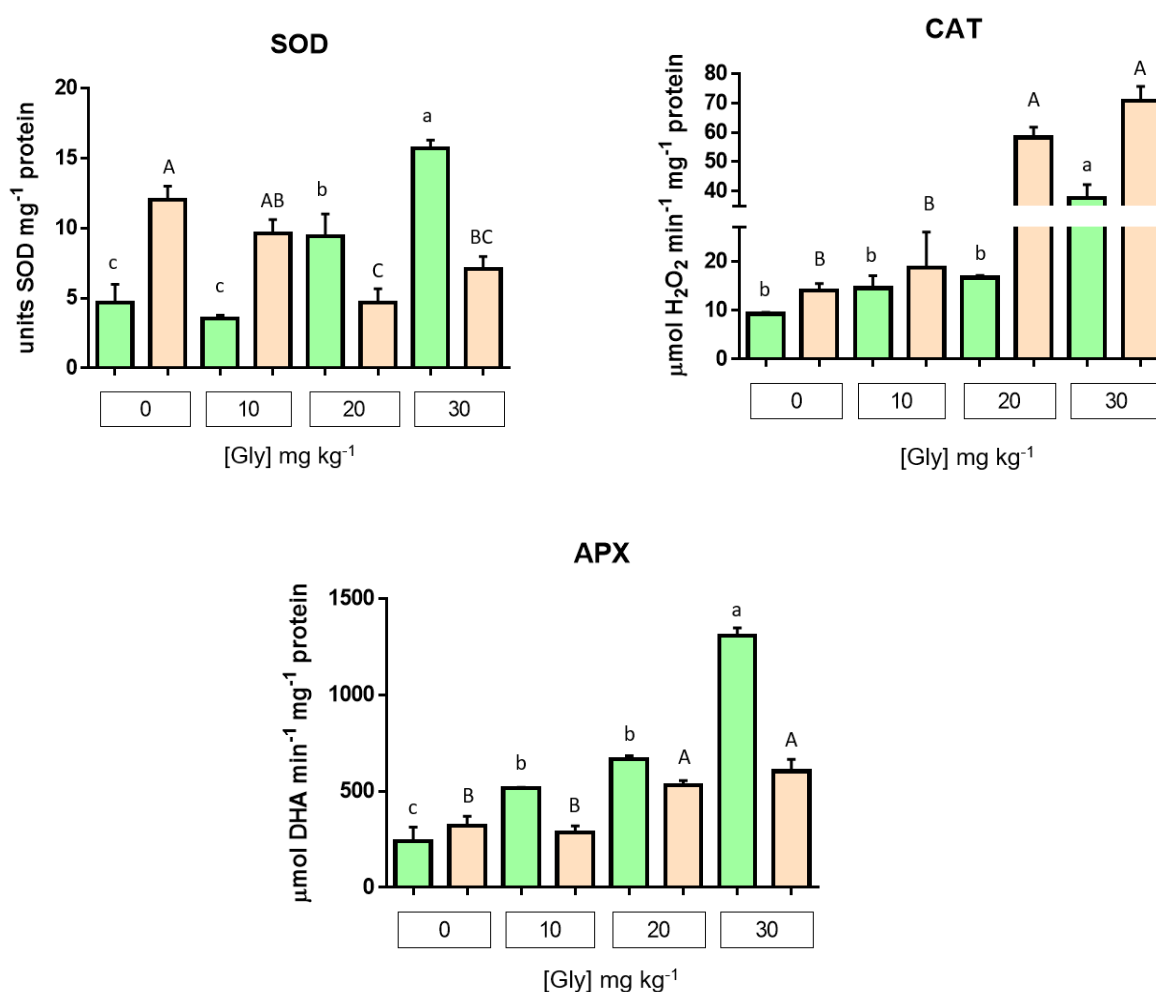


Figure 2. Activity of SOD (left), CAT (right) and APX (bottom) in shoots (green) and roots (pink) of *S. lycopersicum* exposed to increased concentrations of GLY (0, 10, 20 and 30 mg kg⁻¹) after 28 d of growth. Results are expressed as mean $\pm$ standard deviation (SD). Different letters above bars indicate statistic differences at $p \leq 0.05$ (lowercase letters – shoots; capital letters – roots).

4. DISCUSSION

Although studies dealing with GLY foliar application are relatively common, little is known concerning the phytotoxicity of soils contaminated by GLY for non-target plants, where tomato is included. Additionally, the majority of the studies exploring the effects of GLY on non-target plant species did not simulate soil contamination scenarios; instead, GLY was often sprayed in leaves, as it is in target species, or provided to the nutrient solution in high levels (de Freitas-Silva et al., 2017; Gomes et al., 2016a; Gomes et al., 2017a; Mondal et al., 2017; Serra et al., 2015; Tong et al., 2017). Thus, this work is one of the firsts providing important findings related to the specific and realistic phytotoxicity of soil contamination by GLY in one of the most important crops worldwide, tomato. In this way, the main goal of this work was to unveil the effects of GLY (0, 10, 20 and 30 mg kg⁻¹), as part of one of the most used commercial formulations in the world, on the growth and oxidative status of soil-grown *S. lycopersicum* plants.

GLY raised significant disturbances in tomato's growth, particularly in shoot and root apex development

After 28 d of GLY exposure, the herbicide severely repressed the growth of *S. lycopersicum* plants in a concentration-dependent manner, reaching its maximum inhibitory effect in the highest concentration tested (Figure 1). Indeed, knowing that herbicides are purposely developed to impair plant cell's viability, it is not surprising that high concentrations of GLY have the ability to negatively affect plant growth, even of non-weed species (Brito et al., 2018; Gomes et al., 2014; Soares et al., 2018c). Yet, it is presumed that GLY quickly degrades upon contact with soil, being harmless for non-target species and, thereby, assumed as a low-risk agrochemical (Baylis, 2000; Borggaard and Gimsing, 2008). Despite of that, in the present study, our results pinpointed that GLY exerts its herbicidal activity even at doses substantially lower than those recommended for foliar application. Although studies dealing with the phytotoxicity of soil/natural waters contaminated by GLY are limited, equivalent findings have already been reported for several plant species, such as barley, pea and even tomato (Singh et al., 2017a,b; Soares et al., 2018c). Moreover, as reviewed by Gomes et al. (2014), foliar-applied GLY can suffer further exudation from roots to soil, being capable of inhibiting the growth of adjacent plants and seedlings (Kremer et al., 2005).

After reaching the soil, both GLY and AMPA can be uptaken by roots (Gomes et al., 2014), quickly reaching root and shoot meristems through xylem movement. Indeed, works performed with *Helianthus annuus* L., *Sorghum halepense* L., and *Zea mays* L., revealed this herbicide tends to accumulate in highly active metabolic tissues, impairing the

shikimate pathway and, thus, preventing the biosynthesis of several important amino acids (Eker et al., 2006; Hetherington et al., 1999; Vila-Aiub et al., 2012). In line with this, our results clearly showed that, although GLY was added to the soil, it greatly affected the normal development of shoot's apex (Figure 1), especially in the treatments of 20 and 30 mg kg⁻¹. Furthermore, GLY highly hampered root growth, at both length and fresh mass, in a much more pronounced way than in shoots, a fact that was likely related with the direct contact of the roots with the GLY-contaminated soil. Moreover, as GLY can directly reduce the endogenous levels of indole-3-acetic acid (IAA) (Gomes et al., 2014), the observed decrease in growth may have also be related with this disturbance, alongside with GLY interference with plant-water relations. Actually, even using GLY-resistant soybean plants, Krenchinski et al. (2017) proved that GLY evoked a decline in water-use efficiency, along with detrimental effects on photosynthetic-related endpoints.

Besides the inhibition of plants' growth, GLY might also have conditioned the physiological uptake of mineral nutrients, though there is an extensive debate on how GLY can affect this process and further research is required (Zobiole et al., 2010a,b). In fact, up to now, several studies reported that this agrochemical can negatively influence the absorption of different macro and micronutrients, such as calcium (Ca), magnesium (Mg), nitrogen (N), phosphorous (P), iron (Fe), zinc (Zn), among others (reviewed by Gomes et al., 2014), whilst other works did not observe any relationship between GLY and mineral nutrition (reviewed by Gomes et al., 2014). Yet, in a study conducted with sunflower plants, root-to-shoot translocation of micronutrients was highly repressed by GLY (Eker et al., 2006). Furthermore, it appears that GLY toxicity for this physiological process is greatly dependent on the mode-of-application, the concentration used, as well as the plant developmental stage (Zobiole et al., 2011). Also, it cannot be forgotten that GLY may compete with phosphate (PO₄³⁻) to enter the root system possibly affecting *in planta* P levels. Based on our results, especially the observed leaf chlorosis and reduced plant growth, it can be hypothesized that soil contamination by GLY constrained the uptake of nutrients from the soil solution. In the future, in order to test this hypothesis, the levels of the main nutrients must be analysed in both shoots and roots of tomato plants.

GLY-induced oxidative stress was more pronounced in roots than in shoots in a concentration-dependent manner

Once exposed to unfavourable conditions, such as salinity, drought, ultraviolet radiation, metal toxicity, and/or herbicides contamination, plant cell's homeostasis and normal physiology can be austerey conditioned, leading to a series of interconnected events and metabolic adjustments (Gill and Tuteja, 2010; Sharma et al., 2012). Although plant

responses can be stress-specific, the induction of oxidative stress, as consequence of a burst in ROS production, is a common feature to all types of stress (Gill and Tuteja, 2010; Sharma et al., 2012; Tripathi et al., 2017).

In this study, the induction of pro-oxidative conditions was assessed by measuring the levels of two of the main ROS – H_2O_2 and $\text{O}_2^{\cdot-}$ – and by quantifying MDA and thiols (total, protein and non-protein) content. Our results showed that the accumulation of ROS was tissue- and concentration-dependent. In shoots, levels of both H_2O_2 and $\text{O}_2^{\cdot-}$ were not changed with GLY treatments, indicating that the herbicide did not provoke significant oxidative damage in the aerial part of *S. lycopersicum*, at the concentrations tested. Indeed, although previous studies reported an increased accumulation of ROS in GLY-exposed leaves (Ahsan et al., 2008; Gomes et al., 2016b; Radwan and Fayez, 2016; Soares et al., 2018c; Zhong et al., 2018) (with concentrations similar to this study), Moldes et al. (2008) did not find any upsurge induced by GLY in oxidative stress markers in leaves of resistant and susceptible soybean genotypes. Also, the exposure of *Arabidopsis thaliana* (L.) Heynh. to 40 μM GLY resulted in decreased levels of H_2O_2 (de Freitas-Silva et al., 2017), while in *Dimorphandra wilsonii* Rizz. the content of this ROS did not change in response to GLY (0, 5, 25 and 50 mg L^{-1}) (Gomes et al., 2017a). Corroborating the data obtained for ROS content, lipid peroxidation, assessed based on the levels of MDA, did not increase in shoots and even significantly decreased in this organ for the highest applied doses. When studying the responses of *Vallisneria natans* (Lour.) H.Hara to GLY, Zhong et al. (2018) reported that lipid peroxidation did not change after 1 and 7 d of exposure to the herbicide (0-80 mg L^{-1}). However, in the present work, thiols' levels were diminished upon GLY exposure, especially in leaves under the highest applied concentrations (20 and 30 mg kg^{-1}). The evaluation of thiols as an oxidative stress marker is increasingly frequent and they are currently regarded as key elements to enhance plants' tolerance to abiotic stress (Zagorchev et al., 2013). Thus, to really assess if shoots were in the absence of oxidative damage, we decided to evaluate the content of thiols in shoots of tomato plants. Based on our findings, the observed decrease of this oxidative stress biomarker in shoots in response to the herbicide concentrations suggests that, although the other endpoints (lipid peroxidation and ROS) did not increase, substantial changes occurred in the shoots of tomato plants, with thiols possibly playing an important role in detoxification. Indeed, Soares et al. (2018a) also reported changes in thiols' content in barley plants exposed to another emerging contaminant (NiO nanoparticles), confirming that thiols are a sensitive marker of the exposure of plant tissues to oxidative stress. Actually, the decreased levels of free -SH groups may indicate that these compounds are being recruited for detoxification and redox-active processes, consequently reducing the oxidative damage. Thus, based on this set of results, a new hypothesis can be raised – the AOX response of

the shoots of *S. lycopersicum* is able to limit the oxidative damage induced by the herbicide.

Regarding roots, an opposite pattern from that observed in shoots was recorded – ROS production, especially $O_2^{\cdot-}$, was stimulated by the herbicide in almost all treatments, along with LP, whose levels were significantly increased in the highest herbicide concentration. In opposition to the shoots, these observations were not a surprise and were most likely related to i) the highest availability of GLY to the roots given the exposure pathway and/or ii) a less effective AOX response of root tissues to counteract herbicide-induced oxidative stress. As previously mentioned, there are only few studies dealing with GLY effects on plant oxidative metabolism and most of the available records only analyse the responses in aerial organs. However, in line with our data, the exposure of willow (*Salix miyabeana* Seemen) to GLY (1 mg L^{-1}) under hydroponic conditions resulted in higher LP and loss of plasma membrane function in roots (Gomes et al., 2016a,b). Overall, GLY triggered the occurrence of oxidative damage in both organs, especially in roots, which may have largely contributed to the observed growth impairment. Indeed, under pro-oxidative conditions, the activation of different metabolic pathways requires a high ATP demand, leading to a cellular investment in repairing mechanisms rather than in growth and development processes.

Non-enzymatic and enzymatic AOX mechanisms were activated by GLY in both shoots and roots

Although under stressful conditions ROS can be overproduced and damage lipids, proteins and nucleic acids, plants have a powerful and efficient AOX system, responsible for keeping a homeostatic balance between the production and the neutralization of ROS (Gill and Tuteja, 2010). In response to different stresses, the performance of the AOX defences can be enhanced or inhibited, leading to tolerance or susceptibility (Gill and Tuteja, 2010; Sharma et al., 2012; Soares et al., 2019). It is recognised that GLY has the ability to interact with the AOX mechanisms, at both protein synthesis and gene expression levels (Ahsan et al., 2008; Gomes et al., 2014; Moldes et al., 2008; Singh et al., 2017a,b). When GLY was applied as foliar spray or added to the nutrients solution of soybean and pea plants, respectively, changes in both enzymatic (glutathione S-transferase – GST, glutathione reductase – GR, SOD, CAT, APX and guaiacol peroxidase – GPX) and non-enzymatic (GSH) components of the AOX system were recorded (Miteva et al., 2010; Moldes et al., 2008).

Over the last years, Pro has been increasingly gaining special attention due to its AOX potential and it is now recognised that the functions of this amino acid highly exceed its

role as a compatible solute. Indeed, the accumulation of Pro in response to several anthropic-related abiotic stresses, such as xenobiotics and metals, is commonly observed in different plant species (Gill and Tuteja, 2010; Hayat et al., 2012). For instance, results obtained by Soares et al. (2018a, 2018b) revealed that nano-sized Ni and paracetamol led to a higher Pro accumulation in barley plants. Here, our data showed that increased concentrations of GLY potentiated the levels of this non-enzymatic AOX in both plant organs, especially in 20 and 30 mg kg⁻¹ GLY treatments. This observation is paired with a recent study, also conducted with tomato plants, in which foliar-applied GLY (42 mg L⁻¹) stimulated the levels of Pro (Singh et al., 2017a), as a defence mechanism to prevent oxidative damage and/or to contribute to a higher osmotic balance (Hayat et al., 2012). The opposite trend between Pro and lipid peroxidation levels found in shoots is not a surprise, since it is globally recognised that this amino acid effectively prevents the peroxidation of lipids (Hayat et al., 2012). Indeed, acting as a membrane stabiliser and being capable of directly neutralising the most dangerous ROS – $\cdot\text{OH}$ – Pro helps to keep oxidative stress under control and usually enhances plant abiotic stress tolerance (Gill and Tuteja, 2010; Soares et al., 2019). Thus, taking into consideration the observed rise in Pro levels in shoots of plants from GLY-treated soils, it seems that this AOX, acting synergistically with other compounds/mechanisms, was able to limit oxidative damage, possibly explaining the marked decrease in the content of MDA in shoots. However, in roots, the accumulation of Pro was not able to reduce the oxidative damage evidenced by the higher levels of lipid peroxidation in plants exposed to the highest GLY treatment. Indeed, if the stress factor is too pronounced, Pro cannot be enough to prevent the damages.

Alongside with Pro, AsA and GSH are the most important non-enzymatic AOX molecules (Sharma et al., 2012). Our data revealed different responses of both AOX in shoots and roots of tomato plants. In shoots, the levels of GSH and AsA were reduced by the herbicide exposure, being this effect dependent on the concentration for GSH. Actually, this behaviour is commonly detected in plants subjected to various types of adverse growth conditions (Gill and Tuteja, 2010). Under balanced situations, the reduced forms (GSH and AsA) are more abundant than the oxidised (GSSG and DHA) ones, being this index usually regarded as a robust indicator of pro-oxidative conditions (Sharma et al., 2012). Once again, despite levels of ROS and lipid peroxidation remained unchanged or decreased in shoots, the accumulation of free Pro along with the variation pattern recorded for GSH and AsA/DHA levels, unequivocally show that shoots were also under oxidative damage. In contrast, GSH content in roots was increased in GLY treatments in a dose-dependent manner. Paired with these findings, other authors also reported enhanced levels of GSSG in leaves and roots of pea plants treated with GLY (Miteva et al., 2010).

Overall, soil contamination by GLY induced the activation of several non-enzymatic AOX pathways, with Pro, AsA and GSH playing important roles in mitigating oxidative damage.

Acting in coordination with non-enzymatic mechanisms, plant cells also display important enzymatic events to mediate ROS toxicity and to ensure the redox homeostasis. Among all, a great relevance is attributed to SOD, which is considered the first enzymatic line of defence against $O_2^{\cdot-}$, catalysing its dismutation into H_2O_2 , and CAT and APX, both involved in the intracellular detoxification of H_2O_2 (Soares et al., 2019). Globally, in this study, the activity of the three enzymes was enhanced in both organs upon GLY exposure, with the exception of SOD in roots, whose activity was inhibited by the presence of the herbicide. Thus, one can assume that the observed rise in SOD activity has also allowed a better management of GLY-induced oxidative stress in shoots, by preventing the overaccumulation of $O_2^{\cdot-}$. On the contrary in roots, the marked decrease in SOD was much likely linked to the rise of $O_2^{\cdot-}$ content in this tissue. Up to date, there are controversial reports about the effects of different xenobiotics, where herbicides are included, on the performance of several AOX enzymes. Specifically concerning GLY, there are still a few studies exploring the involvement of ROS and AOX enzymes in its associated phytotoxicity. In previous studies, the exposure of pea and tomato plants to this herbicide culminated in an increased activity of SOD, as a protective AOX mechanism against oxidative stress (Singh et al., 2017a,b). Yet, SOD activity, evaluated by native gel staining, in leaves and roots of soybean plants did not change as a consequence of the herbicide treatment (Moldes et al., 2008); however, when studying the potential phytotoxicity of GLY, Soares et al. (2018c) also recorded an increase in SOD activity in leaves and roots of barley plants exposed to 30 mg kg⁻¹ GLY.

As described for SOD and $O_2^{\cdot-}$, the total activity of CAT and APX generally matched the pattern registered for H_2O_2 levels, albeit its levels are not always meaningful of harsh oxidative damage, since this ROS is highly recognised for its signaling properties and involvement in many physiological processes (Soares et al., 2019). Although several other enzymes, like many classes of peroxidases, are also involved in H_2O_2 elimination, APX and CAT are the most effective players in controlling the intracellular levels of this ROS. Based on our data, it seems that the activation of these two enzymes helped to maintain the H_2O_2 content under balanced conditions, since its levels were not changed from the CTL situation in shoots and, in roots, only increased upon exposure to 30 mg kg⁻¹ GLY. Moreover, given the obtained results, it is also evident that APX activation occurred at lower concentrations than CAT, since significant differences from the CTL were only found in the highest GLY treatment. Indeed, although CAT does not require any reducing agent, in the last years several publications are attributing a growing importance to APX over CAT in H_2O_2 elimination inside plant cells (Foyer and Noctor, 2005; Halliwell, 2006). Besides

that, the significantly higher APX activity in shoots of tomato plants exposed to all GLY concentrations, helps to explain the observed decrease in AsA/DHA ratio. The enzymatic reaction catalysed by APX requires the reducing power of AsA, which is oxidised to DHA (Gill and Tuteja, 2010). Thus, it can be suggested that AsA-mediated protection of oxidative stress may arise from its direct involvement in ROS neutralization and/or from its ability to serve as electron donor to APX.

Over the years, the emergence of studies related to the AOX responses of plants to pesticides, such as paraquat, GLY, 2,4-D, atrazine and prometryne, allowed to infer that, generally, there is an upsurge in the activity of different AOX enzymes, being this effect dependent on the exposure conditions and media, duration and magnitude of the stress and, also, the plant species and/or cultivar (Cui et al., 2010; Jun Zhang et al., 2014; Moldes et al., 2008; Shakir et al., 2018, 2016; Wu et al., 2010). Accordingly, in the present study, significant boosts in the AOX performance of tomato shoots and roots under GLY stress were observed. Actually, in a previous record, the treatment of *S. lycopersicum* plants with emamectin benzoate, α -cypermethrin and imidacloprid also culminated in higher activities of SOD, CAT and APX (Shakir et al., 2018).

Overall, after 28 d of growth, low concentrations (10, 20 and 30 mg kg⁻¹) of GLY greatly impaired the growth and physiological performance of *Solanum lycopersicum* L. plants in a concentration-dependent manner, with different effects in shoots and roots (Figure 3). Our data clearly indicated that the AOX system was greatly activated in both organs, although in roots this response was not enough to counteract the oxidative damage induced by the overproduction of ROS, thereby culminating in a very pronounced reduction of growth (Figure 3). As previously discussed in this work, GLY applications can induce harms of multiple magnitudes in different plant species (see review by Gomes et al., 2014). Also, if even GLY-resistant plants are physiologically disturbed by the herbicide (Cakmak et al., 2009; Johal and Huber, 2009; King et al., 2001; Krenchinski et al., 2017; Moldes et al., 2008; Zobiole et al., 2011, 2010), it is not surprising that its phytotoxicity is much higher in susceptible non-target plant species, such as important crops, like tomato, and plants with other functions in agroecosystems (e.g. cover crop species). Additionally, it is worth mentioning that the phytotoxic effects recorded in this study may have also been synergistically or antagonistically modulated by the other components of the commercial formulation used in this work. Actually, in a previous research, it was shown that the toxicity of different pesticide formulations was different from that of their active ingredients (GLY included) for both aquatic and terrestrial species (Pereira et al., 2009). Despite of that, and knowing that the active ingredient is assumed to be the chemical substance that is intentionally produced to be a biocide, it is more environmentally relevant to test it as part of the chemical formulation that attains the soil.

Thus, by simulating soil contamination by GLY residues at environmentally relevant concentrations, this work provides new shreds of evidence of GLY-associated risks to agroecosystems and food chains, reinforcing the urgent need of new studies dealing with GLY effects on susceptible and simultaneous non-target crop and wildlife plant species to better understand the impacts of GLY on agronomic yield, agroecosystem biodiversity and, ultimately, human health.

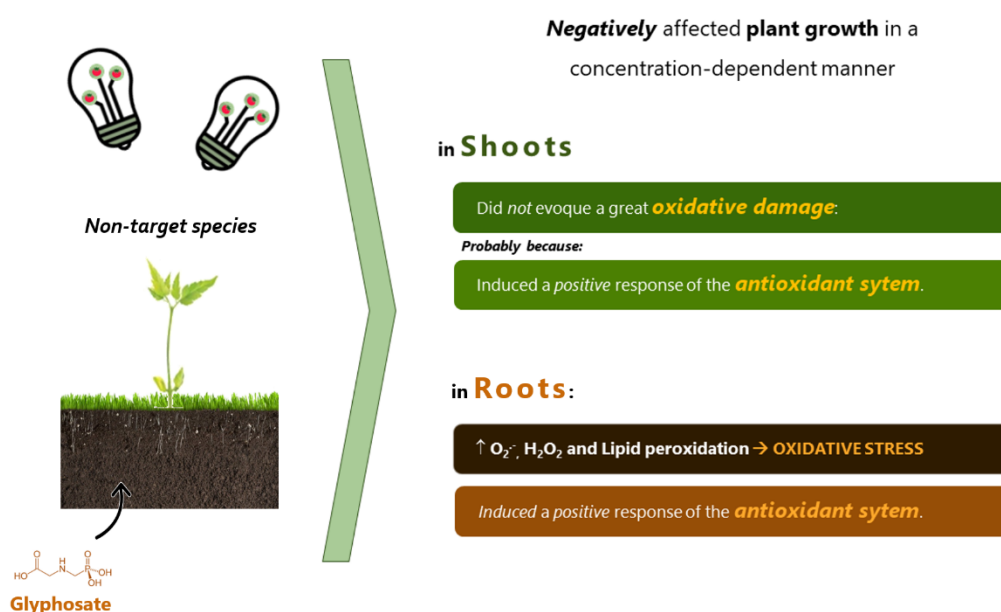


Figure 3. Overview of the main results of the present study.

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Glyphosate-dependent effects on photosynthesis of *Solanum lycopersicum* L. – an ecophysiological, ultrastructural and molecular approach

Abstract

This study aimed to assess the toxicity of glyphosate (GLY; 0, 10, 20 and 30 mg GLY kg⁻¹) in *Solanum lycopersicum* L., particularly focusing on the photosynthetic metabolism. By combining ecophysiological, ultrastructural, biochemical and molecular tools, the results revealed that the exposure of tomato plants to GLY led to changes in leaf water balance regulation [increasing stomatal conductance (g_s) and decreasing water use efficiency (WUE_i) at higher concentrations] and induced slight alterations in the structural integrity of cells, mainly in chloroplasts, accompanied by a loss of cell viability. Moreover, the transcriptional and biochemical control of several photosynthetic-related parameters was reduced upon GLY exposure. However, *in vivo* chlorophyll fluorometry and IRGA gas-exchange studies revealed that the photosynthetic yield of *S. lycopersicum* was not repressed by GLY. Overall, GLY impacts cellular and subcellular homeostasis (by affecting chloroplast structure, reducing photosynthetic pigments and inhibiting photosynthetic-related genes transcription), and leaf structure, but is not reducing the carbon (C) flow on a leaf area basis. Altogether, these results suggest a trade-off effect in which GLY-induced toxicity is compensated by a higher photosynthetic activity related to GLY-mediated dysfunction in g_s and an increase in mesophyll thickness/density, allowing the viable leaf cells to maintain their photosynthetic capacity.

Keywords

Abiotic stress; calvin cycle; chlorophyll fluorometry; gas-exchange; non-target plants; photochemistry.

1. INTRODUCTION

As a result of the accelerated world population growth, as well as of the increased food and feed demands, agriculture is progressively more dependent on the use of chemical products to ensure high yield rates. According to recent data, pesticide application, increasing since 1990, has surpassed, on average, the mark of 2.5 kg ha⁻¹ worldwide and it is expected that this value will be further aggravated in the following years (<http://www.fao.org/faostat/en/#data/EP/visualize>). From all pesticide classes, herbicides

and insecticides are currently the most representative ones, accounting for the highest production volume (Atwood and Paisley-Jones, 2017). Among all herbicides, glyphosate [GLY; N-(phosphonomethyl) glycine] is the most used at the global scale, with application rates exceeding 820 million kg between 1998 and 2014 (<https://www.statista.com/statistics/567250/glyphosate-use-worldwide/>).

GLY is considered as a broad-spectrum herbicide of systemic and non-selective action, commonly applied to leaves of weeds (Franz et al., 1997). Regarding its mode-of-action, GLY inhibits the activity of the 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS; EC 2.5.1.19), blocking the shikimate pathway and consequently the biosynthesis of aromatic amino acids and secondary metabolites in plants and some species of microorganisms (Franz et al., 1997). Due to its low price, great efficacy, along with the development of several GLY-resistant species, such as maize and soybean transgenic cultivars, GLY rapidly turned into the most used herbicide worldwide. Additionally, GLY became regarded as the most innocuous option of weed chemical control for the environment, since, once in contact with the soil, it quickly degrades (DT50 in the field = 23.79 d, <http://sitem.herts.ac.uk/aeru/iupac/Reports/373.htm>) into aminomethyl phosphonic acid (AMPA). However, GLY can remain adsorbed to clay and organic matter, lowering its degradation rates, which are also highly dependent on soil pH (Zhang et al., 2015). All of these factors can potentiate GLY, as well as AMPA, accumulation in the soil (see review by Van Bruggen et al., 2018), where they can persist or move to other environmental compartments. Although there are still few studies reporting the accumulation, fate and transport of GLY and of its degradation products in soils, especially in EU countries (Silva et al., 2018), residual levels of GLY and AMPA have been detected up to $\mu\text{g kg}^{-1}$ and mg kg^{-1} , reaching values as high as 8 mg kg^{-1} in agricultural soils (Primost et al., 2017; Peruzzo et al., 2008). Besides, since GLY residues in surface waters have also reached 15 mg L^{-1} (Wei et al., 2016), it is expected that soil can present even higher amounts due to repetitive applications (Soares et al., 2019b). Thus, given the widespread use of GLY-based herbicides, along with data confirming its accumulation in the environment, there is a growing need to adequately evaluate its potential toxicity to non-target biota. Within this context, in the past few years, scientific evidence has been showing that GLY is not as safe as it was thought to be, being able to negatively affect the environment, either directly or through the production of AMPA, which is also toxic (Bai and Ogbourne, 2016; Borggaard and Gimsing, 2008). Indeed, there is currently a strong debate on this matter amongst the scientific community, since contrasting and divergent data in relation to GLY's non-target effects have been reported, especially on animal species, including mammals (Silva et al., 2018). For instance, the World Health Organization has classified GLY as potential carcinogenic, but, in 2017, the United States Environmental Protection Agency

(US-EPA) stated that GLY does not represent a risk to the human health, with no evidence that GLY is carcinogenic (EPA, 2017). Despite the great number of studies in animals, not much is known regarding the responses of non-target plants to GLY exposure through contaminated soils/waters. Given the high application rates of GLY, this aspect is quite concerning, since contaminated soils can be unable to grow crops, as well as other important plant species. In this way, new studies addressing this issue under realistic and ecologically relevant concentrations of GLY are of special importance to identify the main effects of the environmental contamination by GLY on the growth and development of crops, produced for both human and animal feeding. Recent research unequivocally indicated that the presence of high levels of GLY greatly impaired the growth and performance of non-target plant species (Gomes et al., 2017; Soares et al., 2019b; Spormann et al., 2019), affecting multiple biological mechanisms, from the oxidative metabolism to cellular respiration and photosynthesis (Gomes et al., 2014 and references therein).

From all the processes occurring in a plant cell, photosynthesis is crucial to ensure the cellular homeostasis necessary to the normal plant development (Taiz et al., 2015). Effects on photosynthesis may thus have a major impact on plant productivity, and recent reports have shown that it is seriously affected by herbicides (Parween et al., 2016; Sharma et al., 2018). Although GLY's mode-of-action does not directly block the photosynthetic mechanism, some authors advocate that this herbicide can affect photosynthesis (Mateos-Naranjo et al., 2009; Yanniccari et al., 2012; Zobiole et al., 2012), both indirectly, by preventing the biosynthesis of chlorophylls through the action of AMPA, and directly, by enhancing chlorophyll degradation (Gomes et al. 2014). However, using chlorophyll fluorescence approaches, inhibitory effects of GLY on the photosystem II (PSII) activity, electron transport rate (ETR) and non-photochemical quenching (NPQ) were documented (see review by Gomes et al., 2014). Yet, in the great majority of these studies, GLY was applied on leaves, thus not translating potential effects on non-target plant species exposed to GLY by soil contamination.

Tomato plant (*Solanum lycopersicum* L.) is one of the main agricultural crop species produced worldwide, being also considered as the second most important vegetable, not only due to its excellent nutritional properties, but also to its antioxidant (AOX) and health-promoting characteristics (Branco-Neves et al., 2017; Dorais et al., 2008). Besides its economic importance, tomato is also acknowledged for being a perfect model species for plant stress physiology studies (Gerszberg et al., 2015). Although *S. lycopersicum* is not directly exposed to GLY, since it is a non-target plant species, the environmental contamination by this herbicide may end up affecting tomato plants' growth, development and survival. Actually, recent work from our group revealed that realistic levels of GLY in

the soil greatly impair tomato growth, by inducing severe oxidative damage in both shoots and roots after 28 d of growth (Soares et al., 2019b). However, to the best of our knowledge, no study on the interplay between GLY contamination and the carbon (C) metabolism on non-target plants has been reported so far. In this context, the main goal of this work was to evaluate the effects of soil contamination by GLY, provided as RoundUp® UltraMax, on C assimilation and photosynthetic efficiency of *S. lycopersicum*. Since photosynthesis is a very complex mechanism, involving several processes from gene expression, protein synthesis and enzyme activity, to photoprotective and damage repair mechanisms, at the cellular level, and to gas diffusion, at the leaf level, different methodologies were employed to unveil the mechanism of action of this herbicide and its subsequent effect on non-target plants. For this purpose, 28-d soil grown seedlings exposed to increasing concentrations of GLY (0, 10, 20 and 30 mg GLY kg⁻¹) were used to evaluate: i) the content of photosynthetic pigments and ribulose-1,5-bisphosphate carboxylase oxygenase (RuBisCO), ii) the ultrastructure of mesophyll cells and histochemical detection of cell death, iii) *in vivo* photosynthetic performance by chlorophyll fluorescence and infrared-gas analyses (IRGA), and iv) the expression level of several photosynthetic-associated genes.

2. MATERIALS AND METHODS

2.1. Chemicals and substrate

The herbicide RoundUp® UltraMax (Monsanto Europe, S.A., Belgium), a GLY-based (360 g GLY L⁻¹, potassium salt) herbicide, acquired from a local supplier, was used to prepare a stock solution of 1 g GLY L⁻¹, which was then diluted to achieve the tested concentrations (10, 20 and 30 mg GLY kg⁻¹ soil). The substrate used to grow plants was an artificial soil [pH 6.0 ± 0.5, 5% (m/m) organic matter], composed by sphagnum peat, quartz sand (< 2 mm) and kaolin clay (OECD, 2006).

2.2. Plant material and germination conditions

Seeds of *S. lycopersicum* L. cv. Micro-Tom, obtained from FCUP's seed collection, were surface disinfected with 70% (v/v) ethanol, followed by 20% (v/v) commercial bleach [5% (v/v) active chlorine], containing 0.05% (m/v) Tween-20, for 7 min each, followed by a series of cleanup with deionised water (dH₂O). Seeds were then placed in Petri dishes containing half-strength MS medium (Murashige and Skoog, 1962) solidified with 0.625% (m/v) agar and left to germinate in a growth chamber under controlled conditions

(photoperiod: 16 h light/ 8 h dark; temperature: 25 ± 1 °C; photosynthetic photon flux density – PPFD: $120 \mu\text{mol m}^{-2} \text{s}^{-1}$).

2.3. Experimental setup

At day 8, sets of 6 plantlets were transferred to plastic pots containing 200 g soil. After determining the maximum water hold capacity (WHC_{max}) of the soil, the volume of water required to adjust soils to 40% of their WHC was used to dilute GLY stock solution to attain the final concentrations of 10, 20 and 30 mg GLY kg^{-1} soil. A control with no GLY (CTL; 0 mg GLY kg^{-1}) was also included. The concentrations herein used were selected based on a recent work of our group (Soares et al., 2019b) and all of them are all environmentally relevant, as previously demonstrated. For each treatment, four replicates (pots) were prepared, with 6 plants each. At the beginning of the assay, to ensure the availability of mineral nutrients, 100 mL of modified Hoagland solution (HS; pH 5.8) (Taiz et al., 2015) were added to a box placed under each pot, communicating by a cotton rope, and plants were grown for 4 weeks under the same conditions as described above and irrigated with ddH₂O when necessary. At the end of this period, fully expanded leaves (2nd and 3rd) were randomly collected from 3 plants of each biological replicate, randomly selected, and frozen under liquid nitrogen (N₂) for subsequent biochemical and molecular analyses or immediately processed for transmission electron microscopy (TEM). All the other *in vivo* parameters were measured in fully expanded leaves from at least two plants from each biological replicate.

2.4. Biochemical assays – photosynthetic pigments and relative RuBisCO content

Total chlorophylls (Chl a + b) and carotenoids (Car) were extracted from frozen leaf samples (ca. 100 mg) with 80% (v/v) acetone. After centrifugation (1400 g; 10 min) for clearing the extract, the absorbance (Abs) was recorded at 663, 647 and 470 nm, and Chl a + b and Car contents determined using Lichtenthaler (1987) equations.

Ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO; EC 4.1.1.39) relative content was quantified as in Soares et al. (2016a), from a protocol originally described by Li et al. (2013). Briefly, after protein extraction and quantification (Bradford, 1976), 20 μg of extract from each biological replicate were loaded onto a polyacrylamide gel and separated by electrophoresis under denaturing conditions. Then, following gel staining with BlueSafe (NZYTech®), the portions of the large and small subunits of RuBisCO of each sample were excised and incubated in formamide at 50 °C overnight. The remaining gel was also incubated under the same conditions. Lastly, the Abs of the washing solution

was measured at 595 nm and the relative RuBisCO levels expressed according to a mathematical formula (Soares et al., 2016b).

2.5. Histochemical detection of cell viability

Cell viability of tomato leaves was evaluated as described in Soares et al. (2016a). After 4 h-incubation in dark conditions in 0.25% (m/v) Evans Blue, leaves were boiled in 96% (v/v) ethanol for pigment decolorization, then, carefully rinsed with deionised water and photographed. The presence of blueish spots in the leaf is an indicator of the cell death.

2.6. Gene expression analysis

2.6.1. RNA extraction and cDNA synthesis

Total RNA was extracted from leaf tissue (ca. 80-100 mg) with NZYol® reagent (NZYTech, Lda) according to the guidelines of the manufacturer. After extraction, RNA was spectrophotometrically quantified at 260 nm in a µDrop Plate (Thermo Fisher Scientific) and its integrity assessed by 0.8% (m/v) agarose gel electrophoresis. Each RNA sample was treated with ezDNase enzyme (Invitrogen) to prevent any genomic DNA contamination. Then, cDNA synthesis was performed with SuperScript™ IV VILO™ Master Mix, using 2.5 µg RNA in a final volume of 20 µL. At the end, cDNAs were diluted (1:10) and stored at -20 °C for real-time PCR expression analysis.

2.6.2. Real-time PCR (qPCR) conditions and primers

cDNA from each experimental condition was amplified through qPCR in a CFX96 Real-Time Detection System (Bio-Rad®, Portugal), using the specific primers listed in Table 1. All qPCR reactions were performed in triplicate, using PowerUp™ SYBR™ Green Master Mix (Applied Biosystems) for a final volume of 20 µL, containing 1 µL of diluted cDNA. The qPCR conditions were as follow: 2 min at 50 °C, 2 min at 95 °C, followed by 35 cycles of 3 s at 95 °C and 30 s at 60 °C. At the end of each reaction, a melting curve was carried out by gradually increasing the temperature from 60 to 95 °C in 0.5-s intervals, in order to ensure primer and amplification specificity. For normalization of the expression data, four reference genes previously validated and tested were used (*18S* - Leclercq et al., 2002; *UBI* and *ACTIN* - Løvdaal and Lillo, 2009; and *EF1* – Dzakovich et al., 2016) and the quantification of the transcript levels was executed by applying the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen, 2001).

Table 1. Gene-specific primers used in qPCR analysis.

Gene name	Primer sequence	T _m (°C)	Amplicon (bp)	Reference
<i>D1</i>	Fwd: 5- TGG ATG GTT TGG TGT TTT GAT G -3 Rev: 5- CCG TAA AGT AGA GAC CCT GAA AC -3	Fwd: 54.03 Rev: 54.83	191	Mariz-Ponte 2017
<i>CP47</i>	Fwd: 5- CCT ATT CCA TCT TAG CGT CCG -3 Rev: 5- TTG CCG AAC CAT ACC ACA TAG -3	Fwd: 54.90 Rev: 54.87	142	Mariz-Ponte 2017
<i>RCBL</i>	Fwd: 5- ATC TTG CTC GGG AAG GTA ATG -3 Rev: 5- TCT TTC CAT ACC TCA CAA GCA G -3	Fwd: 54.68 Rev: 54.64	81	Mariz-Ponte 2017
<i>RCBS</i>	Fwd: 5- TGA GAC TGA GCA CGG ATT TG -3 Rev: 5- TTT AGC CTC TTG AAC CTC AGC -3	Fwd: 54.90 Rev: 54.79	148	Mariz-Ponte 2017

2.7. Ultrastructure analysis by TEM

Leaf samples were fixed in a mixture of 5% (v/v) glutaraldehyde and 4% (v/v) paraformaldehyde (PFA) and post-fixed in 2% (m/v) osmium tetroxide (OsO₄), prepared in 0.1 M sodium cacodylate buffer (pH 7.2). Then, dehydration was carried out using increased concentrations of ethanol, followed by embedding in EMBED-812. Finally, ultrathin sections were obtained using a ultramicrotome, contrasted with uranyl acetate and lead citrate, and observed using a Zeiss EM C10 TEM (Zeiss, Göttingen, Germany).

2.8. Chlorophyll fluorescence analyses

2.8.1. Photochemical efficiency of PSII – F_v/F_m , Φ_{PSII} and $rETR$

Chlorophyll fluorescence analysis, by pulse amplitude modulated fluorometry (PAM), was performed in the 2nd and 3rd young fully expanded leaves of tomato plants, using a PAM-210 fluorometer (Heinz Walz GmbH, 1997), controlled via the PAMWin software. The emitter-detector unit comprises a red measuring light LED with short-pass filter (< 690 nm), peaking at ca. 650 nm, an actinic red LED (unfiltered, peaking at ca. 665 nm), a far-red LED, with a long-pass filter (> 710 nm, peaking at ca. 730 nm), and a PIN photodiode and dichroic filter, reflecting fluorescence at 90° towards the detector. Prior to the

measurements, plants were dark-adapted for at least 20 min to open all the PSII reaction centers. Then, after recording the minimal fluorescence (F_0), a saturating light pulse ($3500 \mu\text{mol photons m}^{-2} \text{s}^{-1}$, 800 ms) was applied to determine the maximal fluorescence yield (F_m) and calculate the maximum quantum yield of PSII [$F_v/F_m = (F_m - F_0)/F_m$; Kitajima and Butler, 1975]. In order to estimate the effective quantum yield of PSII [$\Phi_{\text{PSII}} = (F'_m - F_t)/F'_m$; Genty et al., 1989] and the respective relative electron transport rate [$r\text{ETR} = \Phi_{\text{PSII}} \times \text{PPFD}$; Genty et al., 1989], indicative of the electrons pumped through the photosynthetic chain under plant growth light conditions, leaves were adapted for 5 min to actinic light (AL; $128 \mu\text{mol photons m}^{-2} \text{s}^{-1}$) and, then, a saturating pulse was applied to record F'_m and F_t .

2.8.2. Photochemical efficiency recovery study

After the screening of the photosynthetic yield of tomato leaves of plants under GLY contamination, a new PAM chlorophyll fluorometry-based study was designed to investigate GLY effects on the non-photochemical quenching efficiency and F_v/F_m recovery of tomato leaves. All the experiments were performed using an imaging chlorophyll fluorescence fluorometer (FluorCAM 800MF, Photon System Instruments, Brno, Czech Republic), comprising a control unit (SN-FC800-082, PSI) and a CCD camera (CCD381, PSI) with a f1.2 (2.8–6 mm) objective (Eneo, Japan). Multiple samples were exposed simultaneously to AL, by using an LCD digital projector (EB-X14; Seiko Epson, Suwa, Japan), controlled as described by Serôdio et al. (2017). Briefly, five leaf discs (≈ 2 cm) from each experimental condition were placed on the surface of 2 mL of water in a 24-well microplate. After 20 min of dark adaptation, F_v/F_m was measured as described above, and samples were exposed to saturating AL ($1800\text{--}2100 \mu\text{mol m}^{-2} \text{s}^{-1}$) for 1 h. A saturating light pulse was then applied to record F'_m and calculate the non-photochemical quenching [$\text{NPQ} = (F_m - F'_m)/F'_m$], which corresponds to the fraction of light captured by Chl that is converted into heat (Genty et al., 1989). Afterwards, the AL was switched off and saturating pulses were provided every 3 min to evaluate F_v/F_m recovery. Images of chlorophyll fluorescence parameters were captured by applying modulated measuring light ($< 0.1 \mu\text{mol m}^{-2} \text{s}^{-1}$) and saturation pulses ($> 7500 \mu\text{mol m}^{-2} \text{s}^{-1}$) provided by red (612 nm emission peak, 40 nm bandwidth) LED panels. Images (512×512 pixels) were processed using FluorCam7 software (Photon System Instruments). The results were expressed as the proportion of F_v/F_m recovery in relation to the original value.

2.9. Gas exchange measurements

The evaluation of gas-exchange parameters was performed using an infrared gas analyser (IRGA; *LC pro+*, ADC, Hoddersdon, UK), coupled to a broad light source (PPFD of 255 $\mu\text{mol m}^{-2} \text{s}^{-1}$), simulating the greenhouse conditions (atmospheric CO_2 concentration and a PPFD of 120 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$). For each of the 4 replicates, measurements were made in two plants, being each measurement repeated twice to assess the feasibility of the method. Net CO_2 assimilation rate (P_N , $\mu\text{mol m}^{-2} \text{s}^{-1}$), stomatal conductance (g_s , $\text{mmol m}^{-2} \text{s}^{-1}$), transpiration rate (E , $\text{mmol m}^{-2} \text{s}^{-1}$), and intercellular CO_2 concentration (C_i , $\mu\text{mol mol}^{-1}$) were estimated using the equations developed by von Caemmerer and Farquhar (1981). Intrinsic water use efficiency (WUE_i) was determined as follows: $WUE_i = P_N / g_s$. In complement, the specific leaf area [$\text{SLA} = \text{leaf area (cm}^2) / \text{dry mass (g)}$] was also calculated.

2.10. Statistical analyses

All biochemical, molecular and physiological evaluations were performed using 4 experimental replicates ($n = 4$), except for the ultrastructure analysis where $n = 2$ was considered. The results were expressed as mean $\pm$ standard deviation (SD). The effect of different GLY concentrations on the parameters assessed were analysed by one-way ANOVA, assuming a significance level of 0.05, after checking for the normality and homoscedasticity assumptions. Whenever significant differences ($p \leq 0.05$) were found, Dunnet *post-hoc* tests were used to identify differences between each GLY treatment – 10, 20 and 30 mg GLY kg^{-1} – and the CTL. Correlation analyses were performed using Spearman's test. All statistical procedures were executed in Prism 8 (© 2018 GraphPad Software).

3. RESULTS

3.1. Biochemical determinations – photosynthetic pigments, soluble protein and RuBisCO

As shown in Figure 1a,b, Chl a + b and carotenoid contents significantly decreased [$\text{Chl a} + \text{b} - F(3, 10) = 24; p < 0.01$; $\text{Car} - F(3, 11) = 21.03; p < 0.01$] when tomato plants were exposed to the highest GLY concentration (30 mg GLY kg^{-1}) (Dunnet: $p \leq 0.05$) to about 40 and 50% of the control, respectively.

Total soluble protein levels were also significantly reduced [$F(3, 11) = 9.399$; $p = 0.0023$] under GLY exposure, with significant changes from the control detected for all concentrations of GLY (Dunnett: $p \leq 0.05$), even in the lowest one (10 mg GLY kg⁻¹) (Figure 1c). Again, a GLY effect was detected in the relative content of RuBisCO [$F(3, 12) = 4.345$; $p < 0.015$] (Figure 1d). Although a dose-dependent inhibition was apparent, significant differences from the CTL were only found when plants were grown at 30 mg GLY kg⁻¹ (Dunnett: $p \leq 0.05$).

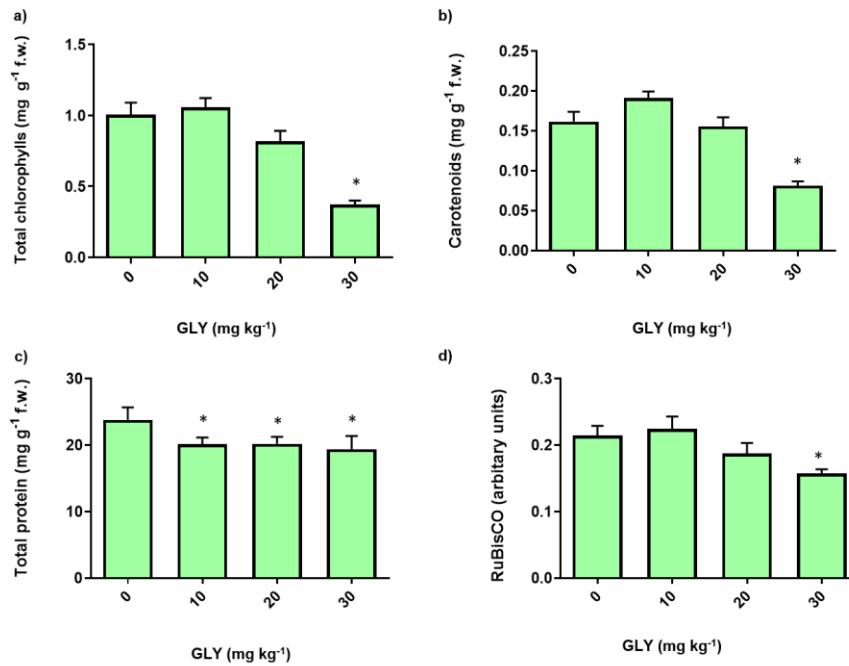


Figure 1. Total chlorophylls (a), carotenoids (b), total protein (c) and RuBisCO (d) levels in leaves of *S. lycopersicum* plants exposed to increased concentrations (0, 10, 20 and 30 mg kg⁻¹) of GLY. * above bars indicate differences from the CTL (0 mg GLY kg⁻¹) at $p \leq 0.05$.

3.2. Cell viability assay

The exposure of tomato plants to GLY induced losses in cell viability of leaves, as can be seen in Figure 2. As the blueish areas are indicative of cell death, it is also clear that this effect was dependent on the concentration of GLY, reaching a maximum in the plants subjected to the highest concentration tested (30 mg GLY kg⁻¹).

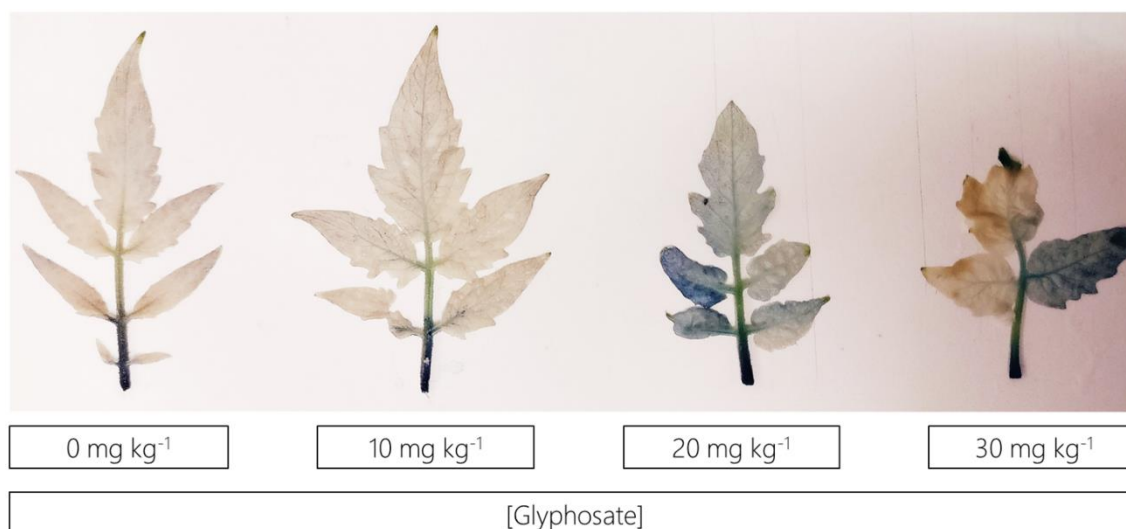


Figure 2. Histochemical detection of cell death in leaves of *S. lycopersicum* plants exposed to increased concentrations (0, 10, 20 and 30 mg kg⁻¹) of GLY. Necrotic areas are manifested as blue spots on the leaf surface.

3.3. Foliar morphology and ultrastructure analysis by TEM

When tomato plants were grown in the presence of increasing concentrations of GLY, alterations in plant growth, leaf morphology and mesophyll structure were registered. As can be observed in Figures 3a-b, the compound leaves of GLY-treated plants suffered profound changes, with less primary and secondary leaflets and with more rounded terminal leaflets at the highest concentration tested. The SLA was also significantly reduced [$F(3, 23) = 10.76$; $p = 0.0001$] upon exposure to the highest GLY concentrations (20 and 30 mg GLY kg⁻¹), to values around 70% of those registered in the CTL (Figure 3c).

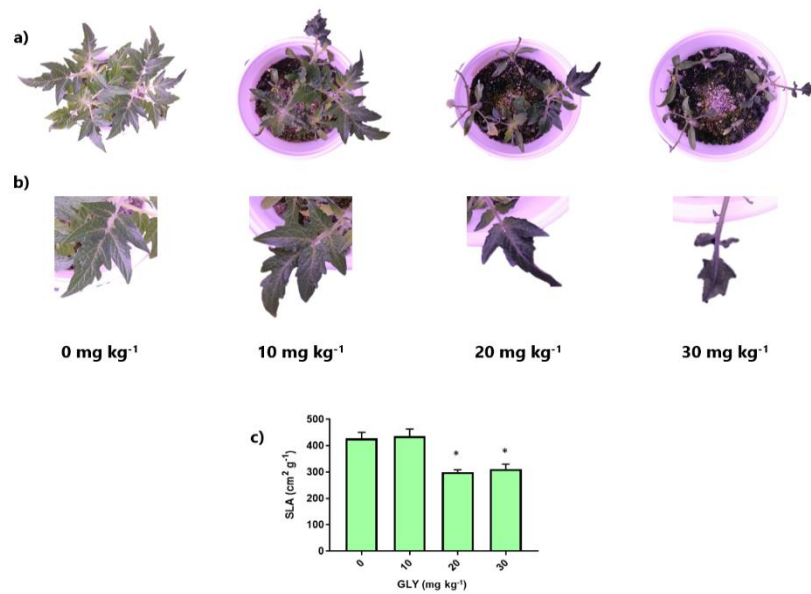


Figure 3. Growth comparison (a), leaf morphology (b) and specific leaf area (SLA; c) of leaves of *S. lycopersicum* plants exposed to increased concentrations (0, 10, 20 and 30 mg kg⁻¹) of GLY. * above bars indicate differences from the CTL (0 mg GLY kg⁻¹) at $p \leq 0.05$.

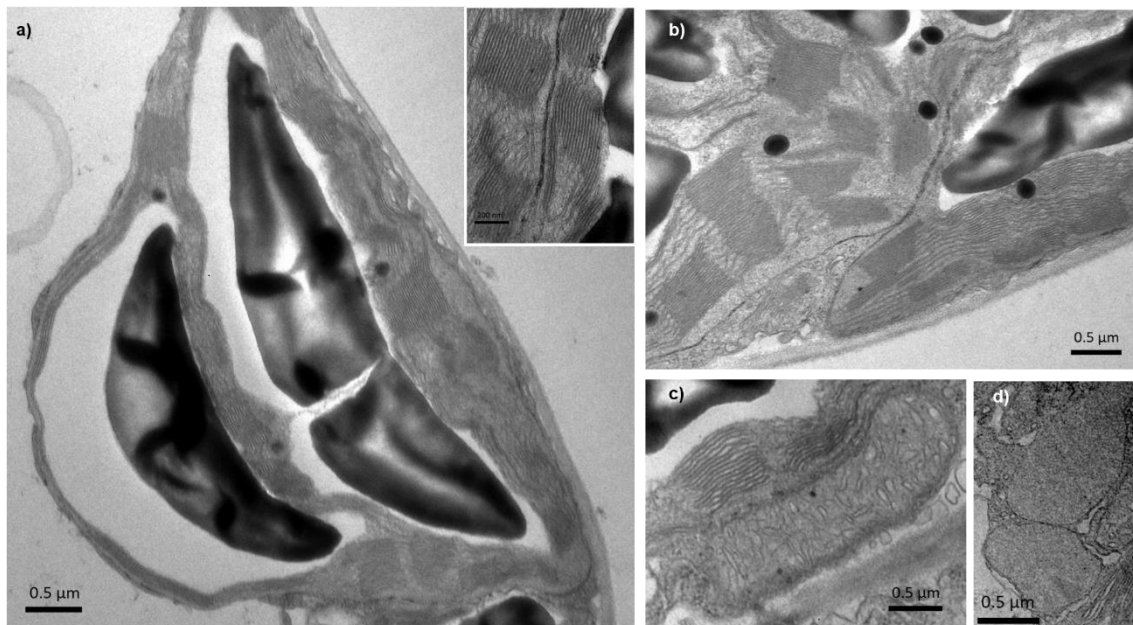


Figure 4. Ultrastructural analysis of the foliar mesophyll of *S. lycopersicum* plants grown under control conditions (no GLY). Region of a mesophyll cell showing well-preserved chloroplasts, which contain huge starch grains (a); high magnification of well-preserved chloroplasts (b), mitochondria (c) and peroxisomes (d)

The ultrastructure of tomato leaves exposed to increased concentrations of GLY (0, 10, 20 and 30 mg GLY kg⁻¹) are depicted in Figures 4-6. As can be observed, mesophyll cells from CTL plants displayed abundant and lens-shaped chloroplasts, with well-organised thylakoid systems, along with the accumulation of multiple starch grains (Figure 4a-b).

Other cellular organelles, such as mitochondria and peroxisomes had also their integrity well preserved (Figure 4c,d). However, upon exposure to GLY, substantial ultrastructural changes occurred in tomato leaves. As illustrated in Figures 5 and 6, as GLY concentration increases, chloroplasts displayed a variable degree of thylakoid swelling and increased damage in thylakoid membranes organization, though no apparent changes in starch accumulation has been noticed. However, the appearance of numerous plastoglobuli (PG) in response to GLY treatments was strongly induced (Figures 5 and 6a-b), along with an increase of peroxisomes and mitochondria abundance, especially in plants exposed to 30 mg GLY kg⁻¹ (Figure 6a-d).

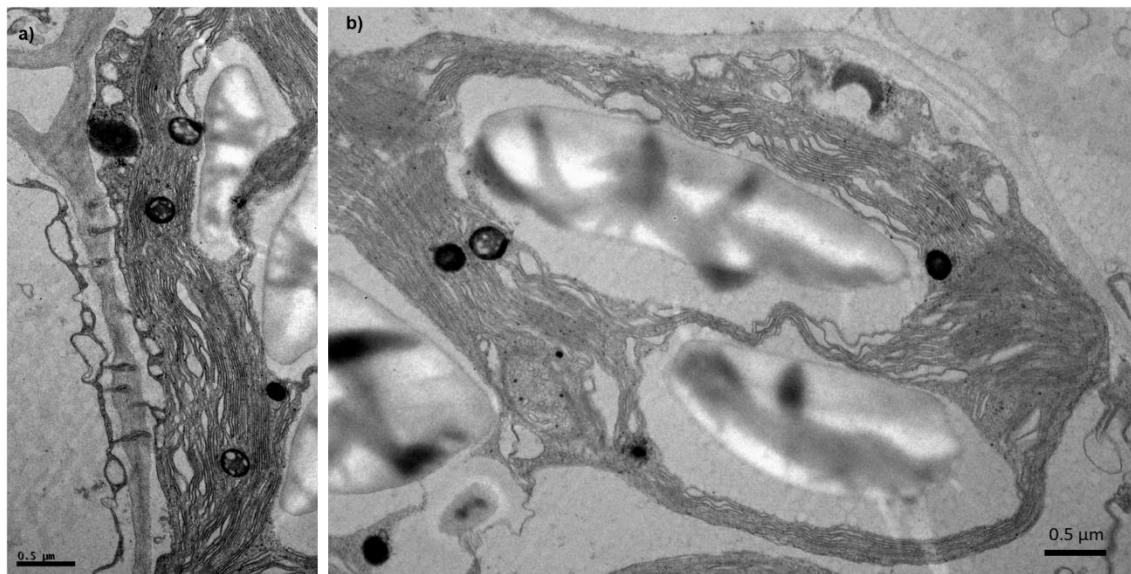


Figure 5. Ultrastructural analysis of the foliar mesophyll of *S. lycopersicum* plants exposed to 20 mg GLY kg⁻¹. Portion of a mesophyll cell displaying marked abnormalities in chloroplast ultrastructure, with a higher incidence of osmiophilic deposits (plastoglobuli) (a); Damaged chloroplast, showing swelling thylakoids, with no apparent change in starch accumulation (b).

