

EVALUATION OF THE ACCUMULATION AND TOXICITY OF PHARMACEUTICALS IN LETTUCE PLANTS

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TESE ELABORADA PARA OBTENÇÃO DO GRAU DE DOUTOR EM
ENGENHARIA DO AMBIENTE

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Abstract

Pharmaceutical products (considered emerging contaminants) are a growing source of environmental contaminants, mainly due to their high daily use by the population.

The main objective of this work was to study the absorption of pharmaceuticals and their main effects on lettuce metabolism. The experimental tests were carried out in hydroponic culture, contaminating lettuce plants with three selected pharmaceuticals, carbamazepine (CBZ), metformin (MTF) and acetaminophen (ACT). Three concentrations (0.1, 1 and 5 mg L⁻¹), of each pharmaceutical were tested as well as three different time points (1, 8 and 15 days).

CBZ and ACT uptake was measured in roots and leaves, and it was possible to observe that both pharmaceuticals were absorbed by roots and translocated to the leaves. MTF quantification was not carried out, yet the effects resulting from its presence in growth medium were analysed. Plants subjected to the highest concentrations of CBZ (5 mg L⁻¹) showed visible symptoms of toxicity, such as a reduction in leaf and root biomass, as well as a decrease in root length. The main results point to different responses according to the pharmaceutical applied. The three contaminants showed to increase the concentrations of stress indicators (hydrogen peroxide and malondialdehyde). Consequently, the enzymes involved in the detoxification of reactive oxygen species showed different patterns over time and according to the applied concentration. The ability to tolerate different concentrations of CBZ, ACT and MTF reflects the global antioxidant response of lettuce. In general, these mechanisms showed to be more effective in contaminations with ACT and MTF than with CBZ.

Proteomic analysis allowed a detailed study of different proteins involved in detoxification processes and cellular regulation processes. CBZ proved to be the contaminant that most influenced proteins abundance in roots, comparing to control, while in the leaves, this trend was observed in contamination with ACT.

Keywords: Acetaminophen, Carbamazepine, Metformin, Oxidative Stress, Proteomics.

Resumo

Os produtos farmacêuticos (considerados contaminantes emergentes) constituem uma fonte crescente de contaminantes ambientais, devido à sua elevada utilização.

Neste trabalho, pretendeu-se estudar a absorção de fármacos por uma planta alimentar, a alface, e os principais efeitos no seu metabolismo. Realizaram-se ensaios de hidroponia, em meios contaminados com carbamazepina (CBZ), metformina (MTF) e acetaminofeno (ACT), com três concentrações de cada fármaco (0,1, 1 e 5 mg L⁻¹) e três tempos de exposição (1, 8 e 15 dias).

A absorção de CBZ e ACT foi quantificada em raízes e folhas, verificando-se a sua absorção pela planta e translocação da raiz para a parte aérea. Para a MTF, a quantificação não foi possível, mas foram analisados os efeitos resultantes da sua presença no meio. As plantas sujeitas às concentrações mais elevadas de CBZ (5 mg L⁻¹) apresentaram sintomas visíveis de toxicidade, como a redução na biomassa de folhas e raízes, e menor comprimento da raiz. Os três contaminantes induziram diferentes respostas ao stresse, com aumento das concentrações de indicadores de stresse (peróxido de hidrogénio e malondialdeído), indicando que estas substâncias causam stresse oxidativo e induzem mecanismos de resposta metabólica da planta. As enzimas envolvidas na destoxificação de espécies reativas de oxigénio apresentaram padrões diferentes ao longo do tempo e da concentração aplicada. A capacidade de tolerar diferentes concentrações de CBZ, ACT e MTF resulta de uma resposta antioxidante global da alface, sendo estes mecanismos mais eficazes nas contaminações com ACT e MTF, apresentando a CBZ maior fitotoxicidade.

A análise proteómica permitiu o estudo mais detalhado de diferentes proteínas envolvidas em processos de destoxificação e processos de regulação celular. A CBZ revelou ser o fármaco com maior influência nas raízes, totalizando um maior número de proteínas mais abundantes comparativamente ao controlo. Por sua vez, nas folhas, esta tendência foi observada na contaminação com ACT.

Palavras-chave: Acetaminofeno, Carbamazepina, Metformina, Stresse oxidativo, Proteómica.

Resumo Alargado

Os produtos farmacêuticos são considerados contaminantes emergentes, e incluem-se na categoria de Fármacos e Produtos de Higiene Pessoal (do inglês: *Pharmaceutical and Personal Care Products*). Estes contaminantes têm como característica serem de grande utilização por parte da população geral. Para além dos fármacos consumidos pela população existem ainda os que são administrados aos animais, constituindo uma elevada taxa de consumo e, conseqüentemente uma via alternativa de libertação deste tipo de produtos para o meio ambiente. A presença destes contaminantes no ambiente, e o estudo dos seus efeitos em organismos presentes nos diferentes ecossistemas (espécies aquáticas, plantas, microrganismos do solo, algas) constituem uma preocupação ambiental e de saúde humana, pelo que recentemente têm sido alvo de investigação.

A alface (*Lactuca sativa* L.) é uma hortícola consumida em elevada escala (na Europa cerca de 22,5 g dia⁻¹). Tanto ao nível de exploração agrícola, como ao nível de hortas domésticas o seu cultivo é pouco exigente, com a característica de ser uma cultura que se adapta às variações climáticas. Esta hortícola é considerada um produto fresco e integra a dieta mediterrânica. A alface é uma planta constituída por 94-95% de água, o que lhe confere um baixo valor calórico e conduz ao aumento do potencial de absorção de nutrientes e outros compostos presentes no meio de cultivo.

A absorção de fármacos por plantas alimentares revela-se uma possível via para a entrada destes contaminantes na cadeia alimentar. Neste sentido, alguns estudos têm vindo a ser desenvolvidos, de modo a perceber qual o potencial de absorção de cada espécie. Também a perspetiva de fitorremediação tem sido alvo de escrutínio, uma vez que o uso de plantas pode ser uma estratégia para a redução destes compostos de águas residuais e/ou solos, sendo já comum a utilização de plantas no tratamento de efluentes. Ainda que a absorção de fármacos pelas plantas tenha relevância ao nível de acumulação e conseqüente entrada na cadeia alimentar, também o metabolismo e a regulação ao nível celular devem ganhar foco, nomeadamente ao nível do stresse oxidativo e dos mecanismos de defesa desenvolvidos.

Os fármacos selecionados para este estudo foram o acetaminofeno (ACT), a carbamazepina (CBZ) e a metformina (MTF). O ACT, também designado como paracetamol, é um dos antipiréticos e/ou analgésicos mais consumidos a nível mundial e foi já detetado tanto em águas subterrâneas como em águas destinadas ao consumo doméstico. A CBZ pertence ao grupo de anticonvulsivos ou antiepiléticos e tanto o composto parental, como os seus metabolitos são frequentemente encontrados em águas residuais. A MTF é um fármaco utilizado no tratamento da diabetes tipo II, sendo um dos medicamentos mais consumidos em

Portugal e, à semelhança dos outros fármacos, já foi detetado tanto em águas superficiais como águas subterrâneas.

Os objetivos deste trabalho consistem na avaliação e acumulação daqueles fármacos na alface, o efeito na absorção de elementos essenciais e a caracterização da resposta antioxidante nas diferentes partes da planta, nomeadamente raiz e folhas. Para estes objetivos foram realizadas diferentes metodologias e determinações analíticas: determinação da concentração de cada fármaco (apenas para ACT e CBZ), análise da composição em elementos minerais, avaliação de indicadores e mecanismos de resposta ao stresse oxidativo, e análise proteómica.

A produção de plantas de alface decorreu em diferentes ensaios de acordo com o fármaco aplicado. Para a germinação das plântulas foi utilizado substrato de germinação, e a transferência de plantas para hidroponia decorreu após o aparecimento da primeira folha. As plantas foram contaminadas com cada um dos fármacos estudados após 36 dias de desenvolvimento em solução nutritiva. As concentrações aplicadas foram $0,1 \text{ mg L}^{-1}$, 1 mg L^{-1} e 5 mg L^{-1} . Todos os ensaios foram acompanhados de um grupo de controlo, decorrendo numa câmara de crescimento sob condições controladas de intensidade luminosa, temperatura e humidade. De modo a avaliar a resposta em função do tempo, foram colhidas amostras de raízes e folhas após 1, 8 e 15 dias de contaminação com cada fármaco.

A CBZ foi detetada tanto em raízes como em folhas, mesmo nas concentrações mais baixas. Os efeitos deste contaminante foram observados através do decréscimo na biomassa total da planta, bem como na diminuição do comprimento da raiz. As folhas de alface apresentaram elevados níveis de coloração avermelhada, resultantes da resposta metabólica que se manifestou pelo aumento do teor de antocianinas e da atividade enzimática da fenilalanina-amoníase. As concentrações de peróxido de hidrogénio aumentaram em ambas as partes da planta, enquanto o teor de malondialdeído aumentou apenas nas folhas, indicando que nas raízes a peroxidação lipídica é mais controlada. A resposta antioxidante teve como principal mecanismo de defesa a ação de enzimas, diretamente envolvidas na remoção de espécies reativas de oxigénio, como a superóxido-dismutase, a catalase, a guaiacol-peroxidase e a ascorbato-peroxidase, e são estas que constituem a proteção de degradação oxidativa nas folhas. Em raízes, os mecanismos de proteção antioxidante revelaram-se um pouco distintos, baseando-se na atividade da catalase, guaiacol-peroxidase e glutathione-redutase.

Os mecanismos não-enzimáticos que envolvem o ascorbato e a glutathione mostraram ter um papel importante no processo de proteção antioxidante, em resposta ao aumento das

concentrações de CBZ. Os mecanismos não-enzimáticos evidenciaram ter maior importância nas folhas do que nas raízes, indicando serem determinantes no controlo dos danos de natureza oxidativa resultante do efeito deste contaminante nas folhas.

As plantas de alface contaminadas com ACT não apresentaram alterações visuais ao longo dos ensaios experimentais. Os teores de clorofila não revelaram diferenças significativas. Na análise dos mecanismos de resposta ao stresse oxidativo as principais diferenças foram observadas em folhas. Os teores de peróxido de hidrogénio aumentaram significativamente no dia 15 de exposição, acompanhados de um aumento da atividade da catalase indicando uma resposta da planta no controlo dos níveis de espécies reativas de oxigénio. Na contaminação com este fármaco, também a superóxido-dismutase revelou um aumento da atividade ao longo do tempo de exposição, que é relevante no controlo dos níveis de radical superóxido. No geral, as raízes foram menos afetadas, no que diz respeito à atividade de enzimas responsáveis pela remoção de espécies reativas de oxigénio. A translocação do fármaco para a parte aérea pode condicionar a resposta registada nas raízes. O ACT não afetou em grande escala a absorção de outros elementos minerais. A resposta global das plantas de alface apresentou níveis de tolerância elevados para a presença de ACT.

A exposição à MTF mostrou alguma tolerância por parte das plantas de alface. Visualmente não foram observados danos radiculares nem foliares. Ainda assim, algumas diferenças significativas foram registadas na massa de folhas e raízes no último dia de contaminação. Apesar da quantificação da MTF não ter sido realizada neste trabalho, a absorção deste fármaco foi já evidenciada em estudos de outros autores, e verificou-se o efeito da contaminação nos indicadores de stresse em folhas, nomeadamente na concentração de peróxido de hidrogénio. A remoção de espécies reativas de oxigénio em folhas foi mais evidente com o aumento da atividade de enzimas como a catalase e guaiacol-peroxidase, mas foi a ascorbato-peroxidase que revelou um aumento significativo, no conjunto dos dias de exposição estudados, indicando a importância desta enzima neste processo e o seu envolvimento nos mecanismos de resposta ao stresse. Enzimas do ciclo da glutatona destacaram-se pelo aumento da sua atividade em raízes no dia 15 de exposição, enquanto em folhas, estas diferenças ocorreram no dia 8. De um modo geral, as raízes foram o tecido menos afetado na exposição a este contaminante. A exposição à MTF causou também alteração na composição em elementos minerais em folhas, tendo contribuído para o aumento dos teores de Zn e de Mn em determinados dias de exposição, e para diminuição dos teores de Ca e Cu em relação ao controlo.

A análise proteómica permitiu uma abordagem mais ampla da influência dos fármacos no proteoma de raízes e folhas. De um modo geral, a contaminação com CBZ mostrou ser a que

mais afetou a abundância de proteínas nas raízes. No entanto, foi a contaminação com ACT a que revelou maior número de proteínas com mais diferenças significativas em relação ao controlo nas folhas. Nas raízes, proteínas relacionadas com a resposta ao stresse (superóxido-dismutase e peroxidases) revelaram uma abundância superior nas plantas contaminadas com CBZ, e a MTF afetou a abundância da proteína ascorbato-peroxidase. Estas respostas salientam importância da análise da sua relação com os mecanismos de defesa anteriormente referidos. Ainda que, diferenças significativas não tenham sido detetadas na atividade de algumas destas enzimas a sua abundância mostra uma resposta ao nível celular. Em folhas, as plantas contaminadas com ACT revelaram um aumento na abundância de proteínas como a tioredoxina-redutase e glutathione-redutase. A abundância da segunda foi também verificada nas outras contaminações e foi superior relativamente à modalidade de controlo. Para além disso, em folhas foi registado um aumento da abundância de proteínas como aquaporinas e citocromos P450, ambas com um papel importante na resposta à destoxificação de espécies reativas de oxigénio e de xenobióticos.

Os resultados obtidos para cada um dos fármacos revelaram diferentes diferenças ao nível de resposta antioxidante, absorção de elementos minerais e análise proteómica. Neste cenário, é possível afirmar que a CBZ foi o contaminante com mais efeitos negativos no metabolismo da erva, o que reflete uma menor tolerância da planta ao seu efeito tóxico. Na contaminação com ACT e MTF foi evidente que, na erva, existem mecanismos antioxidantes que constituem uma resposta eficiente e coordenada ao stresse oxidativo. O potencial de absorção destes contaminantes foi comprovado, evidenciando a possibilidade de entrada na cadeia alimentar através da erva. Ainda assim, dado o carácter emergente destes contaminantes é ainda precoce fazer uma análise do nível de exposição e dos níveis de toxicidade.

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Symbols and Abbreviations

ACT – Acetaminophen	HPLC – High performance liquid chromatography
APX –Ascorbate peroxidase	HSP – Heat shock protein
AsA – Ascorbate	SIGREM – Sistema Integrado de Gestão de Resíduos de Embalagens e Medicamentos
BP – Biological process	ICP – OES - Inductively coupled plasma - optical emission spectrometry technique
BSA – Bovine serum albumin	IDH – Isocitrate dehydrogenase
CAT – Catalase	LC – MS/MS - Liquid chromatography coupled to mass spectrometry
CBZ – Carbamazepine	LoD – Limit of detection
Ctrl – Control	LoQ – Limit of quantification
CYP450 – Cytochromes P450	MDA – Malondialdehyde
DAT – Days after treatment	MDHA – Monodehydroascorbate
DHA – Dehydroascorbate	MDHAR – Monodehydroascorbate–reductase
DHAR – Dehydroascorbate–reductase	MF – Molecular function
DM – Dry matter	MTF – Metformin
DPPH – 2,2–diphenyl–1–picrylhydrazyl	NADPH – Nicotinamide adenine dinucleotide phosphate
EDTA – Ethylenediaminetetraacetic acid	NADP+ – Nicotinamide adenine dinucleotide phosphate oxidized
EEA – European Environment Agency	NDVI – normalized difference vegetation index
ESI – Electrospray ionization	n.q. – Not quantified
EU – European Union	NRAMP – Natural resistance – associated macrophage proteins
FC – Fold change	NSCC - Non-selective cation channels
FW – Fresh weight	OECD – Organisation for Economic Cooperation and Development
GABA – Gamma–aminobutyric acid	PAL – phenylalanine ammonia–lyase
GAD – Glutamate decarboxylase	POX – peroxidases
GPOD – Guaiacol peroxidase	PPCP – Pharmaceutical and personal care products
GPX – Glutathione peroxidase	
GR – Glutathione reductase	
Grx - Glutaredoxins	
GSH – Reduced glutathione	
GSSG – Oxidized glutathione	
GST – Glutathione S transferase	
G6PDH – Glucose–6–phosphate 1–dehydrogenase	
HKT - High-affinity potassium transporters	
HMA – Heavy metal transporting ATPases	

PRI – Photochemical reflectance index

Prx – Peroxiredoxins

PSI – Photosystem I

PSII – Photosystem II

ROS – Reactive oxygen species

SH – Thiol group

SOD – Superoxide dismutase

SOS - Salt overly sensitive

TBA – Thiobarbituric acid

TCA – Trichloroacetic acid

Trx – Thioredoxins

TrxR – Thioredoxin reductase

UGHD – UDP–glucose 6–dehydrogenase

USEPA – United States Environmental
Protection Agency

WHO – World Health Organization

ZIP – Zinc regulated transporter

List of publications

2018 Poster: Leitão, I., Pinto, F.R., Sales, J., Mourato, M.P., Martins, L.L. (2018) Effects on antioxidant defense mechanisms of lettuce plants exposed to contamination by metformin. Chemistry at ULisboa – 3rd Meeting of the College of Chemistry, 3ECQUL. July. Lisbon, Portugal.

2019 Poster: Leitão, I., Pinto, F.R., Sales, J., Mourato, M.P., Martins, L.L. (2019) The presence of pharmaceuticals in irrigation water: a case study of lettuce contaminated with acetaminophen. 2019-4ECQUL-Chemistry at ULisboa - Shaping the Future, July, Lisbon, Portugal.

Poster: Leitão, I., Sales, J., Pinto, F.R., Oliveira, M.C., Mourato, M.P., Martins, L.L. (2019) Effect of carbamazepine on mineral content of lettuce plants. Science 2019, Science and technology Meeting, July 2019, Lisbon, Portugal.

2020 Oral Communication: Leitão, I., Oliveira, M. C., Martins, L.L., Mourato, M.P. (2020). Effects on antioxidant defence mechanisms of lettuce plants exposed to contamination by acetaminophen and carbamazepine. International Conference of Plant Abiotic Stress Tolerance. Wien, Austria.

E-presentation: Leitão, I. Mourato, M. P., Sales, J., Marques, M.M., Oliveira, M.C. Martins, L.L. (2020). Effects of metformin on antioxidative response of *Lactuca sativa* plants. The 1st International Electronic Conference on Plant Science. Plants. MDPI. December. Online.

International Scientific Journal: Leitão I., Mourato, M., Sales, J., Marques, M.M., Oliveira, M.C., Martins, L.L. Effects of metformin on antioxidative response of *Lactuca sativa* plants. Proceedings. 2020. doi:10.3390/IECPS2020-0877.

2021 International Scientific Journal: Leitão I., Mourato M.P., Carvalho, L., Oliveira, M. C., Marques M. M., Martins, L.L. Antioxidative response of lettuce (*Lactuca sativa*) to carbamazepine-induced stress. Environmental Science and Pollution Research. 2021. doi: 10.1007/s11356-021-13979-3.

International Scientific Journal: Leitão I., Leclercq, C.C., Ribeiro, D.M., Jenny Renaut, J., Almeida, A.M., Martins, L.L., Mourato, M.P. Stress response of lettuce (*Lactuca sativa*) to environmental contamination with selected pharmaceuticals: A proteomic study. 2021. doi: 10.1016/j.jprot.2021.104291.

International Scientific Journal: Leitão, I., Mourato, M.P., Carvalho, L., Oliveira, M. C., Marques, M.M., Martins, L.L. Acetaminophen effects on lettuce antioxidative response. *Plants*. 2021, 10(6):1152. doi: [org/10.3390/plants10061152](https://doi.org/10.3390/plants10061152).

Thesis outline

The present thesis comprises a study of the effect of three pharmaceuticals (carbamazepine, acetaminophen and metformin), in lettuce plants (*Lactuca sativa* L). Therefore, this document was organized in order to give a complete picture and a schematic analysis of the work performed, focusing on the effects of the three studied pharmaceuticals on lettuce. The contents of each chapter and the main objectives of each one are described below. Figure I shows a schematic view of the presented work.

Chapter 1 – Introduction and General Objectives – This chapter aims to provide a state of the art regarding the presence of pharmaceuticals in ecosystems, and it may be considered a key chapter for the framing of the emerging contaminants problem, providing new insights about their occurrence in different locations. It also introduces the main consequences of the pharmaceutical's uptake by edible plants, as well as the main drivers of plants defence mechanisms. Oxidative stress and pathways of reactive oxygen species detoxification are also addressed.

Chapter 2 – Material and Methods - This chapter consists in the description of the experimental work performed during this PhD project. It provides a full description of the conditions under which the experiments were carried out and the applied experimental design, as well as a description of the analytical methodologies used.

Chapter 3 – Antioxidative response of lettuce to carbamazepine-induced stress - In this chapter a detailed study of the effects of carbamazepine in lettuce plants is described. Enzymatic and non-enzymatic mechanisms were studied to understand the main changes in the antioxidative system. Oxidative stress was evaluated throughout stress indicators, to get a complete picture of cells reaction to this contaminant. Quantification of carbamazepine was also performed, as well as the mineral content of leaves and roots under carbamazepine contamination. This chapter is published in Environmental Science and Pollution Research Journal (Springer).

Chapter 4 – Antioxidative response of lettuce to acetaminophen-induced stress - This chapter includes a detailed study of the effects of acetaminophen in lettuce plants. Enzymatic and non-enzymatic mechanisms were studied to understand the main changes in antioxidative systems. Oxidative stress was evaluated throughout stress indicators, to get a complete picture of cells reaction to this contaminant. Quantification of acetaminophen was also performed, as well as the mineral content of leaves and roots under acetaminophen contamination. This chapter is published in Plants Journal (MDPI).

Chapter 5 – Antioxidative response of lettuce to metformin-induced stress - This chapter includes a detailed study of the effects of metformin in lettuce plants. Enzymatic and non-enzymatic mechanisms were studied to understand the main changes in antioxidative systems induced by this contaminant. Oxidative stress was evaluated throughout stress indicators, to get a complete picture of cells reaction to this contaminant. Mineral content of leaves and roots under metformin contamination was also studied. An analysis of reactive oxygen species detoxification mechanisms, as well as possible interaction with essential elements uptake is provided. This chapter is published in Proceedings Journal (MDPI).

Chapter 6 – Stress response of lettuce to environmental contamination with selected pharmaceuticals: A Proteomic study – Chapter 6 aims to provide more answers to the main mechanisms identified in chapter 3, 4 and 5 for each contaminant. Therefore, a proteomic approach was carried out in collaboration with Luxembourg Institute of Science and Technology. Roots and leaves of lettuce plants were exposed to carbamazepine, metformin and acetaminophen, and a protein identification was performed. Proteins were identified in different abundances as a response to each contaminant, providing new insights of the main pathways affected by the presence of these emerging contaminants in growth medium. This chapter is published in Journal of Proteomics (Elsevier).

Chapter 7 – Conclusions and Future Perspectives – The main conclusions and a final linkage among all results obtained for each contaminant described in previous chapters are presented. Future perspectives for research work are also presented.

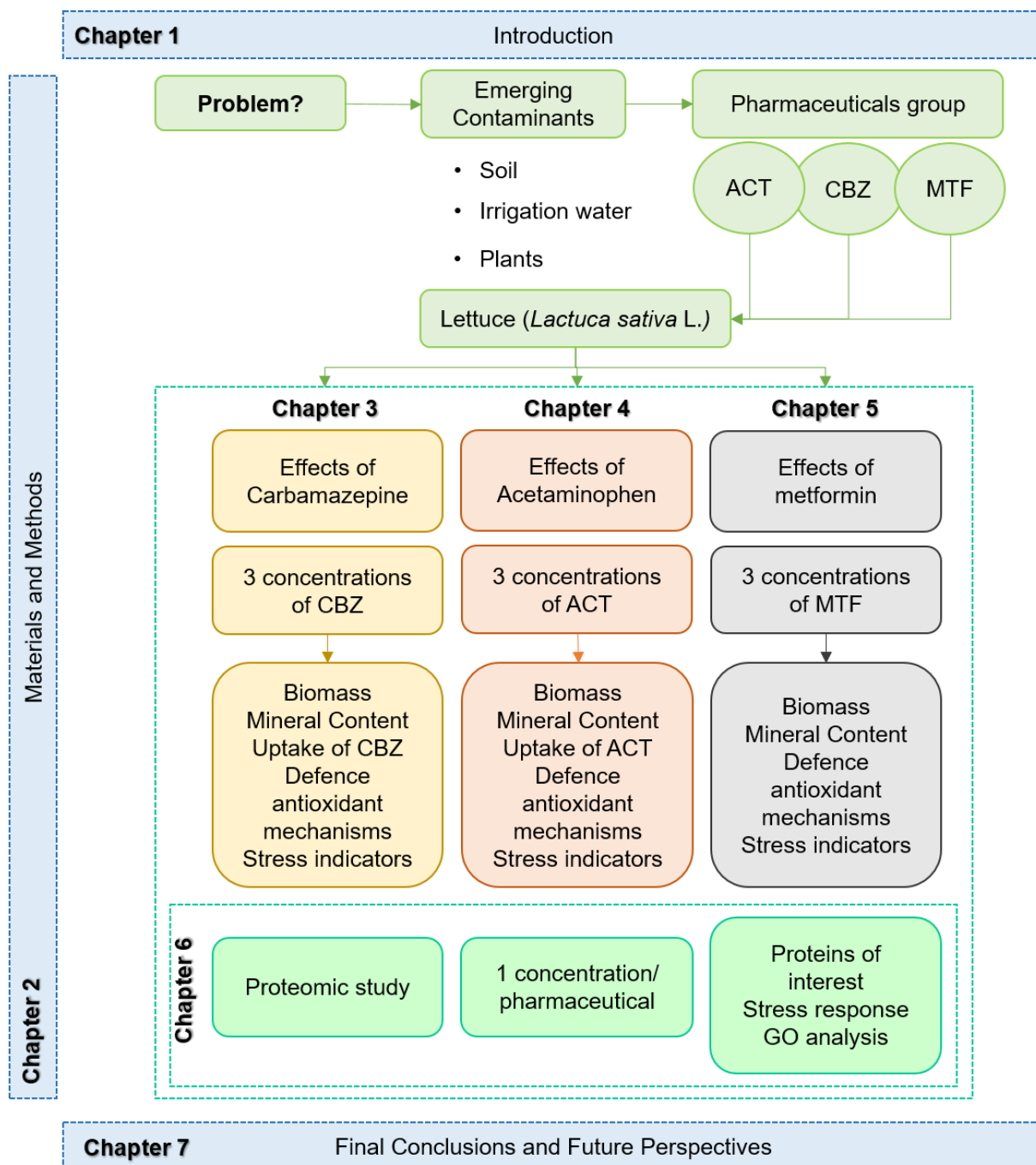


Figure I – Graphical outline of the thesis

CHAPTER I

Introduction

Chapter I - Introduction

1.1. Objectives

The objective of this thesis was the study of the response mechanisms activated by lettuce plants when subjected to the stress induced by three different pharmaceuticals (carbamazepine, acetaminophen, and metformin). The uptake capacity of these three pharmaceuticals by lettuce plants was also analysed. This work involved the following specific objectives:

- Evaluate the accumulation of pharmaceuticals in edible parts of lettuce;
- Determine the influence of pharmaceuticals on plant biomass;
- Determine the influence of pharmaceuticals on the uptake of essential minerals;
- Assess the biochemical response of lettuce to the pharmaceuticals-induced stress;
- Characterize the overall antioxidant defence mechanisms;
- Identify and understand the main proteins involved in response to pharmaceuticals exposure, throughout a proteomic approach.

1.2. Main questions

With the work presented on this thesis we intended to answer the following main questions:

- Why is it important to study these compounds?
- Are they available, absorbed and accumulated by lettuce plants?
- Are lettuce plants able to cope with the toxicity induced by these compounds?
- Which are the main defence mechanisms developed by lettuce plants during exposure?
- Which are the main results to be applied in future research?

1.3. Emerging contaminants: concept and classes

Emerging contaminants are a new class of contaminants which comprise different organic compounds such as pharmaceuticals, illicit drugs, dietetic supplements and personal care products, and also surfactants, nanoparticles and industrial additives. The fact that these compounds remain unregulated or in process to be regulated and have frequent occurrence in waterbodies confers a classification of “emerging” (Riviera-Utrilla *et al.*, 2013). According to NORMAN network (Norman Network, 2020), emerging contaminants are divided in several classes such as naturally produced compounds, bio-terrorism/sabotage agents, human personal care products, emerging persistent organic pollutants, veterinary and human pharmaceuticals, hormones, nanomaterials, metabolites and transformation products of man-made chemicals.

Our study will focus on pharmaceuticals which follow within the pharmaceuticals and personal care products (PPCP) classification. The pharmaceuticals category can include antipyretics, analgesics, lipid regulators, antibiotics, antidepressants, chemotherapeutic agents, contraceptives, and endocrine disruptors. Personal care products include not only hygienic products as shampoos and shower gels, but also sunscreen, lotions, cosmetics, fragrances and soaps (Ahmed *et al.*, 2018; Ebele *et al.*, 2017; Oliveira *et al.*, 2020). This class of products is considered essential for a healthy society and most of the compounds are associated with hygienic and disinfectant conditions required to guarantee a healthy lifestyle. Specifically, pharmaceuticals are crucial to control diseases and assure patients wellbeing.

European Union following the Precautionary Principle demand an efficient removal of all potentially harmful components (Ternes *et al.*, 2006). Different organizations have already expressed their concern about the occurrence of this type of contaminants in the environment (OECD, 2019; WHO, 2012). The United States Environment Protection Agency (2008) defined as contaminants of emerging concern products which include pharmaceuticals and personal care products. In fact, this organization also highlights that these compounds are increasingly being detected at low levels in surface waters. The European Environment Agency (2011) has already considered the potential environmental risk and hazard of pharmaceuticals. While some of these substances have already been detected as being a risk to the environment others still have unknown effects.

1.4. Pharmaceutical products as contaminants: sources and exposure

The World Health Organization (WHO) published a report acknowledging the occurrence of pharmaceuticals in drinking water and a human health risk assessment (WHO, 2012). Pharmaceuticals were already found at trace levels (from nanograms to micrograms per litre) in the environment (Talib *et al.*, 2017).

The presence of pharmaceuticals in the environment has been increasingly studied and reported, mainly in the XXI century. A search in Web of Science for “Pharmaceutical and Personal Care Products” shows an increase in the number of scientific publications since 2002 (Figure 1.1).

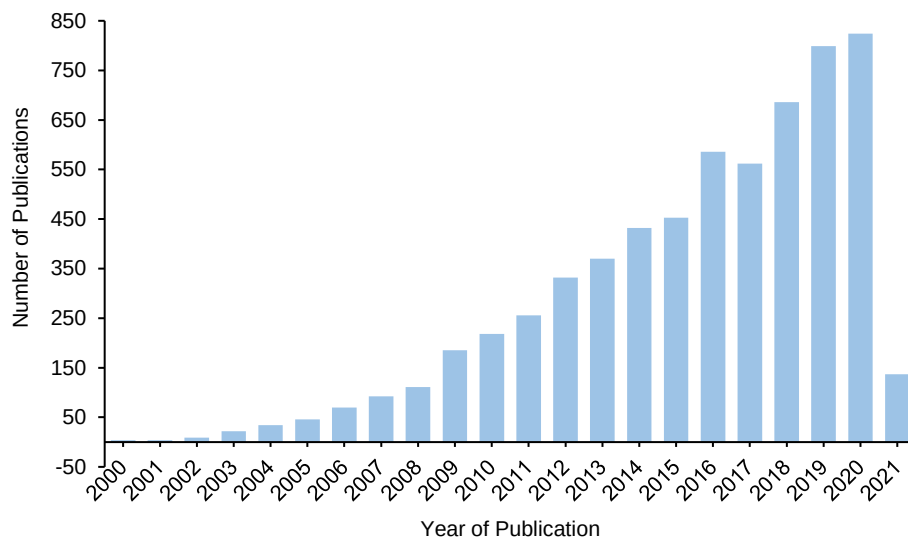


Figure 1.1. - Web of science statistic for “Pharmaceutical and personal care products publication over the past 20 years.

WHO recognized medical (human and veterinary) practices, aquaculture and agriculture or livestock, as the main contributors to the occurrence of pharmaceuticals in the environment. In fact, a wide range of activities can be the source of these contaminants of emerging concern as shown in Figure 1.2.

Pharmaceutical products (PP) can enter the environment through different pathways. One of them is the excretion of pharmaceuticals from human body (urine and faeces) in a metabolised or partly metabolised forms.

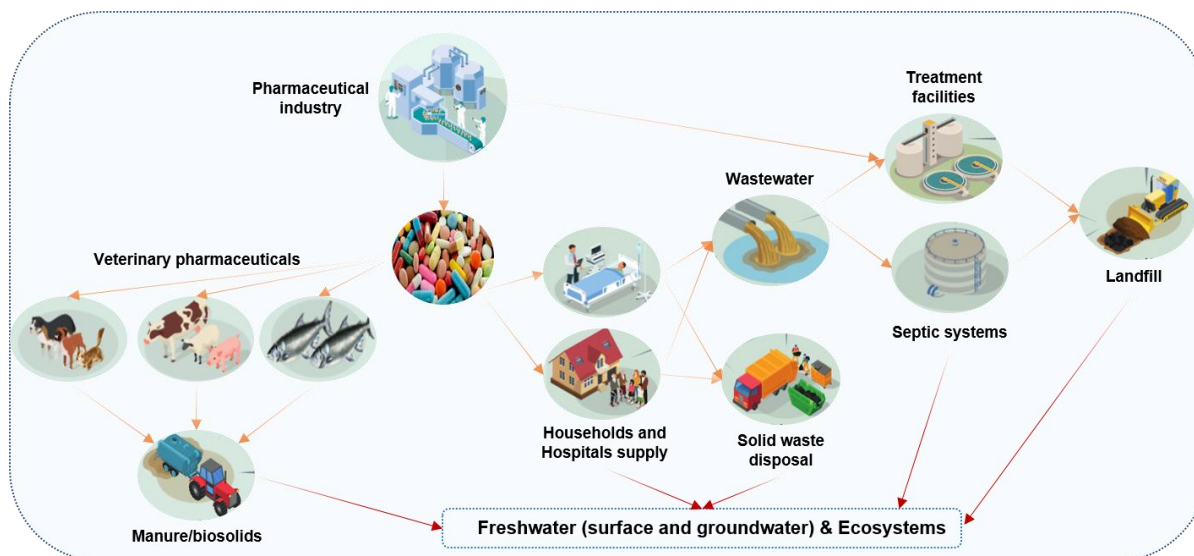


Figure 1.2. - Lifecycle of pharmaceuticals and presence in environment (adapted from OECD, 2019).

The other pathway is the incorrect disposal of pharmaceuticals in the toilet. Personal care products are released via cleaning, showering and bathing (Birch *et al.*, 2015). Wilkinson *et al.* (2017) affirmed that every human constitutes a source of a PPCP in the environment. The presence of pharmaceuticals in trace levels is occurring mainly in developed countries. Data collected supports that millions of people are drinking water contaminated with pharmaceuticals (Benotti *et al.*, 2009). The incorrect disposal of drugs in the sink, rubbish or in the toilet is more frequent than expected and is a contributor to contamination of surface and ground waters. Several studies were already conducted in attempt to understand the behaviour and the main pathways of disposal used by common people. Vellinga *et al.* (2014) conducted a study in Ireland where 51% of people dispose the unused drugs in household waste and 43% in the sink or toilet. Law *et al.* (2015) did a similar study in the US where only 11.2% of people returned unused drugs to the pharmacy. Kusturica *et al.* (2016) reported German and Sweden as the best models in terms of users' behaviour, having more than 40% of drugs returned to the pharmacy. Kinrys *et al.* (2018) defends that in the US the improvement of education on drugs disposal practices will diminished the practise of incorrect disposal of unused drugs. One of the options proposed by the authors is the increasing of takeback programs, where patients are encouraged to return the drugs on hospitals or pharmacies.

In the case of Portugal, several similar programs are already implemented. The collection of unused or expired drugs in pharmacies is already a common practise. There is a recovery system already implemented (SIGREM¹) that covers, not only pharmacies, hospital pharmacies and household pharmaceutical waste, but also veterinary pharmaceuticals and

¹ Sistema Integrado de Gestão de Resíduos de Embalagens e Medicamentos.

packaging produced during industrial activities. This system provides a wide web of collecting points, distributors, and the responsible entity (VALORMED). The collecting points receive waste from empty packaging and unused or expired medicines delivered by the consumers. Besides, the collecting points are responsible for raise awareness and enlighten consumers about the destination that should be given to waste products that have been purchased.

Hospital wastewaters have a significant impact on pharmaceuticals presence in environment, not only concerning the wide variety of pharmaceuticals (antibiotics, anti-cancer agents, among others), but also the doses administrated per day and per patient (Ternes *et al.*, 2006).

Recently, Zillien *et al.* (2019) led a study to understand the contribution of two hospitals in the Netherlands to pharmaceuticals in environment. The predicted Hospital Wastewater emissions was 68157 g year⁻¹ for metformin, 489 g year⁻¹ for carbamazepine and 39408 g year⁻¹ for acetaminophen.

Agriculture and livestock are two other sources of pharmaceuticals in the environment. Veterinary medical practices are often associated with livestock activity, not only to prevent and treat animal diseases, but also to assure the wellbeing of the animals during their life cycle. The use of veterinary antibiotics has been increasing because of the rapid development in animal farming. According with Kuppussamy *et al.* (2018) the consumption of veterinary antibiotics varies from 63000 to 240000 ton worldwide mainly due to a deficient supervision of this activity. Other authors suggested that the consumption of antimicrobials varies among animal species with 148, 45 and 12 mg kg⁻¹ for chicken, cattle and pigs, respectively (Van Boeckel *et al.*, 2015). The authors suggest that between 2010 and 2030 these values will increase 67%.

There are several causes for veterinary antibiotics being present in soils and consequently be a pathway for crops uptake. The contamination sources more frequently addressed are the application of fertilizers, such as manure from medicated animals, sewage sludge, sediments or biosolids, directly or even after composting, and the use of treated irrigation water previously contaminated with pharmaceuticals (Du *et al.*, 2014). Aquaculture activity is also one source of antibiotics and other veterinary pharmaceuticals in the environment.

The number of pharmaceuticals present in manure can fluctuate according to animals' characteristics, diet, and dose of pharmaceutical applied. Pharmacokinetics and animals' metabolism can also interfere with the amount of drugs that are excreted. Despite this, most of them are expelled in urine and faeces not only as metabolites but also in form of parent compounds (Kim *et al.*, 2007; Sarmah *et al.*, 2006). Several authors reported already a range of concentrations in different matrices, such as 46 mg kg⁻¹ of chlortetracycline in pig manure

(Martínez-Carballo *et al.*, 2007) or up to 183 mg kg⁻¹ of oxytetracycline in liquid livestock manure (Hu *et al.*, 2010).

The application of sewage sludge to soil is a practise regularly used to increase the rate of organic matter and nutrients in soil. Despite the widespread advantages of its application, the occurrence of contaminants in this sub product is common, particularly in case of pharmaceuticals (Cucina *et al.*, 2019). Sewage sludge has the capacity to absorb many potential contaminants (phthalates, pesticides, phenols, pharmaceuticals, illicit drugs, and other organic chemical residues). The ecotoxicological properties of these contaminants may comprise a potential risk to the environment due to continuous discharge in the ecosystems (Ivanová *et al.*, 2018; Martín *et al.*, 2015). Recently, the EU included some pollutants such as azithromycin, clarithromycin, erythromycin, diclofenac, estrone, 17 α -ethinylestradiol and 17 β -estradiol in the watch list to provide a monitoring program in water bodies (European Commission, 2018) but no strict regulation was yet provided for sewage sludge. More tools and technologies are being developed to increase the ability to perform analysis of these compounds in several matrices, so it is expected that more regulation will be implemented soon.

Aquaculture is an important activity for fish and shell-fish production in countries such as Norway, Egypt, Chile and several Asian countries (FAO, 2017), and these activities have been increasingly regulated, mainly associated with the use of antibiotics or growth promoters. Different hazards can occur due to veterinary products applied in aquaculture such as the incidence of residues applied above the allowed limits in fish, the use of banned/non-specific drugs for aquaculture and the development of pathogen strains resistant to antibiotics (FAO, 2018).

During the last few years, these regulations have been stricter. However, different production methods are still notorious, comparing Europe to Asia. Liu *et al.* (2017), described that aquaculture in UK only allowed the use of five different veterinary antibiotics, on the other hand in China the list included thirteen different pharmaceuticals. The production of salmon in Norway used 0.0008 kg of antibiotics per ton of salmon comparing to Chilean aquaculture where a tonne of salmon required 1.4 kg. The use of manure or wastes from livestock production in aquaculture improves the efficiency in terms of nutrients content, however this practise may also enhance potential problems concerning pharmaceuticals content or even antibiotic resistance (Cabello *et al.*, 2013, 2016).

Other source of pharmaceuticals in the environment is the use of treated wastewater in irrigation, a practise that is gaining importance to improve water resource practises. Pedrero

et al. (2010) described some parameters that should be taken into account when treated wastewater is used for irrigation (for example: salinity, nutrients and microbiological contents, the presence of metals, metalloids, pathogens, residual drugs, organic compounds, endocrine disruptor compounds and active residues of personal care products).

In Spain, the use of treated wastewater is common on agricultural activities and different regions already implemented this practise in their crops production. For instance, Valencia and Murcia regions use 57% of all the treated wastewater in Spain (Esteban *et al.*, 2008).

In Portugal, the use of treated wastewater for irrigation has being implemented (APA, 2019). These practices are established according to the main classes of products both in agricultural and landscape irrigation systems (Marecos Do Monte, 2008).

The use of treated wastewater has the main advantage of reducing the freshwater consumption of agricultural activities. As mentioned before, pharmaceuticals are frequently detected in influents and effluents from wastewater treatments plants in concentrations typically varying from ng L^{-1} to $\mu\text{g L}^{-1}$. Wastewater treatments are highly variable according to the type of pharmaceuticals and their properties. This also varies among countries and even among facilities within the same country (OECD, 2019). For instance, Deblonde *et al.* (2011), described that analgesics, anti-inflammatories and beta-blockers are extremely resistant to applied treatments, with a removal rate from 30 to 40%. A study conducted by Comber *et al.* (2018) in the UK showed that a high percentage of pharmaceuticals were removed in treatment facilities by comparison among influents and effluents. However, there are still some pharmaceuticals that can persist along the treatments such as ethinyloestradiol, diclofenac, propranolol, the macrolide antibiotics, fluoxetine, tamoxifen, and carbamazepine. For instance, in Asia the concentrations of pharmaceuticals in effluents are usually higher when compared to Europe or North America, mainly due to the high consumption of pharmaceuticals as well as poor wastewater treatment technologies (Carter *et al.*, 2019). Pedrero *et al.* (2010) reported that in Mexico farmers irrigate their crops with diluted, untreated or partly treated wastewater, which will increase the presence of contaminants and uptake by food crops. Tran *et al.* (2018) studied the occurrence of different types of pharmaceuticals and provided information about influents and effluents concentrations in Europe, North America and Asia. In that study, acetaminophen was detected in influents and effluents of wastewater treatment with a maximum concentration of $500 \mu\text{g L}^{-1}$ and $62 \mu\text{g L}^{-1}$ respectively, in North America.