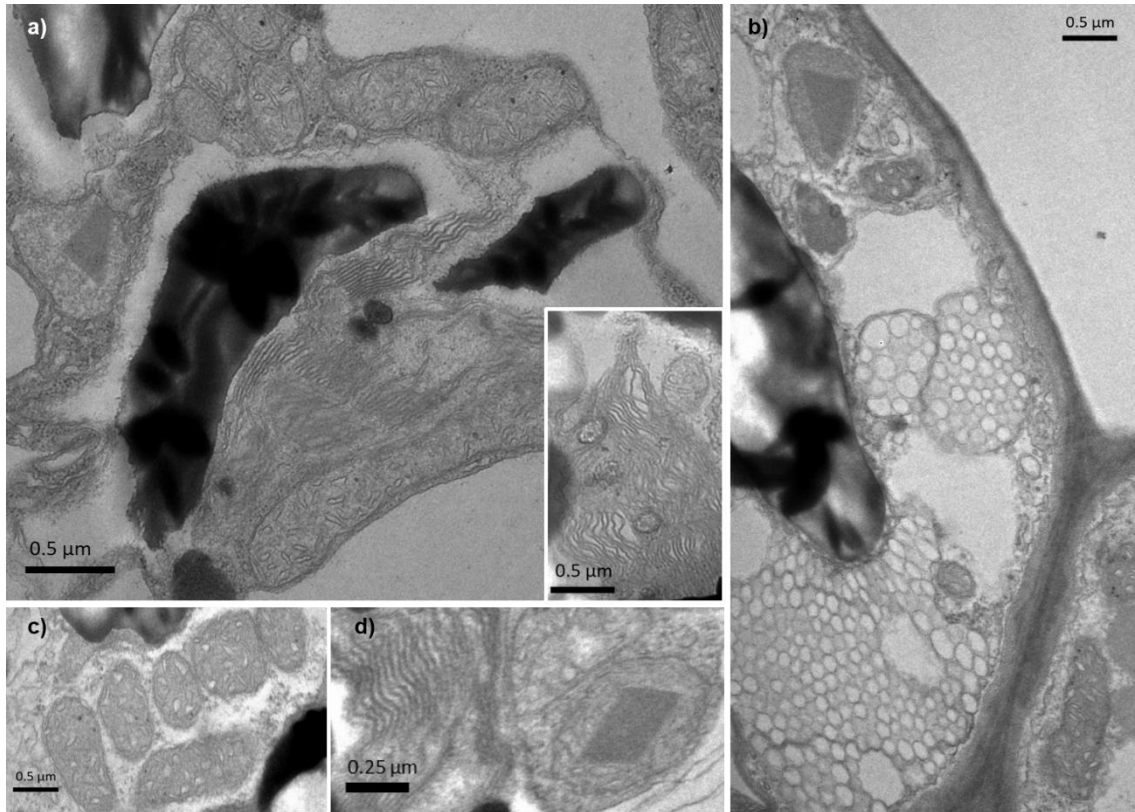


Figure 6. Ultrastructural analysis of the foliar mesophyll of *S. lycopersicum* plants grown exposed to 30 mg GLY kg⁻¹. Region of a mesophyll cell showing damaged chloroplasts and a huge occurrence of mitochondria. Inset: magnification of thylakoid membrane disorganization (a); portion of a cell exhibiting signs of great damage, with the appearance of several vesicular bodies throughout the chloroplast (b); magnification of mitochondria (c) and peroxisome (d) with a paracrystalline inclusion.

3.4. Transcriptional regulation of photosynthesis-related genes

The transcript accumulation of genes coding for PSII proteins (D1 and CP47), as well as for the small and large subunits of RuBisCO, was evaluated by qPCR (Figure 7). Upon exposure to GLY, gene expression of *D1* and *CP47* was strongly repressed in a dose dependent-manner and for all the concentrations tested [*D1*: (F (3, 8) = 437.4; $p < 0.01$ and *CP47*: (F (3, 8) = 530.2; $p < 0.01$], reaching minimal values (up to 15% of the CTL) in plants exposed to 20 and 30 mg GLY kg⁻¹ (Dunnet: $p \leq 0.05$) (Figure 7a). Concerning genes related to RuBisCO, the expression of *RCBL* [F (3, 8) = 234.4; $p < 0.01$] and *RCBS* [F (3, 8) = 43.28; $p < 0.01$] was also affected by GLY, but only under the two highest treatments (Dunnet: $p \leq 0.05$; Figure 7b).

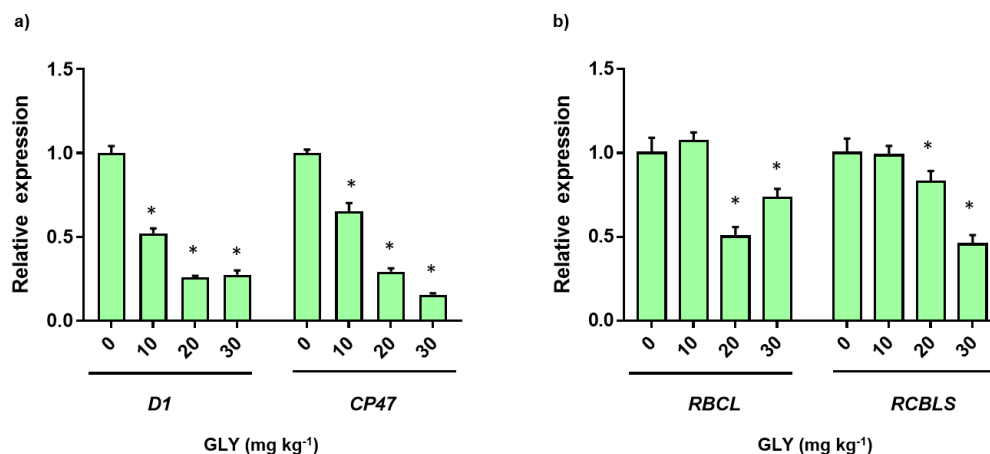


Figure 7. Expression profile of *D1* and *CP47* (a), and *RBCL* and *RBCS* (b) genes in leaves of *S. lycopersicum* plants exposed to increased concentrations (0, 10, 20 and 30 mg kg⁻¹) of GLY. * above bars indicate differences from the CTL (0 mg GLY kg⁻¹) at $p \leq 0.05$.

3.5. Chlorophyll fluorescence analysis

3.5.1. Photochemical and non-photochemical efficiency at plant growth light conditions

GLY induced significant changes in leaf photosynthetic potential quantum yield and photosynthetic activity, as revealed by the results obtained for F_v/F_m [$F(3, 26) = 18.96$; $p < 0.01$], $\Phi PSII$ [$F(3, 26) = 36.78$; $p < 0.01$], $rETR$ [$F(3, 28) = 23.49$; $p < 0.01$], and NPQ [$F(3, 24) = 19.96$; $p < 0.01$] (Figure 8a-d). Actually, after adapting the leaves for 5 min to growth light conditions ($AL \approx 128 \mu\text{mol m}^{-2} \text{s}^{-1}$), GLY induced a positive response for all the analysed parameters, significantly increasing $\Phi PSII$ (up to 45%; Dunnet: $p \leq 0.05$) and $rETR$ (up to 45%; Dunnet: $p \leq 0.05$) and decreasing NPQ (up to 51%; Dunnet: $p \leq 0.05$) in relation to the CTL, in a concentration-independent manner.

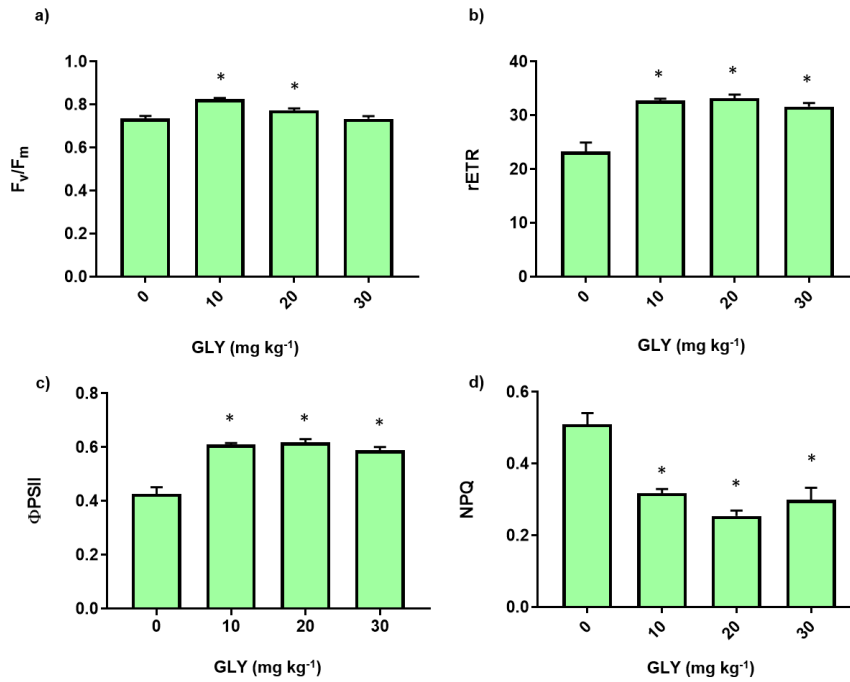


Figure 8. F_v/F_m (a), rETR (b), Φ_{PSII} (c) and NPQ (d) in leaves of *S. lycopersicum* plants exposed to increased concentrations (0, 10, 20 and 30 mg kg⁻¹) of GLY. * above bars indicate differences from the CTL (0 mg GLY kg⁻¹) at $p \leq 0.05$.

3.5.2. NPQ dark relaxation and F_v/F_m recovery studies

The recovery of the maximum quantum yield following light exposure, expressed as % of the initial F_v/F_m values, is represented in Figure 9. The results showed that, after 1 h exposure to saturating high light conditions (1800-2100 $\mu\text{mol m}^{-2} \text{s}^{-1}$), tomato plants exposed to GLY, especially those under the highest concentrations (20 and 30 mg GLY kg⁻¹), were the ones showing the highest F_v/F_m recovery (respectively to 85 and 87% of the initial value), exhibiting a steady increment from min 6 to the last measure (after 30 min). The CTL plants presented the lowest recoveries, reaching recovery values of only 70%.

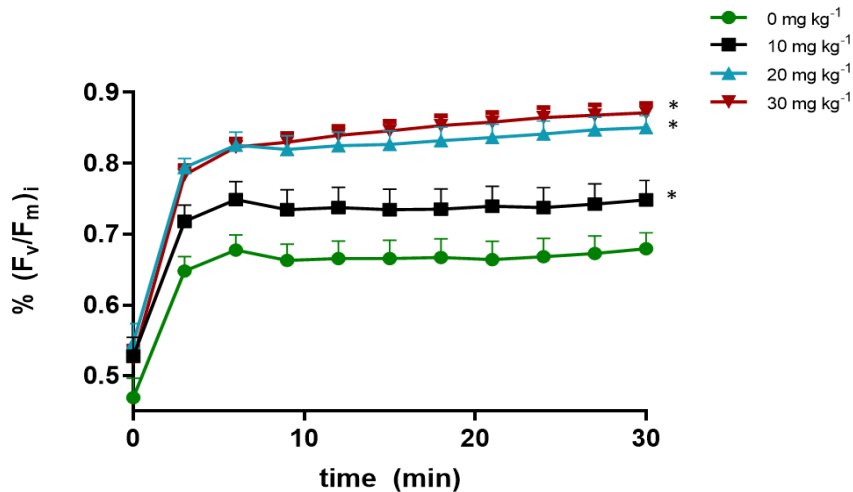


Figure 9. Photochemical recovery of F_v/F_m , expressed as % in relation to the initial F_v/F_m value, in leaves of *S. lycopersicum* plants exposed to increased concentrations (0, 10, 20 and 30 mg kg⁻¹) of GLY after 1 h of exposure to saturating AL ($\approx 1800\text{--}2100 \mu\text{mol photons m}^{-2} \text{s}^{-1}$).

3.6. Gas exchange measurements

GLY exposure increased the stomatal conductance (g_s) [$F(3, 16) = 37.74$; $p < 0.01$] and leaf transpiration (E) [$F(3, 18) = 19.20$; $p < 0.01$] in a dose-dependent manner, though significant differences from the CTL (Dunnett: $p \leq 0.05$) were only recorded in plants exposed to the two highest concentrations (Figure 10a-b). In parallel, GLY treatment also had a significant impact on the net CO₂ assimilation rate (P_N) [$F(3, 17) = 149.9$; $p < 0.01$], with increases of 2.9- and 2.2-fold in plants exposed to 20 and 30 mg GLY kg⁻¹, respectively (Figure 10c). No differences were recorded for intracellular concentration of CO₂ (C_i) among groups (Figure 10d). Regarding the water use efficiency (WUE_i) [$F(3, 16) = 31.9$; $p < 0.05$], a significant decrease (Dunnett: $p \leq 0.05$) of about 50% was recorded in plants under the highest concentration of GLY, in relation to the CTL (Figure 10e).

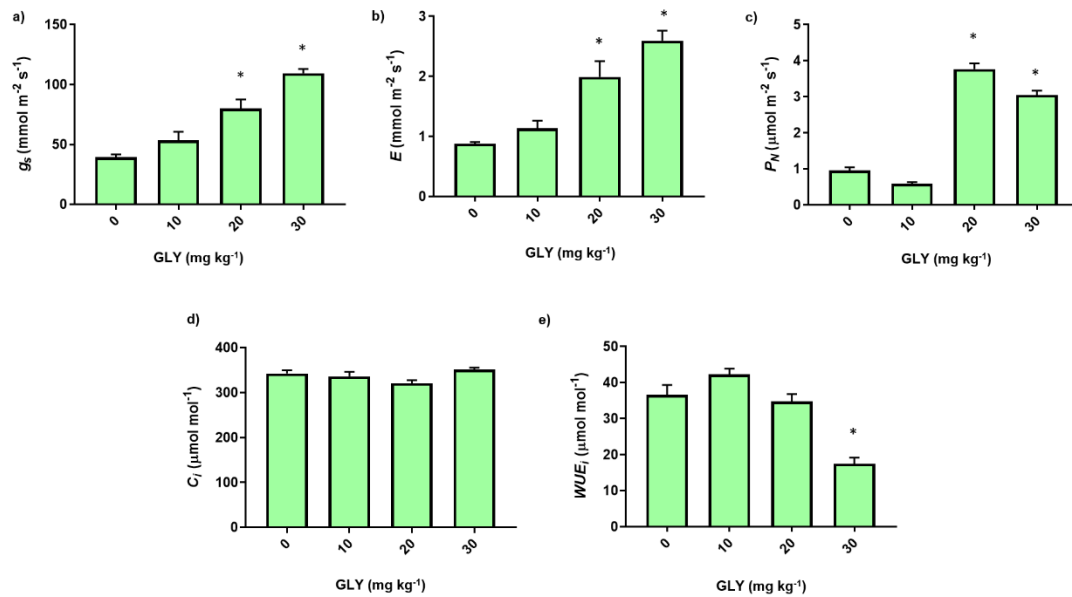


Figure 10. Stomatal conductance (g_s ; a), transpiration rate (E ; b), net CO₂ assimilation (P_N ; c) intracellular concentration of CO₂ (C_i ; d), and water use efficiency (WUE_i - P_N/G_s ; e) in leaves of *S. lycopersicum* plants exposed to increased concentrations (0, 10, 20 and 30 mg kg⁻¹) of GLY. * above bars indicate differences from the CTL (0 mg GLY kg⁻¹) at $p \leq 0.05$.

4. DISCUSSION

Due to their sessile nature, plants' growth and development are largely dependent on their adaptability to an ever-changing environment, where they face constant abiotic fluctuations (e.g. water stress, radiation, temperature) and contact with different contaminants, such as pesticide residues in soil and/or irrigation water (Pessarakli, 2011). Although GLY is the most widely applied herbicide worldwide, comprehensive knowledge regarding its phytotoxicity to non-target species, such as crops, due to residual soil contamination, is still limited. Recently, our research group provided important clues concerning GLY effects on tomato plants, clearly showing that GLY residues in the soil cause oxidative stress, severely compromising plant growth after 28 d of exposure (Soares et al., 2019b). In this line, the present work is a follow-up study and firstly aimed to unravel the effect of GLY added to the soil on photosynthesis in non-target plants, using *S. lycopersicum* as a model species for crops. Although the direct effects of foliar GLY application on the photosynthetic metabolism of target and resistant plants are relatively well described (reviewed by Gomes et al., 2014), studies exploring changes in the photosynthetic metabolism in response to soil contamination by GLY are still scarce, especially in non-target plants, where agricultural crops are included, and for which it is of utmost importance to assess the potential impacts on yield.

The presence of GLY residues in the soil ended up affecting the subcellular organisation of tomato leaves, promoting an increase of cell death

Although it is claimed that GLY in the soil should not represent a risk to non-target plants (<http://www.glyphosate.eu/glyphosate-safety-profile-non-target-wildlife-and-plants>), growing evidence has been showing the opposite for different species, such as barley (*Hordeum vulgare* L.), willow (*Salix miyabeana* Seemen), saltmarsh bulrush (*Bolboschoenus maritimus* L.), pea (*Pisum sativum* L.) and even tomato (*S. lycopersicum*) (Gomes et al., 2017, 2016b; Mateos-Naranjo and Perez-Martin, 2013; Singh et al., 2017; Soares et al., 2019b; Spormann et al., 2019). In line with this, and corroborating our previous work (Soares et al., 2019b), the exposure of tomato plants to increased concentrations of the herbicide resulted in a substantial alteration of leaf morphology and shape (Figure 3), greatly impairing leaf development. These GLY-induced alterations in leaf architecture were previously reported in *Eucalyptus* sp. and *Arachis hypogaea* L. (peanut) plants, even though in these studies the herbicide was sprayed onto the foliage (Radwan and Fayez, 2016; Tuffi Santos et al., 2009). As reviewed by Sukhov et al. (2019), abiotic stressors, such as contaminants and drought, are able to generate different signals that can reach other parts of the plant, triggering systemic physiological adjustments. Thus, the observed effects on leaves' physiological, biochemical and molecular status, can arise due to the production, at the root level, of hydraulic, chemical and/or electric signals, which then may propagate inducing alterations in leaves. However, knowing that GLY is phloem-mobile, direct consequences of GLY on leaves, derived from its translocation to the aerial organs, are most likely occurring. Based on the interference of GLY with shikimate pathway, it can be suggested that the observed phytotoxicity is a direct consequence of blocking aromatic amino acid and protein synthesis, as evidenced by our results (Figure 1c-d). In addition to the macroscopic symptoms, GLY exposure also resulted in substantial changes in leaf ultrastructure, especially in what regards to chloroplast organization and structure (Figures 5 and 6), and in a concentration-dependent manner. Paired with our observations, Vannini et al. (2016) also reported that GLY promoted the occurrence of ultrastructural disturbances in the lichen *Xanthoria parietina* L., when the herbicide was applied to the nutrient solution. Upon GLY treatment, a growing damage of chloroplast integrity was observed, particularly in thylakoid system organization, being this effect accompanied by a rise of PG. According to different studies, these lipoprotein bodies tend to accumulate under stressful conditions, contributing to less damage of cellular sub-structures and to restriction of leaf surface injury (Almeida et al., 2005). Their existence is generally indicative of a high metabolic activity in the chloroplast, being often associated with stress responses and with thylakoid breakdown, but also with

senescence events (van Wijk and Kessler, 2017). Moreover, the observed changes in PG number and size in GLY-exposed plants may reflect the metabolic network established between PG and thylakoids, as well as the synthesis of metabolites, such as quinones and tocopherols (van Wijk and Kessler, 2017), which are powerful AOXs in plant cells (Soares et al., 2019a). This hypothesis makes even more sense considering the results obtained in our previous recent study, where shoots of GLY-treated tomato plants exhibited a prompt and efficient response of the AOX system, limiting the peroxidation of lipids and the accumulation of ROS (Soares et al., 2019b). Supporting this, a higher abundance of peroxisomes with paracrystalline inclusions, indicative of catalase (CAT; EC 1.11.1.6) presence (Frederick and Newcomb, 1969), was observed in plants exposed to the highest GLY concentration (Figure 6a,b,d). Additionally, the maintenance of mitochondria integrity as well as an increased number of mitochondria in leaves of GLY-treated plants (Figure 6c) can also reflect the high energy demand of these plants to counteract the negative effects of the herbicide. Despite of that, the histochemical detection of cell death unequivocally indicated that GLY ends up hampering cellular homeostasis, inducing cell death in tomato leaves, especially under the highest concentrations tested (20 and 30 mg GLY kg⁻¹). Furthermore, as can be observed in Figure 6b, some mesophyll cells from plants grown under 30 mg GLY kg⁻¹ were severely damaged, as evidenced by the generalised appearance of numerous vesicles throughout the cell and organelles. Based on this set of results, and in order to infer how these structural changes were related to the photosynthetic function, additional studies were designed to evaluate GLY's effects on different biochemical and molecular attributes, as well as on photochemical and gas exchange parameters.

GLY-induced reduction of D1, CP47 and RuBisCO genes transcription and pigment levels does not inhibit photochemical reactions of photosynthesis

Photosynthesis begins with the absorption of sunlight energy by photosynthetic antenna pigments localised in the thylakoids (Taiz et al., 2015). Thus, stress conditions leading to variations in the content of chlorophylls and carotenoids may induce negative effects in photosynthesis, obstructing the first step of the whole process (Zhong et al., 2018). Either due to its chelating properties or by decreasing Mg content in plant leaves (Cakmak et al., 2009), one of the indirect effects of GLY on photosynthesis is the inhibition of photosynthetic pigments' biosynthesis (Gomes et al., 2014). From our observations, GLY only led to significant reductions in total chlorophylls and carotenoids under the highest concentration applied. Although quite surprising, this phenomenon may be ascribed to two complementary hypotheses, one related to GLY's application mode (soil vs foliar), and the

other associated with a low production of AMPA, a metabolite derived from GLY's degradation. Accordingly, GLY primary effects on photosynthesis are directly linked to AMPA, and so, being dependent on the degradation rate of GLY (Reddy et al., 2004; Serra et al., 2013). Due to chemical similarities with glycine, AMPA competes with this amino acid, resulting in decreased levels of δ -aminolevulinic acid, an intermediate in chlorophyll biosynthesis (Gomes et al., 2014). In this sense, it can be suggested that, either by the use of a soil with poor microbial activity, as is the case of the artificial OECD soil used and/or by the root application of GLY, only the highest concentration tested (30 mg GLY kg⁻¹) allowed the production of enough AMPA to inhibit chlorophyll biosynthesis. This observation is further supported by the higher number of PG recorded in plants grown under 30 mg GLY kg⁻¹, since it is known that these lipid bodies play a role in chlorophyll degradation (van Wijk and Kessler, 2017).

After pigment excitation by light in the antenna, the energy is transferred to the reaction centers of PSI and PSII, which is used to channel electrons to the electron transport chain (Taiz et al., 2015). Structurally, the PSII reaction center includes two monomeric core reaction center proteins (D1 and D2), two antenna proteins (CP43 and CP47), two cytochromes, an oxygen evolution protein (PsbO), as well as chlorophyll *a* and other co-factors (Taiz et al., 2015). Thus, transcripts accumulation pattern related to these proteins may provide important hints concerning the response of PSII to GLY exposure. Results of the present study revealed a severe downregulation of both *D1* and *CP47* gene expression in a dose-dependent manner, strongly indicating that, at least transcriptionally, GLY is impairing the normal functioning of PSII. Indeed, since D1 and CP47 are essential for pigments binding and act in energy transfer to the reaction center, respectively (Taiz et al., 2015), changes in their transcript levels may result in disturbances during the photochemical reactions of photosynthesis (Gomes et al., 2014). Thus, it can be assumed that, in addition to affecting protein abundance in PSII by impairing amino acid biosynthesis (Gomes et al., 2014), GLY is also capable of reducing gene expression of PSII-related proteins.

Chlorophyll fluorescence measurements can provide quantitative data related to all stages of the photochemical phase of photosynthesis (Kalaji et al., 2016). Thus, after assessing GLY effects on biochemical and molecular endpoints targeted to PSII, it was decided to take a closer look at the photochemical efficiency of tomato plants exposed to GLY. Despite the negative influence of GLY on the levels of chlorophylls, no apparent effects were observed regarding photochemical parameters. Actually, when plants were exposed to light intensities similar to those experienced during growth, the values of Φ PSII and rETR were increased in response to GLY treatments, suggesting that the observed decrease in chlorophyll content, as well as the depletion of gene expression of *D1* and

CP47, did not result in photochemical damage, at least under these conditions. Although the *rETR* ($rETR = \Phi PSII \times PPFD$) does not reflect the absolute electron flow across thylakoids membrane, this formula has been widely used in stress physiology studies to report the electron transport rate occurring at a given light intensity in different photoautotrophic organisms and types of samples (Garrido et al., 2019; Masojídek et al., 2001; Ritchie, 2012; Williams et al., 2009; Zivcak et al., 2013), and more specifically on studies dealing with effects of GLY on photosynthesis (Gomes et al., 2017, 2016a; Vital et al., 2017; Yanniccari et al., 2012; Zobiole et al., 2010b). Besides, even if it is conceivable that GLY, as other stress signals (Sukhova et al., 2017), could have affected *p* (fraction of PPFD absorbed by leaves) and *dII* (multiplication factor since the transport of a single electron requires the absorption of 2 photons), *ETR* is largely determined by $\Phi PSII$ (Genty et al., 1989). Therefore, the results obtained in the current study still translate a significant impact of GLY in the electron transport rate. Although GLY exposure was found to inhibit *PSII* efficiency, *ETR* and non-photochemical energy dissipation (see review by Gomes et al., 2014), it should be stressed out that the majority of those studies evaluated the effects of GLY foliar application in resistant/susceptible plants. However, Cañero et al. (2011) and Gravena et al. (2012, 2009) also observed no negative signs on fluorescence parameters in olive and citrus plants, both considered as non-target species, upon exposure to GLY. Furthermore, in the present study, an inverse relationship between photochemical efficiency and non-photochemical quenching was observed, as revealed by the higher *rETR* and $\Phi PSII$, and lower *NPQ* levels. Indeed, although *NPQ* plays an important role in energy dissipation under excessive light conditions (Ruban, 2016), it may be suggested that, under growing light conditions, plants exposed to the herbicide increased their photochemical efficiency, allowing more power to be re-routed to the *PSII* and, thus, decreasing the *NPQ*. In an attempt to confirm the results obtained, an assay towards the evaluation of photochemical recovery upon exposure to high light saturating conditions for 1 h was performed. In fact, while the fluorescence photochemical parameters (e.g. F_v/F_m and $\Phi PSII$) may bring useful information regarding photosynthetic responses under steady-state, *PSII* photoinactivation and photorepair studies are of utmost importance to the proper understanding of photosynthetic responses to light (Serôdio et al., 2017 and references therein). Upon exposure to high light conditions, plants need to employ distinct compensatory mechanisms to control the excessive energy not used for photochemistry. One of the most common pathways is the *NPQ*, which transforms excitation energy into heat, contributing to a lower production of singlet oxygen ($^1O_2$) and preventing photo-oxidative stress in chloroplasts (Ruban, 2016). *NPQ* is a complex mechanism that comprises, at least, three different components – *qE* (energy dependent component), *qT*

(redistribution of energy from PSII to PSI) and *qI* (photoinhibition) (Latowski et al., 2011). The major NPQ component, *qE*, starts after the activation of the PsbS protein and the xanthophyll cycle, in which violaxanthin is reversibly converted into zeaxanthin, in response to the acidification of thylakoid lumen caused by the operation of the electron transport chain (Latowski et al., 2011; Ruban, 2016). Chemically, xanthophylls belong to the group of the carotenoids, which are recognised as important AOX especially in light stress conditions (Soares et al., 2019a). Thus, knowing that carotenoid and NPQ levels were decreased in response to the herbicide, GLY-treated plants were expected to present a lower photochemical recovery rate. However, as can be observed in Figure 9, plants exposed to increased concentrations of GLY showed a better photochemical recovery after high light saturating conditions. Moreover, although all groups of plants recover faster within the first few minutes (Figure 9), only the plants exposed to the highest GLY concentrations (20 and 30 mg GLY kg⁻¹) continue to recover, reaching values closer to 90% of the initial value. This behaviour supports the hypothesis that NPQ, through the activation of the xanthophyll cycle, is not the only mechanism underlying the higher photochemical recovery in GLY-stressed plants. Indeed, it is recognised that NPQ related to the xanthophyll cycle (*qE*) relaxes within few minutes (≈ 5 min) (Ruban, 2016), so there must be other mechanisms to balance the observed decrease in chlorophylls levels and *D1* and *CP47* transcripts. Indeed, it seems that cells tried to overcome GLY-induced stress by triggering offsetting mechanisms at the expense of a high energy demand (evidenced by the higher abundance of mitochondria). As recently reported, GLY application resulted in a higher efficient response of the plant's AOX system, enhancing the levels of proline and the main AOX enzymes, including ascorbate peroxidase (APX; EC 1.11.1.11) and CAT (Soares et al., 2019b). Thus, it appears that, under GLY exposure, the prompt response of the plant's AOX system helped to mitigate and/or reverse any photooxidative damage, resulting in a higher recovery rate but also explaining the higher photochemical efficiencies of GLY-treated plants. In order to pursue this hypothesis, further experiments will be designed to quantify the total AOX capacity right before and after the saturating light period.

Overall, based on our results, it can be hypothesized that, although GLY greatly impaired photosynthetic metabolism at the transcriptional and biochemical level, the cells were able to activate compensatory mechanisms, which is demonstrated by the stimulation of the photochemical reactions and by the higher energy demand related to the increased number of mitochondria. Despite that, it should be stressed that the substantial investment of cellular energy in protective mechanisms (NPQ and/or AOX defences) to maintain the photochemical efficiency, ends up dysregulating the normal plant metabolism,

possibly resulting in a higher cell death and damaging the ultrastructure of tomato leaves, thereby compromising plant growth.

GLY exposure does not compromise the photosynthetic CO₂ fixation or photosynthesis, but results in reduced water use efficiency (WUE_i)

It is well documented that foliar-applied GLY substantially reduces the chemical yield of photosynthesis, by promoting the malfunctioning of stomata (Gomes et al., 2014). However, no study has elucidated the connection between root-applied GLY and the performance of photosynthetic CO₂ fixation yet. Somewhat unexpectedly, our results showed that the herbicide not only did not apparently hamper this mechanism but instead promotes a 3-4-fold increment in CO₂ fixation in plants treated with the two highest GLY concentrations (20 and 30 mg GLY kg⁻¹). Although this might seem a little surprising, these observations are somewhat in line with the results relative to the photochemical efficiency. Nevertheless, transcript levels and RuBisCO content were reduced upon exposure to GLY, corroborating the observations of previous studies (Servaites et al., 1987), especially at the highest GLY concentration (Figure 1d and 7b). These findings reinforce the premise that the herbicide is affecting subcellular homeostasis at both transcriptional and protein levels, which would probably reflect in a decrease of Calvin cycle yield if the exposure period was longer.

Although our results have shown that exposure to increasing GLY concentrations also resulted in proportionally higher stomatal conductance, at least partially explaining the unexpected increment in P_N , the response of P_N to GLY concentration was not dose-dependent (Figure 10a,c), suggesting that factors other than stomata limitations are governing the measured photosynthetic activity. As can be observed (Figure 10c), although plants under 30 mg GLY kg⁻¹ exhibited a higher C assimilation rate than the CTL, the observed increase was lower than that of plants under 20 mg GLY kg⁻¹. Probably, this phenomenon can be explained by biochemical limitations rather than diffusional ones, perhaps by an impact on RuBisCO content (Figure 1d) and expression (Figure 7b). From what it appears, upon exposure to the highest GLY concentration, the registered inhibition on RuBisCO gene expression and protein content is already impacting the CO₂ assimilation, whose levels were closer to the ones of the CTL. Thus, it can be suggested that, although the intracellular concentration of CO₂ did not change, a lower content of RuBisCO transcripts and polypeptides did not allow the further increase of CO₂ assimilation rate. Moreover, our findings also suggest that, at the highest tested concentration, GLY may compromise plant water balance, since WUE_i was strongly diminished (Figure 10e), what may further impact the photosynthetic activity. In fact,

according to Zobiole et al. (2010a), the application of different GLY rates to GLY-resistant soybean plants ends up blocking the water uptake, reducing the WUE. On the other hand, knowing that the CO_2 fixation rate is expressed per unit leaf area ($\mu\text{mol m}^{-2} \text{s}^{-1}$), the sharp increment in P_N of plants exposed to GLY at 20 and 30 mg GLY kg^{-1} when compared to those in CTL, not paralleled by g_s , can also be explained by the alteration on leaf mesophyll structure caused by GLY treatment, as can be observed by the significant reduction in SLA at those highest GLY concentration (Figure 3c).

Although the interdependence of photosynthetic reactions is unquestionable, it is recognised that the photochemical and chemical phases can be differentially affected by abiotic stress factors (Sharma et al., 2019). Thus, in order to better understand the chain of photosynthetic events affected by GLY, correlation analyses between multiple parameters, namely between components of the two phases of photosynthesis, were performed. Despite not always being possible to find significant correlations, namely when $r\text{ETR}$ and P_N values were plotted ($p > 0.05$; Supplementary Material), it should be noted that, when integrating NPQ and P_N values, the rate of CO_2 assimilation increased, as the energy dissipated in the form of heat decreased ($< \text{NPQ}$), especially in plants treated with GLY. However, for the same NPQ value, plants exposed to 20 mg kg^{-1} show a greater assimilation potential than those treated with 30 mg kg^{-1} . In what concerns the gas-exchange parameters, significant correlations ($p \leq 0.05$; Supplementary Material) were detected between E and P_N . This observation, together with the relationship found between NPQ and P_N , sustains the hypothesis previously raised: the lower CO_2 assimilation rate of plants exposed to the highest concentration (30 mg kg^{-1}), in relation to those exposed to 20 mg kg^{-1} , is not due to stomatal restrictions, but most probably to biochemical and molecular limitations, contributing for a lower WUE_i .

So, studies must consider the alterations caused by GLY treatment, from the subcellular to the physiological level, to have a more realistic picture of the impact at the plant level. Furthermore, the higher values of stomatal conductance and transpiration induced by GLY can clearly affect water relations in the plant, raising its water requirements. Thus, on an agronomic perspective, this can be regarded as an important indirect effect of GLY on crops, which can bring important economic issues and must be carefully analysed.

5. CONCLUSIONS

The results obtained in the current study helped to disclose the consequences of soil contamination by GLY in the photosynthetic performance of one of the main crops worldwide, *S. lycopersicum* (Figure 11). The combination of ecophysiological, ultrastructural, biochemical and molecular tools allowed to achieve a robust and

comprehensive perception of the mechanisms behind GLY-induced stress in plants. From a wide perspective, it can be concluded that, although growth and development of this species is highly compromised by the herbicide exposure (Soares et al., 2019b), the observed toxicity in leaf ultrastructure, cell viability and water use efficiency, as well as in the transcriptional and biochemical control of photosynthetic-related players, seems not to substantially reduce C flow through photosynthesis, at least in a short-term exposure (Figure 11). Based on previous findings from our group, this apparent maintenance of photosynthesis is probably related to the stimulation of the AOX defences (Soares et al., 2019b), which must have been efficient at preventing ROS-induced damage in the viable cells of leaf mesophyll, and also closely related to a higher energetic investment to ensure the homeostasis of the cells. Thus, we hope that this work motivates future research efforts to clearly understand the risks of GLY overuse in non-target crops, not only from a productivity point-of-view, but also focusing on metabolic events which may help to develop ways to minimise GLY toxicity.

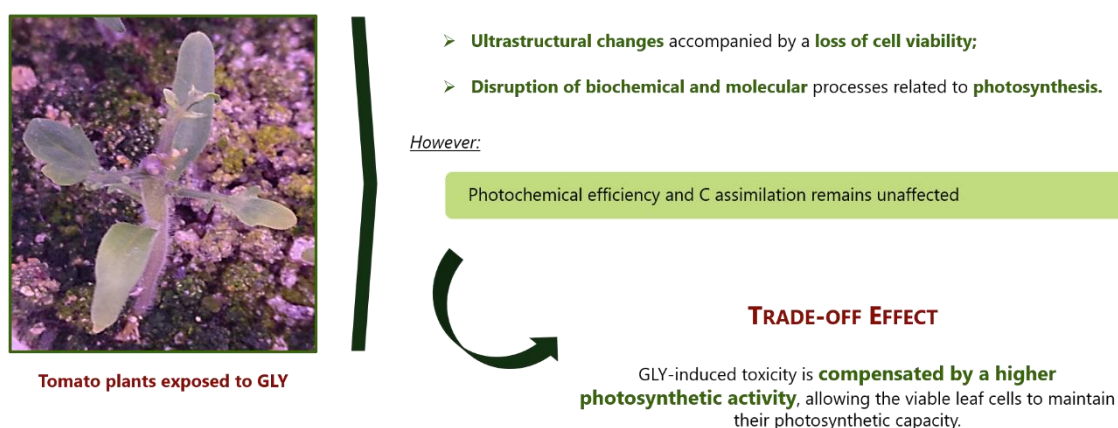


Figure 11. Overview of the main results obtained in this study.

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Supplementary Materials

Table S1. Data concerning the Spearman’s correlation analyses, discriminating r_s , p and N , between gas exchange parameters and chlorophyll fluorescence endpoints.

Correlation	N	p	r_s
rETR x NPQ	28	0.0113	-0.4718
Φ PSII x NPQ	26	0.0451	-0.3962
E x P_N	18	0.0002	0.7709
rETR x P_N	21	0.4263	-
NPQ x P_N	19	0.0114	-0.5667

According to Spearman’s test, correlations are defined according to the r_s value, which can vary from: 0-0.19 – very weak (red); 0.20-0.39 – weak (yellow); 0.40-0.59 – moderate (orange); 0.60-0.79 – strong (light green); 0.80-1.0 – very strong (green).

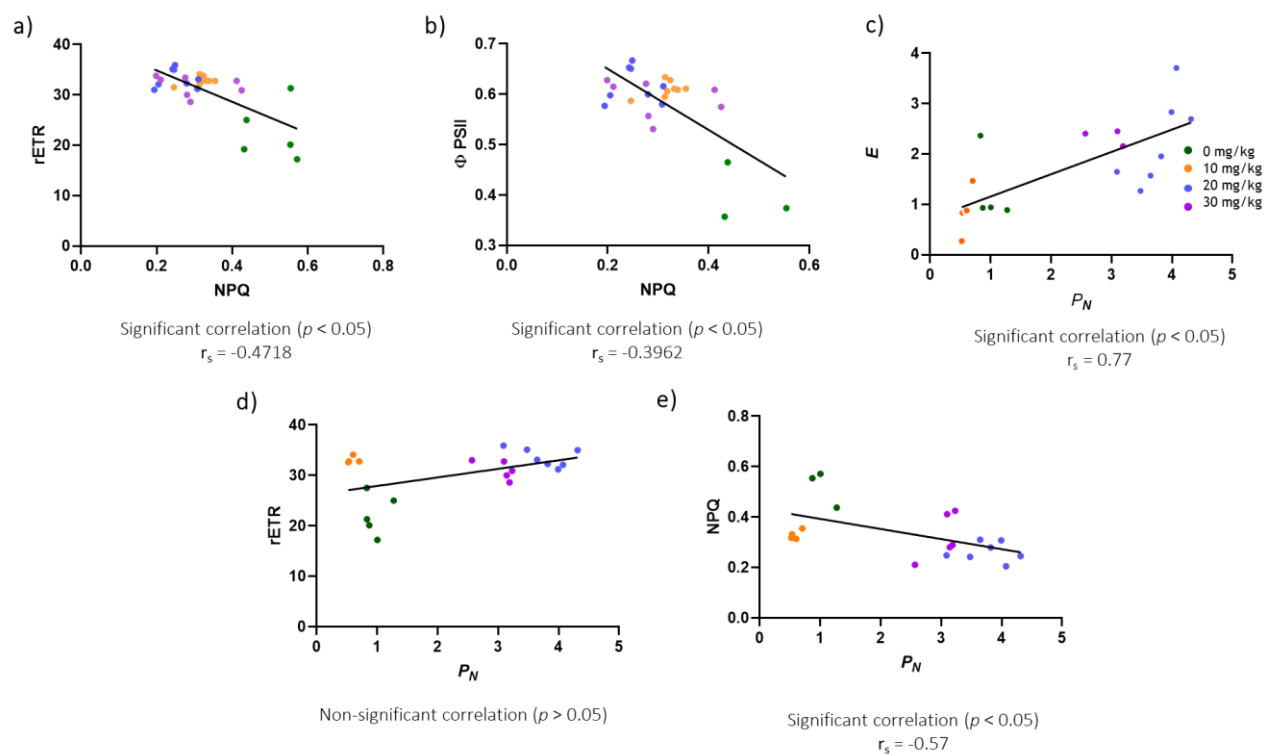


Figure S1. Spearman’s correlation analyses between some gas exchange parameters and chlorophyll fluorescence endpoints.

Ecotoxicological assessment of a glyphosate-based herbicide in cover plants: *Medicago sativa* L. as a model species

Abstract

Despite the several innovations that have been incorporated in agriculture, the use of herbicides, especially glyphosate (GLY), is still the major tool for weed control. Although this herbicide has a notable worldwide representation, concerns about its environmental safety were recently raised, with a lot of divergence between studies on its ecotoxicity. Therefore, it is of utmost importance to understand the risks of this herbicide to non-target plants, including cover crop species, which have a crucial role in maintaining agroecosystems functions and in preventing soil erosion. Thus, this work aims to evaluate the growth and physiological responses of a cover plant species (*Medicago sativa* L.) exposed to increasing concentrations of a GLY-based herbicide (GBH), particularly focusing on the oxidative metabolism. The growth of roots and shoots was affected, being this effect accompanied by a rise of lipid peroxidation, suggesting the occurrence of oxidative stress, and by an activation of the antioxidant (AOX) system. Indeed, the results showed that adverse effects are visible at active ingredient (a.i.) concentrations of 8.0 mg kg⁻¹, with the lowest EC₅₀ being 12.0 mg kg⁻¹, showing that GBH-contaminated soils may pose a risk to the survival of non-target plants in the most contaminated areas. Overall, these findings proved that GBHs greatly impair the growth of a non-target plant, strengthening the need of additional studies to unravel the real risks associated with the overuse of this pesticide, since there is an evident lack of studies performed with contaminated soils.

Keywords

Alfalfa; antioxidant system; herbicides; oxidative stress; reactive oxygen species.

1. INTRODUCTION

Plant protection products, also referred as pesticides, are widely used in agriculture in order to improve productivity, prevent crop losses or yield reduction, and control disease vectors or agents. However, it is known that only a small portion of the applied pesticides reach the target pests, while the remainder will end up in soil or will have the potential to move to other environmental compartments, including ground and surface water (Duke,

2017; Pimentel, 1995). Nevertheless, the mobility of these contaminants in the environment depends on several biotic and abiotic variables, and of their physicochemical properties. Thus, depending on the persistence of each substance, soil contamination can occur, thereby affecting soil quality, compromising its ability to perform its functions and leading to an irreversible degradation of this non-renewable resource (Aktar et al., 2009; Imfeld and Vuilleumier, 2012; Mahmood et al., 2016; Prashar and Shah, 2016; Silva et al., 2019). For this reason, concerns about the use of pesticides are increasing, and the most controversial at the moment is probably glyphosate (GLY), a post-emergence systemic herbicide of broad spectrum (non-selective). Applied to the foliage of weeds, GLY is absorbed by the leaves and is rapidly translocated in the plants through the phloem, particularly accumulating in meristems (root and shoot apex). Right after its discovery in the '70s of the last century, GLY quickly became the most applied herbicide worldwide and in 2014 the volume applied was sufficient to treat between 22 and 30% of globally cultivated cropland (Benbrook, 2016). Despite its great efficiency, several concerns about this herbicide were recently raised, related to the divergence between scientific studies regarding its toxicity to non-target organisms (Pochron et al., 2020; Van Bruggen et al., 2018). Another factor that may turn difficult to evaluate the real impacts of GLY on the environment is that GLY commercial formulations not only contain GLY, but also substances such as polyethoxylated amine (POEA) surfactants (Mesnage et al., 2019). It is known that the first generation of POEA surfactants present in RoundUp® were markedly more toxic than GLY, but since the mid-1990s, these compounds were progressively replaced by other POEA surfactants, ethoxylated etheramines, which exhibit lower non-target toxic effects (Mesnage et al., 2019). However, the composition of non-active ingredients in GLY-based herbicides (GBHs) is not fully known, and while a recent study pointed for a lower toxicity for earthworms of the GBH compared with the active ingredient (a.i.) itself, Pochron et al. (2020), another study concluded the opposite regarding *Dimorphandra wilsonii* Rizzini seed germination (Gomes et al., 2017a). Thus, GLY can be considered an old pesticide, but an emergent problem.

In areas in which high extensions of land are dedicated to intensive agriculture, the dispersion of GLY in the environment can be a serious problem of diffuse contamination, particularly due to its tendency to adsorb to solid particles (Aparicio et al., 2013; Bento et al., 2017). Depending on climatic conditions (especially temperature and humidity), the removal of GLY from soils can be reduced, resulting in its accumulation (Bento et al., 2016). This accumulation and dispersion through the environment, due to its non-selectivity (Herrmann and Weaver, 1999; Zabalza et al., 2017), can cause damage to plants that are not targeted, affecting a great number of species that account both directly and indirectly for soil biodiversity. From the available data, it was suggested that GLY

negative effects on plant growth and development substantially exceed the effects triggered by its mode-of-action as it can induce several metabolic and physiological disorders, favoring the occurrence of oxidative stress as an indirect consequence (Gomes et al., 2014). Indeed, when plants are exposed to stress factors such as soil contamination, oxidative stress occurs due to an overproduction of reactive oxygen species (ROS) (Choudhury et al., 2013; Soares et al., 2019a). Therefore, given their higher sensibility, ROS along with oxidative stress parameters (e.g. lipid peroxidation), can be used as exposure biomarkers allowing an early warning and sensitive evaluation of plants physiological status, representing a potential tool for phytotoxicity studies (Soares et al., 2016). Although ROS are important signaling factors, high levels of these compounds can easily become phytotoxic, damaging proteins, lipids, carbohydrates and nucleic acids. By influencing the cellular gene expression pattern, ROS are involved in many processes such as growth, cell cycle, abiotic stress responses, pathogen defence and systemic signaling and development. Thus, in order to maintain the redox homeostasis of the cell, plants possess a powerful antioxidant (AOX) system, composed of both enzymatic and non-enzymatic mechanisms (Gill and Tuteja, 2010). It is the joint action of these players that prevents the occurrence of redox disorders in the cell, by directly neutralising the toxic effects of ROS and/or by reducing their overaccumulation. However, depending on the plant species, the magnitude of stress and the exposure period, the AOX system may not be able to efficiently counteract ROS-induced toxicity, leading to the establishment of an oxidative stress condition (Soares et al., 2019a).

One group of plants that is particularly exposed to GLY contamination is cover plants, since they can be sown few months after the herbicide application, during off-season. In crops such as vines, they can be sown between the lines and left as a green cover. They are of extreme importance to the management of soil erosion, fertility and quality as well as crop yield (Büchi et al., 2018; Wittwer et al., 2017). Indeed, the European Commission (EC) established that the maintenance of permanent grassland areas is one of the actions that each European Union (EU) country and farmers must put in place, if they want to be rewarded for the protection of natural resources (European Commission, 2015). Thus, by affecting cover plants, GLY may jeopardise the balance of the ecosystem in which they are inserted. An example of a cover plant is *Medicago sativa* L., commonly known as alfalfa, a perennial leguminous, belonging to the family Fabaceae and subfamily Faboideae, well known by its ability to improve both soils' structure and biochemical activity (Hamdi et al., 2012). This cover crop has the potential to establish symbiotic relations with nitrogen-fixing bacteria thus increasing its growth and development, while contributing for the enrichment of soils with nitrogen compounds (Gamal Hassouna et al., 1994; Zhu et al., 2016).

Since little is known about the potential phytotoxicity of GLY contaminated soil, particularly in non-target species, the aim of this work is to unravel the effects of soil contamination by this herbicide on the growth and redox homeostasis of a cover plant species, *M. sativa*. By combining biometrical and biochemical approaches, this study will focus not only on the effects of a GBH on the development and growth performance of *M. sativa*, but also on the assessment of whether its toxicity is mediated by the occurrence of oxidative stress.

2. MATERIALS AND METHODS

2.1. Preparation of the artificial soil

The substrate used in this work consisted in an artificial soil composed of 70% (m/m) sand, 20% (m/m) kaolin and 10% (m/m) peat (OECD, 2006). The pH_{KCl} of the soil (1:5 m/v) was adjusted to 6.0 ± 0.5 by the addition of calcium carbonate (CaCO_3), whenever necessary.

2.2. Glyphosate (GLY) concentrations tested

The herbicide RoundUp UltraMax® (Bayer, Germany), acquired from a local supplier, was used in this study. From the commercial formulation (360 g L^{-1} GLY as potassium salt), a stock solution was prepared and a series of sequential doses of GBH was applied, ranging from 0 to 40 mg kg^{-1} of the active ingredient (a.i.), with a dilution factor of 1.5, giving rise to the following concentrations: 40; 27; 18; 12; 8.0 mg kg^{-1} , which were tested together with a GBH-free control.

2.3. Plant material and growth conditions

The seedling emergence and seedling growth test, performed according to the OECD protocol for terrestrial plants (OCDE, 2006), was carried out in plastic pots containing 200 g of artificial soil, to which the solutions with the desired GLY concentrations were added. Maintenance of soil moisture was ensured by the presence of a pot with deionised water (dH_2O) placed at the base of the soil pots with soil, and by using a cotton rope to ensure the capillarity rise of the water. Twenty seeds of *Medicago sativa* [var. Dimitra, acquired from Flora Lusitana Lda (Cantanhede, Portugal)] were placed in each pot, after sterilization with 70% (v/v) ethanol (7 min) and 20% (v/v) commercial bleach [5% active chloride; supplemented with 0.05% (m/v) Tween 20; 7 min), followed by washing with dH_2O . To ensure the availability of nutrients, a commercial fertiliser (EcoGrow, NPK 3-6-7) was added at the start of the test. A negative control (CTL; absence of contaminant) was also prepared, obtaining a total of 24 pots (4 replicates for each treatment). The assay began

when 50% of the seeds from the CTL germinated. In each pot, only 8 plants were kept, avoiding intraspecific competition. The plants germinated and grew in a growth chamber with controlled temperature (21 °C), photoperiod (16 h light/8 h dark) and photosynthetically active radiation (PAR; 120 $\mu\text{mol m}^{-2} \text{s}^{-1}$). After 21 d of growth, plants from each replicate were collected, used for the estimation of biometric parameters and then, shoots were frozen in liquid nitrogen (N_2) and stored at -80 °C until analyses.

2.4. Analysis of biometric indicators

The biometric analysis was performed as described in the OECD protocol for seedling emergence and seedling growth test (OECD, 2006). Eight plants from each replicate of every experimental group were used. After root and shoot separation, root length, and shoot height were measured, and the fresh mass of roots and shoots was registered.

2.5. Determination of physiological endpoints

Total chlorophylls (a + b) and carotenoids were extracted in 80% (v/v) acetone and quantified by spectrophotometry as described by Lichetenthaler (1987). The absorbance at 470, 647, and 663 nm was recorded, and the results obtained were expressed in mg g^{-1} fresh weigh (fw).

Total soluble protein content and glutamine synthetase (GS; EC 6.3.1.2) were extracted by homogenising, on ice, frozen shoot samples in an extraction buffer, followed by a centrifugation at 4 °C for 20 min and 15 000 *g*. Afterwards, extracts were used to quantify the total soluble protein (Bradford, 1976) and to determine GS activity by the transferase assay (Ferguson and Sims, 1971) by recording the absorbance at 500 nm. GS activity was calculated and expressed as nkat mg^{-1} protein.

2.6. Quantification of oxidative stress biomarkers

The assessment of lipid peroxidation (LP) was performed as described by Heath and Packer (1968), by the quantification of malondialdehyde (MDA). Briefly, plant samples were homogenised in 0.1% (m/v) trichloroacetic acid (TCA) and subsequently centrifuged (5 min; 10 000 *g*). Afterwards, the extracts were incubated with a mixture of 0.5% (m/v) thiobarbituric acid (TBA) in 20% (m/v) TCA for 30 min at 95 °C. At the end, the absorbance of each sample was read at 532 and 600 nm. After this step, the absorbance values of 600 nm were subtracted to the ones obtained at 532 nm to eliminate the effects of unspecific turbidity. The molar extinction coefficient ($\epsilon = 155 \text{ mM}^{-1} \text{ cm}^{-1}$) was used to calculate MDA levels and the results were expressed as $\text{nmol g}^{-1} \text{fw}$.

The determination of hydrogen peroxide (H_2O_2) was performed according to the procedure described by Jana and Choudhuri (1981). Upon homogenization of shoot aliquots in potassium phosphate (PK) buffer (50 mM; pH 6.5) and centrifugation (25 min; 6 000 g), the obtained plant extracts were combined with a mixture containing 0.1% (m/v) titanium sulphate (TiSO_4) in 20% (v/v) H_2SO_4 . Finally, the absorbance at 410 nm of each sample was recorded and the H_2O_2 levels were determined using the molar extinction coefficient of $0.28 \mu\text{M}^{-1} \text{cm}^{-1}$. Results were expressed in $\text{nmol g}^{-1} \text{fw}$.

2.7. Analysis of the AOX response

In order to determine the total antioxidant capacity (TAC) and the total phenolics content (TPC), the procedure described by Zafar et al. (2016) was followed. Firstly, frozen shoot samples were extracted in 80% (v/v) methanol followed by a centrifugation at 2500 g, for 10 min. Regarding TAC, upon dilution of the extracts (1:5), these were mixed with a reaction solution (0.6 M sulphuric acid – H_2SO_4 , 4 mM ammonium molybdate and 28 mM sodium phosphate), incubated at 95 °C for 90 min, and cooled on ice. After that, the absorbance was read at 695 nm. TAC levels were obtained from a calibration curve obtained with dilutions of a standard solution of ascorbic acid (AsA) and the results were expressed in mg equivalents of AsA $\text{g}^{-1} \text{fw}$. Concerning phenolics, their quantification was performed by a colorimetric assay using the Folin-Ciocalteu reagent. Absorbance was registered at 725 nm and TPC was calculated from a calibration curve, prepared with dilutions of a gallic acid solution. The results were expressed in mg of gallic acid $\text{g}^{-1} \text{fw}$.

The extraction and quantification of proline (Pro) was performed as previously described by Bates et al. (1973), using the ninhydrin-based colorimetric assay. Samples were homogenised in 3% (m/v) sulphosalicylic acid and centrifuged (500 g; 10 min). Then, the extracts were incubated, under acid conditions, with a ninhydrin solution for 1 h at 96 °C. At the end, the absorbance of each sample was read at 520 nm and Pro content was obtained from a calibration curve obtained with known Pro concentrations, and the results were expressed as $\mu\text{g g}^{-1} \text{fw}$.

2.8. Statistical analyses

All endpoints were evaluated using, at least three replicates per treatment and results were expressed as mean $\pm$ standard deviation (SD). The effects of the herbicide on the parameters previously mentioned were evaluated using one-way ANOVA, after checking the homogeneity of variances by the Levene Test. Whenever $p \leq 0.05$, the *post-hoc* Dunnet's test was used to compare the mean of each group with the CTL. The EC_{50} (concentration of GLY expected to have an effect in 50% of test organisms) and the

corresponding 95% confidence limits (95% CL) for the biometric parameters, were estimated with a non-linear least squares regression adjustment. All statistical procedures were performed in Graph Pad Prism® 8 (San Diego, CA, USA).

3. RESULTS

3.1. Biometric parameters of *M. sativa*

As shown in Figures 1 and 2, the application of a GBH had a negative impact in both root and shoot length and biomass. By analysing Figure 1a, it is possible to notice that there was a significant decrease in root length [$F(5, 16) = 106.8$; $p \leq 0.05$] for concentrations above the second lowest, with a monotonic dose-response relationship. Between 12 and 18 mg kg⁻¹ of the a.i. there was a drastic reduction of root length: the inhibition values rose from 27% to 68% comparatively to the CTL group. The EC₅₀ was estimated to be 16 mg kg⁻¹ (95% CL:14-19). Regarding shoot length, despite the observed decrease as the concentration increased, significant differences [$F(5, 16) = 36.21$; $p \leq 0.05$] were only recorded when plants were exposed to the highest doses of GBH (18, 26 and 40 mg kg⁻¹ of the a.i.), with inhibition values up to 64% in relation to the CTL. Nevertheless, a similar EC₅₀ was estimated (16 mg kg⁻¹ of the a.i.; 95% CL:14-22).

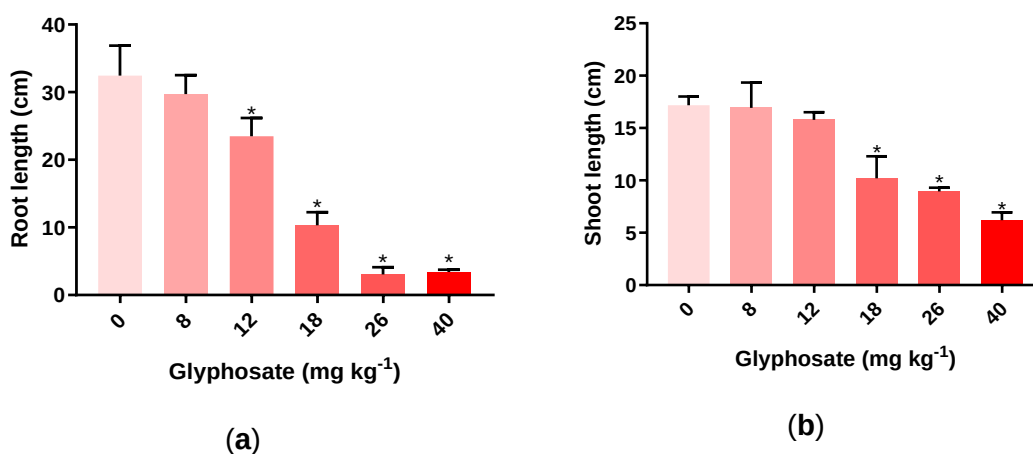


Figure 1. Average root (a) and shoot (b) lengths of *M. sativa* plants, 21 d after exposure to different concentrations of GLY. Error bars correspond to the standard deviation (SD). Statistically significant differences compared to the CTL (no GLY), considering $p \leq 0.05$, are marked with a * above bars.

Regarding fresh biomass (Figure 2), both roots and shoots were affected by GBH exposure in a concentration-dependent manner. Despite both organs exhibited the same global trend, some differences were recorded between them: while in shoots, all concentrations are statistically different from the CTL [$F(5, 15) = 92.02$; $p \leq 0.05$], reaching inhibition values ranging from 36-88%, in roots, significant differences [$F(5, 16) = 16.02$; $p \leq 0.05$] were only detected upon exposure to a.i. concentrations of 18, 26 and 40 mg kg⁻¹.

¹, with reductions of about 62, 79 and 90%, respectively. The highest effects observed in shoots are translated into differences in the EC_{50} values obtained. For root fresh biomass the estimated a.i. concentration was 15 mg kg^{-1} (95 % CL:12-22), whereas for the shoot fresh biomass it was 12 mg kg^{-1} (it was only possible to calculate the lower limit of the CL, which was 8.5).

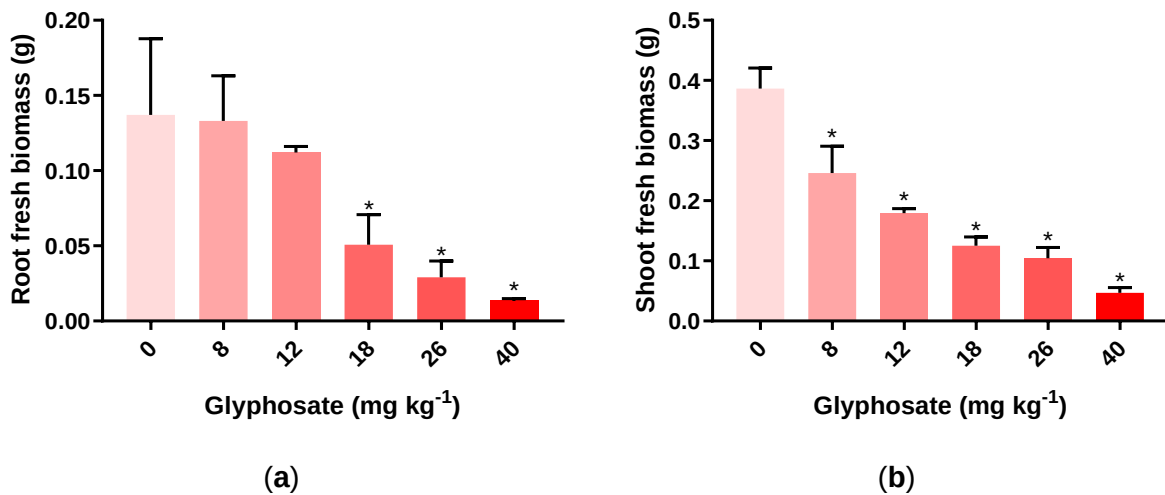


Figure 2. Average biomass of roots (a) and shoots (b) of *M. sativa* plants, 21 d after exposure to increased concentrations of GLY. Error bars correspond to the standard deviation (SD). Statistically significant differences compared to the CTL, considering $p \leq 0.05$, are marked with a * above bars.