Different studies have been conducted to understand the dynamic of pharmaceuticals in the environment and the possible effect in ecosystems and food chain. Figure 1.3 shows a correlation among the use of reclaimed wastewater and possible consequences to human

health and agricultural systems. However, a lot of work has yet to be done in this area, with relation to food and water security. Moreover, the consequences in soil and plants or food crops must be address, and several questions related not only with the uptake and toxicity to herbivores and humans, but also with persistence in different matrices have to be answered.

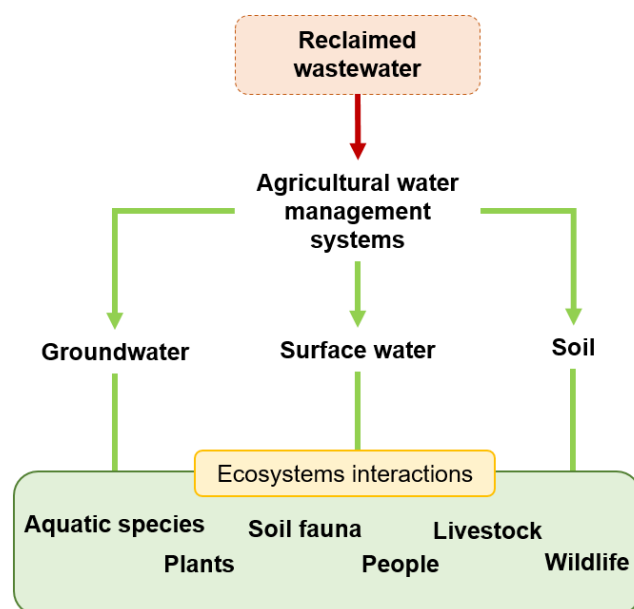


Figure 1.3. - Different pathways of pharmaceuticals in ecosystems showing the main source (reclaimed wastewater) and the Precursors and Receptors. Adapted from Carter *et al.* (2019).

1.4.1. Pharmaceuticals in water

Several pharmaceuticals have already been found in surface waters. Deo *et al.* (2014) described that 93 pharmaceuticals were present in surface waters from the USA. Although this number of pharmaceuticals is just a part of a wide variety of pharmaceuticals intake by humans and used in veterinary activities, it is expected that the effective number of pharmaceuticals occurring in surface waters continues to increase. WHO (2012) reported the occurrence of some pharmaceuticals such as carbamazepine, diclofenac and ibuprofen, in European surface waters (from Austria, Finland, France, Germany and Switzerland), with a maximum of 1200 ng L⁻¹ of diclofenac detected in German surface waters.

Some degradation of pharmaceuticals can occur in surface waters. Different reactions have been described by several authors, among them reactions like photodegradation and photolysis (Fent *et al.*, 2006). However, these reactions are not completely efficient in eliminating these compounds. Usually, concentrations in surface waters are higher compared to ground waters since the first one represents the main sink for environmental discharges. Consequently, populations that use surface water for drinking water are more exposed to

pharmaceuticals concentrations. In any case, the presence of pharmaceuticals in groundwater is also a possibility, and their persistence in this type of sink tends to be higher compared to surface waters (OECD, 2019). Figure 1.4 shows the distribution of pharmaceuticals in different water sources.

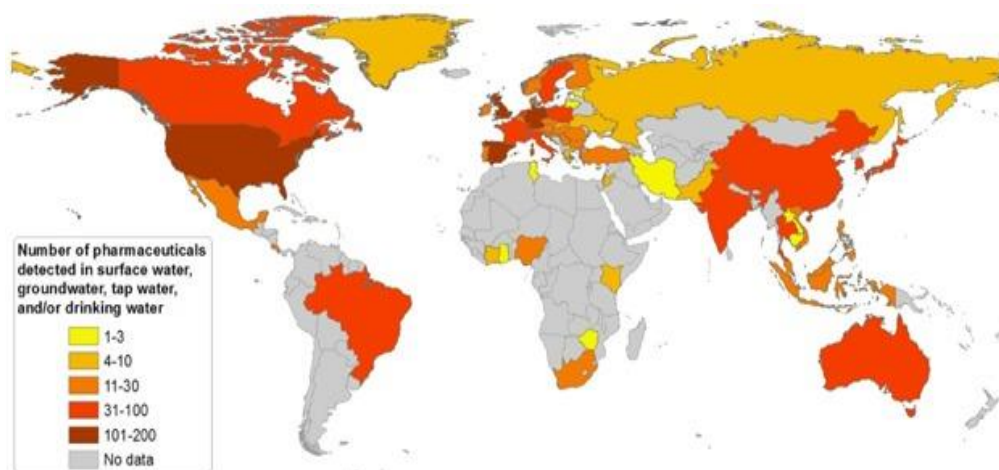


Figure 1.4. - Number of pharmaceuticals detected in surface, ground and drinking waters. Data per country (aus der Beek *et al.*, 2016)

The marine environment is less described and has less impact in human exposure to pharmaceuticals. Nevertheless, different authors described already the impact of pharmaceuticals in marine waters for aquatic organisms (mussels, amphipods, several fish species, algae, among others). The main consequences of this exposure are the reduced feeding rates, impacts on survival, reduced mussel byssus strength, oxidative and neurotoxic effects, and changes in immune response and biochemical markers (Ericson *et al.*, 2010; Gonzalez-Rey *et al.*, 2014; Guler *et al.*, 2010; Mezzelani *et al.*, 2016; Solé *et al.*, 2010).

In terms of human health risks few data is available related to long-term toxicity. Currently, organizations consider that no risk is present in terms of human exposure in drinking water containing pharmaceuticals, given the low concentrations found (OECD, 2019, WHO, 2012). However, many researchers consider that a lack of knowledge concerning long-term and low-level exposures can have different effects in certain parts of populations (children, pregnant women, and non-healthy people). However, it must be considered the exposure from different sources, not only from drinking water but also through the ingestion of plant crops, dairy products, or meat. It is therefore important to understand the bioaccumulation in edible plants and potential impact in the food chain.

1.4.2. Pharmaceuticals in soil

The presence of pharmaceuticals in soil was already confirmed by different studies, described below. After entering the soil, pharmaceuticals are subjected to microbial transformation, photodegradation, surface runoff, leaching, plant uptake or sorption/desorption processes (Jechalke *et al.*, 2014; Kuppusamy *et al.*, 2018). The occurrence of these processes is also related with the chemical properties of pharmaceuticals.

Jechalke *et al.* (2014) outlined the different pathways which are strongly determined by the soil solid phase reactions. The processes of sorption and desorption are the core of pharmaceuticals mobility and possible uptake by plants. These two processes also control pharmaceuticals biotransformation and biological effects. These reactions are mostly controlled by the polarity and water solubility of the pharmaceutical and by the pH of the medium, influencing the charge of the compounds and their speciation. Part of the pharmaceuticals may be removed throughout biological processes and interaction with organic matter or minerals (Jechalke *et al.*, 2014; Ter Laak *et al.*, 2006).

Kuppusamy *et al.* (2018) described the main effects of pharmaceuticals in soil as changes in microbial structure and function, inhibition of microbial activity in soil enzymatic activity, effects on crops growth and accumulation in plant tissues, and runoff and leaching to groundwater.

Different pharmaceuticals have been detected in leachate and runoff from lands irrigated with treated wastewaters indicating a high mobility in soil, including carbamazepine. Wu *et al.* (2015) reviewed several studies where the mobility of pharmaceuticals tends to decrease with soil organic matter content due to enhanced sorption. The fate of pharmaceuticals in soil influences the potential for plant uptake.

1.5. Pharmaceuticals properties and occurrence in environment

Physical and chemical properties of pharmaceuticals may influence their persistence and fate in ecosystems. The knowledge based on these characteristics allow to better understand their behaviour in water, soil, and uptake by plants.

Dissociation constants are important to predict the fate of different pharmaceuticals as they give information about the probability of dissociation of pharmaceuticals on aqueous solution. For high constants values, the dissociation is higher and therefore dissociation of the compound occurs faster than for compounds with lower dissociation constant. Acidity constants are particularly important on the evaluation of pharmaceuticals properties since neutral and ionic forms of a compound frequently behave differently.

Octanol water partition coefficient (K_{ow}) is another parameter considered to evaluate pharmaceuticals in the environment. It represents a measure of the tendency of a compound to move from the aqueous phase into lipids (Voutsas, 2005). The degree of compound uptake is proportional to K_{ow} usually expressed by $\log K_{ow}$ function. This parameter relates also with hydrophobicity, where low values of $\log K_{ow}$ are related with hydrophilic compounds while higher values of $\log K_{ow}$ are linked to more hydrophobic compounds (Carter *et al.*, 2014).

Despite the importance of K_{ow} , recently different authors suggested that pH from growth medium also influenced the uptake of pharmaceuticals by plants (Bhalsod *et al.*, 2018; Carballa *et al.*, 2008; Chuang *et al.*, 2019; Wu *et al.*, 2015). Then, the K_{ow} values have to be adjusted according to the medium pH, resulting in pH adjusted octanol-water coefficient (D_{ow}) which varies according to compounds and medium properties.

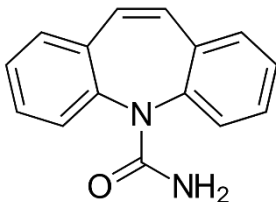
Some correlations can be established between K_{ow} and plant uptake. Jha *et al.* (2015) suggested that organic contaminants with $\log K_{ow} < 1$ are water soluble and plant roots do not accumulate at a rate surpassing passive influx into the transpiration stream, while organic contaminants with $\log K_{ow} > 3.5$ have high sorption to the roots but slow or no translocation to aerial tissues. So, the K_{ow} ranging from 1 to 3.5 is considered to favour organic compounds translocation, including pharmaceuticals.

1.5.1. Carbamazepine

Carbamazepine is an anticonvulsant used to control seizures, epilepsy, and bipolar disorder. It is a tricyclic compound related to tricyclic antidepressants with anticonvulsant and analgesic properties. The route of elimination consists in 72% of the dose in urine and 28% in faeces. Carbamazepine is mainly excreted in conjugated and hydroxylated forms. Only 3% of excreted products corresponds to the parent unchanged compound. The major metabolite of carbamazepine is carbamazepine-10,11-epoxide. In vitro studies suggest that carbamazepine is metabolized by cytochrome P450 (CYP) 3A4 to the epoxide form (National Center for Biotechnology Information, 2019b). Chemical properties of carbamazepine are described in Table 1.1.

Carbamazepine is one of the most studied pharmaceuticals in terms of persistence after wastewater treatments and occurrence in surface and ground waters. Different researchers found already this pharmaceutical in concentrations ranging from μg to ng L^{-1} . Calderón-Preciado *et al.* (2011) collected irrigation water from different streams in the area of Barcelona and found concentrations from 0.063 to 0.275 $\mu\text{g L}^{-1}$.

Table 1.1. - Physical and chemical properties of carbamazepine (National Center for Biotechnology Information, 2019b).

Physical and Chemical Properties	
Molecular Formula	C ₁₅ H ₁₂ N ₂ O
Molecular Structure	
Molecular Weight	236.269 g mol ⁻¹
Density	-
Solubility	17.7 mg L ⁻¹ at 25° C, practically insoluble. Soluble in chloroform, dimethylformamide, methanol. Slightly soluble in ethanol or glacial acetic acid.
Log K _{ow}	2.45
Log D _{ow} (pH 5.8)	2.45
pK _a	2.3 (conjugate acid), 13.9
Melting point	190.2 °C

In surface waters, Vulliet *et al.* (2011) reported a value of 13.9 ng L⁻¹ and a value of 10.4 ng L⁻¹ in groundwaters. The use of treated wastewater in agriculture is a common practise in various countries. The occurrence of carbamazepine in these water sources in USA was reported by Wu *et al.* (2009) in concentrations of 1.2 µg L⁻¹. Aquatic compartments are the most affected by the presence of pharmaceuticals, in particular carbamazepine was previously detected in concentrations ranging from 0.03 to 11.6 µg L⁻¹ in EU countries (Bahlmann *et al.*, 2014; Loos *et al.*, 2009).

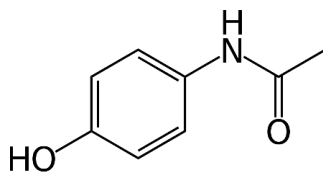
The uptake of carbamazepine by crops is a current issue approached by different authors. Recently, Chuang *et al.* (2019) showed the uptake on lettuce shoots and roots with concentrations of 258.2 ng L⁻¹ and <136.9 ng L⁻¹, respectively. In fact, value log K_{ow} suggest that carbamazepine is highly translocated to aerial parts.

The removal of carbamazepine from nutrient solution by carrot, tomato and lettuce was studied by Dodgen *et al.* (2015). Removal capacity varied from 2.7 to 15% in carrot, -0.6 to 29.6% in lettuce and 11.4 to 48.4% in tomato, depending on environment conditions - cool-humid or warm-dry.

1.5.2. Acetaminophen

Paracetamol or acetaminophen (N-acetyl-p-aminophenol) is included in the analgesics and/or antipyretics group. This pharmaceutical is mainly used for the flu, headaches, migraines, muscle, and joint pain treatments. It is also often used after surgical treatments as a pain reliever (National Center for Biotechnology Information, 2019a). According to the data from Infarmed (2020) acetaminophen is in the top 3 of most used pharmaceuticals. Acetaminophen is also used in combination with other pharmaceuticals. In human organism, 80% is excreted in urine (glucuronide or sulphate conjugates), while 3% remain as the original compound. Part of acetaminophen is metabolized by a cytochrome P450 enzyme resulting in N-acetyl-p-benzoquinoneimine, a toxic compound (National Center for Biotechnology Information, 2019a). Chemical properties of acetaminophen are described in Table 1.2.

Table 1.2. - Physical and chemical properties of acetaminophen (National Center for Biotechnology Information, 2019a).

Physical and Chemical Properties	
Molecular Formula	C ₈ H ₉ NO ₂
Molecular Structure	
Molecular Weight	151.165 g mol ⁻¹
Density	1.3 g cm ⁻³
Solubility	14000 mg L ⁻¹ at 25° C Soluble in ethanol, methanol, ethylene dichloride, acetone, ethyl acetate.
Log K _{ow}	0.46
Log D _{ow} (pH 5.8)	0.46
pK _a	9.38 (acid)
pH in aqueous solution	5.5 - 6.5
Boiling point	>500° C
Melting point	170° C

The value of log K_{ow} suggest that acetaminophen presents low tendency to accumulate in roots, or in other words, high probability of being translocated within the plant.

Although the therapeutic application is the main route of exposure to acetaminophen, exposure by other pathway should also be considered, like environmental exposure. The occurrence of

acetaminophen in environment was also studied by several researchers. Thiebault *et al.* (2017) found a concentration of acetaminophen of $96,7 \mu\text{g L}^{-1}$ in wastewaters. Vulliet *et al.* (2011) found 14.7 ng L^{-1} in surface waters from France. Fram *et al.* (2011) analysed 1231 groundwater samples used for drinking water where a maximum concentration of $1.89 \mu\text{g L}^{-1}$ of acetaminophen was found. The occurrence in streams in the USA (95 in total) was also reported by Zhang *et al.* (2007) with a medium concentration of 110 ng L^{-1} .

The uptake of acetaminophen by plants has also been studied. Li *et al.* (2019) showed that acetaminophen revealed low translocation factor from roots to aerial parts of radish plants. Chuang *et al.* (2019) described that after a period of 48 h lettuce accumulated less than 10 ng g^{-1} in roots and less than 20 ng g^{-1} in shoots. The metabolism of horseradish plants contaminated with acetaminophen was described by Huber *et al.* (2009). The authors found similarities among plants and mammalian metabolism in relation to acetaminophen, describing that the two most significant detoxification mechanisms included glutathione and formation of glucosides.

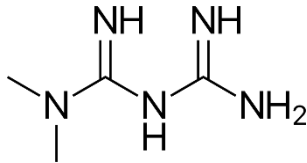
1.5.3. Metformin

Metformin is used in the treatment of adult patients with diabetes type 2. It improves glycaemic control by decreasing hepatic glucose production and glucose absorption and increasing insulin-mediated glucose uptake. Studies revealed that metformin is excreted unchanged in urine. About 90% of metformin is excreted after 24 hours (National Center for Biotechnology Information, 2019c). In Portugal, antidiabetics are one of the most prescribed class of therapeutics and metformin is in the top 3 of most used pharmaceuticals (INFARMED, 2020). Table 1.5 shows the main physical and chemical properties of metformin.

Both metformin and its biodegradation product guanyurea were found in wastewater treatment facilities (Scheurer *et al.*, 2012). Metformin exists in its monoprotonated form when pH range is from 6 to 8. At lower pH values (<3.1) metformin is biprotonated and is neutral at $\text{pH} > 13.8$ (Hernández *et al.*, 2015).

The occurrence of metformin in the environment has been reported not only in wastewater treatment plants influents ($101\text{--}129 \mu\text{g L}^{-1}$) and effluents ($2.2\text{--}21 \mu\text{g L}^{-1}$), but also in surface waters ($1.7 \mu\text{g L}^{-1}$) and sewage sludges ($500\text{--}1600 \mu\text{g kg}^{-1} \text{ DW}$) (Eggen *et al.*, 2012). Blair *et al.* (2013) reported a concentration ranging from 8.7 to 9240 ng L^{-1} in surface waters. Also, in Canada metformin was detected in 48% of the samples from surface water with a maximum concentration of 1487 ng L^{-1} (Blair *et al.*, 2013).

Table 1.3. - Physical and chemical properties of metformin (National Center for Biotechnology Information, 2019c).

Physical and Chemical Properties	
Molecular Formula	C ₄ H ₁₁ N ₅
Molecular Structure	
Molecular Weight	129.16 g mol ⁻¹
Solubility	1 x 10 ⁶ mg L ⁻¹ at 25 °C Freely water soluble as salt
Log K _{ow}	-2.64 -4.30 (pH 7.4)
Log D _{ow} (pH 5.8)	-2.64
pK _a	12.4
Boiling point	268.97 °C
Melting point	223-226 °C

This pharmaceutical was also detected in drinking water from Germany where concentrations between 2 and 61 ng L⁻¹ were found (Trautwein *et al.*, 2014). Generally, hospitals are the main contributors for the increase of metformin in influents. A study of Santos *et al.* (2013) reported that four hospitals in Portugal contributed with 7.9% of the total mass load of metformin to a wastewater treatment plant influent.

The uptake of this pharmaceutical by plants was addressed by Eggen *et al.* (2012), where the accumulation and translocation of metformin in edible part of cereals, oily seeds, beans, tomato, squash and carrots was evaluated. For instance, in case of rapeseed (*Brassica napus* and *Brassica rapa*) metformin revealed high uptake and translocation. However, the low value of log K_{ow} suggests low translocation within the plant parts. Sabourin *et al.* (2012) detected metformin in potatoes, after fertilization with municipal biosolids. Metformin was also absorbed and reached the aerial parts of *Typha latifolia* plants, however in this case the main objective of the study was to understand removal of pharmaceuticals by phytoremediation (Cui *et al.*, 2015).

1.6. Pharmaceuticals toxicity in plants

Plants are organisms that are always under pressure due to different environmental conditions. As mentioned before, different types of stresses may influence plants adaptation to the

environment. In the case of pharmaceuticals, these compounds may have a great impact on plants metabolism, since they can potentially target some ion channels and enzymes, activating some stress response pathways and acclimation processes. Besides, these compounds may also influence the absorption of essential nutrients and influence plant growth and development (Al-Farsi *et al.*, 2005; Bartrons *et al.*, 2017; Carvalho *et al.*, 2014).

Exposure to pharmaceuticals will cause plant damage at different levels, like changes on photosynthetic pigments, roots elongation, change on number and development of leaves. Studies have been carried out in different mediums, testing a wide range of concentrations. Shenker *et al.* (2011) described phytotoxicity effects on cucumber under concentrations higher than 10 mg L⁻¹ of carbamazepine showing a decrease of 50% in total biomass as well as differences in root elongation. On the other hand, concentrations of 58 µg L⁻¹ carbamazepine tested by Winker *et al.* (2010) did not affect biomass of wild species *Lolium perenne*. Carvalho *et al.* (2014) conclude that in general carbamazepine tends to accumulate in leaves in higher concentrations than in roots. Acetaminophen contamination was already studied in plants of *Brassica Juncea* (150 mg L⁻¹) showing different effects along time of exposure, bleaching after 72 h and necrosis after 168 h. For longer periods radicular tissue architecture started to change presenting a dark colour, and oxidative stress was noticed due to glutathione S-transferases and peroxidases activities (Huber *et al.*, 2009). Kotyza *et al.* (2010) studied the effects of acetaminophen in plants of *Armoracia rusticana*, *Linum usitatissimum* (both at 30 mg L⁻¹), *Hordeum vulgare*, *Lupinus luteolus*, *Phragmites australis* (all in a range of 15-181 mg L⁻¹). In all plants, acetaminophen resulted in low or no toxicity effects. Eggen *et al.* (Eggen *et al.*, 2011) carried out a study with carrots subject to metformin contaminant, with a concentration of 10 mg kg⁻¹ in spiked soil, revealing some negative effects in plants development. The same authors conducted a different study to test the uptake of metformin by *Brassica napus* and *Brassica rapa* as well as by tomato and three different cereals (wheat, barley and oat). All tested plants revealed capacity to uptake this pharmaceutical with higher accumulation in *Brassica* species (Eggen *et al.*, 2012).

Kong *et al.* (2007) conducted an experiment with *Medicago sativa* under oxytetracycline exposure at 9.2 mg L⁻¹, showing inhibition on plant growth. Several studies were carried out with *Lactuca sativa* plants. Hillis *et al.* (2011) studied the effects of ten different antibiotics, among them chlortetracycline, levofloxacin, and sulfamethoxazole, at up to 10 mg L⁻¹ concentrations. These three pharmaceuticals presented the major toxic effects, diminishing root length. Germination ratio was not affected. Migliore *et al.* (2003) studied the effects of enrofloxacin on *Lactuca sativa* plants which revealed changes on primary root length and number of leaves at the highest concentration applied (5 mg L⁻¹). More recently Mukhtar *et al.*

(2020) studied the effects of 5 antibiotics in rice culture. The study revealed phytotoxicity and genotoxicity throughout the negative impact on germination rate, seedling root/shoot length, biomass, and vigour index. The effects of the applied antibiotics were also felt on physiological parameters, decreasing proteins, carbohydrates and iron and phosphorus contents. The translocation of acetaminophen, caffeine, tylosin, carbamazepine, among others, were addressed by Chuang *et al.* (2019) showing that different compounds reveal different affinities to roots and shoots. For instance, carbamazepine and caffeine showed to be highly translocated to shoots while tylosin remain in roots tissues. The authors concluded also that acetaminophen was rapidly metabolized in lettuce cells.

Several studies have been presented over the years regarding the uptake of pharmaceuticals by plants (Carla *et al.*, 2018; Dordio *et al.*, 2011; Mzukisi *et al.*, 2018; Shenker *et al.*, 2011). Many of these studies were performed in hydroponic culture since it is the best way to control and evaluate the dynamic plant-pharmaceutical system without other interferences. The application of different treatments such as treated irrigation wastewater, manure or biosolids, or even the irrigation with contaminated solution have been the main practises in soil experiments.

One way to evaluate the uptake by plant is throughout the Bioconcentration Factor (BCF), the ratio among the concentration of pharmaceutical in plants tissues and the spiked concentration in growth medium. The BCF varies according to the pharmaceuticals properties and mobility. For instance carbamazepine and diazepam accumulated more in stems/leaves which indicates a high translocation factor (Wu *et al.*, 2015). Translocation factor (TF) is the ratio of leaf concentration and root concentration, as described by Miller *et al.* (2016).

Transpiration is often considered as the main mechanism for pharmaceuticals uptake (Dodgen *et al.*, 2015). Studies with organic chemicals suggest that hydrophobicity and dissociation are the main precursors for uptake and translocation of pharmaceuticals in plants (S. Trapp, 2000).

The uptake of organic compounds by plants and the transport through xylem have great importance on risk assessments and on the understanding of the potential for uptake and possible removal of xenobiotics by plants, a process known as phytoremediation (Dettenmaier *et al.*, 2009). The layers of plants roots (epidermis, cortex, endodermis, casparian strip, phloem, and xylem) allow different types of compounds absorption from soil solution. According to Al Farsi *et al.* (2017) the three main transportation pathways are apoplastic (between cells along cell walls), symplastic (through cells via plasmodesm) and transmembrane (through cell membranes). The properties of the compounds will influence the type of transportation into plants.

Wu *et al.* (2013) observed a linear correlation between hydrophobicity and root uptake. However, in case of ionisable compounds other factors such as electrical potential and ion trap may affect the accumulation in roots system. The acidity or basicity of pharmaceutical compounds also influence the uptake by plants. Different mechanisms are activated according to the chemical reactions. For instance, acidic species (negative ion conjugate species) are poorly absorbed by roots since plants cells have negative electrical potential at the cell membrane (-71 to -174 mV) leading to repulsion of negative ions. In case of basic species, the positive ion will induce the absorption by cells. Furthermore, two other processes may occur in these species, for example the accumulation in the vacuole by ion capture or the sorption on roots in case of more lipophilic compounds (Goldstein *et al.*, 2014; Trapp, 2009; Wu *et al.*, 2013).

Miller *et al.* (2016) suggested that lipophilicity and speciation of pharmaceuticals affects their ability to cross plant cells membrane through passive diffusion. Usually, higher lipophilicity results in a rapid diffusion across lipid bilayers. When a compound is in an ionic form it has positive or negative charge, and its diffusion is slower.

The uptake of pharmaceuticals can induce damages and changes in plant metabolism. Zhang *et al.* (2017) described as the main effects of exposure to xenobiotics changes in cell structure, damage to DNA and alterations in cell biosynthesis. Furthermore other authors described already changes in growth and development of plants, like carrot, barley and cucumber (Eggen *et al.*, 2011; Shenker *et al.*, 2011). Despite the number of studies conducted to understand the potential uptake of pharmaceuticals, a lack of knowledge constrains a proper assessment in terms of plant metabolism. Huber *et al.* (2012) suggested that xenobiotics metabolism in plants is similar to mammalian cells: phase 1 – activation reaction, phase 2 – compounds conjugate in small biomolecules and phase 3 – storage in plant vacuole, cleavage/degradation of compounds. Later, the same authors suggested an activation of peroxidases in the degradation of diclofenac (Huber *et al.*, 2016). The resulted metabolites may induce different biological activities in plants (Bartha *et al.*, 2014).

Chuang *et al.* (2019) performed a study where several pharmaceuticals were evaluated in terms of uptake and translocation into lettuce plants. The main results obtained are summarized in Figure 1.5. Briefly, pharmaceuticals were divided according to molecular weight and according to their behaviour in the Casparian strip (a region of root cell composed of lining and lamellar suberin). These cell component acts as an hydrophobic barrier between the apoplast and vascular tissues (Miller *et al.*, 2016).

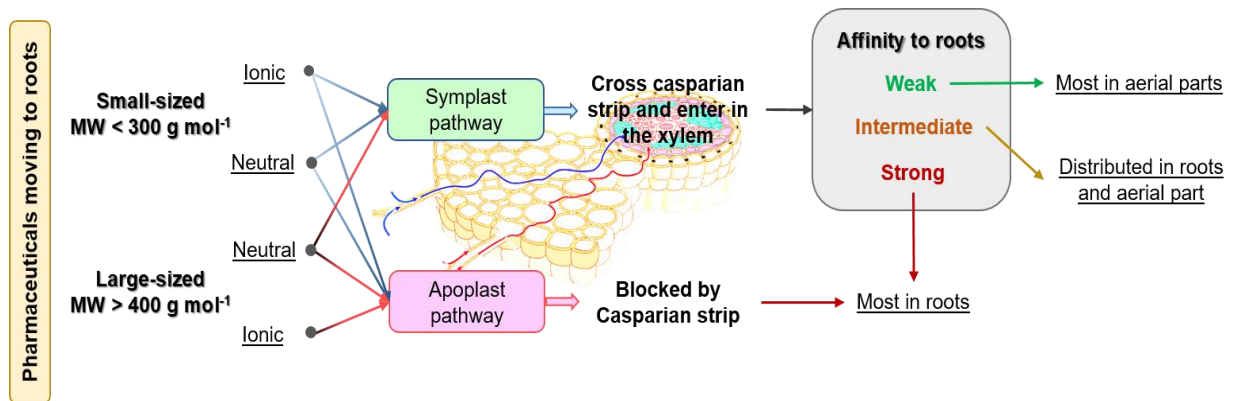


Figure 1.5. - Flow scheme of pharmaceuticals in lettuce plants, according to pharmaceutical properties. Adapted from Chuang *et al.* (2019).

By inducing phytotoxicity, these compounds affect metabolic processes, inhibiting enzymes, blocking specific pathways, with consequences in crop growth and yield, and food security.

As a consequence of phytotoxicity, an imbalance on ROS contents will be promoted causing oxidative stress. Some plants are able to cope and tolerate the presence of these compounds developing different strategies and activating antioxidant mechanisms against oxidative stress which will be described below.

1.7. Plant defence mechanisms

1.7.1. Oxidative stress

The presence of pharmaceuticals in the growth medium can induce oxidative stress in plants which cope with this stress by activating different response mechanisms.

The occurrence of oxidative stress will trigger defence mechanisms and metabolic pathways that can mitigate the oxidizing effect of Reactive Oxygen Species (ROS) preventing serious cell damage.

Mittler (2017) defined oxidative stress as a result of ROS accumulation in cells at levels that exceed the capacity of ROS scavenging. Also, the accumulation of ROS will induce different responses in cellular components, causing oxidative stress or even cell death in extreme cases.

ROS play an important role in cell homeostasis and they include singlet oxygen, superoxide radical, hydrogen peroxide and hydroxyl radical. These species are partially the result of reduced forms of atmospheric oxygen and the sequence of reactions is explained in Figure 1.6. Different species have different stabilities in cells and diverse effects in terms of oxidative stress generating different responses as defence mechanisms.

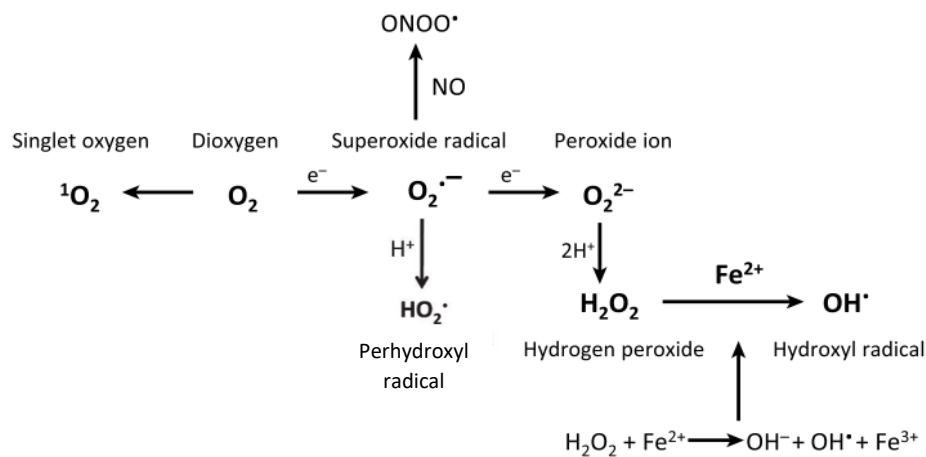


Figure 1.6. - ROS species and sequence of reactions occurring in different cell organelles. Adapted from Mittler (2017).

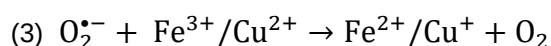
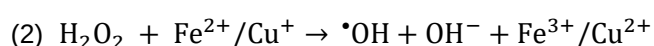
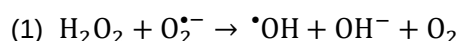
ROS are primarily formed in chloroplasts, mitochondria, and peroxisomes. The antioxidative system keeps the basal non-toxic level of ROS and deviation from this balance can cause oxidative stress.

Superoxide radical ($O_2^{\bullet-}$) has a half-time between 1 and 4 μ S and is produced in the apoplast, chloroplast, mitochondria, and peroxisomes. The main scavenging systems used to remove superoxide radical is superoxide dismutase, ascorbate and flavonoids (Demidchik, 2015; Drzeżdżon *et al.*, 2018; Mittler, 2017). This radical can be produced in several organelles of cells in non-stressed plants, however its production increases under stress producing more $O_2^{\bullet-}$ than antioxidants can detoxify. Del Rio *et al.* (2006) described that $O_2^{\bullet-}$ is produced in peroxisomes under stresses such as salinity, xenobiotics and heavy metals. Dordio *et al.* (2011) described differences in superoxide dismutase activity in *Typha* plants under carbamazepine exposure. This will cause the accumulation of hydrogen peroxide in peroxisomes and cytosol.

Singlet oxygen ($^1O_2$) is obtained through the excitation of an electron in dioxygen and throughout spin inversion. The sequence of redox reactions starts with the reduction of dioxygen with superoxide radical as primary result. The half-time of singlet oxygen ranges from 1 to 4 μ s and its production occurs in chloroplast, nucleus and membrane (Mittler, 2017). Apel and Hirt (2004) described that a high production of singlet oxygen takes place under stress conditions. Singlet oxygen is relatively stable in cells, since some studies suggested that this species can diffuse throughout chloroplast and possibly reach cell walls. The scavenging system of singlet oxygen is often linked with carotenoids, specifically β -carotene in photosystem II reactions, tocopherol, reduced plastoquinone or flavonoids (Asada, 2006; Mittler, 2017).

Hydrogen peroxide (H_2O_2) is the most stable of the reactive oxygen species. The lifetime of this species is around 1000 μ S. Hydrogen peroxide reacts with several proteins (cysteine, GSH, thioredoxins, methionine, among others), Fe enzymes, ascorbate and superoxide radical (Smirnoff *et al.*, 2019). Mitochondria, chloroplast, and cytosol are the sites of H_2O_2 production. The main detoxification pathways are catalase and some peroxidases (Mittler, 2017). Other source of H_2O_2 is the apoplast as a result of superoxide scavenging.

Hydroxyl radical ($^{\bullet}OH$) has a lifetime of 1 ns, nevertheless it is extremely reactive with biomolecules reacting rapidly with lipids, carbohydrates, DNA and proteins (Møller *et al.*, 2007). The main sources of this radical are the Fenton (eq. 1 and 2) and Haber-Weiss (eq. 3) reactions.



The specificity of scavengers for this species is not certain, however Mittler (2017) defined that the scavenging systems for $\cdot\text{OH}$ are flavonoids, proline, sugars, ascorbate, among other metabolites. Hydroxyl radical causes not only lipid peroxidation, but also oxidative damage to proteins and nucleic acids, DNA damage and chemical cross linking.

When ROS accumulates in cells they can react and affect lipids, proteins, carbohydrates and polynucleic acids. In stress conditions lipid peroxidation leads to damage of membranes and collapse of the barrier function and can also lead to the loss of function of proteins, DNA and RNA. Other cell organelles may also be affected (Demidchik, 2015; Mittler, 2002; Mourato *et al.*, 2012).

Despite the toxicity of ROS, these species are also essential to guarantee different cellular functions (Fichman *et al.*, 2019; Foyer *et al.*, 2016). The redox biology describes the regulation of different cellular processes, among them gene expression, metabolic pathways and signal transductions, which are mediated by redox reactions. This redox state is directly linked to ROS levels, mostly due to H_2O_2 capacity to oxidize cysteine residues. Therefore, the basal level of ROS sustains the redox biology keeping the regulation of several processes essential for life (Mittler, 2017) as shown in Figure 1.7.

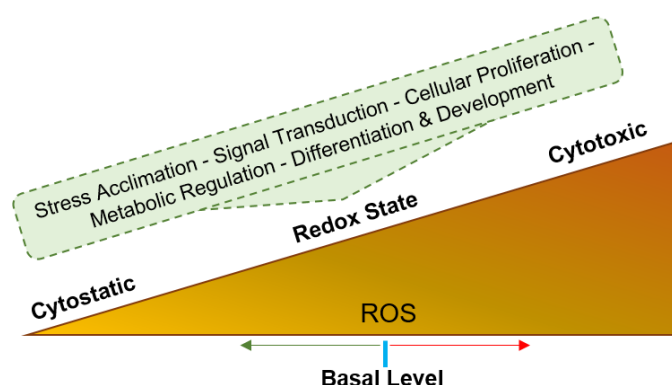


Figure 1.7. - ROS interaction with redox biology and processes related with Basal Level (Adapted from Mittler, 2017).

To respond to changes in ROS equilibrium some metabolic pathways are activated in different organelles. The antioxidant defence system is a highly coordinated mechanism, which involve specific enzymes and respective substrates in redox sequences. These substrates are different compounds that alternate their oxidation state between the reduced and the oxidized forms, which can be regenerated by the enzymatic systems. These defence systems have been described in plants by several authors, and includes the water-water cycle, ascorbate-

glutathione cycle and glutathione peroxidase cycle (Ahmad *et al.*, 2008; Foyer *et al.*, 2011), involving enzymatic and non-enzymatic coordinated reactions.

Non-enzymatic mechanisms are resultant of the presence of other components in plants with antioxidant activity such as phenols, pigments like carotenoids and flavonoids, free amino acids like proline, organic acids, phytochelatins and plants hormones such as abscisic acid, ethylene, cytokinins, salicylic acid, gibberellins, auxins among others, that can also participate in the overall defence mechanism.

The imbalance of ROS has several effects on cells as mentioned before. In fact, the activation of enzymatic and non-enzymatic mechanisms is the key to counteract the imbalance. The affinity of ROS with certain groups of enzymes and metabolites has been described by several authors. All superoxide dismutase complexes (Cu-Zn, Mn, and Fe) in different cell organelles (cytosol, mitochondria, chloroplast) have high affinity for ROS scavenging, as well as catalases, peroxidases, peroxiredoxins, thioredoxins and glutaredoxins. Enzymes such as glutathione reductase, monodehydroascorbate reductase, dehydroascorbate reductase and thioredoxin reductase take part of ROS scavenging through reduction or oxidation of substrates. Ascorbate, glutathione, proline, polyamines, carotenes, flavonoids, and tocopherol are included in group of non-enzymatic antioxidants. Despite there is no ROS specificity for these metabolites, they contribute to the defence mechanisms being substrates for related enzymes.

1.7.2. Antioxidant enzymatic mechanisms

The main enzymatic antioxidant mechanisms involve catalase (CAT, EC 1.11.1.6), superoxide-dismutase (SOD, EC 1.15.1.1), different peroxidases, guaiacol peroxidase (GPOD, EC 1.11.1.7), ascorbate peroxidase (APX, EC 1.11.1.11) and glutathione peroxidase (GPX, EC 1.11.1.9). Also, it is relevant the participation of some enzymes directly related with glutathione pools as glutathione reductase (GR, EC 1.8.1.7) and other enzymes related with the secondary metabolism on phenolic compound, such as phenylalanine ammonia lyase (PAL, EC 4.3.1.24) involved in anthocyanins synthesis.

Superoxide-dismutase (SOD) is responsible for the scavenging of superoxide radical, according to the reaction: $O_2^{\cdot-} + O_2^{\cdot-} + 2H^+ \rightarrow H_2O_2 + O_2$. This enzyme is considered the first line of response to oxidative stress as acting on one of the first types of ROS. SOD is detected in chloroplast, cytosol, mitochondria, apoplast and peroxisomes. SOD have different isoforms according with its location. When in chloroplasts, SOD uses Fe as a cofactor and in case of

mitochondria and peroxisomes the cofactor is Mn. The cofactors Zn and Cu appear in chloroplast, cytosol and apoplast (Demidchik, 2015; Mittler, 2002; Mourato *et al.*, 2012).

Catalases (CAT) are mainly present in peroxisomes, organelles which increase their number under stress conditions (Mourato *et al.*, 2012). Plant catalases monomers have a molecular mass of 55 kDa, approximately. The activity of this enzyme increases linearly with H_2O_2 concentrations. CAT is often associated with higher concentrations of H_2O_2 at mM levels in cell. CAT mediates the reactions between two molecules of hydrogen peroxide, producing water and oxygen according to the equation: $H_2O_2 + H_2O_2 \rightarrow H_2O + O_2$. This enzyme is characterized by not using a specific substrate unlike other enzymes (Mhamdi *et al.*, 2012; Mittler, 2002).

Peroxidases are a class of enzymes that catalyse several oxidative reactions, using hydrogen peroxide as oxidant substrate, but with different types of molecules as reductant substrates. Peroxidases class can be divided in three main classes, but only classes I and III are found in plants (Van Doorn *et al.*, 2014). Guaiacol peroxidase (GPOD), ascorbate peroxidase (APX), glutathione peroxidase (GPX) and thioredoxin peroxidase (Prx) are some of the peroxidases most related with oxidative stress. Some of these enzymes are activated with antioxidative compounds such as ascorbate and glutathione for APX and GPX respectively, while other enzymes such as GPOD and thioredoxin reductase (TrxR), are activated in presence of other metabolites as phenols or thiol groups (Apel *et al.*, 2004; Mata-Pérez *et al.*, 2019; Vieira Dos Santos *et al.*, 2006). In general, peroxidases are involved in the control of cell growth through cell elongation process. Flavonoids, ethylene and auxins may also be targets of peroxidase action (Bela *et al.*, 2015).

Ascorbate peroxidase (class I) is present in cytosol, apoplast, chloroplast, mitochondria, and peroxisomes, and is part of the water-water cycle and ascorbate-glutathione cycle. This enzyme is responsible for the scavenging of H_2O_2 using as electron donor reduced ascorbate (AsA), resulting in water and monodehydroascorbate or oxidized ascorbate, as in the following reaction: $H_2O_2 + AsA \rightarrow 2H_2O + MDHA$. The balance among APX, SOD and CAT activities is extremely important to maintain the steady-state level of superoxide radical and hydrogen peroxide. Ascorbate peroxidase is responsible for the fine modulation in signalling process with low concentrations of ROS, in the range of μM (Cuypers *et al.*, 2011; Mittler, 2002).