3.2. Physiological parameters of *M. sativa*

For the photosynthetic pigments, the behaviour was similar for both carotenoids and total chlorophylls (Figures 3a and 3b, respectively), as no significant statistical differences were registered among treatments and the CTL: $F(5, 8) = 2.920$; $p > 0.05$ for carotenoids; $F(5, 12) = 2.072$; $p > 0.05$ for chlorophylls.

GS levels (Figure 3c) showed a different pattern from that of photosynthetic pigments. Comparatively to the CTL, all GBH concentrations induced a significant reduction in GS activity levels [$F(5, 12) = 7.851$; $p \leq 0.05$]. As can be observed in Figure 3c, when plants were exposed to a.i. concentrations between 8 and 26 mg kg^{-1} , decreases of around 50% were found in comparison with the CTL. Curiously, upon exposure to the highest concentration, GS levels became closer to the ones registered for the CTL.

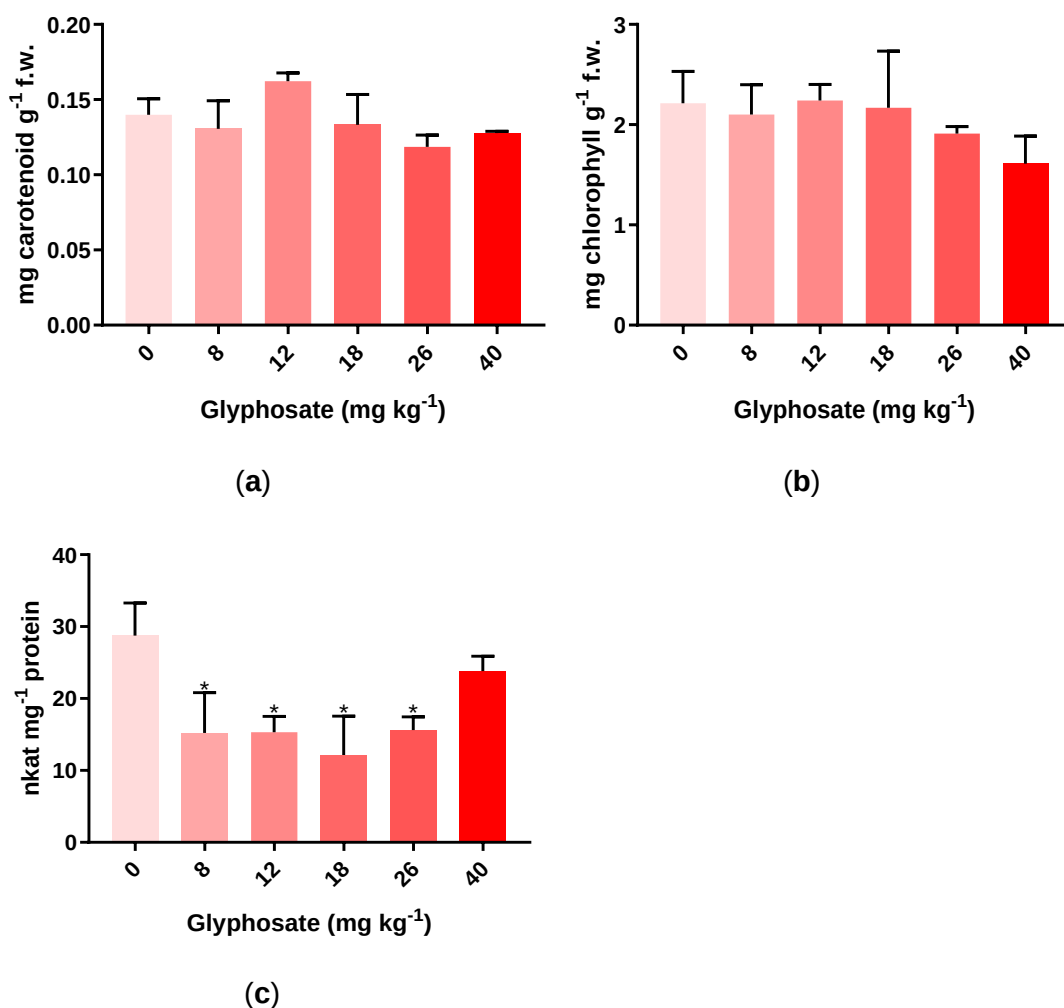


Figure 3. Average concentrations of carotenoid (a) and chlorophyll (b), and GS activity levels (c) in shoots of *M. sativa* plants 21 d after exposure to increased concentrations of GLY. Error bars correspond to the standard deviation (SD). Statistically significant differences compared to the CTL, considering $p \leq 0.05$, are marked with a * above bars.

3.3. Oxidative stress biomarkers of *M. sativa*

The behaviour of the analysed oxidative stress biomarkers, H₂O₂ and LP, is shown in Figure 4. In general, H₂O₂ levels rose along with the increase of GBH concentration (Figure 4a). However, significant differences [$F(5, 11) = 6.294$; $p \leq 0.05$] were only observed for concentrations higher than 12 mg kg⁻¹, compared to the CTL. A similar behaviour was also observed for LP with MDA levels increasing in a concentration-dependent manner (Figure 4b). Despite of this pattern, for LP, statistically significant differences from the CTL [$F(5, 30) = 13.37$; $p \leq 0.05$] were observed only at the highest a.i. concentrations (26 and 40 mg kg⁻¹).

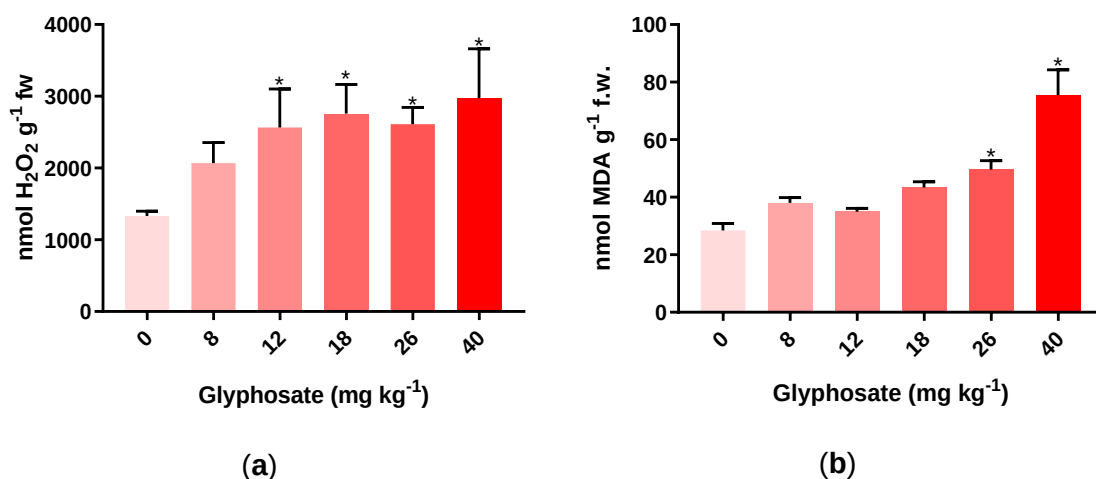
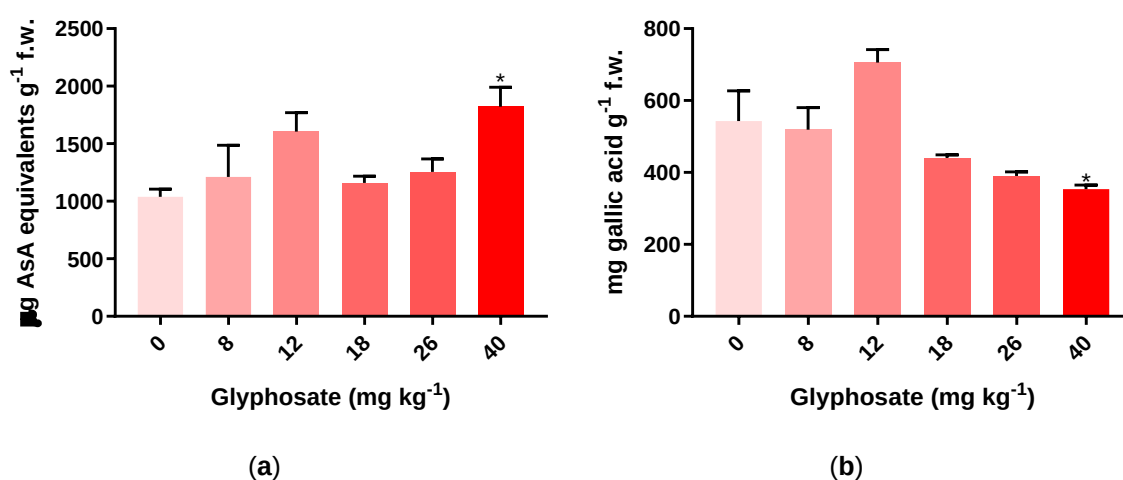
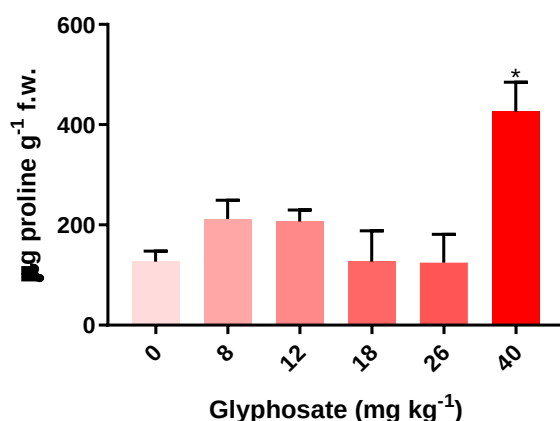


Figure 4. Average concentrations of H₂O₂ (a) and MDA (b) in shoots of *M. sativa* plants 21 d after exposure to increased concentrations of GLY. Error bars correspond to the standard deviation (SD). Statistically significant differences compared to the CTL, considering $p \leq 0.05$, are marked with a * above bars.

The AOX response, evaluated by assessing the TAC, TPC and Pro levels, of *M. sativa* exposed to RoundUp UltraMax[®] is presented in Figure 5. Regarding TAC (Figure 5a), although a tendency for enhanced values as the concentration of the GBH goes up was noticed, statistically significant differences [$F(5, 14) = 3.468$; $p \leq 0.05$] were only found when plants were exposed to 40 mg kg⁻¹ of a.i., with an increase of about 75% above the CTL. On the other hand, TPC (Figure 5b) was reduced upon exposure to increased concentrations of the GBH, especially in the highest dose (decreases up to 36%). Indeed, significant differences [$F(5, 13) = 7.802$; $p \leq 0.05$] comparing to the CTL were observed only for the highest concentration. Concerning Pro (Figure 5c), its content showed a similar pattern to that of TAC, with levels significantly higher [$F(5, 8) = 5.574$; $p \leq 0.05$] than the CTL (by 3-fold) only for the highest concentration of GLY.





(c)

Figure 5. Effect of increased concentrations of GLY, on the AOX system of *M. sativa* shoots after 21 d of exposure. (a) TAC; (b) TPC; (c) Pro. Error bars correspond to the standard deviation (SD). Statistically significant differences compared to the CTL, considering $p \leq 0.05$, are marked with a * above bars.

4. DISCUSSION

Up to date, little is known regarding the phytotoxicity of GLY contaminated soils on non-target plants, including cover crop species, such as *M. sativa*. Although these plants are not intentionally treated with GLY, they can still be affected by its application through leaching, runoffs or even wind in case of spraying. Moreover, GLY strongly adsorbs to solid particles (Aparicio et al., 2013; Bento et al., 2017) and accumulates in soils (Bento et al., 2016), resulting in a serious problem of diffuse contamination. Indeed, several studies were conducted in order to determine GLY levels in soils around the world and despite many of them reporting levels lower than 3 mg kg⁻¹ for agricultural soils or soil located nearby agricultural areas in South America (Alonso et al., 2018; Aparicio et al., 2013; Primost et al., 2017; Soracco et al., 2018) and Europe (Grunewald et al., 2001; Karanasios et al., 2018; Laitinen et al., 2006; Silva et al., 2019, 2018), other studies have reported values of 5.0 mg kg⁻¹ in soybean cultivated areas in Argentina (Peruzzo et al., 2008), reaching values as high as 40.6 mg kg⁻¹ in olive groves from Greece (Karanasios et al., 2018) or even 608 mg kg⁻¹ in a crop field from Mexico (Muñoz et al., 2019). Therefore, the main goal of the present study was to assess the effects of soil contamination by a GBH on the growth responses and redox homeostasis of alfalfa plants, at environmentally relevant concentrations of the a.i. In fact, although recent studies have been conducted to evaluate the effects of GLY application in non-target plants, most of these works applied GLY as foliar spray (Akbulut et al., 2015; Gomes et al., 2016; 2017b; Krenchinski et al., 2017; Radwan and Fayez, 2016; Singh et al., 2017b, 2017a) or as a supplement to the

nutrient solution (De Campos Oliveira et al., 2016; de Freitas-Silva et al., 2017; Gomes et al., 2017a; Mondal et al., 2017; Serra et al., 2015; Tong et al., 2017) rather than simulating soil contamination scenarios.

The present study showed that, after 21 d of exposure, RoundUp UltraMax® severely repressed the growth of *M. sativa*, in a dose-dependent manner, inhibiting both organs' elongation and biomass production. Actually, given the already accentuated reduction of shoot fresh weight upon exposure to the lowest concentration tested (8 mg kg⁻¹ of a.i.), it can be suggested that even lower concentrations would be capable of impairing plant growth. When GLY is absorbed by the plant, it is translocated through vascular tissues, namely by phloem, reaching active metabolite sites, such as root and shoot meristems, following the same pathway as photoassimilates (Gomes et al., 2014; Satchivi et al., 2000) which could explain the repression of shoot growth. The fact that GLY is an herbicide that inhibits an enzyme from the shikimate pathway, 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS; EC 2.5.1.19), can also explain the results obtained. EPSPS plays a role in the synthesis of the aromatic amino acids tryptophan, phenylalanine, and tyrosine that are crucial for the growth and survival of plants and which function as the precursors of many secondary metabolites such as pigments, auxins and lignin (Herrmann, 1995). As a result of the shikimic acid pathway being blocked, there will be an accumulation of shikimate in plant tissues which will lead to a deficit in important end products such as lignin, alkaloids, and flavonoids, and a reduction in carbon dioxide (CO₂) fixation and biomass production in a dose-dependent manner (Olesen and Cedergreen, 2010). The decrease in root and shoot length and biomass can also be due to the impact that GLY has on indole-3-acetic-acid (IAA) metabolism, which is the main endogenous auxin in the plant as well as to the interference with plant-water relations (Clay and Griffin, 2000; Mondal et al., 2017; Soares et al., 2019b). Another hypothesis that can explain these results is the fact that GLY can condition the absorption of several macro and micronutrients such as calcium (Ca), magnesium (Mg), N, phosphorous (P), iron (Fe), zinc (Zn), among others as reviewed by Gomes et al. (2014).

Several studies were conducted in order to evaluate the phytotoxicity of GLY to non-target plants such as: *Pisum sativum* L. (GLY or GBH, applied directly to the seeds or supplemented to the nutrient solution) (Mondal et al., 2017; Singh et al., 2017a); *Hordeum vulgare* L. [GBH supplemented to a mixture of perlite:vermiculite (1:2)] (Spormann et al., 2019); *Solanum lycopersicum* L. (GLY applied by foliar spray) (Singh et al., 2017b); *Vigna radiata* (L.) R. Wilczek (seeds treated with a GBH) (Basantani et al., 2011); *Fagopyrum esculentum* Moench (GLY isopropylamine salt supplemented to the nutrient solution) (Debski et al., 2018); *Lemna minor* L. (GBH supplemented to the nutrient solution) (Sikorski et al., 2019); and *D. wilsonii* (seeds treated with a GBH or analytical grade

glyphosate) (Gomes et al., 2017a). Even though the experimental conditions of the previously mentioned studies were not similar to the present study, they all recorded a decrease in plant growth demonstrating the negative effect that both GLY and GBH have on biometric indicators. Concerning GBH-contaminated soils, a similar decrease was also observed in the work of Soares et al. (2019b), in which tomato plants grew in an artificial soil contaminated by increasing a.i. concentrations (0, 10, 20 and 30 mg kg⁻¹). Their results showed significant statistical differences even at 10 mg kg⁻¹, a concentration pretty much identical to the lowest dose tested in this study.

Photosynthesis, one of the main biochemical process occurring in photoautotrophic organisms, highly depends on light absorption by chlorophylls and carotenoids. The biosynthesis of these pigments as well as fatty acids or amino acids can be affected by GLY exposure (Dewick, 1986; Fedtke and Duke, 2005). Previous studies showed that GLY can impair plastoquinone synthesis, thereby contributing for a lower production of carotenoid precursors (Gomes et al., 2016). Regarding chlorophylls, both GLY and GBH can also directly inhibit its biosynthesis, by reducing δ -aminolevulinic acid (ALA) levels, or increase chlorophyll degradation as reported by several authors (Gomes et al., 2016; Huang et al., 2012; Kitchen et al., 1981; Mateos-Naranjo et al., 2009; Singh et al., 2017a; Zobiole et al., 2011). Based on these results, it was expected to observe a significant decrease of the levels of both chlorophylls and carotenoids. Indeed, even a previous work conducted with the same plant species, but grown in perlite and quartz sand (Muñoz-Rueda et al., 1986), reported that the foliar application of a GBH resulted in a reduction of the total photosynthetic pigments as the a.i. concentration increased. However, in the present study, the herbicide showed no effects on chlorophyll and carotenoid contents, despite the slightly lower contents observed when comparing to the control group (except for 12 mg kg⁻¹). Thus, these results suggest that, at the tested doses, this herbicide did not negatively affect the photosynthetic pigments as also demonstrated in the study performed by Spormann et al. (2019) with a GLY concentration of 30 mg kg⁻¹, applied in the form of RoundUp UltraMax® and using a mixture of perlite:vermiculite as substrate. As discussed by Spormann et al. (2019), these results could be explained by the lack of AMPA production in the artificial medium. Indeed, AMPA, the main metabolite formed upon GLY degradation, is considered as a potent phytotoxin, capable of competing with glycine and consequently inhibiting chlorophyll biosynthesis (Reddy et al., 2004; Serra et al., 2013). Thus, there are two hypotheses for the lack of negative effects, due to GLY exposure, on chlorophyll and carotenoid levels: i) the use of a standard artificial soil with low microbial activity not allowing enough AMPA production to cause negative effects on biosynthesis of these pigments, and/or ii) the mode-of-application of GLY, which, in this study, was added to the soil contrasting to the majority of works which provided GLY as foliar spray.

Nevertheless, and regarding the former hypothesis, this does not mean that an enhanced effect on a natural soil with a more diverse and functionally active soil microbial community would certainly be expected, as the degradation rates of both GLY and AMPA are still not well studied.

As important as photosynthesis, the mineral nutrition of plants highly contributes for a proper growth performance. However, the effect of GLY on plant mineral nutrition is yet to be fully understood (Zobiole et al., 2010). Up to now, no consensus has been reached on the influence that GLY may bring on nutrient uptake, since the studies conducted so far point towards different results: while several authors reported a negative effect of GBH on plants' nutrient uptake (Cakmak et al., 2009; Zobiole et al., 2012, 2011, 2010), other studies concluded that this application does not affect the mineral status of the plants (Bailey et al., 2002; Duke et al., 2012a, 2012b). As reviewed by Duke et al. (2012), these inconsistent results may be due to differences in the type of soil, climatic conditions, and/or GLY-resistant cultivars used. Aiming to assess the nutritional status of *M. sativa* under GLY exposure, the present study evaluated the activity of GS, an enzyme that is involved in the first step of ammonium (NH_4^+) assimilation, not only that absorbed by roots, but also that generated from photorespiration, proteolysis and processes that are increased by several stresses (Gomes Silveira et al., 2003; Pageau et al., 2006). The results revealed that GS was dysregulated for almost all tested concentrations, indicating that, at least under the experimental conditions of the present work, GBH interfered with the nitrogen (N) metabolism. Based on these findings, the hypothesis that GLY conditioned the physiological uptake of mineral nutrients specially nitrogen (N), due to the formation of complexes making them unavailable for biological processes, arises (Zhong et al., 2018). Concerning N uptake, once again, results from different studies, all of them using GBH, are contradictory with no effect in field studies (Bellaloui et al., 2006; Henry et al., 2011) and inconsistencies in greenhouse studies (Cakmak et al., 2009; Zobiole et al., 2010).

As previously reviewed by Gill and Tuteja (2010) and Soares et al. (2019a), plant development can be severely affected by various abiotic stressors such as herbicide application, leading to an overproduction of ROS which in its turn will cause significant damage to cell structures, ultimately resulting in oxidative stress. In order to verify the occurrence of oxidative stress, H_2O_2 levels and LP degree, as a mean to assess membrane damage, were evaluated. According to the results obtained, H_2O_2 accumulation was enhanced upon exposure to GBH, especially at levels of the a.i. higher than 12 mg kg^{-1} . However, when looking at LP results, MDA content was only increased in response to the two highest treatments (26 and 40 mg kg^{-1} of a.i.). Based on this behaviour, one can suggest that ROS overproduction took place earlier than the observed membrane damage, being this possibly related to the dual role played by ROS in plant cells. Indeed,

H₂O₂, as other ROS, can act as a signal molecule at low concentrations as it is involved in acclimation signaling leading to plant tolerance to various biotic and abiotic stresses, becoming toxic above a certain threshold, capable of inducing programmed cell death (Quan et al., 2008). Therefore, it can be hypothesized that, at lower GLY concentrations, H₂O₂ was involved in signaling mechanisms (with no LP increase), while at the highest concentrations (26 and 40 mg kg⁻¹ of the a.i.), H₂O₂ accumulation started to induce oxidative damage, which is reflected by the occurrence of LP.

The induction of oxidative stress by GLY is described as one of its indirect effects on plant physiology, either by the overproduction of ROS or by a depletion of defence mechanisms (Gomes et al., 2016). Although not so explored as in target and resistant species, the influence of this herbicide on the redox status of non-target plants, including crops, willow and aquatic plants (Akbulut et al., 2015; Gomes et al., 2016; 2017b; Gomes and Juneau, 2016; Moldes et al., 2008; Radwan and Fayez, 2016; Singh et al., 2017b, 2017a; Soares et al., 2019b; Spormann et al., 2019; Zhong et al., 2018) is starting to gain attention. Corroborating the results of the present work, several studies reported an increase in H₂O₂ content and MDA levels in plants grown in GBH-contaminated solid substrate (Spormann et al., 2019), or when GLY or GBH were supplied in nutrient solutions (Gomes et al., 2016; 2017a; Gomes and Juneau, 2016; Singh et al., 2017a), or applied as foliar spray (Akbulut et al., 2015; Radwan and Fayez, 2016; Singh et al., 2017b).

In order to defend themselves from oxidative damage caused by ROS, plants developed protective mechanisms by synthesising enzymatic and non-enzymatic AOXs (Gill and Tuteja, 2010). In the context of this work, TAC, TPC and Pro levels were measured to assess the involvement of the non-enzymatic component of the AOX system in limiting GLY-induced stress. The results showed an increase in TAC and Pro levels only at the highest a.i. concentration (40 mg kg⁻¹). Since TAC gives a general idea regarding the cell's AOX status (Pinto et al., 2019) and Pro acts as a strong AOX (Gill and Tuteja, 2010), the elevated TAC and Pro levels suggest that the AOX defence mechanisms were activated due to oxidative stress, but only at the highest concentrations of GLY. Thus, it can be hypothesized that *M. sativa* plants boosted the accumulation of Pro, along with other non-enzymatic players, to counteract the induced oxidative stress by this herbicide; however, bearing in mind that LP remained higher at the two highest concentrations, this response was not enough to counteract the harmful effects observed. Moreover, phenolic compounds, which are known to chelate metals, scavenge ROS and inhibit LP (Sharma et al., 2012), were negatively affected by the presence of the herbicide, since reduced levels of these specialised metabolites were found in treated plants. This effect probably arises as a consequence of GLY-induced impairment of the shikimate pathway, since

phenolic compounds are formed through this biosynthetic process (Santos-Sánchez et al., 2019), and is in accordance with the results obtained for LP.

Up to now, some studies were conducted in order to evaluate the AOX defence mechanisms of plant species exposed to both GLY and GBH (Gomes et al., 2017; Sergiev et al., 2006; Singh et al., 2017b; Soares et al., 2019b; Spormann et al., 2019). These studies demonstrate that there is a dysregulation of the AOX defence system, with records of both increases and decreases of these mechanisms. Particularly, in the study of Soares et al. (2019b), performed with GBH-contaminated soils, it was observed that this formulation stimulated the AOX defence mechanisms of tomato shoots, at concentrations of 20 and 30 mg kg⁻¹ of the a.i.. This suggests that like other environmental stresses, the response to herbicide application depends on several factors such as the plant species, the concentration, and the mode-of-application. However, the results obtained in the present study are in line with those already published by other authors (Gomes et al., 2017; Singh et al., 2017b; Soares et al., 2019b; Spormann et al., 2019) indicating that the increase in Pro levels seems to be the most consistent signal of the activation of the AOX defence against GLY-induced stress, suggesting that this amino acid can be used as a biomarker of exposure to GLY.

5. CONCLUSIONS

Overall, it is possible to conclude that after 21 d of exposure to a GBH, the growth and physiological performance of *M. sativa*, were negatively affected at the tested concentrations. The results also showed an activation of the AOX system, although its action was not enough to counteract the oxidative damage induced by an overproduction of ROS ultimately leading to a decrease in this plant's growth. In the present work adverse effects of GLY are visible at 8 mg kg⁻¹ of the a.i., which is a concentration much lower than the highest levels reported for European and South American soils. However, it should be noted that soil properties such as soil organic matter content, may affect the behaviour of GLY on soils. In addition, the type of formulation can also affect the toxicity, since the presence of surfactants may enhance the negative effects of the a.i.. Thus, considering that plant responses to GLY can be species-specific and vary with distinct experimental conditions, it is of utmost importance to better understand the impacts of GLY-contaminated soils on the survival of non-target plants and subsequently on soils biodiversity, as well as to develop new strategies to minimise its potential risks to agroecosystems.

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CHAPTER V. ECO-FRIENDLY WAYS TO REDUCE GLYPHOSATE-INDUCED OXIDATIVE STRESS IN CROPS



Article

Silicon Improves the Redox Homeostasis to Alleviate Glyphosate Toxicity in Tomato Plants—Are Nanomaterials Relevant?

Cristiano Soares ^{1,*}, Pedro Nadas ¹, Bruno Sousa ¹, Edgar Pinto ^{2,3}, Isabel M. P. L. V. O. Ferreira ², Ruth Pereira ¹ and Fernanda Fidalgo ^{1,*}

- ¹ GreenUPorto—Sustainable Agrifood Production Research Centre and INOV4AGRO, Biology Department, Rua do Campo Alegre s/n, Faculty of Sciences of University of Porto (FCUP), 4169-007 Porto, Portugal; up201604645@fc.up.pt (P.N.); bruno.sousa@fc.up.pt (B.S.); ruth.pereira@fc.up.pt (R.P.); fidalgo@fc.up.pt (F.F.)
- ² LAQV/REQUIMTE, Laboratory of Bromatology and Hydrology, Department of Chemical Sciences, Faculty of Pharmacy, University of Porto (FFUP), Rua de Jorge Viterbo Ferreira no. 228, 4050-313 Porto, Portugal; ecpl@ffup.pt (E.P.); isabel.ferreira@ffup.pt (I.M.P.L.V.O.F.)
- ³ School of Health, Department of Environmental Health, PPorto (ESS-P/Porto), Rua Dr. António Bernardino de Almeida, 400, 4200-072 Porto, Portugal
- * Correspondence: c.souares@fc.up.pt (C.S.); fidalgo@fc.up.pt (F.F.)



Article

Foliar Application of Sodium Nitroprusside Boosts *Solanum lycopersicum* L. Tolerance to Glyphosate by Preventing Redox Disorders and Stimulating Herbicide Detoxification Pathways

Cristiano Soares ^{1,*}, Francisca Rodrigues ^{1,†}, Bruno Sousa ¹, Edgar Pinto ^{2,3}, Isabel M. P. L. V. O. Ferreira ², Ruth Pereira ¹ and Fernanda Fidalgo ¹

- ¹ GreenUPorto—Sustainable Agrifood Production Research Centre & INOV4AGRO, Biology Department, Faculty of Sciences of University of Porto, Rua do Campo Alegre s/n, 4169-007 Porto, Portugal; franciscamrodrigues98@gmail.com (F.R.); bruno.filipe@fc.up.pt (B.S.); ruth.pereira@fc.up.pt (R.P.); fidalgo@fc.up.pt (F.F.)
- ² LAQV/REQUIMTE, Laboratory of Bromatology and Hydrology, Department of Chemical Sciences, Faculty of Pharmacy, University of Porto (FFUP), Rua de Jorge Viterbo Ferreira n.º 228, 4050-313 Porto, Portugal; ecpl@ffup.pt (E.P.); isabel.ferreira@ffup.pt (I.M.P.L.V.O.F.)
- ³ Department of Environmental Health, School of Health, PPorto (ESS-P/Porto), Rua Dr. António Bernardino de Almeida, 400, 4200-072 Porto, Portugal
- * Correspondence: c.souares@fc.up.pt
- † These authors contributed equally to this work and, therefore, should be both considered as first co-authors.



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Research article

Salicylic acid alleviates glyphosate-induced oxidative stress in *Hordeum vulgare* L.

Sofia Spormann ^{a,b,1}, Cristiano Soares ^{a,b,*,1}, Fernanda Fidalgo ^{a,b}

^a GreenUPorto—Centro de Investigação em Produção Agrícola Sustentável, Biology Department, Faculty of Sciences, University of Porto, Rua Campo Alegre 5/n, 4169-007, Porto, Portugal

^b Plant Stress Lab, Biology Department, Faculty of Sciences, University of Porto, Rua Campo Alegre 5/n, 4169-007, Porto, Portugal



Silicon improves the redox homeostasis to alleviate glyphosate toxicity in tomato plants – are nanomaterials relevant?

Abstract

Given the widespread use of glyphosate (GLY), this agrochemical is becoming a source of contamination in agricultural soils, affecting non-target plants. Therefore, sustainable strategies to increase crop tolerance to GLY are needed. Within this perspective, and recalling silicon (Si)'s role in alleviating different abiotic stresses, the main goal of this study was to assess if the foliar application of Si, either as bulk or nano forms, is capable of enhancing *Solanum lycopersicum* L. tolerance to GLY (10 mg kg⁻¹). After 28 d, GLY-treated plants exhibited growth-related disorders in both shoots and roots, accompanied by an overproduction of superoxide anion (O₂^{•-}) and malondialdehyde (MDA) in shoots. Although plants solely exposed to GLY have activated non-enzymatic antioxidant (AOX) mechanisms (proline, ascorbate and glutathione), a generalised inhibition of the AOX enzymes was found, suggesting the occurrence of great redox disturbances. In response to Si or nano-SiO₂ co-application, most of GLY phytotoxic effects on growth were prevented, accompanied with a better ROS removal, especially by an upregulation of the main AOX enzymes, including superoxide dismutase (SOD), catalase (CAT) and ascorbate peroxidase (APX). Overall, results pointed towards the potential of both sources of Si to reduce GLY-induced oxidative stress, without major differences between their efficacy.

Keywords

Antioxidants; herbicides; nanoparticles; oxidative stress; stress alleviation.

1. INTRODUCTION

“It took 200,000 years for our human population to reach 1 billion – and only 200 years to reach 7 billion” (<https://mahb.stanford.edu/blog/human-population-time/>). The message is clear. Human population is on the rise and, therefore, more food must be produced with fewer resources and less available land (FAO, 2017). For this reason, and especially since the last half of the 20th century, agriculture is progressively more dependent on agrochemicals to achieve high yield rates, reason why the pesticide industry has been

continuously growing over the recent decades (Nishimoto, 2019). By definition, agrochemicals are chemical agents used to protect crops from diseases and pests, and/or to enhance plant growth under adverse conditions (Mandal et al., 2020). According to a recent report of the Environmental Protection Agency (EPA) of the United States, among all kinds of pesticides, herbicides account for almost 50% of the total expenditures between 2008-2012 worldwide (Atwood and Paisley-Jones, 2017).

Specifically focusing on this class, glyphosate (GLY)-based herbicides are the most sold formulations and are expected to remain as the leading chemical option for weed control in the following years (<https://www.marketsandmarkets.com/Market-Reports/herbicides-357.html>). Concretely in Europe, GLY use was recently renewed until the end of 2022 (https://ec.europa.eu/food/plant/pesticides/glyphosate_en). Although at the beginning, GLY [N-(phosphonomethyl) glycine] use was restricted to some areas, given its non-selective action, the development of GLY-resistant crops (e.g. maize, soybean, cotton) has largely contributed to the substantial increase of its commercialization and widespread use (Singh et al., 2020; Van Bruggen et al., 2018). In general terms, GLY is classified as a foliar, broad-spectrum, post-emergent and systemic herbicide, acting by blocking the biosynthesis of essential amino acids, such as tryptophan, tyrosine, and phenylalanine (Gomes et al., 2014). Once absorbed by the plant, GLY tends to accumulate in metabolically active sites, mainly in apical meristems, where it inhibits the action of 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS; EC 2.5.1.19). As a consequence, the shikimate pathway is compromised, resulting in an overaccumulation of shikimate and a deficit of chorismate in plant cells, ultimately inhibiting the synthesis of aromatic amino acids (Gomes et al., 2014).

Since the shikimate pathway is exclusively found in plants and some species of microorganisms (Herrmann and Weaver, 1999), GLY was – and still is – considered as one of the most innocuous chemical options for weed control (Duke, 2020). However, especially in the last few years, concerns have been raised regarding the possible toxicity of GLY, not only for vertebrates, including humans, but also for soil organisms and non-target plants (Gomes et al., 2017; Singh et al., 2020; Soares et al., 2019b; Spormann et al., 2019; Van Bruggen et al., 2018). Although it is claimed that, once in contact to the soil, GLY is quickly degraded by the action of microorganisms and/or adsorbed to soil particles (DT₅₀ of around 20 d; <http://sitem.herts.ac.uk/aeru/iupac/Reports/373.htm>), recent findings suggest that GLY can persist in the environment, accumulating in soils and/or being leached to surface waters (Van Bruggen et al., 2018). For this reason, the scientific community has been gathering efforts to unravel the potential hazards of GLY to different trophic levels, from producers to consumers and decomposers (recently reviewed by Van Bruggen et al., 2018). Up to now, although there is no consensus regarding the real toxicity

of GLY to animals, multiple studies have found that soil residues of this herbicide can impair plant growth, possibly inflicting losses in global agronomic yields. For instance, lab-scaled experiments revealed that GLY soil contamination greatly affects the growth of different crop plants, including tomato (*Solanum lycopersicum* L.), barley (*Hordeum vulgare* L.) and pea (*Pisum sativum* L.), contributing for the disruption of the redox homeostasis and imposing a severe oxidative stress condition (e.g. Gomes et al., 2017; Soares et al., 2019b; Spormann et al., 2019). In this way, bearing in mind that GLY use is still approved in the European Union (EU), it is of special importance to develop new ways to enhance the tolerance of non-target plants to this herbicide.

Silicon (Si) is the second most abundant element on Earth crust, being considered as a beneficial element for plant growth (Epstein, 2009). Although there is no consensus about its role as an essential nutrient, the involvement of Si in several metabolic pathways and physiological events is well described in the literature (Epstein, 1999; Kim et al., 2017; Liang et al., 2007), especially in what concerns its ability to improve plant stress tolerance (Guntzer et al., 2012). Si, applied either by soil amendment, foliar spray or seed priming, is highly recognised for its potential to reduce the negative effects of different stressful conditions on plants, acting at different levels of plant physiology, reducing the overproduction of reactive oxygen species (ROS) and boosting the plant antioxidant (AOX) system (Kim et al., 2017). Nowadays, not only bulk forms of Si are considered as promising tools to increase plant resilience, but also their nano-sized counterparts, namely silicon dioxide nanomaterials (nano-SiO₂), which are being pointed as a more efficient way to provide Si (Luyckx et al., 2017; Rastogi et al., 2019). However, to the best of our knowledge, the effects of both Si and nano-SiO₂ on the alleviation of GLY-induced stress are yet to be uncovered. Therefore, this work aims at exploring the beneficial effects of the application of Si, in its bulk and nano forms, on GLY-induced oxidative stress in tomato plants (*S. lycopersicum* cv. Micro-Tom). For this purpose, a set of biometrical, ecophysiological and biochemical approaches were implemented to unravel the potential of Si and nano-SiO₂ to mitigate the stress induced by GLY, in the prevention of oxidative damage and in the efficiency of the AOX system.

2. MATERIALS AND METHODS

2.1. Chemicals and artificial substrate

Sodium metasilicate pentahydrate (Na₂SiO₃·5H₂O) and silicon dioxide nanomaterial (nano-SiO₂) (hydrophilic with a particle size of 7–14 nm, a specific surface area of 200 m² g⁻¹ and a 99.8% purity) were purchased from Merck® and Nanostructured & Amorphous Materials Inc. (Houston, TX, USA), respectively, as powders. The characterization of nano-

SiO₂, in terms of size and shape, was previously performed by our group (Soares et al., 2018a). GLY was acquired in the form of RoundUp® Ultramax (Bayer, Portugal), which is a commercial formulation containing 360 g L⁻¹ GLY as potassium salt. The plant growth substrate was an artificial soil (pH 6.0 ± 0.5), containing 5% (m/m) organic matter, provided as peat, prepared according to the guidelines of an OECD protocol (OECD, 2006).

2.2. Plant material and growth conditions

Seeds of *Solanum lycopersicum* L. cv. Micro-tom, obtained from FCUP's seed collection (Porto, Portugal), were used as the biological material in the present work. Before sowing, seeds were surface disinfected with 70% (v/v) ethanol and 20% (v/v) sodium hypochlorite (5% active chloride), supplemented with 0.05% (m/v) Tween® 20, for 5 min each, and subsequently washed several times with deionised water (dH₂O). Then, seeds were placed in Petri dishes containing half-strength MS medium (Murashige and Skoog, 1962) solidified with 0.625% (m/v) agar, and left for germination in a growth chamber, under controlled conditions of temperature (25 °C), photoperiod (16 h light/8 h dark) and photosynthetic active radiation (120 μmol m⁻² s⁻¹). After 8 d, plantlets were transferred to plastic pots (200 g OECD substrate contaminated, or not, by 10 mg kg⁻¹ GLY). To ensure nutrient availability, 120 mL of modified Hoagland solution (Taiz et al., 2015) were added to a cup placed under each pot at the beginning of the assay. The communication between the cup and the pot was achieved by a cotton rope. Afterwards, dH₂O was added when required, and plants were grown for 28 d in a growth chamber, as described above.

2.3. Experimental design

In order to investigate the possible ameliorating role of Si nutrition on GLY-induced toxicity in *S. lycopersicum*, plants were divided into different experimental groups (Figure 1):

- **CTL** – control plants grown in OECD substrate (negative control);
- **Si** – plants grown in OECD substrate and treated once a week with 1 mM Si by foliar spraying;
- **Nano-SiO₂** – plants grown in OECD substrate and treated once a week with 1 mM nano-SiO₂ by foliar spraying;
- **GLY** – plants grown in OECD substrate contaminated by 10 mg kg⁻¹ GLY (positive control);
- **GLY + Si** – plants grown in OECD substrate contaminated by 10 mg kg⁻¹ GLY and treated once a week with 1 mM Si by foliar spraying;

- **GLY + nano-SiO₂** – plants grown in OECD substrate contaminated by 10 mg kg⁻¹ GLY and treated once a week with 1 mM nano-SiO₂ by foliar spraying.

For each condition, eight experimental replicates were considered, each one with five plants. The selection of Si, provided as Na₂SiO₃·5H₂O, and nano-SiO₂ concentrations was based on previous bibliographic records (Farhangi-Abriz and Torabian, 2018; Hasanuzzaman et al., 2018; Khaliq et al., 2016; Wang et al., 2015) and set as 1 mM of Si. Plants from the CTL and GLY experimental groups were weekly sprayed with dH₂O only. After 28 d of growth, individuals from four replicates, randomly selected, were collected, separated into shoots and roots, and immediately used for biometric analysis and O₂^{•-} content; in parallel, shoots and roots of plants from the other four replicates were frozen in liquid nitrogen (N₂) and stored at -80 °C for posterior use. For all studied parameters, including all biochemical procedures, samples from at least three experimental replicates were used.

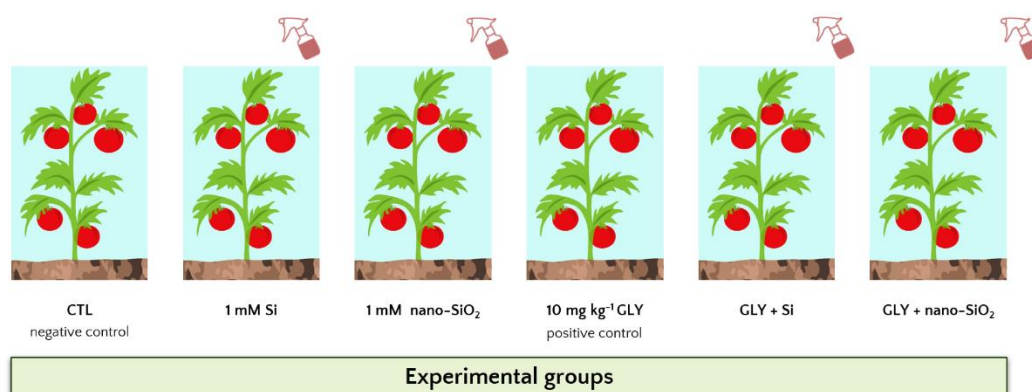


Figure 1. Graphical representation of the experimental design, detailing the main treatments.

2.4. Biometric determinations

At the end of the assay, *i.e.* after 28 d of growth, plants were used for the measurement of root and shoot length and biomass production. Upon separation of roots and shoots, the organ elongation was measured and, then, using a precision balance (KERN®; EWJ 300-3), the fresh biomass of both organs was registered.

2.5. Assessment of lipid peroxidation (LP)

LP was evaluated in terms of malondialdehyde (MDA) content, using frozen samples of roots and shoots, according to Heath and Packer (1968). After homogenization and centrifugation, extracts were mixed with 0.5% (m/v) thiobarbituric acid (TBA) in 20% (m/v) trichloroacetic acid (TCA). Following 30 min at 95 °C, the absorbance of each sample was read at 532 and 600 nm. To avoid unspecific turbidity, the obtained values at 600 nm were

subtracted to those at 532 nm, and the MDA content was calculated using a ϵ of $155 \text{ mM}^{-1} \text{ cm}^{-1}$ and expressed as nmol g^{-1} fresh mass (fm).

2.6. Determination of ROS levels – superoxide anion ($\text{O}_2^{\cdot-}$) and hydrogen peroxide (H_2O_2)

Cellular levels of $\text{O}_2^{\cdot-}$ were determined in samples of fresh roots and shoots, by incubating pieces of plant material (ca. 1 cm^2 ; 200 mg), for 1 h, in 3 mL of a reaction mixture [10 mM sodium phosphate buffer (pH 7.8), 10 mM sodium azide (NaN_3) and 0.05% (m/v) nitroblue tetrazolium (NBT)] (Gajewska and Skłodowska, 2007). At the end, the absorbance (Abs) was registered at 580 nm. The levels of $\text{O}_2^{\cdot-}$ were expressed as $\text{Abs}_{580 \text{ nm}} \text{ h}^{-1} \text{ g}^{-1} \text{ fm}$. Regarding H_2O_2 , its content was evaluated following the protocol of Alexieva et al. (2001), in which the extract reacts with potassium iodide (KI) to form a yellowish complex that can be measured at 390 nm. Levels of H_2O_2 were determined by a linear calibration curve, and expressed in $\text{nmol g}^{-1} \text{ fm}$.

2.7. Quantification of non-enzymatic AOX – proline (Pro), glutathione (GSH) and ascorbate (AsA)

Pro was quantified in frozen plant samples by the ninhydrin-based colorimetric assay (Bates et al., 1973), by measuring the absorbance at 520 nm. Its levels were determined after obtaining a linear calibration curve with solutions of known concentration, and results were expressed in $\text{mg g}^{-1} \text{ fm}$. The quantification of GSH was accomplished by following the procedure described in Soares et al. (2019b), in which GSH reduces 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) to 2-nitrobenzoic acid (TNB), a reaction that can be measured at 412 nm. GSH levels were estimated from a linear calibration curve prepared with known concentrations of this AOX. Results were expressed in a fm basis. Ascorbate content, as well as its reduced (AsA) and oxidised (dehydroascorbate – DHA) forms, were quantified by spectrophotometry at 525 nm, based on the 2,2'-bipyridyl method (Gillespie and Ainsworth, 2007). Levels were estimated using a linear calibration curve obtained with AsA standards, and results expressed in $\mu\text{mol g}^{-1} \text{ fm}$.

2.8. Extraction and quantification of AOX enzymes – superoxide dismutase (SOD; EC 1.15.1.1), catalase (CAT; EC 1.11.1.6), ascorbate peroxidase (APX; EC 1.11.1.11), glutathione reductase (GR; EC 1.8.1.7), and dehydroascorbate reductase (DHAR; EC 1.8.5.1)

The extraction of the main AOX enzymes was performed as previously described (Soares et al., 2018a). After centrifugation, the supernatant (SN) was collected and used for both, protein content quantification (Bradford, 1976) and enzyme's activity assays. In the case of SOD, an aliquot of the SN was complexed with 10 μM sodium azide (NaN_3). Total SOD activity was determined based on the inhibition of the photoreduction of NBT, by spectrophotometry at 560 nm (Donahue et al., 1997). For each sample, an appropriate volume of extract (30 μg of protein) was added to a reaction mixture containing 100 mM potassium phosphate buffer (pH 7.8), 0.093 mM ethylenediaminetetraacetic acid (EDTA), 12.05 mM L-methionine, 0.0695 mM NBT and 0.0067 mM riboflavin in a final volume of 3 mL. The enzymatic reaction was started by adding the riboflavin to the tubes, which were immediately placed under 6 fluorescent 8 W lamps for 10 min. After this period, the light source was removed in order to stop the reaction. Enzyme activity was expressed as units SOD mg^{-1} protein, in which one unit represents the amount of SOD required to inhibit NBT photoreduction by 50%. The evaluation of CAT and APX activity was accomplished by enzyme kinetics, by measuring the decomposition of H_2O_2 ($\epsilon_{240\text{ nm}} = 39.4\text{ M}^{-1}\text{ cm}^{-1}$) (Aebi, 1984) and of AsA ($\epsilon_{290\text{ nm}} = 2.8\text{ mM}^{-1}\text{ cm}^{-1}$) (Nakano and Asada, 1981) during 2 minutes. In both cases, the reaction was started by the addition of H_2O_2 . Regarding DHAR and GR, changes in Abs at 265 and 340 nm were monitored to follow AsA ($\epsilon_{265\text{ nm}} = 14\text{ mM}^{-1}\text{ cm}^{-1}$) production and NADPH consumption ($\epsilon_{340\text{ nm}} = 6.22\text{ mM}^{-1}\text{ cm}^{-1}$), respectively. Results were expressed as $\mu\text{mol min}^{-1}\text{ mg}^{-1}$ protein. The original protocol was adapted to UV microplates, based on the optimization of Murshed et al. (2008).

2.9. Quantification of GLY and aminomethylphosphonic acid (AMPA) accumulation in plant tissues

The extraction of GLY and AMPA from tomato tissues was carried out according to the AOAC Official method 2000.05. Briefly, 100 mg of freeze-dried homogenised tissues (shoots or roots) were extracted with 5 mL of ultrapure water by shaking for 10 min on an end-over-end shaker. Afterward, samples were centrifugated at 10 000 rpm for 10 mins (4 °C) and the supernatant recovered. Samples derivatization and analysis was performed according to Pinto et al. (2018), with some modifications: 1 mL of the SN was diluted with 1 mL of internal standard (200 $\mu\text{g L}^{-1}$ of GLY 1,2- $^{13}\text{C}_2$ ^{15}N and 200 $\mu\text{g L}^{-1}$ of ^{13}C , ^{15}N -AMPA),

to which 120 µL of 1% (m/v) ammonium hydroxide (NH₄OH) solution and 120 µL of 9-fluorenylmethoxycarbonyl chloride (FMOC-Cl; 12 000 mg L⁻¹ in acetone) were added. The tubes were shaken for a few seconds and incubated for 30 min at room temperature. The reaction was stopped by adding 10 µL of 6 M hydrochloric acid (HCl). The derivatized extracts were filtered through a 0.45 µm polytetrafluoroethylene (PTFE) filters into liquid chromatography (LC) vials. GLY and AMPA were determined by liquid chromatography with *tandem* mass spectrometry (LC-MS/MS) using the internal standard method.

The LC-MS/MS system comprised a Waters 2695 XE separation module (Milford, MA) interfaced to a triple quadrupole mass spectrometer (Quattro micro™ API triple quadrupole, Waters Micromass, Manchester, UK). The LC separation was performed using a Kinetex® EVO C18 core-shell column (2.6 µm; 100 x 2.1 mm) at a flow rate of 225 µL min⁻¹. A binary gradient was used, which consisted of solvent A (10 mM ammonium bicarbonate) and solvent B (methanol). The percentage of organic modifier (B) was changed linearly as follows: 0 – 0.5 min, 5%; 0.5 – 5.5 min, 90%; 5.5 – 6.5 min, 90%; 6.5 – 6.7 min, 5%; 6.7 – 14 min, 5%. The injection volume was 20 µL and the column temperature was kept at 40 °C. The mass spectrometry parameters were as follows: ion mode, positive; capillary voltage, 3.00 kV; source temperature, 130 °C; desolvation temperature, 450 °C; desolvation gas flow, 600 L h⁻¹; and multiplier, 650 V. High purity nitrogen (>99.999%) and argon (>99.999%) were used as the cone and collision gases, respectively. The precursor and product ions as well as the cone voltage and collision energy for each GLY-FMOC, AMPA-FMOC and ILIS-FMOC were determined by flow injection analysis and the MRM transitions, cone voltages and collision energies are listed in Table 1. Data acquisition was performed by the MassLynx V4.1 software. Results were expressed on a dry mass (dm) basis.

Table 1. MRM transitions, cone voltages and collision energies for each used compound.

Compound	Precursor ion (m/z)	Product ion (m/z)	Cone voltage (V)	Collision energy (V)
GLY-FMOC	392.2	Q:88.0	20	20
		q:170.0	20	10
1,2- ¹³ C ₂ , ¹⁵ N GLY-FMOC	395.2	91.0	20	20
AMPA-FMOC	334.0	Q:112.1	20	15
		q:179.1	20	20
¹³ C, ¹⁵ N-AMPA	336.0	114.1	20	15

Q: quantification transition; q: confirmation transition

2.10. Statistical analyses

All biometric and biochemical determinations were performed, at least, in three independent replicates ($n \geq 3$), and results are expressed as mean $\pm$ standard deviation (SD). Differences between experimental groups were tested by one-way ANOVA, assuming a significance level (α) of 0.05. In case of significant differences, Tukey's *post-hoc* tests were performed to discriminate differences between groups. Prior to the ANOVAs, data were checked for normality and homogeneity through Shapiro-Wilk and Brown–Forsythe tests, respectively. All statistical procedures were performed in GrahPad®Prism 8 (San Diego, CA).

3. RESULTS

3.1. Biometric and growth-related parameters

As can be seen in Figure 2, the exposure of *S. lycopersicum* to 10 mg kg⁻¹ GLY caused a marked reduction in plant development, significantly impairing the growth of both roots and shoots. This finding was effectively demonstrated when root length [F (5, 13) = 70.90; $p < 0.05$] and fresh biomass of both organs [shoots: F (5, 15) = 5.93; $p < 0.05$; roots: F (5, 15) = 46.76; $p < 0.05$] were evaluated. Inhibitions around 50 and 70% were recorded for shoot and root growth, respectively, in comparison with the CTL (Figure 3). When GLY was not added to the substrate, the foliar spray with both sources of Si, especially nano-SiO₂, positively affected plant growth, significantly increasing the root length (by 114%) and fresh biomass (by 27%) in comparison with the CTL (Figures 2 and 3). Moreover, GLY phytotoxicity was significantly reduced by the foliar application of both Si or nano-SiO₂ (Figures 2 and 3). Indeed, plants from GLY + Si and GLY + nano-SiO₂ groups showed a better ability to grow, reaching values closer to the CTL for root length and shoot fresh weight. In addition, the marked reduction of root biomass in response to GLY (73% lower) was significantly counteracted by Si or nano-SiO₂ treatments, with reduced inhibition values over the CTL (GLY + Si – 48% of reduction; GLY + nano-SiO₂ – 40% of reduction).

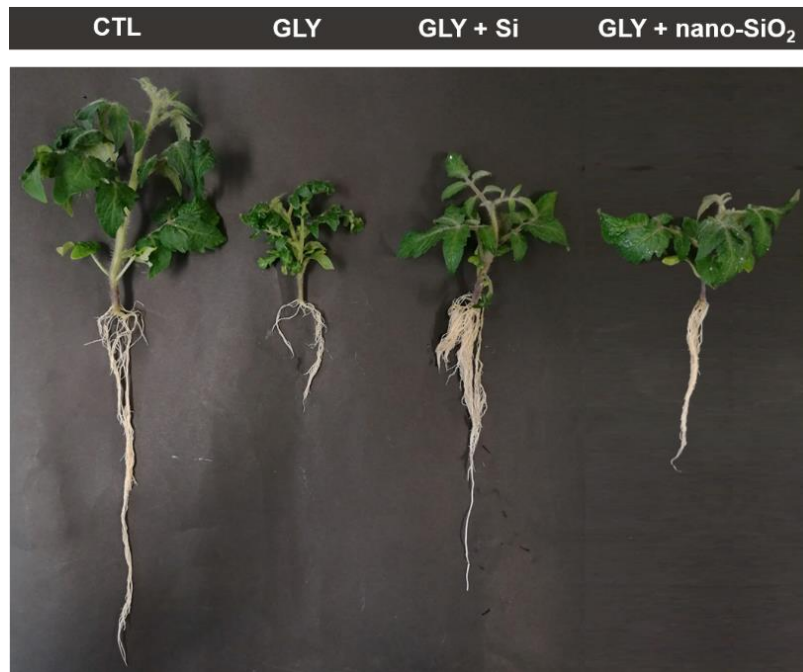


Figure 2. *S. lycopersicum* plants after four weeks of growth (CTL – control plants; GLY – plants exposed to GLY alone; GLY + Si – plants exposed to GLY and treated with Si; GLY + nano-SiO₂ – plants exposed to GLY and treated with nano-SiO₂).

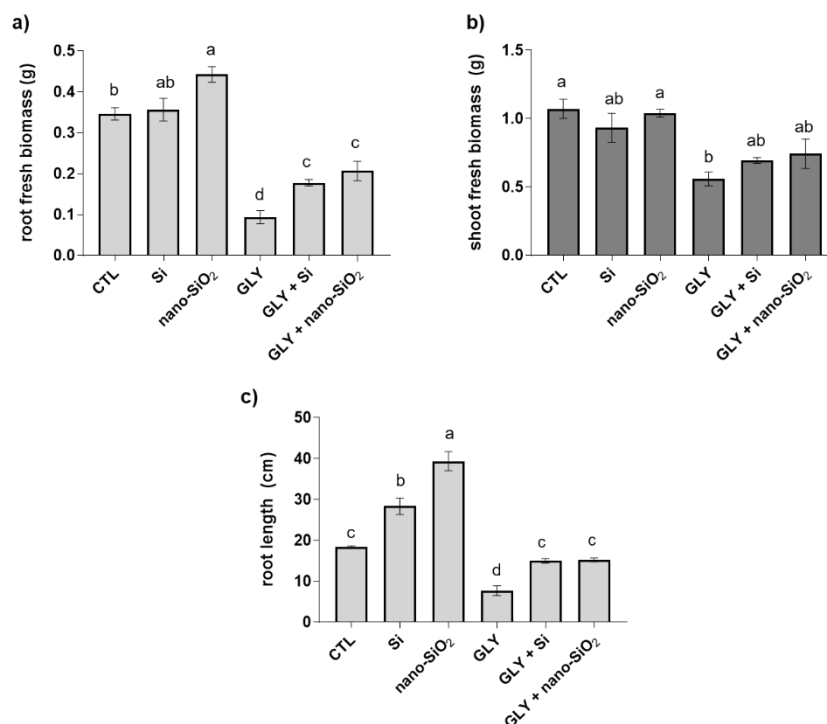


Figure 3. Biometric parameters of *S. lycopersicum* plants after four weeks of growth [CTL – control plants; GLY – plants exposed to GLY alone; GLY + Si – plants exposed to GLY and treated with Si; GLY + nano-SiO₂ – plants exposed to GLY and treated with nano-SiO₂]. (a) root fresh biomass; (b) shoot fresh biomass; (c) root length. Data presented are mean $\pm$ SD ($n \geq 3$). Different letters above bars indicate significant statistical differences between treatments (Tukey: $p \leq 0.05$).

3.2. Lipid peroxidation – MDA content

The MDA content, indicative of LP, showed a distinct behaviour between shoots and roots of tomato plants (Figures 4a,d). Significant differences between treatments were found for both organs [shoots: $F(5, 12) = 8.04$; $p < 0.05$; roots: $F(5, 13) = 6.74$; $p < 0.05$]. In shoots, GLY induced a pronounced increment of MDA levels, with a rise of 32% in relation to the CTL, this effect being significantly counteracted by the application of Si or nano-SiO₂ to levels similar to the CTL. As evidenced in Figure 4d, in general, roots of GLY-exposed plants exhibited a lower LP degree (up to 41%). Despite this pattern not being changed in Si co-treated plants, the co-exposure of GLY and nano-SiO₂ resulted in MDA levels identical to the CTL in roots.

3.3. ROS homeostasis – O₂^{•-} and H₂O₂ content

The production of O₂^{•-} was significantly changed in shoots [$F(5, 23) = 4.25$; $p < 0.05$] and roots [$F(5, 20) = 12.88$; $p < 0.05$] (Figures 4b,e). The levels of O₂^{•-} revealed to be higher in plants exposed to GLY alone, with values exceeding by 75 and 80% those found in shoots and roots of CTL plants, respectively. However, when plants were treated with Si or nano-SiO₂, the levels of this ROS were kept identical to the CTL, especially in roots, where each treatment resulted in a decrease of around 60% in relation to GLY-exposed plants (Figures 4b and e).

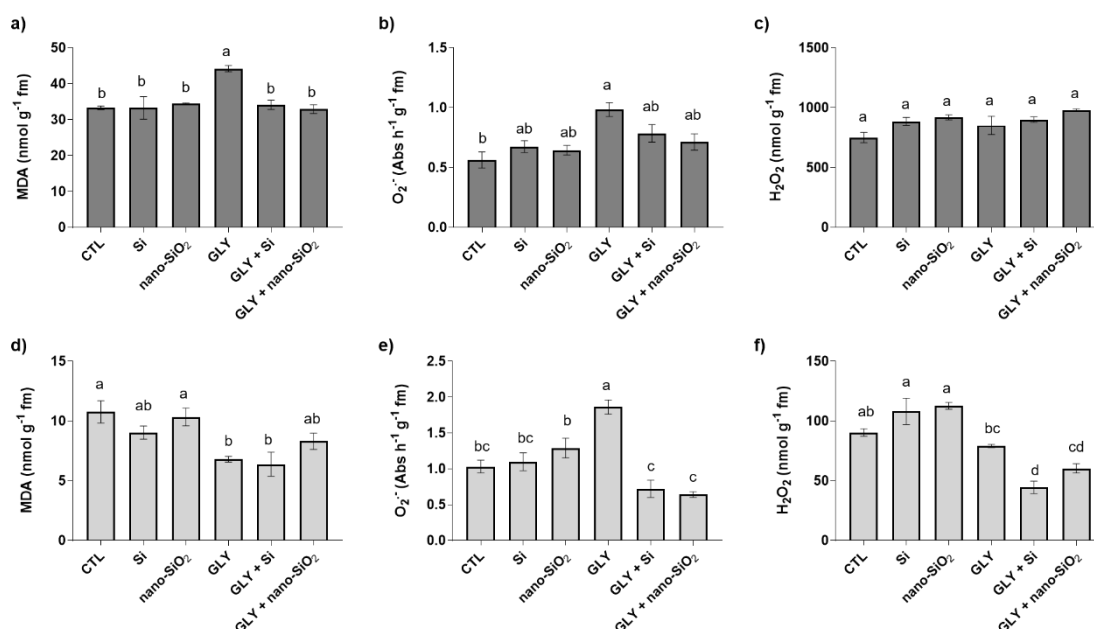


Figure 4. Oxidative stress markers of *S. lycopersicum* plants after 4 weeks of growth. (a,d) malondialdehyde (MDA); (b,e) superoxide anion (O₂^{•-}); (c,f) hydrogen peroxide (H₂O₂). Dark and light bars represent shoots and roots, respectively. Data presented are mean ± SD ($n \geq 3$). Different letters above bars indicate significant statistical differences between treatments (Tukey: $p \leq 0.05$).

Concerning H_2O_2 , no significant changes were found in shoots among all experimental groups [$F(5, 13) = 2.45$; $p > 0.05$; (Figure 4c)]. In roots, significant effects between treatments were found [$F(5, 13) = 22.69$; $p < 0.05$]. However, no statistical differences were detected between GLY and CTL plants (Figure 4f). Under the co-exposure scenario, both Si treatments contributed for decreasing the accumulation of H_2O_2 , especially bulk Si, where a reduction of around 60% in H_2O_2 content was found in relation to the CTL and plants only exposed to GLY. This was observed despite both Si materials *per se* having significantly increased the levels of this ROS (44% and 54%, bulk and nano, respectively), when applied alone.

3.4. Non-enzymatic AOX – Pro, GSH and AsA

Pro levels, illustrated in Figures 5a,d, were changed in response to GLY [shoots: $F(5, 14) = 15.44$; $p < 0.05$; roots: $F(5, 18) = 12.14$; $p < 0.05$], with values around 5.3- and 2.0-fold of those of the CTL, in both shoots and roots, respectively. Upon co-treatment with Si or nano-SiO₂, Pro content was reduced in relation to the plants exposed to GLY alone, showing values identical to the CTL.

Regarding GSH [shoots: $F(5, 20) = 7.52$; $p < 0.05$; roots: $F(5, 15) = 10.80$; $p < 0.05$], its content was significantly higher in shoots of plants from all treatments when compared to the CTL. In roots, GLY alone provoked a significant increment (100%) of GSH (Figures 5b,e). However, plants from the co-exposure with Si or nano-SiO₂ displayed root levels of GSH identical to those found in the CTL plants, especially in GLY + Si treated plants (Figure 5e).

Total AsA levels [shoots: $F(5, 19) = 6.71$; $p < 0.05$; roots: $F(5, 21) = 5.85$; $p < 0.05$] are presented in Figures 5c,f. As can be observed, in shoots, the total levels of this AOX were increased by 34% in GLY- and GLY + nano-SiO₂-treated plants, in relation to the CTL. Regarding roots, differences in total AsA were only found in plants treated with nano-SiO₂ alone, with an increment of 39% when compared to the CTL.

Concerning the ratio between AsA/DHA [shoots: $F(5, 16) = 10.76$; $p < 0.05$; roots: $F(5, 20) = 22.70$; $p < 0.05$], results are compiled in Table 2. Reductions up to 62% over the CTL were detected in the shoots, in all experimental groups. In roots, GLY increased this parameter by 32%, with this effect being counteracted by the application of Si or nano-SiO₂, in which AsA/DHA levels were identical to the CTL. Moreover, both forms of Si, when applied alone, led to a reduction of about 50% in AsA/DHA (Table 2).

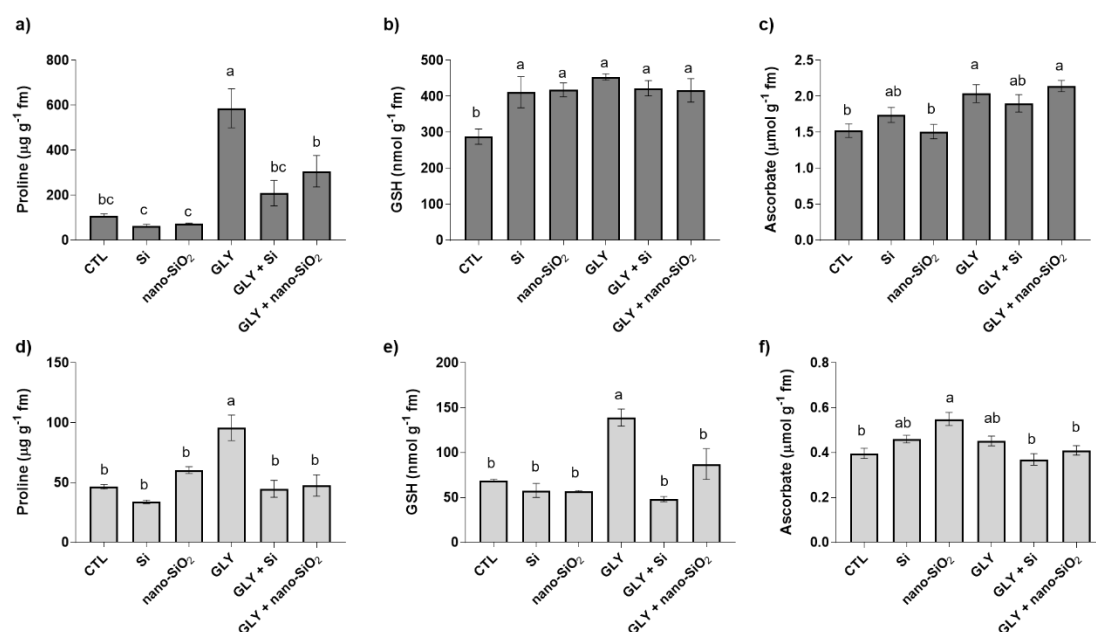


Figure 5. Levels of the main AOX metabolites of *S. lycopersicum* plants after 4 weeks of growth. (a,d) proline (Pro); (b,e) glutathione (GSH); (c,f) total ascorbate. Dark and light bars represent shoots and roots, respectively. Data presented are mean $\pm$ SD ($n \geq 3$). Different letters above bars indicate significant statistical differences between treatments (Tukey: $p \leq 0.05$).

Table 2. Levels of total ASA ($\mu\text{mol g}^{-1} \text{fm}$), along with its reduced and oxidised forms (dehydroascorbate – DHA), of *S. lycopersicum* plants after 4 weeks of growth. Data presented are mean $\pm$ SD ($n \geq 3$). Different letters indicate significant statistical differences between treatments (Tukey: $p \leq 0.05$).

Organ	Treatment	Total AsA	AsA/Total AsA	DHA/Total AsA	AsA/DHA
Shoots	CTL	1.52 $\pm$ 0.09b	0.72 $\pm$ 0.05a	0.28 $\pm$ 0.05a	3.44 $\pm$ 0.66a
	Si	1.74 $\pm$ 0.10ab	0.59 $\pm$ 0.06a	0.41 $\pm$ 0.06a	1.55 $\pm$ 0.40b
	Nano-SiO ₂	1.51 $\pm$ 0.10b	0.56 $\pm$ 0.05a	0.44 $\pm$ 0.05a	1.31 $\pm$ 0.27b
	GLY	2.04 $\pm$ 0.12a	0.56 $\pm$ 0.03a	0.44 $\pm$ 0.03a	1.47 $\pm$ 0.22b
	GLY + Si	1.90 $\pm$ 0.12ab	0.60 $\pm$ 0.05a	0.40 $\pm$ 0.05a	1.48 $\pm$ 0.30b
	GLY + nano-SiO ₂	2.14 $\pm$ 0.08a	0.63 $\pm$ 0.02a	0.37 $\pm$ 0.02a	1.65 $\pm$ 0.19b
Roots	CTL	0.40 $\pm$ 0.02b	0.41 $\pm$ 0.03bc	0.59 $\pm$ 0.03ab	0.77 $\pm$ 0.05b
	Si	0.46 $\pm$ 0.02ab	0.33 $\pm$ 0.04c	0.67 $\pm$ 0.04a	0.42 $\pm$ 0.06c
	Nano-SiO ₂	0.55 $\pm$ 0.03a	0.33 $\pm$ 0.03bc	0.67 $\pm$ 0.03a	0.38 $\pm$ 0.04c
	GLY	0.45 $\pm$ 0.02ab	0.50 $\pm$ 0.02a	0.50 $\pm$ 0.02bc	1.02 $\pm$ 0.04a
	GLY + Si	0.37 $\pm$ 0.03b	0.45 $\pm$ 0.01ab	0.49 $\pm$ 0.02c	0.79 $\pm$ 0.03b
	GLY + nano-SiO ₂	0.41 $\pm$ 0.02b	0.44 $\pm$ 0.01ab	0.56 $\pm$ 0.01abc	0.80 $\pm$ 0.05b

3.5. Enzymatic AOX – activity of SOD, CAT, APX, GR, and DHAR

As can be observed in Figure 6a, SOD activity was significantly changed in the shoots [F (5, 14) = 3.99; $p < 0.05$], but no differences were recorded between GLY and the CTL. Yet, plants co-treated with Si, especially nano-SiO₂, showed an increased activity of this enzyme, in comparison with plants only exposed to the herbicide. In roots [F (5, 13) = 49.60; $p < 0.05$], GLY significantly inhibited SOD activity by 40% over the CTL (Figure 6d). Once again, plants simultaneously exposed to Si or nano-SiO₂ and GLY displayed a significantly improved SOD activity, with values even higher (up to 63%) than those found in the CTL roots (Figure 6d). Moreover, the application of nano-SiO₂ alone reduced the activity of this enzyme in both organs.

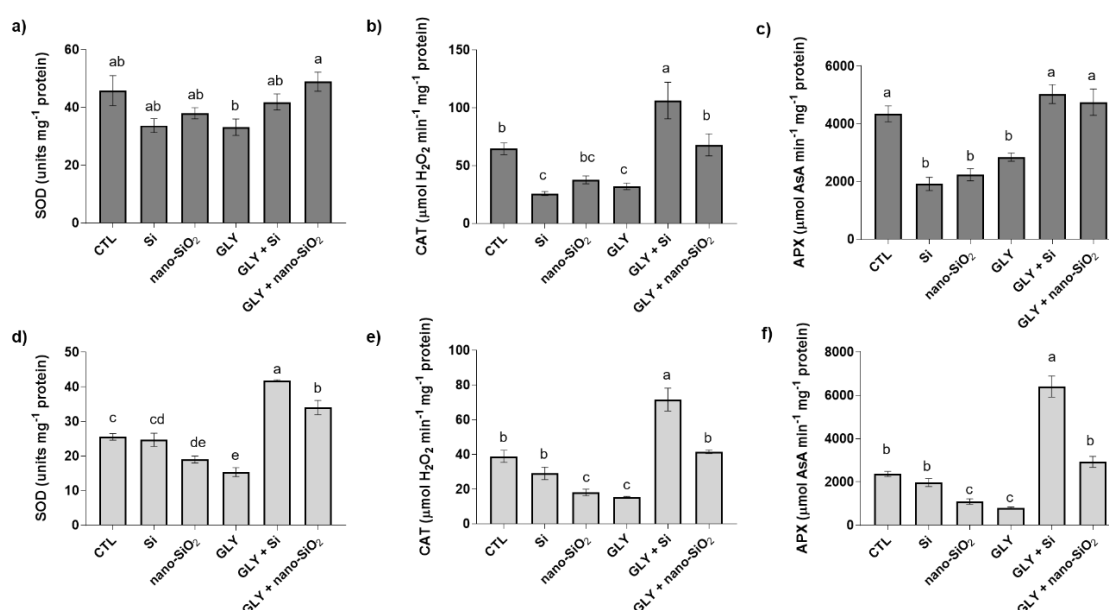


Figure 6. Total activity of superoxide dismutase (SOD; a, d), catalase (CAT; b, e) and ascorbate peroxidase (APX; c, f) of *S. lycopersicum* plants after 4 weeks of growth. Dark and light bars represent shoots and roots, respectively. Data presented are mean $\pm$ SD ($n \geq 3$). Different letters above bars indicate significant statistical differences between treatments (Tukey: $p \leq 0.05$).

Concerning CAT and APX, their activity values are shown in Figures 6b,c, and e,f. As illustrated, both enzymes showed the same pattern in shoots [CAT: F (5, 18) = 11.76; $p < 0.05$; APX: F (5, 12) = 21.47; $p < 0.05$] and roots [CAT: F (5, 12) = 34.75; $p < 0.05$; APX: F (5, 12) = 67.64; $p < 0.05$] of tomato plants. In general, CAT and APX activity were negatively affected by GLY alone, but plants under the co-treatment with Si or nano-SiO₂, especially bulk Si, displayed enzyme activity levels identical or even higher than those of the CTL plants. For instance, while CAT and APX activity in roots suffered a decrease of around 0.6-fold induced by GLY, the foliar application of Si enhanced the total activity of both enzymes, with increments up to 3.6- (CAT) and 7-fold (APX) in relation to the plants only exposed to the herbicide (Figures 6e,f). As in the case of SOD, the foliar spraying of

with Si or nano-SiO₂ seemed to have diminished the activity of these two AOX enzymes in the absence of the herbicide (Figures 6b,c, and e,f).

The quantification of GR and DHAR activity showed distinct patterns in shoots [GR: $F(5, 12) = 55.33$; $p < 0.05$; DHAR: $F(5, 12) = 26.49$; $p < 0.05$] and roots [GR: $F(5, 13) = 2.94$; $p > 0.05$; DHAR: $F(5, 13) = 4.41$; $p < 0.05$] (Figure 7). Regarding GR, GLY did not affect its activity in the shoots; nevertheless, the foliar spraying of plants to Si or nano-SiO₂, independently of GLY co-exposure, significantly improved the activity of this AOX enzyme, with values even higher than those found in the CTL (Figure 7a). In roots, no changes were recorded (Figure 7c).

Lastly, in what concerns DHAR, GLY induced a decrease of its activity (ca. 30%) over the CTL in shoots; however, the application of Si or nano-SiO₂ alone or in combination with GLY exposure contributed for an enhanced activity of this enzyme, with values exceeding those of GLY-treated plants by 37% (Figure 7b). In general, no major changes were found in roots (Figure 7d).

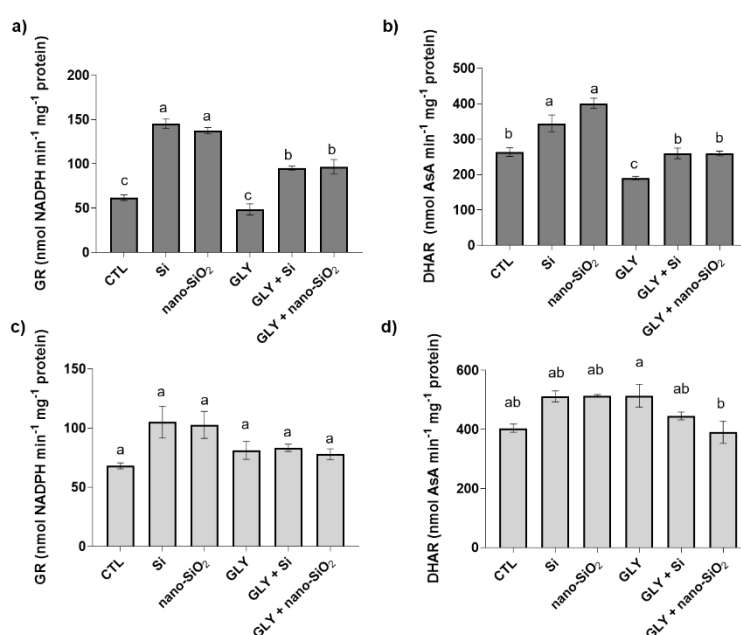


Figure 7. Total activity of glutathione reductase (GR; a, c) and dehydroascorbate reductase (DHAR; b, d) of *S. lycopersicum* plants after 4 weeks of growth. Dark and light bars represent shoots and roots, respectively. Data presented are mean $\pm$ SD ($n \geq 3$). Different letters above bars indicate significant statistical differences between treatments (Tukey: $p \leq 0.05$).

3.6. Bioaccumulation of GLY in shoots and roots

As can be observed, GLY was not detected in shoots of any experimental group (Figure 8). In opposition, roots of plants exposed to the herbicide alone displayed GLY levels up to 14.2 $\mu\text{g g}^{-1}$ d.w. However, in response to the foliar application of Si or nano-SiO₂, GLY

bioaccumulation was reduced, with a significant decrease of 17 and 13%, respectively. AMPA was not detected in any sample, being below the detection limit.

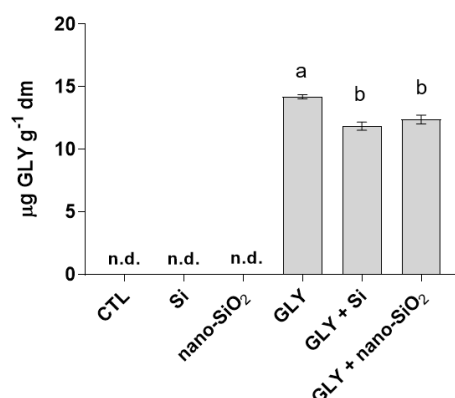


Figure 8. GLY levels in roots of *S. lycopersicum* plants after 4 weeks of growth. n.d.: non-detected, which means below the detection limit. Data presented are mean $\pm$ SD ($n \geq 3$). Different letters above bars indicate significant statistical differences between treatments (Tukey: $p \leq 0.05$).

4. DISCUSSION

Recently, our research group, alongside with other worth mentioning studies (Gomes et al., 2017, 2016; Singh et al., 2017b; Zhong et al., 2018), has provided important findings concerning GLY non-target phytotoxicity in important plant species, such as barley (*Hordeum vulgare* L.) (Soares et al., 2018c), alfafa (*Medicago sativa* L.) (Fernandes et al., 2020) and tomato (*Solanum lycopersicum* L.) (Soares et al., 2020, 2019b). However, more than understanding GLY-associated environmental risks, it is also essential to develop new approaches to increase plant tolerance to this herbicide (Spormann et al., 2019). Therefore, the main goal of the present study was to explore the potential of Si, either in its bulk or nano formulations, to overcome GLY-induced oxidative stress in tomato plants.

GLY-mediated inhibition of plant growth is efficiently counteracted by the foliar application of Si or nano-SiO₂

The occurrence of phytotoxic symptoms, along with the inhibition of plant growth performance, is among the most common effects of soil contamination on plants (Gratão et al., 2005; Zhang et al., 2017). As expected, the exposure of tomato plants to 10 mg kg⁻¹ GLY [levels already found in agricultural fields (Fernandes et al., 2020 and references therein)] resulted in a marked decrease of growth traits, with significant reductions at both root and shoot levels. These observations go in agreement with our previous report, in

which the development of *S. lycopersicum* cv. Micro-Tom was severely hampered by increasing concentrations (10, 20 and 30 mg kg⁻¹) of GLY residues in soil (Soares et al., 2019b). The same pattern has already been observed for other plant species grown in GLY-contaminated media, with negative impacts in both dicots (pea, willow and alfalfa plants) and monocots (barley and rice plants) species (Ahsan et al., 2008; Fernandes et al., 2020; Gomes et al., 2017; Singh et al., 2017a; Spormann et al., 2019). The phytotoxic hazards of GLY in plant growth are probably related to its mode-of-action, where amino acid biosynthesis is hampered, thus compromising cell homeostasis and plant growth, but can also reflect the interference of GLY with mineral nutrition. By acting as a metal chelating agent, GLY may reduce the uptake of different essential nutrients and/or decrease their bioavailability inside plant tissues (reviewed by Gomes et al., 2014). Albeit not consensual, with studies reporting contrasting findings on this matter (see review by Mertens et al., 2018), GLY was found to reduce the leaf levels of calcium (Ca), manganese (Mn), iron (Fe) and magnesium (Mg) in a non-resistant soybean genotype (Cakmak et al., 2009). This suggests that it can affect not only the root uptake of these elements, but also root-to-shoot transport. Moreover, GLY is known to form complexes with divalent cations (e.g. Ca²⁺, Mg²⁺, Fe²⁺), resulting in nutrient immobilization inside plant tissues (Bellaloui et al., 2009; Cakmak et al., 2009; Su et al., 2009; Zobiole et al., 2011a; 2011b). Although EPSPS is a chloroplastidial enzyme (della-Cioppa et al., 1987; Tzin and Galili, 2010), the phytotoxic action of GLY was preferentially observed in roots, where a much sharper decrease of biomass production was observed. Accordingly, bioaccumulation data showed that roots were the preferential organ for GLY storage, with highly limited translocation for the aerial parts. Even when foliarly-applied, GLY has been found to spread to different plant organs, particularly accumulating in tissues with a high metabolic activity, such as root and shoot apices (Gomes et al., 2014). Thus, it can be suggested that GLY-mediated stress in tomato plants is not strictly related to its specific herbicidal activity, which was designed to target the EPSPS enzyme, but also emerges as the result of GLY secondary effects on plant physiology. Moreover, although GLY was not detected in the aerial organs of tomato plants, and thus seems to not represent a threat to food safety, its risks in terms of food security should not be neglected, since plant growth was majorly impaired.

The potential of Si to enhance plant abiotic stress tolerance is widely recognised, being its action efficient against several types of environmental stresses, including abiotic (drought, salinity, metals) and biotic (pathogens, virus, herbivore attack) factors (Imtiaz et al., 2016; Luyckx et al., 2017; Meharg et al., 2015). However, up to now, this is the first study reporting the effective potential of Si to increase GLY tolerance in crops. Upon Si foliar spray, GLY-induced phytotoxic effects were partially or almost completely inhibited

(Figures 1 and 2). Actually, not only did plants show a better growth potential at the macroscopic level, but also the results concerning biomass production and organ elongation further confirmed this trend. Curiously, the only available record exploring the ameliorative features of Si nutrition against herbicide toxicity was recently published (Tripathi et al., 2020). In accordance with our data, Tripathi et al. (2020) also observed a positive effect of Si, provided as 10 μM sodium silicate, when 25 d-old rice plants were exposed to butachlor (4 μM). Furthermore, Si-mediated alleviation of GLY phytotoxicity seems to be also related to a reduced herbicide bioaccumulation, since GLY levels decreased in roots of Si and nano-SiO₂ treated plants. The ability of Si to prevent contaminants accumulation by different plant species has been often reported in the literature (Liang et al., 2007; Guntzer et al., 2011), which goes in accordance with our data.

Si-mediated stimulation of plant growth, either under optimal or stressful conditions, can result from its role in maintaining a proper water balance in plants (Malhotra and Kapoor, 2019; Souri et al., 2020). In fact, Si application can modulate the transcript levels of aquaporin-related genes, contributing for a better water absorption (Liu et al., 2014; Sonobe et al., 2011) and, consequently, an improved nutrient uptake and translocation (Chen et al., 2011). As can be observed, the co-treatment of plants with GLY and Si or GLY and nano-SiO₂ contributed to a better plant growth, lowering the observed damage induced by GLY in root length and root and shoot fresh biomass. Despite the lack of studies, this result was a fairly expected outcome given the widely recognised ability of Si, both at bulk and nano forms, to enhance plant stress tolerance, namely to metal(loid)s, whose detoxification pathways are somewhat identical to those of organic xenobiotics (Schröder et al., 2009). Indeed, Si supplementation (2 mM sodium silicate) to the nutrient solution helped to reduce cadmium (Cd) phytotoxicity in tomato plants (Wu et al., 2015); in parallel, when studying the potential of Si to alleviate the effects of nickel oxide nanomaterials (nano-NiO) in *H. vulgare*, Soares et al. (2018a) reported an increased plant growth performance upon soil amendment with nano-SiO₂ (3 mg kg⁻¹). Equivalent observations were also described for other plant species, treated with Si, exposed to different metals, including Cd (Alzahrani et al., 2018; Hussain et al., 2019), chromium (Cr) (Ashfaq et al., 2017; Tripathi et al., 2015) and aluminium (Al) (de Sousa et al., 2019; Pontigo et al., 2017).

Apart from the overall beneficial effects of Si against GLY-induced toxicity in tomato plants, no substantial differences were detected between the two applied forms of Si, though different studies report that nanotechnological-based tools can be more efficient than their bulk counterparts (Liu et al., 2015; Tripathi et al., 2017). Yet, and as herein reported, a previous study also conducted with *S. lycopersicum* plants revealed that Si-mediated salinity tolerance did not differ between bulk (silicate – 1 and 2 mM) and

nanoformulations (nano-SiO₂ – 1 and 2 mM) (Haghighi and Pessarakli, 2013). However, since no solid conclusions can be drawn only based on biometric parameters, analyses then focused on the evaluation of the redox homeostasis of GLY-exposed plants treated, or not, with Si or nano-SiO₂.

The foliar application of Si or nano-SiO₂ reduces GLY-induced oxidative stress, particularly stimulating the enzymes of the AOX defence system

Although GLY primary target is not related to redox disorders, it is widely accepted that, once in plant cells, GLY is able to disturb the redox homeostasis (Caverzan et al., 2019; Gomes et al., 2014). Our results clearly showed that GLY residues in the soil ended up affecting the overall redox state of tomato plants. In general, data concerning ROS quantification and LP degree evaluation seemed to suggest the occurrence of oxidative stress as a response to GLY exposure, which agrees with our previous study (Soares et al., 2019b). Indeed, and although LP and H₂O₂ have not followed the same trend in roots and shoots, O₂^{•−} content revealed to be greatly enhanced upon exposure to GLY. Although this ROS is considered to be a moderate reactive radical, since it has a short half-life, is negatively charged and does not have the ability to cross biological membranes, O₂^{•−} can further give rise to the production of other more oxidising agents, including hydroxyl radical ([•]OH), through the Haber-Weiss reaction, and hydroperoxyl (HO₂[•]), through protonation, the latter being permeable and highly reactive (Demidchik, 2015). Moreover, and considering that EPSPS is located in chloroplasts (della-Cioppa et al., 1987; Tzin et al., 2010), the main source of O₂^{•−} in plant cells, the burst of this ROS in GLY-exposed plants suggests, once again, that tomato plants failed to prevent the occurrence of oxidative stress. Accordingly, in our former study, a completely altered ultrastructure of chloroplasts following GLY treatment was observed (Soares et al., 2020), reinforcing that plastid-mediated changes can be overall indicators of GLY-induced stress in plants.

Although the involvement of Si in alleviating pesticide-induced oxidative damage is somehow unexplored, the widely recognised ability of this element to counteract the toxic effects of ROS on membrane and organelle damage have made us to hypothesize that beneficial effects would be recorded upon the application of Si to GLY-exposed plants. Indeed, supporting the results of the biometric assessment, tomato plants grown under GLY exposure but simultaneously treated with Si or nano-SiO₂ were capable of maintaining the redox homeostasis, with lower levels of O₂^{•−} in both organs and MDA in shoots. One of the ways by which Si is able to increase plant abiotic stress tolerance is through the reduction of the oxidative stress, given its ability to enhance the AOX performance (Kim et al., 2017). Positive effects of Si, along with nano-SiO₂, on the

prevention of ROS overproduction and membrane damage in stressed plants are quite common in the literature and strongly point towards the potential of this beneficial element for plant stress management (Guntzer et al., 2012; Luyckx et al., 2017). Up to now, the potential of Si to overcome pesticide-induced toxicity is limited to a very recent work, conducted by Tripathi et al. (2020) in rice plants treated with butachlor. In that study, authors observed that Si's ameliorative action was linked with an improvement of nutrient uptake, maintenance of the photosynthetic potential and prevention of oxidative damage, through an upregulation of the AsA-GSH cycle.

GLY exposure resulted in differential responses between the enzymatic and non-enzymatic AOX components. In general, an overaccumulation of Pro, AsA and GSH took place in both organs, but an overall downregulation of the enzymatic mechanisms was perceived. Often, plant responses to abiotic stress factors, including xenobiotic exposure, result in differentially activated/inhibited players. Pro stimulation as a consequence of GLY is a common response of different plant species (Fernandes et al., 2020; Gomes et al., 2017; Singh et al., 2017b; Soares et al., 2019b; Spormann et al., 2019). Accordingly, one of the most recurrent symptoms of GLY at the cellular level is the overaccumulation of this proteinogenic amino acid, whose involvement in stress tolerance has been recurrently highlighted (Soares et al., 2019a). The observed rises in Pro content against GLY underpin that plant cells are able to sense and respond to GLY intracellularly, attempting to limit its toxicity. However, not always an upsurge of this AOX is synonym of an enhanced tolerance, but rather a stress signal. Indeed, the magnitude of these increases should be carefully interpreted. Interested in unravelling the role of Pro accumulation against salt stress in 30 wheat (*Zea mays* L.) cultivars, Poustini et al. (2007) found that all of them enlarged Pro levels, but the most sensitive were the ones reporting the highest increases. Hence, Pro is believed to act not always as a tolerance mechanism, but also as one of the earliest metabolic signals upon exposure to stress, capable of inducing other regulatory networks (Hare and Cress, 1997). In accordance to this hypothesis, the prevention of GLY-mediated burst of $O_2^{\cdot-}$ and LP, as well as H_2O_2 in roots, by both sources of Si was not accompanied by a great increase of Pro accumulation as in plants only exposed to GLY. Thus, and although several reports state that Si application can boost Pro levels to increase the AOX efficiency (Abdel-Halim et al., 2017), the findings herein reported confirm the hypothetical role of Pro as a stress signal, rather than as a tolerance mechanism against herbicide exposure. Similarly, Tripathi et al. (2020), Spormann et al. (2019) and Sousa et al. (2020) found out that pesticide- and zinc (Zn)-mediated increases in Pro levels were efficiently counteracted by the application of Si, salicylic acid, and brassinosteroids (24-epibrassinolide), respectively. Thus, it appears that Si and nano-SiO₂

ameliorative effects on ROS homeostasis and LP in shoots are not related to the action of Pro, but rather to other AOX components.

In the current study, the activity of the main ROS-detoxifying enzymes was severely repressed by GLY. Especially in shoots, where MDA and $O_2^{\cdot-}$ levels rose, the explanation of this inhibition can be ascribed to the effects of oxidative stress itself on enzyme activity. Indeed, it is known that some AOX enzymes are particularly sensitive to oxidation, resulting in a reduced catalytic capacity (Kapoor et al., 2019). Moreover, the significant decrease of protein-bond thiols (-SH groups) (data not shown) in shoots further supports that AOX enzymes failed to prevent the negative effects of GLY on tomato plants. In contrast to plants only exposed to GLY, $O_2^{\cdot-}$ and H_2O_2 -neutralising AOX enzymes were found to be generally increased upon the co-treatment with Si, especially when this element was provided in its bulk form. SOD is usually considered as the first enzymatic line of defence against oxidative stress, being capable of neutralising the toxic effects of $O_2^{\cdot-}$. While SOD activity was restored back to CTL values in shoots and stimulated in roots, thus explaining the generalised reduction of $O_2^{\cdot-}$ in both organs, CAT and APX performance were upregulated, particularly when compared to plants exposed only to GLY, helping to maintain the controlled levels of H_2O_2 . In agreement with APX activity, the other studied AsA-GSH cycle-related enzymes were also enhanced in shoots in response to the co-treatments, reinforcing Si-mediated activation of the AOX system, particularly the enzymatic one. Integrating these responses as a whole, it can be strongly suggested that Si promoted a positive redox balance that allows protein stability and proper redox state. Actually, in plants exposed to GLY, but simultaneously treated with Si or nano-SiO₂, protein thiols were increased in shoots (data not shown), despite GLY negative effects on this parameter. In previous studies, the involvement of Si in stimulating the thiol-based redox network was also suggested (Soares et al., 2018a, 2018b). Although studies exploring ways to increase GLY tolerance of non-target plants are quite few, some of them highlight the importance of the enzymatic AOX system in this response. When studying the potential of salicylic acid (100 μ M) and nitric oxide (NO; 250 μ M) to ameliorate GLY-induced oxidative stress in barley and pea plants, respectively, Spormann et al. (2019) and Singh et al. (2017a) reported that different AOX enzymes, including CAT, SOD and APX, were much more efficient under the co-treatments, limiting GLY harmful effects on ROS overproduction. In opposition, by unravelling the modulation of GLY toxicity by the supplementation of phosphate (PO_4^{3-}), a general inhibition of the activity of the main AOX enzymes (e.g. SOD, CAT, APX) was found in GLY-treated *Hydrocharis dubia* (Blumer) Backer upon P supplementation (Backer et al., 2018).

Being considered as the main AOX buffers of plant cells, the reduced state of AsA and GSH determines the whole redox balance of the cell (Foyer and Noctor, 2011). As shown,

GSH and AsA accumulation were stimulated in response to the presence of the herbicide. Following the herbicide detoxification system, it is not a surprise that GSH levels rose in shoots and roots of GLY-treated plants. Indeed, the conjugation of herbicides with GSH, via glutathione-S-transferase (GST; EC 2.5.1.18) is one of the most common reactions occurring during xenobiotic detoxification (Basantani and Srivastava, 2007). This increase was particularly higher in roots than in shoots, suggesting that the main organ responsible for GLY degradation is the root system, in an attempt to limit its translocation to the aerial parts. Acting *in tandem* with GSH, through the involvement of the AsA-GSH cycle, ascorbate forms (AsA and DHA) were also significantly altered by GLY, generally showing increased levels. Thus, recalling that H_2O_2 did not differ between CTL and GLY plants, it can be hypothesized that non-enzymatic mechanisms, rather than enzymatic ones, have dealt with the excess of this ROS, preventing its accumulation. Regarding this matter, both AsA and GSH are capable of directly eliminating this ROS (Soares et al., 2019a).

Despite this marked stimulation of the AOX metabolites towards GLY exposure, upon co-treatment with both forms of Si, their content did not substantially differ in relation to plants exposed only to the herbicide. Actually, and regardless of the overall upregulation of the AsA-GSH cycle enzymes, the levels of AsA and GSH were somehow identical to those of GLY-treated plants. However, one should highlight that despite the total levels of both AOXs not having changed, their biological relevance is pretty much distinct, given the recorded effects on enzyme kinetics and ROS content. Indeed, while total AsA increased in GLY-treated plants, the observed decreases in AsA/DHA ratio suggest different regulatory phenomena: while in GLY single treatments, this lower ratio was not accompanied by a stimulation of APX activity, but rather a rise in MDA and $O_2^{\cdot-}$, the same was not verified in response to the co-treatments. With effect, under the joint action of Si and GLY, APX and DHAR activities were increased in shoots, aligned with a decrease of the same oxidative stress markers. Concerning GSH, although some reports suggest that both Si and nano-SiO₂ are able to boost its levels as a protective effect against the toxic action of ROS, an identical (in the shoots) or even lower (in the roots) content of this AOX was found in response to the simultaneous application of Si and GLY. Bearing in mind the proved role of GSH as a xenobiotic-conjugating agent (Singh et al., 2016), these results agree with the hypothesis that both forms of Si were efficient at limiting GLY uptake. From a similar way, and knowing that metals and xenobiotics share a common process of detoxification, including sensing, uptake and storage mechanisms (Ramel et al., 2012), this finding is not surprising. Thus, while in shoots GSH is probably being recruited for AOX defence, in roots, its role is probably linked to GLY detoxification, especially in plants solely exposed to the herbicide.

In terms of efficiency, both sources of Si exhibited interesting potential to increase tomato plants' tolerance to GLY, sharing mechanisms of action and enhancing similar AOX players, especially at the enzymatic level. Since Si nanomaterials display a greater surface area and a higher reactivity, some reports suggest that nano-based solutions are often more appealing than their bulk counterparts (Liu et al., 2015; Tripathi et al., 2016, 2017). However, other studies found no substantial differences (Haghighi and Pessarakli, 2013) or, quite the opposite, have indicated that bulk sources of Si are a better option for boosting plant abiotic stress tolerance (Abdel-Hallem et al., 2017). Yet, in order to fully unravel the potential of nanotechnological-based solutions, additional studies are needed, namely to test other types of Si nanomaterials (NMs), as well as several concentrations and modes-of-application. Actually, it is known that NMs physical-chemical features (*e.g.* size, ionic charge, morphology), by modulating the formation of aggregates, can possibly limit their biological effects (Andreani et al., 2021, 2020). So, one cannot exclude that, at the concentration tested, nano-SiO₂ could have formed aggregates, lowering their ameliorative potential, and approaching it to that found for conventional Si. Nevertheless, from a wide perspective, and although in some cases bulk Si led to a more proactive performance of the AOX enzymes, such as SOD, CAT and APX, our data suggest that no major aspects differed between the two applied sources of Si, with both of them showing promising effects for increasing crop resilience to GLY residues.

5. CONCLUSIONS

Overall, results herein reported highlight the urgency for additional studies attempting in preventing GLY-associated risks to non-target plants, especially crops, since residual levels of this herbicide are still capable of inducing phytotoxicity and impairing plant growth. Moreover, being the first report on the effect of Si against GLY toxicity, our data strongly suggest that Si or nano-SiO₂ can be good candidates for plant stress management approaches, especially from an eco-friendly and sustainable perspective (Figure 9). By applying different complementary approaches, the main mechanisms behind Si-mediated protection towards GLY were unravelled, being the limitation of GLY uptake and the efficiency of the enzymatic AOX system the main factors behind the higher tolerance of plants sprayed with Si, either as bulk or nano formulations (Figure 9).

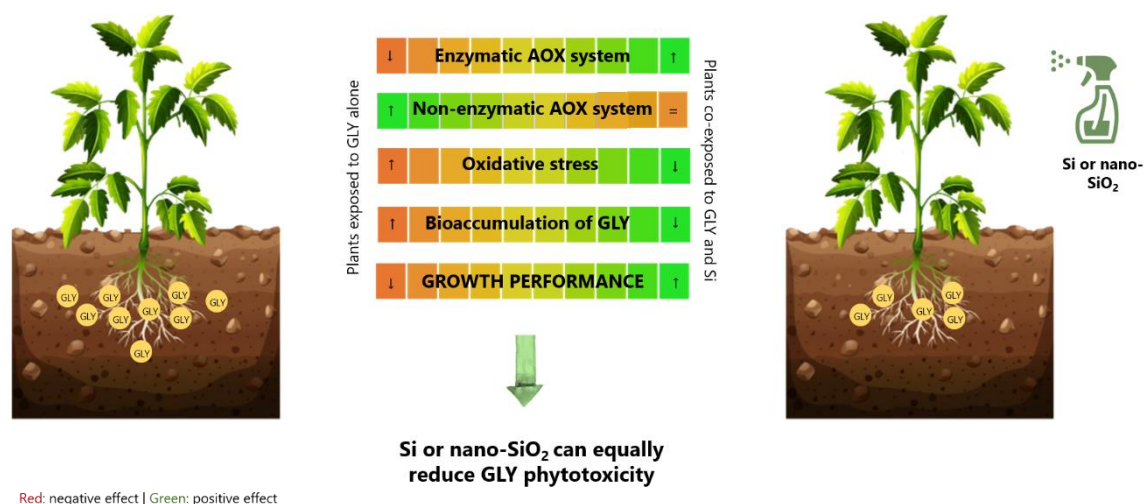


Figure 9. Overview of the main benefits of the foliar application of Si or nano-SiO₂ against GLY-mediated impacts in *S. lycopersicum*.

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Foliar application of sodium nitroprusside boosts *Solanum lycopersicum* L. tolerance to glyphosate by preventing redox disorders and stimulating herbicide detoxification pathways

Abstract

Strategies to minimise the effects of glyphosate (GLY), the most used herbicide worldwide, on non-target plants need to be developed. In this context, the current study was designed to evaluate the potential of nitric oxide (NO), provided as 200 μ M sodium nitroprusside (SNP), to ameliorate GLY (10 mg kg⁻¹ soil) phytotoxicity in tomato plants. Upon herbicide exposure, plant development was majorly inhibited in shoots and roots, followed by a decrease in flowering and fruit set; however, the co-application of NO partially prevented these symptoms, improving plant growth. Concerning redox homeostasis, lipid peroxidation (LP) and reactive oxygen species (ROS) levels rose in response to GLY in shoots of tomato plants, but not in roots. Additionally, GLY induced the overaccumulation of proline and glutathione, and altered ascorbate redox state, but resulted in the inhibition of the antioxidant (AOX) enzymes. Upon co-treatment with NO, the non-enzymatic AOXs were not particularly changed, but an upregulation of all AOX enzymes was found, which helped to keep ROS and LP under control. Overall, data point towards the benefits of NO against GLY in tomato plants by reducing the oxidative damage and stimulating detoxification pathways, while also preventing GLY-induced impairment of flowering and fruit fresh mass.

Keywords

Antioxidants; antioxidant system; herbicides; non-target toxicity; redox homeostasis; stress alleviation.

1. INTRODUCTION

Glyphosate [GLY; N-(phosphonomethyl)glycine], the active compound of several commercial herbicides, was introduced on the pesticide market by Monsanto Company (S.A., Belgium, Europe) in the mid-1970s and has been in a leading position since then (Duke and Powles, 2008; Gomes et al., 2014; Myers et al., 2016). As a broad-spectrum

herbicide, GLY's use was initially restricted for weed removal from cultivated fields, meadows and non-crop areas (Myers et al., 2016). However, since 1996, the introduction of transgenic GLY-resistant crops has led to a general upward trend of GLY-based herbicides application (Duke and Powles, 2008). Indeed, currently, GLY is the most applied herbicide worldwide, accounting, in 2014, for more than 90% of the total herbicide market targeting the agricultural sector (Antier et al., 2020).

Paired with this increasing popularity, emerging concerns on GLY accumulation across the environment have begun to arise. With effect, it has been reported that this agrochemical can accumulate in soil due to leaching losses through the action of rain and/or wind during and after foliar application (Ellis and Griffin, 2002; Gomes et al., 2014; Neumann et al., 2006). Moreover, once applied to weeds' foliage, GLY can be translocated to the roots and gradually released, leading to its accumulation in the rhizosphere (Alves et al., 2008; Neumann et al., 2006). When in soil, residual amounts of GLY can then affect non-target plant species (Soares et al., 2019b; Spormann et al., 2019) since, even upon its metabolization by microorganisms and/or adsorption to soil components, the byproduct of its degradation, aminomethylphosphonic acid (AMPA), is also a recognised phytotoxin (Gomes et al., 2014; Soares et al., 2019b).

Once taken up by plants, GLY is promptly transported to meristems, young roots and leaves, storage organs and any other actively growing tissues through xylem and phloem loading (Duke and Powles, 2008). In terms of action, GLY acts by inhibiting the activity of 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS; EC 2.5.1.19), consequently blocking the shikimate pathway involved in the biosynthesis of phenolic compounds and essential aromatic amino acids, such as phenylalanine, tyrosine and tryptophan (Gomes et al., 2014). Moreover, aside from its primary target effect, increasing evidence has suggested that this herbicide can induce oxidative bursts in plant cells, while also affecting the uptake of essential nutrients (Gomes et al., 2014). Thus, as there is a high demand for agriculture to exponentially increase food production, it is imperative to develop sustainable approaches to increase crops' tolerance to GLY contamination.

Nitric oxide (NO), due to its small size and ability to easily diffuse across biological membranes, is recognised as a remarkable signalling molecule involved in the response of plants to different environmental constraints (Siddiqui et al., 2011). In fact, numerous studies conducted with several plant models have been pointing towards the important role of NO as an ameliorative agent against abiotic stresses (Beligni and Lamattina, 2001; Domingos et al., 2015; Krasnylenko et al., 2017; Nabi et al., 2019; Sharma et al., 2019; Siddiqui et al., 2011). Accordingly, the exogenous application of NO may result in an enhanced crop yield under adverse conditions, due to its role in regulating mechanisms related to increased tolerance to abiotic stress (Nabi et al., 2019). One of the most

commons ways to study NO-mediated effects on plants is through the exogenous application of chemical donors, such as sodium nitroprusside (SNP). Chemically, it is an inorganic molecule composed of Fe (II) and NO⁺, being a derivate of iron-nitrosyl compounds (Filippou et al., 2013; Floryszak-Wieczorek et al., 2006). When in solution, SNP releases NO⁺, Fe (II) and cyanide (CN⁻), which can sometimes mask the effects of NO (Keisham et al., 2019). Either way, this molecule, compared to others, has a relatively lower cost and is recognised for allowing a continuous and enduring production of NO (Floryszak-Wieczorek et al., 2006; Planchet and Kaiser, 2006). Even though NO is a gaseous reactive nitrogen species (RNS), it has the ability to limit reactive oxygen species (ROS)-induced damages by acting as a chain breaker and by activating gene expression of antioxidant (AOX) enzymes (Beligni and Lamattina, 2001; Domingos et al., 2015; Siddiqui et al., 2011). The involvement of NO in enhancing the AOX network in plants is well described in the literature and strongly suggests that NO-mediated increase of plant abiotic stress tolerance is related to a greater ROS detoxification by defence mechanisms (Sharma et al., 2019). Additionally, NO itself is known to have AOX properties, being involved in ROS detoxification and subsequently helping in the inhibition of lipid peroxidation (LP) and protein oxidation (Fancy et al., 2017). Despite the role of NO being relatively well understood in situations of drought, salinity and metal contamination (Arora et al., 2016; Hasanuzzaman et al., 2020; Mazid et al., 2011; Sharma et al., 2019), its involvement in herbicide-induced phytotoxicity, including GLY, remains poorly explored. Regarding this matter, only a recent study conducted by Singh et al. (2017a) is available, in which the potential of this RNS to alleviate GLY-induced stress in *Pisum sativum* L. was evaluated. In spite of the positive outcomes, this study only focused on the early development of seedlings (7 d old) and applied a high concentration of GLY (40 mg L⁻¹) under a hydroponic system, not mimicking a real scenario of soil contamination. Moreover, the precise involvement of NO on the interplay between plant growth and productivity, GLY bioaccumulation and the modulation of AOX and detoxification pathways is yet to be uncovered.

Within this perspective, and as previous studies from our research group have shown that soil contamination by GLY can negatively affect the growth and physiology of non-target plant species, such as tomato (*Solanum lycopersicum* L.) (Soares et al., 2020, 2019b) and barley (*Hordeum vulgare* L.) (Spormann et al., 2019), the main objectives of this study were (i) to evaluate the potential protective role of NO in counteracting GLY-induced stress in crops; and (ii) to pinpoint the main physiological and biochemical mechanisms behind NO action in GLY-exposed plants. Since *S. lycopersicum* (tomato) is one of the most important species worldwide and has been widely used as a model

organism for fleshy-fruited plants (Kimura and Sinha, 2008), this species was selected for this study.

2. MATERIALS AND METHODS

2.1. Chemicals and test substrate

RoundUp® UltraMax (Monsanto Europe, S.A., Belgium), whose active compound is GLY (360 g GLY L⁻¹, potassium salt), was acquired from a local supplier. This formulation was diluted in deionised water (dH₂O) to prepare a stock solution of 1 g L⁻¹ GLY, later used for obtaining the required amount of GLY to be added to the soil (10 mg kg⁻¹ GLY). Sodium nitroprusside (SNP; Sigma-Aldrich®), used as NO donor, was diluted in dH₂O to obtain a solution of 200 µM. An artificial soil (pH 6.0 ± 0.5), composed by sphagnum peat, quartz sand (< 2 mm) and kaolin clay (5:72.5:22.5), prepared according to OECD standards (OECD, 2006), was used in this study.

2.2. Plant material, plant growth conditions and experimental design

Seeds of *S. lycopersicum* cv. Micro-Tom were surface disinfected for 7 min with 70% (v/v) ethanol, followed by 5 min with 20% (v/v) commercial bleach (5% active chloride) mixed with 0.05% (m/v) Tween-20, and then rinsed several times with dH₂O. Afterwards, seeds were germinated in Petri dishes (10 cm diameter) with 0.5 x Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) solidified with 0.625% (m/v) agar, in a growth chamber [temperature: 25 °C; photoperiod: 16 h light/8 h dark; photosynthetic active radiation (PAR): 60 µmol m⁻² s⁻¹]. After 10 d, seedlings were selected and transferred to plastic pots (5 seedlings per pot) filled with 200 g_{dry} OECD soil, which was moistened with dH₂O to obtain 40% of its maximum water holding capacity (WHC_{max}), previously determined according to ISO (2012). To acquire a homogenous mixture, the soil was manually mixed. For GLY-contaminated soils, the amount of herbicide needed to obtain a concentration of 10 mg kg⁻¹ GLY was taken from the stock solution of 1 g L⁻¹. The selection of the GLY concentration was based on our previous work, the recommended dosage used in agriculture and studies on soil contamination by GLY (Soares et al., 2019b). The first watering was done with a half-strength modified Hoagland solution (pH 5.8) (Taiz et al., 2015) in order to avoid nutrient deficiency. Deionised water was then added as needed to maintain soil moisture.

With the purpose of understanding the potential ameliorative role of NO against GLY-induced toxicity, the following experimental groups were considered: CTL — control plants, grown in the absence of GLY and foliar sprayed with dH₂O once a week (negative control);

NO — plants grown in the absence of GLY and foliar sprayed with SNP (200 μM) once a week; GLY — plants grown in the presence of GLY (10 mg kg^{-1}) (positive control); GLY + NO — plants grown in the presence of GLY and weekly sprayed with SNP.

For each experimental group, 12 experimental replicates were prepared (8 pots each one with 5 seedlings). After 28 d of growth in a growth chamber (PAR: 120 $\mu\text{mol m}^{-2} \text{s}^{-1}$; photoperiod 16 h light/8 h dark; temperature: 25 $^{\circ}\text{C}$), plants were harvested and divided into shoots and roots. Part of the biological material (4 replicates) was immediately used to evaluate the biometric parameters, and to determine the levels of superoxide anion ($\text{O}_2^{\cdot-}$), while the plant material from other four replicates was frozen in liquid nitrogen and kept at -80°C for further analyses. The remaining set of plants ($n = 4$) were grown until maturity for the estimation of productivity traits (number of flowers, and number and fresh mass of fruits). For all biometric, biochemical and productivity-related endpoints evaluated, aliquots from at least three experimental replicates were used ($n \geq 3$).

2.3. Biometric and productivity-related analysis

After the growth period (28 d), the roots were washed with tap and dH_2O , and their length was measured. Following the separation of roots and shoots, the fresh biomass of both organs (roots and shoots) was determined using a precision balance (KERN® EWJ 300-3; KERN & SOHN GmbH, Balingen, Germany). Concerning productivity-related traits, a set of plants was left until maturity, in order to monitor the total number of flowers and fruits, and the total fresh mass of produced tomatoes.

2.4. Total protein content and nitrate reductase (NR; EC 1.7.1.1) activity

Total soluble protein and nitrate reductase (NR) from shoots and roots were extracted in frozen aliquots (ca. 200 mg) by homogenising samples in an appropriate extraction buffer [50 mM HEPES-KOH (pH 7.8), 1 mM phenylmethylsulfonyl fluoride (PMSF) and 10 mM magnesium chloride (MgCl_2)] under cold conditions. After centrifugation (25 min; 15 000 g ; 4 $^{\circ}\text{C}$), the supernatants (SN) were collected and used for protein quantification (Bradford, 1976) and for NR activity measurements. The determination of NR activity was performed through enzyme kinetics in accordance to Kaiser and Brendle-Behnisch (1991). The proposed procedure was scaled-down to an ultraviolet (UV) microplate and the assays were performed in a microplate reader (Thermo Scientific™ Multiskan™ GO Microplate Reader). Activity levels were expressed as $\text{mmol min}^{-1} \text{mg}^{-1}$ of protein, using the NADH extinction coefficient (ϵ ; 6.22 $\text{mM}^{-1} \text{cm}^{-1}$).

2.5. Biomarkers of oxidative stress

2.5.1. Superoxide anion ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2)

The levels of $O_2^{\cdot-}$ were quantified according to the method described by Gajewska and Skłodowska (2007), using fresh plant material of roots and shoots (200 mg). After a 1 h reaction at dark conditions in a reaction mixture (2 mL), containing nitroblue tetrazolium (NBT) and sodium azide (NaN_3), an incubation period of 15 min at 85 °C was followed. At the end, the absorbance (Abs) of the obtained solution was recorded at 580 nm and $O_2^{\cdot-}$ levels were expressed in $Abs_{580nm} \text{ h}^{-1} \text{ g}^{-1}$ fresh mass (f.m.). The quantification of H_2O_2 levels was performed in frozen samples, following the spectrophotometric assay of Alexieva et al. (2001), which is based on the reaction between H_2O_2 and potassium iodide (KI), forming a yellowish complex, measurable at 390 nm. Its content was determined through a standard curve, using known concentrations of H_2O_2 and later expressed in $\text{nmol g}^{-1} \text{ f.m.}$

2.5.2. LP

LP was estimated by the evaluation of malondialdehyde (MDA) content, via spectrophotometry, following the procedure described by Heath and Packer (1968). Abs was recorded at 532 and 600 nm. The difference between Abs_{532} and Abs_{600} was calculated to eliminate non-specific turbidity. Considering the ϵ of $155 \text{ mM}^{-1} \text{ cm}^{-1}$, MDA content was determined and expressed in $\text{nmol MDA g}^{-1} \text{ f.m.}$

2.6. Evaluation of antioxidant (AOX) metabolites

2.6.1. Quantification of ascorbate (AsA), glutathione (GSH) and proline

The quantification of total and reduced ascorbate (AsA), as well as its oxidised (dehydroascorbate; DHA) form, was accomplished by following the procedure proposed by Gillespie and Ainsworth (2007). This method allows the quantification of reduced AsA, through the 2,2'-bipyridyl method. Total AsA was determined via the same method, but with the addition of dithiothreitol (DTT) to reduce DHA. After 1 h at 37 °C, the Abs of each sample was read at 525 nm and DHA content was determined by the difference between total and reduced AsA levels. Results were expressed as $\mu\text{mol AsA g}^{-1} \text{ f.m.}$ by comparison with a standard curve prepared with stock solutions of AsA.

To determine free glutathione (GSH) levels, a spectrophotometric assay adapted from a commercial kit was followed as described by Soares et al. (2019b). After the extraction procedure [3% (m/v) sulphosalicylic acid], samples were centrifuged at 4 °C and the SN

was mixed with 5,5-dithio-bis-(2-nitrobenzoic acid) (DTNB; 1.5 mg mL^{-1}). After 10 min, the Abs at 412 nm was registered and GSH levels were expressed as $\text{nmol GSH g}^{-1} \text{ f.m.}$ with the aid of a calibration curve prepared with known GSH concentrations.

Proline levels were estimated through a colorimetric ninhydrin-based assay described by Bates et al. (1973). Samples from shoots and roots (200 mg) were homogenised with 3% (m/v) sulphosalicylic acid and centrifuged (500 g; 10 min). Then, an incubation of 1 h at 95°C was performed, in which the SN reacted with ninhydrin in an acidic medium. In the end, the Abs was recorded at 520 nm and the results were expressed in $\mu\text{g g}^{-1} \text{ f.m.}$, using known concentrations of proline to establish a standard curve.

2.6.2. Determination of total phenolic content (TPC), total flavonoids and total antioxidant capacity (TAC)

The estimation of total phenolic content (TPC), flavonoids and total antioxidant capacity (TAC) was achieved by adapting the procedure described by Zafar et al. (2016). For that, frozen samples were homogenised, on ice, with 80% (v/v) methanol and centrifuged (10 min; 250 g). Regarding TPC, the SN reacted with Folin–Ciocalteu reagent and, after 5 min at room temperature (RT), 7.5% (m/v) sodium carbonate (Na_2CO_3) was added. Samples were then incubated for 1 h in dark conditions at RT. Lastly, the Abs was recorded at 725 nm and results were expressed in $\text{mg gallic acid equivalents g}^{-1} \text{ f.m.}$, using a calibration curve prepared with standard solutions of gallic acid. Concerning total flavonoids, the methanolic extracts were incubated with 10% (m/v) aluminium chloride (AlCl_3) and 1 M potassium acetate ($\text{CH}_3\text{CO}_2\text{K}$), for 30 min at RT. Afterwards, the Abs of each sample was read at 415 nm and the levels extrapolated from a linear calibration curve, prepared with quercetin standards. For TAC, the methanolic extracts were properly diluted (1:3 in methanol) and added to a reagent solution containing 0.6 M sulphuric acid, 4 mM ammonium molybdate and 28 mM sodium phosphate, followed by incubation for 90 min at 95°C . Afterwards, the Abs was recorded at 695 nm. Results were expressed in $\text{mg AsA equivalents g}^{-1} \text{ f.m.}$ (TAC), using a calibration curve prepared with standard solutions of AsA.

2.7. Extraction of AOX enzymes

The main ROS-scavenging enzymes were extracted in accordance with the method described by Soares et al. (2019b), using frozen aliquots of shoots (200 mg in 1.5 mL of extraction buffer) and roots (200 mg in 1.2 mL of extraction buffer). Upon centrifugation (16 000 g; 25 min; 4°C), SN was collected and transferred to new tubes for enzyme activity assessment and soluble protein quantification (Bradford, 1976).

2.8. Spectrophotometric activity quantification of superoxide dismutase (SOD; EC 1.15.1.1), catalase (CAT; EC 1.11.1.6), ascorbate peroxidase (APX; EC 1.11.1.11) and glutathione S-transferase (GST; EC 2.5.1.18)

Total activity of superoxide dismutase (SOD) was estimated through spectrophotometry (Abs at 560 nm), based on the inhibition of the photochemical reduction of NBT, according to Donahue et al. (1997). Results were expressed as units of SOD mg^{-1} of protein, in which one unit of SOD corresponds to the amount of enzyme required to cause 50% inhibition of the NBT photoreduction rate.

Glutathione S-transferase (GST) activity was estimated following the procedure described by Teixeira et al. (2011), by measuring the increase of the GSH-2,4-dinitrochlorobenzene (CDNB) complex at 340 nm. Results were expressed in nmol conjugated CDNB $\text{min}^{-1} \text{mg}^{-1}$ of protein, using an ϵ of $9.6 \text{ mM}^{-1} \text{ cm}^{-1}$.

Both catalase (CAT) and ascorbate peroxidase (APX) activity were determined by enzyme kinetics (Abs at 240 and 290 nm, respectively), as described by Aebi (1984) and Nakano and Asada (1981), following the degradation of H_2O_2 ($\epsilon_{240 \text{ nm}} = 39.4 \text{ M}^{-1} \text{ cm}^{-1}$) and AsA ($\epsilon_{290 \text{ nm}} = 2.8 \text{ mM}^{-1} \text{ cm}^{-1}$), respectively, and expressed as $\mu\text{mol H}_2\text{O}_2 \text{ min}^{-1} \text{mg}^{-1}$ of protein or $\mu\text{mol AsA min}^{-1} \text{mg}^{-1}$ of protein, respectively. In either case, the reaction was started by the addition of H_2O_2 . The original protocols were adapted to UV microplates, based on the optimization of Murshed et al. (2008).

2.9. Analytical quantification of GLY and AMPA

The extraction of GLY from roots and shoots of tomato samples was performed as described elsewhere (AOAC official method 2000.05) and fully detailed by Soares et al. (2021). All subsequent analyses were performed based on Pinto et al. (2018), with some modifications: 1 mL of the extract (SN) was diluted with 1 mL of internal standard ($200 \mu\text{g L}^{-1}$ of GLY 1,2- $^{13}\text{C}_2$ ^{15}N and $200 \mu\text{g L}^{-1}$ of ^{13}C , ^{15}N -AMPA), and then added to 120 μL of 1% (m/v) ammonium hydroxide (NH_4OH) solution and 120 μL of 9-fluorenylmethoxycarbonyl chloride (FMOC-Cl; $12\,000 \text{ mg L}^{-1}$ in acetone). Afterwards, samples were vortexed and incubated for 30 min at RT. To stop the reaction, 10 μL of 6 M hydrochloric acid (HCl) were added. The samples derived were filtered through a 0.45 μm polytetrafluoroethylene (PTFE) filters into liquid chromatography (LC) vials. GLY and AMPA were determined by liquid chromatography with tandem mass spectrometry (LC–MS/MS) using the internal standard method.

The LC–MS/MS system included a Waters 2695 XE separation module (Milford, MA) interfaced with a triple quadrupole mass spectrometer (Quattro micro™ API triple

quadrupole, Waters Micro-mass, Manchester, UK). The LC separation was performed using a Kinetex® EVO C18 core-shell column (2.6 μm ; 100 $\times$ 2.1 mm; flow rate of 225 $\mu\text{L min}^{-1}$). A binary gradient was used: solvent A (10 mM ammonium bicarbonate) and solvent B (methanol). The percentage of organic modifier (B) was gradually modified as follows: 0–0.5 min, 5%; 0.5–5.5 min, 90%; 5.5–6.5 min, 90%; 6.5–6.7 min, 5%; 6.7–14 min, 5%. A total of 20 μL of each sample was injected and the analyses were performed at 40 °C. The mass spectrometry parameters were as follows: ion mode, positive; capillary voltage, 3.00 kV; source temperature, 130 °C; desolvation temperature, 450 °C; desolvation gas flow, 600 L h^{-1} ; and multiplier, 650 V. High purity nitrogen (>99.999%) and argon (>99.999%) were used as the cone and collision gases, respectively. The precursor and product ions, along with the cone voltage and collision energy for each GLY-FMOC, AMPA-FMOC and ILIS-FMOC, were measured by flow injection analysis and the MRM transitions, cone voltages and collision energies are listed in Table S1. Data acquisition was performed by the MassLynx V4.1 software. Results were expressed as $\mu\text{g g}^{-1} \text{ d.m.}$

2.10. Statistical analyses

All biometric and biochemical analyses were performed considering at least three experimental replicates ($n \geq 3$). Results were expressed as mean $\pm$ standard deviation (SD). After checking data homogeneity (Brown–Forsythe test), one-way ANOVA was performed in conjunction with Tukey's post hoc test, assuming 0.05 as a significance level (α). All statistical analyses were performed in GraphPad Prism® 8 (San Diego, CA, USA). In order to execute a principal component analysis (PCA), all evaluated parameters (biometric and biochemical) from each experimental group were plotted to investigate the main factors behind the observed differences. These procedures were performed in the software XLSTAT 2021.2.2 (<http://www.xlstat.com>, Addinsoft New York, USA). The statistical data reporting the results of ANOVA analyses can be found in Tables S2–S4 of the Supplementary Material.