Guaiacol peroxidase (class III) is responsible for removing H_2O_2 , however in this enzyme a specific substrate, guaiacol, is required as electron donor (Fijalkowski *et al.*, 2020; Hiraga *et al.*, 2001). This enzyme can be found in the cell wall and vacuole. In the cell wall, GPODs are

responsible for lignin and suberin metabolism, auxin metabolism, and participates in defence response. The presence of phenolic compounds in vacuole allows the activity of GPOD in this organelle using phenols as substrates (Van Doorn *et al.*, 2014).

Glutathione peroxidase also removes hydrogen peroxide using glutathione as substrate. This enzyme occurs in cytosol, chloroplast, mitochondria and endoplasmic reticulum, and is part of glutathione metabolism and glutathione cycle, playing an important role in cellular antioxidant mechanisms (Gechev *et al.*, 2006; Mittler, 2002). Oxidized glutathione disulphide (GSSG) is one of the products of the following reaction catalysed by GPX: $H_2O_2 + GSH \rightarrow H_2O + GSSG$ (Apel *et al.*, 2004). GPX also has an important role as redox sensors (Bela *et al.*, 2015; Passaia *et al.*, 2015).

Peroxidase activity is also characteristic of the peroxiredoxins family, or thioredoxin peroxidases, which are thiol-specific proteins. Peroxiredoxins play a role in the regulation of hydrogen peroxide in cells contributing for oxidative stress defence mechanism (Dietz, 2011; Espinosa-Diez *et al.*, 2015), as shown in Figure 1.9. It is possible to find these enzymes in organelles as chloroplast, mitochondria and in cytosol (Mittler, 2002).

Glutathione reductase (GR) is responsible for the maintenance of glutathione (GSH) pools in cells. This enzyme catalyses the reaction – $GSSG + NAD(P)H \rightarrow GSH + NAD(P)^+$ – being part of ascorbate-glutathione cycle and glutathione peroxidase cycle described by Mittler (2002). Glutathione produced in this reaction will be the substrate for GPX activity responsible for ROS scavenging. Thus, despite the fact that this enzyme does not remove ROS directly, it is essential to the process of ROS removal completed by GPX (Apel *et al.*, 2004). Around 70 to 80% of GR is located in chloroplast while the remaining amount is located in mitochondria and in cytosol (Gill *et al.*, 2013). Figure 1.8 shows the main pathways of ROS detoxification.

The equilibrium of the activity of different enzymes is the key to control the oxidative stress processes. For instance, Mittler (2002) described that in cases where APX is suppressed, SOD, CAT and GR will compensate it inducing their activity. In plants with CAT suppression, the activities of APX and GPX will increase.

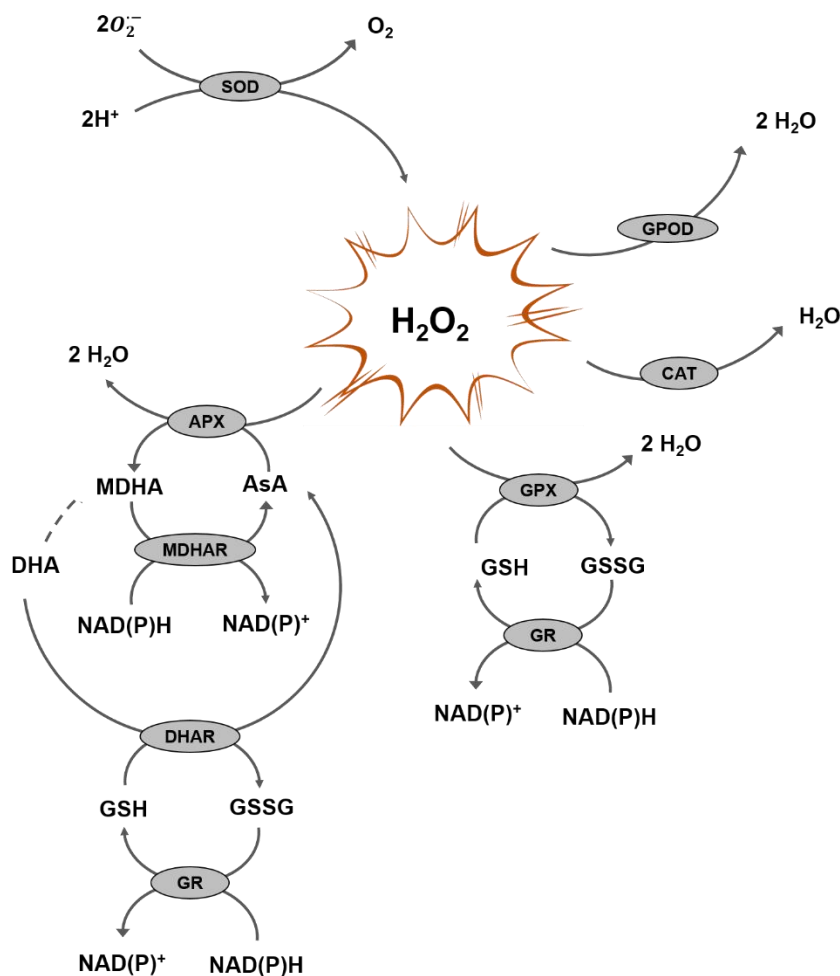


Figure 1.8. - Pathways of ROS scavenging in plants. Adapted from Mittler (2002).
 Legend: SOD – superoxide-dismutase; APX – ascorbate-peroxidase; GPOD – guaiacol-peroxidase; CAT – catalase; DHAR – dehydroascorbate-reductase; MDHAR – monodehydroascorbate-reductase; GPX – glutathione-peroxidase; GR – glutathione-reductase; AsA – reduced ascorbate; MDHA – monodehydroascorbate; DHA – dehydroascorbate.

Glutaredoxins and thioredoxins can also be linked with peroxiredoxins and glutathione antioxidant systems. Glutathione-S-transferases are also involved in these reactions, using GSH pools to detoxify cells from reactive electrophilic compounds and oxidative stress products or xenobiotics (Espinosa-Diez *et al.*, 2015; Vieira Dos Santos *et al.*, 2006). These enzymes are responsible for synthesis and scavenging of GSH and Nicotinamide Adenine Dinucleotide Phosphate (NADPH) interfering with pools available for other mechanism of defence as the ascorbate-glutathione cycle.

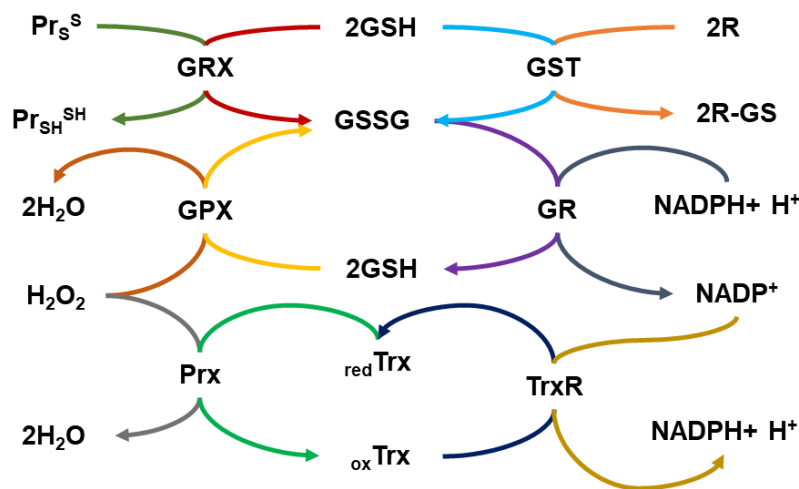


Figure 1. 9. - Glutaredoxin, peroxiredoxin, thioredoxin, and glutathione antioxidant cycle (Espinosa-Diez *et al.*, 2015).

Legend: Hydrogen peroxide (H_2O_2) Peroxiredoxins (Prx), glutathione peroxidases (GPX), glutathione (GSH), thioredoxins (Trx), thioredoxin reductase (TrxR), Oxidized glutathione disulfide (GSSG), glutathione reductase (GR), glutaredoxins (Grx) disulfide (S–S) bonds, proteins (Pr), glutathione S transferase (GST) reactive electrophilic compounds (R).

1.7.3. Non-enzymatic antioxidant metabolites

Non-enzymatic compounds have also an important role to regulate ROS homeostasis. Shulaev *et al.*, (2008) described three types of compounds important for acclimation processes: compounds as antioxidants and osmoprotectants, stress by-products resultants of disruption in cell homeostasis and signal transduction molecules. Some examples of these compounds are ascorbate, glutathione, tocopherols, anthocyanins and carotenoids which are directly related with ROS scavenging. Other molecules produced during stress processes such as salicylic acid and jasmonic acid serve as signalling molecules.

Ascorbate is a low molecular compound, essential as a cofactor in several metabolic pathways and an important component of ROS scavenging in stress response (Wang *et al.*, 2019). Different forms of AsA molecule are presented in Figure 1.10. This metabolite scavenge directly some ROS such as $^1O_2$, $O_2^{\cdot-}$ and $\cdot OH$ and regenerates α -tocopherol from tocopherol radical, which improves membrane protection (Ahmad *et al.*, 2008).

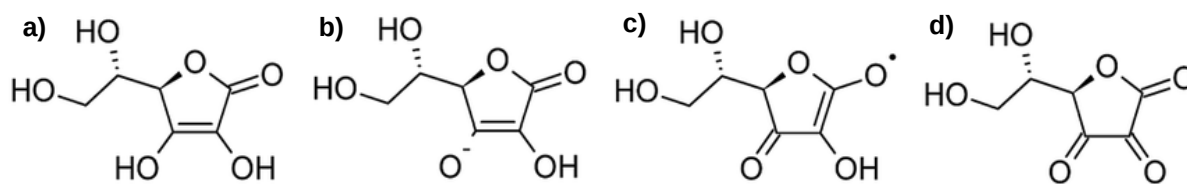


Figure 1.10. - Chemical structures of (a) acid ascorbic, (b) ascorbate, (c) monodehydroascorbate and (d) dehydroascorbate.

Furthermore, due to its high concentration in plant cells, AsA is also involved in the removal of $O_2^{\cdot-}$ leading to monodehydroascorbate and H_2O_2 production (Smirnov *et al.*, 2019). AsA is present in the cytosol, chloroplast, mitochondria, apoplast and peroxisomes (Potters *et al.*, 2002). APX activity is determined by ascorbate as substrate. Moreover, this metabolite acts as electron donor in redox reactions resulting in monodehydroascorbate or even in dehydroascorbate, both species will take part in enzymatic antioxidant systems (Mourato *et al.*, 2012). The ascorbate pool is at reduced state in normal cell conditions, however this state shifts to oxidized state under stress (Foyer *et al.*, 2011).

Glutathione is a small intracellular thiol present in plant, animal, and fungi cells. It occurs in high abundance in cells and is found in two forms, a reduced and an oxidized form. GSH participates in several physiological reactions, like detoxification of xenobiotics compounds, defence against oxidative stress and sulphur metabolism (Potters *et al.*, 2002). In terms of molecular structure, glutathione is a tripeptide which includes a bond between the carboxyl group of glutamate and the amine group of cysteine attached to a glycine. The production of GSH (Figure 1.11a) occurs mainly in chloroplast, cytosol, mitochondria, apoplast and peroxisomes. Once synthesized a rapid oxidation of GSH can occur resulting in oxidized glutathione or glutathione disulphide (GSSG) (Figure 1.11b). In oxidation processes, the thiol group of cysteine is the main target (Hasanuzzaman *et al.*, 2017; Mittler, 2002). The ratio among GSH and GSSG is extremely important in terms of cell regulation and is controlled by glutathione reductase (GR) and glutathione peroxidase (GPX) activities – the glutathione cycle (Foyer *et al.*, 2005).

AsA and GSH together are important participants in the ascorbate-glutathione cycle. Foyer and Noctor (2011) described these two metabolites as key players in a redox hub responsible for metabolic and cellular signalling responses to environmental stimuli. The high concentrations of AsA (5- 20 mM) and GSH (1- 5 mM) in chloroplast and other cellular organelles play an important role in response to oxidative stress. Mittler (2017) described that the maintenance of the ratio among these two metabolites is essential for ROS scavenging in cells. This ratio is balanced by the activity of different enzymes like GR,

monodehydroascorbate reductase (MDAR) and dehydroascorbate reductase (DHAR) using NADPH as electron donor (as can be seen in Figure 1.8). In any case, the concentration of both antioxidants molecules will serve as signalling for oxidative stress.

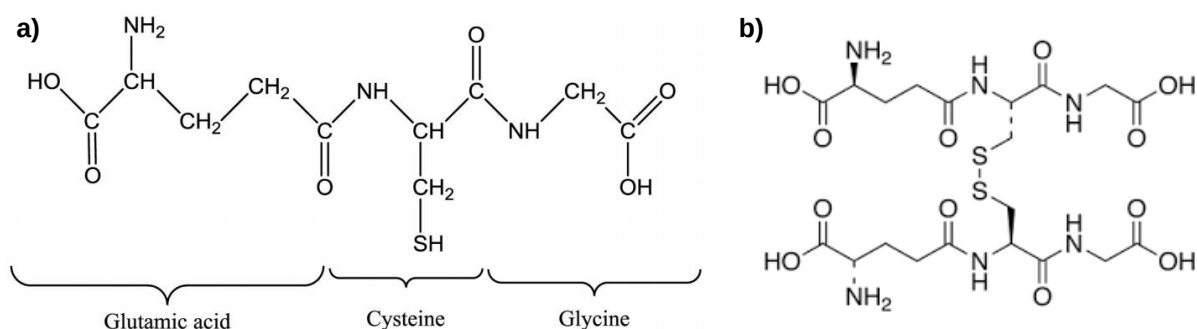


Figure 1.11. - Chemical structure of (a) reduced glutathione (GSH) and (b) oxidized glutathione or glutathione disulphide (GSSG).

Polyphenols are known as secondary metabolites to normal regulation of plant cells. Phenolic compounds are divided into several groups such as flavonoids, stilbenes, linings and phenolic acids according to their physical and chemical properties (Pandey *et al.*, 2009). For instance, lignin and suberin are classified as structural polyphenols. On the other hand, flavonoids groups are classified as non-structural polyphenols.

Different phenolic compounds are produced throughout phenylpropanoid pathway. The role of phenolics in plants is related with several physiological processes, not only growth but also synthesis of photosynthetic pigments, signal transduction, cell division, hormonal regulation, germination and reproduction rate (Sharma *et al.*, 2019).

Regarding stress response, phenolics are able to scavenge different ROS and plants are able to biosynthesize more phenolic under stress acting as an antioxidant response. The scavenging of ROS by phenolics will reduce the cell membrane peroxidation (Schroeter *et al.*, 2002). Also, according to different authors, these metabolites with antioxidant properties improve plant response in stress conditions (Cheynier *et al.*, 2013; Soares *et al.*, 2019).

According to Castañeda-Ovando *et al.* (2009) phenolic compounds have a high capacity of electron donation which will improve their antioxidant capacity when compared to AsA and α -tocopherol. Therefore the combination of AsA, peroxidases and phenolic compounds guarantee the most efficient response to stress (Van Doorn *et al.*, 2014).

As shown in Figure 1.12 flavonoids are a class of phenolic compounds. In this specific case, several subclasses can be defined according to specific properties.

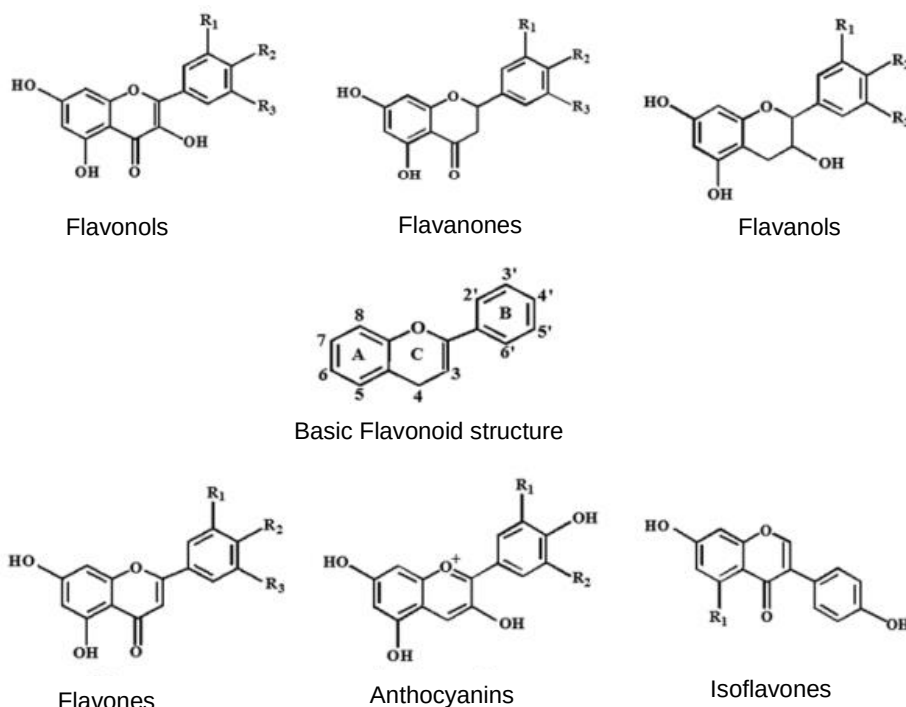


Figure 1.12. - Subclasses of flavonoids (Pandey & Rizvi, 2009).

Anthocyanins are a specific subclass of flavonoids and are characterized by a wide range of pigment colours, such as orange, red, purple or even blue in some fruits or flowers, due to the high electron mobility in molecular structure in response to visible radiation (Brewer, 2011; Liu *et al.*, 2018). In plants, the most common anthocyanins are anthocyanidins, namely pelargonidin, cyanidin, delphinidin, peonidin, petunidin, and malvidin (Kong *et al.*, 2003). These pigments are produced in cytoplasm and move into vacuole across the tonoplast (Gould *et al.*, 2002). One of the most important properties of these compounds is the high antioxidant activity and several authors described the role of anthocyanins on abiotic and biotic stresses (Ahmed *et al.*, 2014; Kong *et al.*, 2003).

The synthesis of phenolics starts with the elimination of nitrogen, in ammonia form, from L-phenylalanine, this reaction is mediated by PAL. The product is trans-cinnamic acid. PAL is crucial in secondary phenylpropanoid metabolism and is frequently associated with plant abiotic and biotic stress. The regulation among primary and secondary metabolism is determined on the first step of phenylpropanoid pathway, the synthesis of cinnamic acid. This enzyme is involved in the biosynthesis of salicylic acid which is an important signal molecule

in stress response (Huang *et al.*, 2010; Kim *et al.*, 2014; Vogt, 2010). Several other reactions take place resulting in different phenolic compounds, including anthocyanins.

Thioredoxins are small proteins with a molecular weight of approximately 12 kDa, present in all organisms, which take part of redox reactions on cells. The disulphide group present in these proteins is responsible for the control the thiol-disulfide exchange of proteins involved in different biological processes (Meyer *et al.*, 2009; Yano, 2014). These proteins are involved with different enzymatic systems such as ferredoxin-thioredoxin reductase, thioredoxin reductases and the glutathione/glutaredoxin system. Thioredoxins may be found in chloroplast, mitochondria and cytosol (Gelhaye *et al.*, 2005; Meyer *et al.*, 2012).

A wide range of applications may be addressed in order to study plants adaptation to environment. Omics technologies are one of the most important approaches providing knowledge to this field. Environmental impact of drought, salinity, herbicides and pesticides, as well as radiation or temperature, genomic and genetic characterization allowing to improve breeding strategies for crop improvement and post-harvest analysis throughout the evaluation of shelf life and special compounds stability (Witzel *et al.*, 2015). The plant proteome is not static, suffering some modifications on cell cycle and physiological states. Therefore, proteomics analyses biochemical pathways and complex response of plants to abiotic stresses. Stress response is highly connected to ROS status in cell and mechanisms of production or scavenging of these species. Consequently, a set of proteins related to the antioxidative mechanism (CAT, SOD, GPX, GR, GST, THX, POX, HSP, BZIP, among others) are important in proteomics studies that focus on oxidative stress.

1.8. Lettuce - *Lactuca sativa* L.

Lettuce is one of the most consumed crops worldwide. In 2018, the production of lettuce around the world was 27256 Mton, with 1270 Mha of harvested area. In Europe, Spain, Italy, and The Netherlands are the top 3 lettuce producers. The relation among lettuce production (ton) and yield (ha) in Europe, from 2010 to 2018, is illustrated in Figure 1.13.

In the case of Portugal, in 2019, the production reached 45219 ton, the area of harvesting was 1990 ha and the yield was 233811 hg ha⁻¹ (FAOSTAT, 2020). Lettuce is on the 7th place of horticultures with more area and production indexes for years 2016, 2017 and 2018 (INE, 2018).

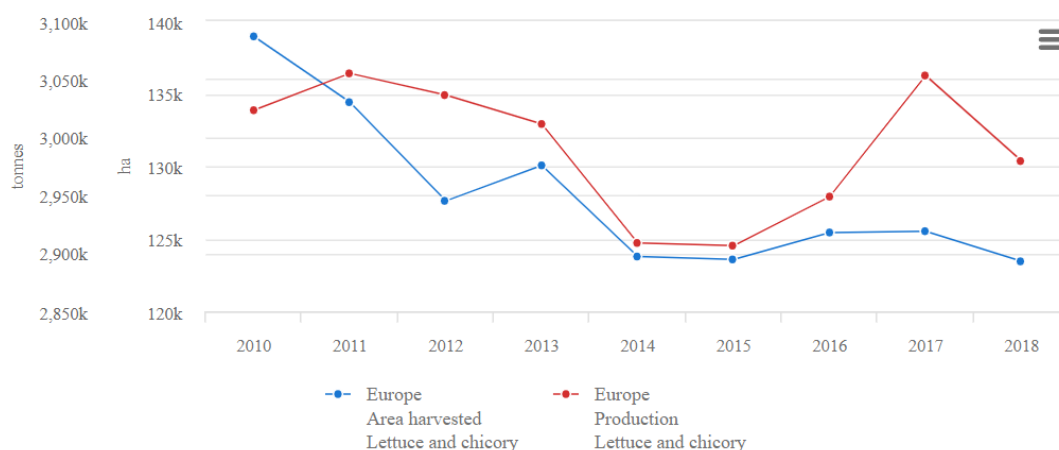


Figure 1.13. - Production/yield of lettuce and chicory in Europe from 2010 to 2018 (FAOSTAT, 2020).

Lettuce (*Lactuca sativa*) belongs to the *Asteraceae* family in the specific genus of *Lactuca*, which integrates around 100 species. Despite different families, it is clear the similarity with *Cichorium* genus. Most *Asteraceae* are cultivated due to their edible leaves, being lettuce one of the main horticultural crops of this family.

Lettuce cultivars are selected based on several criteria such as variety, leaves appearance, (smooth or curled), colour (green or red), and consistency, ability to form cabbage and adaptation to the growing season, which may be autumn-winter or spring-summer. Also, the aptitude for growing system, the susceptibility to marginal necrosis and disease resistance are factors that influence cultivars selection (Almeida, 2015).

Lettuce family include different varieties which are represented in Table 1.4.

Lettuce is an annual herbaceous crop suitable for all growing regions and known as a leaf vegetable. The root system is upright, slightly branched and remains as superficial. The root can reach more than 60 cm. In cabbage lettuce, the stem is short, with 2 to 5 cm during the vegetative phase. The aerial part is very polymorphic. During vegetative growth phase, leaves are arranged in a rosette. The leaves are elongated in the early stages and widen when the formation of cabbage begins (Almeida, 2015).

Table 1.4. - Characterization of different groups of lettuce cultivars.

Cultivars and Variety		Characterization
Butterhead var. capitata		Rounded and not very compact cabbage, with smooth and tender leaves. Susceptible to mechanical damage.
Batavia var. capitata		Rounded or ovoid cabbage or ovoid with curly leaves with sinuous or dented margins. Differences on cabbage among European and American Batavia.
Romaine var. longifolia		Smooth, elongated, upright leaves. Leaves are in the center of the plant, but no cabbage is formed.
Crisphead var. acephala var. crispa		Whole or lobed leaves, with different morphologies according to the type <i>Lollo</i> or <i>Oak Leaf</i>
Stem var. asparagina var. augustana		Characterized by the fleshy stem. It is widely cultivated and consumed in China.

According to Almeida (2015), the lettuce culture cycle is relatively short. It is an annual species and the duration of its cultural cycle varies, depending on the type of lettuce and, above all, the season of production. The greenhouse cultural cycle lasts about 6 to 8 weeks in spring-summer and 10 to 12 weeks in winter.

In this work, the variety chosen was *capitata*, more precisely a butterhead lettuce known as “Maravilha Quatro Estações”. This lettuce is fast maturing, slow to bolt, and tolerant of both high and low temperatures. The leaves are reddish green, wavy and very tender and the whole plant has a medium to large size. The cultural cycle extends for 70 days.

The growth cycle can be divided into five stages of development: germination and emergence, development of leaves, cabbage appearance, spike, flowering, and maturation of achenes. The last three phases are not part of the cultural cycle of lettuce for consumption (Almeida, 2015). Part of this cycle is presented in Figure 1.14.

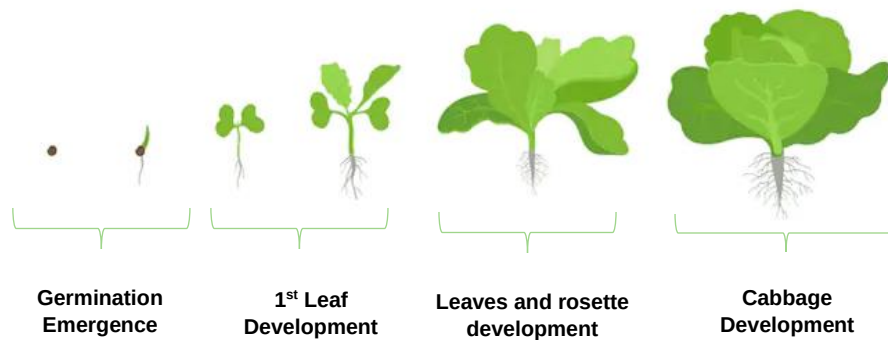


Figure 1.14. - Lettuce growth cycle from germination to harvesting.

Regarding climate requirements, there are varieties adapted to the different seasons and various climates, allowing them to be easily adapted to the climatic conditions of Portugal, at any time of year.

To germinate, lettuce seeds have a minimum temperature of 2-5 °C and a maximum of 30 °C. Germination of lettuce may occur in 2-3 days at temperatures of 20-25 °C, taking 4-6 days at 15 °C. The seeds can take more than 2 weeks to germinate when temperature is around 5 °C.

Lettuce is a plant well adapted to low light intensity although depending also on temperature conditions. The average temperatures between 15 and 20 °C are the optimal range for lettuce production and extreme temperatures affect the quality of lettuce (Gent, 2017). *Lactuca sativa* is a relatively undemanding crop in terms of nutrients. For a productivity of 45 ton ha⁻¹ of *Butterhead* and *Batavia* cultivars, the requirements of N, P₂O₅, K₂O, CaO and MgO are, 80, 40, 170, 40 and 10 kg ha⁻¹, respectively. In specific case of N, lettuce development is possible with N provided by soil organic matter mineralization (Almeida, 2015).

The lettuce crop is demanding in water, being especially important at the beginning of the crop cycle. The installation of the transplanted culture with protected root causes a very superficial root system, with 10 to 25 cm of depth in the soil. The water supply should be increased during the summer, to avoid bolting. When transplantation phase is completed, irrigation needs to be adjusted according to evapotranspiration (Almeida, 2015).

The culture can be installed by direct sowing or transplantation. The protected culture is always installed by transplantation. The substrate used is generally peat, but lettuce can also be grown in several soil-free growing systems (Barbosa *et al.*, 2015).

The harvest period of lettuce is not restricted. In fact, lettuce can be harvested at any time during vegetative development, depending on market demands and prices, or a compromise among yield and quality parameters. The main attributes of lettuce for market and consumers

are the turgidity and colour of leaves. The minimum weight requirements for lettuce are 150 g for lettuce produced in open-air conditions and 100 g when production occurs in a greenhouse (Almeida, 2015).

The composition of lettuce according to nutritional parameters is represented in Table 1.5. Lettuce crop is considered of great importance on a daily basis mainly because of biologically active substances, such as phenolic compounds, ascorbic acid, vitamins A and K, carotenoids, and folates (Kapoulas *et al.*, 2017).

Table 1.5. - Lettuce leaves composition. Values expressed for 100 g of edible part (INSA, 2017).

Water	95.9 g	Folates	55 µg	Na	3 mg
Energy	15 kcal	Caroten	688 µg	K	310 mg
Lipids	0.2 g	α-tocopherol	0.6 mg	Ca	70 mg
Carbohydrates	0.8 g	Thiamine	0.6 mg	P	46 mg
Fiber	1.3 g	Riboflavine	0.02 mg	Mg	22 mg
Protein	1.8 g	Niacin	0.4 mg	Fe	1.5 mg
Vitamin A	115 µg	Niacin equivalent	0.5 mg	Zn	0.4 mg
Vitamin B6	0.04 mg				
Vitamin C	4 mg				

CHAPTER II

Material and Methods

Chapter II - Material and Methods

In this chapter, all materials and methods regarding the laboratory work will be described. The production of plant material and the analytical determinations carried out in each part of the work will be adequately described referring the followed methodologies.

Plants production for all experiments and the major part of laboratory work was carried out in the Plant's Chemistry and Biochemistry Laboratory and at Horto de Química Agrícola of LEAF – Linking Environment, Agronomy and Food Research Centre from Instituto Superior de Agronomia, University of Lisbon.

Quantification of pharmaceuticals was performed at Mass Spectrometer Laboratory, Instituto Superior Técnico, University of Lisbon.

Proteomic studies were performed at Luxembourg Institute of Science and Technology, Green Tech platform, Environmental Research and Innovation Department (ERIN).

2.1. Lettuce plants production and plant contamination conditions

Lactuca sativa var. *capitata* was selected as case study plant. The common name of this variety is “Maravilha Quatro Estações”, as mentioned before. All the seeds used for plants production belong to the same lot and are commercialized by Vilmorin.

Lettuce plants (*Lactuca sativa*) were developed in a growth chamber with automatic control of light, humidity and temperature (Aralab) and in an air-conditioned room with control of light, temperature and humidity. The development of lettuce plants occurred in a temperature range between 20 °C and 25 °C and with a relative humidity of 62%. In addition, the photoperiod was set to 12 h. These conditions were followed for the **metabolic response** and **proteomics analysis** experiments.

The procedure for plants production followed three phases: germination, development in hydroponic or soil-free culture and harvesting of plant material.

Germination of lettuce plants occurred two days after sowing. The seeds were disposed in three trays with commercial substrate for germination (Bio Siro) - composed of humus, peat and fertilizer at pH 6. Seedlings remained in the substrate for 15 days and were then transferred to hydroponic medium. The main criteria were the appearance of the first leave.

The hydroponic medium was Hoagland Solution (Hoagland *et al.*, 1950), which composition is described in table 2.1. Lettuce seedlings were placed in styrofoam plates floating over the aerated nutrient solution, in 6.5 L recipients. During this phase, solution parameters such as

pH (5.5-6.6) and temperature (≈ 20 °C) were monitored. The solution was renewed every 5 days. This phase lasted for 21 days.

Table 2.1. - Composition of Hoagland Solution.

Macronutrients	Micronutrientes
Nitrogen (N) - 18,16 mM	Manganese (Mn) – 100 μ M
Potassium (K) – 7,00 mM	Boron (B) – 100 μ M
Calcium (Ca) – 6,00 mM	Iron (Fe) – 81,7 μ M
Sulphur (S) – 2,63 mM	Zinc (Zn) – 30,0 μ M
Magnesium (Mg) – 2,50 mM	Molibdenium (Mo) – 1,00 μ M
Phosphorus (P) – 1,00 mM	Cobalt (Co) – 0,10 μ M
	Copper (Cu) – 0,10 μ M

After those 21 days, completing 36 days of growth, plants were selected ensuring a homogenous stage of development. At this moment, plants were contaminated with concentrations of each pharmaceutical, individually. The concentrations applied were 0.1 mg L⁻¹, 1 mg L⁻¹ and 5 mg L⁻¹. On 1, 8 and 15 days after treatment (DAT) plant material was collected/harvested. The main steps of this process are illustrated in Figure 2.1.

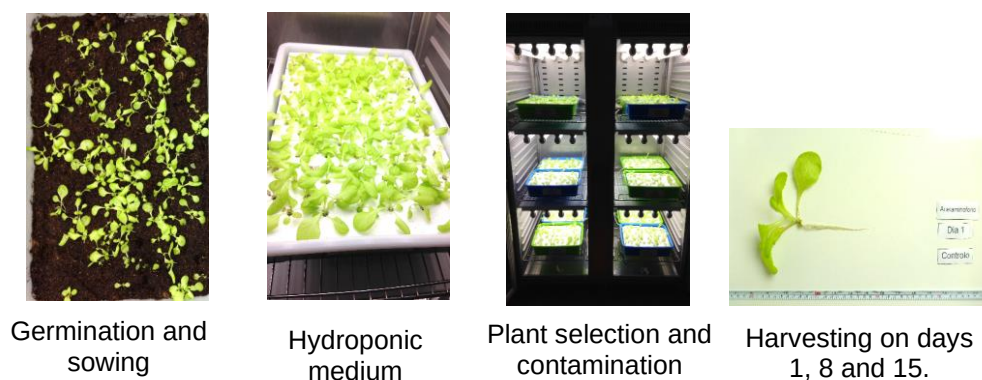


Figure 2.1. - Lettuce plants at different production stages.

Contaminating solutions were prepared according to different protocols taking into account the different pharmaceuticals properties. A stock solution of 0.1 g L⁻¹ was prepared for each pharmaceutical.

In case of metformin contamination, the solution was prepared with Generis Metformin tablets. Tablets were crushed and dissolved in water. For acetaminophen solution the procedure of dissolution was similar to Metformin, however in this case, the solution was prepared with Acetaminophen reagent (Sigma-Aldrich). In the case of carbamazepine (Sigma-Aldrich),

considering its low solubility in water, the solution was prepared with a solution of 3% of ethanol in water.

The stock solutions of each pharmaceutical were then diluted with Hoagland solution in order to obtain a final concentration of 0.1 mg L⁻¹, 1 mg L⁻¹ and 5 mg L⁻¹.

The experiments were carried out in the presence of a control treatment. The control encompassed Hoagland solution with no contamination under the same temperature and light conditions. During experiments, an assay with the different contaminated solutions, but in the absence of lettuce plants was also performed to test the possible degradation of pharmaceuticals.

2.2. Harvesting of plant material

Plant material was harvested on different days to better understand the accumulation and metabolic response during different development stages. On the days of harvest different morphological parameters were noted.

Data on biomass and number of leaves was collected. Chlorophyll was measured using a non-destructive method - Hansatech CL-01 - as well as photosynthetic parameters, Normalized Difference Vegetation Index (NDVI) - PlantPen's NDVI 200 - and Photochemical Reflectance Index (PRI) - PlantPen's PRI 300 - which allow an assessment of chlorophyll a and b, and carotenoids production levels by lettuce plants.

The plants from each contamination were separated into roots and aerial part (leaves) and dried or frozen for further analysis. For each pharmaceutical, lettuce leaves and roots were obtained for 4 concentrations (0, 0.1, 1 and 5 mg L⁻¹) and 3 days (1, 8 and 15 DAT).

The procedure for obtaining the dry material followed different steps. The leaves and roots were separated and their fresh mass (g) was determined. After being washed with deionized water, they were placed in a ventilated drying oven at 50 °C. This material remained in the drying oven for 8 days, until constant mass. When removed, the masses of dried leaves and roots were measured to obtain the dry mass (g). This method made it possible to obtain the percentage of dry matter (DM) of the lettuce for each contamination, based on the following equation:

$$\% \text{ DM} = \frac{\text{Dry mass (g)}}{\text{Fresh mass (g)}} \times 100$$

The measurements of leaves and roots masses were performed in a scale with a precision of 0.01.

To obtain the fresh material, leaves and roots were separated and roots were rinsed with deionized water. This rising is related to the subsequent determination of the pharmaceutical content in the roots, therefore eliminating the possibility of having some pharmaceuticals on the roots surface. The leaves and roots were later divided into packages of approximately 1.50 g each, and immediately frozen in liquid nitrogen. This material was stored at -80 °C until further analysis.

Monitoring of contaminated solutions was performed during the experiment to guarantee the pharmaceutical and essential mineral elements concentrations. Thus, the control and contaminated nutrient solutions were changed every 5 days. An aliquot of 10 mL of contaminated solution was collected (standard solution - 0.1 g L⁻¹, and diluted solutions - 0.1, 1 and 5 mg L⁻¹) to confirm the presence of pharmaceuticals.

2.3. Analytical Determinations on Lettuce Tissues

2.3.1. Quantification of mineral elements

To determine the mineral content in leaves and roots, dry fraction previously described was used. The concentrations of Ca, Mg, K, Na, S, P, Fe, Cu, Zn and Mn were determined.

The methodology involves three steps: measurement of mass of roots and leaves, acid digestion and the determination itself, through inductively coupled plasma - optical emission spectrometry technique (ICP-OES).

The mass of leaves and roots varied between 0.0500 and 0.3000±0.0001 g, depending on available dry material. These measurements were performed in triplicate.

After mass determination, the procedure for acid digestion was performed. A mixture of 7.5 mL of concentrated nitric acid (HNO₃) and 2.5 mL of concentrated hydrochloric acid (HCl) was added to each sample tube. The tubes were placed on a block digestion system *DigiPREP MS*. The temperature increased to 90 °C over 30 min followed by another 30 min at this temperature, followed by another 60 min at 105 °C.

At the end of the digestion process, the tubes were collected and kept in the fume hood until room temperature. When cooled, the samples were diluted to 25 mL or 10 mL, depending on the initial mass. Afterwards, samples were filtered to eliminate possible interferences on ICP-OES equipment. In each digestion, certified samples (*Lactuca sativa* L. WEPAL-IPE-776) and a blank followed the same procedure.

The elements were then quantified using a Thermo Scientific iCap 7200 ICP-OES.

The device was previously calibrated with adequate calibration curves for each element prepared from stock solution where all the elements are present at a concentration of 100 ppm or 1000 ppm. Dilutions from stock solution were prepared to the calibration curve. (Ca, Mg, K, Na, S and P: 5, 18, 50, 150 and 300 mg L⁻¹, Cu, Mn, Fe, Zn: 0.05, 0.1, 0.25, 0.5, 2.0, 5.0 and 20.0 mg L⁻¹). The calibration curves obtained showed a $R^2 > 0.98$.

Lettuce samples were combined with standards, blanks, and certified samples in order to ensure better control over the equipment accuracy. The values obtained for standards and reference samples never differed by more than 10% when compared to the reference values.

The concentration of the different elements was obtained through the relation presented by the calibration curves ($y = ax + b$) and is expressed in mg kg⁻¹ DM.

2.3.2. Quantification of pharmaceuticals in roots and leaves

2.3.2.1. Carbamazepine

To evaluate the uptake of carbamazepine, frozen leaf and root tissues (≈500 mg) were crushed in a mortar with 1.5 mL of 30:70 water:acetonitrile (v:v). The samples were centrifuged at 20000 x g for 20 min at 4 °C. The supernatant was collected and kept at 4 °C until further analysis.

Identification and quantification of CBZ in plant extracts was performed on an LC-MS system composed by an UltiMate 3000 RSLCnano (Thermo Scientific) interfaced with a QqTOF Impact II mass spectrometer, with an electrospray ion source (Bruker Daltonics). Chromatographic separation was carried out on a C18 reverse-phase Halo column (4.6 x 150 mm, 2.7 µm particle size; Advanced Materials Technology). The mobile phase consisted of 0.1% formic acid in water v/v (A) and in acetonitrile v/v (B). The elution gradient was as follows: 0-0.5 min, 0% B; 0.5-2 min, 5% B; 2-4 min, 60% B; 4-4.5 min, 80% B; 4.5-5 min, 90% B; 5-7 min, 90% B; 7-12 min, 0% B. The injected volume was 10 µL, the flow rate was 250 µL min⁻¹, and the temperature of the column and autosampler were maintained at 40 °C and 8 °C, respectively. The mass spectrometer was operated in the ESI positive mode under the following general conditions: ion spray voltage, 4.5 kV; end plate offset, -500 V, nebulizer gas pressure (N₂), 2.8 bar; dry gas flow (N₂), 8 L min⁻¹; and dry heater temperature, 200 °C. To ensure the accuracy and reproducibility of the mass analyser, an internal calibration was achieved with a solution of ammonium formate 10 mM introduced to the ion source via a 20 µL loop at the beginning of each analysis, using a six-port valve. Acquisition was performed in full scan mode in the m/z 70-1000 range at a scan rate of 1 Hz, Data acquisition and processing were performed using Data Analysis 4.2 and TASQ 1.4 software (Bruker Daltonics).

CBZ was identified based on accurate m/z values released as protonated molecules $[M+H]^+$, taking into account the accuracy and precision of the measurement parameters, such as error (ppm) and mSigma. The molecular formula is validated by extracting the ion chromatograms from the raw data, and the accurate mass and isotopic pattern were evaluated.

Estimation of the CBZ concentrations was done on the basis of a calibration curve of carbamazepine (CAS: 298-46-4, Sigma Aldrich) prepared in the concentration range of 5 to 5×10^3 ppb, and in 30:70 water:acetonitrile (v:v), to match the medium to be used for analysis of the plant materials.

The quantitation of CBZ was performed on TASQ Bruker software. The regression data for CBZ have a good linear relationship with $R^2 > 0.98$ over the whole concentration range studied, as shown in Figure 2.2. Limit of detection (LoD) and limit of quantification (LoQ) were determined using a signal-to-noise ratio of 3:1 and 10:1, respectively were 0.0002 and 0.0005 $\mu\text{g g}^{-1}$ FW, using an average mass of 0.24 g (average of the lower quantified samples).