3. RESULTS

3.1. Biometric analysis — fresh biomass and root length

The presence of soil residues of GLY inhibited plant growth, as evidenced by a significant decrease in root length (49%), and fresh biomass of roots and shoots (73% and 48%, respectively), in relation to the CTL (Figure 1). However, after co-exposure to NO, GLY phytotoxic effects were partially prevented in all growth-related parameters, especially when root fresh biomass is concerned (107% increase when compared to the GLY

treatment). This NO-mediated increase in root growth was also noticed when plants were treated only with this molecule, with significant rises up to 65% in relation to the CTL.

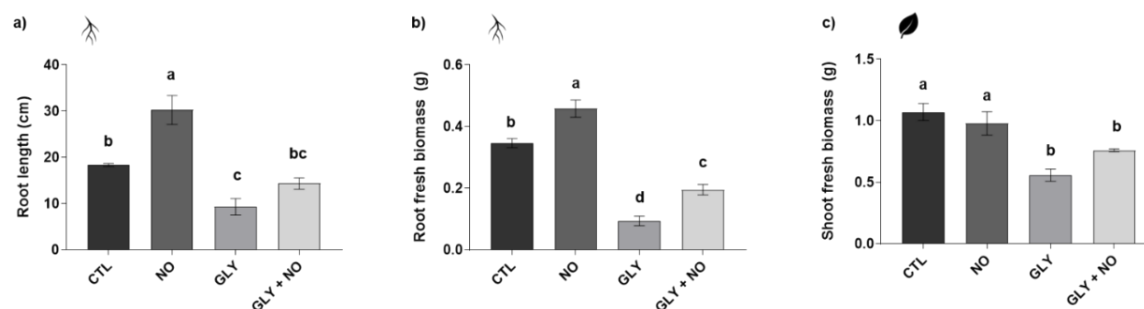


Figure 1. Growth traits of *Solanum lycopersicum* L. cv. Micro-Tom grown for 28 d in an artificial soil contaminated by GLY (10 mg kg⁻¹) and/or foliar-sprayed with SNP (200 μM): (a) root length; (b) root fresh biomass; (c) shoot fresh biomass. CTL — control plants, grown in the absence of GLY and foliar sprayed with dH₂O once a week (black); NO — plants grown in the absence of GLY and foliar sprayed with SNP once a week (dark grey); GLY — plants grown in the presence of GLY (grey); GLY + NO — plants grown in the presence of GLY and weekly sprayed with SNP (light grey). Results are presented as mean ± standard deviation (SD) and result from the evaluation of at least three experimental replicates (n ≥ 3). Different letters above bars indicate significant differences between groups (CTL, NO, GLY and GLY + NO) at $p \leq 0.05$, according to the one-way ANOVA followed by Tukey's post hoc test.

3.2. Soluble protein levels and NR activity

Results referring to total protein content and NR activity are shown in Tables 1 and 2. As can be observed, in the shoots, GLY led to a significant increase in protein levels (27%), regardless of the co-exposure to NO. Nevertheless, in the roots, herbicide treatment resulted in decreased protein levels by 50%, in relation to the CTL, being this effect significantly counteracted by the foliar application of NO (Table 2). Concerning NR, its activity significantly decreased in shoots among treatments, with inhibition values up to 40% compared to the CTL; in the roots, only plants co-exposed to GLY and NO showed a decline in the activity of this enzyme by 24% and 37%, in relation to the CTL and to the plants exposed to GLY alone, respectively (Tables 1 and 2).

Table 1. Biochemical parameters [total protein, nitrate reductase (NR) activity, proline, total ascorbate, ascorbate:dehydroascorbate (AsA/DHA) ratio, glutathione (GSH), total antioxidant capacity (TAC), total phenols and flavonoids] of shoots of *Solanum lycopersicum* L. cv. Micro-Tom grown for 28 d in an artificial soil contaminated by GLY (10 mg kg⁻¹) and/or foliar-sprayed with SNP (200 µM). CTL — control plants, grown in the absence of GLY and foliar sprayed with dH₂O once a week; NO — plants grown in the absence of GLY and foliar sprayed with SNP once a week; GLY—plants grown in the presence of GLY; GLY + NO — plants grown in the presence of GLY and weekly sprayed with SNP. Results are presented as mean ± standard deviation (SD) and result from the evaluation of at least three experimental replicates (n ≥ 3). Different letters indicate significant differences between groups (CTL, NO, GLY and GLY + NO) at *p* ≤ 0.05, according to the one-way ANOVA followed by Tukey's post hoc test.

Parameter	CTL	NO	GLY	GLY + NO
Total protein (mg g⁻¹ f.m.)	3.03 ± 0.20 b	3.47 ± 0.19 ab	3.85 ± 0.14 a	3.68 ± 0.21 ab
NR (mmol NADH min⁻¹ mg⁻¹ protein)	41.67 ± 3.18 a	26.67 ± 1.76 b	28.33 ± 3.18 b	25 ± 2.88 b
Proline (µg g⁻¹ f.m.)	110 ± 8 b	88 ± 11 b	587 ± 87 a	163 ± 26 b
Total ascorbate (µmol g⁻¹ f.m.)	1.52 ± 0.09 bc	1.29 ± 0.18 c	2.05 ± 0.18 ab	2.27 ± 0.21 a
AsA/DHA	2.94 ± 0.66 a	0.37 ± 0.10 b	1.47 ± 0.22 a	2.120 ± 0.40 a
GSH (nmol g⁻¹ f.m.)	288 ± 21 b	310 ± 20 b	454 ± 9 a	426 ± 20 a
TAC (µg AsA equivalents g⁻¹ f.m.)	1067 ± 141 a	928 ± 133 a	717 ± 84 a	895 ± 112 a
Total phenols (µg gallic acid equivalents g⁻¹ f.m.)	960 ± 27 a	381 ± 10 c	542 ± 28 bc	652 ± 54 b
Flavonoids (µg quercetin equivalents g⁻¹ f.m.)	424 ± 43 a	217 ± 14 b	298 ± 8 b	326 ± 16 ab

f.m.: fresh mass.

Table 2. Biochemical parameters [total protein, nitrate reductase (NR) activity, proline, total ascorbate, ascorbate:dehydroascorbate (AsA/DHA) ratio, glutathione (GSH), total antioxidant capacity (TAC), total phenols and flavonoids] of roots of *Solanum lycopersicum* L. cv. Micro-Tom grown for 28 d in an artificial soil contaminated by GLY (10 mg kg⁻¹) and/or foliar-sprayed with SNP (200 µM). CTL — control plants, grown in the absence of GLY and foliar sprayed with dH₂O once a week; NO — plants grown in the absence of GLY and foliar sprayed with SNP once a week; GLY — plants grown in the presence of GLY; GLY + NO — plants grown in the presence of GLY and weekly sprayed with SNP. Results are presented as mean ± standard deviation (SD) and result from the evaluation of at least three experimental replicates (n ≥ 3). Different letters indicate significant differences between groups (CTL, NO, GLY and GLY + NO) at *p* ≤ 0.05, according to the one-way ANOVA followed by Tukey's post hoc test.

Parameter	CTL	NO	GLY	GLY + NO
Total protein (mg g⁻¹ f.m.)	5.09 ± 0.55 a	3.66 ± 0.21 b	2.53 ± 0.10 c	4.91 ± 0.14 a
NR (mmol NADH min⁻¹ mg⁻¹ protein)	110 ± 12 a	122 ± 11 a	132 ± 15 a	83 ± 10 a
Proline (µg g⁻¹ f.m.)	46.44 ± 1.98 b	46.38 ± 3.73 b	95.74 ± 10.68 a	58.17 ± 7.52 b
Total ascorbate (µmol g⁻¹ f.m.)	0.40 ± 0.05 b	0.64 ± 0.05 a	0.41 ± 0.04 b	0.38 ± 0.03 b
AsA/DHA	0.77 ± 0.05 bc	0.68 ± 0.12 c	1.02 ± 0.07 ab	1.11 ± 0.07 a
GSH (nmol g⁻¹ f.m.)	68.96 ± 1.31 b	65.69 ± 4.67 b	139.1 ± 9.53 a	58.49 ± 2.88 b
TAC (µg AsA equivalents g⁻¹ f.m.)	422 ± 35 a	362 ± 14 a	309 ± 21 b	315 ± 20 b

Total phenols (μg gallic acid equivalents g^{-1} f.m.)	243 $\pm$ 9 a	273 $\pm$ 32 a	280 $\pm$ 38 a	273 $\pm$ 17 a
Flavonoids (μg quercetin equivalents g^{-1} f.m.)	19.2 $\pm$ 0.9 a	21.0 $\pm$ 0.2 a	24.1 $\pm$ 2.8 a	24.3 $\pm$ 4.4 a

f.m.: fresh mass.

3.3. Biomarkers of oxidative stress

3.3.1. $\text{O}_2^{\cdot-}$ and H_2O_2

$\text{O}_2^{\cdot-}$ levels were enhanced in shoots (75%) and roots (81%) of plants exposed to GLY (Figure 2a,d), compared to the CTL. With the simultaneous application of NO, the levels of this ROS showed a significant decrease of 74% in shoots and 55% in roots, in relation to the GLY treatment; in shoots, $\text{O}_2^{\cdot-}$ content from GLY + NO plants were even lower than those found in the CTL (decrease of 55%). Regarding H_2O_2 , differences were detected only in the roots, where plants grown in GLY-contaminated soil, but treated with NO, experienced a sharp reduction over the CTL (44%) and GLY (36%) groups (Figure 2b,e).

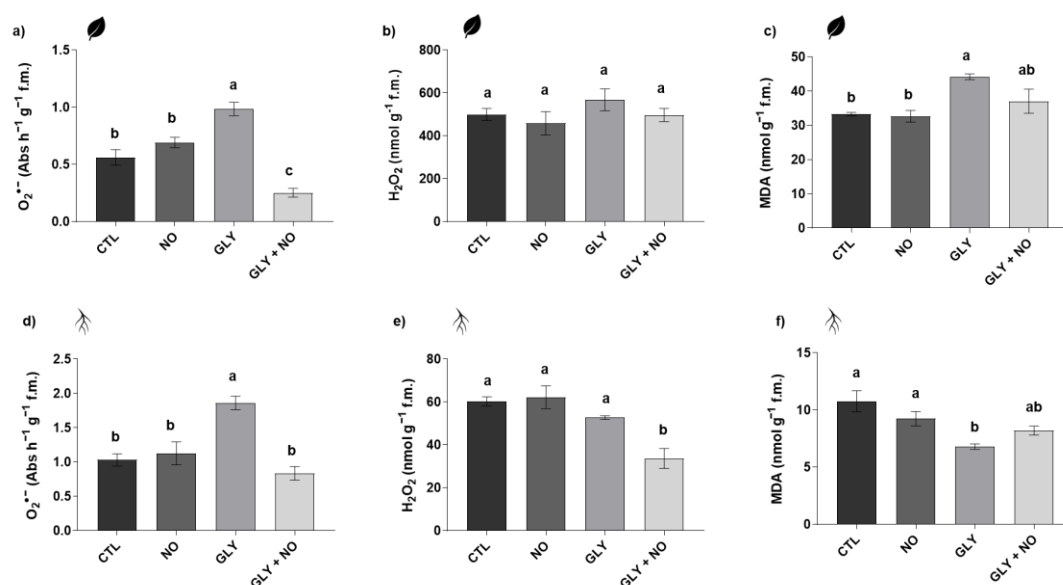


Figure 2. Redox status of *Solanum lycopersicum* L. cv. Micro-Tom grown for 28 d in an artificial soil contaminated by GLY (10 mg kg^{-1}) and/or foliar-sprayed with SNP (200 μM): (a,d) superoxide anion ($\text{O}_2^{\cdot-}$) content; (b,e) hydrogen peroxide (H_2O_2) content; (c,f) malondialdehyde (MDA) levels. CTL — control plants, grown in the absence of GLY and foliar sprayed with dH_2O once a week (black); NO — plants grown in the absence of GLY and foliar sprayed with SNP once a week (dark grey); GLY — plants grown in the presence of GLY (grey); GLY + NO — plants grown in the presence of GLY and weekly sprayed with SNP (light grey). Results are presented as mean $\pm$ standard deviation (SD) and result from the evaluation of at least three experimental replicates ($n \geq 3$). Different letters above bars indicate significant differences between groups (CTL, NO, GLY and GLY + NO) at $p \leq 0.05$, according to the one-way ANOVA followed by Tukey's post hoc test.

3.3.2. MDA content

LP, evaluated in terms of MDA content, was diminished by 37% in roots and increased by 33% in shoots upon GLY single exposure. In response to NO co-application, MDA levels were restored back to the levels found in the CTL (Figure 2c,f).

3.4. Evaluation of the non-enzymatic AOX response

3.4.1. AsA, GSH and proline

Total AsA levels in shoots exhibited a tendency to increase in response to GLY, especially under NO co-exposure, where a significant rise of 49% compared to the CTL was recorded (Tables 1 and 2). In roots, total AsA levels did not vary among treatments, with the exception of NO-treated plants, which showed an increment of 59% in relation to the CTL (Tables 1 and 2). Concerning the ratio between AsA and DHA, in shoots, only NO promoted a significant decrease (87%) of this parameter, though GLY-treated plants also showed a tendency to have reduced values of AsA/DHA by 50%; in the roots, a significant increase of 44% of this ratio was found, over the CTL, when plants were exposed to GLY but simultaneously treated with NO (Tables 1 and 2).

The results of GSH accumulation are presented in Tables 1 and 2. As shown, GLY-treated plants present increased levels of this AOX in shoots (58%) and roots (102%), in relation to the CTL. The co-application of NO did not significantly alter this response in the shoots; however, in the roots, the GSH content was restored to that found in the CTL.

Concerning proline levels, plants' response to GLY was similar in shoots and roots (Tables 1 and 2). As can be observed, proline was severely increased in both organs (1-fold in roots and 4.3-fold in shoots), but the co-treatment with NO was able to inhibit this effect, since no significant differences were registered in relation to the CTL (Tables 1 and 2).

3.4.2. TPC, flavonoids and TAC

In shoots, all treatments led to significantly lower levels of total phenolics, in comparison to the CTL (Table 1); in roots, however, their content did not vary among treatments (Table 2). Flavonoids, as shown in Tables 1 and 2, followed the same trend of TPC, being overall diminished in response to GLY and/or NO, in shoots, and showing no variations in roots. The TAC values, compiled in Tables 1 and 2, presented a similar pattern to that found for TPC with a general decrease in the roots of tomato plants exposed to GLY (inhibition around 33%), regardless of the co-application of NO, and with no major changes in the

shoots. Even so, when GLY-exposed plants were sprayed with NO, TAC was only 16% lower than the CTL (Tables 1 and 2).

3.5. AOX enzymes' activity – SOD, GST, APX and CAT

Data reporting SOD, GST, APX and CAT total activities are presented in Figures 3 and 4. As shown, SOD was only significantly altered in the roots by exposure to GLY alone, where a 41% inhibition was found in relation to the CTL plants (Figure 3a,c). GST activity was also substantially reduced in both shoots (30%) and roots (58%) upon exposure to the herbicide. In response to the co-application of NO, GLY-exposed plants exhibited higher activity values of this enzyme in the roots and, especially, in the shoots, without differences from the CTL situation (Figure 3b,d).

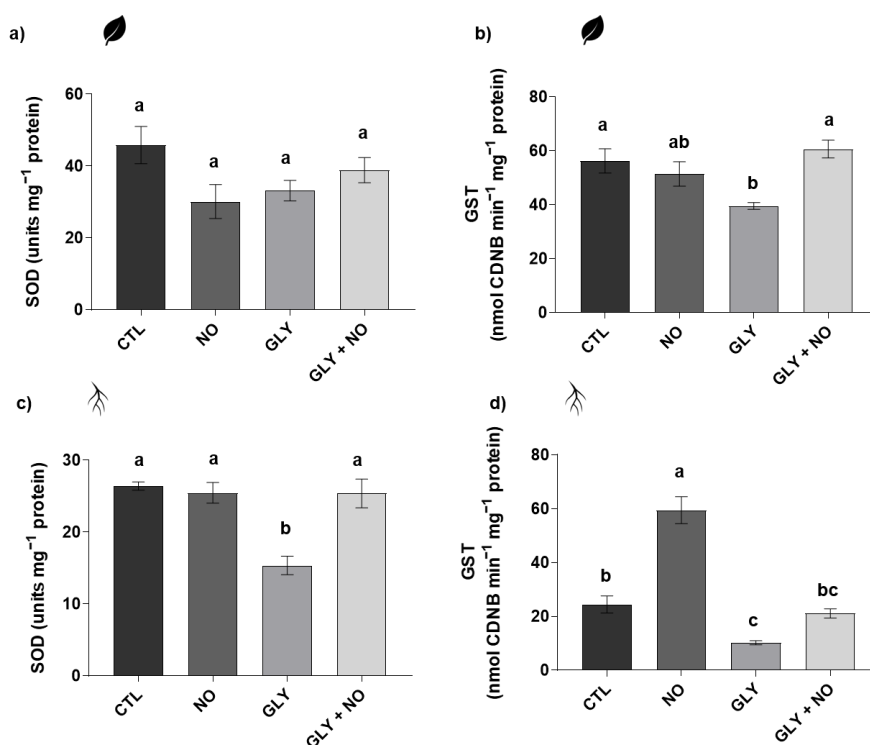


Figure 3. Activity of AOX enzymes of *Solanum lycopersicum* L. cv. Micro-Tom grown for 28 d in an artificial soil contaminated by GLY (10 mg kg⁻¹) and/or foliar-sprayed with SNP (200 μM): (a,c) superoxide dismutase (SOD) and (b,d) glutathione-S-transferase (GST). CTL — control plants, grown in the absence of GLY and foliar sprayed with dH₂O once a week (black); NO — plants grown in the absence of GLY and foliar sprayed with SNP once a week (dark grey); GLY — plants grown in the presence of GLY (grey); GLY + NO — plants grown in the presence of GLY and weekly sprayed with SNP (light grey). Results are presented as mean ± standard deviation (SD) and result from the evaluation of at least three experimental replicates (n ≥ 3). Different letters above bars indicate significant differences between groups (CTL, NO, GLY and GLY + NO) at p ≤ 0.05, according to the one-way ANOVA followed by Tukey's post hoc test.

APX activity suffered a significant decrease in shoots (34%) and roots (66%) of plants exposed only to GLY; once again, the exogenous application of NO increased APX activity, re-establishing its values to those found in the CTL (Figure 4a,c). Regarding CAT, GLY led to a significant inhibition of its activity in both shoots (53%) and roots (63%), in comparison with the CTL. However, in response to the co-treatment, these negative effects were efficiently counteracted, since no differences were recorded between GLY + NO and CTL plants in shoots and an even higher catalytic activity (1.2-fold increase over the CTL) was found in roots (Figure 4b,d).

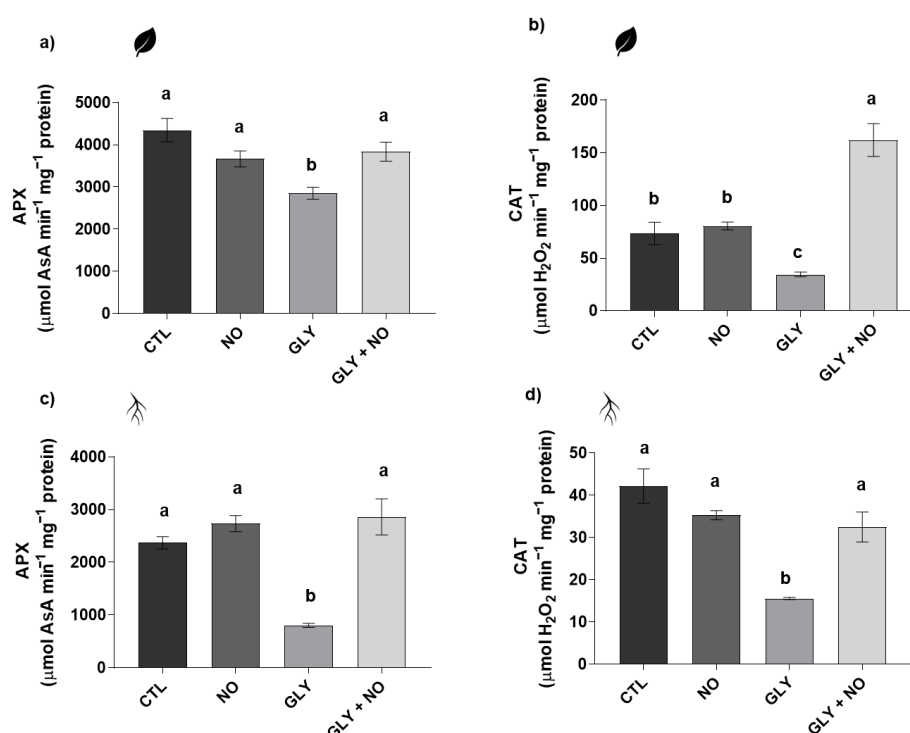


Figure 4. Activity of AOX enzymes of *Solanum lycopersicum* L. cv. Micro-Tom grown for 28 d in an artificial soil contaminated by GLY (10 mg kg⁻¹) and/or foliar-sprayed with SNP (200 μ M): (a,c) ascorbate peroxidase (APX) and (b,d) catalase (CAT). CTL — control plants, grown in the absence of GLY and foliar sprayed with dH₂O once a week (black); NO — plants grown in the absence of GLY and foliar sprayed with SNP once a week (dark grey); GLY — plants grown in the presence of GLY (grey); GLY + NO — plants grown in the presence of GLY and weekly sprayed with SNP (light grey). Results are presented as mean $\pm$ standard deviation (SD) and result from the evaluation of at least three experimental replicates ($n \geq 3$). Different letters above bars indicate significant differences between groups (CTL, NO, GLY and GLY + NO) at $p \leq 0.05$, according to the one-way ANOVA followed by Tukey's post hoc test.

3.6. Bioaccumulation of GLY

As can be observed in Figure 5, GLY was only detected in roots of tomato plants exposed to the herbicide, regardless of the co-treatment with NO. Actually, results show that the application of SNP enhanced the root uptake of GLY, with a significant increase of around

33% in comparison with plants grown in the presence of GLY alone. AMPA was not detected in neither roots nor shoots (data not shown).

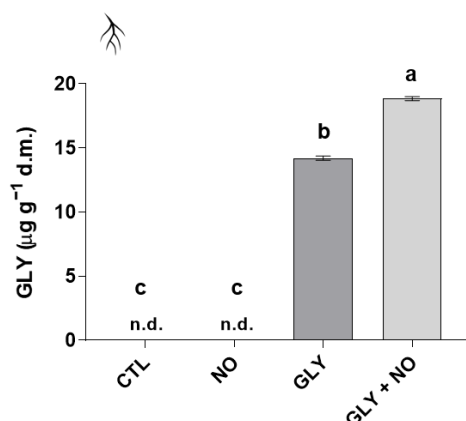


Figure 5. Bioaccumulation of GLY in roots of *Solanum lycopersicum* L. cv. Micro-Tom grown for 28 d in an artificial soil contaminated by GLY (10 mg kg⁻¹) and/or foliar-sprayed with SNP (200 µM). CTL — control plants, grown in the absence of GLY and foliar sprayed with dH₂O once a week (black); NO — plants grown in the absence of GLY and foliar sprayed with SNP once a week (dark grey); GLY — plants grown in the presence of GLY (grey); GLY + NO — plants grown in the presence of GLY and weekly sprayed with SNP (light grey). Results are presented as mean ± standard deviation (SD) and result from the evaluation of at least three experimental replicates (n ≥ 3). Different letters above bars indicate significant differences between groups (CTL, NO, GLY and GLY + NO) at $p \leq 0.05$, according to the one-way ANOVA followed by Tukey's post hoc test; n.d.: non-detected, which means below the detection limit.

3.7. Productivity-related traits

The appearance of the first flower buds occurred upon around 40 d of growth, independently of the presence of GLY in the substrate (data not shown). However, as shown in Table 3, the total number of produced flowers was significantly diminished (51%) by the herbicide, when compared to the CTL. As expected, this reduction in the number of flowers also translated into a decreased fruit set (< 55%), whose development was delayed by one week. However, the foliar application of NO prevented some of these effects, as no differences from the CTL were observed for the total number of flowers. Yet, concerning average fruit production, NO was unable to counteract GLY-mediated effects (Table 3), showing values 46% lower than the CTL. Lastly, although no statistical relevance was achieved for the average fresh mass of fruits, a clear tendency can be observed, in which plants exposed to the herbicide alone tend to produce smaller tomatoes in terms of fresh mass (Table 3).

Table 3. Productivity-related characteristics of *Solanum lycopersicum* L. cv. Micro-Tom grown for 28 d in an artificial soil contaminated by GLY (10 mg kg⁻¹) and/or foliar-sprayed with SNP (200 µM). CTL — control plants, grown in the absence of GLY and foliar sprayed with dH₂O once a week; NO — plants grown in the absence of GLY and foliar sprayed with SNP once a week; GLY — plants grown in the presence of GLY; GLY + NO — plants grown in the presence of GLY and weekly sprayed with SNP. Results are presented as mean ± standard deviation (SD) and result from the evaluation of at least three experimental replicates (n ≥ 3). Different letters indicate significant differences between groups (CTL, NO, GLY and GLY + NO) at *p* ≤ 0.05, according to the one-way ANOVA followed by Tukey's post hoc test.

Parameter	CTL	NO	GLY	GLY + NO
Number of flowers per plant	13.3 ± 2.3 a	10 ± 3.4 ab	6.5 ± 1.0 b	10.0 ± 2.3 ab
Number of fruits per plant	8.0 ± 0.9 a	3.7 ± 1.2 b	3.6 ± 0.4 b	4.3 ± 0.8 b
Fruit fresh mass (g)	3.7 ± 1.1 a	3.0 ± 0.2 a	2.3 ± 0.2 a	3.2 ± 0.8 a

3.8. PCA

In order to determine how all analysed variables explained the differences among experimental groups, a PCA was performed (Figure 6). Results showed that the first component accounted for 43 and 53% of variance in shoots and roots, respectively, and the second for 19% in both organs. Moreover, as can be seen, for roots, CTL and NO plants were clearly grouped together (first quadrant), suggesting that NO alone did not majorly change the growth and physiological status of the plants. In shoots, however, CTL and NO plants were located in distinct quadrants, namely in the second (CTL) and in the third (NO). On the other side, plants exposed to GLY alone were distinctly separated from all other experimental groups, with sample scores being found in the first and second quadrants in shoots and roots, respectively. According to the Figure, the parameters that most contributed for this behaviour were the accumulation of proline and GSH, along with ROS overproduction. When plants were grown in the presence of the herbicide, but treated by foliar spraying with NO, an evident effect was also noticed, as this group remained distant from GLY, but closer to the CTL and NO treatments, being the sample scores located in the first/second and third/fourth quadrants in shoots and roots, respectively.

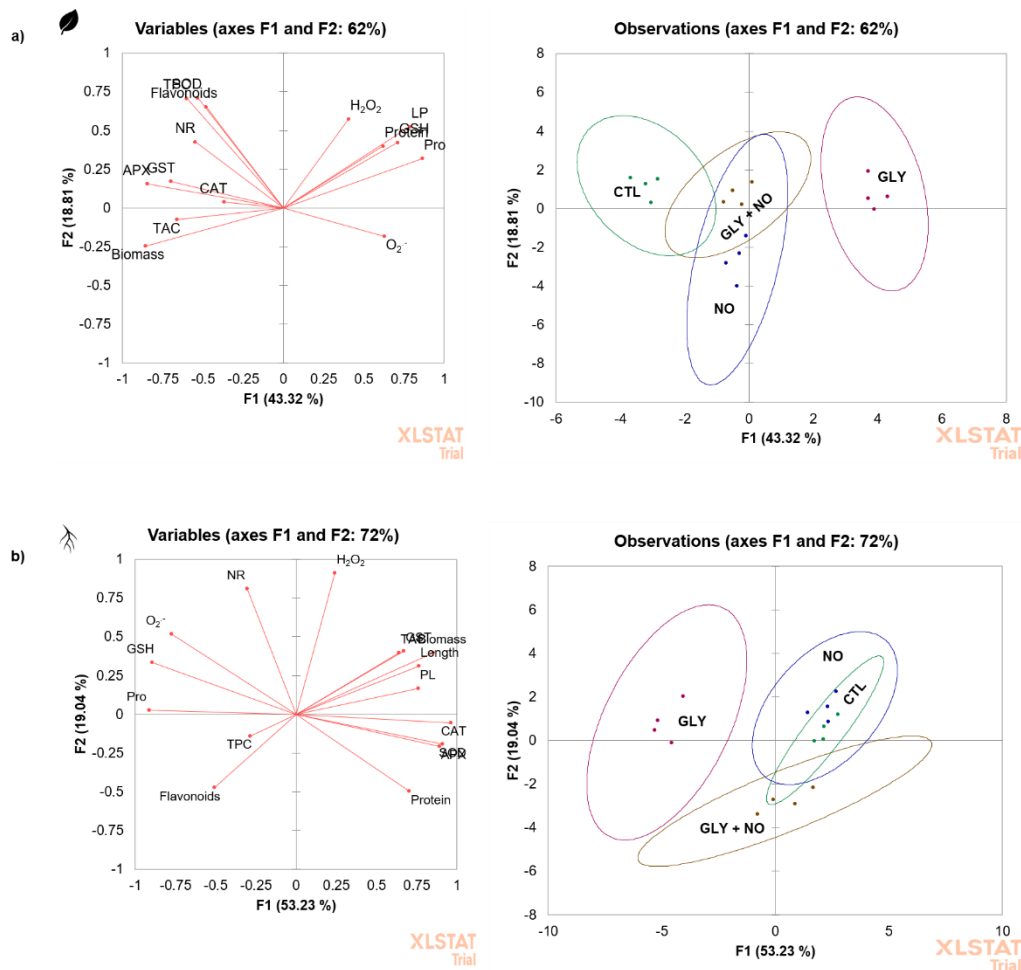


Figure 6. Principal component analysis (PCA) (xx axis—first component, yy axis—second component) of all evaluated endpoints (biometrical and biochemical) in (a) shoots and (b) roots of *Solanum lycopersicum* L. cv. Micro-Tom grown for 28 d in an artificial soil contaminated by GLY (10 mg kg⁻¹) and/or foliar sprayed with SNP (200 μM). CTL — control plants, grown in the absence of GLY and foliar sprayed with dH₂O once a week (green points); NO — plants grown in the absence of GLY and foliar sprayed with SNP once a week (blue points); GLY — plants grown in the presence of GLY (purple points); GLY + NO — plants grown in the presence of GLY and weekly sprayed with SNP (brown points).

4. DISCUSSION

Given the practical and economical relevance of GLY-based herbicides, more than understanding its non-target phytotoxicity, it is also of particular interest to develop new eco-friendly ways to mitigate its risks to agroecosystems and, in particular, to economically important crops. Yet, work focusing on the implementation of mitigation strategies are still in the beginning. By applying a set of ecophysiological and biochemical endpoints, we show that the foliar application of SNP, a NO donor, can boost *S. lycopersicum*'s tolerance to GLY-contaminated soils (10 mg kg⁻¹), improving plant growth by actively controlling the cell redox hub.

GLY disrupted tomato plants' growth, but NO partially reduced its macroscopic phytotoxicity

Here, it was hypothesized that the exogenous application of NO could protect tomato plants from GLY-induced phytotoxicity. In fact, and in accordance with the before mentioned studies, our results suggest that NO neutralises, at least to some extent, the negative effects caused by GLY contamination, as shown by a less pronounced growth inhibition in comparison to the CTL. The registered growth inhibition of plants grown in GLY contaminated soil, largely reported by several authors in different plant models (Gomes et al., 2017, 2016; Singh et al., 2017b, 2017a; Soares et al., 2019b; Spormann et al., 2019), can result from the ability of GLY to decrease the levels of endogenous indole-3-acetic acid (IAA), consequently perturbing cell enlargement and root nodulation (Gomes et al., 2014). In addition, it can be a consequence of its influence on the synthesis of NR and/or nitrate availability, causing a reduction of the enzyme's activity (Bellaloui et al., 2006; Reddy et al., 2010; Singh et al., 2017b, 2017a), as it was reported in roots.

Aligned with this, data from bioaccumulation studies showed that tomato plants were capable of absorbing GLY from the soil solution, and that roots were the preferential organ for GLY storage in plant cells, independently of the NO co-application. Despite several studies having detected GLY in the aerial parts of plants grown under herbicide exposure (Gomes et al., 2016), our data strongly suggest a very limited rate of GLY translocation and/or an efficient detoxification mechanism of GLY. Unexpectedly, when SNP was foliar-applied to GLY-exposed tomato plants, endogenous levels of the herbicide were increased in roots. Although no report is available concerning NO-mediated effects on GLY uptake and partition in plant tissues, a study aimed at evaluating the phytoremediation potential of *Pistia stratiotes* L. to atrazine ($150 \mu\text{g L}^{-1}$) showed that NO supplementation, via SNP (0.05 mg L^{-1}), contributed for a lower phytotoxicity but enhanced the bioaccumulation of this compound (Vieira et al., 2021). Thus, it appears that NO ameliorative features are most likely related to its function as a signalling molecule, capable of inducing a coordinated crosstalk of distinct metabolic chains, rather than inhibiting herbicide uptake and accumulation.

GLY disrupted the cellular redox state, but NO managed to keep ROS under control

Despite being a RNS, the exogenous application of NO to plants exposed to a wide variety of abiotic stresses has been found to prevent the occurrence of oxidative stress (Sharma et al., 2019). Corroborating the data obtained for biometric analysis, we show that the foliar

treatment with NO of GLY-exposed plants results in better ROS management, as evidenced by generally reduced levels of $O_2^{\cdot-}$ (in shoots and roots) and H_2O_2 (in roots), when compared to plants only exposed to GLY. Indeed, increased ROS accumulation in response to GLY exposure has been largely documented in different plant species (reviewed by Gomes et al., 2014). Despite the maintenance of H_2O_2 levels in the shoots, the MDA content, which reflects the degree of LP in cellular membranes, was significantly increased upon GLY single treatment, revealing the occurrence of oxidative damage in the aerial part of tomato plants. This finding, paired with the enhanced accumulation of $O_2^{\cdot-}$, suggests that downstream-formed ROS can be mediating the occurrence of LP. Although $O_2^{\cdot-}$ radicals are described as moderate oxidising agents and cannot be easily diffused through cellular and organelle membranes, evidence suggest that excess of this ROS can indirectly induce substantial oxidative damage by giving rise to more powerful oxidant agents, including the hydroxyl radical ($\cdot OH$) and the hydroperoxyl ($HO_2^{\cdot-}$, a very reactive and stable compound), both able to cross biological membranes and involved in the peroxidation of membrane phospholipids (Gill and Tuteja, 2010; Sharma et al., 2012; Soares et al., 2019a).

Due to its lipophilic features, NO can interact with $O_2^{\cdot-}$ ions, leading to the subsequent formation of peroxynitrite ($ONOO^{\cdot-}$), a less toxic compound, thus limiting the downstream production of other ROS capable of inducing LP. Moreover, as reviewed by Arora et al. (2016), the reaction between NO and superoxide radicals is far faster than the action of $O_2^{\cdot-}$ -degrading enzyme SOD. In accordance, the increased levels of this ROS in response to GLY were restored back to CTL levels upon co-exposure to NO, and actually decreased to lower values in the shoots. Furthermore, the NO co-treatment even promoted a reduction of H_2O_2 content in roots of GLY-exposed plants. In fact, two recent studies by Vieira et al. (2021) and Singh et al. (2017a) have shown that SNP application [0.05 mg L^{-1} ($168 \text{ }\mu\text{M}$) and $250 \text{ }\mu\text{M}$] led to decreased ROS content in *Pista stratiotes* treated with atrazine and *P. sativum* exposed to 0.25 mM GLY, respectively.

Following the same trend observed for ROS, in the shoots, where the herbicide caused a higher proportion of lipid peroxides, the treatment with NO restored MDA values back to those found in the CTL group. The positive role of NO in LP prevention is most likely related to its ability to act as an AOX agent, breaking the reactive chains involved in the LP process (Mazid et al., 2011), which involves activation, propagation and termination steps (Soares et al., 2019a). In a work conducted with soybean (*Glycine max* L.) plantlets, Ferreira et al. (2010) demonstrated that lactofen (0.7 L ha^{-1}) boosted the production of lipoperoxides, suggesting the occurrence of LP, but the co-application of SNP (50 , 100 and $200 \text{ }\mu\text{M}$ SNP; two foliar sprays with a 24 h interval) managed to revert this effect, reducing the accumulation of these subproducts. Curiously, in the roots, data suggested that GLY was

not inducing major oxidative damage since MDA levels were diminished; however, also based on our previous record (Soares et al., 2019b), where we have shown that roots underwent a clear state of oxidative stress, this result was somehow unexpected. Yet, it should be stressed out that the decrease of MDA does not necessarily equals to redox homeostasis. In fact, it is known that ROS, especially $\cdot\text{OH}$, which is formed by the Haber–Weiss reaction via $\text{O}_2^{\cdot-}$, H_2O_2 and transition metals (e.g., copper–Cu), are dangerous for all kinds of biomolecules, namely proteins and nucleic acids (Soares et al., 2019a). Accordingly, when looking to the protein content of roots, a major reduction was found in response to GLY. Moreover, plants simultaneously treated with GLY and NO did not present any significant differences from the CTL in what concerns MDA and total protein content, indicating the re-establishment of homeostasis-promoting conditions.

AOX metabolites are not directly related to NO-mediated restoration of the redox balance disrupted by GLY

According to the data of the current study, a decrease in the TAC of plants subjected to GLY was perceived, with NO treatment not being able to neutralise this effect. Thus, it appears that the non-enzymatic AOX system is not actively involved in the alleviation of GLY-induced stress by NO, although a more detailed approach was followed in order to pinpoint the specific response and interaction of different non-enzymatic AOXs. Due to the nature of phenols biosynthetic process, *i.e.*, the shikimate pathway — the main target of GLY toxicity — it is not surprising that total phenol content was diminished when plants were exposed to this herbicide. In fact, GLY-mediated reduction of phenolic compounds has already been documented by some authors (Hoagland, 1990; Ulanov et al., 2009). Curiously, we report that NO application, with or without GLY co-presence, also led to a decrease in plant phenols and flavonoids in shoots, in contrast to what has been found in the literature.

Proline and GSH, two important players in the non-enzymatic component of the AOX system, have already been shown to be strongly induced in plants exposed to GLY (Gomes et al., 2017; Soares et al., 2019b; Spormann et al., 2019), in accordance with what is herein reported for both analysed organs. However, despite the observed increases in GSH and proline levels, ROS accumulation took place in shoots and roots, revealing that the modulation of their redox state is not able to limit the toxic effects of GLY on tomato's oxidative status. In opposition, plants exposed to GLY but simultaneously treated with NO presented proline and GSH levels similar to the CTL, this being accompanied by a better growth performance. The reduction of free GSH levels in GLY + NO treated plants, in comparison to GLY plants, can be related to GSH ability to chemically

react with different ROS or its use as a substrate in the enzymatic regeneration of AsA. Indeed, it is known that GSH can eliminate ROS excess, such as $O_2^{\cdot-}$, which was clearly reduced in plants co-exposed to GLY and NO. Moreover, and confirming our previous hypothesis raised in Fernandes et al. (2020), it becomes apparent that proline may not be a key player in modulating tolerance to this herbicide, and the reduction of its levels in NO-treated plants can be a consequence of stress alleviation through other mechanisms. Additionally, it is also important to highlight that the exacerbated accumulation of proline in GLY-exposed plants could also have prevented a disbalance in the cellular osmotic potential, as the water status of plants was not altered by the herbicide, as previously reported in tomato plants exposed to 10 mg kg^{-1} GLY (Soares et al., 2020). Following the trend recorded for proline and GSH, the accumulation of AsA in response to the co-treatment with NO was somewhat distinct from that of plants exposed to the herbicide alone. Indeed, the higher AsA/DHA ratio found in shoots and roots of co-treated plants suggests that, upon application of NO, AsA is being actively recruited by APX, as there appears to be an upregulation of the AsA–GSH cycle, possibly pointing towards a tightly regulated enzymatic regeneration mechanism focused on maintaining a sufficient AsA pool to fulfil the AOX needs of *S. lycopersicum* plants. In fact, the stimulation of AsA production when plants were treated with NO during the exposure to different contaminants, such as metals (Ahmad et al., 2018; Chen et al., 2010; Hasanuzzaman et al., 2020) and herbicides (Hasanuzzaman et al., 2018; Qian et al., 2009), has been extensively reported.

NO-mediated alleviation of GLY phytotoxicity involves the upregulation of the main AOX enzymes

For both organs, there was a striking pattern that shows GLY acting as a powerful inhibitor of enzyme activity, as SOD, CAT and APX action were severely hindered when *S. lycopersicum* plants were grown in GLY-contaminated soils. Up to now, distinct findings have been published concerning the effects of GLY on the performance of the plant AOX system (Gomes et al., 2014). Here, the inhibition of SOD is tightly related to the observed increase in $O_2^{\cdot-}$ in both shoots and roots of tomato plants grown in GLY-treated soils. However, CAT- and APX-reduced activity did not result in an overaccumulation of H_2O_2 , reinforcing the idea that tomato plants depend primarily on their non-enzymatic defences to deal with GLY toxic levels intracellularly. Despite the overall inhibition of the main AOX enzymes in response to GLY, when exogenous NO was supplied, all enzymes (SOD, CAT and APX) were restored, or even increased. Accordingly, the upregulation of several enzymatic AOX players by the exogenous application of NO has been reported by different

authors and studies (Ahmad et al., 2018; Hasanuzzaman et al., 2020, 2018; Laspina et al., 2005; Manai et al., 2014; Qian et al., 2009), including in plants exposed to metals such as cadmium (Chen et al., 2010) and copper (Hu et al., 2007), and herbicides, for example, atrazine, glufosinate (Qian et al., 2009) and even GLY (Singh et al., 2017a). In this sense, it is possible to hypothesize that NO-induced redox balance of GLY-treated plants is tightly related to a stimulation or a restoration of the enzymatic component of the AOX system. Moreover, taking into account the possible impact of GLY on the activity of metalloenzymes, by chelating their important co-factors, it is possible that not only NO could be acting by enhancing the efficiency of the enzymatic AOX system, but also by stimulating GLY detoxification pathways, protecting the protein structure of SOD, CAT and APX. Nonetheless, to further prove this hypothesis, subsequent studies to be done should use native polyacrylamide gel electrophoresis to disclose the activity of specific isoenzymes (Azevedo et al., 1998; Gratão et al., 2008; Spormann et al., 2019). This is especially important for SOD, since its various isoforms differ in their metallic co-factors, which are known to be affected by GLY (Gomes et al., 2014).

Detoxification pathways impaired by GLY are stimulated by the exogenous application of NO

Throughout evolution, plants have developed an efficient xenobiotic detoxification system (Coleman et al., 1997; Sandermann, 1992), which involves the conjugation of the transformed compound to GSH or glucose, through the action of GST or glucosyl-transferases (EC 2.4.-.-), respectively. This process depends on the original characteristics of the xenobiotic, but GST-mediated GLY conjugation has already been suggested by several authors (Jain and Bhalla-Sarin, 2001; Miteva et al., 2010). Curiously, our results show an opposite effect, in which plants grown under GLY contamination had a marked decrease in GST activity in both organs, refuting the hypothesis raised in Soares et al. (2021) and suggesting that GLY-mediated elevation of GSH is not related to a higher detoxification mechanism. Thus, it appears that, under GLY exposure, roots of tomato plants failed to employ efficient detoxification systems. From what it appears, it is possible that GLY can be interfering with the structure and activity of GST, which results in a poor detoxification process and increased phytotoxic potential, reflected by the severe impairment of plant growth when exposed to this herbicide. A similar finding was also reported in *Lemna minor* L. exposed to diclofenac (Alkimi et al., 2020). In shoots, surprisingly, the activity levels of GST were also decreased in GLY-exposed plants, even though GLY was not detected in this organ. However, following the same trend recorded for the other AOX enzymes, this finding can reflect the harsh oxidative status that shoots

underwent. In fact, it is known that GST can be highly inactivated by ROS, including $O_2^{\cdot-}$ (Letelier et al., 2010). In response to the co-application of NO, GST activity in roots was restored back to CTL levels, even though a higher bioaccumulation of GLY has been found. This improved, or at least re-established detoxification process made use of the existing GSH pool, to conjugate this thiol with GLY, forming fewer toxic metabolites. Through this process, NO-treated *S. lycopersicum* plants seemed to have been able to reduce GLY toxicity and to improve their growth and performance under these adverse conditions. In fact, increased GST activity in plants treated with this molecule has been reported after exposure to paraquat (Hasanuzzaman et al., 2018) and several metals (Sharma et al., 2019), which share a common detoxification mechanism with xenobiotics.

GLY-mediated effects on crop productivity are partially prevented by the co-application of NO

In addition to affecting plant growth and biomass production, soil residues of GLY (10 mg kg⁻¹) have also resulted in a declined number of flowers and fruits, impacting the fresh mass of the produced tomatoes. Accordingly, a recent study conducted by Strandberg et al. (2021) concluded that, while GLY spray-drift had no effect on flowering time, it adversely affected the cumulative number of flowers of native non-target species (*Trifolium pratense* L. and *Lotus corniculatus* L.). Yet, the assessment of *Brassica* sp. reproductive responses to a GLY-based herbicide (RoundUp®) pointed towards the occurrence of major changes in the flowering time and reproductive function, especially male gametophytes (Londo et al., 2014). Actually, it is known that even GLY-resistant crops can experience substantial changes in their reproductive traits, with major consequences on fruit production (Plin-Srnic, 2005). Aligned with this significant reduction in the number of flowers, fruits from GLY-exposed plants were fewer and smaller than those produced from CTL plants, revealing that soil residues of this herbicide also negatively impact the overall productivity of the plant (Donnini et al., 2016). Up to now, studies dealing with the possible effects of GLY soil contamination on fruit production of non-target crops are scarce (Donnini et al., 2016; Lieten, 2006), this being one of the first records exploring this issue. Based on the data herein collected, one can hypothesize that GLY-mediated impacts on tomato plants' productivity mostly arise as a consequence of the physiological disturbances induced by the herbicide, rather than the effects of GLY itself, since no bioaccumulation was found in shoots. In accordance to our hypothesis, recent findings suggest that composts obtained from earthworms exposed to GLY can disrupt tomato development and ability to flower (Owagboriaye et al., 2020), especially due to GLY-mediated chelation of essential nutrients, which become unavailable for plant growth.

The overall positive effects of NO against GLY-mediated toxicity on the growth and AOX response of tomato plants were also evident in the flowering process. As reviewed by Sun et al. (2021), NO was already proved to benefit plant reproductive traits, inducing the expression of several flowering-related genes. Moreover, although the total number of produced tomatoes was still lower than that of the CTL, fruits' average fresh mass was improved and remained identical to unexposed plants. Indeed, NO application has been found to modulate fruit quality features, contributing for a better firmness and to delay fruit ripening, by inhibiting ethylene biosynthesis (Sun et al., 2021).

5. CONCLUSIONS

As can be seen in the PCA (Figure 6), tomato plants responded differentially to the presence of GLY in the soil, undergoing a state of oxidative stress and impaired growth, especially in the non-green tissues. However, the foliar application of NO successfully improved tomato plant growth and development, with a clear separation from plants exposed to the herbicide alone. According to the biochemical data, this NO-mediated protection was mainly due to its features as radical scavenger and stimulator of AOX mechanisms, contributing for the restoration of the cellular redox status and, consequently, leading to an increased growth potential under herbicide co-exposure (Figure 7). Moreover, the phytoprotective role of NO was also evident when reproductive and productivity traits were evaluated, since the number of flowers and fresh mass of produced tomatoes was increased in comparison with plants only exposed to the herbicide. Overall, this is the first study exploring the benefits of NO supplementation for non-target crops growing in GLY-contaminated soils using an environmentally relevant approach, covering growth- and productivity-related endpoints (Figure 7). In the future, in order to concretely assess if the foliar application of NO, through its donor SNP, can represent an effective tool for plant stress management, it would be of great interest i) to test other modes-of-application and concentrations of this molecule throughout the plant's life cycle (vegetative and reproductive phases) and ii) to study the influence of GLY and NO co-exposure on tomato nutritional and AOX profile to ensure food safety, quality and security.

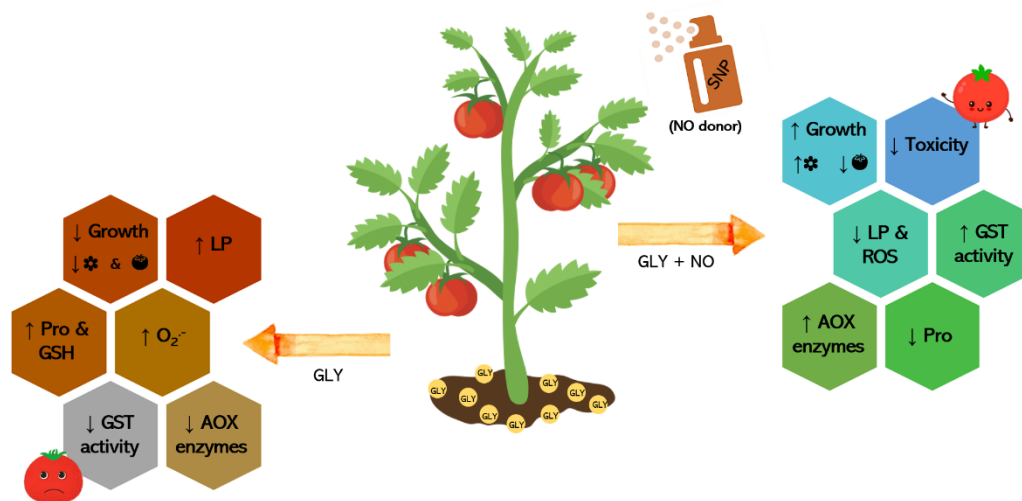


Figure 7. Overview of the main benefits of the foliar application of SNP, a NO donor, against GLY-mediated impacts in *S. lycopersicum*.

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Supplementary Materials

Table S1. MRM transitions, cone voltages and collision energies for each used compound.

Compound	Precursor ion (m/z)	Product ion (m/z)	Cone voltage (V)	Collision energy (V)
GLY-FMOC	392.2	Q:88.0	20	20
		q:170.0	20	10
1,2- ¹³ C ₂ , ¹⁵ N GLY-FMOC	395.2	91.0	20	20
AMPA-FMOC	334.0	Q:112.1	20	15
		q:179.1	20	20
¹³ C, ¹⁵ N-AMPA	336.0	114.1	20	15

Q: quantification transition; q: confirmation transition

Table S2. Detailed ANOVA results for all evaluated parameters in roots of *Solanum lycopersicum* L. cv. Micro-Tom grown for 28 d in OECD soil contaminated by GLY (10 mg kg⁻¹) and/or foliar treated with SNP (200 µM). Parameters where significant differences ($p \leq 0.05$) were recorded are highlighted at bold.

Parameter	ANOVA
Root length	F (3, 10) = 19.11; $p < 0.01$
Fresh biomass	F (3, 9) = 64.36; $p < 0.01$
NR	F (3, 8) = 3.013; $p > 0.05$
Total protein	F (3, 10) = 19.21; $p < 0.01$
LP	F (3, 9) = 9.339; $p < 0.01$
O ₂ ⁻	F (3, 16) = 12.03; $p < 0.01$
H ₂ O ₂	F (3, 8) = 12.29; $p < 0.01$
Proline	F (3, 12) = 14.17; $p < 0.01$
GSH	F (3, 8) = 46.08; $p < 0.01$
Total AsA	F (3, 8) = 7.842; $p < 0.01$
AsA/DHA	F (3, 13) = 5.991; $p < 0.01$
TAC	F (3, 8) = 4.792; $p < 0.05$
TPC	F (3, 8) = 0.3788; $p > 0.05$
SOD	F (3, 9) = 10.36; $p < 0.01$
CAT	F (3, 9) = 13.45; $p < 0.01$
APX	F (3, 8) = 23.07; $p < 0.01$
GST	F (3, 9) = 50.51; $p < 0.01$

Table S3. Detailed ANOVA results for all evaluated parameters in shoots of *Solanum lycopersicum* L. cv. Micro-Tom grown for 28 d in OECD soil contaminated by GLY (10 mg kg⁻¹) and/or foliar treated with SNP (200 µM). Parameters where significant differences ($p \leq 0.05$) were recorded are highlighted at bold.

Parameter	ANOVA
Fresh biomass	F (3, 10) = 13.74; $p < 0.01$
NR	F (3, 8) = 7.339; $p < 0.05$
Total protein	F (3, 27) = 3.902; $p < 0.05$
LP	F (3, 8) = 14.02; $p < 0.01$
O ₂ ^{•-}	F (3, 17) = 20.23; $p < 0.01$
H ₂ O ₂	F (3, 10) = 1.127; $p > 0.05$
Proline	F (3, 9) = 28.02; $p < 0.01$
GSH	F (3, 15) = 14.83; $p < 0.01$
Total AsA	F (3, 9) = 7.387; $p < 0.01$
AsA/DHA	F (3, 9) = 5.532; $p < 0.05$
TAC	F (3, 8) = 1.445; $p > 0.05$
TPC	F (3, 5) = 49.63; $p < 0.01$
SOD	F (3, 9) = 2.874; $p > 0.05$
CAT	F (3, 7) = 27.41; $p < 0.01$
APX	F (3, 9) = 7.474; $p < 0.01$
GST	F (3, 9) = 6.360; $p < 0.05$

Table S4. Detailed ANOVA results for productivity-related parameters of *Solanum lycopersicum* L. cv. Micro-Tom grown for 28 d in OECD soil contaminated by GLY (10 mg kg⁻¹) and/or foliar treated with SNP (200 µM). Parameters where significant differences ($p \leq 0.05$) were recorded are highlighted at bold.

Parameter	ANOVA
Number of flowers	F (3, 10) = 4.444; $p < 0.05$
Number of fruits	F (3, 14) = 4.370; $p < 0.05$
Fruit fresh biomass	F (3, 16) = 0.571; $p > 0.05$

Salicylic acid alleviates glyphosate-induced oxidative stress in *Hordeum vulgare* L.

Abstract

Glyphosate (GLY) is considered the most used herbicide in the world and has been associated with several environmental contamination risks. Despite being partially degraded by soil microorganisms, its residues can negatively affect the growth of valuable non-target plants. Thus, there is a need to find new strategies that minimise its impacts and enhance crop tolerance to GLY, allowing a more advantageous and safer use of this herbicide. Salicylic acid (SA) is a hormone-like substance, able to enhance the efficiency of the antioxidant (AOX) system in plants and their tolerance to oxidative stress. This study aimed to unveil the effects of SA (100 μ M) on the oxidative status of *Hordeum vulgare* L. in response to GLY (30 mg kg⁻¹). After 14 d of growth, the presence of GLY led to a significant inhibition of growth, accompanied by an accumulation of hydrogen peroxide (H₂O₂) and superoxide anion (O₂^{•-}) and an increase in lipid peroxidation (LP). In spite of a reduced ascorbate (AsA) content, GLY also resulted in elevated levels of proline and non-protein thiols, and an upregulation of AOX enzymes. The exogenous application of SA mitigated the effects of GLY on growth, amount of H₂O₂ and degree of LP. It has also contributed for the reduction of AsA content and for the stimulation of non-protein thiols and the AOX enzymatic system, particularly superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) and glutathione S-transferase (GST). These results show a positive role of SA against GLY induced oxidative stress, by modulating the AOX capacity of barley plants. However, given the observed phytotoxicity of GLY was so pronounced, the ameliorating effect of SA on AOX defences was not always enough to overcome the herbicide-induced oxidative damage. Yet, part of the macroscopic phytotoxicity was reverted, suggesting its beneficial role against GLY exposure.

Keywords

Antioxidant system; barley; herbicides; oxidative stress; phytohormones; reactive oxygen species.

1. INTRODUCTION

Glyphosate (GLY), first tested as herbicide in 1970 (Duke and Powels, 2008), quickly became the world's best-selling herbicide, commercialised as anisopropylammonium salt

formulation (Székács and Darvas, 2012). Initially, the application of GLY was intended to eliminate emergent herbaceous plants on agricultural land and/or to control weed growth in gardens and walks. However, the introduction of GLY-resistant crops has greatly enhanced the use of this agrochemical all over the world (Gianessi, 2008). In recent years, several studies have been carried out on the effects of GLY on soil and infiltration waters, particularly addressing GLY bioaccumulation patterns on its risks to consumer health (Buffin and Jewell, 2001; Cerdeira and Duke, 2006; Myers et al., 2016; “Poisoned Field: Glyphosate, the Underrated Risk?”, 2016). GLY has been proven to be toxic to numerous soil organisms and to important arthropod predators in agroecosystems, increasing crop susceptibility to pests and diseases (Buffin and Jewell, 2001). Contradictory results have been reported regarding the bioavailability of GLY residues in soil and the potential risks for non-target organisms, since there are a number of factors that alter the solubility and rate of degradation of GLY in the substrate (Myers et al., 2016; Tesfamariam et al., 2009). Indeed, the fate and transport of herbicides in the environment, particularly in soils and water, are a complex phenomenon, since they are dependent on the nature of the herbicide and the properties of the media matrix (Borggaard and Gimsing, 2008). Moreover, although research on effective methods to remediate herbicide-polluted water bodies is gaining relevance (Kyriakopoulos and Doulia, 2007; 2006; Kyriakopoulos et al., 2003; 2005; 2006a; 2006b), the cumulative and overuse of these phytopharmaceuticals highly contribute to their continuous discharge into the environment.

The herbicidal effects of GLY are due to its action as a substrate analog of phosphoenolpyruvate (PEP), within the shikimate pathway, detaining its activity on the chloroplast enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS; EC 2.5.1.19) (Duke and Powels, 2008). The inhibition of this enzyme leads to excessive accumulation of shikimate, preventing the biosynthesis of the essential aromatic amino acids phenylalanine, tyrosine and tryptophan, which participate in the formation of several important secondary metabolites in plant growth (Siehl, 1997; Yamada and Castro, 2007). Once inside the plant, GLY is rapidly translocated to all organs, but tends to accumulate more in the meristematic regions, blocking the development of new organs. The peroxidation of essential molecules, changes in photosynthesis, carbon (C) metabolism and mineral nutrition, and the occurrence of oxidative stress have also been associated with GLY phytotoxicity (Gomes et al., 2014).

Oxidative stress can occur in response to various adverse conditions, leading to increased production of reactive oxygen species (ROS) (Sharma et al., 2012). The production of ROS and oxidation of essential molecules in plants exposed to GLY have already been described by several authors, both in sensitive and herbicide resistant species (see review by Gomes et al., 2014). This oxidative unbalance can be explained

as a side effect of the prevention of the shikimate pathway, due to the action of herbicide degradation in the plant, or by effects of nutritional interference (Ahsan et al., 2008; Sergiev et al., 2006). Additionally, the chelating properties of GLY may deprive cells of important cofactors to the enzymatic antioxidant (AOX) system, exacerbating oxidative stress. In addition to causing direct damage, ROS may also alter signaling pathways and the action of plant hormones, contributing to the delayed growth and development of plants (Gomes et al., 2014). However, studies focusing on the effects of residual GLY in soil on crops and other non-target plants are limited. Moreover, based on the recent approval of its use by Europe Union (EU) for five more years, it is also of utmost importance to develop effective, eco-friendly and economically rentable strategies to increase crop's tolerance to GLY contamination.

Numerous studies on different plant models point to the important role of salicylic acid (SA) as a signaling molecule in the local resistance reactions and in the expression of defence-related genes (Wani et al., 2017). The accumulation of salicylates (SA and their derivatives) in tissues is indicative of plant defences activation in response to stressful conditions (Makandar et al., 2012). SA has been reported to induce proline accumulation in plants under abiotic stress (Parashar et al., 2014, Hayat et al., 2010) and to stimulate the ascorbate-glutathione (AsA-GSH) cycle, contributing for the improved performance and efficacy of AOXs such as catalase (CAT), peroxidases, superoxide dismutase (SOD), ascorbic acid (AsA) and metal detoxification systems (Wani et al., 2017, Belkadi et al., 2014, Hayat et al., 2010, Shi et al., 2009). Although in recent years there has been a considerable increase in studies related to SA's protection potential, its mechanisms of action in plants remain unclear.

Among crops, *Hordeum vulgare* L. (barley) is one of the most economically important species, considered as an excellent model for studies of agronomy, plant physiology and abiotic stress, with a rapid growth rate and a high adaptability to various habitats (Katerji et al., 2006). In this way, the primary objectives of this study were to i) unravel the main cellular and biochemical mechanisms behind GLY toxicity in barley and to ii) assess the potential of SA to alleviate GLY-induced stress, especially focusing on the AOX network and on the prevention of oxidative damage.

2. MATERIALS AND METHODS

2.1. Plant material, treatments and experimental design

Seeds of *Hordeum vulgare* L., obtained from a local retailer, were surface sterilised with 70% (v/v) ethanol (10 min) and 20% (v/v) sodium hypochlorite [5% (v/v) active chloride; 6 min] and washed multiple times with deionised water (dH₂O). Then, 20 seeds were placed

in plastic pots filled with 300 g of a mixture of perlite:vermiculite (1:2). For each pot, 50 mL of dH₂O supplemented or not with the adequate amount of GLY to achieve the final concentration of 30 mg kg⁻¹ were added. The stock solution of GLY (1 g L⁻¹) was obtained by diluting the commercial herbicide RoundUp UltraMax® (360 g L⁻¹ GLY as the potassium salt). The selected GLY concentration was based on our recent study with tomato plants (Soares et al., 2019b). Moreover, the concentration of 30 mg kg⁻¹ intends to simulate the cumulative effects of repeated applications and/or overuse of the herbicide in agricultural fields (Nguyen et al., 2016).

At this moment, different treatments were included: CTL – plants watered with 0.5 x Hoagland solution (HS; Taiz et al., 2015); SA – plants watered with 0.5 x HS supplemented with 500 µM SA; GLY – plants exposed to 30 mg kg⁻¹ GLY and watered with 0.5 x HS; GLY + SA 500 - plants exposed to 30 mg kg⁻¹ GLY and watered with 0.5 x HS supplemented with 500 µM SA; GLY + SA 100 - plants exposed to 30 mg kg⁻¹ GLY and watered with 0.5 x HS supplemented with 100 µM SA. Since the application of SA alone did not negatively affect the growth of barley plants, and knowing that its effects are more prominent when plants are under stress (reviewed by Wani et al., 2017), after evaluating biometric and some physiological parameters (details in Supplementary Materials), a concentration of 100 µM SA was selected to study its potential to ameliorate GLY-induced stress.

For each experimental condition, four biological replicates (defined as the pot) were considered. The assay started after half of the seeds from the CTL germinated and only seven plants were left in each pot to avoid intraspecific competition. Plants were grown in a growth chamber under controlled conditions of temperature (25 °C), photoperiod (16 h light/8 h dark) and photosynthetically active radiation (PAR; 120 µmol m⁻² s⁻¹). After 14 d of growth, plants were collected and separated into roots and leaves. Some leaves and roots, randomly chosen from each replicate, were immediately selected for the quantification of O₂^{•-} levels, while the remaining plant material was used for biometric evaluations, then frozen under liquid N₂ and stored at -80 °C for posterior analysis.

2.2. Biometric evaluation

After separating the plants into roots and leaves, the maximum root length was recorded, along with the fresh biomass of both organs.

2.3. Quantification of total chlorophylls and carotenoids

Photosynthetic pigments of frozen leaf samples were extracted in 80% (v/v) acetone and quantified according to Lichtenthaler (1987). After centrifugation, the absorbance (Abs) was read at 663, 647 and 470 nm and the content in chlorophylls (a and b) and carotenoids

was estimated based on the formulas of Lichtenthaler (1987). Results were expressed in mg g^{-1} fresh weight (fw).

2.4. Evaluation of oxidative stress endpoints

2.4.1. Lipid peroxidation (LP) and thiols

Lipid peroxidation (LP) and thiols levels were estimated in frozen samples of leaves and roots and quantified following the procedure of Heath and Packer (1978) and Zhang et al. (2009), respectively. For LP, after homogenising the material in 0.1% (m/v) trichloroacetic acid (TCA), malondialdehyde (MDA) content was determined using the extinction coefficient (ϵ) of $155 \text{ mM}^{-1} \text{ cm}^{-1}$ and expressed in $\text{nmol g}^{-1}\text{fw}$. Total and non-protein thiols were spectrophotometrically assayed using the Ellman's reagent and calculated using the ϵ of $13\,600 \text{ M}^{-1} \text{ cm}^{-1}$. Protein thiols were obtained by subtracting the non-protein fraction to the total thiols. Results were expressed in a fw basis.

2.4.2. Superoxide anion ($\text{O}_2^{\cdot-}$) and hydrogen peroxide (H_2O_2)

$\text{O}_2^{\cdot-}$ was extracted from fresh plant material and quantified by the reduction of the nitroblue tetrazolium (NBT) reagent (Gajeweska and Sklodowska, 2007). The levels of $\text{O}_2^{\cdot-}$, represented by the reduction of NBT, were expressed as the $\text{Abs h}^{-1} \text{ g}^{-1} \text{ fw}$. Regarding H_2O_2 , its levels were quantified according to Jana and Choudhuri (1982) by a spectrophotometric assay (410 nm). H_2O_2 levels were calculated using the ϵ of $0.28 \text{ }\mu\text{M}^{-1} \text{ cm}^{-1}$ and expressed in $\text{nmol g}^{-1} \text{ fw}$.

2.5. Quantification of proline and ascorbate (AsA)

Proline and AsA were quantified following the methods of Bates et al. (1973) and Gillespie and Ainsworth (2007), respectively, using leaf and root frozen aliquots. Levels of both AOX were calculated through a calibration curve, prepared with standard solutions of proline and AsA, respectively, and expressed in terms of fw.

2.6. Extraction of total soluble protein and AOX enzymes

Total protein and AOX enzymes were extracted, on ice, by homogenising frozen aliquots of plant material in an extraction buffer, composed of potassium phosphate (PK) (100 mM; pH 7.3), 1 mM ethylenediaminetetraacetic acid (EDTA), 8% (v/v) glycerol, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 5 mM L-ascorbic acid, and 2% (m/v) polyvinylpolypyrrolidone (PVPP). Then, all homogenates were centrifuged at 16 000 g, for 25 min at 4 °C and the supernatant (SN) collected and transferred to new tubes for soluble

protein quantification (Bradford, 1976) and enzyme activity evaluation. Samples for discontinuous PAGE under denaturing and non-denaturing conditions were conditioned as previously described (Fidalgo et al., 2013) and then stored at -80 °C.

2.7. RuBisCO (ribulose-1,5-bisphosphate carboxylase/oxygenase) content

After performing a sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970) on leaf extracts (25 µg protein), relative RuBisCO content was determined (Soares et al., 2016b). Briefly, for each sample, gel slices corresponding to the large and small subunits of RuBisCO were incubated overnight in 2 mL of formamide at 50 °C. Results were expressed as $Abs_{595}RC/Abs_{595}PC$, in which RC is the RuBisCO content and PC is the total protein content.

2.8. Gel blot analysis of superoxide dismutase (SOD; EC 1.15.1.1) activity

Electrophoretic analysis of SOD isoenzymes and activity was accomplished by non-denaturing PAGE, as described by Laemmli (1970) with sodium dodecyl sulphate (SDS) omitted and supplemented with 10% (v/v) glycerol. For the separation of SOD isoenzymes, samples (25 µg protein per slot, for both roots and leaves) were subjected to electrophoresis at 4 °C in 4% stacking and 10% separating gels under constant current (15 mA per gel). After electrophoretic separation, SOD activity staining was performed as described by Donahue et al. (1997).

2.9. Spectrophotometric activity of catalase (CAT; EC 1.11.1.6), ascorbate peroxidase (APX; EC 1.11.1.11) and glutathione S-transferase (GST; EC 2.5.1.18)

The total activity of CAT and APX was spectrophotometrically assayed as described by Soares et al. (2018a), by monitoring the change in Abs at 240 and 290 nm, respectively. GST activity was also estimated by enzyme kinetics (340 nm) as previously reported (Teixeira et al., 2011), using the 2,4-dinitrochlorobenzene (CDNB) reagent.

2.10. Statistical analyses

The experiment was a randomised complete factorial block design, with four biological replicates. Results were expressed as mean ± standard deviation (SD). After checking the homogeneity of variances (Levene's test), data from biometric and biochemical analyses were subjected to analysis of variance (ANOVA), followed by Tukey's *post-hoc* test,

whenever significant differences were found ($p \leq 0.05$). All statistical procedures were performed in GraphPad® Prism 7 (GraphPad Software Inc., USA).

3. RESULTS

3.1. Biometric evaluation – fresh biomass and root length

GLY (30 mg kg⁻¹) in soil caused a significant decrease in leaf (79%) and root (83%) fresh biomass of barley plants compared to those of the control (CTL). In response to the co-treatment with SA (100 and 500 µM), there was an increase of the biometric parameters in relation to plants exposed to GLY only, particularly in root fresh weight (only 67% than that of the CTL; Table 1). Regarding root length, the same pattern of fresh weight was recorded, with marked reductions upon GLY exposure, independently of SA co-exposure (Table 2).

Table 1. Effect of salicylic acid (SA) on root length, root and leaf fresh biomass, and total chlorophylls and carotenoids of barley plants exposed to glyphosate (GLY; 30 mg kg⁻¹). Data presented are mean ± SD (n ≥ 3); different letters indicate significant statistical differences between treatments at $p \leq 0.05$.

Endpoint	CTL	500 µM SA	30 mg kg ⁻¹ GLY	GLY + 100 µM SA	GLY + 500 µM SA
Root lenght (cm)	22.6 ± 0.1a	17.8 ± 0.9b	5.1 ± 0.4c	6.3 ± 0.6c	5.2 ± 0.7c
Root fresh biomass (g)	0.22 ± 0.01a	0.19 ± 0.02a	0.037 ± 0.003b	0.068 ± 0.004ab	0.045 ± 0.002b
Leaf fresh biomass (g)	0.89 ± 0.05a	0.81 ± 0.05a	0.19 ± 0.01b	0.28 ± 0.02b	0.28 ± 0.08b
Total chlorophylls (mg g⁻¹ f.w.)	1.27 ± 0.06a	0.97 ± 0.15ab	1.06 ± 0.04ab	0.67 ± 0.01b	0.71 ± 0.14b
Carotenoids (mg g⁻¹ f.w.)	0.21 ± 0.01a	0.17 ± 0.03ab	0.18 ± 0.01ab	0.12 ± 0.01b	0.11 ± 0.02b

Table 2. Effect of salicylic acid (SA) on RuBisCO, ascorbate (AsA and DHA), proline and thiols (protein and non-protein) content in leaves and roots of barley plants exposed to glyphosate (GLY; 30 mg kg⁻¹). Data presented are mean ± SD (n ≥ 3); different letters indicate significant statistical differences between treatments at $p \leq 0.05$.

Endpoint		CTL	GLY	GLY + 100 µM SA
Leaves	RelativeRuBisco	18.2 ± 2.3a	20.9 ± 0.6a	23.7 ± 3.4a
	AsA/DHA	6.80 ± 1.41a	2.50 ± 0.34b	2.32 ± 0.08b
	AsA/Total ascorbate	0.86 ± 0.02a	0.71 ± 0.03b	0.70 ± 0.01b
	DHA/Total ascorbate	0.14 ± 0.02b	0.29 ± 0.03a	0.30 ± 0.01a
	Total thiols (µmol g⁻¹fw)	1.32 ± 0.09a	1.08 ± 0.07a	1.16 ± 0.01a
	Protein thiols/total thiols	0.98 ± 0.01a	0.94 ± 0.01b	0.89 ± 0.01c
	Non-protein thiols/total thiols	0.02 ± 0.01c	0.06 ± 0.01b	0.11 ± 0.01a
	Proline (µg g⁻¹fw)	37.7 ± 7.0c	200.8 ± 25.7a	106.8 ± 13.6b
Roots	Total thiols (µmol g⁻¹fw)	0.16 ± 0.01b	0.27 ± 0.01a	0.27 ± 0.02a

Protein thiols/total thiols	0.79 ± 0.02a	0.70 ± 0.03ab	0.68 ± 0.02ab
Non-protein thiols/total thiols	0.21 ± 0.02b	0.30 ± 0.03ab	0.32 ± 0.02a
Proline (µg g ⁻¹ fw)	86.5 ± 1.9c	232.3 ± 5.8a	194.8 ± 12.8b

3.2. Physiological performance – photosynthetic pigments and relative RuBisCO content

Although some chlorotic zones were observed in the leaves of the plants subjected to GLY treatments (data not shown), the chlorophyll and carotenoid contents were not significantly affected by the presence of the herbicide alone, presenting only slight declines of 17% and 14%, respectively, in comparison with CTL (Table 1). Controversially, the results indicated that there was an even more pronounced reduction in the amount of photosynthetic pigments when SA was applied alone (chlorophylls: reduction of 24%; carotenoids: reduction of 19%) and in combination with GLY (decreases up to 47%), in comparison to the CTL situation (Table 1).

RuBisCO levels did not significantly vary among treatments (Table 2). However, there was a tendency for increased levels of this enzyme in response to the herbicide, with an even more marked effect under the joint action of the SA.

3.3. LP and thiols content

LP was enhanced in plants exposed to GLY compared to the CTL, with a 45% increase in MDA content in the leaves and 104% in the roots (Figure 1a). Exogenous application of SA (100 and 500 µM) failed to pointedly attenuate GLY-induced oxidative damage, since no significant differences were found between GLY and GLY + SA treatments (Figure 1a and Supplementary Material).

Total, protein and non-protein thiols were measured (Table 2 and Figure 1b). Based on the obtained results, it was possible to observe a decrease in the relative content of protein thiols in both leaves (4%) and roots (11%) of barley plants exposed to GLY alone or in the presence of SA, which further decrease protein thiol content (9% in leaves and 14% in roots, in relation to the CTL). However, accumulation of non-protein thiols was markedly increased in response to GLY (up to 2-fold), with an even more pronounced effect upon SA co-exposure (up to 4.5-fold), in both leaves and roots compared to the CTL (Figure 1b and Table 2).

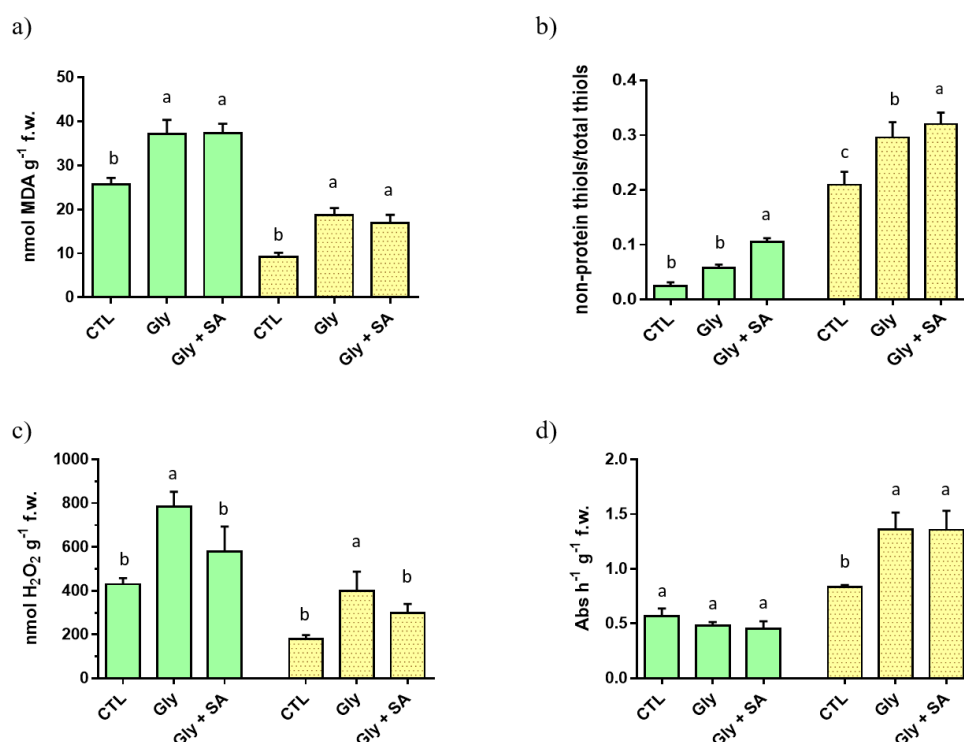


Figure 1. Effects of salicylic acid (SA; 100 µM) on lipid peroxidation (a), non-protein/protein thiols ratio (b), H₂O₂ levels (c) and O₂^{•-} content in leaves (green) and roots (yellow) of barley plants exposed to glyphosate (GLY; 30 mg kg⁻¹). Data presented are mean ± SD (n ≥ 3). Different letters above bars indicate significant statistical differences between treatments at p ≤ 0.05.

3.4. ROS (O₂^{•-} and H₂O₂) levels

ROS production was evaluated through the quantification of H₂O₂ and O₂^{•-} levels. H₂O₂ was increased in leaves (82%) and roots (123%) of plants exposed to GLY compared to the CTL (Figure 1c). As a result of the application of SA, H₂O₂ levels decreased, approaching those obtained in CTL (Figure 1c). Production of O₂^{•-} remained unchanged among treatments in leaves; however, GLY increased its content in roots, regardless of the presence of SA, with increases in the range of 60% (Figure 1d).

3.5. Proline and AsA levels

Proline content increased considerably in the presence of GLY, both in leaves (4.3-fold) and roots (1.7-fold), in comparison to the CTL. When SA was simultaneously applied with GLY, levels of proline were diminished in relation to plants only exposed to GLY (decreases of 47% and 16% in leaves and roots, respectively), yet presenting higher levels than the CTL (Table 2).

The contents of AsA and its oxidised portion (dehydroascorbate - DHA) were quantified in leaves of barley plants. AsA and DHA decreased up to 66% upon herbicide exposure regardless of the presence of SA (Table 2).

3.6. SOD, CAT, APX and GST activities

The activity of SOD was evaluated by electrophoretic separation of its isoforms - Cu/Zn-, Fe- and Mn-SOD. Barley plants appeared to have only the Cu/Zn type of SOD (Fig 2). Furthermore, from the analysis of Figure 2, GLY increased SOD activity in both leaves and roots (Figure 2). In the presence of SA, SOD activity remained higher than in CTL, and slightly higher than that observed upon exposure to GLY alone (Figure 2)

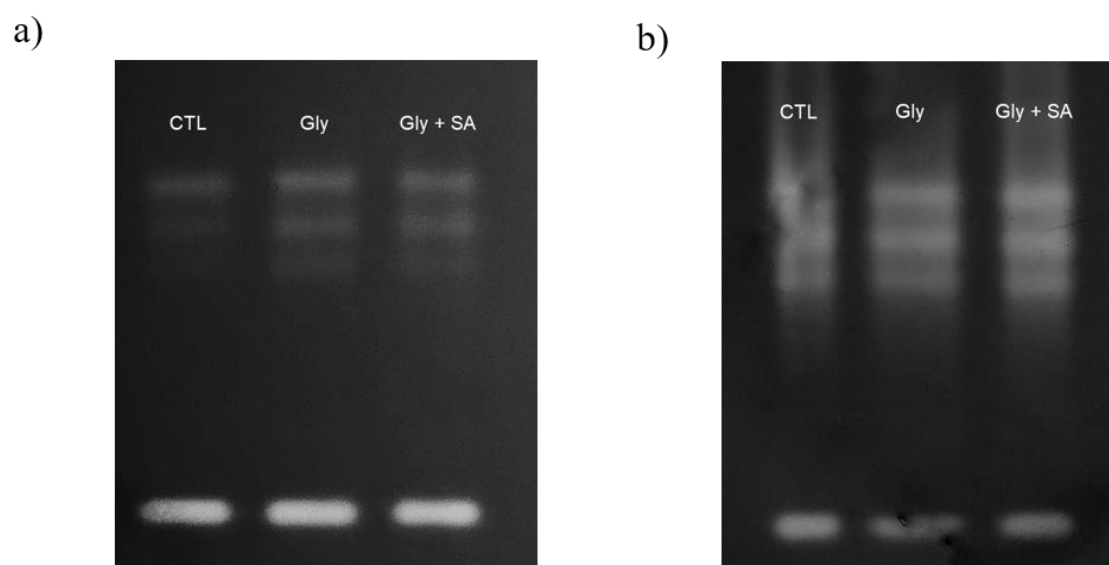


Figure 2. Effects of salicylic acid (SA; 100 μ M) on the activity of SOD in leaves (a) and roots (b) of barley plants exposed to glyphosate (GLY; 30 mg kg⁻¹). Evaluation of enzyme activity was performed under native electrophoresis conditions and the identification of SOD isoenzymes was achieved by pre-incubation of gels with 4 mM potassium cyanide (KCN) or 5 mM H₂O₂ in the incubation buffer.

CAT and APX activity values are shown in Figures 3a,b. Except for CAT activity in roots (which decreased 62%), GLY increased the activity of both enzymes in leaves (CAT by 58% and APX by 89%) and APX in roots (162%) compared to the CTL. SA and GLY co-

application further enhanced the activity of both enzymes, with a more pronounced rise of CAT (80% and 46% in leaves and roots, respectively) and APX (106% and 97% on leaves and roots, respectively) levels in relation to the CTL.

GST activity was positively affected by GLY in both organs (36% and 61% in leaves and roots, respectively), with respect to the CTL (Figure 3c). GST activity increased even further in the co-presence of GLY and SA, with more marked increments of enzyme activity on leaves and roots of barley plants (61% and 95%, respectively).

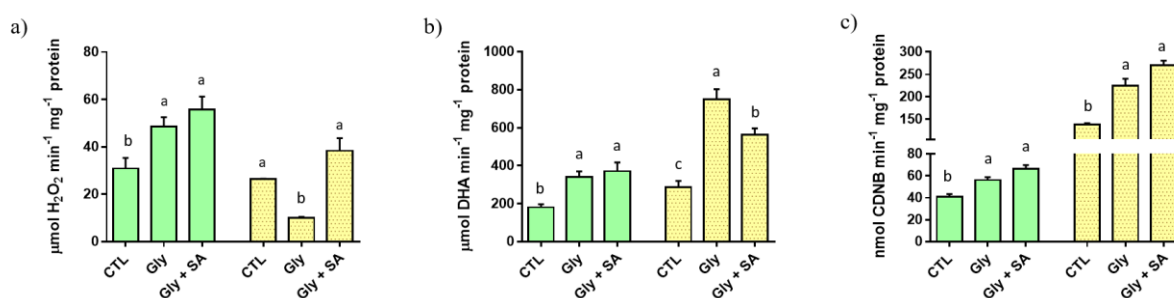


Figure 3. Effects of salicylic acid (SA; 100 μ M) on the activity of CAT (a), APX (b) and GST (c) in leaves (green) and roots (yellow) of barley plants exposed to glyphosate (GLY; 30 mg kg⁻¹). Data presented are mean $\pm$ SD ($n \geq 3$). Different letters above bars indicate significant statistical differences between treatments at $p \leq 0.05$.

4. DISCUSSION

Environmental contamination by GLY is a worldwide problem. During or even after application, a significant fraction of GLY reaches soil, where it can exert side effects on different biological processes and organisms. In this sense, the understanding of GLY impacts on the survival of non-target plants, including crops, is a matter of special interest that needs to be carefully answered. Residues of this herbicide were already detected in soils at concentrations of μ g and mg kg⁻¹ (Busse et al., 2001; Peruzzo et al., 2008), strongly indicating that GLY could pose a serious threat to the global dynamics of ecosystems. As referred by Soares et al. (2019b), levels up to 8 mg kg⁻¹ GLY were recently reported in agricultural soils (Primost et al., 2017; Peruzzo et al., 2008). Moreover, the concentration used in the present study (30 mg kg⁻¹) can be considered as environmentally relevant, and intends to simulate the effects of cumulative herbicide applications and/or overuse practices (Nguyen et al., 2016). Thus, the concentration of 30 mg kg⁻¹ GLY was chosen to study the effects of GLY soil contamination on development and physiological responses of *H. vulgare* L., as well as the possible protective effect of SA.

GLY impairs the growth and development of barley plants, but SA partially alleviates its macroscopic phytotoxicity

The presence of GLY residues (30 mg kg^{-1}) in the substrate compromised plant development, by significantly affecting leaf and root growth. Equivalent findings were recently reported in a study with tomato plants (*Solanum lycopersicum* L.) growing in a GLY-contaminated soil ($0\text{-}30 \text{ mg kg}^{-1}$) (Soares et al., 2019b). The results of the present study are consistent with the hypothesis that plants have a root transport pathway for GLY (Ricordi et al., 2007). Therefore, it is important to explore mitigation strategies to decrease GLY phytotoxicity. SA is a signaling molecule directly involved in the response of plants to different kinds of abiotic stress (Wani et al., 2017), including water stress and heavy metal contamination. However, its potential effects on xenobiotic stress mostly remain to be elucidated. Both positive and negative effects of SA have been reported and appear dependent on plant species, GLY treatments and growth conditions (Gomes et al., 2014). Furthermore, up to date and to the best of our knowledge, no study concerning the potential of SA to alleviate soil contamination by GLY is available. Thus, we hypothesized that the exogenous application of SA could alleviate GLY-induced phytotoxicity. Indeed, results suggested that this compound allowed, at least in part, to reduce the stress induced by GLY, with less accentuated decreases in growth when compared to the CTL, proposing a potential role of SA in increasing plants tolerance to the herbicide. These observations are in agreement with results obtained by other authors (Akbulut et al., 2015; Deef, 2013; Wani et al., 2017) regarding the possible protective effects of SA, in concentrations similar to those tested in this work, in various abiotic stresses and using different plant models. At this point, two complementary hypotheses can be raised to understand SA-mediated protection against GLY: i) SA reduced the uptake of GLY and/or ii) SA stimulated internal defences by limiting the occurrence of oxidative stress. The rationale behind the first hypothesis relies on SA ability to inhibit phosphate (PO_4^{3-}) root uptake, by altering membrane polarization and permeability (Glass, 1974), as well as on the interaction between GLY and PO_4^{3-} (Rose et al., 2017). Given their alike binding ability to soil particles, GLY and phosphate compete for the same adsorption sites and high PO_4^{3-} levels can increase GLY soil remobilization, which can further be uptaken through the same cellular carriers of phosphate. However, due to the possible membrane permeability disturbance caused by SA, a reduced GLY uptake could have occurred in response to the exogenous application of SA. In order to confirm this hypothesis, GLY and PO_4^{3-} levels should be determined in the media and plant tissues in future studies.

Photosynthetic-related endpoints were not substantially affected by GLY exposure

Indirect effects of GLY include the inhibition of biosynthesis and accumulation of pigments, such as chlorophyll a and b and carotenoids, either by the obstruction of the shikimate metabolic pathway or by the phytotoxic action of the secondary metabolite aminomethylphosphonic acid (AMPA) (Gomes et al., 2014; Kitchen et al., 1981; Székács and Darvas, 2012; Wong, 2000). Indeed, decomposition of the herbicide by soil microorganisms yields AMPA, also a phytotoxin, that can reduce the levels of several amino acids. This metabolite competes with glycine and, consequently, inhibits the biosynthesis of chlorophylls (Reddy et al., 2004; Serra et al., 2013). However, in the present study, GLY did not negatively affect chlorophylls and carotenoids content. This observation may be related, in part, to the soil application of GLY, but also to the short duration of the test and the use of a substrate with low microbial activity, not allowing the enough production of AMPA to negatively affect pigment biosynthesis. Controversially, when SA was co-applied with GLY, a reduction in total chlorophylls and carotenoids was observed, though no more macroscopic chlorotic spots were found in leaves in relation to the GLY treatment (data not shown). Although SA was found to prevent pigment degradation (Wani et al., 2017), Moravcova et al. (2018) also reported that exogenous application of SA resulted in decreased photosynthetic pigments plants exposed to copper (Cu). Corroborating the data obtained for chlorophyll and carotenoid levels, GLY did not appear to have detrimental effects on the relative content of RuBisCO subunits, as compared to the control. The results even suggest a tendency for the levels of this enzyme to increase in response to the herbicide, with an even more noticeable effect under the joint action of the SA. Several studies pinpoint a decrease in the RuBisCO levels and loss of photosynthetic efficiency, when GLY is sprayed on the plants (reviewed by Gomes et al., 2014). Again, the results obtained may be related to the root application of the herbicide, since it is known that stress magnitude and effects are largely dependent on the mode-of-exposure (Branco-Neves et al., 2017; Soares et al., 2016b; Taiz et al., 2015). Moreover, although SA co-exposure resulted in reduced levels of chlorophylls, it also induced the synthesis of RuBisCO, possibly suggesting that SA-mediated photoprotection is being targeted to the chemical reactions of photosynthesis.

GLY triggered oxidative stress by an overproduction of ROS, but SA ameliorated this condition by improving thiol redox-based network

Abiotic stress can exacerbate ROS production, leading to oxidative damage to lipids, proteins and DNA (Gill and Tuteja, 2010; Sharma et al., 2012; Soares et al., 2019a). In order to verify whether the observed decrease in growth induced by GLY was related to the establishment of pro-oxidative conditions, the levels of the main ROS were quantified. The content of $O_2^{\cdot-}$, which is usually the first ROS to be produced, was only increased in roots of GLY-treated plants. However, GLY promoted the accumulation of H_2O_2 in both organs, with significant increases from the CTL. Indeed, although the primary effects of GLY are not related to ROS metabolism and oxidative events, it is currently accepted that this herbicide can lead to oxidative injury in plants (Gomes et al., 2014; 2016; Soares et al., 2019b).

Upon co-exposure to GLY and SA, barley plants exhibited lower values of H_2O_2 , while the $O_2^{\cdot-}$ content did not majorly change comparatively to plants only treated with GLY. Thus, it appears that, in the presence of SA, the amount of H_2O_2 may have been scavenged by the increased APX, CAT and/or other peroxidases. Accordingly, the effects of SA on the induction of AOX defences have already been described for several plant models (Akbulut et al., 2015, Belkadhi et al., 2014, Fayez and Bazaid, 2014, Khan et al., 2015, Li et al., 2014), suggesting that the application of SA, up to the order of mM, can stimulate the AOX enzymatic activity, attenuating the production and accumulation of ROS.

The degree of LP, evaluated by the MDA content, is widely used as an indicator of oxidative stress, since it reflects the degree of oxidative degradation of membranes (Sharma et al., 2012). The results obtained indicated that 30 mg kg^{-1} GLY was enough to induce oxidative stress in barley plants, impairing the organization and stabilisation of biological membranes. Similarly, increases in the degree of LP as a consequence of exposure to GLY have been reported for several plant species, such as rice and maize (Ashan et al., 2008; Sergiev et al., 2006). The quantification of thiol (-SH groups) levels is, together with LP, used as a good biomarker of oxidative stress, providing an overview of the oxidative status of the cell, including the redox state of proteins and other non-protein molecules (amino acids, glutathione, among others) (Zagorchev et al., 2013). Although the exposure of barley plants to GLY did not change the content of total thiols, it was possible to observe a shift between the ratio of protein/non-protein thiols. Actually, in response to the herbicide, it appears that both roots and leaves stimulated the non-protein thiols, with a more marked effect upon co-treatment with SA. Currently, the importance of thiols in the abiotic stress attenuation is recognised, with particular emphasis on their participation in

AOX defence pathways. In fact, the role of thiols as attenuators of oxidative stress has largely been documented, especially for the most abundant non-protein thiol, glutathione (GSH) (Zagorchev et al., 2013). Thus, overall, it can be suggested that GLY imposed a harsh oxidative stress condition, resulting in an upsurge in ROS levels and LP; nevertheless, even not always statistically supported, SA played a role in limiting the oxidative damage, by lowering the levels of ROS and LP, along with the stimulation of the cellular redox pathways.

GLY activated several AOX defence mechanisms, whose performance was even more notorious upon SA co-treatment

GSH is considered one of the most important defences in cells against oxidative stress induced by ROS, since it regulates the intracellular redox state, directly removes $^1\text{O}_2$ and H_2O_2 , controls the expression of several defence genes and plays an active and essential role in the process detoxification of several xenobiotics, serving as substrate to GST (Anjum, 2010; Soares et al., 2019a). In the current study, the significant increase of non-protein thiols, especially in chlorophyllin tissues, reflects an increased synthesis and/or regeneration of GSH, ensuring a higher availability of GSH in response to oxidative stress induced by the herbicide, as reported by Miteva et al. (2010) in pea plants treated with GLY. Moreover, the addition of SA, when acting as a signaling molecule, seems to have upregulated the thiol-based network, strengthening the results of Wani et al. (2017) regarding the accumulation of GSH, in the presence of SA, under conditions of oxidative stress. When looking into our results, it may be suggested that, in a joint response to SA and GLY, there has been a remobilization of -SH groups of dysfunctional proteins, contributing to increase the pool of non-protein thiols as a defence strategy against the stress induced by the herbicide (Zagorchev et al., 2013).

Proline, an amino acid constituent of proteins, has revealed beneficial characteristics in plants under stress, which extend far beyond its functions as a compatible solute. Given its chelating properties, its involvement in cell signaling mechanisms, as well as its active participation in the non-enzymatic component of the plant AOX system, the potential of proline in increasing plant tolerance to various abiotic stresses has been progressively recognised (Hayat et al., 2012), by its ability to eliminate some ROS, such as $\cdot\text{OH}$, and to prevent LP (Ashraf et al., 2007; Matysik et al., 2002; Smirnov et al., 1989). Proline content increased in the presence of GLY, evidencing the activation of the non-enzymatic AOX system. A similar response was also reported in *Zea mays* leaves treated with GLY (Sergiev et al., 2006). Recently, and in agreement with our results, Gomes et al. (2017) and Soares et al. (2019b), when studying the effects of GLY (foliar and soil applied,

respectively) on the oxidative metabolism of *Salix miyabeana* (willow) and *Solanum lycopersicum* (tomato), observed the accumulation of proline, most likely as a response to GLY-induced inhibition of the shikimate pathway (Gomes et al., 2017). Although SA has been reported to promote proline accumulation under conditions of oxidative stress (Wani et al., 2017), the co-treatment of barley plants with GLY and SA did not trigger a significant rise in the levels of proline. The biochemical and physiological responses of plants are highly dependent on several factors, including plant species, intensity and duration of exposure, as well as the nature of the stressing agent (Gratão et al., 2008; Soares et al., 2016a, b; 2018a; 2018,b). The findings that the co-treatment of barley plants with GLY and SA did not trigger a significant rise in proline levels could support the previously mentioned hypothesis that SA causes a reduction in GLY uptake.

Along with GSH and proline, AsA is also one of the main AOX molecules across plant kingdom (Gill and Tuteja, 2010). Being only present in photosynthetic organisms, AsA plays several major roles in plant stress tolerance, being able to directly eliminate some ROS, such as $O_2^{\cdot-}$ and serving as the substrate for APX (Sharma et al., 2012). Here, upon herbicide exposure, levels of total AsA decreased, along with significant declines in its reduced portion and rises in the oxidised form (DHA), being this effect even more notorious in the presence of SA. Although in cases of different types of stress, the increase in DHA over AsA might be a sign of toxicity, one cannot completely exclude that SA promoted the decrease of AsA as a consequence of an increased APX activity and/or by its direct reaction with the $O_2^{\cdot-}$ (Ushimaru et al., 1997). Bearing in mind the results obtained for $O_2^{\cdot-}$ levels in leaves, this hypothesis makes even more sense, suggesting the direct involvement of this AOX in the response of barley plants to GLY.

Acting together with the non-enzymatic defences, plant cells possess different enzymes involved in ROS scavenging and xenobiotic detoxification. Since SOD is the main enzyme responsible for $O_2^{\cdot-}$ elimination, it is commonly considered as the first enzymatic line of defence against oxidative stress. Indeed, the activation of SOD in response to different stresses, like pesticides and metals, has been largely reported (see references by Soares et al., 2019a). Data from the current work revealed that SOD activity was increased in both organs upon exposure to GLY. Besides this, and bearing in mind the levels of $O_2^{\cdot-}$, it seems that the upsurge in SOD activity in leaves was enough to prevent an overproduction of $O_2^{\cdot-}$, but not in roots, since the levels of this ROS remained higher than the CTL. In the presence of SA, SOD activity also increased over the CTL plants, but kept practically identical to the treatment with GLY alone. Although SA is supposedly able to increase the activity of SOD (Wani et al., 2017), in the present study, it can be assumed that SA may have been involved in the direct removal of $O_2^{\cdot-}$ and/or activation of other AOX defences, including non-enzymatic molecules, such as GSH and AsA. When studying the

polymorphism of this AOX enzyme, it was only possible to identify one type of isoenzyme – Cu/Zn-SOD – in both analysed organs. Although it may seem surprising, the absence, or negligible presence, of Fe-SOD and Mn-SOD isoforms in barley plants was already reported (Azevedo et al., 1998; Rao et al., 1995; 1996).

In addition to SOD, the plant AOX system also encompasses several enzymes involved in the intracellular detoxification of H_2O_2 , generated either by the activity of SOD, by several metabolic processes and/or as a result of several stressors (Soares et al., 2019a). Among these enzymes, and given their subcellular localization and high catalytic activity, CAT and APX are particularly noteworthy. Our observations, such as those that have been reported in previous studies with this herbicide (Kielak et al., 2011; Miteva et al., 2010; Sergiev et al., 2006; Soares et al., 2019b), suggest that barley plants increased their AOX defences in the presence of GLY, in order to counteract oxidative stress (Sharma et al., 2012), and that SA has partly enabled the amplification of the response, providing cells with an even more efficient AOX performance. The increase in the activity of these AOX enzymes, induced by SA, has already been described for several abiotic stresses and plant models, emphasising the benefits that come from the application of SA in stressed plants (reviewed by Wani et al., 2017).

Exposure of plants to xenobiotics activates detoxification processes to transform the contaminant to more innocuous and easily metabolizable substances (Gill and Tuteja, 2010; Soares et al., 2019a). Conjugation of the xenobiotic to the AOX tripeptide GSH often occurs via a reaction catalysed by a glutathione-S-transferase (GST). Our results indicate that *H. vulgare* activated the herbicide detoxification process, with GST playing a key role. Similar findings have been previously reported (Basantani et al., 2011; Miteva et al., 2010 and references therein). Simultaneous treatment with GLY and SA potentiated increases in GST activity, strongly suggesting the activation of cellular detoxification processes. Moreover, the observed decrease of H_2O_2 levels in co-treated plants can also be related to GST, since it is known that this enzyme can also act as peroxidase (Soares et al., 2019a). Thus, gathering all data obtained, it was possible to observe that SA-mediated protection against GLY phytotoxicity is mainly attributed to an increased AOX performance, especially at the enzymatic level, and herbicide detoxification; furthermore, based on the interactions between SA, and PO_4^{3-} and GLY absorption, the hypothesis of a reduced uptake of GLY due to SA should also be considered and must be the core of future studies.

5. CONCLUSIONS

With this work, new evidence regarding the phytotoxicity of GLY to non-target plants was highlighted, strongly suggesting that oxidative stress occurs even when the herbicide is applied to the plant growth substrate (Figure 4). Overall, the foliar application of SA to plants grown under the presence of 30 mg kg⁻¹ GLY allowed to revert, at least partially, the detrimental effects of the herbicide on growth traits and redox homeostasis, substantially contributing to an enhanced AOX efficiency (Figure 4). Thus, since there are already different commercial formulations incorporating SA in the agroindustry, it can be concluded that SA may represent a promising tool to overcome the side-effects of GLY on crop plants in a real agricultural context. However, as discussed in detail by Janda et al. (2017), additional research is needed to optimise SA concentration, mode and number of applications, along with field-scaled experiments to validate its practical use. Only in this way it will be possible to effectively transfer technology into the market scene, by the development of new products containing SA and whose application is directed to specific stresses and not in an empirical and generalised way.

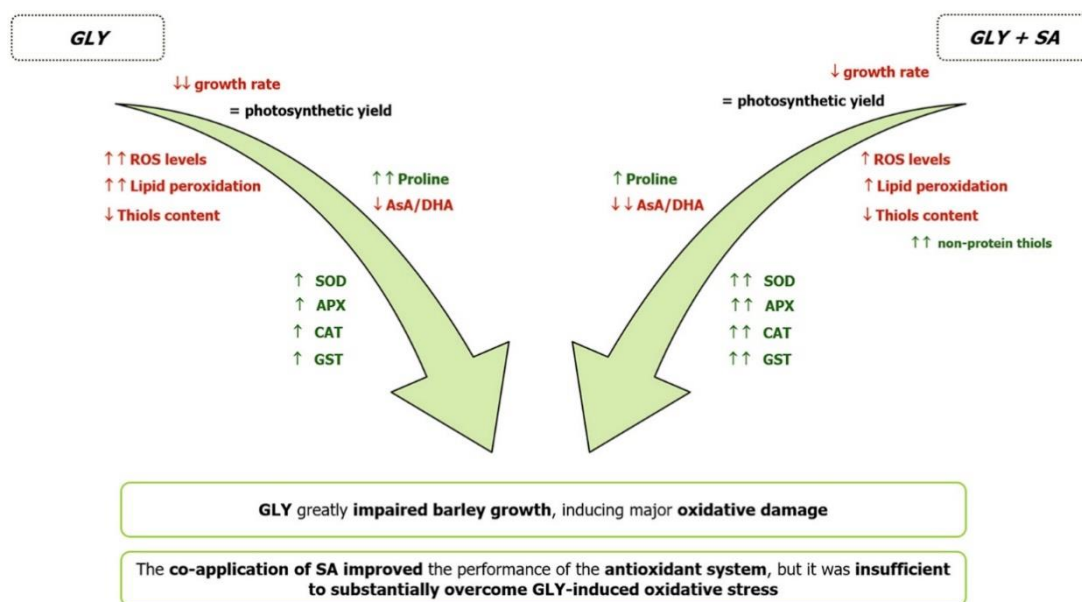


Figure 4. Overview of the effects of SA supplementation on GLY-induced stress in *H. vulgare*.

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Supplementary Materials

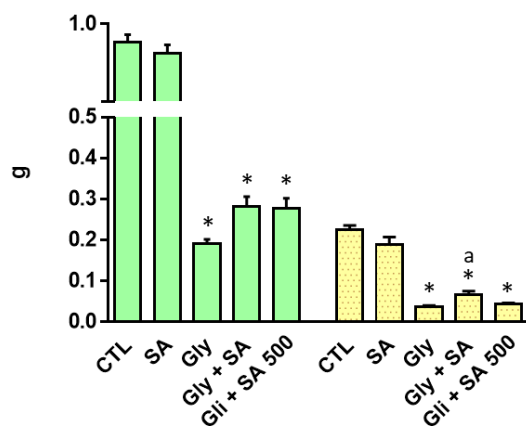


Figure S1. Effect of salicylic acid (SA) on root (yellow) and leaf (green) fresh biomass of barley plants exposed to glyphosate (GLY; 30 mg kg⁻¹). * and ^a above bars indicate statistical differences ($p \leq 0.05$) from the CTL and Gly treatments, respectively.

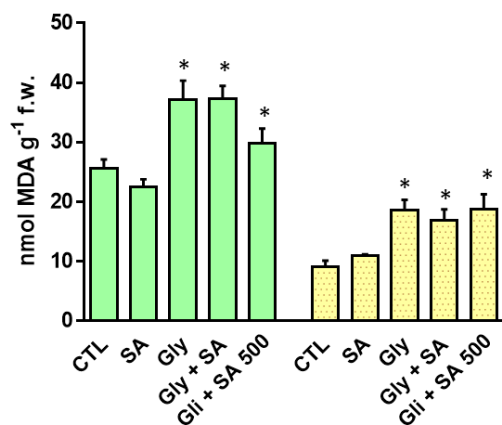


Figure S2. Effect of salicylic acid (SA) on root (yellow) and leaf (green) lipid peroxidation of barley plants exposed to glyphosate (GLY; 30 mg kg⁻¹). * above bars indicate statistical differences ($p \leq 0.05$) from the CTL plants.

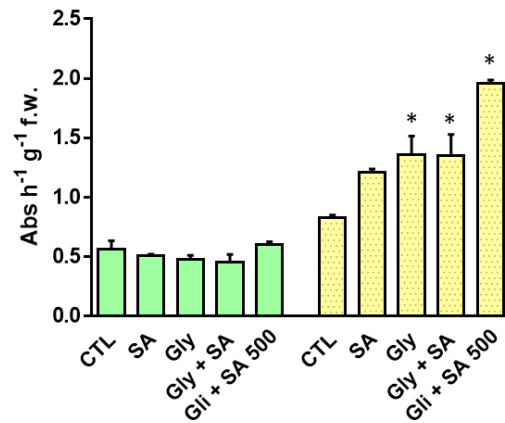


Figure S3. Effect of salicylic acid (SA) on root (yellow) and leaf (green) $O_2^{\cdot -}$ content of barley plants exposed to glyphosate (GLY; 30 mg kg⁻¹). * above bars indicate statistical differences ($p \leq 0.05$) from the CTL plants.

Since the application of SA alone did not negatively affect the growth of barley plants, as evidenced by Figure S1, and that the results obtained for 100 μ M SA were more promising than those of 500 μ M SA (Figure S1-S3), the concentration of 100 μ M was selected for the subsequently analyses and evaluations.

Modulation of the non-target phytotoxicity of glyphosate by soil organic matter in tomato (*Solanum lycopersicum* L.) plants

Abstract

Glyphosate (GLY)-based herbicides are the most widely used and, although it is admitted that, when in contact with the soil, GLY degrades rapidly, recent studies show that the accumulation of its residues in soils can negatively affect the growth of non-target plants. Knowing that soil properties, such as organic matter (OM) content, influence the bioavailability of pesticides, this study aimed to study the role of soil OM in preventing GLY phytotoxicity, using tomato (*Solanum lycopersicum* L.) as a model crop species. For this, plants grew for 28 d in soils with different concentrations of OM [2.5; 5.0; 10 and 15% (m/m)] contaminated, or not, by GLY (10 mg kg⁻¹). Afterwards, biometric parameters, oxidative stress markers [lipid peroxidation (LP); hydrogen peroxide (H₂O₂); proline] and several physiological indicators [total sugars, amino acids and soluble proteins; glutamine synthetase (GS) and nitrate reductase (NR)] were evaluated. According to the results, GLY significantly reduced plant growth in all tested soils, especially in those with lower OM content (2.5 and 5.0%), this being accompanied by an upsurge of LP in shoots and proline in shoots and roots, and a decrease of total sugars in both organs. In contrast, the exposure of plants to GLY in OM-enriched soils (10 and 15%) did not substantially alter the cellular redox status, while contributing to a higher content of total amino acids in shoots. Nitrogen (N) metabolism-related endpoints were not substantially affected by GLY independently of the soil OM. Overall, the results seem to suggest that soils with a higher OM content, 10 and 15%, can mitigate the non-target phytotoxicity of GLY, possibly by decreasing herbicide bioavailability and/or by stimulating defence mechanisms, thereby improving plant growth and physiological performance.

Keywords

Crops; herbicides; oxidative stress; pesticides; RoundUp; soil contamination.

1. INTRODUCTION

Conventional agricultural practices are heavily dependent on the application of numerous plant protection products (PPPs), where pesticides (e.g. herbicides, fungicides and

insecticides) are included (FAOSTAT, 2019). Despite their undeniable role in granting higher agronomic yields and ensuring food safety against emerging pests and diseases, the widespread and uncontrolled use of these synthetic chemicals has also been accompanied by worrying environmental consequences – the contamination of soils and surface waters, where PPPs can accumulate and pose risks to non-target organisms, like crops used for human and animal feeding (Geissen et al., 2021; Sharma et al., 2019). From all pesticides, glyphosate [N-(phosphonomethyl)glycine; GLY] is one of the most used worldwide, being typically applied by foliar spraying and acting in a non-selective and systemic manner after seedling emergence (Duke and Powles, 2008). Since its commercialization, it has been used in agriculture and in urban contexts to control the vegetation of roads and pavements (Silvia et al., 2020; Vereecken, 2005), quickly assuming a leading position in the pesticide industry especially since the biotechnological development of GLY-resistant crops during the 90's of the last century (Benbrook, 2016). As a result of this steady growth in terms of global application rates, residues of this herbicide, as well as of its main degradation product (aminomethylphosphonic acid - AMPA), have been frequently detected in urban and agricultural soils from all over the world, including in Europe (Maggi et al., 2020). In fact, based on a recent report, GLY and AMPA were found in 21 and 42%, respectively, of samples collected from agricultural soils of 11 European countries (Silva et al., 2019). Moreover, according to Geissen et al. (2021), GLY and AMPA were the most frequent and abundant compounds in topsoils from Portugal, Spain and the Netherlands.

When in the soil, the fate and behaviour of GLY are determined by the joint action of different variables, such as physicochemical properties of the soil, with regard to mineral composition, texture, organic matter (OM) and pH, and soil microbial diversity and activity (Gimsing et al., 2007; Laitinen et al., 2006; Sørensen et al., 2006). Likewise, other aspects, including soil water content and temperature, have also been found to modulate GLY persistence and degradation, whose half-life in the soil can vary from several weeks to months or even one year (DT_{50} under field conditions around 23 d; <http://sitem.herts.ac.uk/aeru/iupac/Reports/373.htm>) (Bento et al., 2016). GLY is recognised for being quickly degraded by the action of microorganisms and for its ability to adsorb to different soil components. Accordingly, adsorption is often stated to have a major influence on pesticide behaviour and environmental fate (Wauchope et al. 2002), determining its bioavailability and movement in soils (Pérez-Lucas et al., 2021). Due to the high hydrophobicity of most pesticides, they are usually adsorbed by OM. In contrast, GLY differs from the majority of these compounds, as the three polar functional groups (carboxyl, amino, and phosphonate) in its chemical structure and its high hydrophilicity make it ideal for interacting with inorganic elements such as aluminum (Al) and iron (Fe)

(Borggaard and Gimsing, 2008), rather than with organic complexes. Thus, it is often stated that soil OM does not play a role in the fate of GLY in the soil (De Jonge et al., 2001; Gerritse et al., 1996; Mamy and Barriuso, 2005). However, previous reports have suggested interaction between humic acids and GLY (Albers et al., 2009). Even so, it should be stressed out that such findings cannot be easily transposed to all soils, which hampers the understanding of the role of OM in mediating GLY adsorption (Albers et al., 2009). Albers et al. (2009) also reported that, after 80 d, almost 40% of GLY was adsorbed to humic and fulvic acids in sandy soils, despite these interactions being more easily broken than those between GLY and inorganic elements. Considering these outputs, additional data exploring the behaviour and possible risks of GLY towards non-target species in soils differing in their OM content is a matter of special interest.

In the last decade, a growing number of publications has been advising that soil contamination by GLY may result in toxicity to non-target plants, capable of significantly inhibiting their development (Gomes et al., 2017, 2016; Shahid and Khan, 2018; Zhong et al., 2018). Based on different studies, GLY-mediated impacts on plant growth can arise as a consequence of its direct herbicidal action, by blocking one of the key-steps of the shikimate pathway, but also as a result of its interference with other physiological and biochemical processes, such as nutritional status, redox homeostasis and photosynthetic performance (Gomes et al., 2014). Indeed, our research group, along with other remarkable studies in the field, have been providing evidence that GLY residues in soil greatly compromise plant growth (Fernandes et al., 2020; Soares et al., 2020, 2019b). Thus, recognising the leading position of GLY in the agroeconomic scenario, new tools need to be developed to limit its non-target phytotoxicity, thereby protecting crops and other important plant species that should be part of the agroecosystems, in particular under conservation agriculture practices. Within this perspective, the main objective of the current study was to assess the potential of OM in limiting GLY bioavailability to plants, using *Solanum lycopersicum* L. (tomato plant) as a model crop species. For achieving this goal, some questions have been raised: 1) How is GLY phytotoxicity governed by soil OM? 2) Can the enrichment of soil with OM be an effective tool to reduce GLY phytotoxicity to non-target species? To answer these questions, tomato seedlings grew in soils with different levels of OM and contaminated by GLY (10 mg kg⁻¹) for one month. After the growth period, plants were used to assess growth parameters, as well as biochemical indicators related to oxidative metabolism [hydrogen peroxide – (H₂O₂), lipid peroxidation (LP), proline and sugars] and physiological status [total proteins and amino acids, and total activity of nitrate reductase (NR; EC 1.6.6.1) and glutamine synthetase (GS; EC 6.3.1.2)].

2. MATERIAL AND METHODS

2.1. Preparation of the artificial soil and GLY treatments

In this study, an artificial soil, composed of quartz sand, kaolin and sphagnum peat, was prepared in accordance with standard guidelines (OECD, 1984) and used as substrate for plant growth. The percentage of sphagnum peat was changed to prepare soils with 2.5, 5, 10 and 15% (m/m) OM. Upon the manual preparation, batches of soil (1 kg) were: i) hydrated with the volume of deionised water (dH₂O) required to attain a maximum water capacity (WHC_{max}) of 40%; or ii) hydrated with the same volume of dH₂O, to which 10 mL of a GLY stock solution (1 g L⁻¹), prepared from a commercial formulation [RoundUp®UltraMax (Bayer, Germany); 360 g L⁻¹ GLY], were added to obtain a final concentration of 10 mg kg⁻¹ GLY. The pH and OM content of each soil were determined by ignition according to ISO (2005) and SPAC (2000), respectively. Afterwards, and considering the half-life of GLY in soils (mean values around 23 d in the field; <http://sitem.herts.ac.uk/aeru/iupac/Reports/373.htm>), a stabilization period of two weeks was followed. Since soil residues of this herbicide have been found in the range of mg kg⁻¹ (Primost et al., 2017; Peruzzo et al., 2008), reaching levels as high as 40 mg kg⁻¹ (Karanasios et al., 2018; Muñoz et al., 2019), the tested concentration of GLY is considered environmentally relevant, and was selected based on our previous studies (Soares et al., 2020, 2019b).

2.2. Plant material, growth conditions and experimental setup

Seeds of *Solanum lycopersicum* L. cv. Micro-Tom, obtained from FCUP's botanical collection, were surface disinfected with 70% (v/v) ethanol for 7 min, and with 20% commercial bleach (containing 0.5% active chlorine), supplemented with 0.05% (v/v) Tween-20, for 7 min, followed by a cleanup series with dH₂O to remove the excess of disinfectants. Then, seeds were randomly selected and germinated under *in vitro* conditions in Petri plates, containing half-strength MS (Murashige and Skoog, 1962) medium solidified with 0.625 % (m/v) agar. After 10 d in a growth chamber (25 °C, 16 h light/8 h dark, 120 μmol m⁻² s⁻¹), seedlings were transferred to plastic pots containing 200 g_{dw} OECD soils with different levels of OM, contaminated or not by 10 mg GLY kg⁻¹. At this point, different experimental groups were considered, as illustrated in Figure 1. For all situations, a total of four experimental replicates were considered, each one with five plants. Assays were conducted for 28 d in a growth chamber with controlled conditions, as described above. At the end of the growth period, plants were collected, separated into shoots and roots and immediately used for the assessment of biometric parameters (organ

elongation and fresh biomass production). Then, the plant material was frozen and grinded under liquid nitrogen and stored at -80 °C for biochemical endpoints.

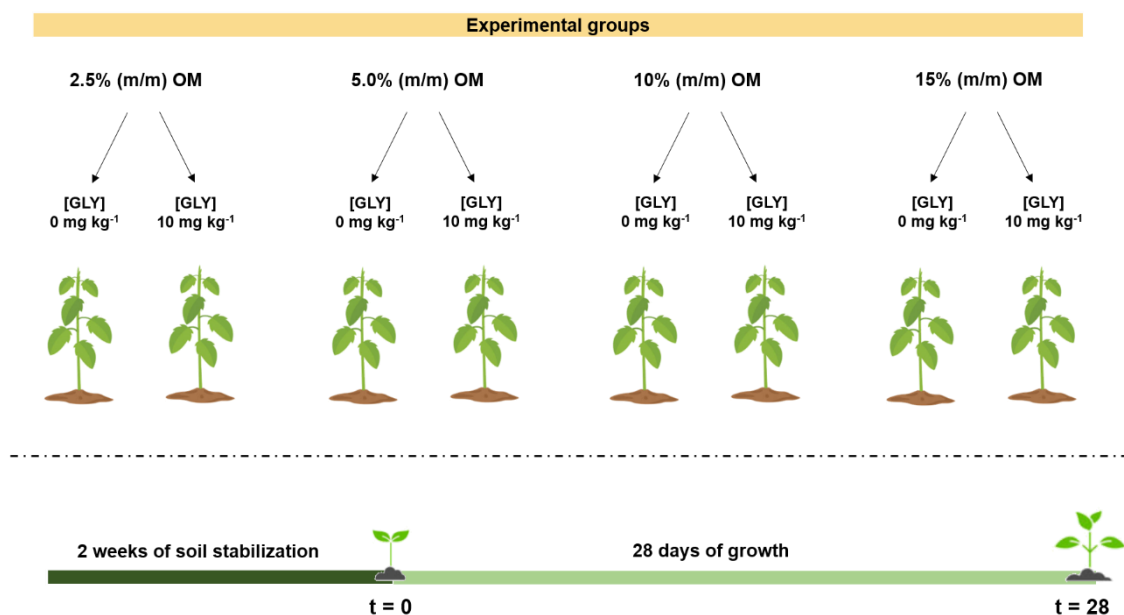


Figure 1. Graphical representation of the experimental design of the current research. Soils containing increasing levels of OM [2.5, 5.0, 10 and 15% (m/m)] were contaminated, or not, by GLY at 10 mg kg⁻¹. After a two-week stabilization period, seedlings of tomato plants were sown in each soil and grown for 28 d under controlled conditions.

2.3. Evaluation of the redox status – lipid peroxidation (LP), hydrogen peroxide (H₂O₂), and proline

Malondialdehyde (MDA), a sub-product of LP, and H₂O₂ were quantified according to the procedures of Heath and Packer (1968) and Alexieva et al. (2001), respectively. For this, shoot and root aliquots (0.200 g) were extracted with 0.1% (m/v) trichloroacetic acid (TCA), using a bead miller homogeniser (Bead Ruptor 12, OMNI, INC[®]), and centrifuged for 15 min at 10 000 *g*. Then, for MDA quantification, samples were mixed with 0.5% (m/v) thiobarbituric acid in 20% (m/v) TCA and incubated, for 30 min, at 95 °C. Lastly, after a cool-down period, the absorbances of each sample were registered at 532 and 600 nm, being the values of the latter subtracted from those of the former to minimise unspecific turbidity effects. MDA levels were calculated based on the molar extinction coefficient (ϵ) of 155 mM⁻¹ cm⁻¹, and the results were expressed as nmol g⁻¹ fresh mass (f.m.).

Regarding H₂O₂, the extracts reacted with 1 M potassium iodide (KI) for 1 h, at dark conditions, and the absorbance at 390 nm was then registered. Levels of H₂O₂ were estimated based on a linear standard curve, prepared with fresh solutions of H₂O₂, and results expressed on a f.m. basis.

The extraction and quantification of proline were performed as previously described by Bates et al. (1973). Briefly, frozen samples of shoots and roots (ca. 0.200 g) were homogenised in 3% (m/v) sulphosalicylic acid, using a bead mill homogeniser (Bead Ruptor 12, OMNI, INC[®]), and centrifuged for 15 min at 15 000 *g*. Afterwards, the supernatant (SN) reacted, in acidic conditions, with ninhydrin for 1 h at 96 °C, followed by an extraction with toluene to obtain the proline-ninhydrin complex. Lastly, absorbances were registered at 520 nm and the results were calculated based on a calibration curve, prepared with standard solutions of proline, and expressed in a f.m. basis.

2.4. Evaluation of physiological endpoints

2.4.1. Quantification of total soluble sugars

Total soluble sugars were quantified from shoots and roots of tomato plants according to Irigoyen et al. (1992), using frozen aliquots of around 0.200 g. After an extraction with 80% (v/v) ethanol in a bead mill homogeniser (Bead Ruptor 12, OMNI, INC[®]), samples were incubated for 10 min at 50 °C and centrifuged (7 500 *g*; 20 min). Then, 100 µL of SN, properly diluted in 80% (v/v) ethanol, were added to 1.5 mL anthrone, and the mixtures incubated at 100 °C for 10 min. At the end, the absorbance of each sample was read at 625 nm. The results were calculated from a linear standard curve, prepared with solutions of known-concentration of glucose, and data were expressed as µg g⁻¹ f.m..

2.4.2. Quantification of total amino acids and soluble protein

The quantification of total amino acids was based on the method of Lee and Takahashi (1966), being the extraction procedure identical to that of total sugars (2.4.1). Upon centrifugation, 75 µL of SN were added to 1430 µL of a reaction solution, containing 1% (m/v) ninhydrin, 99% (v/v) glycerol and 0.5 M sodium citrate buffer (pH 5.5) in a proportion of 5:12:3, respectively. Samples were incubated for 15 min at 100 °C and subsequently cooled on ice. Lastly, the absorbance was registered at 570 nm. Total amino acid levels were calculated through a linear standard curve, prepared with standard solutions of glycine. Results were expressed in a f.m. basis.

Total soluble protein was extracted from roots and shoots (0.200 g) of tomato plants by homogenisation of the samples, under cold conditions, in an appropriate extraction buffer, composed of 50 mM HEPES-KOH buffer (pH 7.8), 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 mM magnesium chloride (MgCl₂) and 1% (m/v) polyvinylpolypyrrolidone (PVPP). The homogenization was performed in the Bead Ruptor 12, OMNI, INC[®]. Then, extracts were centrifuged for 25 min, at 15 000 *g* and 4 °C, and the obtained SN were used

to quantify the total soluble protein, as formerly described by Bradford (1976). Results were calculated from a standard curve, prepared with stock solutions of bovine serum albumin (BSA) and expressed as $\text{mg g}^{-1} \text{ f.m.}$.