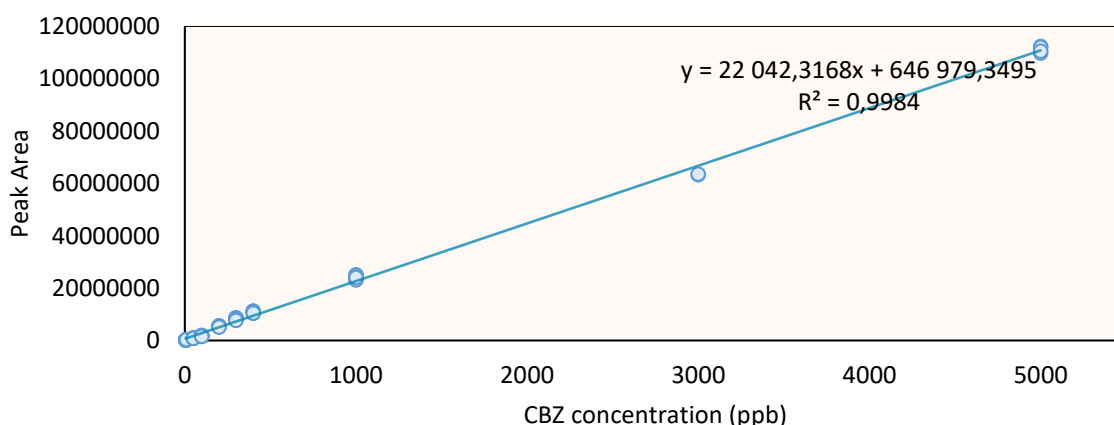


Figure 2.2. - Linear regression between concentration and signal (peak area). CBZ standards in the concentration range of 5 to 5×10^3 ppb.

Results of CBZ contamination in lettuce were presented in $\mu\text{g CBZ g}^{-1}$ of fresh weight (FW) of roots or leaves.

2.3.2.2. Acetaminophen

To evaluate the uptake of acetaminophen, frozen leaf and root tissues (≈ 500 mg) were crushed in a mortar with 1.5 mL of methanol. The samples were centrifuged at $20000 \times g$ for 20 min at 4°C . The supernatant was collected and kept at 4°C until further analysis.

Identification and quantification of ACT in plant extracts was performed in an LC-MS system composed by an Elute UHPLC system and tandem Impact II QTOF mass spectrometer (Bruker Daltonics, Bremen, Germany) with an electrospray ion source (ESI). Chromatographic separation was carried out on a Luna C18 reversed-phase column $100 \text{ \AA}$ ($150 \times 3 \text{ mm}$, $3 \text{ \mu m}$ particle size; Phenomenex). The mobile phase consisted of 0.1% (v/v) formic acid in water (A) and in acetonitrile (B). The elution gradient was as follows: 0-0.5 min, 5% B; 0.5-2 min, 5% B; 2-7 min, 40% B; 7-11 min, 95% B; 11-11.5 min, 95% B; 11.5-15 min, 5% B. The injected volume was $10 \text{ \mu L}$, the flow rate was $300 \text{ \mu L min}^{-1}$, and the temperatures of the column and autosampler were maintained at 40°C and 8°C , respectively. The mass spectrometer was operated in the ESI positive mode under the following general conditions described in section 2.3.2.1. Sodium formate (10 mM) was used as internal calibrant before each analysis. Acquisitions were performed in full scan mode in the m/z 70–1000 mass range, at a scan rate of 1 Hz. Data acquisition and processing were performed using Data Analysis 4.2, Target Analysis 1.3 and TASQ 1.4 software (Bruker Daltonics).

ACT was identified based on accurate m/z values released as protonated molecules $[\text{M}+\text{H}]^+$, taking into account the accuracy and precision of the measurement parameters, such as error (ppm) and mSigma, as described in previously section. As internal standard it was used a solution of acetaminophen-D₄, ($100 \text{ \mu g mL}^{-1}$), to correct for experimental variability during sample preparation and analysis,

Estimation of the ACT concentrations was performed based on an external standard curve of acetaminophen (CAS: 103-90-2, Sigma Aldrich) prepared in the concentration range of 10 to 3×10^3 ppb in methanol, to match the medium to be used for analysis of the plant materials. The regression data for ACT had a good linear response, with $R^2 > 0.98$ over the whole concentration range studied.

The quantitation of ACT was performed on TASQ Bruker software and the obtained calibration curve is shown in Figure 2.3. The regression data for ACT had a good linear response, with $R^2 > 0.98$ over the whole concentration range studied. Limit of detection (LoD) and limit of quantification (LoQ) were 0.0042 and $0.0013 \text{ \mu g g}^{-1}$ FW, using an average mass of 0.75 g (average of the lower quantified samples).

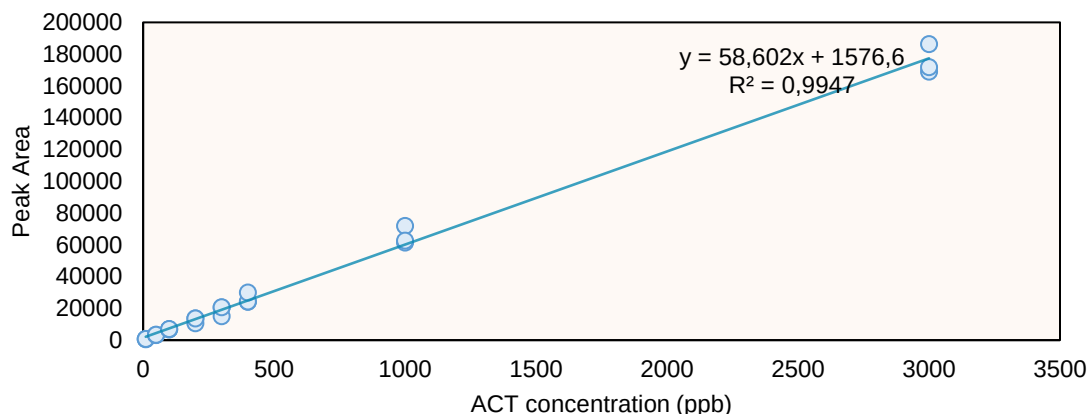


Figure 2.3. - Linear regression between concentration and signal (peak area). ACT standards in the concentration range of 10 to 3×10^3 ppb.

Results of ACT contamination in lettuce were presented in $\mu\text{g ACT g}^{-1}$ FW of roots or leaves.

2.3.2.3. Bioconcentration and Translocation Factors

To evaluate plant exposure and uptake to pharmaceuticals, bioconcentration factors (leaf, BCF_{leaf} or BCF_{root}) and translocation factors (TF) were determined by the following expressions as described by Miller *et al.* (2016).

$$\text{BCF} = \frac{\text{concentration in roots or leaves}}{\text{medium growth concentration}}$$

$$\text{TF} = \frac{\text{concentration in leaves}}{\text{concentration in roots}} = \frac{\text{LCF}}{\text{RCF}}$$

2.3.3. Photosynthetic parameters

The photosynthetic parameters chlorophyll, Normalized Difference Vegetation Index (NDVI) and Photochemical Reflectance Index (PRI) were determined with a non-destructive method, performed on harvestings days (1, 8 and 15).

To determine chlorophyll the equipment used was the Hansatech CL-01. This device allows the estimation of photosynthetic pigments by measuring the absorbance at two wavelengths, 620 and 940 nm. Units of chlorophyll content are usually referred to as Hansatec units and are arbitrary. This indirect and non-destructive method allowed a continuous assessment of the chlorophyll content, both in control and contaminated plants. For this, 5 leaves of each modality

were selected and on each 4 distinct spots measurements were performed. The device was calibrated at the beginning of each measurement.

NDVI and PRI indexes were also measured on the harvest days, similar to the chlorophyll content. For these indexes, 5 leaves of each modality were also selected, but picking only 2 distinct points on the leaf.

For NDVI measurement, PlantPen's NDVI 200 equipment was used. The equipment allows the comparison among the reflected light at two wavelengths - 677 and 774 nm. The first wavelength is related to the peak of visible light absorption by chlorophyll, while the second one is related to infrared radiation reflected by the cellular structure of the leaves. The absorbance differences are thus used to determine the NDVI (Cordon *et al.*, 2016), according to the formula:

$$NDVI = \frac{\rho_{774} - \rho_{677}}{\rho_{774} + \rho_{677}}$$

PRI was determined using PlantPen's PRI 300 equipment. This equipment obtains the absorbance at two wavelengths - 531 and 570 nm. PRI is responsive to changes in carotenoid pigments which often are indicators of photosynthetic light use efficiency or the rate of carbon dioxide uptake by leaves. The PRI values vary between -1 and 1 and are obtained using the following formula:

$$PRI = \frac{\rho_{531} - \rho_{570}}{\rho_{531} + \rho_{570}}$$

The quantification of photosynthetic pigments was also performed using a method adapted from Sims & Gamons (2002).

For sample extraction 0.06 g of leaves were crushed with 10 mL of 80% acetone solution in 100 mM Tris-HCl buffer, in a dark environment. The determination was performed in triplicate. The extracts were centrifuged at 10000 x g for 15 minutes at 6 °C (*Sigma 03-18 K*). The supernatant was collected and the absorbances were acquired at 470, 537, 647 and 663 nm in glass cuvettes, by molecular absorption spectrophotometry (AnalyticJena SPECORD 200).

The concentrations of different pigments were estimated throughout the relationship with the Lambert-Beer Law and with the following equations:

$$[\text{Chlorophyll a}] = 0.01373\text{Abs}_{663} - 0.000897\text{Abs}_{537} - 0.003046\text{Abs}_{647} \quad (\mu\text{mol mL}^{-1})$$

$$[\text{Chlorophyll b}] = 0.02405\text{Abs}_{647} - 0.0004305\text{Abs}_{537} - 0.005507\text{Abs}_{663} \quad (\mu\text{mol mL}^{-1})$$

$$[\text{Total chlorophyll}] = [\text{Chlorophyll a}] + [\text{Chlorophyll b}] \quad (\mu\text{mol mL}^{-1})$$

To express the results on fresh weight, the following equation was applied:

$$[pigment] = \frac{[P] \times volume_{acetone}}{mass_{sample}} \quad (\mu\text{mol pigment } g^{-1} \text{ FW})$$

Where $[P]$ is the concentration of the pigment obtained from equations previously described ($\mu\text{mol mL}^{-1}$).

The results of pigments concentrations were expressed in $\mu\text{mol g}^{-1}$ FW.

2.3.4. Analysis of Non-Enzymatic parameters

2.3.4.1. Lipid Peroxidation

Lipid peroxidation was obtained following the method adapted from Demiral & Turkan (2005), through molecular absorption spectrophotometry to determine malondialdehyde (MDA) concentration present in lettuce tissues.

Sample extraction was performed by macerating 0.5 g of fresh material with 2.5 mL of trichloroacetic acid (TCA) at 0.10% (w/v), followed by centrifugation during 15 min at 12000 x g. Afterwards, 1 mL of the supernatant and 4 mL of TCA 20% + thiobarbituric acid (TBA) 0.5% solution were heated at 95 °C for 30 minutes, and cooled with an ice bath to stop the reaction, followed by centrifugation for 15 minutes at 10000 x g (*Sigma 03-18 K*). The absorbance was measured at 532 and 600 nm (a correction for unspecified turbidity) by molecular absorption spectrophotometry (AnalyticJena SPECORD 200). The concentration of MDA was obtained using the equation:

$$[MDA]_{mM} = \frac{Abs_{532} - Abs_{600}}{\epsilon}$$

Where ϵ is molar absorptivity coefficient ($155 \text{ mM}^{-1} \text{ cm}^{-1}$).

To convert the concentration of MDA to fresh mass, the following formula was applied:

$$[MDA] = \frac{[MDA]_{mM} \times 1000}{mass_{sample}} \quad (\mu\text{M MDA } g^{-1} \text{ FW})$$

The MDA concentrations were express $\mu\text{M g}^{-1}$ FW.

2.3.4.2. Hydrogen Peroxide

The determination of hydrogen peroxide H_2O_2 was performed using a method adapted from Singh *et al.*, (2006). The sample extraction was performed as described previously for the MDA

assay. Following the extraction and centrifugation, a supernatant aliquot of 100 to 500 μL was taken and 900 to 500 μL of phosphate buffer solution 0.1 M (pH 7.6) plus 1000 μL of potassium iodide solution (KI) 1 M were added to each sample. This solution was added to the extract+buffer because the iodide ions slowly oxidize in the presence of H_2O_2 , causing a variation on absorbance. The absorbance of supernatant was measured at 390 nm by molecular absorption spectrophotometry (AnalyticJena SPECORD 200).

The correlation between absorbance and H_2O_2 concentration was obtained throughout a calibration curve. A stock solution 0.16 mM H_2O_2 was prepared and diluted to obtain the required final concentrations: 2.2; 4.4; 6.6; 8.8; and 11.0 $\mu\text{g mL}^{-1}$. Each standard was prepared in triplicate to obtain a triplicate of the calibration curve. The equation used as correlation was $y = 0.0699x + 0.0083, R^2 = 0.9984$.

The concentration of H_2O_2 was obtained using the following equation:

$$[\text{H}_2\text{O}_2] = \frac{[\text{H}_2\text{O}_2] \times \text{volume}_{\text{TCA}}}{\text{mass}_{\text{sample}}} \times \frac{0.5}{\text{volume}_{\text{ext}}} \quad (\mu\text{g H}_2\text{O}_2 \text{ g}^{-1} \text{FW})$$

Where $[\text{H}_2\text{O}_2]$ ($\mu\text{g mL}^{-1}$) is the concentration of H_2O_2 from the calibration curve.

2.3.4.3. Antioxidant Activity

The presence of antioxidants is crucial in some of the reactions with radicals present in cells. The method to determine total antioxidant activity described by Swain *et al.* (1959) is based on the capture of the radical 2,2-diphenyl-1-picrylhydrazil (DPPH) by the antioxidants of the leaves or roots under analysis. The DPPH radical is characterized by its intense purple colour, however when it contacts with antioxidant compounds this colour becomes less intense, thus revealing a greater or lesser antioxidant activity.

For determination of antioxidant activity, sample extraction was performed by macerating 0.5 g of fresh material (roots or leaves) with 2 mL of methanol followed by centrifugation at 8000 x g for 10 min at 4 °C (*Sigma 03-18 K*). The extraction was performed in triplicate and in a low light environment. Samples were kept at 4 °C in the dark for 24 h.

Afterwards, samples were once again centrifuged (10 min at 4 °C). A volume of supernatant (150 μL) was collected and 2850 μL of DPPH (2,2-diphenyl-1-picrylhydrazyl) were added. The DPPH solution was prepared from a stock solution of 0.61 mM of DPPH, with a 5.5 x dilution factor. This solution was prepared 2 hours before the procedure. Tubes were incubated in the dark for 40 min, and the absorbance at 517 nm was determined with a blank test with methanol

(Brand-Williams *et al.*, 1995). The absorbance was obtained by molecular absorption spectrophotometry (AnalyticJena SPECORD 200).

In parallel to sample preparation, a calibration curve with 8 standards was also prepared from a stock solution of 6-hydroxy-2,5,7,8-tetramethyl chroman-2-carboxylic acid or Trolox (reference antioxidant compound). The stock solution of Trolox at 1 mM was diluted to obtain the following standards: 0, 75; 150; 300; 500; 600; 700 and 1000 μM . The same procedure of the samples was applied to the standards. The calibration curve was $y = -0.001x + 1.1256$, $R^2 = 0.9895$.

The Trolox Equivalent Antioxidant Capacity (TEAC) was obtained applying the following equation:

$$\text{TEAC} = \frac{[x] \times \text{volume}_{\text{ext}}}{\left(\frac{\text{mass}_{\text{sample}}}{\text{volume}_{\text{methanol}}} \right) \times 0.15} \quad (\mu\text{M Trolox g}^{-1} \text{FW})$$

Where $[x]$ is the concentration value obtained from the calibration curve (μM).

The results were expressed in TEAC or $\mu\text{M Trolox g}^{-1} \text{FW}$. This designation indicates the capacity of inhibition of Trolox by the antioxidants present in samples.

2.3.4.4. Total Phenolic Compounds

The phenolic compounds determination followed a method adapted from Ainsworth *et al.* (2007). On this method, phenolic compounds are oxidized by the Folin-Ciocalteu reactant originating molybdenum and tungsten oxides, characterized by green colour. The colour intensity is an indicator of phenolic compounds concentration.

The sample extraction for antioxidant activity and total phenol content followed the same procedure. An aliquot of 150 μL of supernatant was collected and 140 μL of Folin solution were added. This reaction occurred for 3 min, followed by a dilution with 2400 μL of water to each sample tube. Afterwards 300 μL of 1 M Na_2CO_3 were added. Samples were incubated for 2 hours in dark environment at room temperature. This period is essential to guarantee the total oxidation of phenolic compounds. The absorbance was determined at 720 nm with a blank test by molecular absorption spectrophotometry (AnalyticJena SPECORD 200), where the supernatant was replaced by methanol (Waterhouse, 2002).

A calibration curve with 7 standards was prepared. A stock solution of gallic acid at 5 g L⁻¹ was diluted to obtain the following concentrations: 0, 50; 100; 150; 200; 250 and 500 mg L⁻¹. The equation of the calibration curve was $y = 0.0039x + 1.0794$, $R^2 = 0.99945$, allowing the correlation amongst absorbance and phenolic compounds concentrations.

The following equation was used to determine the concentration in mg g⁻¹ FW.

$$[\text{phenolic compounds}] = \frac{[x] \times \text{volume}_{\text{ext}}}{\left(\frac{\text{mass}_{\text{sample}}}{\text{volume}_{\text{methanol}}} \right) \times 0.15} \quad (\text{mg g}^{-1} \text{ FW})$$

Where $[x]$ is the concentration value obtained from the calibration curve (μM).

The results of phenolic compounds concentration were expressed in mg g⁻¹ FW.

2.3.4.5. Ascorbate

The ascorbate content was determined following the method described by Singh *et al.* (2006) allowing the quantification of total ascorbate and reduced ascorbate. The oxidized form (DHA) is obtained by the difference between total and reduced forms. The quantification is possible due to the reduction of Fe³⁺ to Fe²⁺ by ascorbic acid. This reaction will induce a red complex between Fe²⁺ ion and α, α' -bipyridile as described in the reaction: $\text{Fe}^{3+} + \text{AsC} \rightarrow \text{Fe}^{2+} \alpha, \alpha' - \text{Bipiridil}$ (red complex).

Sample extraction was performed by macerating 0.5 g of fresh material with 2.5 mL of 100 mM Tris-HCl, 1.5 mM dithiothreitol and 1 mM EDTA buffer at pH 7.5. The extraction was performed in triplicate for each sample. After extraction procedure, samples were centrifuged at 22000 x g for 20 min at 4 °C (*Sigma 03-18 K*).

To determine total ascorbate, 200 μL of supernatant were added with 500 μL 150 mM KH₂PO₄, 5 mM EDTA solution and 100 μL 10 mM dithiothreitol (DTT) and stirred. The DTT allows the reduction of DHA to AsA. After stirring for 10 min at room temperature 100 μL of 0.5% m/v N-ethylmaleimide were added to remove the excess of DTT.

The reduced ascorbate content was determined using the same procedure but replacing the addition of DTT and N-ethylmaleimide by deionized water.

To complete the colour development, 400 μL of 10% TCA, 400 μL of 44% orthophosphoric acid, 400 μL of 0.26 M 2,2'-bipyridyl in 70% ethanol and 200 μL of 11 mM FeCl₃ were added to both mixtures. Both were stirred and incubated at 40 °C for 1 h. Absorbance readings were

performed at 525 nm, by molecular absorption spectrophotometry (AnalyticJena SPECORD 200).

A calibration curve with 8 standards (0; 0.2; 0.4; 0.6; 0.8; 1; 1.2 and 1.4 $\mu\text{mol mL}^{-1}$) was prepared from a stock solution of 10 mM ascorbic acid diluted as necessary. The correlation between absorbance and concentration of ascorbate was accomplished through the calibration curve equation: $y = 1.0304x + 0.032$, $R^2 = 0.9972$.

To express the results in terms of fresh weight the following equation was applied:

$$[\text{ascorbate}] = \frac{[\text{ascorbate}] \times \text{volume}_{\text{TrisHCl}} \times 0.2}{\text{mass}_{\text{sample}} \times \text{volume}_{\text{ext}}} \quad (\mu\text{mol ascorbate g}^{-1} \text{FW})$$

Where [ascorbate] is the concentration of ascorbate forms obtained from the correlation and 0.2 is the dilution factor.

The results of AsA and AsC were expressed in $\mu\text{mol ascorbate g}^{-1} \text{FW}$.

2.3.4.6. Glutathione

Glutathione was determined by the method adapted from Anderson *et al.* (1987) and Carvalho *et al.* (2016). This method allowed the quantification of glutathione in the three forms: total (TGSH), reduced (GSH) and oxidized (GSSG). Two of them are directly determined – TGSH and GSSG. The main reaction of this method occurs between GSH and 5,5-dithiobis (2-nitrobenzoic acid) (DTNB) that synthesize the glutathione-TNB complex (GSTNB): $GSH + DTNB \rightarrow TNB + GSTNB$.

This complex is subsequently reduced to 5-thio-2-nitrobenzoic acid (TNB) by the GR enzyme, according to the following reaction: $GSTNB + GSH \xrightarrow{GR} TNB + GSSG$.

However, it is important to note that the GR enzyme also catalyses the reaction between GSSG and the electron donor Nicotinamide Adenine Dinucleotide Phosphate (NADPH), producing GSH again: $GSSG + NADPH \rightarrow GSH + NADP^+$.

To perform sample extraction, 0.50 g of plant material were crushed with 2.5 mL of 6% metaphosphoric acid solution and 1 mM EDTA. After extraction, the samples were centrifuged at 22000 x g for 15 minutes at a temperature of 4 °C (Sigma 03-18 K).

For glutathione determination the extract was diluted 7x with Na_2HPO_4 and divided in two microplates (TGSH and GSSG plates) each one with 50 μL of diluted extract per well. In the GSSG plate the extract was incubated for 1 hour with 2 μL of 2-vinilpyridine. This procedure

was performed under the fume hood. After this, 50 μL of reagent 1 (15 mM EDTA, 0.3 mM Ellman reagent, 0.04% BSA, 110 mM $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$, 40 mM $\text{Na}_2\text{HPO}_4 \cdot \text{H}_2\text{O}$) and 40 μL of reagent 2 (1 mM EDTA, 50 mM Imidazole, 0.02% BSA, 1.5 U GR) were added to each well of both plates. Afterwards the absorbance was measured (blank) at 412 nm. Following the blank test, 10 μL of 9 mM NADPH were added to each well. One and two hours later the reduction of DTNB to TNB ($\text{GSH} + \text{DTNB} \rightarrow \text{TNB} + \text{GSTNB}$) produced a yellow colour. The absorbances were determined with a Biotek Sinergy HT plate reader. GSH was calculated by subtracting the amount of GSSG to the total glutathione.

The absorbance values were converted to concentration, according to Lambert-Beer Law equation. The concentrations of GSH were obtained by difference between TGSH and GSSG. To express the results regarding fresh weight, the following equation was applied:

$$[\text{glutathione}] = \frac{[\text{TGSH; GSH; GSSG}] \times \text{volume}_{\text{ext}}}{\frac{\text{mass}_{\text{sample}}}{\text{volume}_{\text{metaphosphoric acid}}} \times 0.05} \times f_d \quad (\mu\text{mol glutathione g}^{-1} \text{FW})$$

Where $[\text{TGSH; GSH; GSSG}]$ is the value of concentration obtained from Lambert-Beer Law with a -SH molar absorptivity coefficient ($\varepsilon = 0.0136 \mu\text{M}^{-1} \text{cm}^{-1}$) and f_d is the sample dilution factor (7x).

The results of GSH were expressed in $\mu\text{mol glutathione g}^{-1} \text{FW}$.

2.3.4.7. Anthocyanin content

The concentration of anthocyanins was determined according to the method from Sims & Gamon (2002).

To perform sample extraction, 0.06 g of leaves were macerated with 10 mL of a 90% methanol and 10% hydrochloric acid solution. Then, the samples were centrifuged at 10000 x g for 15 minutes at 6 °C (*Sigma 03-18 K*). After collecting the supernatant, the absorbances were acquired at 529 and 650 nm in glass cuvettes, by molecular absorption spectrophotometry (AnalyticJena SPECORD 200).

To determine the concentration of anthocyanins the next equation was applied.

$$[\text{anthocyanins}] = \frac{[A] \times \text{volume}_{\text{methanol}}}{\varepsilon \times L \times \text{mass}_{\text{sample}} \times 1000} \quad (\mu\text{mol anthocyanins g}^{-1} \text{FW})$$

Where $[A]$ represents the corrected absorbance ($A = \text{Abs}_{529} - 0,228\text{Abs}_{650}$), ε represents the molar absorptivity coefficient of anthocyanins at 529 nm ($30000 \text{ L mol}^{-1} \text{cm}^{-1}$) and L the length of the cuvette (cm).

The concentration of anthocyanins in lettuce leaves was expressed in μmol anthocyanins g^{-1} FW.

2.3.5. Analysis of Enzymatic Activity

The enzymatic activities of CAT, SOD, GPOD, GR, GPX, APX and PAL were performed as indicated below.

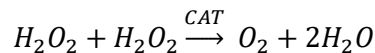
The enzymatic activities evaluate the amount of substrate consumed or product formed in a reaction catalysed by an enzyme and were determined by molecular absorption spectrophotometry. They were evaluated using a rate assay determination and measured in triplicate for each test modality and for both plant tissue (roots and leaves). The results were expressed in Enzyme Unit (U), defined as the amount of enzyme needed to catalyse the decomposition/formation of 1 μmol of substrate per unit of time, at room temperature. The results were expressed in U g^{-1} FW.

2.3.5.1. Enzyme extraction procedure

The extraction of CAT, SOD, GPOD and GR enzymes follow the same procedure. Approximately 0.50 g of fresh material were crushed in cold mortar with 2 mL of extraction buffer, 100 mM Tris-HCl at pH 7.5, 1.5 mM DTT, 1 mM EDTA e 0.1% (w/v) Triton X-100. Also, the procedure includes the addition of inert sand and 0.02 g of 1% w/v insoluble polyvinylpolypyrrolidone (PVPP). After extraction, the samples were centrifuged at $12000 \times g$ (Sigma 03-18 K) for 15 min at 4 °C. For GPX enzyme the extraction uses the same extraction buffer with the addition of 0.1 mM phenylmethylsulfonyl fluoride (PMSF) and 5 mM cysteine. For APX enzyme the extraction buffer is a solution of 100 mM Tris-HCl at pH 7.5 with 1.5 mM DTT, 1 mM EDTA and 10 mM ascorbate. Regarding PAL enzyme, approximately 0.5 g of fresh material were crushed with inert sand and a mixture of 3 mM DTT and 25 mM of sodium borate buffer (pH 8.8). Samples were centrifuged at $20000 \times g$ (Sigma 03-18 K) for 20 min at 4 °C. After centrifugation, the supernatant was collected and used for further determinations, according to each enzyme.

2.3.5.2. CAT enzymatic activity

Catalase (EC 1.11.1.6) is involved in the formation of two molecules of water and one molecule of oxygen, through the degradation of two molecules of hydrogen peroxide.



CAT determination activity was adapted from Aebi (1974) and is based on the decrease of absorbance at 240 nm due to the H_2O_2 consumption, for 2 minutes. The activity of CAT was determined in a reaction medium with the sample extract, 11 mM H_2O_2 in 100 mM phosphate buffer at pH 7.6.

The results of CAT enzyme activity are expressed using the following equation:

$$\text{Enzymatic activity} = \frac{\Delta \text{Abs}}{\Delta t} \times \frac{V_{\text{total}}}{\epsilon L V_{\text{ext}}} \times \frac{V_{\text{extraction}}}{\text{mass}_{\text{sample}}} \quad (\text{U g}^{-1} \text{FW})$$

Where $\frac{\Delta \text{Abs}}{\Delta t}$ is the slope of the linear relation between time and absorbance; ϵ represents the molar absorptivity coefficient of H_2O_2 at 240 nm (39.4 mM cm^{-1}), L represents the length of the cuvette and v the volume for each component.

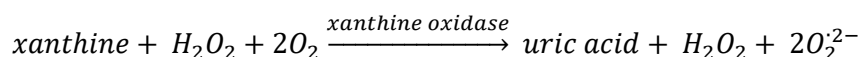
One unit of enzymatic activity (U) is defined as the amount of catalase needed to catalyse the decomposition of $1 \mu\text{mol}$ of $H_2O_2 \text{ min}^{-1} \text{ mL}^{-1}$, at room temperature. The results were expressed in $\text{U g}^{-1} \text{FW}$.

2.3.5.3. SOD enzymatic activity

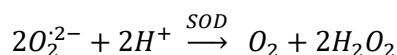
The determination of the enzymatic activity of SOD (EC 1.15.1.1) followed the method adapted from Rubio *et al.* (2002). This method is based on the competition between the SOD enzyme and the cytochrome-C-ferric species (CitC-Fe^{3+}) for the superoxide radical ($O_2^{\bullet-}$). The system xanthine/xanthine-oxidase (EC 1.17.3.2) is responsible for the synthesis of $O_2^{\bullet-}$ radicals.

CitC-Fe^{3+} species are reduced to CitC-Fe^{2+} in presence of $O_2^{\bullet-}$ radicals. However, the greater the SOD activity, the greater the competition with CitC-Fe^{3+} and the lower the production of CitC-Fe^{2+} . This competition benefits the SOD activity, leading to $O_2^{\bullet-}$ removal and consequent production of H_2O_2 . These reactions are described by the following equations.

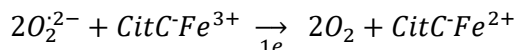
Superoxide radicals ($O_2^{\bullet-}$) synthesis:



Reaction catalysed by SOD:



Reduction of CitC-Fe³⁺:



The reaction medium for this determination consists in a solution A (0.05 mM EDTA, 0.25 mM xanthine and 0.025 mM C-ferric cytochrome), which produces O₂^{•-} radicals, and solution X (0.025 U xanthine-oxidase) both prepared in 100 mM phosphate buffer at pH 7.6. The Δabsorbance was acquired at 550 nm for 2 minutes.

The results of the SOD enzymatic activity were expressed using the following equation:

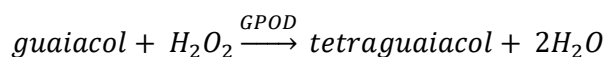
$$\text{Enzymatic Activity} = \frac{0,5 \times \frac{\Delta Abs_{ER}}{\Delta t}}{\frac{\Delta Abs_{sample}}{\Delta t}} \times \frac{v_{total}}{v_{ext}} \times \frac{v_{extraction}}{mass_{sample}} \quad (U \text{ g}^{-1} \text{ FW})$$

Where $\frac{\Delta Abs_{ER}}{\Delta t}$ is the slope of the linear regression between time and absorbance for the reference assay, $\frac{\Delta Abs_{sample}}{\Delta t}$ is the slope of the linear relation between time and absorbance for the sample assay, 0,5 represents the percentage of reduction of CitC-Fe³⁺ (50%) and v the volume for each component.

The enzymatic activity unit, U, is defined as the amount of SOD required to inhibit 50% reduction of ferric cytochrome-C. The results were expressed in U g⁻¹ FW.

2.3.5.4. GPOD enzymatic activity

The enzyme guaiacol peroxidase (GPOD) catalyses the H₂O₂ scavenging reaction using guaiacol (C₇H₈O₂) as a reducing agent, resulting in a coloured compound, tetraguaiacol (oxidized product) and water, as described in the following equation:



The determination of GPOD activity is based on the method described by Gajewska *et al.* (2006). The reaction medium consists in 31 mM guaiacol, 4 mM H₂O₂ in 200 mM sodium acetate buffer at pH 6.0 and sample extract. GPOD activity is obtained by measuring the increase in absorbance at 470 nm, for 2 min.

The results of GPOD enzymatic activity were expressed applying the following equation:

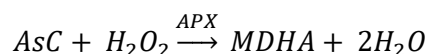
$$\text{Enzymatic activity} = \frac{\Delta \text{Abs}}{\Delta t} \times \frac{V_{\text{total}}}{\epsilon L v_{\text{ext}}} \times \frac{V_{\text{extraction}}}{\text{mass}_{\text{sample}}} \quad (\text{U g}^{-1} \text{FW})$$

Where $\frac{\Delta \text{Abs}}{\Delta t}$ is the slope of the linear regression between time and absorbance, ϵ is the molar absorptivity coefficient guaiacol at 470 nm (26.6 mM cm⁻¹), L represents the length of the cuvette and v the volume for each component.

One unit of enzymatic activity (U) is defined as the amount of GPOD needed to catalyse the decomposition of 1 μmol of guaiacol min⁻¹ mL⁻¹ at room temperature. The results were expressed in U g⁻¹ FW.

2.3.5.5. APX enzymatic activity

Similarly to CAT and GPOD enzymes, ascorbate peroxidase (APX) acts on the scavenging of H₂O₂, but using ascorbate as the reducing agent, according to the following reaction:



The ascorbate is oxidized to the form of monodehydroascorbate product (MDHA). This oxidation leads to a change in absorbance and corresponds to the initial reaction rate, and is determined as described by Sharma *et al.* (2004).

The sample extract was added to a reaction medium consisting in 0.25 mM ascorbate, 0.025 mM EDTA and 0.25 mM H₂O₂ in 100 mM phosphate buffer at pH 7.6. APX activity was obtained by measuring the decrease in absorbance at 290 nm, for 2 min.

The results of APX's enzymatic activity were expressed using the following equation:

$$\text{Enzymatic activity} = \frac{\Delta \text{Abs}}{\Delta t} \times \frac{V_{\text{total}}}{\epsilon L v_{\text{ext}}} \times \frac{V_{\text{extraction}}}{\text{mass}_{\text{sample}}} \quad (\text{U g}^{-1} \text{FW})$$

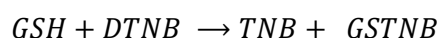
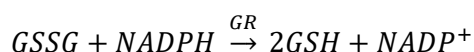
Where $\frac{\Delta \text{Abs}}{\Delta t}$ is the slope of the linear regression between time and absorbance; ϵ is the molar absorptivity coefficient ascorbate at 290 nm (2.8 mM cm⁻¹), L represents the length of the cuvette and v the volume for each component.

A unit of enzymatic activity (U) is defined as the amount of APX needed to catalyse the decomposition of 1 μmol of ascorbate min⁻¹ mL⁻¹, at room temperature. The results were expressed in U g⁻¹ FW.

2.3.5.6. GR enzymatic activity

The enzyme glutathione reductase (GR) is responsible for the synthesis of reduced glutathione (GSH). The enzymatic activity of GR was determined using the method described by Shanker *et al.* (2004).

GR catalyses the reaction between oxidized glutathione (GSSG) and NADPH, resulting in two GSH molecules. This method allows a change in absorbance, as a result of 5-thio-2-nitrobenzoic acid (TNB) production, as described by the following equations:



The reaction medium includes 0.75 mM DTNB, 0.1 mM NADPH and 1 mM GSSG in 100 mM potassium phosphate buffer solution with 1 mM EDTA at pH 7.6. The absorbance variation was measured at 412 nm for 2 minutes.

The GR enzymatic activity is calculated using the following equation:

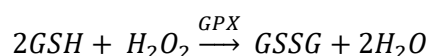
$$\text{Enzymatic activity} = \frac{\Delta \text{Abs}}{\Delta t} \times \frac{V_{\text{total}}}{\epsilon L v_{\text{ext}}} \times \frac{V_{\text{extraction}}}{\text{mass}_{\text{sample}}} \quad (\text{U g}^{-1} \text{FW})$$

Where $\frac{\Delta \text{Abs}}{\Delta t}$ is the slope of the linear regression between time and absorbance; ϵ is the molar absorptivity coefficient of NADPH at 412 nm (6.2 mM cm^{-1}), L represents the length of the cuvette and v the volume for each component.

One unit of enzymatic activity (U) is defined as the amount of GR needed to catalyse the reduction of $1 \mu\text{mol}$ of DTNB $\text{min}^{-1} \text{mL}^{-1}$ at room temperature. The results were expressed in $\text{U g}^{-1} \text{FW}$.

2.3.5.7. GPX enzymatic activity

Glutathione peroxidase (GPX) belongs to the group of peroxidases and plays a role at glutathione pathway. GPX enzyme is also responsible for the scavenging of H_2O_2 through the following reaction:



The enzymatic activity of GPX was determined according to the method described by Aravind *et al.* (2005). GSH is used as substrate and is synthesized by GR activity added to the reaction

medium. The consumption of NADPH by GR activity leads to a decrease in the absorbance, which controls the initial GPX reaction rate.

The reaction medium consists in a mixture of sample extract and 114 mM NaCl, 1.5 mM GSH, 0.3 mM NADPH, 0.01 U of GR and 0.25 mM H₂O₂ on 50 mM Tris-HCl buffer at pH 7.9. The absorbance is obtained at 340 nm for 2 min.

The GPX activity is calculated by the following equation:

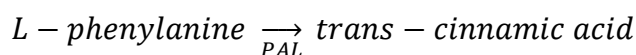
$$\text{Enzymatic activity} = \frac{\Delta \text{Abs}}{\Delta t} \times \frac{V_{\text{total}}}{\epsilon L v_{\text{ext}}} \times \frac{V_{\text{extraction}}}{\text{mass}_{\text{sample}}} \quad (\text{U g}^{-1} \text{ FW})$$

Where $\frac{\Delta \text{Abs}}{\Delta t}$ is the slope of the linear regression among time and absorbance; ϵ is the molar absorptivity coefficient NADPH at 340 nm (6.2 mM cm⁻¹), L represents the length of the cuvette and v the volume for each component.

One unit of enzymatic activity (U) is defined as the amount of glutathione peroxidase needed to catalyse the reduction of 1 μmol of NADPH min⁻¹ mL⁻¹ at room temperature. The results were expressed in U g⁻¹ of FW.

2.3.5.8. PAL enzymatic activity

Enzymatic activity of PAL was determined following the method described by Boo *et al.* (2011) and Nagaranthna *et al.* (1993) which consist in the production of trans-cinnamic acid in the presence of phenylalanine.



Samples were placed in tubes and the reaction medium was composed of 5 mM of L-phenylalanine and 25 mM of sodium borate buffer (pH 8.8). In parallel a blank assay was performed. The samples were incubated for 2 hours at 40 °C. To stop the reaction a solution of 5 M HCl was added to each sample. The absorbance was measured at 290 nm against the blank assay and the enzymatic activity calculated according to equation:

$$\text{Enzymatic activity} = \frac{\Delta \text{Abs}}{\Delta t} \times \frac{V_{\text{total}}}{\epsilon L v_{\text{ext}}} \times \frac{V_{\text{extraction}}}{\text{mass}_{\text{sample}}} \quad (\text{U g}^{-1} \text{ FW})$$

One unit of enzymatic activity (U) is defined as the amount of Phenylalanine ammonia lyase needed to catalyse the production of 1 mmol of trans-cinnamic acid h⁻¹ mL⁻¹ at room temperature. Results were expressed in mmol of trans-cinnamic acid h⁻¹ mL⁻¹ g⁻¹ FW.

2.4. Proteomic analysis

To carry out a careful overall evaluation of lettuce metabolism, a proteomic analysis was conducted. This part of the work was performed in a collaboration with Luxembourg Institute of Science and Technology. The main objective of this approach was to understand and improve the knowledge of how proteins were affected by the presence of pharmaceuticals, corroborating some results obtained in enzymatic and non-enzymatic analysis.

2.4.1. Protein extraction and sample preparation

To carry out the proteomics analysis, 8 samples of lettuce from each experimental group (4 leaves and 4 roots each) were analysed for a total of 32 samples (16 per tissue). The sample extraction procedure followed the method described by Ribeiro *et al.* (2019). First, approximately 0.3-0.4 g of fresh tissue were weighted and grinded in order to homogenize the sample. Protein extracts were obtained by adding 1.5 mL of extraction buffer of 20% w/v Trichloroacetic Acid (TCA) and 0.1% w/v of Dithiothreitol (DTT) in acetone. The crushed samples were placed at -20 °C overnight to ensure protein precipitation. A centrifugation at 30000 x g during 45 min at 4 °C was performed for all sample extracts. After centrifugation, the pellet was collected and washed three times with rinsing buffer of 0.1% w/v DTT in acetone and vacuum dried. Finally, the protein pellet was well solubilized with 115 µL of lysis buffer (7 M urea, 2 M thiourea and 0.5% (w/v) 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS).

To estimate the protein amount obtained, the method of Reducing Agent Compatible/Detergent Compatible quantification (Bio-Rad RC DC™ Protein Assay Kit II) was performed following the manufacturer's instructions.

For the sample preparation, 20 µg of total proteins were loaded according to manufacturer instructions on a Criterion™ XT precast 1D gel (12% Bis-Tris SDS-PAGE, 18 wells, Bio-Rad). A short migration was achieved for 10 min. The protein gels were stained in a bath with Instant Blue™ (Expedeon). Afterwards the gels were divided in two equal parts, for each sample, and cut into small pieces. Each part was placed in a microplate well. Then, the proteins were reduced with DTT, alkylated with iodoacetamide and destained. Thereafter, trypsin (5 ng µL⁻¹, mass grade Promega) was added to carry on in-gel protein digestion. The samples were then placed in an ice bath for 30 min followed by incubation for 5 h at 37 °C after which the peptides were extracted from the gels. The extracted peptides were dried and stored at -20 °C until LC-MS/MS analysis.

2.4.2. LC-MS

Samples were analysed with Eksigent NanoLC 425 system (SCIEX, Belgium) coupled with SCIEX TripleTOF® 6600 MS system. Peptides were loaded onto a C18 trap column (5 x 0.5 mm YMC triart) using a loading buffer (2% acetonitrile (ACN), 0.1% formic acid (FA)) at a flow rate of 10 $\mu\text{L min}^{-1}$ and were separated on a C18 LC column (Luna® Omega 3 μm polar C18 100 Å 150 x 0.3 mm, Phenomenex) using a linear binary gradient with a mobile phase A composed with 0.1% FA in LC-MS grade water and mobile phase B of ACN with 0.1% FA. Peptides were eluted at a flow rate of 5 $\mu\text{L min}^{-1}$ with 3% B, followed by an increase from 3 to 30% during 45 min, increase to 40% in 7 min, then reaching 80% to wash the column. Prior to the next injection the column was re-equilibrated for a few minutes with 3% B.

The mass range of full scan MS spectra was set to 400-1250 Da. The maximum accumulation time for precursor ions was set for 250 ms and a dynamic exclusion was set to 10 s. The 30 most intense ions with a charge state of 2⁺ to 4⁺ were selected and fragmented using the rolling collision energy. The mass range of MS/MS scans was set to 100-1500 Da with an accumulation time of 50 ms.

2.4.3. Protein Identification

MS data were processed with Progenesis QI for Proteomics software (version 4.2, Nonlinear Dynamics, Waters).

The protein and peptide identification were performed via Mascot Daemon interface (version 2.6.0. Matrix Science, UK) searching against *Lactuca sativa* database on UniprotKB (released 27th of November 2019, 39366 sequences). Different search parameters were used: trypsin enzyme, a maximum of 2 missed cleavages, a peptide tolerance of 20 ppm, and a fragment mass tolerance of 0.3 Da. Carbamidomethylation at cysteine was set as fixed modification and the variable modifications included oxidation at methionine, N-terminal protein acetylation and conversion of tryptophan to kynurenine. The identified proteins with a significant Mascot-calculated confidence of 95% were selected, for further analysis.

2.4.4. Data analysis and interpretation

To ensure the significant differences between conditions in Progenesis QIP, the following filters were applied on the identified protein list: an ANOVA p-value < 0.05, at least 1 unique and 2 significant peptides per protein, and a fold change higher than 1.5. Data analysis and visualization were carried out using Progenesis QIP.

Significant proteins were selected for each condition and the normalized abundance obtained on Progenesis QIP was applied to comparisons between control and pharmaceutical contamination (Control vs MTF, Control vs ACT, Control vs CBZ).

After data analysis, the list of significant proteins was imported to Blast2GO in order to blast and obtain more information about “uncharacterized proteins”, and to complete proteome characterization of *Lactuca sativa*, default parameters were applied. The protein sequences were mapped and annotated with sequence identity against existing functional annotation from yet uncharacterised sequences. The differentially identified proteins were classified based on Gene Ontology (GO) identifications according to their biological processes, cellular component and molecular functions.

To obtain protein information about metabolic functions and biological processes, differentially significant proteins from contaminations vs control condition, were loaded in the PANTHER tool (<http://www.pantherdb.org/>). The proteins differentially expressed (in comparison to control) for roots and leaves of each condition were displayed in a Venn diagram using the bioinformatics and evolutionary genomics tool (Gent University, Belgium): <http://bioinformatics.psb.ugent.be/webtools/Venn/>. In order to obtain a relation of proteins abundance among contaminants for both tissues, heatmaps were obtained in R Studio 3.6.3 software applying the packages "tidyverse", "bitops", "heatmap.plus", and "RColorBrewer".

The MS proteomics data has been deposited to the ProteomeXchange Consortium (Deutsch *et al.*, 2017) via the PRIDE (Vizcaíno *et al.*, 2016) partner repository with the dataset identifier PXD021474 and 10.6019/PXD021474.

Figure 2.4 shows a workflow of the proteomics analysis from extraction procedure to data analysis.

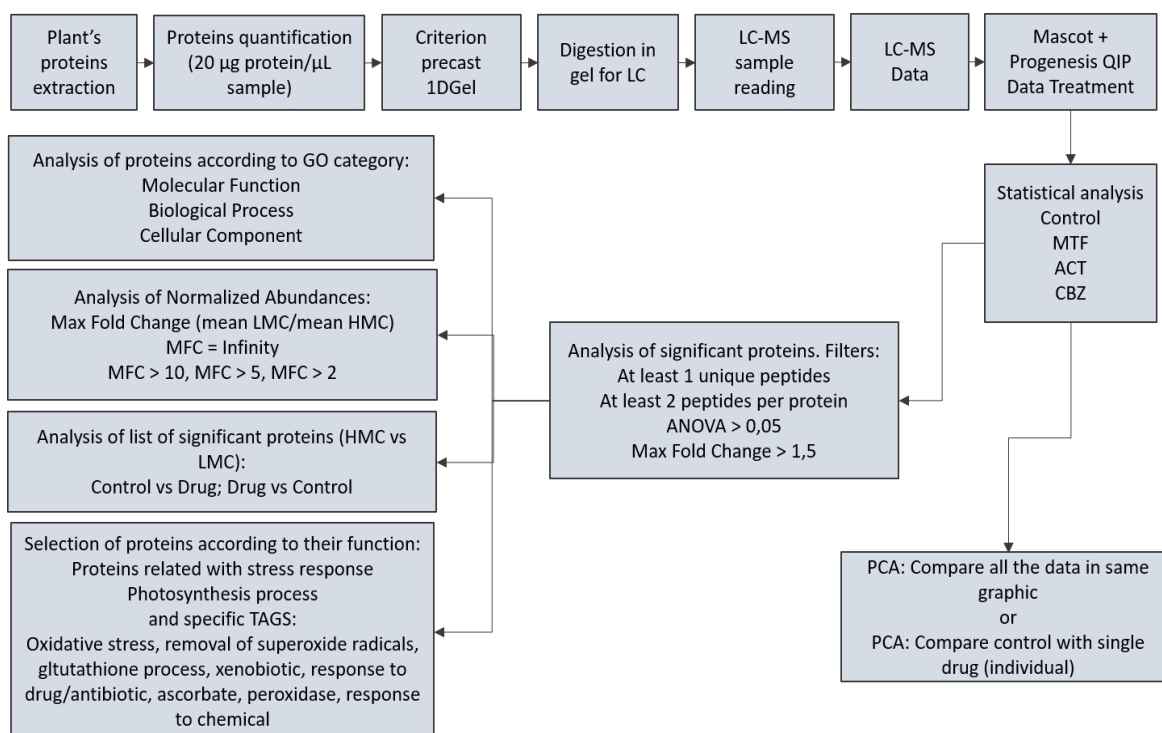


Figure 2.4. - Proteomics approach workflow.

2.5. Data Analysis

All the analytical determinations described above were performed in triplicate. To assess significant differences between the different experimental conditions, the results were subjected to a one-way ANOVA using the Tukey test to check for significant differences between means ($p < 0.05$). In the figures, error bars represent mean $\pm$ one standard deviation, different uppercase letters represent significant differences between different pharmaceutical concentrations at the same time point and different lowercase letters represent significant differences between treatment duration for the same pharmaceutical concentration.

Principal Component Analysis (PCA) were performed with R Studio 3.6.3 software as well as correlation analysis with the package "ggplot2".

CHAPTER III

Antioxidative response of lettuce to carbamazepine-induced stress

Leitão I., Mourato M.P., Carvalho, L., Oliveira, M. C., Marques M. M., Martins, L.L. Antioxidative response of lettuce (*Lactuca sativa*) to carbamazepine-induced stress. Environmental Science and Pollution Research. 2021. doi: 10.1007/s11356-021-13979-3.

Chapter III - Antioxidative response of lettuce to carbamazepine-induced stress

Abstract

Carbamazepine (CBZ) is a widely used anti-epileptic drug that has been detected in wastewaters from sewage treating plants and thus appears in rivers, streams, and other water bodies. As plants can absorb this compound, it can also appear in edible plants like lettuce, entering in the food chain. In this study, the effect of CBZ in lettuce plants grown in hydroponic solution is analysed. CBZ was detected both in roots and in leaves and is shown to induce oxidative stress. Hydrogen peroxide levels increased both in leaves and in roots while malondialdehyde increased only in leaves. Regarding the activity of antioxidative enzymes in the leaves, it is shown that superoxide dismutase, catalase, guaiacol peroxidase and ascorbate peroxidase have a relevant role in quenching reactive oxygen species induced by oxidative stress. In roots, the only enzymes that showed increased activity were catalase, guaiacol peroxidase and glutathione reductase. Ascorbate and glutathione also appear to have an important role as antioxidants in response to increased concentrations of CBZ. Although the roots are in direct contact with the contaminant, the leaves showed the strongest oxidative effects. CBZ also affected anthocyanins content as well as phenylalanine ammonia lyase activity, suggesting a possible interference on secondary metabolism. Besides, mineral content in both parts of plants was affected by the presence of this contaminant (mainly Cu, K, Mn and Mg).

Keywords: abiotic stress, antioxidative enzymes, hydrogen peroxide, reactive oxygen species, oxidative stress, pharmaceuticals.

3.1. Introduction

Pharmaceuticals and personal care products (PPCP) are considered a very diverse group of contaminants of emerging concern in environmental samples (Bartrons *et al.*, 2017). In this classification are included pharmaceuticals for both human and veterinary use and products used for human basic hygiene or cosmetics. The worldwide increase in the prescription and consumption of pharmaceutical products has led to their appearance in different environmental compartments (Carvalho *et al.*, 2014; Dodgen *et al.*, 2013). These contaminants can enter the environment through several sources. One of them is wastewater from households and hospitals since, after being administered to humans, the parent compound or its metabolites are partially excreted in urine and faeces or, in lower proportion, incorrectly disposed in the toilet. Other sources of improper disposal are through garbage that is allocated to a landfill. When in a landfill, the PCPPs can reach groundwater reservoirs by lixiviation processes (Lapworth *et al.*, 2012; Vulliet & Cren-Olivé, 2011).

Although wastewaters are subjected to different treatments, most of these treatments are not 100% effective in the elimination of all PPCPs (Lamastra *et al.*, 2016). Thus, it is possible to find them in concentrations from ng L^{-1} to $\mu\text{g L}^{-1}$ in surface waters or ground waters, depending on the elimination pathway (Rossmann *et al.*, 2014; Xu *et al.*, 2007).

The presence of PPCPs in soil, surface and ground waters can also take these compounds to irrigation waters (Christou *et al.*, 2017) and thus they can reach plants and be accumulated in different organs, depending on the bioavailability and translocation of each compound along the plant. This way, it is possible that food crops will be affected by PPCP accumulation, causing a food security problem (Dodgen *et al.*, 2013; Herklotz *et al.*, 2010; Shenker *et al.*, 2011)

CBZ is an anticonvulsant pharmaceutical used worldwide in epileptic patients. In the human body, it is metabolized leading to several known metabolites, which are then excreted through urine and faeces. Both the parent compound and its metabolites are frequently found in wastewaters (Bahlmann *et al.*, 2014).

Although there have been several reports of CBZ uptake by different plants (Bartrons *et al.*, 2017; Bhalsod *et al.*, 2018; Dordio *et al.*, 2011; Hurtado *et al.*, 2016; Mordechay *et al.*, 2018; Paz *et al.*, 2016; Winker *et al.*, 2010) there is still a lack of information about the overall defence response in different organisms. For instance, Bhalsod *et al.* (2018) described a root concentration factor of 0.80 and a translocation factor of 8.15 in greenhouse lettuce with surface irrigation. Mordechay *et al.* (2018) presented results regarding not only the accumulation of CBZ but also some of its metabolites like 10,11-dihydro-10,11-dihydroxy-

carbamazepine (dihydroxy-CBZ) and 10,11-carbamazepine epoxide (epoxide-CBZ), after irrigation with treated wastewater.

As plants do not excrete these types of compounds, they are accumulated in different plant parts, like vacuoles or cell walls, and may cause oxidative stress through mechanisms that are not yet fully understood (Bartha *et al.*, 2014; Carla *et al.*, 2018). Regarding CBZ, several studies in different organisms suggest an increase in ROS and ROS scavenging enzyme activities induced by CBZ intake. Wang *et al.* (2019) showed several acute responses to CBZ such as increased ROS levels and cell membrane permeability in different bacteria. Dordio *et al.* (2011) showed an increase in the activities of SOD, CAT and GPOD in *Typha latifolia* leaves with increasing CBZ concentrations (between 0.5 and 2.0 mg L⁻¹). Naziroglu & Yurekli (2013) suggested that some antiepileptic drugs, like CBZ, induce ROS production in human and animal cells.

The main objective of this study was to identify the main mechanisms used by lettuce plants against oxidative stress induced over time by different concentrations of CBZ in hydroponic solution. The overall antioxidant response to oxidative stress was evaluated. Enzymatic activities of CAT, SOD, GPOD, APX, GPX and GR were measured at different time points, together with antioxidant metabolites like GSH and AsA. The study was designed to elucidate the different metabolic pathways involved, thus contributing to improve the knowledge on how lettuce copes with CBZ aggression.

3.2. Material and Methods

Seeds of the cultivar *Lactuca sativa* L. “Maravilha das Quatro Estações” were germinated in substrate (Bio Siro – peat, humus and fertilizer). During the first 15 days the seedlings were maintained in germination substrate until the development of the first leaf. Afterwards, young lettuce plants were transferred to hydroponic culture with Hoagland solution and developed in this medium for 21 days. On the 36th day of growth, lettuce plants were contaminated with different concentrations of CBZ: 0 (control), 0.1, 1 and 5 mg L⁻¹. These concentrations were based on previous studies from the literature (Dordio *et al.*, 2011; Herklotz *et al.*, 2010; Shenker *et al.*, 2011) and known to be capable of inducing oxidative stress in different plants. Three pots were used for each concentration, with 12 plants each, for a total of 36 plants per dose. Growing conditions and more detailed experimental data are described in Chapter II Material and Methods.

The following procedures were performed, all described in Chapter II:

- Harvesting of plant material (2.2.)
- Determination of mineral elements (2.3.1.)
- Determination of carbamazepine in roots and leaves (2.3.2.1.)
- Determination of Photosynthetic parameters (2.3.3.)
- Determination of Lipid Peroxidation (2.3.4.1.)
- Determination of Hydrogen Peroxide content (2.3.4.2.)
- Determination of Antioxidant activity (2.3.4.3.)
- Determination of Phenolic Compounds (2.3.4.4.)
- Determination of Ascorbate (2.3.4.5.)
- Determination of Glutathione (2.3.4.6.)
- Determination of Anthocyanins (2.3.4.7.)
- Determination of Enzymatic Activities (2.3.5.)

Data analysis is described in section 2.5, from Chapter II.

3.3. Results and Discussion

3.3.1. Carbamazepine uptake by plants

Several pharmaceutical compounds are known to be absorbed by plants in considerable amounts (Christou *et al.*, 2017). Carbamazepine uptake studies have been performed in different plants like *Typha* spp (Dordio *et al.*, 2011), carrots and sweet potatoes (Malchi *et al.*, 2014), wheat (Paz *et al.* 2016), cucumber (Shenker *et al.*, 2011), several macrophyte species (Tejeda *et al.* 2017), ryegrass (Winker *et al.* 2010), celery, carrot and pak choi (Li *et al.*, 2018), and *Scirpus validus* (Zhang *et al.*, 2013). In order to confirm the presence of CBZ in lettuce plants used in the current work, an LC-MS analysis was performed in leaf and root extracts. Two typical ion chromatograms obtained in the ESI positive mode, are shown in Figure 3.1. The peak at the retention time of 5.6 min, displaying an accurate m/z 237.1022 value (error 0.5 ppm and mSigma 6.6), was unmistakably assigned to the protonated molecule of CBZ.

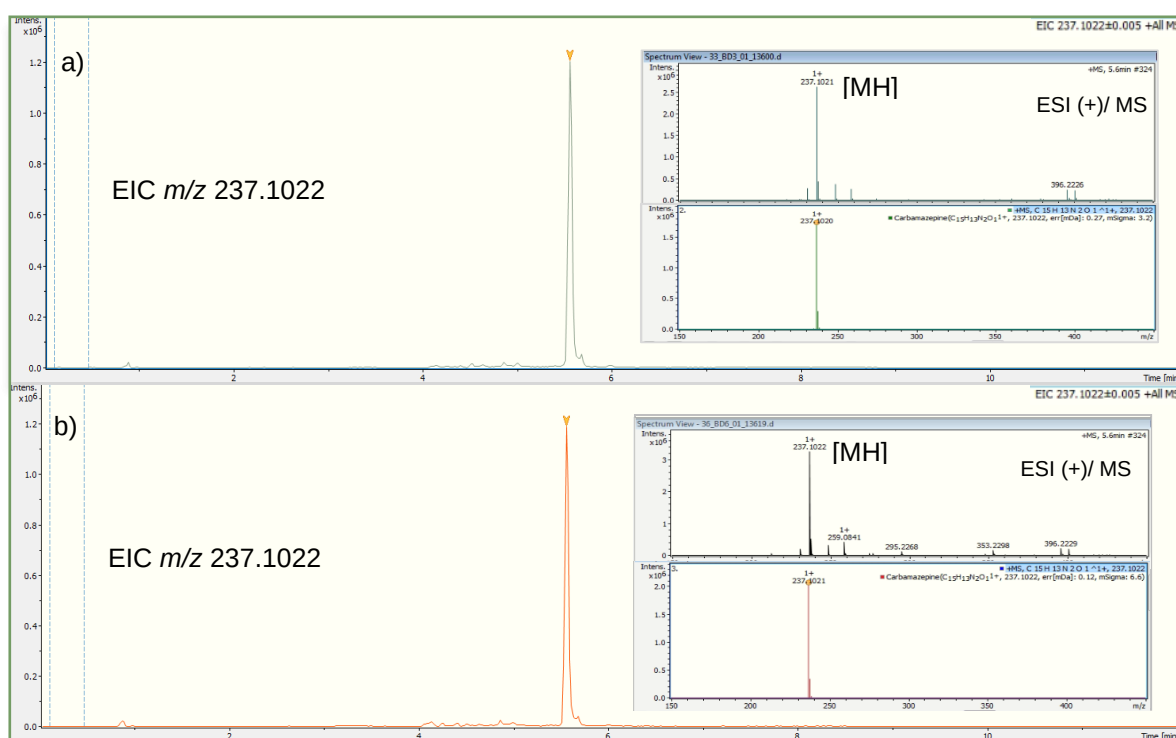


Figure 3.1. - Typical ion chromatograms obtained in the ESI positive mode for root (a) and leaf (b) extracts of plants growing with 0.1 mg L^{-1} CBZ, showing the presence of the protonated molecule (m/z 237.1022) of the pharmaceutical compound after 1 day of exposure. The experimental conditions are outlined in Materials and Methods.

This pharmaceutical was identified in all analysed plant parts, at all concentrations used, confirming the uptake of CBZ and its translocation to the upper plant parts.

Regarding the accumulation of CBZ in roots and leaves, Figures 3.2 to 3.4 show some examples of the extracted ion chromatograms for every concentration on days 1, 8 and 15 of exposure to CBZ. In Figure 3.5 are presented the concentrations of CBZ in roots and in leaves for the three time periods analysed.

In case of contamination with 0.1 mg L^{-1} of CBZ, observing Figure 3.2, it is possible to confirm that both tissues showed an accumulation of CBZ on every day of exposure. A higher intensity of the peak of protonated CBZ is showed for day 15, meaning that for longer periods of exposure the accumulation of CBZ tends to be higher, as expected.

In roots under 1 mg L^{-1} the concentration of CBZ was almost constant with time, while in leaves an increasing pattern was observed reaching the highest value on day 15 of exposure. Looking at the chromatograms (Figure 3.3) it is possible to observe that the intensity of CBZ peak remains similar in roots over time of exposure, whereas in leaves on day 15 intensity reaches the highest values. These results are in accordance with previous studies showing a trend of accumulation or translocation of CBZ to aerial parts. Pursuant to statistical analyses, significant differences on concentration in leaves were found over time of exposure to CBZ, while in roots days 8 and 15 remained similar.

In the case of contamination with 5 mg L^{-1} of CBZ (Figure 3.4), the accumulation of CBZ increased over time, showing significant differences between day 1 and days 8 and 15, in roots and leaves. The comparison to other concentrations revealed significant differences for every day in both tissues. The CBZ content in roots, after 15 days, was significantly higher. This may be explained by the breakdown of plants defence mechanisms at this concentration, after a longer period, or as a mean of protecting the plant, avoiding the translocation to the upper part that was considerably affected by the induced toxicity.

The bioaccumulation factors (BCF) and translocation factor (TF) of CBZ are presented in Table 3.1. The TF was higher than 1 on day 15. This result may indicate that for this concentration the translocation from roots to leaves only occurs for longer exposure. The TF is generally higher than 1, meaning that for most time periods and most concentrations the leaves accumulate more CBZ than the roots confirming that this pharmaceutical is easily translocated to the upper plant parts. There were two exceptions to this, the lower time period at 0.1 mg L^{-1} and the higher time period (15 days) and higher concentrations. The first one is probably because of the small time passed and the low concentration, while in the second case it is due to the high CBZ content in roots and the fact that the plant presented a deficient growth, since translocations mechanisms were probably affected.

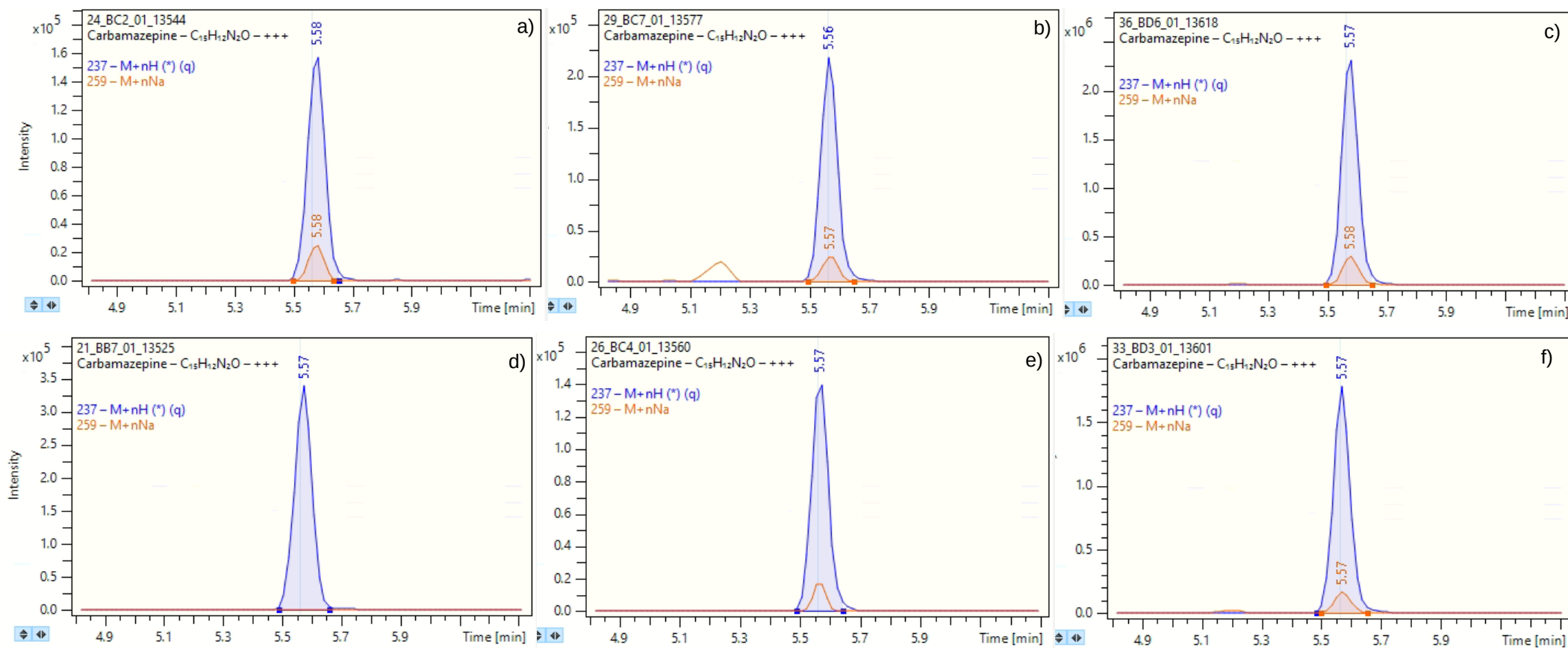


Figure 3.2. – Extracted ion chromatograms representing carbamazepine peak [237 – M +nH(*)q – blue] used for quantification of CBZ uptake by leaves (a, b, c) and roots (d, e, f) from lettuce contaminated with 0.1 mg L⁻¹ on days 1 (a,d), 8 (b,e) and 15 (c,f) of exposure.

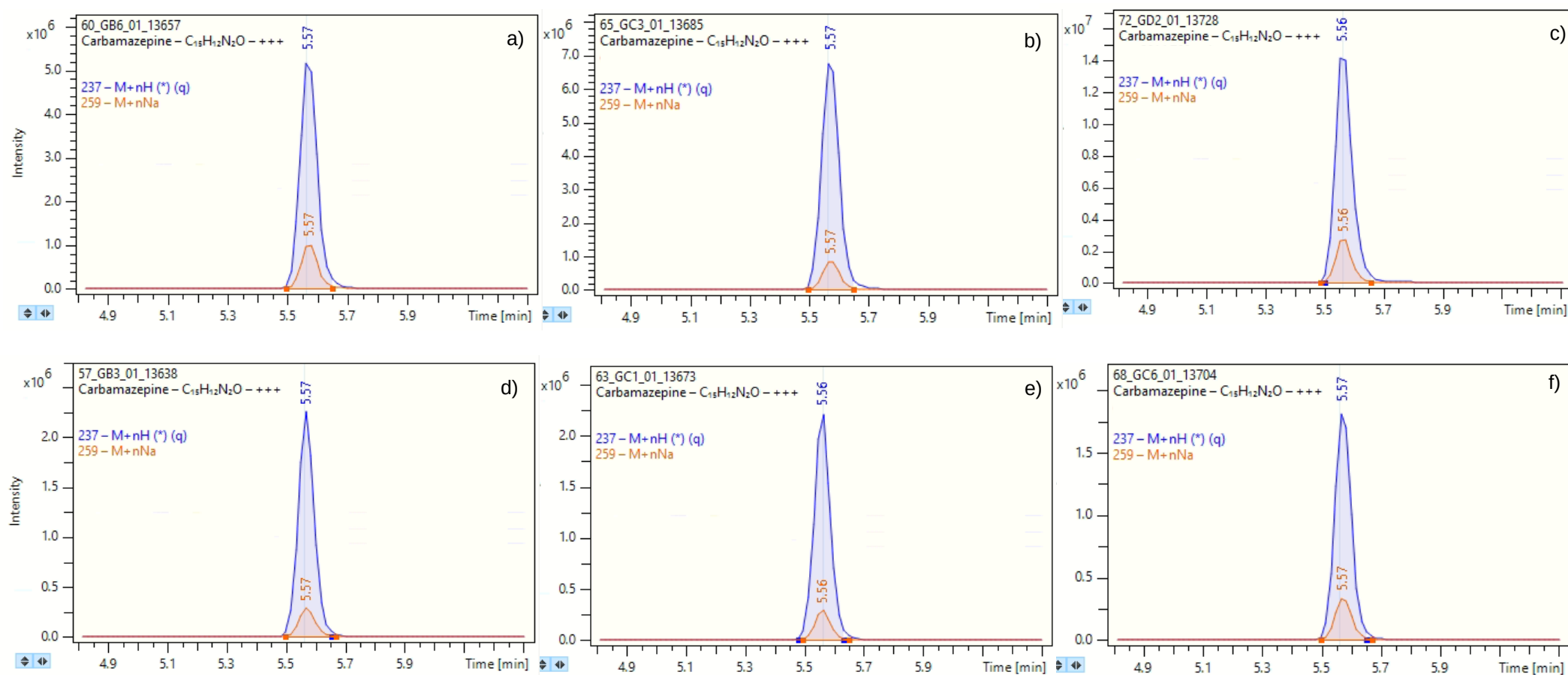


Figure 3.3. - Extracted ion chromatograms representing carbamazepine peak [237 – M + nH(*)q – blue] used for quantitation of CBZ uptake by leaves (a, b, c) and roots (d, e, f) from lettuce contaminated with 1 mg L⁻¹ on days 1 (a,d), 8 (b,e) and 15 (c,f) of exposure.

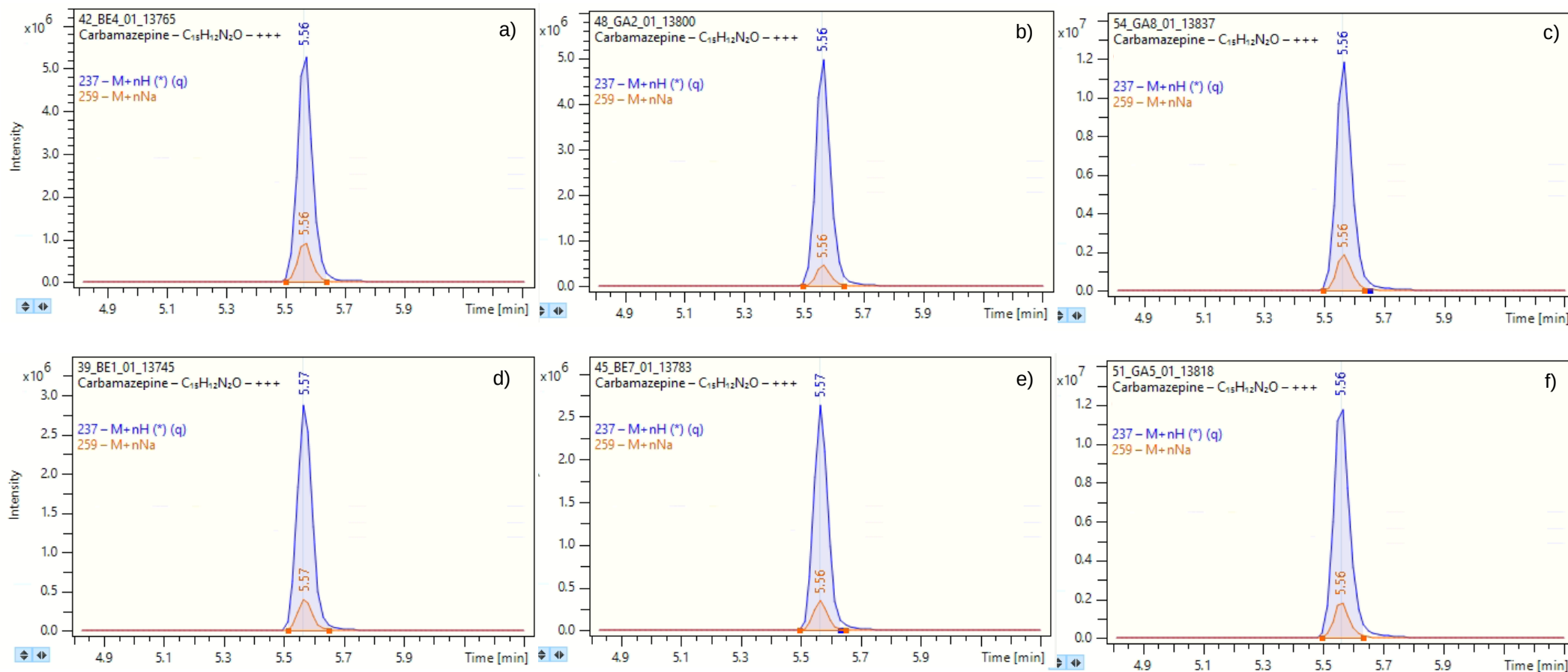


Figure 3.4. - Extracted ion chromatograms representing carbamazepine peak [237 - M + nH(*)q - blue] used for quantitation of CBZ uptake by leaves (a, b, c) and roots (d, e, f) from lettuce contaminated with 5 mg L⁻¹ on days 1 (a,d), 8 (b,e) and 15 (c,f) of exposure.

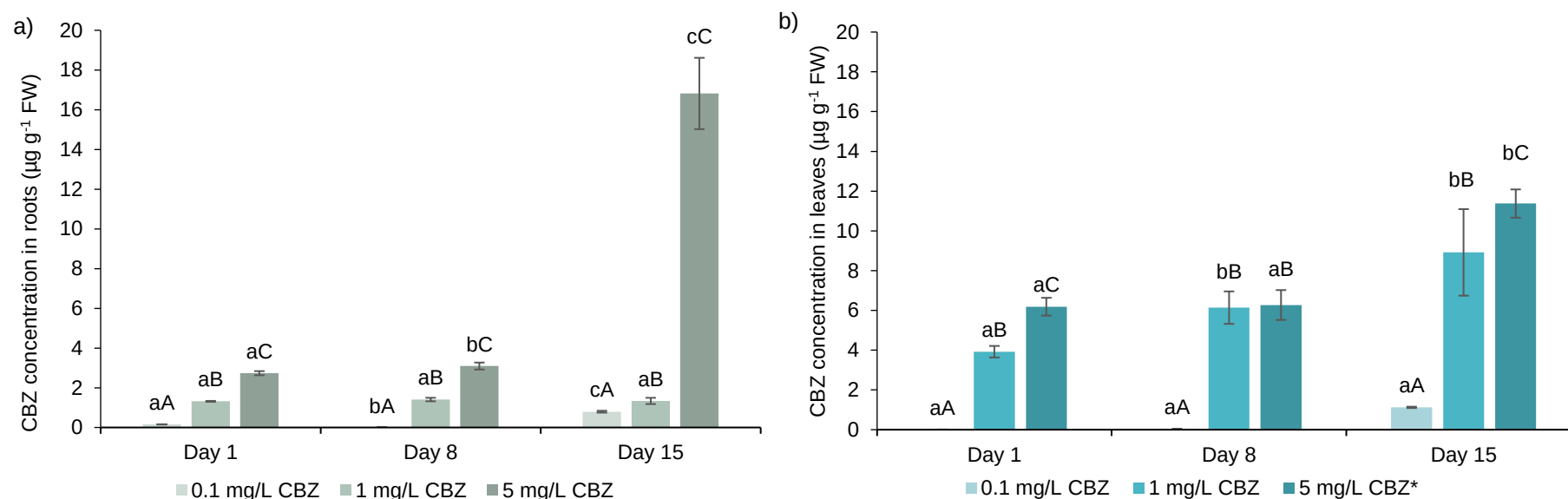


Figure 3.5. - CBZ concentration ($\mu\text{g g}^{-1}\text{ FW}$) in roots (a) and leaves (b) of *Lactuca sativa* on days 1, 8 and 15 of exposure. Different uppercase letters represent significant differences between different CBZ concentrations at the same time point and different lowercase letters represent significant differences between treatment duration for the same CBZ concentration.

Table 3.1. – Bioaccumulation factor (BCF) and translocation factor (TF) of CBZ in roots and leaves during 15 days of exposure.

	Day 1			Day 8			Day 15		
	BCF _{Roots}	BCF _{Leaves}	TF	BCF _{Roots}	BCF _{Leaves}	TF	BCF _{Roots}	BCF _{Leaves}	TF
0.1 mg L ⁻¹ CBZ	0.0015	0.0001	0.0531	0.0000	0.0003	-	0.0079	0.0112	1.4137
1 mg L ⁻¹ CBZ	0.0013	0.0039	2.9800	0.0014	0.0061	4.3656	0.0013	0.0089	6.6700
5 mg L ⁻¹ CBZ	0.0005	0.0012	2.2628	0.0006	0.0013	2.0269	0.0034	0.0023	0.6763

3.3.2. Biomass and chlorophyll parameters

Compared to the plants grown in control medium, those contaminated with CBZ revealed significant differences in both leaf and root biomass, mainly for the higher CBZ concentrations (1 and 5 mg L⁻¹), as can be seen in Figure 3.6.

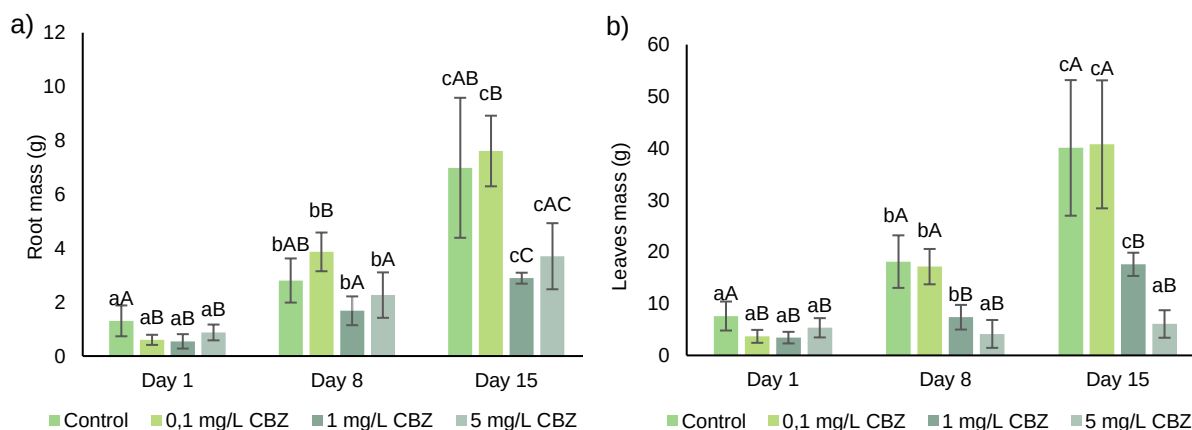


Figure 3.6. - Leaf (a) and root (b) mass of *Lactuca sativa* under contamination with CBZ for up to 15 days. Different uppercase letters represent significant differences between different CBZ concentrations at the same time point and different lowercase letters represent significant differences between treatment duration for the same CBZ concentration.

Root development appears to be highly affected by this compound in terms of visual effects (Figure 3.7), leading to impaired plant growth. A 73% reduction in leaf biomass was observed at 5 mg L⁻¹ after 15 days. In fact, plants contaminated with 5 mg L⁻¹ CBZ showed visual toxicity symptoms and development damages.

At the lower concentration of 0.1 mg L⁻¹ both root and leaf biomass were only affected at day 1 of the experiment. This shows that, for this concentration, plant growth and development was initially affected but the plants were later able to compensate the effects of CBZ and after the 8th day of contamination, growth was similar to control levels. Similar effects were also observed by Dordio *et al.* (2011) where *Typha latifolia* plants subjected to 2.0 mg L⁻¹ CBZ showed a decrease on relative growth rates and by Carter *et al.* (2015) that reported a decrease in biomass of zucchini under carbamazepine contamination. Also, other pharmaceuticals were reported as affecting the growth and development of plants, such as ciprofloxacin and metformin in carrots (Eggen *et al.*, 2011).



Figure 3.7. - Lettuce plants on days 1, 8 and 15 of contamination with different concentrations of CBZ - Control, 0.1 mg L⁻¹, 1 mg L⁻¹ and 5 mg L⁻¹.

Plant photosynthesis has also been reported to be affected by different pharmaceutical compounds (Carvalho *et al.*, 2014). Therefore, chlorophyll content was determined (Figure 3.8). As can be seen in Figure 3.8, chlorophyll levels actually increased for the longest treatment periods and higher concentrations of CBZ. While, on one hand, this could be due to a decreased leaf area (leading to an increase in chlorophyll concentration), on the other hand it also suggests that chlorophyll production and degradation are not affected by this compound. In order to verify if the photosynthetic ability of lettuce plants was affected by CBZ the NDVI, and the PRI (Peñuelas *et al.*, 2011) were measured (Figure 3.9). While NDVI correlates with photosynthetic rates, PRI has been used to evaluate changes in plant response to stress, as it measures plant pigment pools, such as the ratio between carotenoid and chlorophyll content (Mänd *et al.*, 2010).

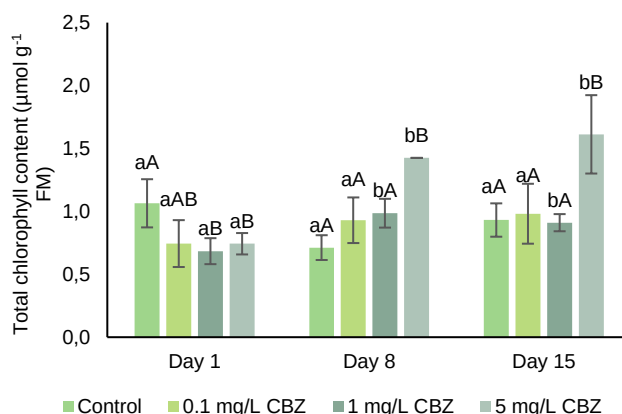


Figure 3.8. - Chlorophyll content of *Lactuca sativa* leaves under contamination with CBZ for up to 15 days. Different uppercase letters represent significant differences between different CBZ concentrations at the same time point and different lowercase letters represent significant differences between treatment duration for the same CBZ concentration.

The fact that the observed changes in the NDVI parameter were slight and did not show a clear time- or dose-dependent trend (Figure 3.9a) reinforces the hypothesis that the main photosynthetic machinery is not affected by this pharmaceutical. The observed increase in the PRI parameter for the highest doses and longer treatment period (Figure 3.9b) could indicate that stress, induced by CBZ, leads to a higher turnover of xanthophyll cycle pigments, which together with SOD activity are an efficient protective measure, as described by Esteban *et al.* (2015).

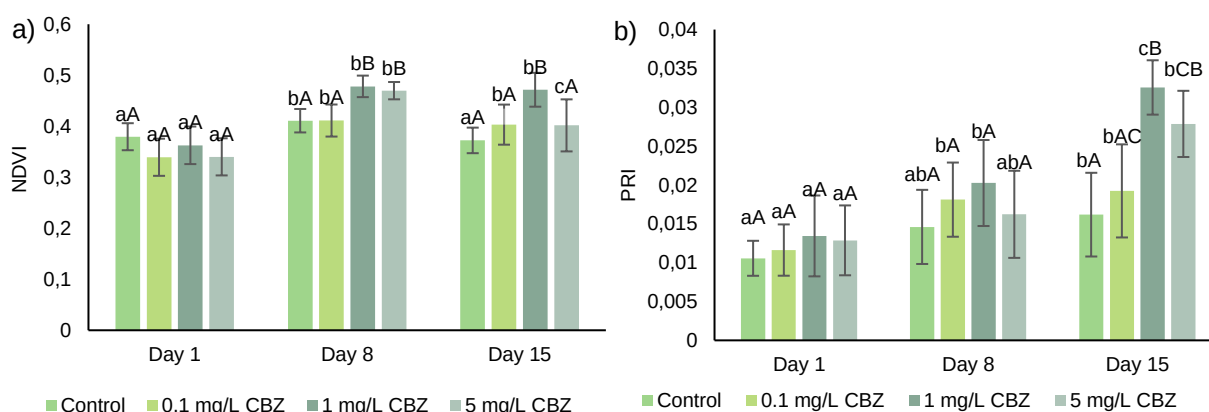


Figure 3.9 - NDVI (a) and PRI (b) of *Lactuca sativa* leaves under contamination with CBZ for up to 15 days. Different uppercase letters represent significant differences between different CBZ concentrations at the same time point and different lowercase letters represent significant differences between treatment duration for the same CBZ concentration.

Even though photosynthesis was apparently not affected, pharmaceutical compounds are known to induce oxidative stress in plants (Christou *et al.*, 2016) and so different oxidative stress indicators were analysed, in order to identify the main antioxidative mechanisms used by lettuce plants.

3.3.3. Stress indicators analysis

Hydrogen peroxide is commonly used as an indicator of the occurrence of oxidative stress in plants. ROS, like H_2O_2 , are toxic at high concentrations but also have an important function as signalling molecules (Sies *et al.*, 2017). As can be seen in Figure 3.10, there was a general increase in H_2O_2 levels in lettuce leaves for the longer time points and higher CBZ concentrations. Even for the lower CBZ concentration of 0.1 mg L^{-1} a significant increase in hydrogen peroxide levels was detected, though only after 15 days. This shows that the plant has defence mechanisms capable of quenching the excess H_2O_2 but only up to a certain point, which suggests that the repair pathways may become saturated. Hydrogen peroxide in the roots increased significantly in relation to the control for the two highest concentrations of 1 and 5 mg L^{-1} of CBZ at the 8th day of the experiment and only for 5 mg L^{-1} at the 15th day (Figure 3.10). This shows that these CBZ concentrations induced the presence of this ROS but the root defence mechanisms were efficiently activated after a certain period of time for the 1 mg L^{-1} concentration. Interestingly, Christou *et al.* (2016) also detected a more pronounced effect of different pharmaceutical compounds in the leaves of alfalfa than in the roots, showing that, although roots are in direct contact with the contaminant, the oxidative effect is more pronounced after these contaminants being translocated to the upper plant parts.