2.5. Extraction and quantification of N metabolism-related enzymes activity

2.5.1. Glutamine synthetase (GS; EC 6.3.1.2)

The extraction and quantification of GS was accomplished by following the protocols described by Martins et al. (2020) and Ferguson and Sims (1971), respectively. Briefly, plant samples were homogenised in an appropriate extraction buffer [25 mM Tris-HCl (pH 6.4), 10 mM MgCl_2 , 1 mM dithiothreitol (DTT), 10% (v/v) glycerol, 0.05% (v/v) Triton X-100 and 1% (m/v) PVPP], using a bead mill homogeniser (Bead Ruptor 12, OMNI, INC[®]), under cold conditions. After centrifugation (15 000 g ; 20 min; 4 °C), SNs were collected and used to determine the total protein content (as in 2.4.2.) and GS activity. Enzyme activity levels were evaluated by the transferase reaction, based on a colorimetric assay, in which the production of γ -glutamylhydroxamate can be monitored at 500 nm. The assays were scaled-down to UV-microplates (Greiner UV-Star) and the absorbances were read in a microplate reader (Thermo Scientific™ Multiskan™ GO). GS activity levels were estimated by linear regression from a standard curve according to the total amount of γ -glutamyl hydroxamate produced and expressed as $\mu\text{mol min}^{-1} \text{ mg}^{-1} \text{ protein}$.

2.5.2. Nitrate reductase (NR; EC 1.7.5.1)

NR was extracted and quantified from shoots and roots according to Kaiser and Brendle-Behnisch, (1991). For that purpose, samples were homogenised and centrifuged as previously described (2.4.2). Then, the activity of NR was evaluated through enzyme kinetics, by following the consumption of NADH at 340 nm for 2 min, in 5 s-intervals. As in GS, the assays were scaled-down to UV-microplates (Greiner UV-Star) and the absorbance variation was monitored in a microplate reader (Thermo Scientific™ Multiskan™ GO). NR activity values were calculated by using the ϵ of NADH ($6.22 \text{ mM}^{-1} \text{ cm}^{-1}$) and the results were expressed as $\text{mmol NADH min}^{-1} \text{ mg}^{-1} \text{ protein}$.

2.6. Statistical analyses

All biometrical and biochemical parameters were performed in, at least, three independent replicates ($n \geq 3$), and the results expressed as mean $\pm$ standard deviation (SD). Prior to any statistical analyses, data were checked regarding normality and homogeneity

assumptions. In order to check for significant differences between factors (OM – 2.5, 5, 10 and 15%; GLY – 0 and 10 mg kg⁻¹), a two-way ANOVA was performed, assuming a significance level (α) of 0.05. Whenever $p \leq 0.05$, Tukey's *post-hoc* test was used to identify differences between groups. In case of significant interactions, a correction for simple main effects was performed. All statistical data were generated in Prism® 8 (GraphPad, San Diego, California, USA). Details on the results of the ANOVAs can be found in the Supplementary Material (Tables S1 and S2).

3. RESULTS

3.1. Biometrical assessment

After 28 d of growth, plant development was significantly modulated by the combination of both factors – OM and GLY – as evidenced in Figures 2 and 3 and Tables S1 and S2. Soil OM did not significantly change the growth of plants alone, *i.e.* in the absence of GLY (0 mg kg⁻¹ GLY). However, as can be observed, the exposure of *S. lycopersicum* to 10 mg kg⁻¹ GLY resulted in an overall reduction of plant growth (Figure 2), manifested by inhibitions in root elongation (Figure 3c) and root and shoot fresh biomass production (Figure 3b,d).

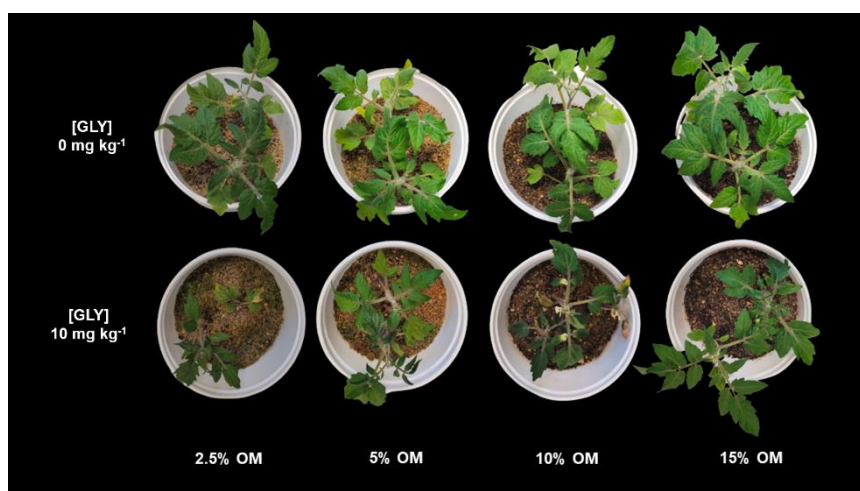


Figure 2. Visual effects of GLY (10 mg kg⁻¹) on the growth of *Solanum lycopersicum* L. cv. Micro-Tom grown in OECD soils containing increasing contents of OM [2.5, 5.0, 10 and 15% (m/m)] for 28 d. 0 mg kg⁻¹ GLY – plants grown in the absence of GLY; 10 mg kg⁻¹ GLY – plants grown in the presence of GLY.

The increasing levels of OM in the soil partially contributed to decrease GLY phytotoxicity, as plants exposed to the herbicide in OM-enriched soils [especially 15% (m/m)] showed an enhanced growth, when compared to soils with lower levels of OM (Figure 2). As illustrated, root fresh biomass of GLY-exposed plants in soils with 10 and 15% OM did not significantly vary from the treatments without GLY with the same level of OM, pointing for a reduction of the bioavailability of GLY to non-phytotoxic levels. In the presence of GLY, root and shoot fresh biomass was decreased by 48 and 30%, respectively, in plants exposed to GLY in soils with the lowest (2.5%) OM content, in relation to those with the highest (15%) (Figure 3b,d). When compared with uncontaminated soils, plants exposed to the herbicide upon the presence of 2.5% OM had their growth decreased, with reductions of 55% (root length), 54% (root biomass) and 47% (shoot biomass).

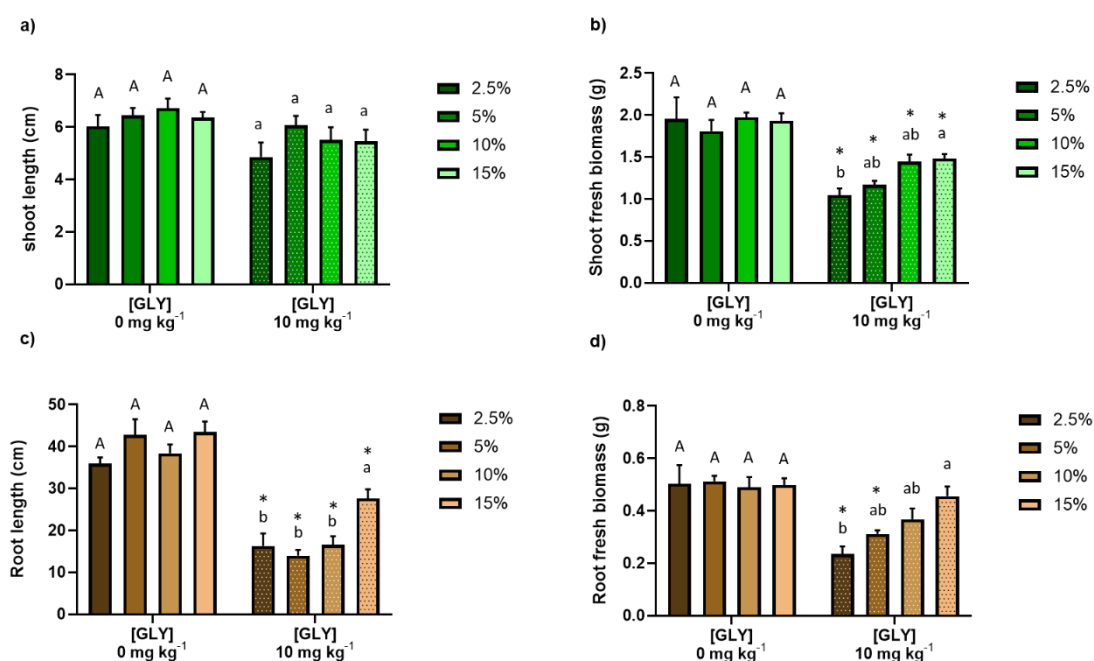


Figure 3. Shoot and root length (a,c) and fresh biomass (b,d) of *S. lycopersicum* L. cv. Micro-Tom grown for 28 d in OECD soils containing increasing contents of OM [2.5, 5.0, 10 and 15% (m/m)]. 0 mg kg⁻¹ GLY – plants grown in the absence of GLY; 10 mg kg⁻¹ GLY – plants grown in the presence of GLY. Results are expressed as mean $\pm$ SD ($n \geq 3$). Different letters above bars indicate differences between soils with different OM contents for each group (uppercase letters – without GLY; lowercase letters – GLY) (Tukey: $p \leq 0.05$). * above bars indicate significant differences between treatments with and without GLY for each OM level [2.5, 5.0, 10 and 15% (m/m)] (Tukey: $p \leq 0.05$).

3.2. Redox status – LP, H₂O₂ and proline

According to the ANOVA results, significant effects were found in LP for both factors and for their interaction (Tables S1 and S2), in shoots and roots of tomato plants. Based on

the data collected, soil OM *per se* did not significantly change MDA levels in the absence of GLY (0 mg kg⁻¹ GLY). Nevertheless, under GLY contamination, a clear pattern could be observed in both shoots and roots – as the soil OM increases, MDA tend to decrease to levels similar to those found in the treatments without GLY, especially in shoots. However, the same is not observed when only 2.5% of OM is added to soils, as shown in Figure 4a. In roots, although no upsurges were found, a significant reduction (46%) of MDA levels was found for GLY-treated plants grown in soils with 15% OM, not only when compared with GLY treatments with 5% OM, but also with uncontaminated soils (Figure 4b).

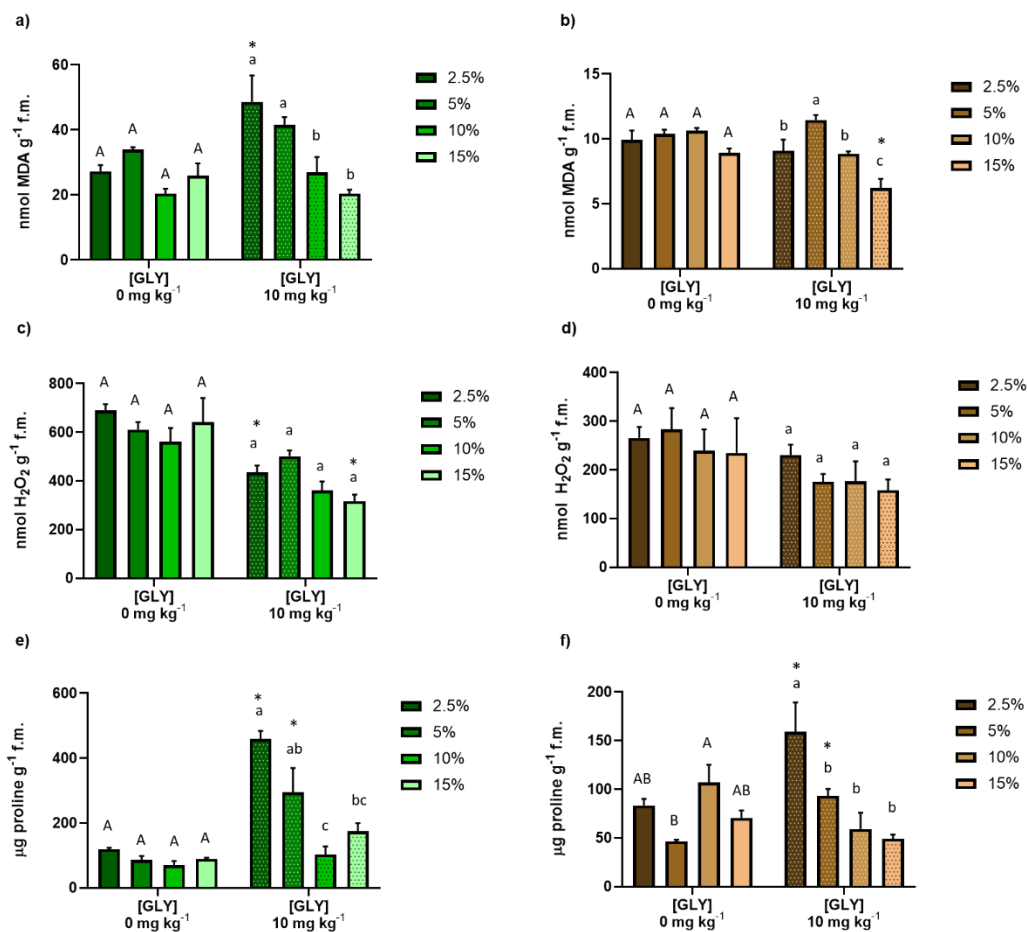


Figure 4. MDA (a,b), H₂O₂ (c,d) and proline (e,f) levels of shoots (green bars) and roots (brown bars) of *S. lycopersicum* L. cv. Micro-Tom grown for 28 d in OECD soils containing increasing contents of OM [2.5, 5.0, 10 and 15% (m/m)]. 0 mg kg⁻¹ GLY – plants grown in the absence of GLY; 10 mg kg⁻¹ GLY – plants grown in the presence of GLY. Results are expressed as mean ± SD (n ≥ 3). Different letters above bars indicate differences between soils with different OM contents for each group (uppercase letters – without GLY; lowercase letters – GLY) (Tukey: $p \leq 0.05$). * above bars indicate significant differences between treatments with and without GLY for each OM level [2.5, 5.0, 10 and 15% (m/m)] (Tukey: $p \leq 0.05$).

Concerning H_2O_2 accumulation, its levels are represented in Figure 4 and, as detailed in Tables S1 and S2, only GLY had a significant effect, particularly in shoots. As shown, H_2O_2 levels were diminished as a consequence of GLY exposure in shoots, and only the intermediary OM amendments (5 and 10%) were able to re-establish the levels found in non-contaminated soils (Figure 4c).

Proline accumulation was significantly changed by both factors and their interaction in shoots. The OM and the interaction between both factors also had a significant effect in roots of tomato plants (Tables S1 and S2). As Figure 4 suggests, in general, plants grown without GLY presented identical proline levels despite of the content of OM in shoots. However, in roots, a different pattern was observed, in which plants growing in soils with 5% OM were significantly different from those of 10% OM; yet, it is not excluded the hypothesis that such difference may have occurred by chance. Concerning GLY-mediated impacts, an overall trend for proline levels to be increased in shoots and roots of tomato plants grown in soils with the lower OM contents (2.5 and 5%) was found, with rises up to 2.8- (shoots) and 1-fold (roots) in relation to the treatment without GLY and the same level of OM. On the contrary, Pro levels remained unaffected in plants exposed to GLY in OM-enriched soils (10 and 15%) when compared with GLY-free treatments (Figure 4e,f).

3.3. Physiological indicators – total sugars, total amino acids and soluble protein

The levels of total soluble sugars were significantly changed in response to both factors in shoots, while in roots significant effects were observed for OM and its interaction with GLY (Tables S1 and S2). In general, the same pattern was observed for both organs (Figure 5a,b). In the absence of the herbicide, increasing levels of OM resulted in a lower accumulation of soluble sugars in shoots and roots, with significant decreases up to 57%, when plants were grown in soils with 15% OM (Figure 5a,b). Upon GLY exposure, the effects of OM prevailed, with plants from enriched soils showing lower values of soluble sugars. For amendments of OM > 2.5% GLY did not contribute for decreasing even more total sugars, as no differences were found when compared with treatments without GLY (Figure 5a,b).

Total amino acid content was significantly changed in shoots (significant effects for OM and interaction; Table S1). As can be seen, as the soil OM increases so does the total content in amino acids upon exposure to GLY (Figure 5c,d). With effect, at the highest OM content (15%), total amino acid levels in shoots significantly surpassed those registered in the absence of the herbicide (Figure 5c,d). In roots, significant differences were found for

the factor GLY, but, according to the multiple comparison tests, such effects were not relevant to the goal of the current study.

Concerning total protein content, data suggested that both OM and GLY did not significantly affect this endpoint in shoots and roots (Figure 5e,f), although a significant interaction between both factors was found in shoots (Tables S1 and S2). Yet, this interaction did not result in any significant difference relevant to the scope of the present research (Figure 5e).

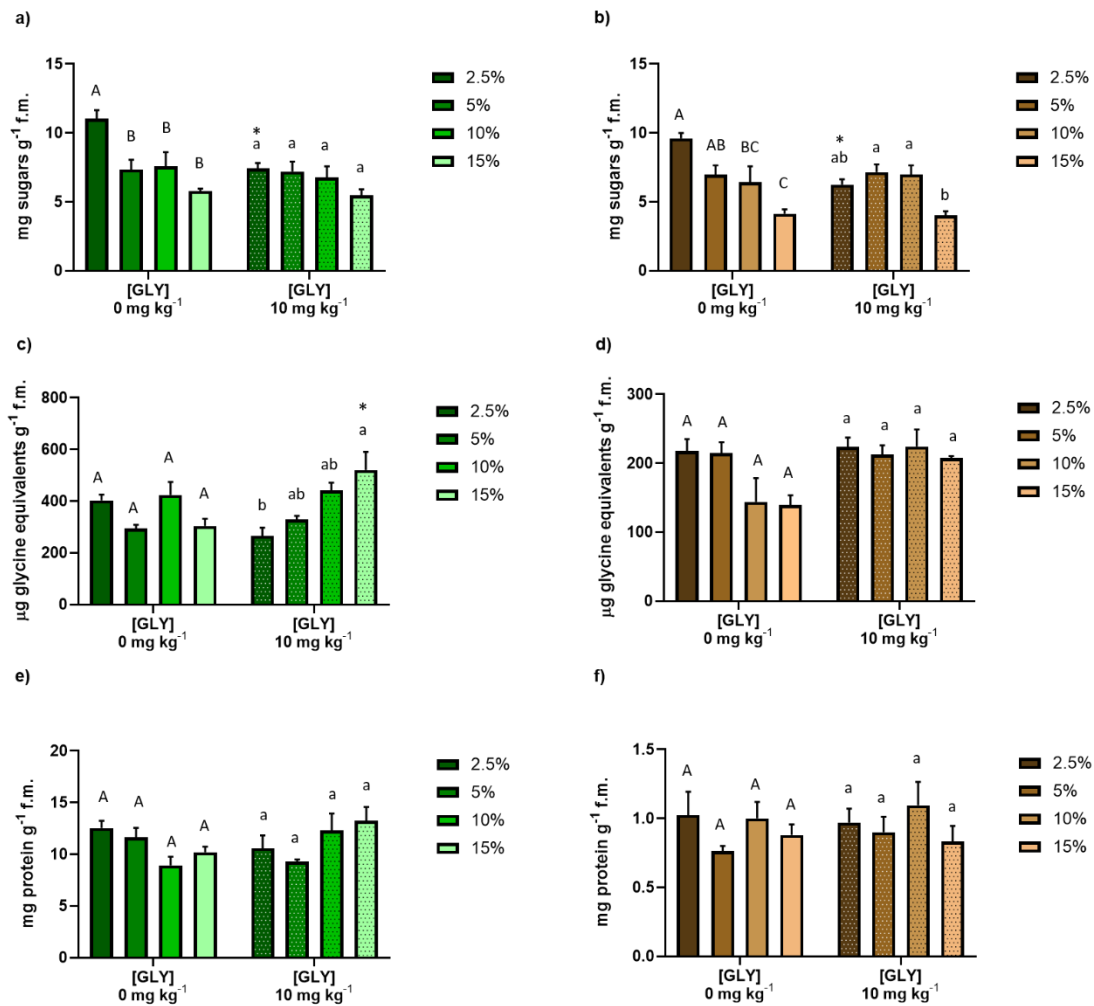


Figure 5. Soluble sugars (a,b), amino acids (c,d) and protein (e,f) levels of shoots (green bars) and roots (brown bars) of *S. lycopersicum* L. cv. Micro-Tom grown for 28 d in OECD soils containing increasing contents of OM [2.5, 5.0, 10 and 15% (m/m)]. 0 mg kg⁻¹ GLY – plants grown in the absence of GLY; 10 mg kg⁻¹ GLY – plants grown in the presence of GLY. Results are expressed as mean ± SD (n ≥ 3). Different letters above bars indicate differences between soils with different OM contents for each group (uppercase letters – without GLY; lowercase letters – GLY) (Tukey: *p* ≤ 0.05). * above bars indicate significant differences between treatments with and without GLY for each OM level [2.5, 5.0, 10 and 15% (m/m)] (Tukey: *p* ≤ 0.05).

3.4. N metabolism-related enzymes – NR and GS

The activity of NR was only significantly changed by the OM content under the absence and presence of the herbicide, as detailed in Tables S1 and S2 and Figure 6. As can be observed, in shoots, plants from soils with the highest levels of OM (10 and 15%) showed an enhanced activity of NR, in relation to those grown in soils with 2.5 and 5% OM (Figure 6a). The opposite was recorded for roots, as the highest OM levels contributed for decreasing NR activity. The effect of OM in both organs persisted even in the presence of GLY. (Figure 6b).

Lastly, GS activity was significantly modulated by the interaction of both factors in shoots, while, in roots, only OM contributed to the recorded differences (Tables S1 and S2). In the presence of GLY, the increasing level of OM reduced the activity of GS, with this being particularly relevant for shoots. In fact, in this organ, a decrease of around 70% was recorded for plants grown in soil amended with 15% of OM in comparison with plants exposed to the same level of OM, but in the absence of the herbicide (Figure 6c,d).

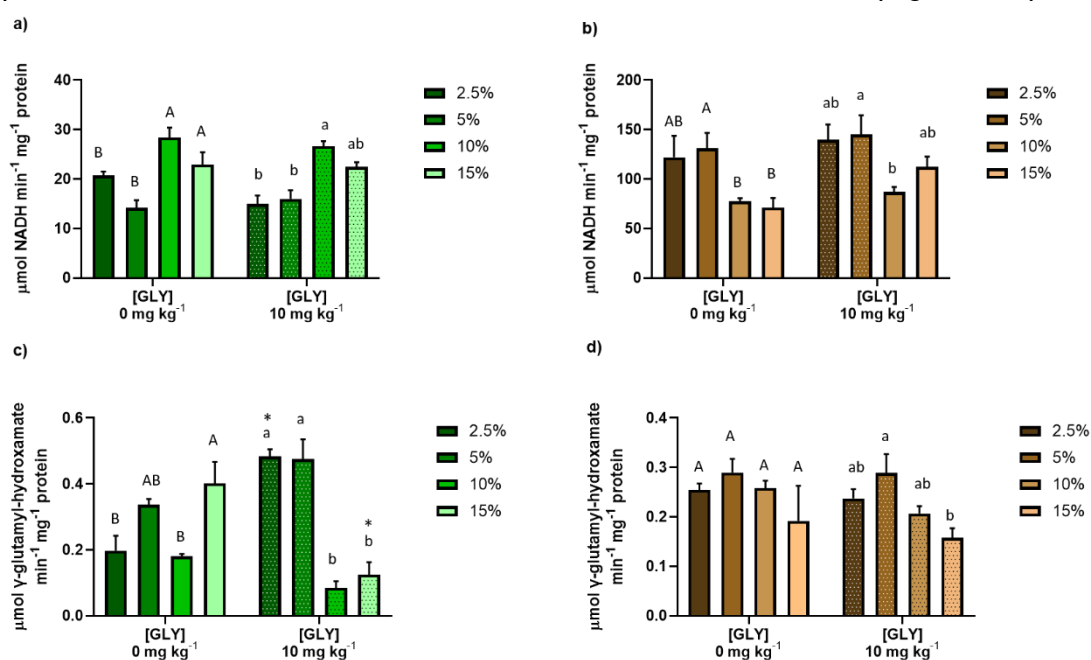


Figure 6. Activity levels of NR (a,b) and GS (c,d) in shoots (green bars) and roots (brown bars) of *S. lycopersicum* L. cv. Micro-Tom grown for 28 d in OECD soils containing increasing contents of OM [2.5, 5.0, 10 and 15% (m/m)]. 0 mg kg⁻¹ GLY – plants grown in the absence of GLY; 10 mg kg⁻¹ GLY – plants grown in the presence of GLY. Results are expressed as mean ± SD (n ≥ 3). Different letters above bars indicate differences between soils with different OM contents for each group (uppercase letters – without GLY; lowercase letters – GLY) (Tukey: $p \leq 0.05$). * above bars indicate significant differences between treatments with and without GLY for each OM level [2.5, 5.0, 10 and 15% (m/m)] (Tukey: $p \leq 0.05$).

4. DISCUSSION

OM is known to play a key role in modulating pesticides' mobility and persistence in soils, generally exhibiting binding sites for adsorption events (Pérez-Lucas et al., 2021). Despite the polar nature of GLY, and its preferential adsorption to inorganic materials, such as clays and Al and Fe ions, previous research, conducted under laboratory and field conditions, has suggested that OM constituents, such as humic and fluvic acids, can also play a role in the immobilization of GLY residues in soil (Albers et al., 2009; Yu and Zhou, 2005). Therefore, soil enrichment in OM can represent an effective practice to reduce the impacts of GLY contamination in non-target plants. Accordingly, the main goal of this work was to evaluate the role of OM in the modulation of GLY phytotoxicity, using tomato plants as a model non-target crop species.

Growth-related parameters

When evaluating the non-target effects of pesticides on plants, two of the most relevant endpoints are fresh and/or dry biomass and organ's elongation, as they express the impacts on biomass production (OECD, 2006). Here, and in accordance with our previous records (Soares et al., 2020, 2019b), which allowed us to select the appropriate herbicide concentration, results showed that GLY residues (10 mg kg⁻¹) in soils greatly compromise *S. lycopersicum* growth, with adverse impacts on both roots and shoots. Although GLY is typically applied at the foliar level, and should not pose a threat when in soil, recent evidence has been suggesting that root cells are able to uptake GLY from the soil solution, since it competes with phosphate for the same transporters (Gomes et al., 2014). Afterwards, GLY is rapidly transported to highly active metabolic tissues, such as shoot and root apices, where it starts to exert its toxicity (reviewed by Gomes et al., 2014). Aligned with this, and corroborating the data of the present study, environmental contamination by GLY also resulted in severe growth disorders in a wide range of non-target plants, including dicot (Fernandes et al., 2020; Khan et al., 2020; Singh et al., 2017b) and monocot species (Helander et al., 2019; Spormann et al., 2019).

Although studies dealing with soil GLY dynamics are relatively common, not much is known about the consequences of GLY adsorption to soil components for non-target plants. To the best of our knowledge, this is the first report exploring the role of soil OM in modulating GLY non-target phytotoxicity. Our data clearly suggested that GLY-mediated impacts were less pronounced in soils with a higher content of OM, reinforcing the premise that GLY can be adsorbed to the organic fraction of soil matrix, as previously hypothesized (Bai and Ogbourne, 2016 and references therein). Nevertheless, this study does not diminish the role of other mineral components of soil in retaining GLY residues. With effect,

a prior study found out that GLY adsorption in three soils with distinct physicochemical characteristics was mostly related to iron and aluminium amorphous oxides and OM (Morillo et al., 2000). From the obtained results, shoot and root growth, especially in what concerns biomass production, were particularly affected by GLY exposure mostly in soils with a lower OM input (2.5 and 5.0%). On the contrary, as the OM content of soil increased, the side negative effects of the herbicide were gradually reduced. Despite the absence of studies with an approach similar to ours, a previous work explored the sorption and desorption mechanisms of four common pesticides (2,4-dichlorophenoxyacetic acid, lindane, paraquat and GLY) in different soils. Based on their data, the sorption of all pesticides followed the Freundlich equation, which represents the adsorption of pesticides by soil OM and clay minerals (Copaja and Gatica-Jeria, 2021). Moreover, GLY was strongly adsorbed by muck soil samples, which are characterised for possessing a high content of OM, mainly peat (Cheah et al., 1997). Concomitantly, Yu and Zhou (2005) also found that GLY behaviour in soils was tightly associated with the levels of OM. Overall, by considering the biometrical endpoints assessed, one can suggest that soils enriched with OM, here provided as sphagnum peat (at 10% and especially at 15%), may help to prevent GLY-mediated risks to non-target crops most likely by decreasing herbicide mobility and bioaccumulation by plants. Given the microbial activity of the artificial soil used in this study, which is expected to be poor, it can be hypothesized that the reduction of GLY phytotoxicity by OM was mainly linked to adsorption events rather than by microbial degradation.

From complementary perspectives, other studies have also been addressing the impacts of soil amendment with other types of OM in the fate of pesticides in the soil. One of the most common examples is the use of biochar – composed by pyrolysed carbon, with a prolonged turnover in soils – which has been shown to limit pesticides' bioavailability, by increasing its adsorption and decreasing its presence in the soluble fraction of the soil (reviewed by Safaei Khorram et al., 2016). When studying the potential of biochar to improve wheat (*Triticum aestivum* L.) growth under soils contaminated by fomesan (herbicide), Meng et al. (2019) observed that concentrations up to 4% (m/m) biochar significantly reduced the bioaccumulation of the herbicide, accompanied by a better physiological status and growth performance of plants, in relation to non-amended soils.

The role of OM in preventing GLY-induced redox disorders

Although the primary effect of GLY is not strictly related to oxidative bursts in plant cells, increasing evidence suggest that this herbicide, once accumulated in plants, can affect the

redox homeostasis, altering the normal equilibrium between reactive oxygen species (ROS) generation and elimination (Gomes et al., 2014). Previous records from our research group clearly showed that tomato plants grown in GLY-contaminated soils (10, 20 and 30 mg kg⁻¹), containing 5.0% OM, underwent a situation of oxidative imbalance, through a deregulation between the production and scavenging of ROS (Soares et al., 2020, 2019b). However, to the best of our knowledge, how GLY-induced oxidative stress is modulated by soil OM is not yet known. Here, the redox homeostasis of tomato plants was significantly changed by the interaction between GLY and soil OM. With effect, the presence of higher levels of OM in the soil significantly improved the redox status of tomato plants under herbicide stress. In general, MDA, H₂O₂ and proline were decreased in soils contaminated with GLY but with higher OM contents (10 and 15%) reinforcing the premise that OM can, indeed, prevent GLY non-target phytotoxicity.

These beneficial effects on the plant redox status upon exposure of GLY in soils containing 10 and 15% OM can also be linked to the abundance of complex organic molecules present in the OM fraction of the soil. Actually, sphagnum peat, whose physical and chemical properties considerably vary with its botanical origin, environmental forming conditions and decomposition degree, is generally composed by bitumen, soluble sugars and phenolics, humic and fluvic acids, cellulose and lignin, and ash (Fuchsman, 2012). Thus, the higher abundance of phenolics and sugars in peat composition (Naumova et al., 2015) may have aided in plant defence against GLY. These metabolites are considered as powerful non-enzymatic antioxidants, being able to limit the burst of ROS in plant cells under unfavourable conditions, diminishing their toxic action towards lipids and proteins (Soares et al., 2019a). In contrast to these fairly positive outcomes, and following the pattern observed for the growth-related parameters, plants from soils with lower inputs of OM (2.5% and 5%) were much more sensitive to GLY action, with significant changes in the two oxidative stress markers assessed, especially in shoots, revealing the occurrence of redox disorders. Moreover, plants grown in soils with 2.5% OM contaminated with GLY had their total soluble sugar levels diminished in both organs. Soluble sugars are recognised as emerging components of the non-enzymatic antioxidant (AOX) system, due to their ability to react directly with various ROS (Soares et al., 2019a), besides their pivotal role in adenosine triphosphate (ATP) production through the cellular respiration. These carbohydrates, such as glucose and sucrose, can also induce the expression of genes that result in the production of other metabolites of the AOX system, aiding in the regulation of the redox homeostasis (Keunen et al., 2013). On the contrary, proline – one of the most important non-enzymatic AOXs in plant cells – revealed to be dramatically increased in GLY-contaminated soils containing 2.5 and 5.0% OM. The overaccumulation of this compatible solute seems to be a key signature symptom of GLY phytotoxicity, probably

indicating a higher sensitivity degree rather than a tolerance mechanism. This hypothesis was formerly raised in Fernandes et al. (2020) and further supported by Soares et al. (2021) and other worth-mentioning studies (Han et al., 2015; Singh et al., 2017b).

The influence of soil OM on the physiological status and N metabolism-related enzymes under GLY stress

Here, the results suggested that the protein and amino acid content did not greatly vary in response to the presence of the herbicide in the soil in both analysed organs. However, the amendment of GLY-contaminated soils with OM, especially 15%, led to an increase of total amino acids in shoots of tomato plants. Although protein catabolism during stress can result in an increased production of free amino acids, as deeply investigated by Batista-Silva et al. (2019), the observed rise of amino acids was not accompanied by a decrease of total protein, suggestive of a different phenomenon. Indeed, under stressful conditions, plants can rely on amino acids to serve as intermediates for the production of other secondary metabolites involved in signalling, defence and other cellular functions (Pratelli and Pilot, 2014). Moreover, there are other amino acids, rather than proline, whose role in stress responses is being increasingly popular, such as the case of cysteine and methionine (Soares et al., 2019a). According to different authors, these molecules can participate in multiple redox reactions, namely in the direct scavenging of ROS, such as H_2O_2 , due to the presence of a thiol (-SH) group in their molecular skeleton (Soares et al., 2019a). In fact, shoots of plants exposed to the herbicide in the soil with 15% OM exhibited a higher amino acid content followed by a significant reduction in the levels of H_2O_2 , with MDA levels not being changed. Alongside, Moldes et al. (2008), by studying the impacts of GLY application in resistant and susceptible soybean (*Glycine max* L.) genotypes, reported that amino acid levels were increased, probably as a mechanism to enhance the AOX efficiency of plants, since LP remained unaffected. Therefore, our results suggest that MO may have protected tomato plants from GLY phytotoxicity through diverse mechanisms, including by a stimulation of the production of other amino acids, rather than proline, with a possible role against oxidative stress.

Since GLY is recognised for affecting several physiological processes in plants, namely N metabolism (Gomes et al., 2014), focus was also paid to the modulation of N metabolism-related enzymes by GLY in soils differing in their OM content. For this purpose, the evaluation of the activity of two enzymes involved in the N assimilation were evaluated: NR, which catalyses the reduction of nitrate (NO_3^-) to nitrite (NO_2^-) (Rohilla and Parkash Yadav, 2020), and GS, responsible for incorporating the ammonia (NH_4^+) produced in the process of assimilating N or from photorespiration into amino acids

(Bernard and Habash, 2009). A recent study from our research team shown that the exposure of *Medicago sativa* L. plants to increasing concentrations of GLY (8-26 mg kg⁻¹) resulted in an inhibition of GS activity in shoots (Fernandes et al., 2020). Alongside, in the current work, the inhibition of GS caused by GLY was also more evidently recorded for shoots, but only for high levels of OM (10 and 15%), this being concomitant with a decrease of proline levels in shoots. In spite of the marked reduction in this enzyme activity, no major consequences were found in nitrogenous compounds in plants, since total amino acid and soluble protein levels were not decreased. On the other side, GS activity was significantly higher in shoots of plants exposed to GLY in the soil with the lowest OM level. This observation emerges perfectly aligned with the higher proline content also found in shoots of these plants. In fact, it is known that the accumulation of proline is closely related to the N cycle in plants (Díaz et al., 2010), since this amino acid is mostly produced from glutamate, especially under stress conditions (Brugière et al., 1995; Szabados and Savoure, 2010).

According to the results obtained in this work, the activity of NR was only affected by the OM content of the soil. Moreover, from what can be seen, a differential response was observed between roots and shoots – in the aerial parts of tomato plants, NR was stimulated in OM-enriched soils, which goes in agreement with the higher content of amino acids found; however, in roots, a sharp reduction in NR activity was observed in the soils with 10 and 15% OM. This finding seems quite controversial, since humic acids were already shown to induce the uptake of NO₃⁻ from the soil, by stimulating the gene expression of key transporters (Quaggiotti et al., 2004). However, the peat used in this study (H2-H4) is not particularly rich in humic acids, according to the scale of Von Post humification scale (Kellner, 2003), so direct extrapolations cannot be made. Moreover, as the levels of NR in leaves are commonly recognised for providing a direct assessment on the status of inorganic N in plants (Vaccaro et al., 2009), no major consequences regarding N metabolism are foreseen if plants grow in an OM-enriched soil, at least at the conditions herein tested, namely the duration of the assay.

5. CONCLUSIONS

Despite the undeniable role of GLY in agriculture, its accumulation in soils and its potential impacts on non-target species are an emerging issue which farmers have to deal with. Here, we have shown that soil OM differentially modulates GLY non-target phytotoxicity. Overall, tomato plants exposed to GLY in soils with a higher input of OM (10 and, especially, 15%) showed a higher growth performance and a better physiological status than those grown in OM-depleted soils (2.5 and 5.0%), which had their development

hampered in a wider extent (Figure 7). Based on the parameters assessed, high levels of OM in the soil, especially 15%, were effective in limiting GLY phytotoxicity either by promoting its adsorption and/or by preventing redox disorders, with no major impacts in N metabolism. Thus, our data strongly suggest that soil amendments with organic residues may provide an effective tool to reduce GLY-associated risks to agroecosystems, as described for other classes of pesticides (Carpio et al., 2021). Although in the present study OM was provided as sphagnum peat, as it is the organic component of the artificial soil [in accordance to the OECD (2006) standard protocols], from a practical and ecological point-of-view, other sources of organic carbon, rather than peat, must also be tested in the future, in order to minimise the impacts on bogs, essential players in the C sequestration (Hemes et al., 2019; <https://www.wildernesscommittee.org/peat>). Ultimately, by taking a leading step into a new direction, we hope to motivate further studies focused on the relationship between OM and GLY in the soil, namely the involvement of soil microbiome (Kepler et al., 2020) and the testing of agricultural soils in long-term experiments.

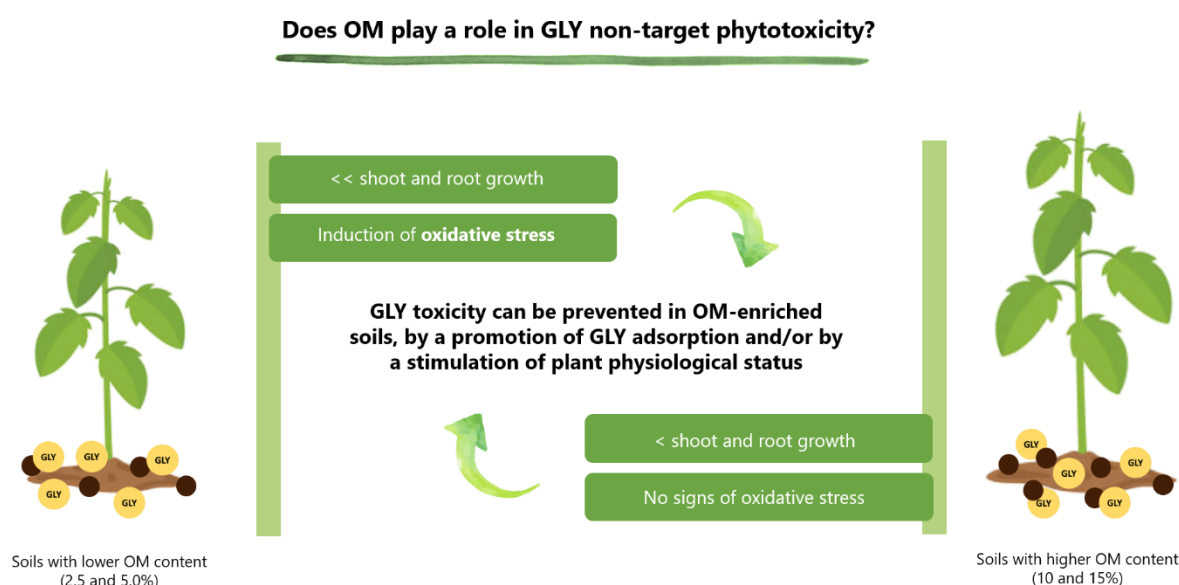


Figure 7. Overview of the main results obtained in this work.

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Supplementary Materials

Table S1. Results of the two-way ANOVA for all evaluated parameters in roots of *Solanum lycopersicum* L. cv. Micro-Tom grown for 28 d in OECD soils, contaminated or not by GLY (10 mg kg⁻¹) and containing increasing contents of OM [2.5, 5.0, 10 and 15% (m/m)]. Parameters where significant differences ($p \leq 0.05$) were recorded are highlighted at bold.

Parameter	Factors		Interaction
	GLY	OM	
Root length	F (1, 39) = 152.7; $p \leq 0.05$	F (3, 39) = 5.212; $p \leq 0.05$	F (3, 39) = 2.489; $p > 0.05$
Root biomass	F (1, 41) = 30.45; $p \leq 0.05$	F (3, 41) = 2.366; $p > 0.05$	F (3, 41) = 2.874; $p \leq 0.05$
Lipid peroxidation	F (1, 36) = 7.834; $p \leq 0.05$	F (3, 36) = 12.40; $p \leq 0.05$	F (3, 36) = 4.384; $p \leq 0.05$
H ₂ O ₂	F (1, 25) = 4.856; $p \leq 0.05$	F (3, 25) = 0.5385; $p > 0.05$	F (3, 25) = 0.2213; $p > 0.05$
Proline	F (1, 20) = 2160; $p > 0.05$	F (3, 20) = 8.827; $p \leq 0.05$	F (3, 20) = 9.437; $p \leq 0.05$
Total sugars	F (1, 34) = 2.446; $p > 0.05$	F (3, 34) = 13.92; $p \leq 0.05$	F (3, 34) = 4.335; $p \leq 0.05$
Total amino acids	F (1, 24) = 7.953; $p \leq 0.05$	F (3, 24) = 2.972; $p > 0.05$	F (3, 24) = 2.419; $p > 0.05$
Total protein	F (1, 19) = 0.1253; $p > 0.05$	F (3, 19) = 1.301; $p > 0.05$	F (3, 19) = 0.2704; $p > 0.05$
GS	F (1, 15) = 1.835; $p > 0.05$	F (3, 15) = 5.711; $p \leq 0.05$	F (3, 15) = 0.3506; $p > 0.05$
NR	F (1, 16) = 4.347; $p > 0.05$	F (3, 16) = 7.991; $p \leq 0.05$	F (3, 16) = 0.5207; $p > 0.05$

Table S2. Results of the two-way ANOVA for all evaluated parameters in shoots of *Solanum lycopersicum* L. cv. Micro-Tom grown for 28 d in OECD soils, contaminated or not by GLY (10 mg kg⁻¹) and containing increasing contents of OM [2.5, 5.0, 10 and 15% (m/m)]. Parameters where significant differences ($p \leq 0.05$) were recorded are highlighted at bold.

Parameter	Factors		Interaction
	GLY	OM	
Shoot height	F (1, 44) = 9.883; $p \leq 0.05$	F (3, 44) = 1.427; $p > 0.05$	F (3, 44) = 0.4474; $p > 0.05$
Shoot biomass	F (1, 87) = 64.96; $p \leq 0.05$	F (3, 87) = 2.534; $p \leq 0.05$	F (3, 87) = 1.537; $p > 0.05$
Lipid peroxidation	F (1, 29) = 6.155; $p \leq 0.05$	F (3, 29) = 7.632; $p \leq 0.05$	F (3, 29) = 3.095; $p \leq 0.05$
H ₂ O ₂	F (1, 28) = 35.43; $p \leq 0.05$	F (3, 28) = 1.949; $p > 0.05$	F (3, 28) = 1.576; $p > 0.05$
Proline	F (1, 28) = 32.38; $p \leq 0.05$	F (3, 28) = 8.070; $p \leq 0.05$	F (3, 28) = 4.997; $p \leq 0.05$
Total sugars	F (1, 37) = 5.945; $p \leq 0.05$	F (3, 37) = 8.211; $p \leq 0.05$	F (3, 37) = 2.623; $p > 0.05$
Total amino acids	F (1, 36) = 1.319; $p > 0.05$	F (3, 36) = 4.230; $p \leq 0.05$	F (3, 36) = 6.513; $p \leq 0.05$
Total protein	F (1, 16) = 0.5325; $p > 0.05$	F (3, 16) = 0.7741; $p > 0.05$	F (3, 16) = 4.545; $p \leq 0.05$
GS	F (1, 16) = 0.2067; $p > 0.05$	F (3, 16) = 17.53; $p \leq 0.05$	F (3, 16) = 19.68; $p \leq 0.05$
NR	F (1, 17) = 1.446; $p > 0.05$	F (3, 17) = 18.67; $p \leq 0.05$	F (3, 17) = 1.559; $p > 0.05$

CHAPTER VI. ECOTOXICOLOGICAL RELEVANCE OF GLYPHOSATE-BASED HERBICIDES

Ecotoxicological relevance of glyphosate and flazasulfuron to soil habitat and retention functions – single vs combined exposures

Abstract

In order to face the increasing weed resistance, farmers rely on repetitive applications of herbicides, resulting in their accumulation in the environment, where their residues exert non-target toxicity. Glyphosate (GLY) and flazasulfuron (FLA) are two non-selective herbicides commonly applied together in vineyards and crop fields. However, ecologically relevant research aimed at understanding their single and combined impacts from an ecotoxicological perspective is still scarce. Therefore, this study was designed to i) test the single effects of each compound on soil's habitat and retention functions; and ii) unravel the impacts of GLY and FLA co-exposure to earthworms and terrestrial plants, based on their application doses and relevant residue concentrations. For this purpose, ecotoxicological assays were performed to test the effects of both pesticides on terrestrial non-target species (higher plants – *Medicago sativa*; oligochaetes – *Eisenia fetida*; collembola – *Folsomia candida*). In parallel, soil elutriates were prepared to assess the effects towards freshwater aquatic organisms (macrophytes – *Lemna minor*; microalgae – *Raphidocelis subcapitata*). Results showed that FLA (up to 413 $\mu\text{g kg}^{-1}$) is much more toxic than GLY (up to 30 mg kg^{-1}). Increasing concentrations (82-413 $\mu\text{g kg}^{-1}$) of FLA greatly reduced the reproduction ability of earthworms and collembola and severely impaired the growth of *M. sativa*. In contrast, GLY only significantly affected plant growth ($\geq 9 \text{ mg kg}^{-1}$) and earthworms ($\geq 13 \text{ mg kg}^{-1}$), especially at high concentrations, with no effects on collembola. In what regards soil retention function, only elutriates prepared from FLA-contaminated soils significantly impacted the growth of *L. minor* and *R. subcapitata*, revealing a higher risk of this pesticide towards freshwater ecosystems. In a scenario of a co-exposure, GLY and FLA combined toxicity was comparable to that of FLA for earthworms, whilst for terrestrial plants, mixtures of both herbicides resulted in the amplification of their individual effects. Globally, this work underpins that the risk assessment of herbicides should take into consideration their mixtures, since the ecotoxicity of individual compounds may underestimate the effects under field-conditions.

Keywords

Agriculture; ecotoxicology; herbicides; non-target species; RoundUp; Zagaia.

1. INTRODUCTION

Modern agriculture is heavily dependent on the application of plant protection products (PPPs), such as pesticides, to control emerging diseases, animal pests, and weeds and to achieve high yield rates (Gunstone et al., 2021). Globally, from all applied pesticides, herbicides are the ones accounting for the largest market volume, representing almost 46% of the total sales (<https://www.fao.org/faostat/en/#data/RP/visualize>). Given the wide and massive application of herbicide formulations, weed resistance is arising as an emergent issue faced by farmers, which often rely on mixtures incorporating several active ingredients (a.i.) to maximise their efficacy. It is now estimated that almost 97% of farmers employ mixtures of several PPPs (Gazziero, 2015). These so-called “tank mixtures” – combination of two or more agrochemicals in a single application tank (Tornisiello et al., 2013) – have been gaining increasingly relevance, saving economical costs and labour hours (Gandini et al., 2020), while also allowing a stronger pest management control.

Based on Gandini et al. (2020), from all tank mixtures, 60% of them usually contain glyphosate (GLY)-containing formulations. GLY is, since its discovery during the 70s of the 20th century, the most applied herbicide worldwide. In terms of action, GLY interferes with the shikimate pathway by blocking the activity of 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS; EC 2.5.1.19), thus preventing the biosynthesis of aromatic amino acids (Gomes et al., 2014). Given that this biochemical route is exclusively found in plants and some microorganisms, GLY is allegedly environmentally safe, not posing a threat to other organisms besides plants, when applied according to manufacturers’ recommendations. However, overuse and cumulative practises are leading to environmental contamination of soils by GLY, whose levels can reach the mark of mg kg⁻¹ (Karanasios et al., 2018; Muñoz et al., 2019; Peruzzo et al., 2008; Primost et al., 2017). When accumulating in soils, GLY can suffer adsorption to soil particles, both of organic and inorganic nature, move to deeper soil fractions, or undergo a microbial-mediated degradation, arising the production of its main metabolite, aminomethylphosphonic acid (AMPA) (Kaniserry et al., 2019; Padilla and Selim, 2020). Although considered as the main players of GLY biodegradation, many species of bacteria and fungi also share the shikimate pathway (Van Bruggen et al., 2018), meaning that they can be affected by GLY (Arango et al., 2014; Banks et al., 2014; Cherni et al., 2015; Druille et al., 2016; Schafer et al., 2014), with potential impacts to the overall soil health and productivity, including the biodegradation of soils (Van Bruggen et al., 2018). Moreover, as a result of some agricultural practices, such as tillage and phosphorous (P) fertilization, GLY and AMPA can become bioavailable once again (Borggaard and Gimsing, 2008). In this way, emerging concerns about GLY, as well as AMPA, possible toxicity towards the environment have been raised. Still, no consensus

has been reached among scientists, with a lot of divergent data regarding their ecotoxicity (Niemeyer et al., 2018; Pereira et al., 2009; Pochron et al., 2020; Santos et al., 2012; Singh et al., 2020; Van Bruggen et al., 2018).

Flazasulfuron (FLA; N-[4,6-dimethoxypyrimidin-2-yl)-3-(3-trifluoromethyl-2-pyridylsulfonyl)urea]), is a recent herbicide, grouped into the sulfonylurea family, frequently applied together with GLY, especially on vineyards (Couderchet and Vernet, 2003). Concerning its herbicidal activity, FLA targets the enzyme acetolactase synthase (ALS; EC 2.2.1.6), present in plants and some microorganisms, which catalyses the first step of branched-chain amino acids biosynthesis, such as valine, leucine, and isoleucine. According to a document prepared by European Food Safety Authority (EFSA, 2016), FLA shows a low-to-medium persistence and a low-to-high mobility in soils, with a higher degradation under acidic conditions ($\text{pH} < 6$) (DT_{50} between 3 and 22 d). As a result of FLA's degradation, many metabolites can be formed, as extensively detailed in EFSA (2016). Also based on this report, FLA is expected to be safe for soil macro and mesofauna, including earthworms, soil mites and collembola. However, to the best of our knowledge, no scientific records dealing with FLA's ecotoxicity to non-target biota, such as soil and water organisms, are available, in particular for commercial formulations. Regarding a.i. itself, according to the International Union of Pure and Applied Chemistry (IUPAC), FLA-mediated toxicity towards soil invertebrates, such as oligochaetes and springtails, would only occur at concentrations in the range of mg kg^{-1} , with "*No Observed Effect Concentration*" (NOEC) for reproduction of 8 and 125 mg kg^{-1} , respectively (<http://sitem.herts.ac.uk/aeru/iupac/Reports/319.htm>).

Therefore, a systematic research, covering multiple trophic levels and environmental matrices, reporting the real consequences of GLY and FLA, either alone or in combination, at environmentally relevant concentrations, is urgently needed to clarify their ecotoxicological relevance and possible data needs. Within the frame of risk assessment, studies usually target single chemicals, not paying much attention to the potential effects of their combination with other compounds (Owagboriaye et al., 2020; Weisner et al., 2021). However, from an ecotoxicological perspective, focus must also be driven to herbicide-herbicide interactions (Bopp et al., 2016; Tornisiello et al., 2013), since the simultaneous action of different PPPs can result in different consequences towards non-target biota (Brühl and Zaller, 2019; Topping et al., 2020).

With this in mind, this study aims to obtain a clear and robust perception on the real hazards of GLY and FLA, both single and combined, to soil habitat and retention functions, covering different trophic levels. To reach this objective, some questions need to be answered: i) how does GLY, at environmentally relevant concentrations, affect non-target soil biota? ii) are earthworms capable of recolonise GLY-contaminated soils? iii) does FLA

represent a risk to soil non-target species and to freshwater ecosystems? iv) can the single effects of each pesticide underestimate their real ecotoxicity when their residues occur simultaneously in agricultural soils? To meet these goals, a series of experiments for each substance alone was designed, by evaluating their effects on terrestrial plants and invertebrates (earthworms and collembola), as well as the toxicity of their elutriates towards macrophytes and microalgae. The effects of a co-exposure scenario were assessed by evaluating impacts of both herbicides in plants and earthworms.

2. MATERIALS AND METHODS

2.1. Chemicals and test substrate

GLY and FLA were acquired from local suppliers in the form of commercial formulations – RoundUp® UltraMax (containing 360 g L⁻¹ GLY as potassium salt; Bayer®) and Zagaia (containing 250 g kg⁻¹ FLA; Ascenza®). Before testing, appropriate stock solutions of each herbicide were prepared in deionised water (dH₂O): GLY – 1 g L⁻¹; FLA – 100 mg L⁻¹. In terms of commercial purposes, GLY is described as a non-selective and post-emergent herbicide, while FLA can be used in both post- and pre-emergence scenarios, being directly applied to the soil. All assays were conducted in an artificial soil [70% (m/m) sand; 20% (m/m) kaolin; 10% (m/m) sphagnum peat; pH_{KCl} 6.0 ± 0.5], manually prepared according to the standard guidelines (OECD, 2006a).

2.2. Tested concentrations

To evaluate GLY and FLA single effects, a range of increasing concentrations for each herbicide was defined, based on i) recommended application doses and, ii) environmental relevance in the case of GLY (Karanasios et al., 2018; Peruzzo et al., 2008; Silva et al., 2018). No reports are available concerning FLA environmental levels. According to manufacturer's guidelines, the maximum application dosage of the herein tested commercial formulations of GLY and FLA is 10 L ha⁻¹ and 200 g ha⁻¹, respectively. Considering the area of the pots (110 cm²) used in the assays, these application rates correspond to 20 mg GLY kg⁻¹ and 413 µg FLA kg⁻¹. Thus, the following treatments were considered: GLY – 0, 6, 9, 13, 20 and 30 mg kg⁻¹; FLA – 0, 82, 122, 184, 275, 413 µg kg⁻¹. For co-exposure experiments, based on the reported GLY residues in soils (Primost et al., 2017; Peruzzo et al., 2008), the increasing concentrations of this herbicide (0-30 mg GLY kg⁻¹) were tested together with the recommended application dose of FLA (275 µg FLA kg⁻¹).

2.3. Ecotoxicological tests with soil organisms

2.3.1. Seedling emergence and growth tests

Seedlings' emergence and growth tests were performed based on OECD (2006a) guidelines. For this purpose, seeds of *Medicago sativa* L., a common cover crop species, were obtained from Flora Lusitana Lda (Cantanhede, Portugal). Before sowing, seeds were surface disinfected with 70% (v/v) ethanol and 20% (v/v) commercial bleach (containing 5% active chlorine), supplemented with 0.05% (m/v) Tween-20, for 7 min each, followed by five series of washing with dH₂O. Afterwards, 20 seeds were placed in plastic pots, containing 200 g_{dry} of artificial OECD soil (see section 2.1) previously spiked with the selected concentrations of each herbicide. Soil's maximum water holding capacity (WHC_{max}), determined as recommended by ISO (2005), was adjusted to 40%, being the volume of water required used to prepare a solution of each herbicide to obtain the desired soil concentrations. At the beginning of the assay, to ensure nutrients availability, 120 mL of Hoagland's nutritive solution (Taiz et al., 2015) were added to a cup placed under each pot, both communicating through a cotton rope. Throughout the assay, dH₂O was added whenever necessary to the cup. For each treatment, including the negative control (CTL; OECD soil without herbicides), four experimental replicates were prepared, obtaining a total of 24 pots per assay (GLY, FLA and mixtures). The assays started after the germination of, at least, 50% of the seeds from the control. After that, to avoid intraspecific competition, only 7 seedlings were left to grow for 21 d in each pot, under controlled conditions of light (photosynthetic photon flux density of 120 $\mu\text{mol m}^{-2} \text{s}^{-1}$), photoperiod (16 h light/8 h dark) and temperature (23 ± 1 °C). Upon the growth period, plants from each replicate were collected, separated into shoots and roots and used to record biometric parameters (shoot and root length) and fresh biomass. Then, the plant material was dried at 60 °C until constant weight and used to calculate the dry biomass.

2.3.2. Reproduction tests with *Eisenia fetida*

Oligochaetes of the species *Eisenia fetida* Savigny (Oligochaeta: Lumbricidae) were obtained from laboratory cultures [LabRisk; Faculty of Sciences of University of Porto (FCUP)] maintained under controlled conditions (temperature: 20 ± 2 °C; photoperiod: 16 h light/8 h dark). The individuals grew in plastic boxes in a medium composed of peat, horse manure and dH₂O, being fed defaunated horse manure (previously sterilised by autoclave – 121 °C; 30 min) and oat, moistened with dH₂O. The reproduction tests with *E. fetida* were carried out according to ISO (2012). For this purpose, plastic containers (11.7 cm in diameter and 13 cm in height) containing 500 g of OECD soil contaminated, or not,

by the selected concentrations of GLY, FLA and GLY + FLA were used. For each treatment, five experimental replicates were prepared, each with 10 individuals with well-developed clitella and a mass between 300 and 600 mg. Prior to the test, animals were acclimated for 48 h to OECD soil. At the end of 28 d, the adult oligochaetes were removed from the containers, while the cocoons were left for another 28 d. After this period, juveniles from each pot were removed and counted. During the test, earthworms were fed, once a week, with 5 g of defaunated horse manure per box. At the same time, the moisture content of the soil was controlled and regulated whenever necessary with dH₂O. According to the standardised protocol, some requirements must be met for the validation of the assay: the % of CTL (0 mg kg⁻¹) mortality must be less than 10% in the first four weeks of the trial; the number of juveniles in the controls must be greater than 30 for each replicate.

2.3.3. Recolonization tests with *Eisenia fetida*

In order to understand the ability of *E. fetida* to recolonise soils contaminated by GLY, after, for instance, a season of treatments with this herbicide, an experiment adapted from the avoidance test (ISO, 2008) was developed. For this purpose, plastic boxes (18 cm long, 5 cm high and 10 cm wide) were divided in half, adding 250 g of control soil (0 mg kg⁻¹) to one side and 250 g of contaminated soil to another. Then, 10 adult earthworms with a fresh mass between 300 and 600 mg were added per box in the control side. The selected individuals were acclimated to OECD soil under controlled conditions for at least 24 h before starting the experiment. After 48, 96 h and 7 d of exposure, the individuals present on each side were counted. Whenever an individual was found at the separation line, the direction of movement was taken into consideration. In the 7-day trial, to assess the feed activity of *E. fetida*, two bait-lamina per box were added to the GLY side. The use of bait-lamina, firstly developed by Torne (1990), allows to evaluate changes in the feeding activity of soil organisms to unravel the impacts of different contaminants. The preparation of the bait-laminas was performed as described elsewhere (André et al., 2009). For each concentration of GLY tested, four experimental replicates were prepared. A dual CTL was also included, where non-contaminated soil (0 mg kg⁻¹ GLY) was placed in both sides of the box.

2.3.4. Reproduction tests with *Folsomia candida*

Arthropods of the species *Folsomia candida* Willem (Collembola: Isotomidae) were obtained from laboratory cultures maintained under controlled conditions (temperature: 20 ± 2 °C; photoperiod: 16 h light/8 h dark), at LabRisk, FCUP. The individuals were kept in plastic containers in a mixture composed of moisten plaster of Paris and charcoal 8:1

(m:m), being fed with dry yeast granules, hydrated with dH₂O, twice a week. Before starting the test, cultures were synchronised to obtain juveniles aged 9 to 12 d. Reproduction tests were performed as described by ISO guidelines (ISO, 2014). Then, 10 individuals were placed in plastic pots containing 30 g of soil contaminated by the selected concentrations of GLY and FLA, tested independently. In parallel, a control situation, with no herbicides, was also prepared. For each concentration, five replicates were considered. Once a week, food was added, and the humidity adjusted with dH₂O. After 28 d, the number of adults and juveniles was counted. To enable an easier visualization, dH₂O and few drops of Chinese ink (for contrast increase) were added so that, after homogenisation, individuals could float. Digital images were acquired and processed using the Image J free software (<https://imagej.nih.gov/ij/download.html>). The validation of the assay assumes that control replicates cannot have a mortality higher than 20% and must have more than 100 juveniles.