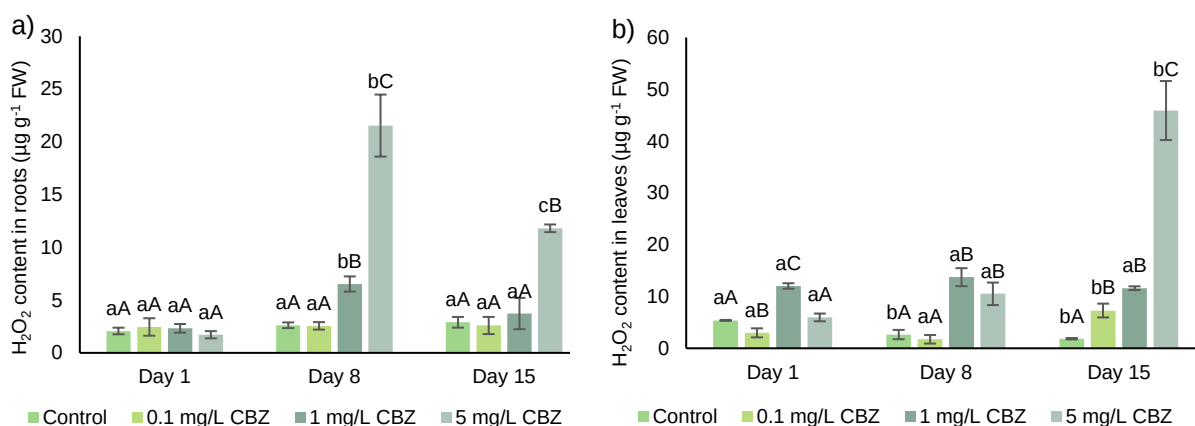


Figure 3.10. - H₂O₂ content (µg g⁻¹ FW) in roots (a) and leaves (b) of *Lactuca sativa* under contamination with CBZ for up to 15 days. Different uppercase letters represent significant differences between different CBZ concentrations at the same time point and different lowercase letters represent significant differences between treatment duration for the same CBZ concentration.

To evaluate if the detected levels of hydrogen peroxide correlated with oxidative damage, the levels of MDA were measured both in the leaves and in the roots (Figure 3.11 a/b) since its production is linked with lipid peroxidation (Mourato *et al.*, 2012). In the roots, there was no increase in MDA levels showing that no lipid peroxidation occurred, probably due to the efficient antioxidant system in this plant part. In leaves, an increase in lipid peroxidation was confirmed on days 8 and 15 for the highest CBZ concentration. This effect has also been reported with other pharmaceuticals, like diclofenac and sulfamethoxazole in alfalfa plants (Christou *et al.*, 2016) but, contrary to the present work, only in roots and not in leaves. This can be due either to different ROS-generating mechanisms induced by other pharmaceutical compounds or to the different antioxidative responses in distinct plant species.

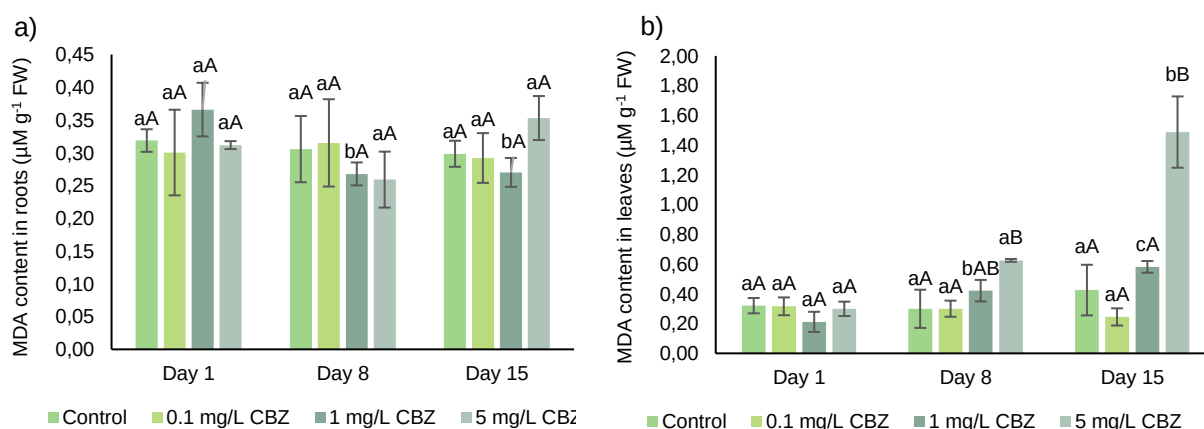


Figure 3.11. - MDA content (µM g⁻¹ FW) in roots (a) and leaves (b) of *Lactuca sativa* under contamination with CBZ for up to 15 days. Different uppercase letters represent significant differences between different CBZ concentrations at the same time point and different lowercase letters represent significant differences between treatment duration for the same CBZ concentration.

The total antioxidant activity was measured using the TEAC assay (Figure 3.12). There was a spike in the antioxidant activity in the roots at the highest CBZ concentration at day 15. This was probably induced by the content of CBZ and high hydrogen peroxide levels and can justify the fact that, although the content of this ROS was higher than in the control, no lipid peroxidation effects were detected. An increase in TEAC was also measured in the leaves, mainly for the two highest CBZ concentrations in all time periods. This shows that CBZ induced a general antioxidative response in the plant. The fact that an increase in MDA in the leaves was only detected for 5 mg L⁻¹ CBZ confirms an efficient mechanism quenching the induced ROS at the lowest CBZ concentrations.

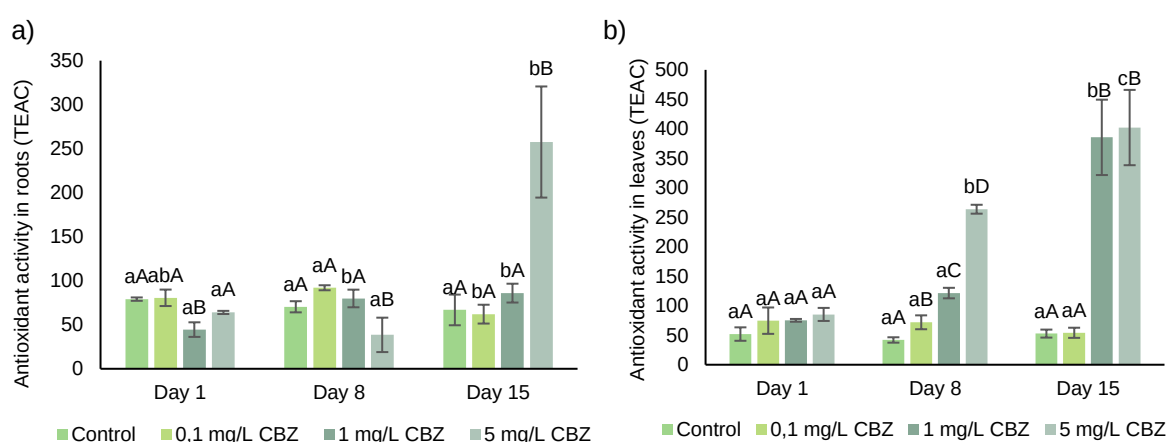


Figure 3.12. - Total antioxidant activity, expressed as TEAC, in roots (a) and leaves (b) of *Lactuca sativa* under contamination with CBZ for up to 15 days. Different uppercase letters represent significant differences between different CBZ concentrations at the same time point and different lowercase letters represent significant differences between treatment duration for the same CBZ concentration.

3.3.4. Enzymatic activities and metabolites related with stress responses

In order to identify the main antioxidative mechanisms used by roots and leaves of lettuce plants, the activities of several antioxidant enzymes and the levels of certain redox-active metabolites were measured.

The activities of different enzymes involved in the antioxidative response are shown in Figures 3.13. to 3.14. SOD is an important enzyme that catalyses the dismutation of superoxide radicals into hydrogen peroxide and oxygen (Mourato *et al.*, 2012). As SOD activity only increased in the leaves during the earliest time periods (Figure 3.13 a/b), its activity cannot fully explain the observed increase in the H₂O₂ levels, which leads to the conclusion that H₂O₂ production resulted directly from CBZ-induced stress (Apel *et al.*, 2004).

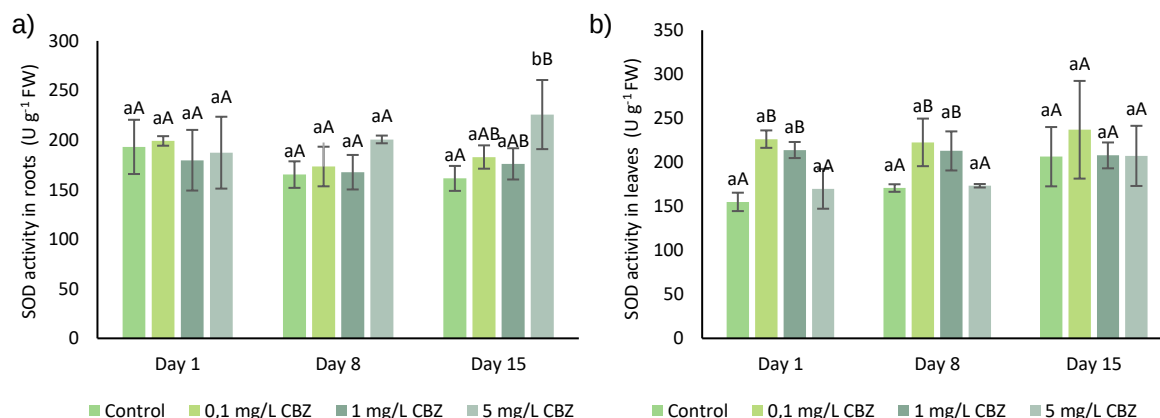


Figure 3.13. - Enzymatic activity (U g⁻¹ FW) of SOD in roots (a) and leaves (b) of *Lactuca sativa* under contamination with CBZ for up to 15 days. Different uppercase letters represent significant differences between different CBZ concentrations at the same time point and different lowercase letters represent significant differences between treatment duration for the same CBZ concentration.

To get a complete picture of the active enzymatic defence mechanisms, the activities of the enzymes responsible for H₂O₂ scavenging (CAT, GPOD and APX) were analysed (Figure 3.14).

Peroxidases are involved in a wide range of reactions, including the regulation of ROS levels, mainly H₂O₂ (Passardi *et al.*, 2005). CAT is an important enzyme in the removal of excess ROS produced as a result of oxidative stress (Mittler, 2002) and has the characteristic of not requiring any electron donor to scavenge H₂O₂ (Mhamdi *et al.*, 2010). Our results (Figure 3.14 a/b) show a large increase in CAT activity for days 8 and 15 at all studied CBZ concentrations in both roots and leaves (except for day 8 in the leaves, where this effect was only observed at 1 and 5 mg L⁻¹ CBZ). This confirms that there was a strong enzymatic response to the oxidative stress induced by CBZ. Although there was a slight increase in the H₂O₂ levels at day 1 in leaves under 1 mg L⁻¹ CBZ, and an increase in SOD activity, CAT activity did not change at this time point. This shows that this enzyme had a long-term response and only to higher concentrations of H₂O₂, as described by Mittler (2002). In roots, the increase in CAT activity was not enough to lower the H₂O₂ levels at higher CBZ concentrations, as can be seen in Figure 3.10.

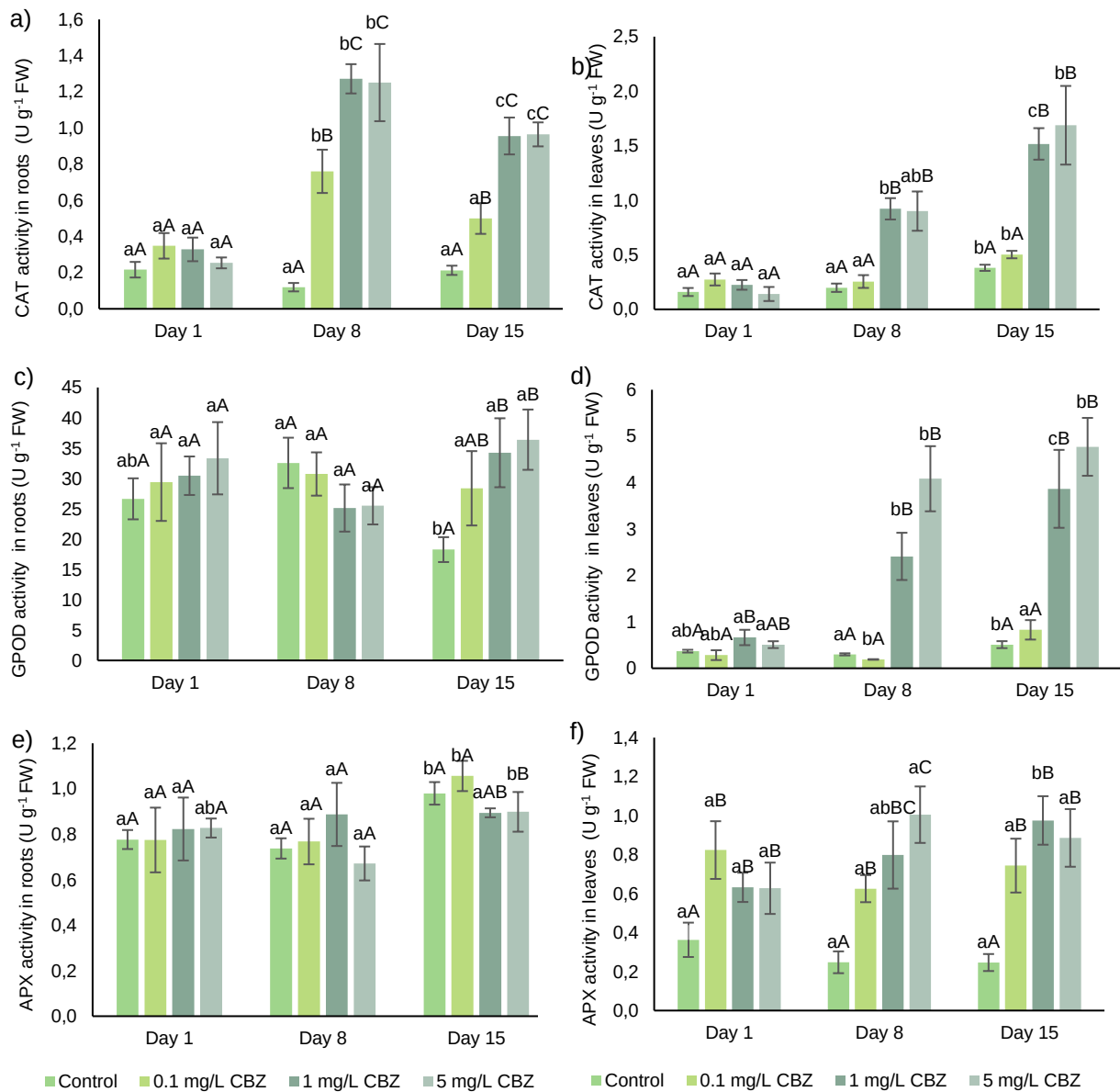


Figure 3.14. - Enzymatic activity (U g⁻¹ FW) of CAT, GPOD and APX in roots (a, c, e) and leaves (b, d, f) of *Lactuca sativa* under contamination with CBZ for up to 15 days. Different uppercase letters represent significant differences between different CBZ concentrations at the same time point and different lowercase letters represent significant differences between treatment duration for the same CBZ concentration.

Regarding CAT activity similar results were reported by Dordio *et al.* (2011) where differences on *Typha latifolia* were described under 0.5, 1 and 2.0 mg L⁻¹ CBZ for 7, 14 and 21 days of exposure. Alkimin *et al.* (2019) conducted an experiment to understand the ecotoxicology of three different pharmaceuticals (diclofenac, acetaminophen and chlorpromazine) on *Lemna Minor* and *Lemna gibba* plants. This study showed an increase of CAT activity at some concentrations of the three pharmaceuticals (even in lower concentrations).

GPOD uses phenolic electron donors to eliminate H_2O_2 . In leaves of lettuce plants an increase in the activity of this enzyme was detected at the two highest concentrations of CBZ (Figure 3.14 c/d) analogous to what was observed for CAT activity. This suggests that this enzyme is also used, in conjunction with CAT, to contribute to scavenge the excess H_2O_2 in other cellular compartments. The increase in GPOD activity in the roots, detected only at the latest time point of 15 days for all the CBZ concentrations used, is probably a late response of the plant to the CBZ-induced stress, involving cell wall lignification as a defence mechanism (Cuypers *et al.*, 2002).

Regarding CAT and GPOD activities, Figure 3.15 shows the correlation between these two enzymes in roots and leaves. It is possible to affirm that the higher contents of H_2O_2 on the most contaminated leaves induced higher activities of both enzymes working together on H_2O_2 scavenging.

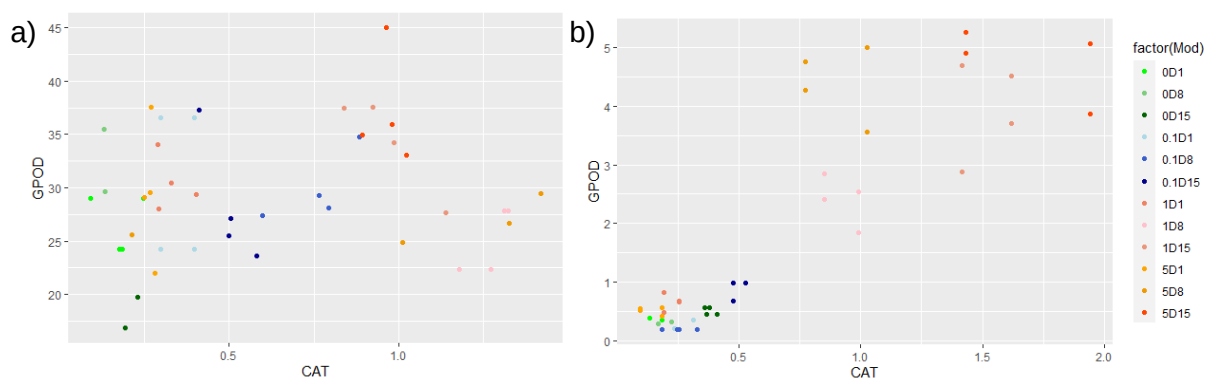


Figure 3.15. - Correlation between GPOD and CAT in roots (a) and leaves (b) of *Lactuca sativa* under contamination with CBZ for up to 15 days.

The analysis in roots is not so clear. It is possible to observe a pattern of correlation between CAT, SOD and H_2O_2 contents (Figure 3.16), however no trend was observed between GPOD and CAT activities. Even in terms of GPOD and H_2O_2 content correlation is not as evident as it was in leaves tissues. In fact, GPOD was not so effective in roots during the experiment, as mentioned before.

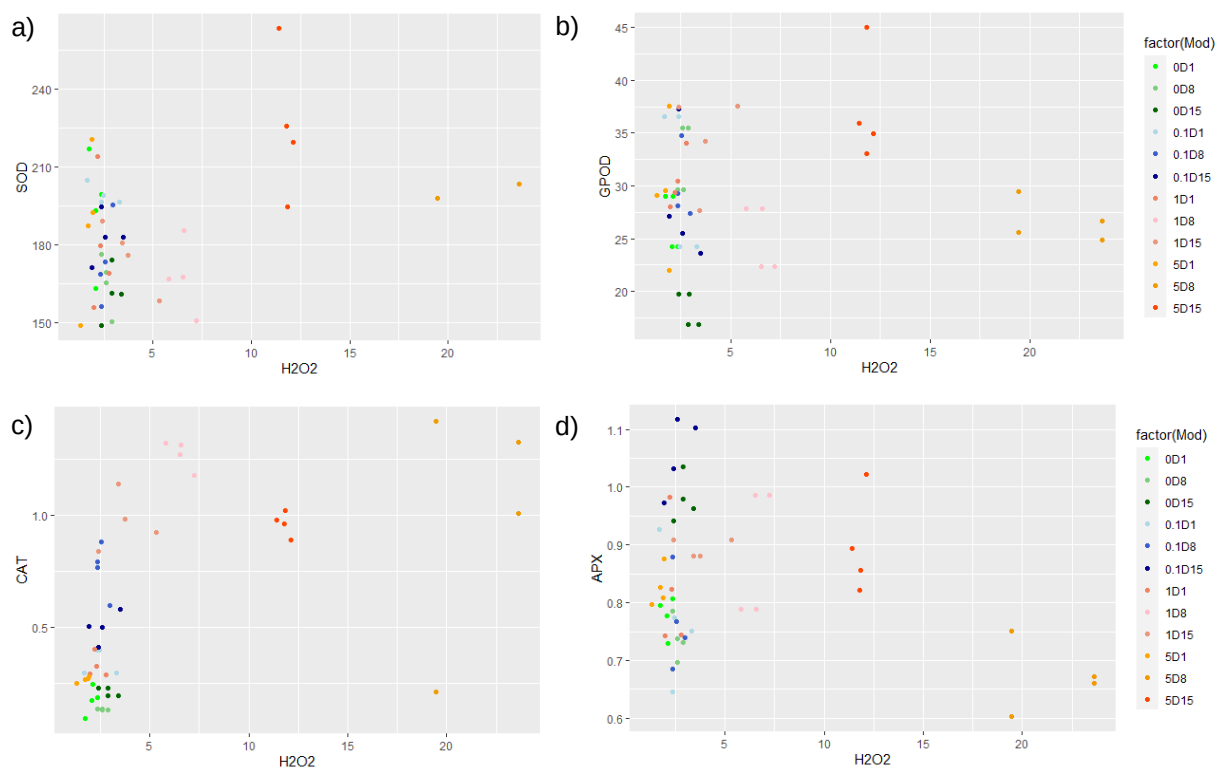


Figure 3.16.- Correlation between SOD/H₂O₂ (a), GPPOD/H₂O₂ (b), CAT/H₂O₂ (c) and APX/H₂O₂ (d) in roots of *Lactuca sativa* under contamination with CBZ for up to 15 days.

APX is an enzyme that is usually active in the removal of H₂O₂ using ascorbate as the reducing substrate (Foyer *et al.*, 2011). As can be seen in Figure 3.14e, there was no change in the activity of APX in relation to the control in lettuce roots, showing that the other enzymes referred above are more important in ROS removal. On the other hand, in lettuce leaves (Figure 3.14 f) there was a significant increase of APX activity at all the CBZ concentrations and time points under study, confirming that the induced oxidative stress caused a coordinated response in several antioxidative enzymes. According to Shigeoka *et al.* (2002), APX activity generally increases together with CAT and SOD activity but in our study the global increase in APX activity in leaves was not extensively followed by the other enzymes. In the roots neither SOD nor APX activity changed (except for SOD at the longest time point and highest CBZ concentration). Bartha *et al.* (2014) described some differences on *Typha latifolia* APX activity under diclofenac contamination. However, as in our study, the authors did not observe a time-dependent trend. In fact, significant differences were only found on days 1 and 7 on leaves, and no differences were found in roots.

As APX uses ascorbate as substrate, the concentration of this metabolite was also determined (Figure 3.17 a/b).

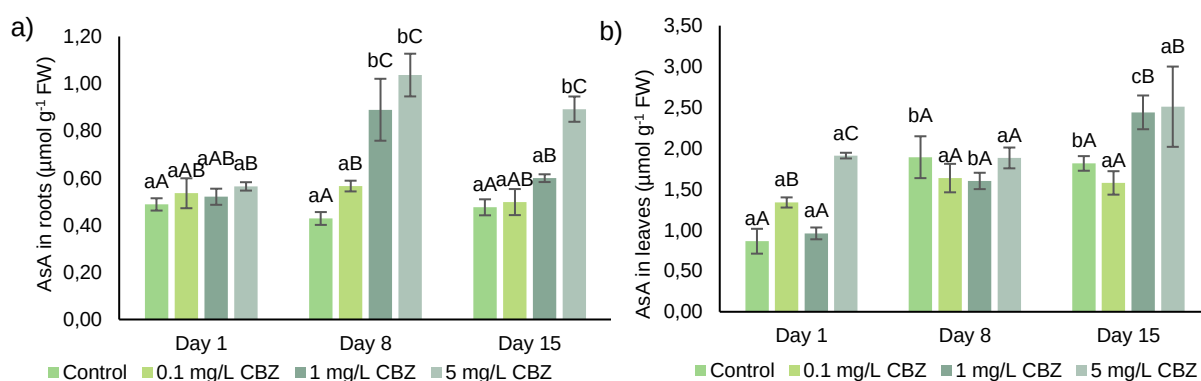


Figure 3.17. – Ascorbate content ($\mu\text{mol g}^{-1}$ FW) in roots (a) and leaves (b) of *Lactuca sativa* under contamination with CBZ for up to 15 days. Different uppercase letters represent significant differences between different CBZ concentrations at the same time point and different lowercase letters represent significant differences between treatment duration for the same CBZ concentration.

An increase in root ascorbate levels in relation to the control was observed but, as APX activity did not increase in this plant part, it can be concluded that ascorbate is acting directly as an antioxidant, justifying, in part, the general increase in antioxidant activity described earlier. In the leaves there was an early (day 1) and late (day 15) response of ascorbate levels that probably reflects the activation of the ascorbate-glutathione cycle for H_2O_2 removal.

Glutathione, GPX and GR are also involved in the ascorbate-glutathione cycle. Generally, the oxidation of reduced glutathione by H_2O_2 is catalysed by GPX, followed by the conversion of glutathione disulphide back to the reduced form, catalysed by GR (Apel & Hirt, 2004). GSH takes part in detoxification of ROS and xenobiotics (GPX and GST activities), thus playing an important role in cells defence mechanism (Hasanuzzaman *et al.*, 2017). Results regarding GSH contents, GR and GPX activities are shown in Figure 3.18.

No increase in the activity of GPX was detected at any CBZ concentration or period analysed, both in roots and in leaves. It thus seems that this enzyme is not important in this plant detoxification mechanisms. However, there was an increase in GSH levels both in roots and in leaves of lettuce (Figure 3.18 a/b). This increase was noticeable in roots at the highest CBZ concentration (for the two longest time points studied) and in the leaves also at days 8 and 15. As there was no concomitant increase in GPX activity, it can be inferred that GSH was being used directly as a ROS scavenger. Reinforcing this hypothesis is the fact that GR activity increased (Figure 3.18 c/d), although only transiently at day 8, for the two highest CBZ concentrations, both in roots and in leaves, as a way to regenerate the levels of reduced glutathione (in fact, this correlates well with the measured increase in glutathione levels at the same concentrations and time point). This is probably not an early response, as there were no

changes in the levels of GSH and no increase in GPX and GR activity on day 1. This trend was already observed in Poplar species under diclofenac contamination, where GR activity presented an increase on the 7th day of the experiment, followed by a decrease at longer time periods (Carla *et al.*, 2018). Interestingly, a decrease in GSH levels was detected in the roots on the first day, at the concentration of 5 mg L⁻¹. This is probably due to an early depletion of GSH levels being used in the defence mechanisms described above, that was compensated in later stages.

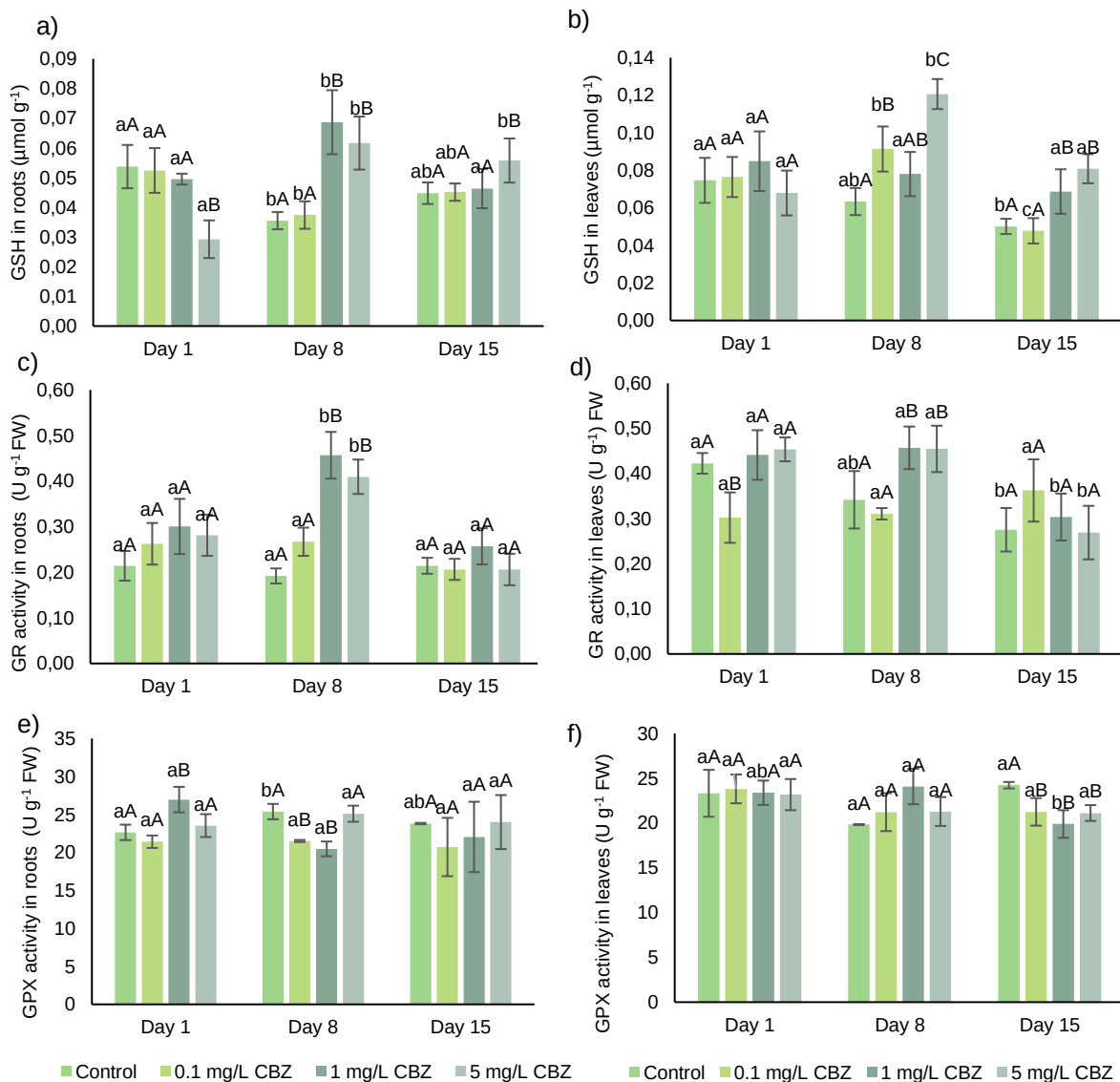


Figure 3.18. – GSH contents and enzymatic activity (U g⁻¹ FW) of GR and GPX in roots (a, c, e) and leaves (b, d, f) of *Lactuca sativa* under contamination with CBZ for up to 15 days. Different uppercase letters represent significant differences between different CBZ concentrations at the same time point and different lowercase letters represent significant differences between treatment duration for the same CBZ concentration.

Concerning GR and GPOD activities, He *et al.* (2017) carried out a study evaluating the effect of ibuprofen on *Phragmites australis* and no differences were found in terms of enzymatic activities, except on day 3 in roots and aerial tissues. The authors suggested a growth adaptation as a response to these differences.

Regarding anthocyanin content (Figure 3.19a), CBZ affected the content of anthocyanins in leaves at the higher concentration. For longer periods of time (days 8 and 15), 1 mg L⁻¹ of CBZ also induced a production of anthocyanins, showing significant differences to control. In terms of comparison among days, it is possible to observe that differences were found from day 1 to day 8 remaining similar on day 15 on the concentration of 1 and 5 mg L⁻¹ of CBZ. For the lower concentrations, no differences were registered among days of exposure and neither to control condition.

The same pattern was verified in PAL activity (Figure 3.19b), which is correlated to phenolic compounds and anthocyanins production. However, in case of 1 mg L⁻¹ of CBZ differences over control were found only on day 1 and 15 of exposure, while with 5 mg L⁻¹ of CBZ, significant differences were observed with time. Differences between days for the same concentration occurred for every condition on day 15, and on every days for concentrations of 1 and 5 mg L⁻¹ of CBZ. This trend reveals a clear influence of CBZ in secondary metabolism which is often related as another strategy of ROS scavenging in cells. The H₂O₂ contents in roots and leaves showed significant differences in 1 and 5 mg L⁻¹ of CBZ suggesting a great influence of CBZ on induced oxidative stress. Therefore, the content of anthocyanins in leaves is intimately related. Also, the high antioxidant activity observed on leaves, on the same days, are correlated with the high content of anthocyanins. Figure 3.20 shows a clear correlation between PAL activity and anthocyanins content in leaves under 5 mg L⁻¹ CBZ (pink and orange dots). Significant differences in PAL activity may be a reflection of the antioxidant activity observed in leaves, as suggested by Oh *et al.* (2009) that related the antioxidant activity response with the increase in the transcript level of PAL. Besides, red pigments as anthocyanins are known to alleviate stress factors throughout ROS neutralization (Cheynier *et al.*, 2013). Then the high content verified in anthocyanins may consist in a secondary metabolism response to ROS presence already shown.

The concentration of phenolic compounds was also measured (Figure 3.21) however no differences were found in leaves. The total levels of polyphenols did not change in relation to the control, demonstrating that these compounds were not as crucial in the antioxidant response.

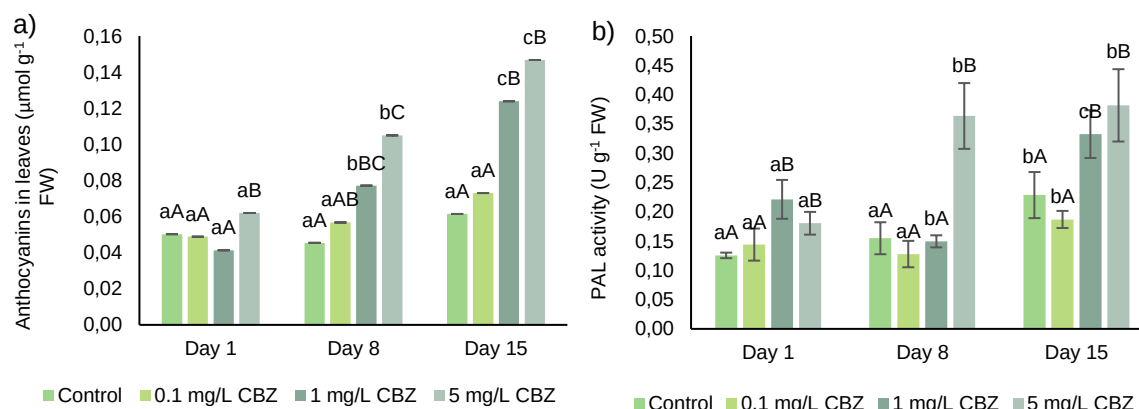


Figure 3.19. – Anthocyanins content ($\mu\text{mol g}^{-1}$ FW) (a) and PAL activity (U g^{-1} FW) (b) in leaves of *Lactuca sativa* under contamination with CBZ for up to 15 days. Different uppercase letters represent significant differences between different CBZ concentrations at the same time point and different lowercase letters represent significant differences between treatment duration for the same CBZ concentration.

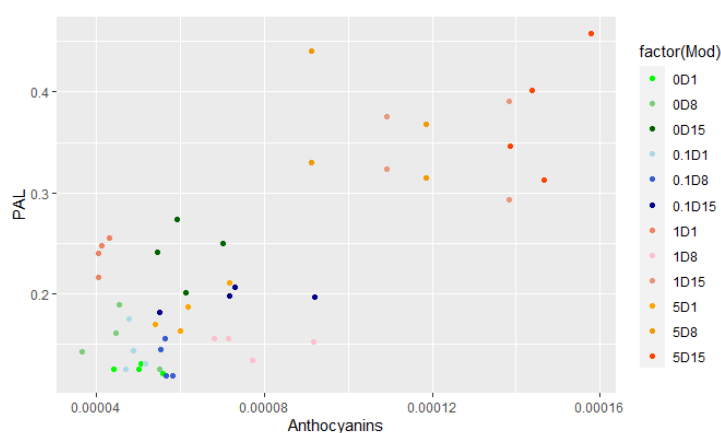


Figure 3.20. - Correlation between Anthocyanins content and PAL in leaves of *Lactuca sativa* under contamination with CBZ for up to 15 days.

On the other hand, in roots significant differences were detected on day 1 under 5 mg L^{-1} of CBZ, where a decrease in phenolic compounds was measured. Usually, abiotic stresses are known to cause an increase in phenolic compounds (Sofa *et al.*, 2017), however in this study this trend was not verified. This might be associated with the different type of contamination which may not be comparable to heavy metals or other nutrients deficiency or toxicity. Phenolic compounds protect cell against ROS and DNA damage, protein denaturation and lipid peroxidation. In fact, some authors suggest that the synthesis of phenolic compounds help on the creation of new homeostasis levels which will improve the response to oxidative stress (Carvalho *et al.*, 2015).

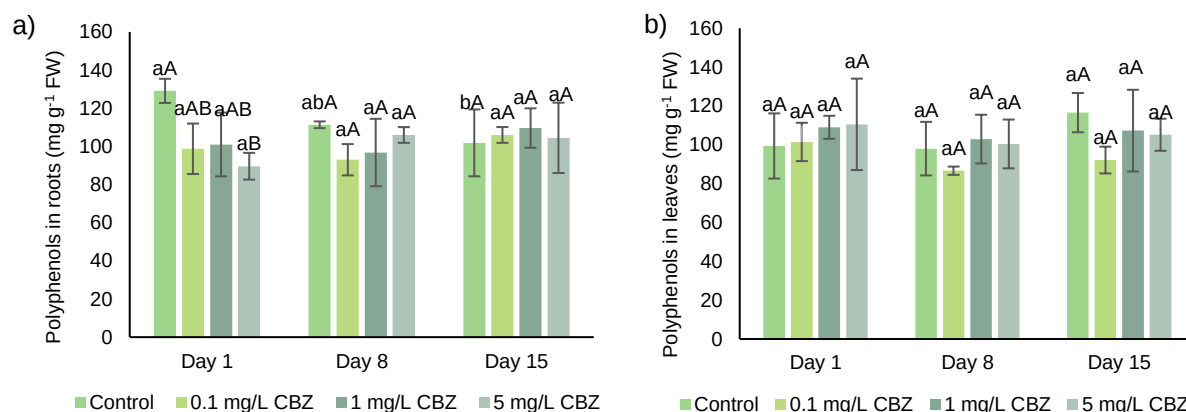


Figure 3.21. - Polyphenols content (mg g⁻¹ FW) in roots (a) and in leaves (b) of *Lactuca sativa* under contamination with CBZ for up to 15 days. Different uppercase letters represent significant differences between different CBZ concentrations at the same time point and different lowercase letters represent significant differences between treatment duration for the same CBZ concentration.

A Principal Component Analysis (Figure 3.22) of enzymatic activities results was performed in order to understand the main trend of the contaminations used in this experiment. Enzymes referred above showed a trend to group some individuals according to contamination and day of exposure. It is possible to observe that lettuce plants showed differences among tissues under different CBZ concentrations. However, there is still an overlap among classes in roots, which may reflect the absence of significant differences in comparison to control, in some enzymes, particularly on day 8. In leaves, classes show a clear differentiation among control and most contaminated tissues (black class). Then, it is possible to affirm that enzymes involved in oxidative stress revealed a stronger response in leaves when compared to roots.

In Figure 3.23, a schematic representation of the main metabolic effects observed in lettuce leaves and roots under CBZ contamination is presented.

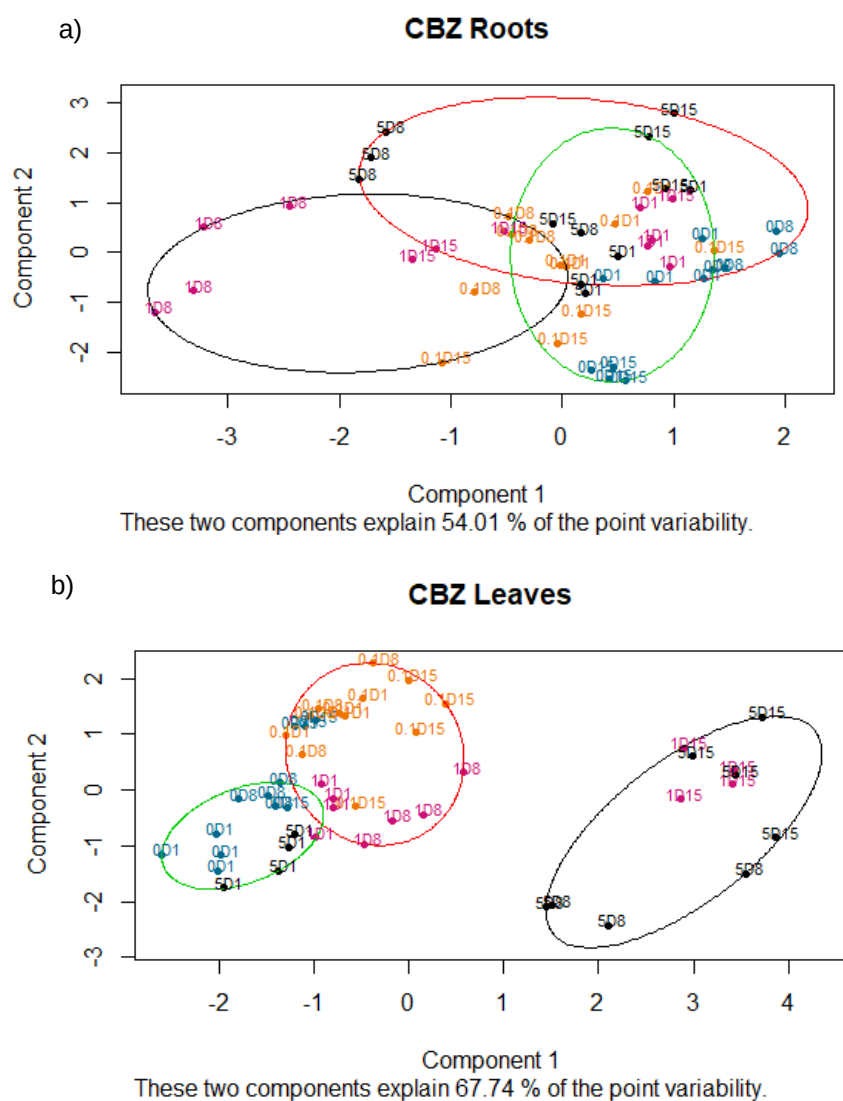


Figure 3.22. – Principal Component Analysis (PCA) of enzymatic activities (CAT, SOD, GPOD, APX, GR, GPX) in roots (a) and leaves (b) of *Lactuca sativa* under contamination with CBZ for up to 15 days. Legend: Control – blue dots, 0.1 mg L⁻¹ CBZ – orange dots, 1 mg L⁻¹ CBZ – pink dots and 5 mg L⁻¹ CBZ – black dots.

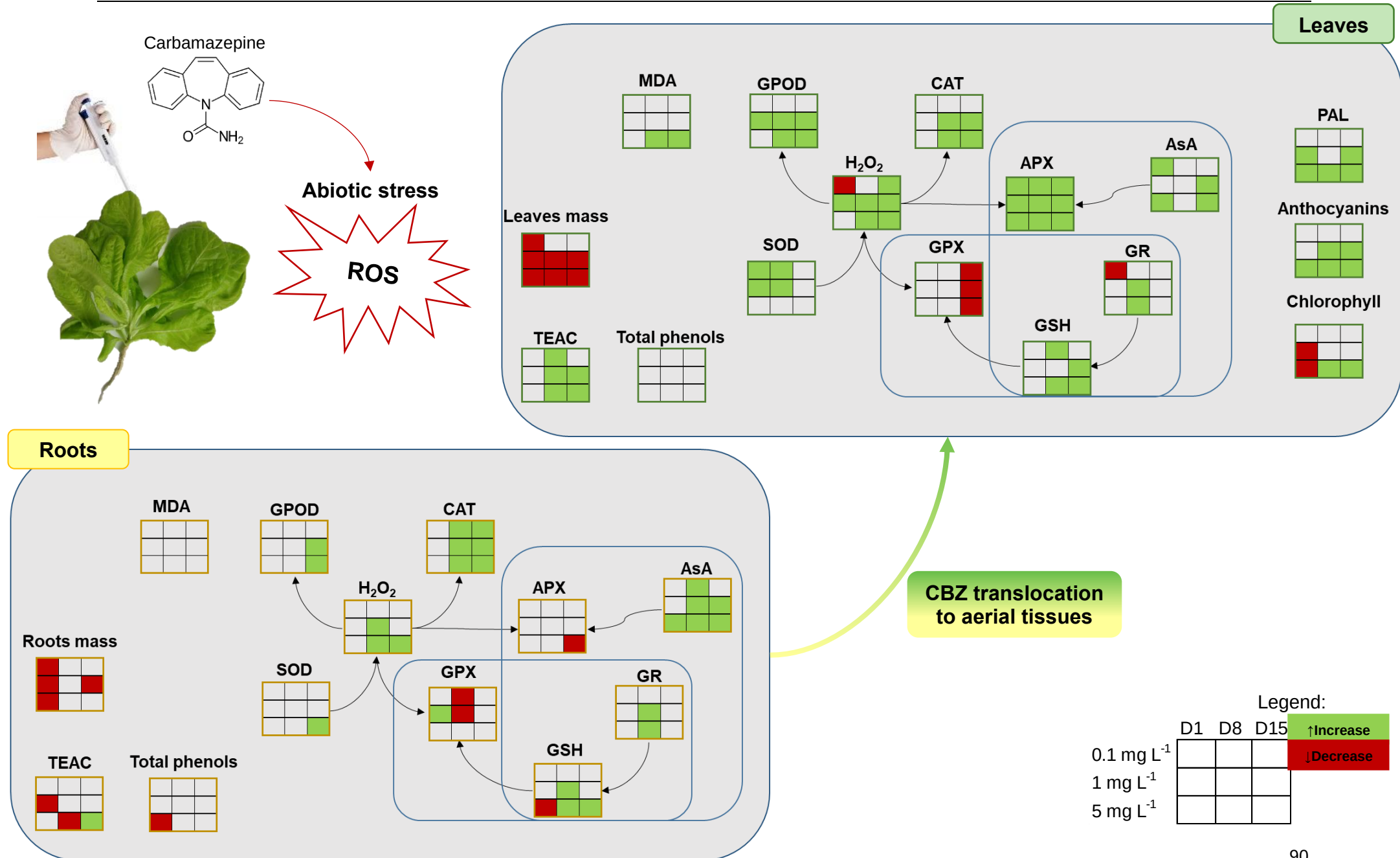


Figure 3.23. – General scheme with significant differences to control over different defence mechanisms against oxidative stress caused by CBZ. Significant differences from control are represented in green (increase) and red (decrease) squares.

3.3.5. Mineral elements analysis

Mineral content on roots and leaves contaminated with CBZ revealed slight differences to control, as presented in Tables 3.2 and 3.3. Some minerals such as, manganese and potassium in roots, and copper, manganese, zinc, potassium, magnesium, and phosphorus in leaves, showed changes over the time of exposure. In some of these cases, differences were only found in last day of exposure, for instance for copper, iron, potassium, and calcium in roots. To assess differences among different concentrations of CBZ, two types of analysis were established: (1) direct statistical analysis and correlation between control and each CBZ concentration for each mineral (Table 3.2); and (2) a translocation factor between leaves and roots for each mineral (Table 3.3). In the latter case, values higher than 1 mean that the content in leaves is higher than in roots while a value lower than 1 means the opposite.

Macronutrients in general presented higher concentrations in leaves compared to roots, while in case of micronutrients higher contents were observed in roots. This first analysis may indicate that macronutrients (K, Ca and Mg) supply was not affected in terms of aerial parts of plants. However, the analysis of variations along time and concentrations showed some differences, some of them with statistical significance. Quantification of each mineral is available in Table A.1 of Appendix section.

Regarding copper, different patterns were observed in its concentration among roots and leaves, under CBZ contamination. In roots, a significant decrease on Cu content was only observed on day 15 of exposure (1 and 5 mg L⁻¹), while in leaves, a significant difference was observed already on day 8. In both tissues a decrease in copper content was measured.

Different trends were observed in roots and leaves regarding manganese contents. In roots, an increasing pattern was observed with CBZ concentration. Indeed, significant differences were observed in 1 and 5 mg L⁻¹ on day 8 and for all concentrations of CBZ on day 15. Mn increased its contents in roots under CBZ contamination. According to proteomic analysis (see Chapter VI) proteins related with Mn (BP: cellular response to manganese ion and/or MF: manganese ion binding) such as HATPase_c domain-containing protein and Mn-SOD were more abundant in CBZ contamination over control. Considering the involvement of Mn in TCA cycle, the Mn-containing enzyme isocitrate dehydrogenase was found as more abundant in roots under CBZ contamination in comparison to control. Leaves under 1 and 5 mg L⁻¹ of CBZ presented significantly lower concentrations of Mn. Despite the decrease of Mn concentration in leaves, the measures of total chlorophyll content (Figure 3.8) did not show lack of production suggesting that the observed lower Mn contents did not affect chlorophyll production. In fact, several chlorophyll a-b binding proteins were identified as more abundant in CBZ condition

over control. In the proteomic analysis proteins such as pyruvate dehydrogenase and malate dehydrogenase revealed more abundance in control leaves compared to CBZ. This result may be a reflection of lower concentration of Mn in contaminated leaves, suggesting that Mn affected part of TCA cycle regulation in leaves.

Under CBZ contamination, roots and leaves did not displayed a specific trend over time and concentrations in relation to Fe contents. In case of roots, significant decrease in Fe was only observed on day 15 for the concentrations of 1 and 5 mg L⁻¹ while in leaves, iron pools showed significant differences on day 1. In fact, according to total chlorophyll content results, significant differences were measured on leaves under 1 and 5 mg L⁻¹ of CBZ. Thus, the lower content of iron in leaves may explain the lower production of chlorophyll on day 1. Although visual differences were observed in roots and leaves over time of exposure, they cannot be linked specifically to Fe deficit. Regarding translocation factors it is possible to observe that values are closer to 0, meaning that values observed in roots are much higher than the ones detected in leaves, thus CBZ may be affecting the translocation of this element to aerial parts.

Zinc concentrations in roots did not show significant differences between control and CBZ treatments. On the other hand, in leaves, Zn present a high reduction on its concentration over time and at all concentrations of CBZ. This may be explained through low translocation of Zn from roots to leaves or by an interaction among CBZ and Zn on cells. In fact, the latter was detected already in human cells. The authors compared the uptake of Zn (serum) in treated patients (CBZ treatment) and control groups (no treatment) and observed that in treated patients the concentration of Zn was lower (Castro-Gago *et al.*, 2011; Sözüer *et al.*, 1995). Despite different organisms, this result may explain some interaction among CBZ and Zn in our study.

Sodium contents were not so influenced by CBZ contamination as other elements, as no statistical significances were found in these results.

In relation to K, significant differences over control were found in concentrations of 1 and 5 mg L⁻¹ of CBZ, for every day of exposure and in both tissues. This pattern points to a clear influence of CBZ intake on K pools, which may have an impact in normal regulation of some proteins and processes such as pyruvate kinases (glycolysis), membrane transporters as PPase, or proteins that contribute to starch synthesis. In fact, according to the proteomic analysis in roots contaminated with 1 mg L⁻¹ of CBZ, differences were found in proteins related with potassium regulation such as calcium-dependent protein kinase 1 (regulation of inward rectifier potassium channel activity), aldo_ket_red domain-containing protein (voltage-gated potassium channel complex, potassium channel regulator activity and negative regulation of

delayed rectifier potassium channel activity) and OBG-type G domain-containing protein (potassium ion binding).

An increase over time in Ca contents was observed in comparison to control. In particular for roots, significant increases were observed for day 15 under 1 and 5 mg L⁻¹ of CBZ. Previous studies suggested that Ca²⁺ can stimulate the activity of some enzymes and alleviate lipid peroxidation under abiotic stresses (Wang *et al.*, 2009). Although, in roots no differences were found in MDA content regarding lipid peroxidation. In leaves under 5 mg L⁻¹ on days 8 and 15 an increase of MDA content was detected.

CBZ contamination influenced root uptake of Mg on day 1 of exposure, showing significant decrease under 5 mg L⁻¹. Yet, this trend was not verified on days 8 and 15 where slight increases were observed, with significant differences on day 15. However, in case of leaves a decrease pattern, showing significant differences, was observed for longer periods of exposure to CBZ. In any case, chlorophyll content was not affected for longer times of exposure.

Regarding phosphorus concentration, this element suffered a decrease in both tissues. In roots, significant differences were observed only on day 15 for 5 mg L⁻¹ concentration. In leaves, these differences were found in every concentration of CBZ on days 8 and 15. Therefore, it is possible to say that CBZ interfere in uptake and possible translocation of this element in lettuce leaves. Some authors suggested that the decrease of P contents may influence the production of anthocyanins (Schachtman *et al.*, 1998) and this trend was verified in leaves under CBZ.

CBZ contamination negatively influenced the concentrations on S in both tissues for longer days of exposure. In roots, significant differences to control were only observed on the last day of contamination with 5 mg L⁻¹ of CBZ and on leaves under 1 mg L⁻¹ CBZ. Proteins related with sulphur activity on cells such as ferredoxin-nitrite reductase, chloroplastic and cystathionine beta-lyase, chloroplastic were identified in lower abundance in CBZ-contaminated roots. In leaves, thioredoxin-like 2, chloroplastic and ferredoxin-nitrite reductase, chloroplastic were also identified as less abundant in plants growing in CBZ contaminated solutions. These proteins are linked to sulphur activity on cells presenting a correlation with the lower S contents of contaminated leaves.

Table 3.2. - Mineral content variations along time and exposure to CBZ in roots and leaves of *Lactuca sativa*. Significant differences are represented in red (decreases) and green (increases).

			Cu			Mn			Fe			Zn			Na			K			Ca			Mg			P			S		
			Day 1	Day 8	Day 15	Day 1	Day 8	Day 15	Day 1	Day 8	Day 15	Day 1	Day 8	Day 15	Day 1	Day 8	Day 15	Day 1	Day 8	Day 15	Day 1	Day 8	Day 15	Day 1	Day 8	Day 15	Day 1	Day 8	Day 15	Day 1	Day 8	Day 15
CBZ	Leaves	0.1 mg L ⁻¹	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
		1 mg L ⁻¹	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
		5 mg L ⁻¹	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	Roots	0.1 mg L ⁻¹	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
		1 mg L ⁻¹	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
		5 mg L ⁻¹	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—

Table 3.3. - Translocation factor of mineral content in roots and leaves of *Lactuca sativa* under CBZ contamination. Brown represent highest accumulation in roots and green represents highest accumulation in leaves.

CBZ		Cu			Mn			Fe			Zn			Na			K			Ca			Mg			P			S		
		Day 1	Day 8	Day 15	Day 1	Day 8	Day 15	Day 1	Day 8	Day 15	Day 1	Day 8	Day 15	Day 1	Day 8	Day 15	Day 1	Day 8	Day 15	Day 1	Day 8	Day 15	Day 1	Day 8	Day 15	Day 1	Day 8	Day 15	Day 1	Day 8	Day 15
Translocat ion Factor	Control	0.75	0.66	0.62	1.27	0.91	0.82	0.04	0.05	0.03	0.29	0.29	0.28	0.67	0.66	1.28	1.42	1.17	1.07	2.08	1.85	1.77	2.60	3.26	3.78	0.34	0.34	0.36	0.54	0.64	0.60
	0.1 mg L ⁻¹	0.66	0.46	0.35	1.12	1.06	0.49	0.05	0.06	0.03	0.20	0.18	0.14	0.66	0.72	0.96	1.33	1.22	1.05	1.89	1.86	1.78	2.77	2.52	2.86	0.36	0.23	0.19	0.57	0.64	0.57
	1 mg L ⁻¹	0.63	0.35	0.45	0.52	0.21	0.17	0.04	0.05	0.04	0.22	0.19	0.22	0.86	0.74	1.08	1.15	1.25	1.25	1.86	1.48	1.40	2.58	2.20	1.49	0.36	0.27	0.31	0.60	0.71	0.67
	5 mg L ⁻¹	0.76	0.65	1.12	0.78	0.31	0.18	0.03	0.07	0.06	0.29	0.26	0.27	0.78	0.85	1.33	1.33	1.60	1.43	1.99	1.56	1.37	3.14	1.81	1.15	0.35	0.18	0.20	0.57	0.68	0.66

3.4. Conclusions

This study was conducted to answer the question: how lettuce plants cope with carbamazepine contamination? An overall analysis of our results allows to confirm the quick reaction of lettuce plants to CBZ exposure. Despite several studies regarding CBZ uptake, few studies report differences in oxidative stress as a main objective and so, our study contributes to improve this knowledge. Differences in terms of time and dose response were addressed and the results suggest an increase of oxidative stress, with the activation of different stress response pathways.

This study contributes to understand the interactions between lettuce, a highly consumed vegetable, and CBZ. Carbamazepine is shown to be present both in roots and in leaves of lettuce plants and to induce oxidative stress, as demonstrated by the levels of hydrogen peroxide (found both in leaves and in roots) and MDA (found only in leaves). In lettuce leaves SOD, CAT, GPOD, APX and, to a lesser extent, GR, are shown to be working together to quench the ROS produced by CBZ-induced oxidative stress. In roots, only CAT, GPOD and GR have a relevant role in the antioxidative system. Ascorbate and glutathione appear to be important antioxidants that respond to increased concentrations of CBZ. Our study also shows that, although the roots are in direct contact with the contaminated media, the strongest oxidative effects appear in the leaves. Despite all the changes in stress-related enzymes under CBZ contamination, photosynthetic activity was not affected by the presence of this pharmaceutical. Regarding mineral content, leaves presented the main differences with significant decrease of K, Mg, Mn and Zn. These results may restrict lettuce nutrition supply, and normal development of plant.

CHAPTER IV

Antioxidative response of lettuce to acetaminophen-induced stress

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Chapter IV - Antioxidative response of lettuce to acetaminophen-induced stress

Abstract

Contaminants of environmental concern, like pharmaceuticals, are being detected in increasing amounts in ground and irrigation waters and can thus be taken up by plants. In this work the uptake of acetaminophen (ACT) by lettuce plants was evaluated through a hydroponic experiment at different concentrations (0, 0.1, 1 and 5 mg L⁻¹). The pathways related with oxidative stress induced by ACT were studied in leaves and roots of lettuce at 1, 8 and 15 days after exposure. Stress indicators such as hydrogen peroxide and malondialdehyde contents were analysed, revealing differences in plants contaminated with ACT in comparison to control and confirming the occurrence of oxidative stress. Enzymatic activities of catalase, superoxide dismutase, guaiacol peroxidase, ascorbate peroxidase and glutathione peroxidase, directly involved in the antioxidative system, showed significant differences when compared to control plants, with a general trend to an increase in both tissues. Glutathione reductase revealed a decrease in contaminated leaves, which may imply a specific impact of ACT in the glutathione cycle. Significant increases were found in the anthocyanin content of leaves, both with exposure time and ACT concentration, indicating an antioxidative response induced by ACT contamination. No effect in lettuce biomass was observed nor in the visual aspect of the plants confirming the efficiency of the defence mechanisms in protecting the plant. ACT contamination did not affect mineral content.

Keywords: Acetaminophen, *Lactuca sativa*, emerging pollutants, pharmaceuticals, oxidative stress.

4.1. Introduction

Plants can suffer different abiotic stress conditions such as soil and water contamination by inorganic or organic products. Pharmaceuticals are documented to be present in surface and ground waters, manure and soil (Kim *et al.*, 2016; Li *et al.*, 2015; Vulliet & Cren-olivé, 2011). Despite the recognition that this group of contaminants may pose environmental concerns, there is still a lack of information on how plants cope with them.

The presence of pharmaceutical products in different environmental compartments results from different activities, including incorrect disposal of drugs by humans, non-efficient removal of drugs during wastewater treatments, and the use of a wide range of drugs in veterinary and livestock activity (Wu *et al.*, 2015).

Since the presence of organic contaminants, like pharmaceutical products, is growing in different ecosystems it is important to address how plants deal with this exposure. Previous studies reported that the uptake of pharmaceuticals by different plants can occur due to their ubiquitous presence in irrigation water, drinking water and wastewaters (Fram *et al.*, 2011; Gaffney *et al.*, 2015; Kang *et al.*, 2013; Herklotz *et al.*, 2010; Singh *et al.*, 2019; Song *et al.*, 2019).

Depending on species, type of contaminant and growing conditions, plants develop distinct strategies to deal with stress conditions as a way to prevent or slow down the assimilation of contaminants and consequently avoid their excessive accumulation in tissues. ROS occur naturally in cells; however, under adverse conditions the equilibrium is disturbed, causing oxidative stress (Apel *et al.*, 2004; Gapper *et al.*, 2006). As a result of plant-contaminant interaction, a disturbance in ROS content, such as hydrogen peroxide, singlet oxygen, superoxide, and the hydroxyl radical may occur (Noctor *et al.*, 2018).

Most ROS species are produced in mitochondria, chloroplasts, peroxisomes, the plasma membrane and the apoplast (Ahmad *et al.*, 2008; Noctor *et al.*, 2018). Particularly in the apoplast, different enzymes such as oxalate and amine oxidases are sources of hydrogen peroxide. Some enzymes act directly on the production or scavenging of ROS while others constitute a part of more complex systems such as the ones involved in the ascorbate-glutathione cycle. For instance, SOD activity is in the frontline of defence mechanisms since its function is to produce hydrogen peroxide as a result of superoxide radical degradation (Mittler, 2002; Mourato *et al.*, 2012).

CAT function is related with the scavenging of hydrogen peroxide and is mostly present in peroxisomes. Despite its crucial action on ROS and its fast turnover, CAT affinity to the

hydrogen peroxide molecule is lower compared to that of APX (Mhamdi *et al.*, 2010; Mittler, 2002). Peroxidases including, APX, GPOD and GPX are also responsible for the degradation of hydrogen peroxide from cells. GPOD, located in vacuoles and apoplast, uses different phenolic substrates in the scavenging of ROS (Bindschedler *et al.*, 2006; Cosio *et al.*, 2009). The elimination of hydrogen peroxide through GPX activity occurs using reduced glutathione as electron donor, resulting in a molecule of oxidized glutathione. GPX competes directly with CAT since it is responsible for hydrogen peroxide scavenging when low levels of ROS occur in cells. Moreover, GPX and APX increase their activity when CAT activity is inhibited (Mittler, 2002; Noctor *et al.*, 2012). Since GPX interferes with the ascorbate-glutathione pathway, it is important to assess the GR enzyme as well. GR is responsible for the production of GSH resulting from the oxidation of NADPH. The regeneration of GSH is one of the most important factors in plants antioxidant defence mechanisms (Fernández *et al.*, 2013; Mittler, 2002). According to Mhamid *et al.* (2010), the ascorbate-glutathione pathway may increase its activity, contributing to the elimination of ROS excess in plants with lower CAT and GR levels.

ACT, also known as paracetamol, is one of the most widely used drugs in the world (Sharma *et al.*, 2014). It is used in the treatment of fever and pain and has been found in groundwater used for public drinking water in California, USA (Fram *et al.*, 2011). Studies to assess acetaminophen uptake by different cultures have been reported with lupine (*Lupinus luteolus*), barley (*Hordeum vulgare*), reed (*Phragmites australis*) and Indian mustard (*Brassica juncea* L. Czern.) (Huber *et al.*, 2009; Kotyza *et al.*, 2010).

In this work, we studied the effect of acetaminophen on *Lactuca sativa* L. plants. The choice of this pharmaceutical product was based on its significant consumption in Portugal according with reports from INFARMED, (2020). We intended to address further issues related to lettuce metabolism to understand how plants cope with low concentration and short-term exposure to pharmaceuticals. A full characterization of the enzymatic antioxidant system was thus carried out in lettuce leaves and roots, in order to identify the main defence mechanisms used by lettuce against the ACT-induced stress.

4.2. Material and Methods

Seeds of the cultivar *Lactuca sativa* L. “Maravilha das Quatro Estações” were germinated in substrate (Bio Siro – peat, humus and fertilizer). During the first 15 days the seedlings were maintained in germination substrate until the development of the first leaf. Afterwards, young lettuce plants were transferred to hydroponic culture with Hoagland solution and developed in this medium for 21 days. On the 36th day of growth, lettuce plants were contaminated with different concentrations of ACT: 0 (control), 0.1, 1 and 5 mg L⁻¹. These concentrations were based on previous studies from the literature (Dordio *et al.*, 2011; Herklotz *et al.*, 2010; Huber *et al.*, 2009) and known to be capable of inducing oxidative stress in different plants. Three pots were used for each concentration, with 12 plants each, for a total of 36 plants per dose.

Growing conditions and more detailed experimental data are described in point 2.1. Lettuce plants production and plants contamination conditions, from Chapter II Material and Methods.

The following procedures were performed, all described in Chapter II:

- Harvesting of plant material (2.2.)
- Determination of mineral content (2.3.1.)
- Determination of acetaminophen in roots and leaves (2.3.2.2.)
- Determination of Photosynthetic parameters (2.3.3.)
- Determination of Lipid Peroxidation (2.3.4.1.)
- Determination of Hydrogen Peroxide content (2.3.4.2.)
- Determination of Antioxidant activity (2.3.4.3.)
- Determination of Phenolic Compounds (2.3.4.4.)
- Determination of Ascorbate (2.3.4.5.)
- Determination of Glutathione (2.3.4.6.)
- Determination of Anthocyanins (2.3.4.7.)
- Determination of Enzymatic Activities (2.3.5.)

Data analysis is described in section 2.5, from Chapter II.

4.3. Results and discussion

4.3.1. Acetaminophen uptake by plants

Acetaminophen is a drug widely consumed all over the world and its consumption in Portugal reached 21% of the total of packaging sold between January and June 2020, on non-prescription drugs classification (INFARMED, 2020). Different studies have detected acetaminophen in rivers and groundwaters (Fram *et al.*, 2011; Gros *et al.*, 2010). Several studies pointed for the uptake of acetaminophen by different plants, for instance Kotyza *et al.* (2010) showed a total removal of acetaminophen from growth medium by *Lupinus* species. The same study showed different results in terms of toxicity varying from no toxicity to low toxicity levels (visual effects) in different plants. Huber *et al.* (2009), confirmed not only the uptake of acetaminophen by *Armoracia rusticana* L. but also the presence of three metabolites, suggesting a similarity among plants and mammals detoxification systems.

In this study the effect of acetaminophen in lettuce was evaluated and its presence was found in roots and leaves (Figure 4.1).

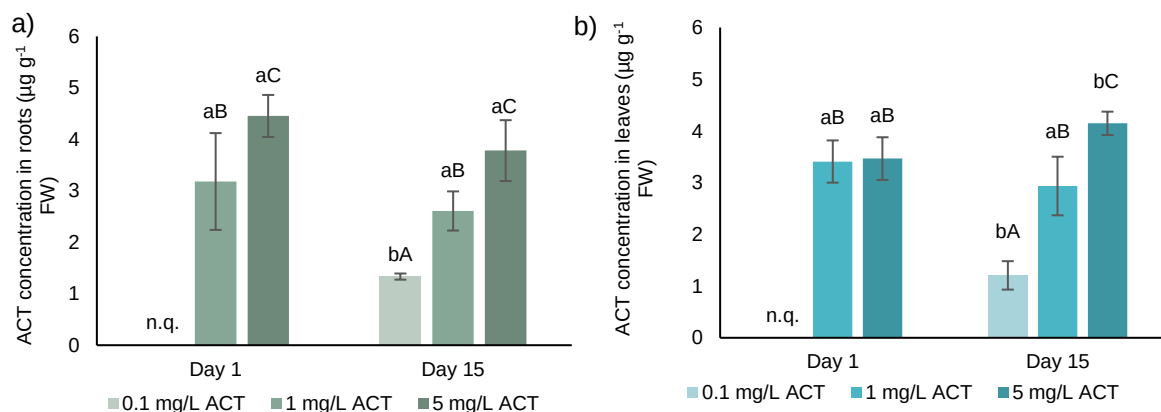


Figure 4.1. - Accumulation of ACT ($\mu\text{g g}^{-1}$) in roots (a) and leaves (b) of *Lactuca sativa* on days 1, 8 and 15 of exposure.

Acetaminophen was detected in roots and leaves exposed to 0.1 mg L^{-1} after one day of exposure as can be seen in Figure 4.2, however its concentration was below the limit of quantification ($0.0013 \mu\text{g g}^{-1}$). On day 15, roots presented a concentration of $1.33 \pm 0.06 \mu\text{g g}^{-1}$ and leaves $1.21 \pm 0.28 \mu\text{g g}^{-1}$ under 0.1 mg L^{-1} ACT. On day 1, in case of 1 mg L^{-1} concentration, ACT was detected in roots and leaves, $3.18 \pm 0.94 \mu\text{g g}^{-1}$ and $3.41 \pm 0.41 \mu\text{g g}^{-1}$, respectively. For the higher concentration of 5 mg L^{-1} ACT, the values of $4.45 \pm 0.41 \mu\text{g g}^{-1}$ and

3.47±0.41 µg g⁻¹ were found in roots and leaves, respectively. Higher levels of accumulation of ACT occurs for longer days of exposure, in all tested conditions. Figures 4.2 to 4.5 show the chromatograms obtained in roots and leaves under 0.1, 1 and 5 mg L⁻¹ of ACT for 1 and 15 days of exposure.

As can be seen in Table 4.1 by the translocation factor, ACT was translocated to the upper plant parts (TF values are all near 1), showing that lettuce can absorb ACT and easily translocate it to the leaves as suggested by Chuang *et al.*, (2019). Values of TF vary among days, yet it is possible to observe that for the two higher concentrations of ACT (1 and 5 mg L⁻¹) on day 15, ACT reached leaves which may suggest a possible tolerance of lettuce to this contaminant. Besides, this may indicate that even for longer periods of exposure translocation pathways were not affected by ACT.

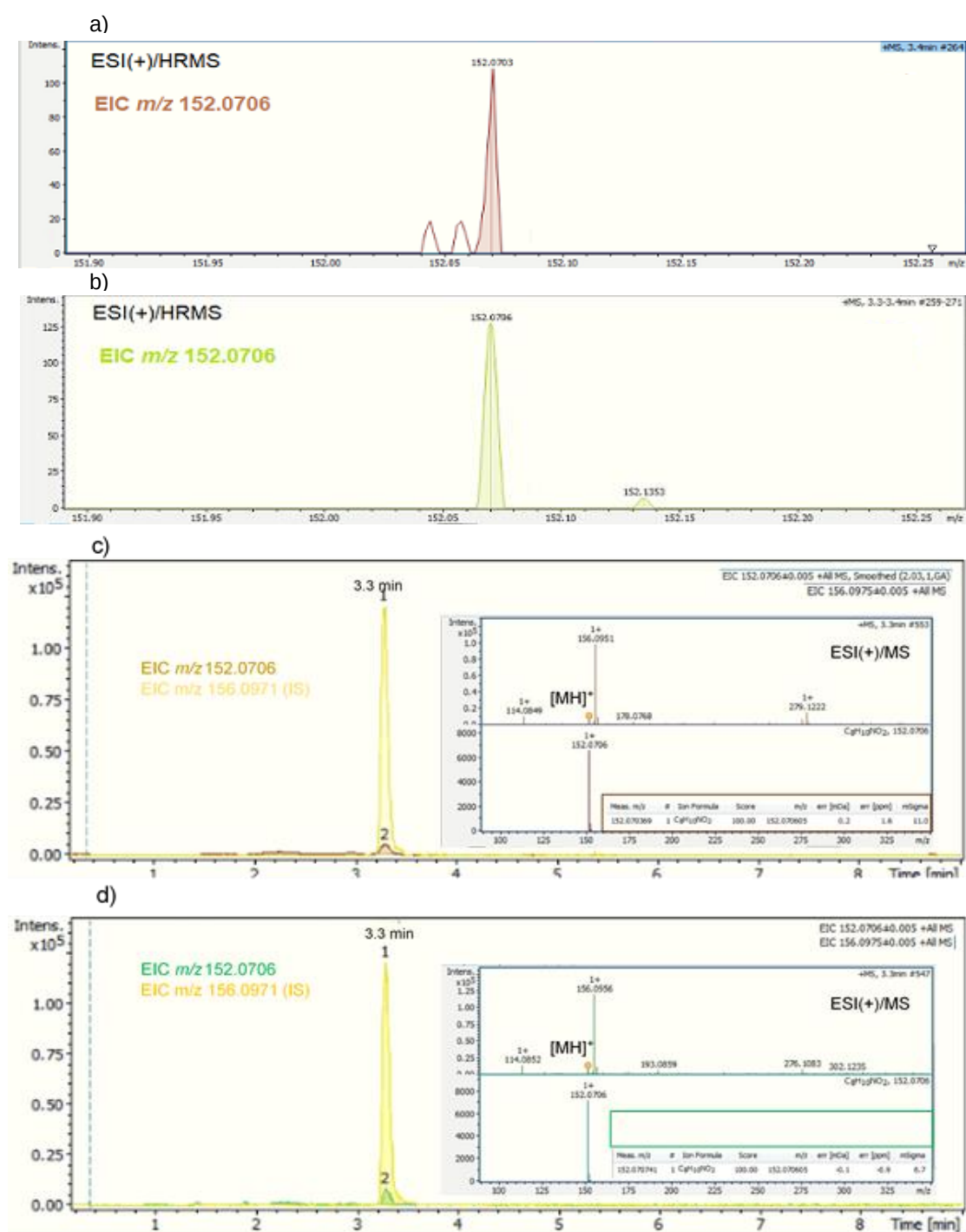


Figure 4.2.- Typical extracted ion chromatograms obtained in the ESI positive mode for the protonated molecules of ACT (m/z 152.0706) identified: (a) and (c) in roots; (b) and (d) in leaves, of the extracts of lettuce growing with 0.1 mg L^{-1} ACT, after 1 day (a, b) and 15 days (c, d) of exposure, respectively. Peak 1 is due to the protonated molecule m/z 156.0975 of the internal standard ACT-D4.

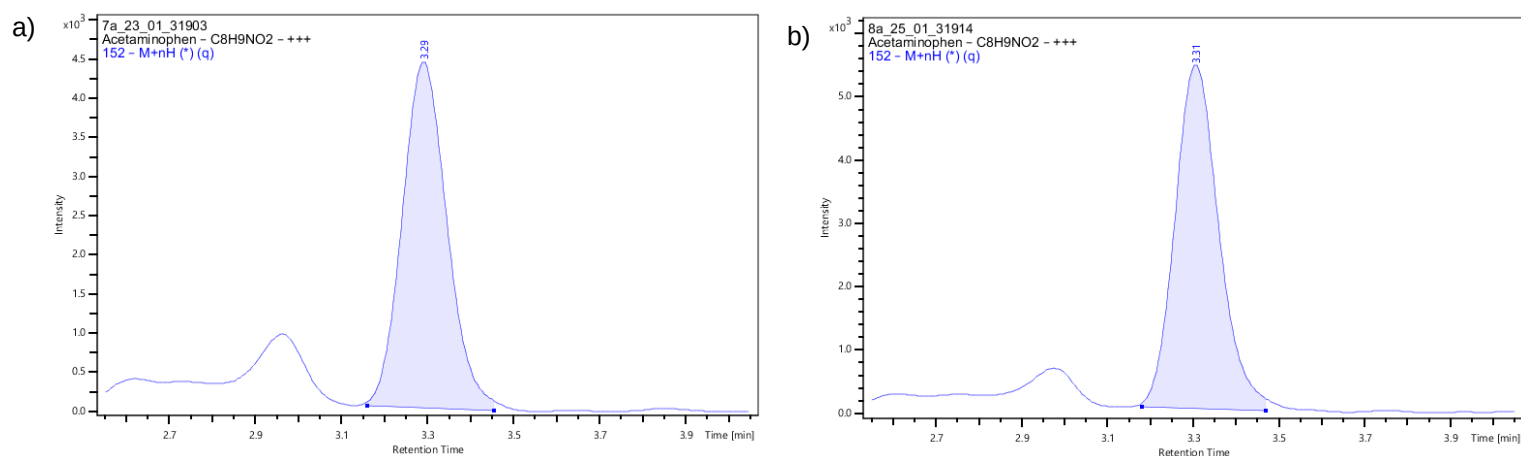


Figure 4.3. – Extracted ion chromatograms representing acetaminophen peak [152 – M +nH(*)q – blue] used for quantitation of ACT uptake by roots (a) and leaves (b) from *Lactuca sativa* contaminated with 0.1 mg L^{-1} on day 15 of exposure.

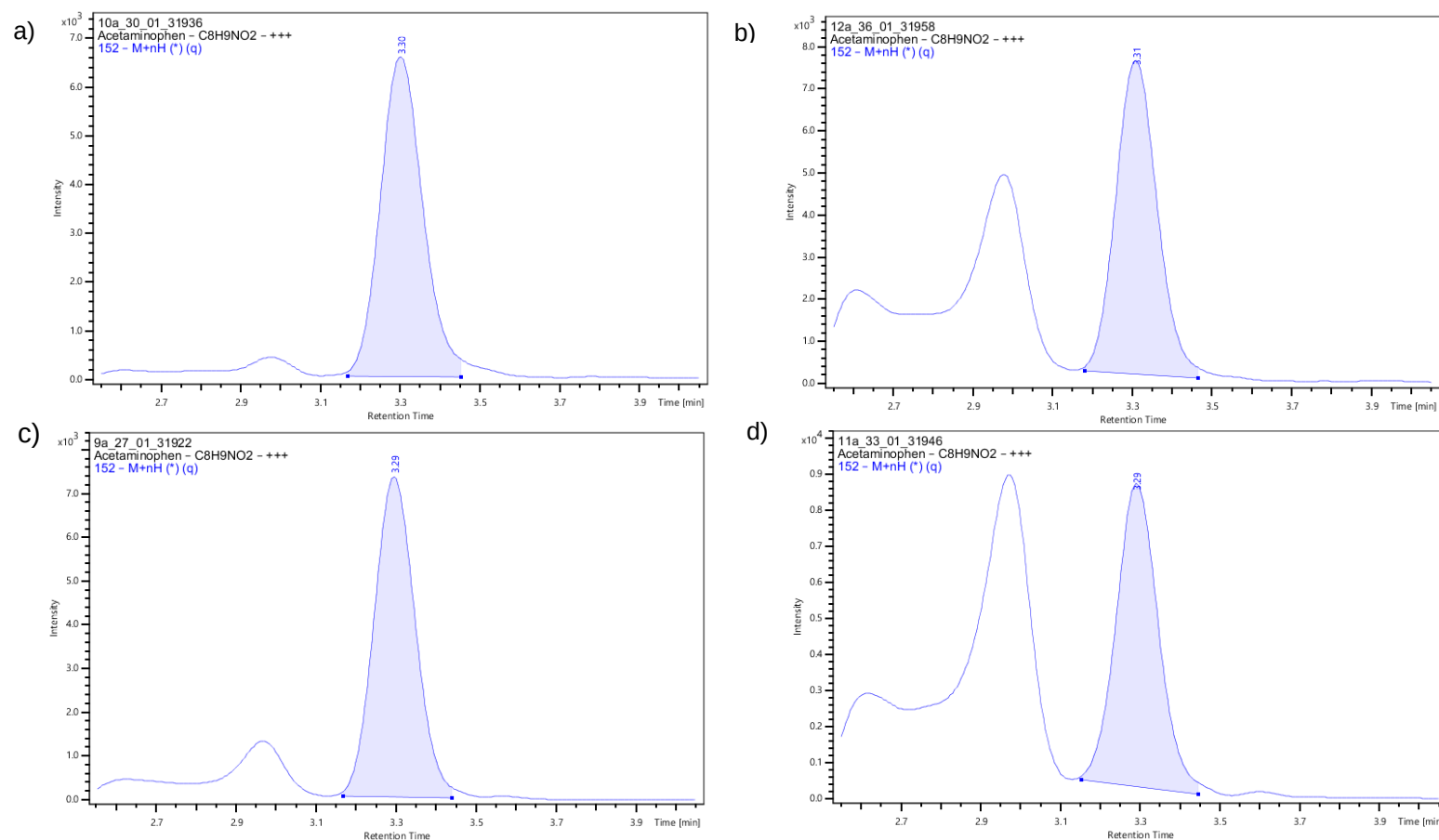


Figure 4.4. - Extracted ion chromatograms representing acetaminophen peak [152 – M + nH(*)q – blue] used for quantitation of ACT uptake by leaves (top) and roots (down) from *Lactuca sativa* contaminated with 1 mg L⁻¹ on days 1(a,c) and 15 (b,d) of exposure.

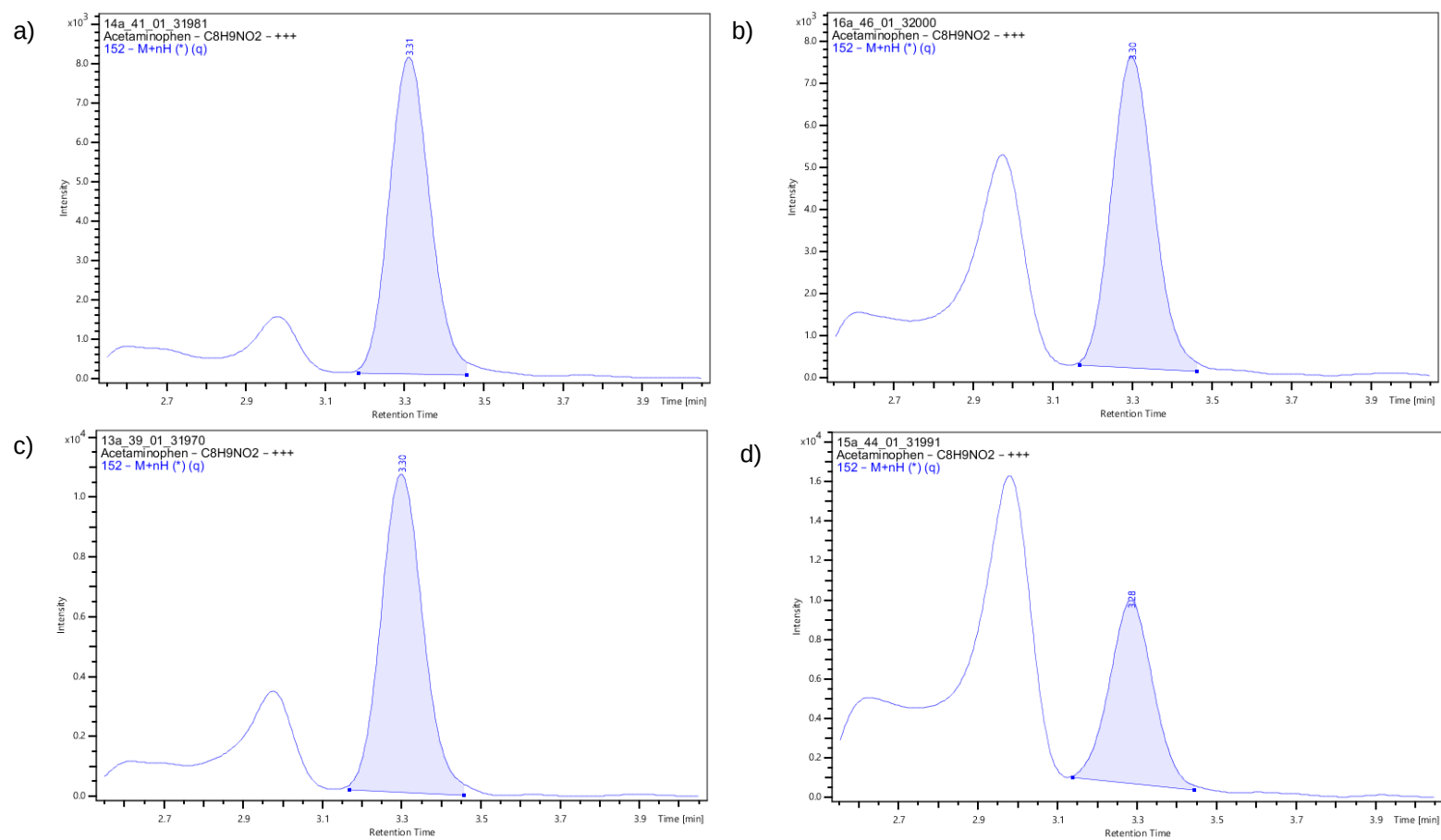


Figure 4.5. - Extracted ion chromatograms representing acetaminophen peak [152 – M +nH(*)q – blue] used for quantitation of ACT uptake by leaves (top) and roots (down) from *Lactuca sativa* contaminated with 5 mg L⁻¹ on days 1(a,c) and 15 (b,d) of exposure.

Table 4.1. - Bioaccumulation factor (BCF) and translocation factor (TF) of ACT roots and leaves on days 1 and 15 of exposure.