2.4. Ecotoxicological tests with aquatic organisms

2.4.1. Preparation of soil elutriates

In order to evaluate the effects of GLY and FLA on soil's retention function, batches of OECD soil contaminated by the highest concentration of GLY (30 mg kg⁻¹) or FLA (413 µg kg⁻¹) were used to prepare soil elutriates. To this end, after the incorporation of the herbicide into the soil (added in the volume of water needed to attain 40% of the WHC_{max}), the samples were left for stabilization for 24 h (to test for a worst-case scenario of availability). Then, elutriates were obtained by preparing soil suspensions (1:4; m/v) with the adequate media for each test-species: Woods Hole for *Raphidocelis subcapitata* (Korshikov) Nygaard et al. (Nichols, 1973) and Steinberg medium for *Lemna minor* L. (OECD, 2006b). After 12 h of mechanical agitation under dark conditions, the suspensions were settled for 24 h. Thereafter, the liquid phase (elutriate) was collected and immediately tested. For each elutriate, a series of dilutions was defined, by applying a factor of 1.5, arising the final concentrations: 100, 66.7, 44.4, 29.6, 19.8 and 13.2% (v/v).

2.4.2. Growth inhibition tests with *Lemna minor*

Cultures of the macrophyte *Lemna minor* L. were maintained in Steinberg nutritive medium, under laboratory-controlled conditions of light (84 – 140 µmol m⁻² s⁻¹; white fluorescent lamps), photoperiod (16 h light/8 h dark) and temperature (21 ± 2 °C). Assays were performed as described in the standard protocol of OECD (2006b). At the beginning of the assay, three plants with three fronds each were selected from the culture and placed

in sterile plastic cups filled with 80 mL of each elutriate dilution (prepared in Steinberg medium). A total of three replicates for each dilution was considered. After 7 d of exposure, under the same conditions described above, the number of fronds of each replicate was counted.

2.4.3. Growth inhibition tests with *Raphidocelis subcapitata*

Individuals from the microalgae *Raphidocelis subcapitata* (Korshikov) Nygaard et al. were obtained from laboratory cultures, maintained in sterile nutritive medium [Woods Hole MBL; (Nichols, 1973)] with constant agitation and light intensity ($84 - 140 \mu\text{mol m}^{-2} \text{s}^{-1}$; white fluorescent lamps) under controlled conditions of temperature ($21 \pm 2^\circ\text{C}$). The effect of soil elutriates on this species was assessed based on the standard microalgae growth inhibition protocol of OECD (2011). Prior to the assay, a stock solution of microalgae [cultured for 72 h under controlled conditions (as above) with a photoperiod of 16 h light/8 h dark], was diluted in MBL until reaching a density of $10^5 \text{ cells mL}^{-1}$, calculated through optical microscopy with the aid of a Neubauer chamber. Assays were conducted in sterile 24-well microplates, considering a total of three replicates per dilution. In each well, 900 μL of elutriate were mixed with 100 μL of microalgae inoculum. The assays lasted 72 h and were kept under the same conditions described above. At the end, the optical density of each well was measured at 440 nm and the number of cells was calculated by linear regression using the following equation: Cells density (cells mL^{-1}) = $(\text{Abs}_{440 \text{ nm}} - 2.5 \times 10^{-3}) / 5.0 \times 10^{-8}$. The growth inhibition, expressed as %, was calculated in relation to the control samples.

2.5. Statistical analyses

All results are expressed as mean $\pm$ standard deviation (SD) and result from the evaluation of the corresponding independent replicates tested. Before any statistical analyses, data were checked for variances homogeneity (Levene's test). However, even when it was not possible to meet these criteria, parametric tests were still used given the robustness of ANOVA (Zhar, 1996). A one-way analysis of variance (one-way ANOVA), followed by Dunnet's *post-hoc* test, was used to assess significant differences between treatments and the control, assuming α value of 0.05. Concerning the recolonization experiments, the Fisher exact test was used to analyse 2x2 contingency tables (<http://graphpad.com/quickcalcs/contingency1.cfm>); a one-tailed test for each concentration of GLY and a two-tailed test for the dual controls were selected, assuming an identical distribution of earthworms between the two sides of the box.

3. RESULTS

3.1. Plant growth assays

As can be observed in Figures 1-3, the growth of *M. sativa* was negatively affected by the tested herbicides, both alone and in combination.

Concerning GLY single effects, significant differences were found for root and shoot length [root: $F(5, 16) = 43.62$; $p < 0.001$]; shoot: $F(5, 16) = 8.167$; $p < 0.001$], as well as for biomass production, either in terms of fresh [root: $F(5, 13) = 1.62$; $p < 0.001$; shoot: $F(5, 19) = 19.64$; $p < 0.001$] and dry [root: $F(5, 17) = 10.92$; $p < 0.001$; shoot: $F(5, 20) = 3.166$; $p < 0.05$] mass (Figure 1). Moreover, it is also evident that roots were the most affected organ by GLY, usually displaying higher inhibition percentages (up to 72% in roots vs 54% in shoots for dry biomass) even at lower concentrations of the herbicide (Figure 1). Indeed, in shoots, GLY only adversely affected their growth upon exposure to concentrations equal and higher than 20 mg kg⁻¹, in comparison with the CTL.

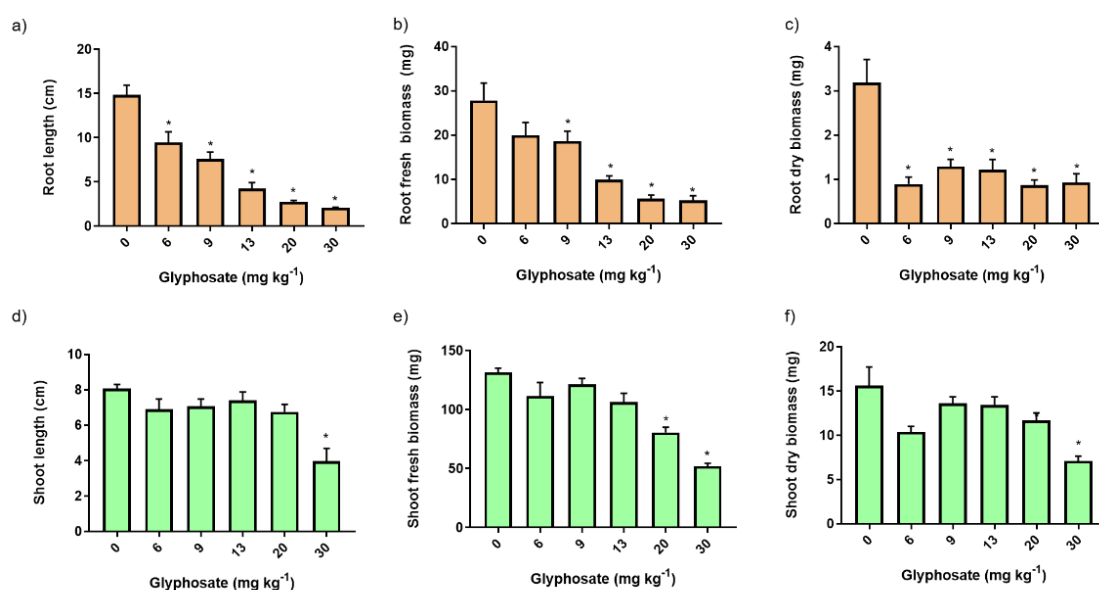


Figure 1. Biometric parameters of roots (orange bars) and shoots (green bars) of *M. sativa* plants grown for 14 d after germination under increasing GLY concentrations (0, 6, 9, 13, 20 and 30 mg kg⁻¹) in OECD soil. (a) root length; (b) root fresh biomass; (c) root dry biomass; (d) shoot length; (e) shoot fresh biomass; (f) shoot dry biomass. Results are expressed as mean $\pm$ SD ($n \geq 3$). * above bars indicate statistical differences from the CTL (0 mg kg⁻¹), at $p \leq 0.05$.

When FLA is regarded, a similar pattern of that of GLY was registered, where significant impacts of this herbicide on plant growth were found [root length: $F(5, 18) = 149.8$; $p < 0.001$; shoot length: $F(5, 18) = 240.2$; $p < 0.001$; root fresh biomass: $F(5, 16) = 29.05$; $p < 0.001$; root dry biomass: $F(5, 16) = 4.769$; $p < 0.01$; shoot fresh biomass: $F(5, 22) = 346.0$; $p < 0.001$; shoot dry biomass: $F(5, 20) = 13.00$; $p < 0.001$]. Moreover, as can be

seen, effects were detected from the lowest applied concentration ($82 \mu\text{g kg}^{-1}$) in all tested parameters and in both organs, when compared to the CTL ($0 \mu\text{g kg}^{-1}$) (Figure 2).

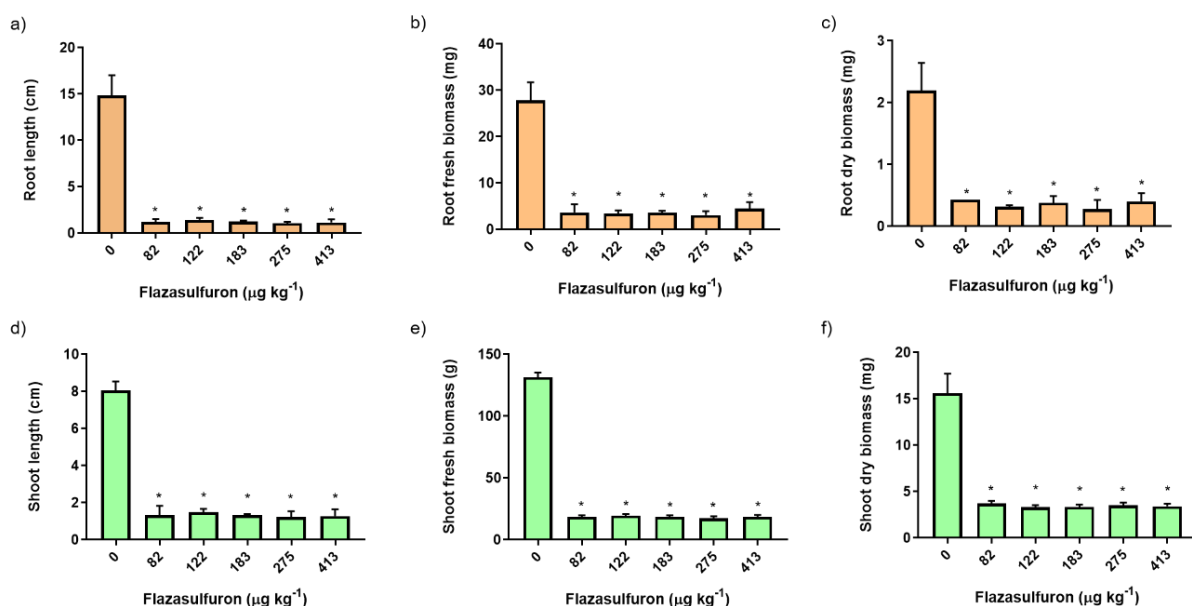


Figure 2. Biometric parameters of roots (orange bars) and shoots (green bars) of *M. sativa* plants grown for 14 d after germination under increasing FLA concentrations ($82, 122, 184, 275, 413 \mu\text{g kg}^{-1}$) in OECD soil. (a) root length; (b) root fresh biomass; (c) root dry biomass; (d) shoot length; (e) shoot fresh biomass; (f) shoot dry biomass. Results are expressed as mean $\pm$ SD ($n \geq 3$). * above bars indicate statistical differences from the CTL ($0 \mu\text{g kg}^{-1}$), at $p \leq 0.05$.

Upon a situation of a co-exposure, plant growth and development were also severely affected [root length: $F(5, 21) = 73.94$; $p < 0.001$; shoot length: $F(5, 18) = 283$; $p < 0.001$; root fresh biomass: $F(5, 16) = 39.40$; $p < 0.001$; shoot fresh biomass: $F(5, 22) = 377.8$; $p < 0.001$; root dry biomass: $F(5, 18) = 5.203$; $p < 0.001$; shoot dry biomass: $F(5, 22) = 14.44$; $p < 0.001$], with significant effects higher than those recorded in the single experiments for all tested concentrations (Figure 3). For instance, while under individual exposure, the maximum inhibition of plant growth was 86% by GLY (30 mg kg^{-1}) and 93% by FLA ($275 \mu\text{g kg}^{-1}$), their combination resulted in a significant decrease up to 98% in relation to the CTL. This same pattern, described for root fresh biomass, was also found for the remaining evaluated biometric endpoints (Figure 3).

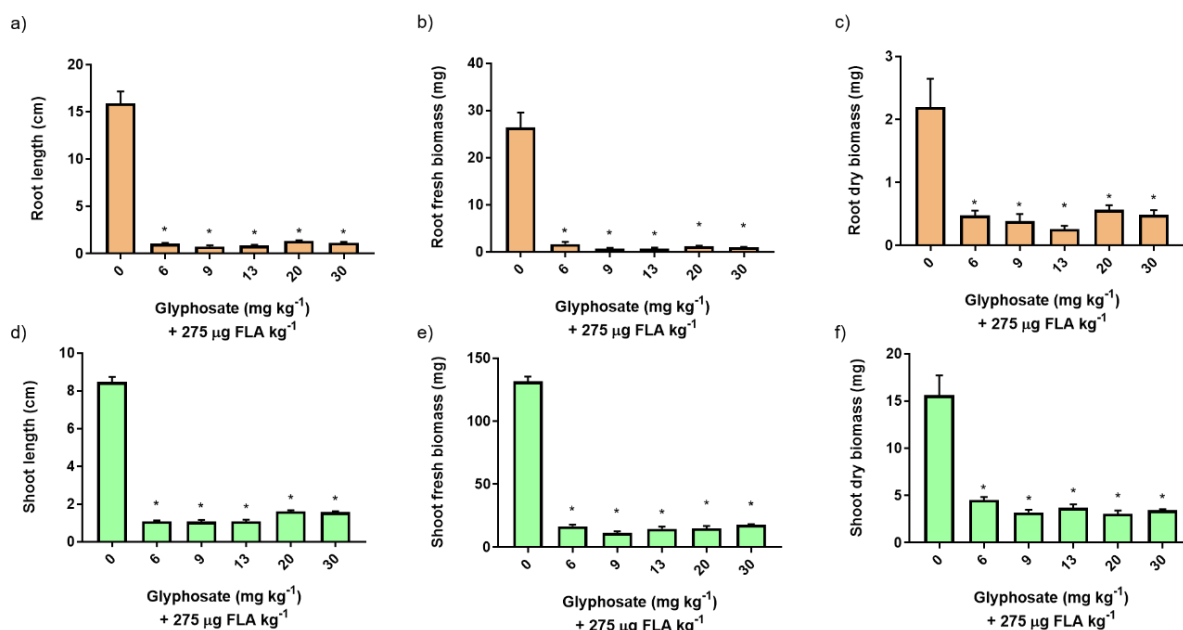


Figure 3. Biometric parameters of roots (orange bars) and shoots (green bars) of *M. sativa* plants grown for 14 d after germination under increasing GLY concentrations (0, 6, 9, 13, 20 and 30 mg kg⁻¹) mixed with FLA at 275 µg kg⁻¹ in OECD soil. (a) root length; (b) root fresh biomass; (c) root dry biomass; (d) shoot length; (e) shoot fresh biomass; (f) shoot dry biomass. Results are expressed as mean $\pm$ SD ($n \geq 3$). * above bars indicate statistical differences from the CTL (0 mg kg⁻¹), at $p \leq 0.05$.

3.2. Reproduction assays with *E. fetida*

The reproduction of *E. fetida* adults was affected by both herbicides, GLY and FLA, in scenarios of single [GLY: $F(5, 19) = 6.036$; $p < 0.01$; FLA: $F(5, 27) = 21.77$; $p < 0.001$] and co-exposure [$F(5, 27) = 9.762$; $p < 0.001$]. However, as can be seen in Figure 4a, GLY only affected the reproduction of worms at the highest concentrations (13, 20 and 30 mg kg⁻¹). In opposition, FLA induced a significant reduction in the offspring of these organisms at all tested doses (Figure 4b), being this pattern also found when GLY treatments were mixed with FLA's application dose (275 µg kg⁻¹) (Figure 4c).

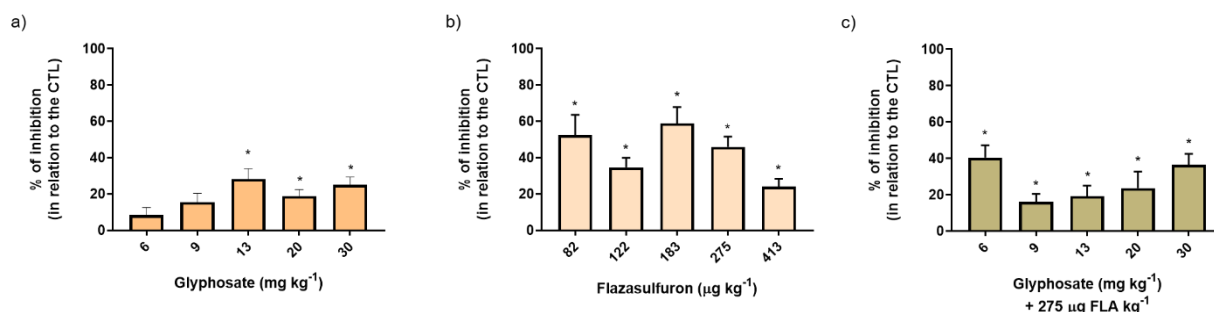


Figure 4. Percentage (%) of inhibition, in relation to the CTL (0 mg kg⁻¹), of the number of juveniles of *E. fetida* exposed to increasing concentrations of: (a) GLY (6, 9, 13, 20 and 30 mg kg⁻¹); (b) FLA (82, 122, 184, 275, 413 µg kg⁻¹); and (c) GLY mixed with FLA at 275 µg kg⁻¹ in OECD soil. Results are expressed as mean $\pm$ SD ($n \geq 4$). * above bars indicate statistical differences from the CTL (0 mg kg⁻¹), at $p \leq 0.05$.

3.3. Recolonization assays with *E. fetida*

The experiment performed to monitor the ability of earthworms to recolonise GLY-contaminated soils revealed that, after 48 h, *E. fetida* individuals remained in the CTL soil (0 mg kg⁻¹) only when exposed to the highest herbicide concentration (30 mg kg⁻¹) (Fischer's exact t-test: $p \leq 0.05$) (Figure 5a). However, upon 96 h and 7 d of exposure, the percentage of recolonization did not show any significant differences (Fischer's exact t-test: $p > 0.05$) between the experimental groups and the dual CTL (Figure 5b,c). Moreover, the feeding activity of earthworms, evaluated by the bait-lamina test, was not significantly different upon exposure to GLY, when compared to the CTL, after 7 d of exposure ($p > 0.05$; data not shown), thus confirming that earthworms were in fact freely moving in the contaminated side of the test boxes.

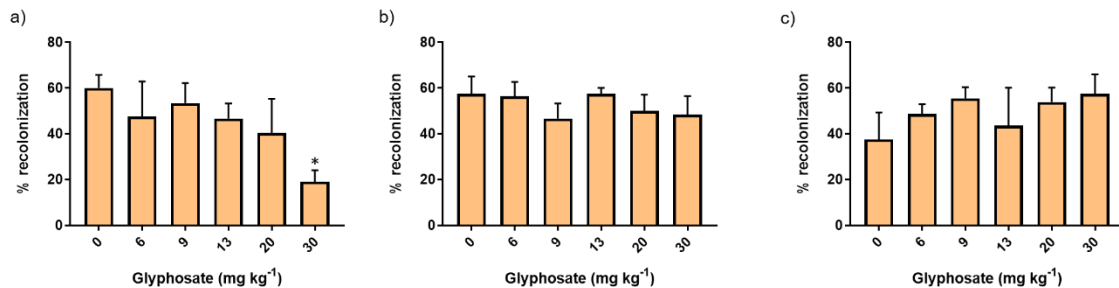


Figure 5. Percentage (%) of the recolonization of GLY-contaminated soils by *E. fetida* after 48 h, 96 h and 7 d of exposure. Results are expressed as mean $\pm$ SD ($n \geq 3$). * above bars indicate statistical differences from the CTL (0 mg kg⁻¹), at $p \leq 0.05$ (Fisher's exact t-test).

3.4. Reproduction tests with *F. candida*

As shown in Figure 6a, the number of juveniles of *F. candida* was not significantly affected by GLY [F (5, 21) = 0.8912; $p > 0.05$], since any of the tested concentrations induced significant differences from the CTL (0 mg kg⁻¹). However, when FLA is concerned, a significant effect on springtails' reproduction was registered [F (5, 21) = 6.469; $p < 0.001$], with impacts occurring upon the second lowest concentration (122 µg kg⁻¹) tested (Figure 6b).

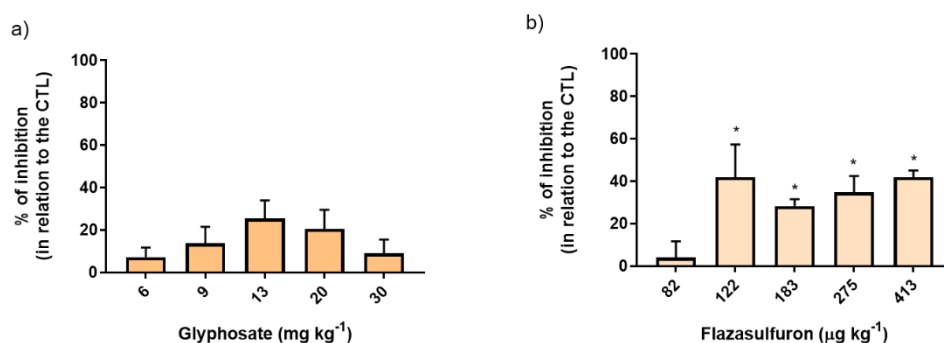


Figure 6. Percentage (%) of inhibition, in relation to the CTL (0 mg kg⁻¹), of the number of juveniles of *F. candida* exposed to increasing concentrations of (a) GLY (6, 9, 13, 20 and 30 mg kg⁻¹) and (b) FLA (82, 122, 184, 275, 413 µg kg⁻¹) in OECD soil. Results are expressed as mean $\pm$ SD ($n \geq 4$). * above bars indicate statistical differences from the CTL (0 mg kg⁻¹), at $p \leq 0.05$.

3.5. *L. minor* and *R. subcapitata* growth inhibition tests

As shown in Figure 7a, elutriates of GLY contaminated soil did not affect the growth of *L. minor* [F (6, 14) = 0.3931; $p > 0.05$], since no significant differences from the CTL (0 mg kg⁻¹) were recorded for the number of fronds. Yet, elutriates induced a significant stimulation in the development of *R. subcapitata* [F (6, 17) = 12.45; $p < 0.001$] (Figure 7b). Concerning the exposure to FLA elutriates of contaminated soil, significant effects on the growth of both organisms [*L. minor*: F (6, 14) = 252.1; $p < 0.001$; *R. subcapitata*: F (6, 13) = 175.5; $p < 0.001$] were observed, with significant inhibitions found for all dilutions tested (Figure 7c,d).

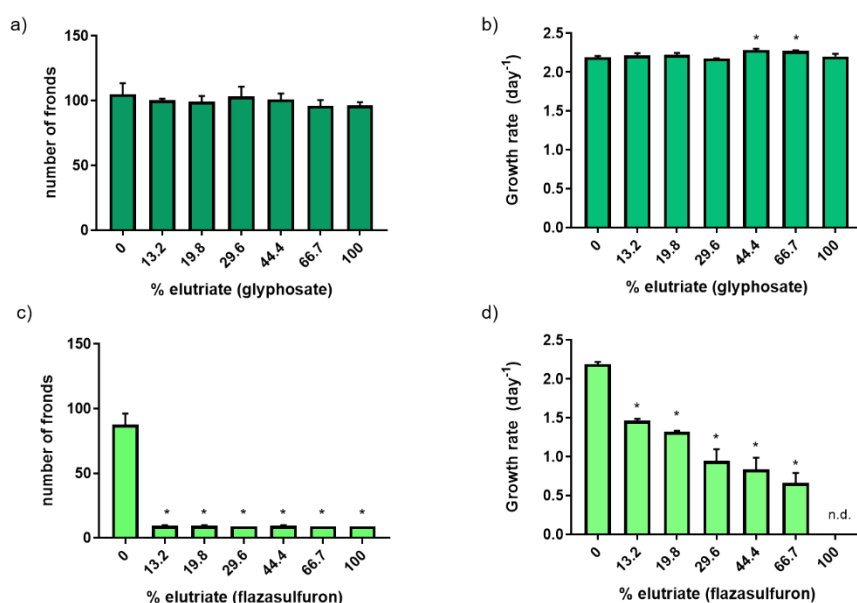


Figure 7. (a,c) Number of fronds of *L. minor* (a,c) and (b,d) growth rate of *R. subcapitata* exposed to serial dilutions [100, 66.7, 44.4, 29.6, 19.8 and 13.2% (v/v)] of elutriates prepared from GLY- or FLA-contaminated soils at 30 mg kg⁻¹ and 413 µg kg⁻¹, respectively. n.d.: non-detected, indicative of total death of microalgae in the sample. Results are expressed as mean ± SD (n ≥ 3). * above bars indicate statistical differences from the CTL (0 mg kg⁻¹), at p ≤ 0.05.

4. DISCUSSION

Risk assessment of PPPs requires a battery of tests with non-target organisms belonging to different trophic levels, in an attempt to estimate the impacts on the ecosystems (Allan et al., 2006; Hagner et al., 2018; Materu and Heise, 2019). However, up to now, most research has focused on the single effects of isolated compounds or on the evaluation of commercial formulations integrating several a.i. (Weisner et al., 2021). Thus, a lack of attention has been driven to the co-occurrence of their residues resulting from the use of tank mixtures and/or successive applications of pesticides in soils, which are already the sink of residues of other a.i. and their metabolites. Here, by evaluating the single and combined ecotoxicity of GLY (6, 9, 13, 20 and 30 mg kg⁻¹) and FLA (82, 122, 184, 275 and 413 µg kg⁻¹), at environmentally relevant concentrations, we provide a holistic vision of their effects on soil and aquatic organisms, discussing their impacts towards soil production and retention functions.

Is FLA less toxic than GLY for soil and aquatic non-target organisms?

Considered as the main producers of terrestrial ecosystems, plants are essential components of the pesticide risk assessment, especially when herbicides are concerned

(Boutin et al., 2014). The germination index is, according to several authors, an easy and expedite parameter sensitive enough to evaluate the phytotoxicity of stressors, including organic and inorganic contaminants (Miralles et al., 2012; Soares et al., 2016). However, according to the data herein obtained, none of the tested herbicides significantly affected the emergence of *M. sativa* (data not shown), a relevant species used as cover plant in crop fields, to improve soil fertility and prevent erosion. Equivalent results have been frequently reported by numerous studies, suggesting that this parameter not always allows a correct and accurate estimation of the impacts of specific contaminants (Bouguerra et al., 2016; Fernandes et al., 2020a; Gavina et al., 2013; Soares et al., 2016). Based on one of the first reviews on sulfonylurea herbicides (where FLA is included), seed germination is not usually impaired by these compounds, with their effects being mostly evident upon the emergence of the cotyledons (Blair and Martin, 1988). Indeed, it is known that during the early steps of germination and seedling emergence, the development of shoot and root primordia is mostly dependent on the resources located in the endosperm and all embryogenic structures are protected by the integument, a physical barrier that not all chemical compounds can cross (Taiz et al., 2015). Despite the maintenance of the germination rate, our data unequivocally showed that plant growth was severely repressed by the presence of residues of both herbicides in the soil, with effects being detected even in the lowest applied concentration of GLY, mainly in roots, and of FLA, in both analysed organs. Actually, although this is the first study exploring the phytotoxicity of FLA towards non-target plants, it is widely acknowledged that GLY, even in residual amounts, is able to inhibit the development of non-target plants, such as crops and other ecologically relevant species (Gomes et al., 2016; Soares et al., 2019; Spormann et al., 2019). For instance, Soares et al. (2019) also found that increasing concentrations of this herbicide, similar to those here tested, resulted in a lower growth performance of *Solanum lycopersicum* L., this being accompanied by an overall deregulation of several physiological and metabolic processes (Soares et al., 2020, 2019). Moreover, a very recent study from our group also highlighted that the exposure of *M. sativa* to increasing soil levels of GLY hampered its growth and induced oxidative stress, while also impacting the nitrogen metabolism (Fernandes et al., 2020b). According to our data, FLA-mediated effects were much more pronounced than those of GLY in terms of shoot and root biomass production (fresh and dry) and elongation. Although not a single report is available on the effects of this herbicide on the growth of non-target plants, FLA belongs to the sulfonylurea family, a class of compounds recognised for interfering with plant growth at low concentrations, which goes in agreement with the data herein obtained. Curiously, the mode-of-action of both herbicides relies on the inhibition of biosynthetic pathways involved in amino acid production. GLY targets the EPSPS enzyme, blocking its activity and preventing the

synthesis of tryptophan, tyrosine and phenylalanine (Gomes et al., 2014). On its turn, FLA's herbicidal action is centred on the repression of ALS, downstream affecting the biosynthesis of branched-chain amino acids (valine, leucine and isoleucine), and completely arresting protein build up and cell proliferation (Chkanikov et al., 2020). In fact, by inhibiting ALS, the mitotic process is abruptly stopped between the G2 of the interphase and the M phase (Singh and Shaner, 1995), which helps to explain the higher phytotoxicity of FLA than GLY towards alfafa plants. In a previous work aimed at unravelling the non-target toxicity of halosulfuron-methyl (HSM), also a sulfonylurea-based herbicide, to crops, Pan et al. (2017) found out that increasing doses of this agrochemical added to the nutrient solution (0, 0.1, 10, 100, 1000 and 10,000 $\mu\text{g L}^{-1}$) greatly impaired the growth of maize (*Zea mays* L.) and soybean (*Glycine max* L.) plants, with effects being detected in the lowest applied level, especially for soybean seedlings.

In this study, attention was paid to the behavioural and reproductive responses of two important soil invertebrate groups – earthworms and springtails, using *E. fetida* and *F. candida* as model species. Recognised as indicators of soil quality, effects recorded in these species as a consequence of herbicide exposure strongly indicate that soil's habitat function is compromised (van Leeuwen et al., 2019). Up to now, a large set of authors has been interested in unravelling the ecotoxicological relevance of GLY-based herbicides, as well as of the a.i. itself, towards collembola and earthworms (Hackenberger et al., 2018; Niemeyer et al., 2018; Pereira et al., 2009; Pochron et al., 2021; Santos et al., 2012; Simões et al., 2018). However, as stated before, there is a lot of divergent data, and frequently effects are not reported at environmentally realistic concentrations. According to our results, the number of springtail juveniles was not significantly changed, and the ability of *E. fetida* individuals to reproduce was only impaired at relatively high GLY concentrations ($\geq 13 \text{ mg kg}^{-1}$). Moreover, we also demonstrate that *E. fetida* adults can recolonise GLY-contaminated soils in less than four days, revealing the low sensitivity of these species to the presence of GLY residues in the soils, at levels already reported by some authors (Primost et al., 2017; Peruzzo et al., 2008). Previous studies, conducted by Buch et al. (2013), showed that GLY (provided as Pica-Pau® 480 SC) did not induce toxic effects on two species of earthworms (*E. andrei* and *Pontoscolex corethrurus*) in concentrations of 7, 14, 21, 30 and 47 mg a.i. kg^{-1} , with animals only avoiding OECD artificial soils (10% OM and pH 6.0 ± 0.5) contaminated by GLY at the highest levels (30 and 47 mg kg^{-1}). In contrast, a former work, where soil samples (pH 5.64–5.79 and organic C around 1.6%) from a GLY-treated soybean agricultural field (GLY applied as RoundUp® FG at 1,440 g a.i. ha^{-1}) were collected, found significant effects on the production and hatchability of cocoons, with a substantial reduction in the number of juveniles of *E. fetida* (Casabé et al., 2007). In parallel, Santadino et al. (2014), by employing a matrix population

model, have concluded that sub-lethal concentrations of GLY (RoundUp® 48%: 6 and 12 L ha⁻¹) impaired the population dynamics of *E. fetida*. Yet, both studies failed to calculate the final nominal concentrations applied to the soil, which hampers a fair comparison with our data. Curiously, Pochron et al. (2020) recently showed that this species was sensitive to GLY (at 26.3 mg kg⁻¹), but it was not affected by two of its commercial formulations (RoundUp Super Concentrate® and RoundUp Ready-to-Use-III®), an observation which disagrees with our results, although we have tested a different commercial product. Also, Niemeyer et al. (2018) studied the ecotoxicity of several GLY-containing agrochemicals and, overall, no major changes were observed for both *E. fetida* (RoundUp® Original and Trop® – 89.29 mg a.i. kg⁻¹; Zapp® Qi 620 – 64.29 mg a.i. kg⁻¹; Crucial® – 59.52 mg a.i. kg⁻¹) and *F. candida* reproduction potential (RoundUp® Original and Trop® – 6, 12, 24 and 48 mg a.i. kg⁻¹; Zapp® Qi 620 – 7.75, 15.5, 31 and 62 mg a.i. kg⁻¹; Crucial® – 8.73, 17.45, 34 and 69.8 mg a.i. kg⁻¹) when exposed to different GLY-based herbicides. Interestingly, our data shows that earthworms were more sensitive to GLY than collembola, with significant impacts on the number of juveniles upon exposure to levels higher than 9 mg kg⁻¹, a concentration much lower than those tested by Pochron (2020) and Niemeyer et al. (2018). Indeed, data herein collected suggest that *F. candida* does not appear to be sensitive to GLY soil contamination, with no effects on their reproduction yield. Based on Simões et al. (2019) the toxic effects of a GLY commercial formulation (Montana®) on the reproduction ability of this species were probably not due to GLY itself and vary according to the commercial formulation used. Overall, given the high variability found in the literature, it appears that results are highly dependent on the employed conditions, not only in terms of the tested concentrations, but also on the test media, as different types of soils are being used as substrate. Different soil properties, such as the pH and the organic matter content, highly influence the fate and availability of pesticides in soils (Bai and Ogbourne, 2016). This already high variability further intensifies when considering the differences between testing the a.i. or different commercial formulations. Indeed, it is widely recognised that the presence of other ingredients, such as surfactants and adjuvants, in the commercialised products can result in different toxic effects when compared to the a.i. alone (Mesnage and Antoniou, 2018). For instance, according to Pereira et al. (2009), the toxicity of different commercial formulations of pesticides ended up over- or underestimate the impacts of the a.i. itself. Thus, and acknowledging the practical relevance of commercial products, studies should pay attention to this aspect, not only in terms of experimental design, but also when comparing different studies.

In relation to GLY, FLA-spiked soils were more toxic to springtails and earthworms, significantly inhibiting the abundance of the offspring. In fact, the exposure of these two species to residues of FLA impaired their reproduction potential, with effects detected for

all tested concentrations in *E. fetida* (81-413 $\mu\text{g kg}^{-1}$) and right after the second one in *F. candida* (122-413 $\mu\text{g kg}^{-1}$). To the best of our knowledge, this is the first study devoted to assess the ecotoxicity of FLA-contaminated soils to soil biota and, therefore, no exact comparisons can be fairly made. Nevertheless, this result is quite surprising since sulfonylurea compounds are thought to only have a narrow and low ecotoxicity towards non-target species (EFSA, 2016). With effect, based on IUPAC database, FLA was found to be innocuous to the reproduction ability of these species at concentrations as high as 8.0 (earthworms) and 125 mg kg^{-1} (collembola), which strongly contrasts with our findings. Yet, according to Pelosi (2021), other sulfonylurea pesticides have also been categorised as toxic to earthworms. With effect, the ecotoxicological assessment of sulfonylurea-containing herbicides suggest that these compounds can, indeed, affect the behaviour and growth of earthworms, inducing mortality and inhibiting their offspring production (Chen et al., 2018; Kalkhoran et al., 2021). Given that FLA's herbicidal action relies on the inhibition of a plant enzyme, absent in the animal metabolism, its toxic effects mostly likely arise as an indirect consequence of FLA in the cellular homeostasis. Though not much is known about FLA-induced physiological, biochemical and molecular disorders, the exposure of an earthworm species, *Dendrobaena veneta*, to another sulfonylurea herbicide, nicosulfuron (provided as the commercial formulation Samson extra) at concentrations of 0.3, 3.0 and 30 $\mu\text{g kg}^{-1}$ impacted the oxidative metabolism of the animals, inducing significant rises in lipid peroxidation and changes in several defence enzymes (Hackenberger et al., 2018). Thus, one can hypothesize that, at the concentrations tested, FLA is inducing major cellular and metabolic disorders, negatively affecting the physiological status of earthworms and collembola, with significant effects on their reproduction ability.

Once in the soil, herbicide residues can persist adsorbed to soil particles, but can also experience leaching or runoff processes, ending up contaminating freshwater courses, where they can represent an additional threat to aquatic organisms. For this purpose, elutriates prepared from soils contaminated by the highest concentration of GLY (30 mg kg^{-1}) or FLA (413 $\mu\text{g kg}^{-1}$) were used to evaluate the ability of soil to retain these herbicides and their potential ecotoxicological impacts in the development of a macrophyte (*L. minor*) and a microalgae (*R. subcapitata*). Based on our data, GLY elutriates did not impose significant effects on any of the tested species, revealing that, at least under the current experimental condition, soil contamination at 30 mg kg^{-1} does not present an increased risk to these freshwater producers. Given that GLY is a non-selective herbicide, impairing the biosynthesis of aromatic amino acids, it was expected that both duckweed and microalgae would be greatly affected, under the availability of the herbicide. However, up to now, the influence of GLY on the growth and development of aquatic plants and

microalgae seems to be ambiguous and not consensual (Gomes and Juneau, 2016; Kielak et al., 2011; Ma et al., 2006; Sikorski et al., 2019; Sobrero et al., 2007; Tsui and Chu, 2003), generally reporting negative effects upon exposure to high levels of the contaminant. For instance, Sikorski et al. (2019) found out that duckweed's growth was majorly hampered by GLY (40 μM , which corresponds to 7 mg L^{-1}), due to its bioaccumulation. Concomitantly, and from a physiological perspective, the exposure of *L. minor* to increasing concentrations of GLY (up to 500 mg L^{-1}) resulted in serious physiological disturbances, with significant rises in ROS accumulation and lipid peroxidation and an inhibition of the photosynthetic potential, with effects upon exposure to levels $\geq 10 \text{ mg L}^{-1}$ (Gomes and Juneau, 2016). When referring to more environmentally relevant concentrations, a prior study conducted by Dosnon-Olette et al. (2011) revealed that 80 $\mu\text{g L}^{-1}$ GLY did not evoke substantial differences in the growth of *L. minor*. Equivalent outputs were also reported a long time ago by Lockhart et al. (1990), whose results revealed that *L. minor* was insensitive to GLY ($\approx 3 \text{ mg L}^{-1}$) added to the nutrient solution, but not when the herbicide was foliar-applied by spraying. Interestingly, although this species is a standard test organism for ecotoxicological assays, it was previously reported that *L. gibba*, another species of the same genus, was more sensitive than *L. minor* and even than some species of microalgae (Burns et al., 2015; Sobrero et al., 2007; Zaltauskaite and Kaciene, 2020). Following the pattern registered for duckweed, *R. subcapitata* did not show any inhibition in terms of growth even in response to the non-diluted elutriate prepared from GLY-contaminated soils. In opposition, an earlier study performed by Ma et al. (2006) observed adverse effects of this herbicide on the growth ability of *R. subcapitata* (effective concentration causing a 50% of effect – EC_{50} : 5.55 mg L^{-1}). In parallel, Tsui and Chu (2003) proposed an IC_{50} value (the concentration causing 50% inhibition) of 1.85 and 5.81 mg L^{-1} for RoundUp® for two species of microalgae, *Selenastrum capricornutum* and *Skeletonema costatum*, respectively. However, once again, it should be highlighted that, in both of these studies, the organisms were directly exposed to GLY and not to soil elutriates. In the present study, the elutriates prepared from GLY-contaminated soils (30 mg kg^{-1}) were diluted (1:4, soil/medium), arising a maximum theoretical concentration of GLY of 7.5 mg L^{-1} , which is unlikely to have occurred, given the interaction of this herbicide with the soil. In fact, the lack of toxicity of elutriates prepared from GLY-treated soils may arise as a consequence of the persistence of GLY adsorbed to soil particles, such as organic matter and clay components, limiting its transference to the aqueous media (Albers et al., 2009; Gunarathna et al., 2018; Nguyen et al., 2018). Although GLY is supposedly quickly degraded by microbial action, it can also be strongly adsorbed by soil, especially by the interaction between GLY's functional groups (e.g. carboxyl, amino and phosphonate) and soil's cations, namely Al^{3+} and Fe^{3+} .

(Borggaard and Gimsing, 2008; Gunarathna et al., 2018; Mamy and Barriuso, 2005).. Despite the elutriate being prepared after 24 h of GLY addition to the soil, previous research suggests that GLY adsorption to the soil components mostly occurs within the first two hours, with a stabilization over 24 h (Mamy and Barriuso, 2005). Equivalent findings were also reported elsewhere (Ozbay et al., 2018; Wang et al., 2009). Thus, this hypothesis gains strength and can, at least partially, explain the absence of toxicity of GLY elutriates towards aquatic plants and microalgae.

The elutriates prepared from FLA-contaminated soil samples were very toxic towards *L. minor* and *R. subcapitata*. As can be observed, all tested dilutions induced significant inhibitions on both organisms, suggesting that, besides affecting soil invertebrates and plants, FLA can represent a high risk to aquatic biota because of runoffs or leaching events. In spite of the few available records, there are reports suggesting a higher persistence of FLA in the soil (Tejada and Benítez, 2017). However, at least under the conditions tested, one can suggest that i) FLA was not strongly adsorbed to soil components and presents a high solubility (2.1 g L^{-1} ; <http://sitem.herts.ac.uk/aeru/iupac/Reports/319.htm>), which contributed for its transfer to the aqueous media; and/or ii) even with adsorption events, the extractable residues of FLA to the liquid phase were capable of inducing phytotoxicity. The exact mechanisms to explain these two premises are hard to establish, especially because not much is known concerning FLA's ecotoxicity. Indeed, to the best of our knowledge, this is one of the first studies unravelling the impacts of FLA to the water compartment. Supporting our data, Olette et al. (2008) observed that FLA (up to $100 \text{ } \mu\text{g L}^{-1}$, provided as Katana®, Zeneca Sopra) was able to affect duckweeds growth and photosynthetic pigments. Furthermore, previous works conducted with other commercial sulfonylurea herbicide (composed of amidosulfuron and iodosulfuron methyl, sodium salt) revealed that *L. minor* growth was majorly hampered by concentrations in the range of $\mu\text{g L}^{-1}$. Additionally, the authors also observed that, even after transfer to clean media, the individuals did not recover or, if so, presented significantly lower values of biomass than the initial ones, being this accompanied by a deregulation of different biochemical parameters, with rises in lipid peroxidation (Zaltauskaite and Kaciene, 2020). Concerning microalgae, Couderchet and Vernet (2003) reported that FLA (0.1, 1, 10, 100 and $1000 \text{ } \mu\text{g L}^{-1}$; provided as Katana®, Zeneca Sopra) decreased the photosynthetic pigments of the alga *Scenedesmus obliquus*. Furthermore, when evaluating the toxicity patterns of several herbicides, Zhao et al. (2018) reported that halosulfuron-methyl, which has the same mode-of-action as FLA, was the most toxic one to *Selenastrum capricornutum*. Overall, considering FLA's chemical properties, which can promote its transference to the aqueous media, and its

herbicidal efficacy at low levels, data suggest a higher risk of this herbicide for nearby freshwater ecosystems.

Can the single effects of each pesticide underestimate their real ecotoxicity when their residues occur simultaneously in agricultural soils?

As a consequence of the application of multiple agrochemicals in the context of intensive agricultural practises, soils can be the sink of residues of different pesticides, capable of inducing non-target effects that can be more complex to predict than those derived from single substances. Based on a recent report from Geissen et al. (2021), complex mixtures of several pesticides were found in batches of soils derived from European agricultural soils, with GLY being the a.i. most frequently found. Thus, although challenging and time-consuming, efforts to disclose the consequences of mixtures of pesticides are currently a priority for international regulatory organisations (More et al., 2019). Aiming at unravelling the impacts of a scenario where GLY is applied in soils with residues of other pesticides, as in the case of vineyards (Mandl et al., 2018), co-exposure experiments were conducted using model species of terrestrial producers (plants) and detritivores (earthworms). As described above in detail, the growth and development of alfalfa seedlings was almost completely arrested in response to the co-presence of GLY and FLA in the soil. Actually, significant inhibitions of organ elongation and biomass production for both shoots and roots were found even for the lowest GLY concentration (6 mg kg^{-1}) tested together with FLA's recommended application dose ($275 \text{ } \mu\text{g kg}^{-1}$), suggesting a prevalence of FLA effects. Despite being the most applied herbicide worldwide, the number of studies dealing with the possible effects of GLY toxicity in combination with other pesticides is still limited. Actually, works dealing with GLY mixtures are generally focused on ways to maximise weed control and to study weed resistance traits (e.g. Bianchi et al., 2020; Fernández-Escalada et al., 2019; Palma-Bautista et al., 2021), not paying attention to the ecotoxicological relevance of these mixtures towards non-target plants that can co-exist in the field. Regarding this matter, a former study devoted to assess the impacts of GLY (applied as RoundUp Original®) and dicamba (provided as Banvel®) mixtures in native plants under laboratory conditions, revealed that plant responses to the combination of both herbicides were identical to those induced by the single treatments (Olszyk et al., 2015).

In spite of the higher toxicity of GLY and FLA co-exposures towards terrestrial plants, results of *E. fetida* reproduction assays revealed that the observed effects were mostly a consequence of FLA presence, since the reduction in juveniles' number was similar to that found when FLA ($275 \text{ } \mu\text{g kg}^{-1}$) was tested alone. Interestingly, studies of such nature (*i.e.*

exploring the ecotoxicological relevance of mixtures of herbicides) are rare. To the best of our knowledge, research on PPP mixtures mostly focus on the combination of residues of different pesticide classes, like herbicides and insecticides. Furthermore, the few available records not always observe a clear pattern in what regards the higher or lower toxicity of mixtures when compared to single compounds (Santos et al., 2011; Yang et al., 2018; Yasmin and D'Souza, 2007; Yu et al., 2019). According to Yasmin and D'Souza (2007), GLY (2 mg kg⁻¹; provided as Glycel 41% S.L.) alone did not affect the reproduction ability of *E. fetida*, but, when combined with carbendazim (0.8 mg kg⁻¹; provided as Bavistin) and dimethoate (0.4 mg kg⁻¹; provided as Rogor 30% E.C.), a significant inhibitory effect was found in earthworms reproduction function upon exposure to contaminated soils. From what it appears, as in the case of our study, the impacts of the mixture most probably arouse as a consequence of the presence of other applied compounds, and not GLY itself. Curiously, when assessing the ecotoxicological relevance of GLY and Cu mixtures towards earthworms, Zhou et al. (2013) found out that GLY (20, 50 100 and 200 mg kg⁻¹ OECD soil) even helped to reduce Cu-mediated effects in what concerns cocoon production and body mass. Moreover, as far as we know, this is the first report exploring the combined impacts of two herbicides towards terrestrial invertebrates, which, supposedly, do not target any metabolic chain present in animal cells. Yet, major changes were found, especially due to the toxic action of FLA, which prevailed in relation to GLY.

5. CONCLUSIONS

The critical evaluation of the single and combined ecotoxicological relevance of pesticides to non-target species at relevant environmentally concentrations is of utmost importance to achieve a balanced and realistic risk assessment. Here, by studying the effects of two herbicides commonly applied in the agricultural context, we provide, for the first time, new ecotoxicological data for FLA, and expand our knowledge on the possible non-target toxicity of GLY, pretty much needed to clarify doubts and conflicting results. Moreover, findings from the current study unequivocally show that FLA is much more toxic than GLY for non-target biota, including terrestrial plants and soil organisms (Figure 8). Also, FLA elutriates greatly impacted the growth of microalgae and macrophytes, suggesting that residues of this herbicide can pose an increased risk to freshwater habitats, in contrast to what was observed for elutriates prepared from GLY-contaminated soils (Figure 8). In fact, despite all the controversy around GLY risks to the agroecosystems, it should be emphasised that, when respecting the recommended application dose, no major effects are expected for soil habitat function and nearby located freshwater courses. Nevertheless, since farmers often rely on the application of mixtures of herbicides, the risk

assessment must clearly address the impacts of their combination for non-target organisms considering both ecologically relevant concentrations of residues in soils as well as of application doses. Our co-exposure studies clearly indicated that soil habitat and production functions were severely compromised, especially due to the prevalence of FLA toxicity, with a synergistic effect of both herbicides on cover crop plants, which usually occur in the proximity of the application areas. In our case, only the application dose of FLA was tested, to the detriment of soil residual concentrations, as there are no data available for the later values. However, since FLA is also used as a pre-emergent herbicide, our approach is still realistic.

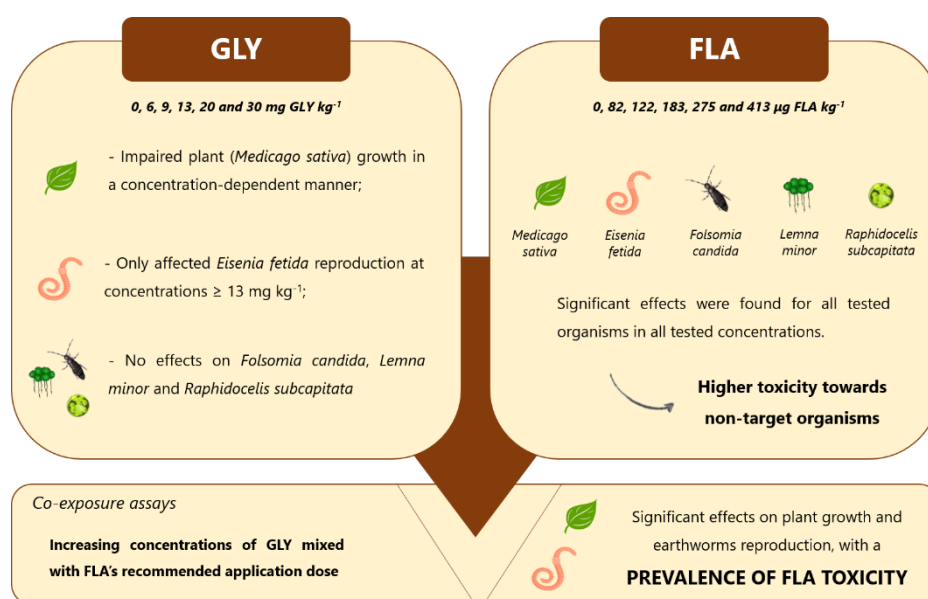


Figure 8. Overview of the main results obtained in this work.

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CHAPTER VII. CONCLUDING REMARKS

General conclusions and Perspectives

In order to respond to the present demographic conjecture and the emerging climate crisis, the agri-food sector, especially agriculture, is currently facing unprecedented challenges (Korres et al., 2016; Malhi et al., 2021). While it is necessary to implement strategies that ensure an increase in food production globally, this increase must be accompanied by the adoption of more sustainable agricultural practices to curb the effects of climate change and environmental degradation (Agovino et al., 2019). Aware of the importance and relevance of this apparent dichotomy, the EU is now committed in making its agricultural systems more profitable and sustainable. On the basis of the European Green Deal initiative (<https://ec.europa.eu/info/strategy/priorities-2019-2024/european-green-deal>), Europe intends to establish "the link between healthy people, healthy societies and a healthy planet", recognising that, for this purpose, agri-food production must be one of the priority axes. Thus, the "Farm to Fork" (https://ec.europa.eu/food/horizontal-topics/farm-fork-strategy_en) strategy seeks to promote the transition to more sustainable practices across all stages of the agri-food chain, from agricultural production to waste management. The pathway towards agriculture sustainability must also comprise a strict regulation of the use of synthetic pesticides, as reflected in the 2030 Agenda for Sustainable Development. In fact, within this action (Directive 2009/128/EC), emphasis is placed on the consequences of the use of agrochemicals, whose impacts extend from the dynamics of ecosystems to human health itself.

From all utilised pesticides, glyphosate (GLY) is arguably the most applied compound at the global scale for the control of weeds' undesired growth, not only in agricultural areas, but also in urban and industrial contexts (Duke, 2018; Gomes et al., 2014). Although GLY's importance cannot be ignored, emerging concerns over its potential effects on non-target organisms – from bacteria and fungi, to plants and animals – have prompted additional efforts from the scientific community to carefully unravel the real impacts derived from the prolonged and systematic use of GLY-based herbicides (Singh et al., 2020).

Since its commercialization, it has always been assumed that, once in contact with the soil, GLY would quickly become inactive – either by adsorption to organic and inorganic components or by microbial degradation –, no longer representing a risk to other organisms, including plant species growing in soils where residues of the herbicide can persist. Nevertheless, recent studies have shown that GLY, either in soil or irrigation media, is able to affect plant development (Gomes et al., 2017; Khan et al., 2020; Himani Singh et al., 2017). Yet, the mechanisms responsible for such effects remain to be characterised, especially with regard to the main metabolic pathways and physiological processes involved. In this sense, the first major objective of this thesis was to understand,

through ecologically relevant approaches, how non-target plants (*i.e.* agricultural crops and cover plants) were affected by the presence of GLY (10, 20 and 30 mg kg⁻¹) residues in the soil, not only in terms of vegetative performance, but also at the (sub)cellular level, with special emphasis on the oxidative and photosynthetic metabolism (**Chapter IV**). Supporting the few data already available in the bibliography (Gomes et al., 2017, 2016; Khan et al., 2020; Singh et al., 2017b, 2017a), the results herein obtained unequivocally reveal that soil contamination by GLY residues represents a hazard threat to the growth of non-target plants, such as *Medicago sativa* L. (alfafa plant) and *Solanum lycopersicum* L. (tomato plant) (Fernandes et al., 2020; Soares et al., 2020, 2019), with noticeable effects on growth and development at concentrations already found in the environment (Peruzzo et al., 2008). Hence, great crop losses, with biomass reductions ascending to 80-90% in worst-case scenarios, can be anticipated in GLY-contaminated soils. Furthermore, by combining ecophysiological, biochemical, ultrastructural and molecular tools, a robust and integrative insight of the main cellular processes involved in the response of plants to GLY-induced stress was achieved. Overall, the strong inhibition of plant growth (Fernandes et al., 2020; Soares et al., 2019) was associated with severe ultrastructural damages, loss of cell viability and a reduced water use efficiency, with repercussions on the transcriptional and biochemical control of diverse cellular agents (including proteins and pigments) involved in photosynthesis (Soares et al., 2020). Still, these negative impacts did not appear to have substantially reduced plants' carbon (C) flux, at least on a short-term exposure; probably, this apparent maintenance of photosynthesis was linked to: i) a greater energetic investment to ensure cell homeostasis and/or ii) the modulation of the oxidative metabolism as a way to prevent reactive oxygen species (ROS)-induced damage in the few viable cells of the leaf mesophyll. Even so, at the root level, the exposure to the herbicide induced strong redox imbalances, reinforcing the premise that the occurrence of oxidative stress is one of the indirect effects of GLY-mediated toxicity.

Still within this context, and transversally to all the studied species [alfalfa, tomato and barley (*Hordeum vulgare* L.)], it is important to highlight the very strong accumulation of proline, a compatible solute and ROS-neutralising agent (Hayat et al., 2012), in response to the herbicide exposure. Based on the gathered data, it can be suggested that, contrary to what would be expected, this large increase in proline is probably related to a response of sensitivity rather than tolerance. Currently, functional and molecular biology studies are underway, using mutant lines of *Arabidopsis thaliana* (L.) Heynh. for genes related to proline metabolism, to understand the specific role of this amino acid in GLY-induced stress.

Recognising that GLY remains – and likely will continue to be – the most widely used herbicide, with its license being approved until the end of 2022 by the EU

(https://ec.europa.eu/food/plants/pesticides/approval-active-substances/renewal-approval/glyphosate_en), more than understanding its impacts in real agronomical contexts, it is also essential to design and develop green strategies to increase the tolerance of non-target plants to GLY. However, up to date, only few studies have been carried out with this objective. In this sense, the second main goal of this PhD thesis was to test several approaches with practical relevance that could reduce the risks of GLY (10 mg kg⁻¹) towards plant species, using tomato and barley as models. To this end, experiments were carried out to assess the phytoprotective potential of several compounds, namely salicylic acid (SA), silicon (Si) – both at bulk and nano (nano-SiO₂) forms - and nitric oxide (NO) (**Chapter V**). In general, the exogenous application of the various compounds allowed to attenuate part of the phytotoxic effects imposed by the herbicide, contributing for a better physiological status and triggering several defence mechanisms, not only related to the antioxidant system, but also to xenobiotic detoxification pathways (e.g. glutathione – GSH, and glutathione S-transferase – GST) (Soares et al., 2021a, 2021b; Spormann et al., 2019). From a holistic perspective, the co-treatment with Si or NO, via foliar spraying, seemed to be the most promising strategy to be implemented in a real agricultural scenario. According to the data herein collected, both elicitors were efficient in stimulating the antioxidant defence mechanisms, especially those of the enzymatic component, reducing the oxidative damage induced by GLY and, thus far, limiting its impacts on growth. Furthermore, in order to gain a preliminary insight into the impacts on productivity-related traits, the foliar application of NO was also able to neutralise part of the effects of GLY with respect to flowering and fruit set (**Chapter V**). In the future, additional studies should be planned not only to understand GLY-mediated effects on the nutritional quality and food safety of the fruits, but also to assess if the protection provided by Si and/or by NO can also benefit fruit development and characteristics from a nutritional and antioxidant perspective.

In addition to the use of biostimulants, another component of the present work aimed at studying whether soil enrichment in organic matter (OM) would result in a lower availability of GLY in the soil and, hence, a lower risk for plant growth. The interaction of OM components with organic pollutants, such as herbicides, is well described in the literature (Pérez-Lucas et al., 2021). However, unlike most pesticides, GLY is a polar compound, being generally assumed that OM does not play an important role in its adsorption (De Jonge et al., 2001; Gerritse et al., 1996; Mamy and Barriuso, 2005). However, as previous research had already suggested the ability of GLY to be adsorbed by humic acids (Albers et al., 2009), it was hypothesized that GLY-contaminated soils with a higher content of OM would sustain a better plant growth. Combining biometric and biochemical approaches, the results suggested that soil enrichment in OM reduces the

negative impacts of the herbicide (10 mg kg^{-1}) on tomato plants, either by decreasing the availability of GLY, or by promoting a better physiological status, further allowing plants to respond more efficiently to the presence of the herbicide (**Chapter V**). Later on, in order to validate these strategies under real conditions, other sources of OM – such as natural residues, including wrack – should be tested, with analyses throughout the life cycle of the plant, from vegetative growth to reproductive development.

Under real conditions, plants are not isolated and are integrated into an ecosystem, where they co-inhabit with other species with equally relevant roles in maintaining soil functions. Thus, another specific objective of this PhD thesis was to assess the impacts of environmental contamination by GLY, both alone or in co-exposure with residues of another herbicide, on habitat and soil retention functions (**Chapter VI**). Following a realistic experimental design, where soil contamination by GLY ($0\text{-}30 \text{ mg kg}^{-1}$) and/or flazasulfuron (FLA; $0\text{-}413 \text{ } \mu\text{g kg}^{-1}$) was simulated, the results allowed us to conclude that, in general, GLY does not present a threat to the tested species, only affecting the reproduction ability of soil oligochaetes at high concentrations ($\geq 13 \text{ mg kg}^{-1}$); still, these organisms were able to recolonise GLY-treated soils after 72 h of soil contamination, without significant changes in their feeding activity. In contrast, data of the current study have unequivocally shown that FLA is far more toxic than GLY to non-target biota, including terrestrial plants, soil organisms and freshwater species. In fact, despite all the controversy surrounding the risks of GLY to agroecosystems, it should be emphasised that, by respecting the recommended application rates and safety intervals, major effects on soil ecosystems and in nearby located freshwater streams are not expected. However, long-term studies dealing with potential transgenerational effects on soil biological communities. Additionally, as farmers often rely on the application of herbicide mixtures, the risk assessment must address the impacts of combined active ingredients (a.i.) on non-target organisms. The co-exposure approach used in this study clearly indicated that soil habitat and production functions are largely compromised, mainly due to the prevalence of FLA toxicity, with an intensification of the negative impacts of GLY in plants.

Overall, the interdisciplinary approach of this work has proven to be strongly effective, allowing a harmonious integration of all the data obtained over the four years of research, leading to the successful fulfilment of the initially proposed objectives with the following key conclusions emerging:

- GLY, when present in soils at environmentally relevant concentrations, continues to pose a risk to non-target plants' growth, being capable of inducing physiological, biochemical and molecular disturbances with macroscopic repercussions;

- The exogenous application of biostimulants, namely Si and NO, represent feasible strategies to reduce the phytotoxic effects of GLY for non-target plants;
- Soil enrichment with OM limits and prevents the impacts of GLY on non-target plants, such as crops, either by decreasing its availability or by promoting a better physiological state of the plant;
- The evaluation of the ecotoxicological relevance of GLY revealed that, when respecting the application rates, no risks for terrestrial invertebrates and aquatic organisms from freshwater systems nearby located are expected;
- Herbicides risk assessment must necessarily contemplate the coexistence of residues of several a.i. – a situation each day more representative of the current phytosanitary treatments –, whose effects may differ from those observed in an individual exposure situation.

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