	Day 1			Day 15		
	BCF _{Roots}	BCF _{Leaves}	TF	BCF _{Roots}	BCF _{Leaves}	TF
0.1 mg L ⁻¹ ACT	-	-	-	0.0133	0.0121	0.9048
1 mg L ⁻¹ ACT	0.0032	0.0034	1.0716	0.0026	0.0029	1.1253
5 mg L ⁻¹ ACT	0.0009	0.0007	0.7781	0.0008	0.0008	1.0968

4.3.2. Biomass and chlorophyll parameters

The root and leaf mass were measured on each harvest day for the three ACT concentrations under study but despite the wide range of concentrations, from 0.1 mg L⁻¹ to 5 mg L⁻¹, no differences in these masses were detected in relation to control plants (Figure 4.6). Also no visible damage was detected as all plants looked healthy even at the highest concentration of ACT (Figure 4.7).

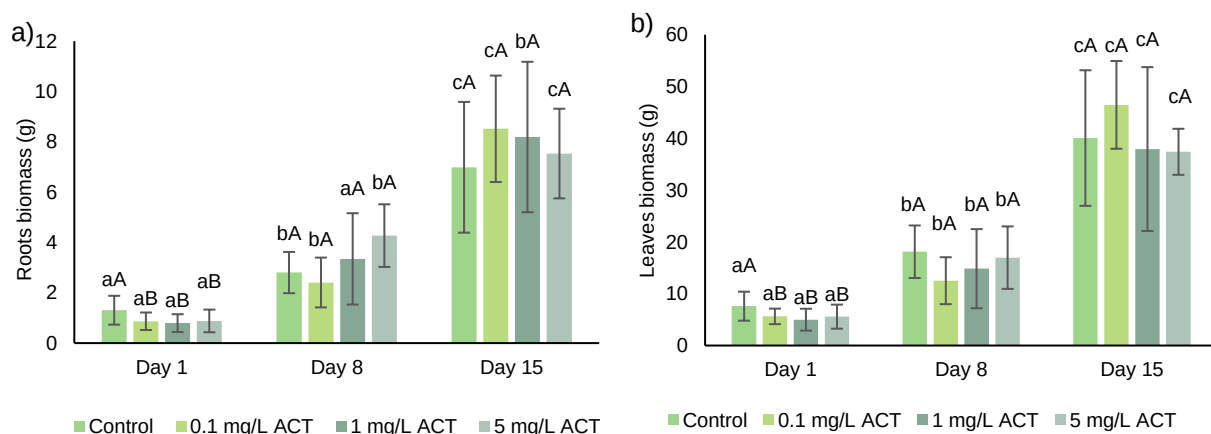


Figure 4.6. - Root (a) and leaf (b) biomass of *Lactuca sativa* under contamination with ACT for 15 days. Different uppercase letters represent significant differences between different ACT concentrations at the same time point and different lowercase letters represent significant differences between treatment duration for the same ACT concentration.

Biomass parameters were already documented by Favaro *et al.* (2011) where no differences in fresh weight were found after the exposure of lettuce to organic contaminants (biopesticides and deltamethrin). It is thus shown that, in this range of concentrations, the effect of ACT in lettuce plants is not severe enough to affect plant growth and that lettuce has efficient defence mechanisms. Hence, it is important to clarify the metabolic effects and the main defence mechanisms activated by lettuce plants under ACT stress.

In order to understand if ACT uptake could affect the photosynthetic system, total chlorophyll content was measured (Figure 4.8). It can be seen that no differences were found between control and ACT contaminated plants.

Alkimini *et al.* (2019), described some differences in chlorophyll b content for *Lemna minor* plants under acetaminophen contamination, even in lower concentrations (5 and 25 $\mu\text{g L}^{-1}$) but they reported no differences in total chlorophyll content. Different results were reported by Huiqing *et al.* (2014), revealing an increasing pattern in lettuce plants exposed to 1.5 mg L^{-1} bisphenol A (an organic contaminant) for 30 days.

Figure 4.9 shows results acquired for NDVI and PRI parameters. Results of NDVI and PRI were measured *in situ*. NDVI is related with plant photosynthetic capacity and photosynthetic rates. For each ACT concentration this parameter remained similar to control plants, which confirms no changes in photosynthetic activity. PRI measurements show significant differences for longer periods of exposure, both in time (8 and 15 days) and concentrations. PRI was originally developed to correlate changes on xanthophyll cycle pigments (Gamon *et al.*, 1995). It stands as an indicator, not only of carotenoids/chlorophyll ratio, but also for anthocyanins presence, since it compares the reflectance in the red and blue regions of the spectrum (Sims *et al.*, 2002). The PRI values indicates that ACT contamination interferes with the pigment content and distribution, and can reflect the variations in chlorophyll, carotenoids and anthocyanins contents.

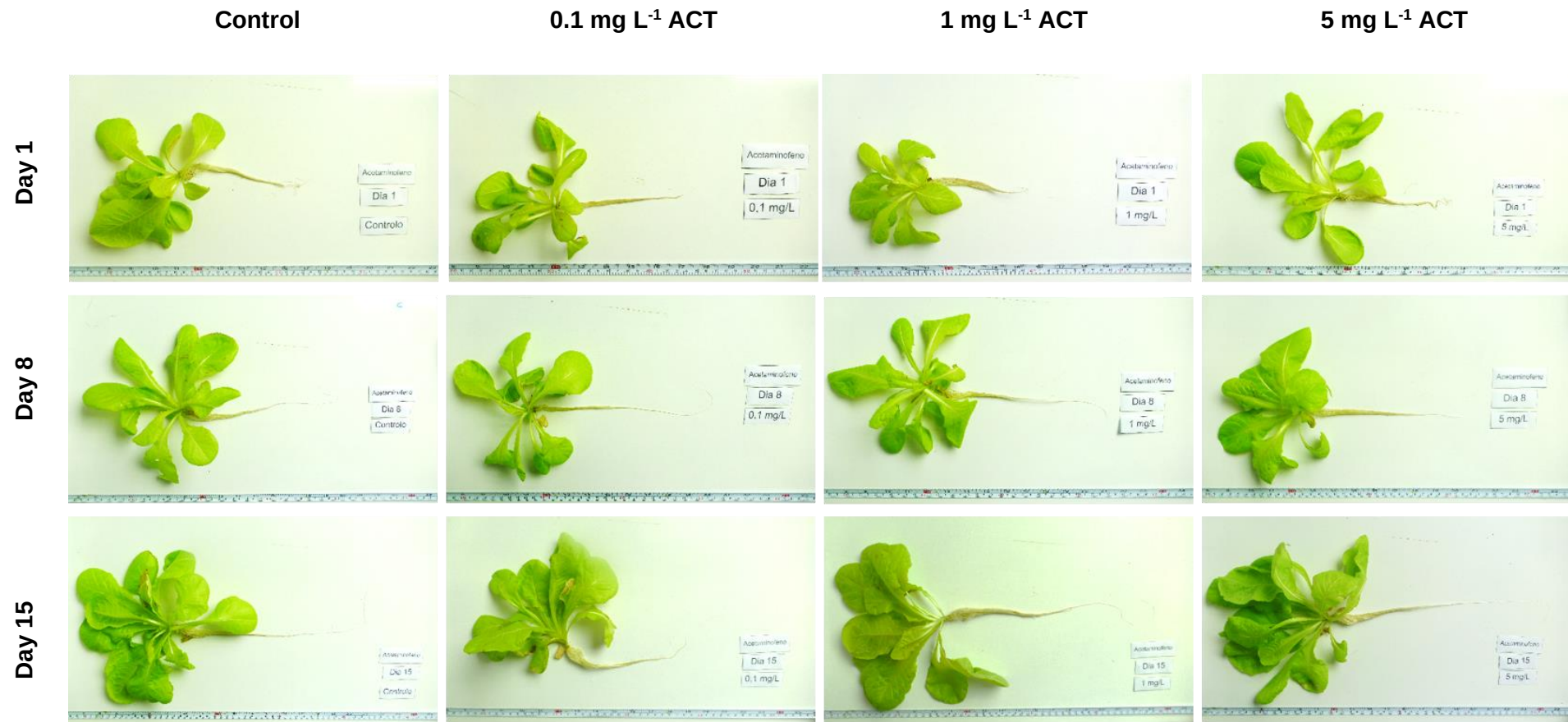


Figure 4.7. - Lettuce plants on days 1, 8 and 15 of contamination with different concentrations of ACT - Control, 0.1 mg L⁻¹, 1 mg L⁻¹ and 5 mg L⁻¹.

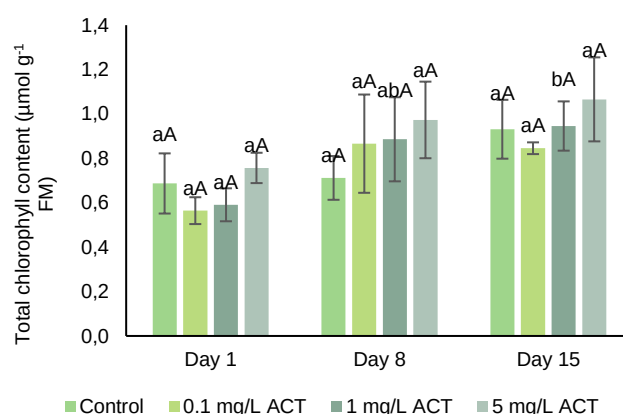


Figure 4.8. - Chlorophyll content of *Lactuca sativa* leaves under contamination with ACT for up to 15 days. Different uppercase letters represent significant differences between different ACT concentrations at the same time point and different lowercase letters represent significant differences between treatment duration for the same ACT concentration.

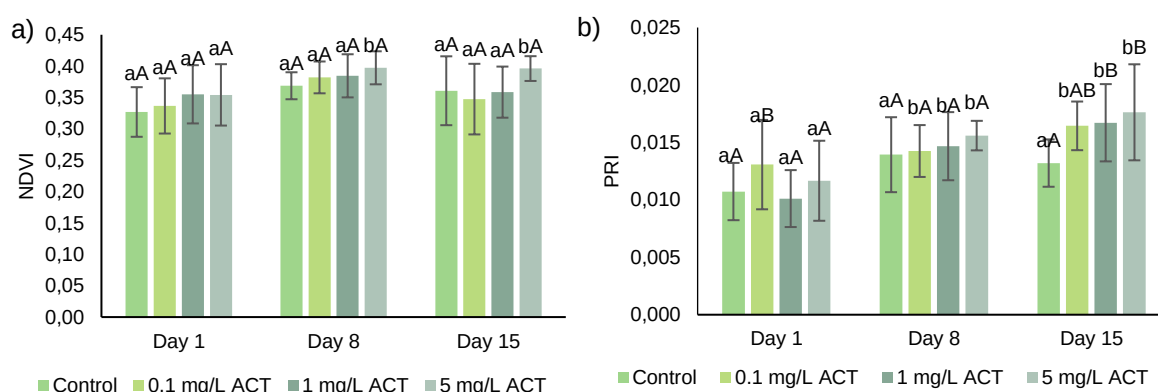


Figure 4.9. - NDVI (a) and PRI (b) measurements of *Lactuca sativa* leaves under contamination with ACT for up to 15 days. Different uppercase letters represent significant differences between different ACT concentrations at the same time point and different lowercase letters represent significant differences between treatment duration for the same ACT concentration.

4.3.3. Stress indicators analysis

In order to evaluate the occurrence of oxidative stress induced by ACT, MDA and H₂O₂ levels were measured. With respect to MDA content, significant differences were found in roots but not in leaves (Figure 4.10). On days 8 and 15, roots showed higher contents of MDA when contaminated with 5 mg L⁻¹ ACT.

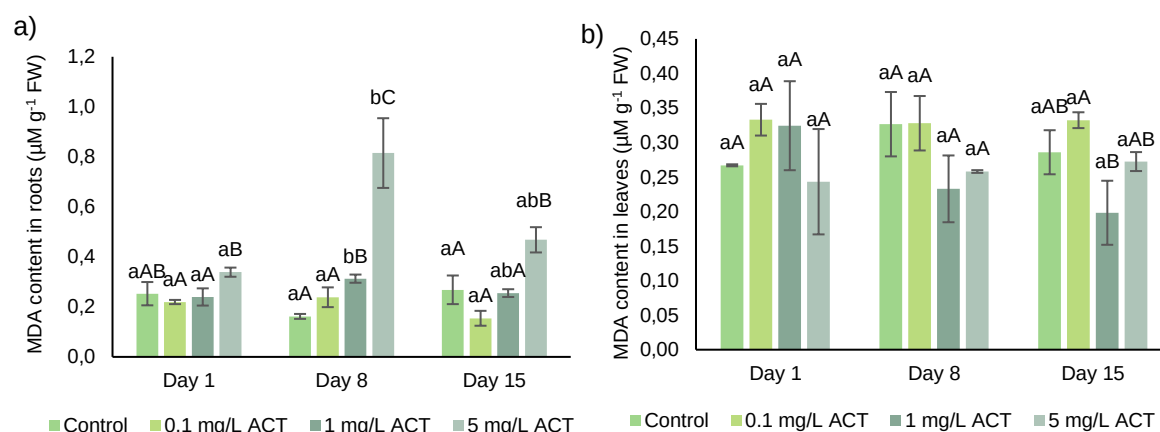


Figure 4.10. - MDA content ($\mu\text{M g}^{-1}\text{ FW}$) in roots (a) and leaves (b) of *Lactuca sativa* under contamination with MTF for up to 15 days. Different uppercase letters represent significant differences between different ACT concentrations at the same time point and different lowercase letters represent significant differences between treatment duration for the same ACT concentration.

Since MDA is considered an indicator of lipid peroxidation, modifications will occur mainly on cell membranes. As roots are in direct contact with the contaminated solutions, the lipid peroxidation effects are more noticeable in this part of the plant comparing to the leaves (although ACT was also detected in leaves as seen in Figure 4.1). According to Dias *et al.* (2014) lettuce leaves exposed to mancozeb, a fungicide, showed differences in lipid peroxidation. However, in terms of roots mass and visible effects, no differences were found in comparison to control roots. The results obtained for MDA can be an indicator that other mechanisms are responsible for the cell membrane structure and stability in roots.

The presence of ACT has also affected H_2O_2 content (Figure 4.11) and this was more evident in leaves compared to roots. It is possible to observe, in leaves, an increase in H_2O_2 content for the highest ACT concentration on the 1st day of exposure, for the concentrations of 0.1 and 1 mg L^{-1} ACT on day 8 and for 1 and 5 mg L^{-1} ACT on day 15. These results confirm that ACT induces the increase of ROS levels (as measured by H_2O_2 contents). In roots, the differences occur mainly at the 8th day of contamination and not later, indicating that the plant developed an efficient antioxidative mechanisms for a long-term response. In leaves, it seems that there was a delayed response as the increase in H_2O_2 was only detected for the longer times of exposure.

To clarify the involved defence mechanisms, a full antioxidative enzyme analysis was performed.

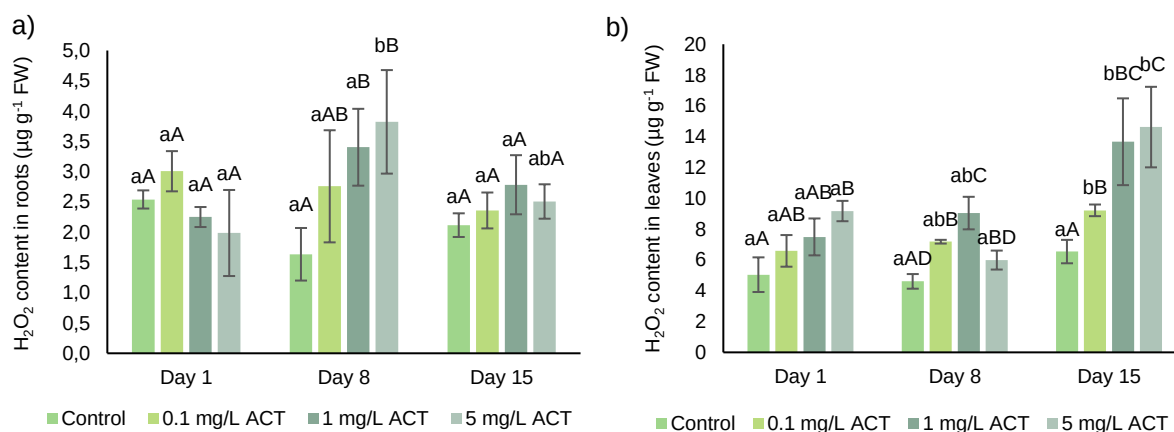


Figure 4.11. - H₂O₂ content (µg g⁻¹ FW) in roots (a) and leaves (b) of *Lactuca sativa* under contamination with ACT for up to 15 days. Different uppercase letters represent significant differences between different ACT concentrations at the same time point and different lowercase letters represent significant differences between treatment duration for the same MTF concentration.

4.3.4. Enzymatic activities and metabolites related with stress response

The increase in H₂O₂ content reflects the increase of ROS and the evidence of oxidative conditions that can cause oxidative damage. This may also be linked to the increase of SOD activity in leaves (as shown in Figure 4.12) which shows significant differences for the same days and concentrations of ACT. The increase in SOD activity is also an evidence of oxidative stress and intracellular increase of ROS.

SOD increase is a frequently triggered response to abiotic stress (Dordio *et al.*, 2011). In lettuce plants from the present work, it was found that SOD activity is not so pronounced in roots as in leaves, suggesting that the presence of O₂^{•-} is not so prominent in the roots. In fact, in roots SOD does not seem to be induced by oxidative stress as its activity did not increase with ACT, compared to control. On the other hand, the increase in SOD activity in leaves is correlated with ACT (with both time and concentration) and it might be a reflection of the increase of O₂^{•-} content. O₂^{•-} is a common ROS in non-stressed cells, but its presence may increase under biotic or abiotic stresses. Since SOD is present in different organelles, the content of H₂O₂ found in leaves might be a result of SOD activation in different parts of the cells such as chloroplast and mitochondria (Asada, 2006; Møller, 2001).

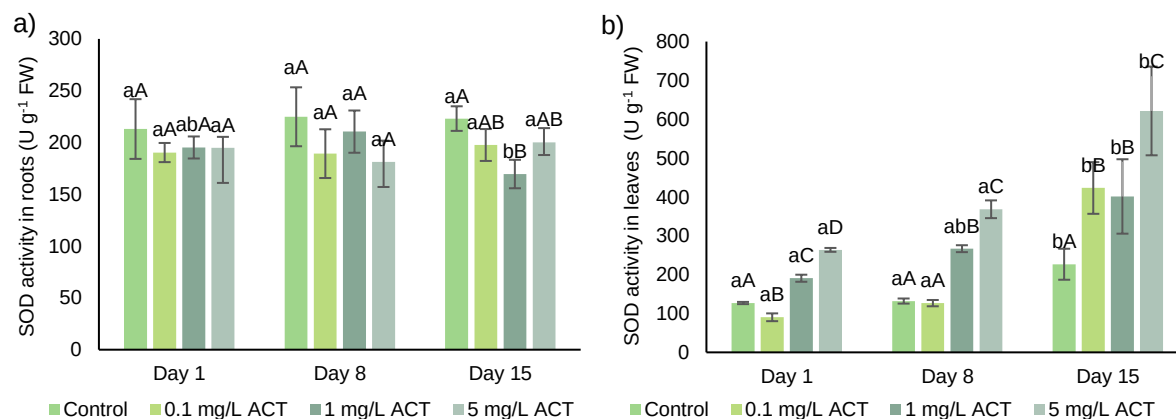


Figure 4.12. - Enzymatic activity (U g⁻¹ FW) of SOD in roots (a) and leaves (b) of *Lactuca sativa* under contamination with ACT for up to 15 days. Different uppercase letters represent significant differences between different ACT concentrations at the same time point and different lowercase letters represent significant differences between treatment duration for the same ACT concentration.

Depending on which organelle H₂O₂ production occurs, its scavenging may be performed by APX (chloroplast) or by GPX and CAT (mitochondria) (Foyer *et al.*, 2003; Møller, 2001). CAT and GPOD activity results are present in Figure 4.13. In the present work catalase activity shows significant differences compared to control plants. For instance, an increase on days 8 and 15 for 5 mg L⁻¹ ACT is found both in leaves and roots. In case of leaves, this result can be justified by the high content of H₂O₂ mostly related to mitochondria and chloroplasts. Otherwise, it is possible to observe that H₂O₂ content in roots, for day 15, was lower when compared to day 8 and that should be related to mitochondria. So, the increase in CAT activity may be the pathway of H₂O₂ reduction in roots, for the longer period of exposure, showing a good antioxidant response to ROS in root cells. For the same tissue, an increase in GPOD activity was also observed.

An interesting correlation among CAT, SOD and H₂O₂ contents in leaves may be observed on Figure 4.14. A good correlation between SOD and H₂O₂ contents can be observed together with a clear differentiation of most contaminated leaves. The same trend is possible to observe between CAT and H₂O₂. Therefore, while high activity of SOD lead to higher content of H₂O₂, high content of H₂O₂ increase CAT activity.

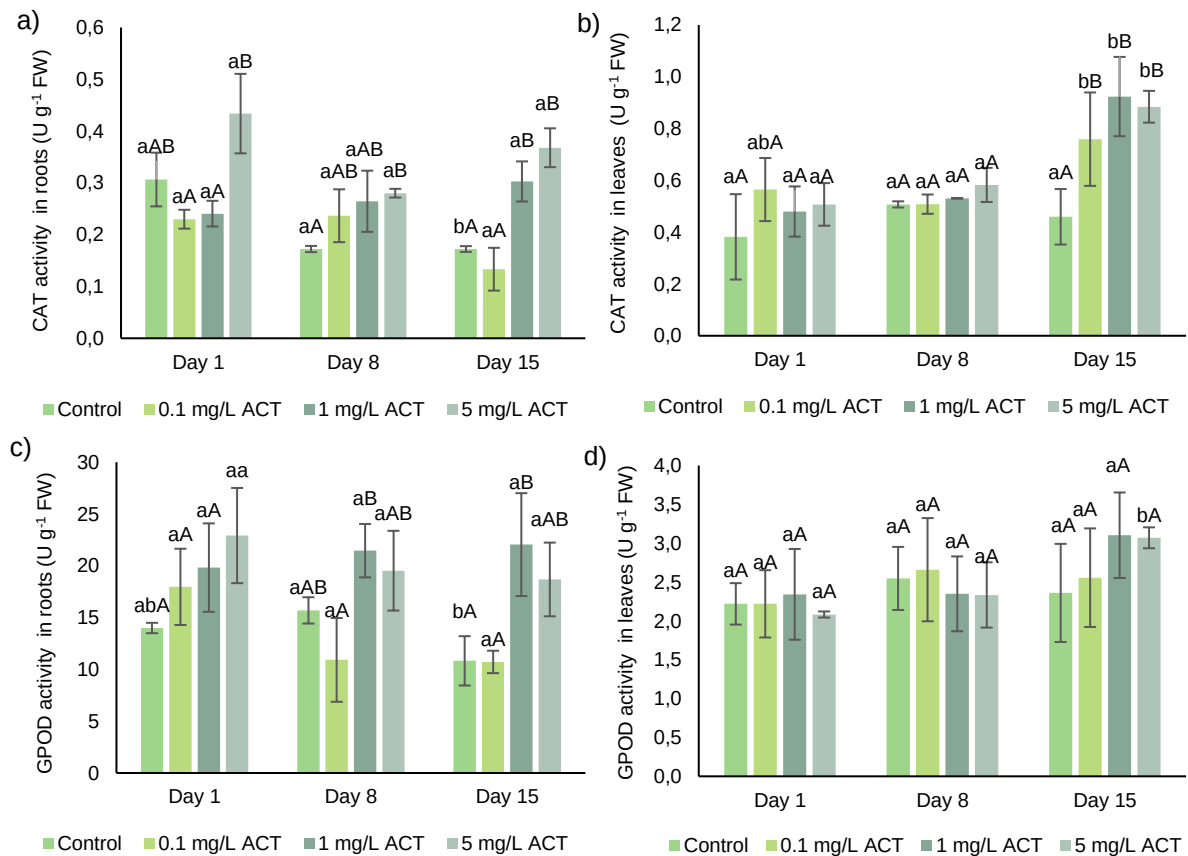


Figure 4.13. - Enzymatic activity (U g⁻¹ FW) of CAT and GPOD in roots (a, c) and leaves (b, d) of *Lactuca sativa* under contamination with ACT for up to 15 days. Different uppercase letters represent significant differences between different ACT concentrations at the same time point and different lowercase letters represent significant differences between treatment duration for the same ACT concentration.

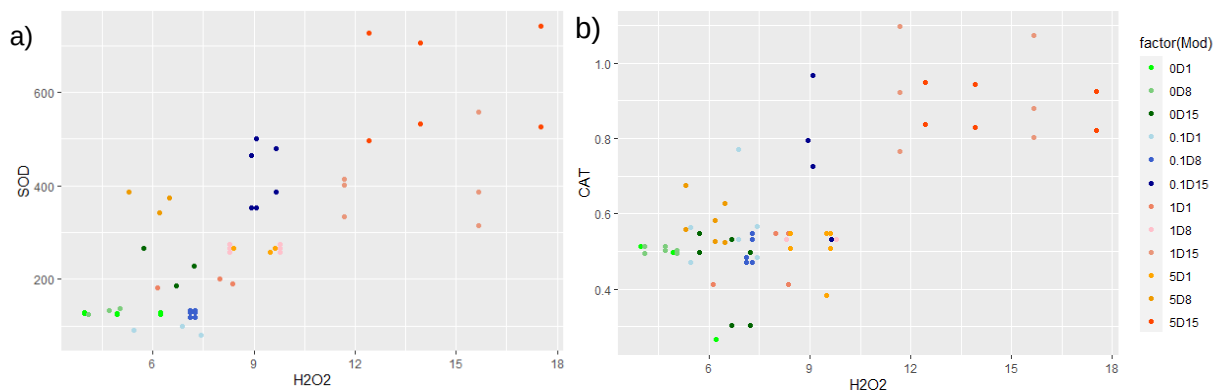


Figure 4.14. - Correlation analysis between SOD and H₂O₂ (a) and CAT and H₂O₂ (b) in leaves of *Lactuca sativa* under contamination with ACT for up to 15 days.

Figure 4.15 shows the relation between these two enzymes. Higher activity of SOD led to higher activity of CAT playing a role in cell equilibrium in leaves through ROS scavenging processes.

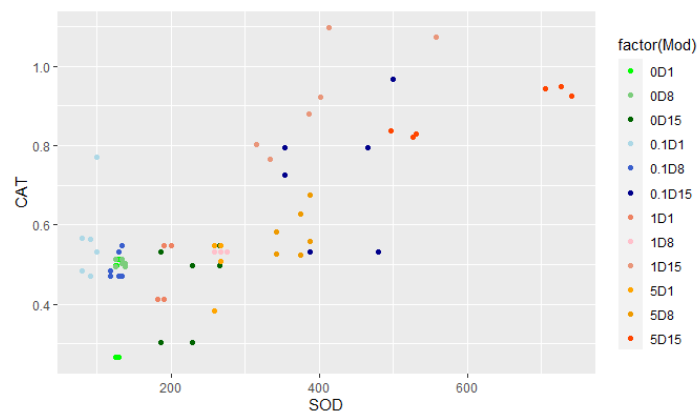


Figure 4.15. - Correlation analysis between CAT and SOD in leaves of *Lactuca sativa* under contamination with ACT for up to 15 days.

In roots, the correlation between GPOD and CAT showed an interesting trend (Figure 4.16). These enzymes seem to work together in H_2O_2 scavenging with proportional activity ratios.

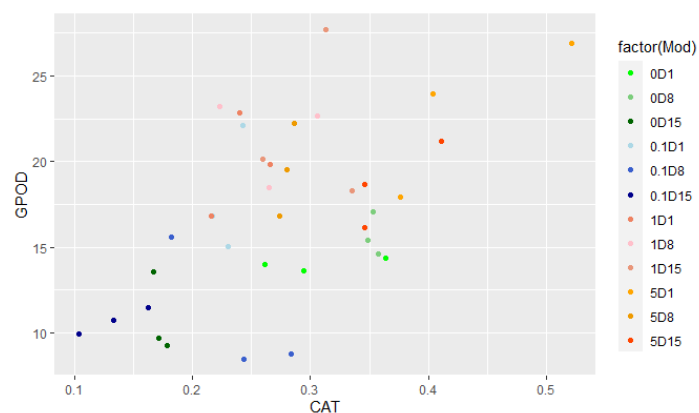


Figure 4.16. - Correlation analysis between GPOD and CAT in roots of *Lactuca sativa* under contamination with ACT for up to 15 days.

In fact, both CAT and GPOD act on H_2O_2 removal using different substrates. In case of GPOD, an aromatic compound is used as electron donor to trigger the reaction of H_2O_2 elimination (Van Doorn *et al.*, 2014). GPOD activity is also related with cell wall properties since this enzyme is linked to cell wall lignification, so the increase in GPOD activity may confer a better stability to root cells (Cori *et al.*, 2013; Lewis *et al.*, 1990). Indeed, as no visible damages were observed in leaves and roots during the exposure to ACT this may indicate an efficient cell

lignification, a result that can be linked to GPOD activity. This result will then justify why lipid peroxidation did not cause visible damage in roots.

As stated before, APX also takes part in H_2O_2 scavenging. During the exposure of lettuce to ACT, a significant increase was found for APX, on day 8 for roots. This result points to a possible response to oxidative stress. A similar behaviour was described by Bartha *et al.* (2010) in *Brassica juncea* plants. However, it is important to highlight that APX activity was not as clear as observed in CAT and GPOD (Figure 4.16).

The results for ascorbate content are shown in Figure 4.17.

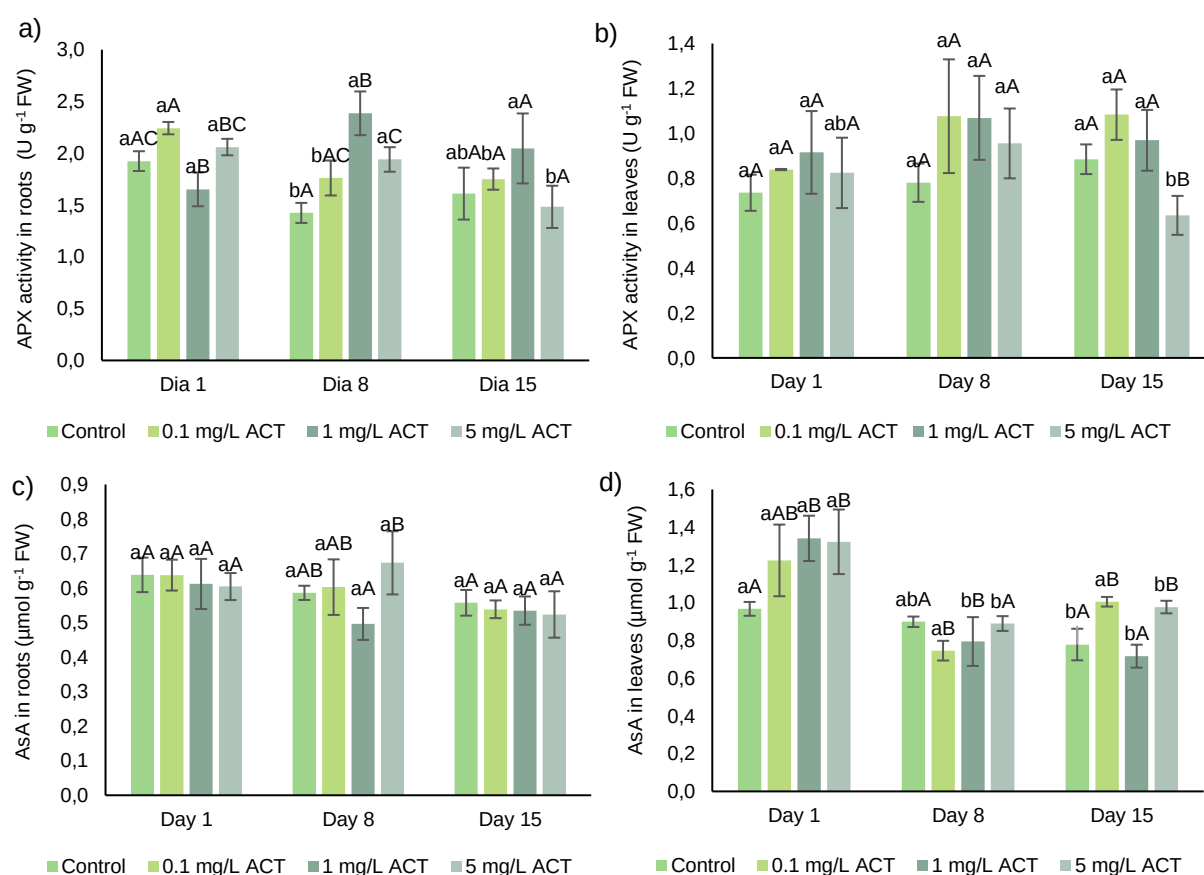


Figure 4.17. – Enzymatic activity (U g⁻¹ FW) of APX in roots (a) and leaves (b) and Ascorbate content (μmol g⁻¹ FW) in roots (c) and leaves (d) of *Lactuca sativa* under contamination with ACT for up to 15 days. Different uppercase letters represent significant differences between different ACT concentrations at the same time point and different lowercase letters represent significant differences between treatment duration for the same ACT concentration.

In detail, for day 1, a significant difference was found in AsA content in leaves. For roots, a decrease, not significant, of AsA content is observed for day 8 at 1 mg L⁻¹ ACT agreeing with an intensification in APX activity for the same concentration on day 8. Ascorbate is involved in

several reactions and is considered one of the most significant species controlling ROS contents in cells together with GSH (Noctor *et al.*, 2015). APX is known to play an important role in terms of H_2O_2 removal and, as mentioned before, APX is activated when H_2O_2 contents are lower, while other enzymes such as CAT will scavenge H_2O_2 when its concentrations are higher (Mittler, 2002). Nevertheless, in this work, APX activity and AsA content did not display as much differences as expected. This observation may be justified by the fact that AsA pools are less affected than GSH contents in some plants (Vanderauwera *et al.*, 2011).

GSH content in leaves found some oscillations between days and concentrations (Figure 4.18), but they always remained at the same levels or lower than control plants.

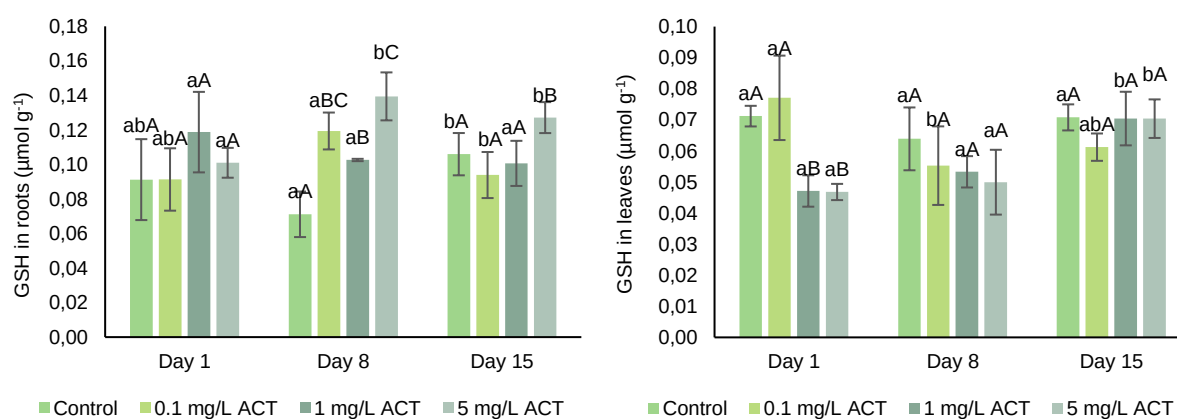


Figure 4.18. - GSH content ($\mu\text{mol g}^{-1}$ FW) in roots (a) and leaves (b) of *Lactuca sativa* under contamination with ACT for up to 15 days. Different uppercase letters represent significant differences between different ACT concentrations at the same time point and different lowercase letters represent significant differences between treatment duration for the same ACT concentration.

According to Noctor *et al.* (2015), GSH status is considered a good indicator of the cellular redox state, in particular when the contents of ROS, such as H_2O_2 increase. The ascorbate-glutathione cycle is one of the most important pathways to regulate the redox state of cells, and the link between these two metabolites is done by the enzymes APX and GR. GR activity results (Figure 4.19) revealed a relation with the differences in GSH content, meaning that when GR activity decrease it is possible to observe a decrease on GSH levels.

This behaviour is mostly observed in leaves, as the activity of GR was lower in contaminated leaves when compared to control. Despite different types of contamination, a similar behaviour (in lettuce, under arsenate and arsenite exposure has been documented by Gusman *et al.* (2013). In fact, GSH may be triggered by different pathways, so depending on the type of stress, the accumulation of this metabolite may occur in oxidative stress conditions (Gómez *et*

et al., 2004). The lower contents of GSH may be explained by the lower activity of GR during ACT exposure or by its use as substrate of GPX activity in ROS scavenging processes. Relating GR and GSH results with APX and AsA, the APX activity increased (not significantly) on day 8 for 1 and 5 mg L⁻¹ in leaves, reducing the ascorbate content and producing more dehydroascorbate (DHA) which will serve as substrate for the reaction mediated by dehydroascorbate reductase (DHAR). At the same time DHAR mediates the oxidation of GSH to GSSG which will be the substrate for GR (Do *et al.*, 2016). In leaves, this conversion is limited by the low GR activity, but otherwise in roots the GSH recycling showed a better performance. In roots, the GSH contents increased with ACT contamination, over time and concentration, as well as GR activity, revealing dissimilar behaviour between roots and leaves cells.

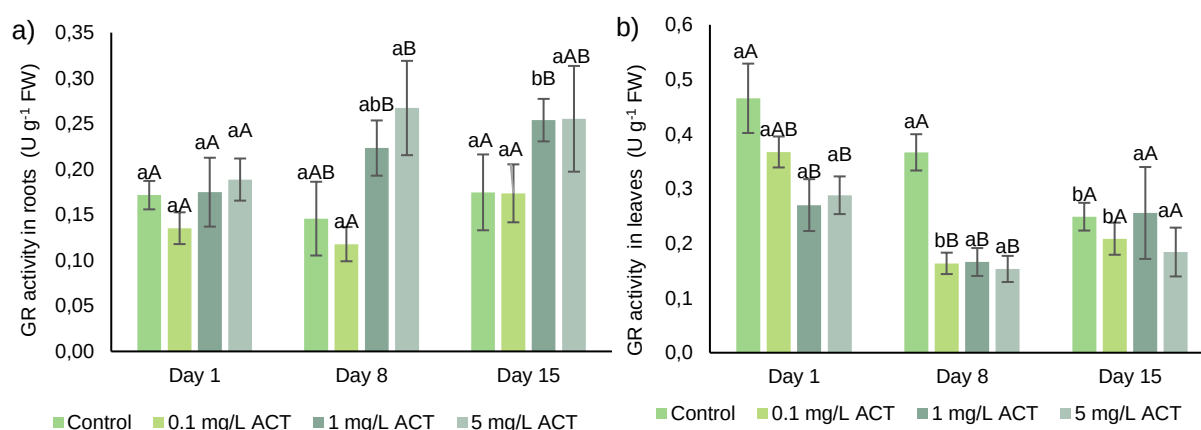


Figure 4.19. - Enzymatic activity (U g⁻¹ FW) of GR in roots (a) and leaves (b) of *Lactuca sativa* under contamination with ACT for up to 15 days. Different uppercase letters represent significant differences between different ACT concentrations at the same time point and different lowercase letters represent significant differences between treatment duration for the same ACT concentration.

Glutathione peroxidase (GPX) plays a role in GSH/GSSG conversion as well. The results (Figures 4.20) for its activity show more significant differences for the days 1 and 8 in leaves and only on day 1 in roots.

The activity of GPX is related with H₂O₂ scavenging (Passaia *et al.*, 2015), nevertheless in lettuce plants the link between H₂O₂ content and GPX activity is not so evident. This may indicate that GPX is not so involved in ROS removal, probably because GSH is involved in other oxidative defence related reactions. It is possible to observe that for day 15, GPX activity remained similar to the control, in both plant parts. However, the concentration of H₂O₂ in leaves increased for this period, leading to the conclusion that GPX does not play a relevant role in H₂O₂ scavenging, while CAT and GPOD are the enzymes that most contributed for H₂O₂

removal. As ROS contents must be kept stable in order to reduce the negative impact in cells, the equilibrium between ROS scavenging enzymes is essential. This can justify why some enzymes are more stimulated (CAT, SOD, GPOD) in ROS removal when compared to others (APX, GPX) (Gratão *et al.*, 2005).

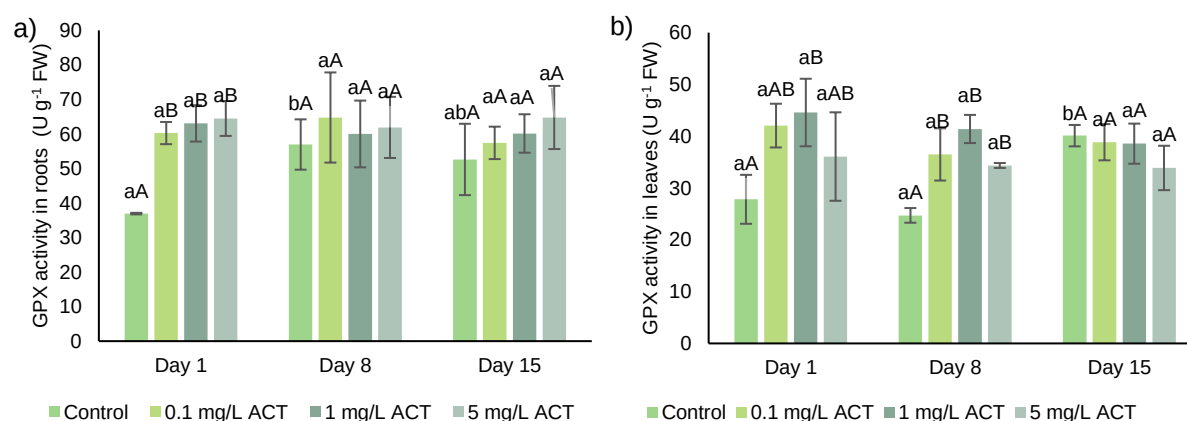


Figure 4.20. - Enzymatic activity (U g⁻¹ FW) of GPX in roots (a) and leaves (b) of *Lactuca sativa* under contamination with ACT for up to 15 days. Different uppercase letters represent significant differences between different ACT concentrations at the same time point and different lowercase letters represent significant differences between treatment duration for the same ACT concentration.

Anthocyanins content (Figure 4.21a) was measured and significant differences were found on days 8 and 15 with a major increase in leaves contaminated with 1 and 5 mg L⁻¹ of ACT. This was observed by other authors confirming that under abiotic stress anthocyanins content tend to increase (Mobin *et al.*, 2007; Mourato *et al.*, 2012; Sytar *et al.*, 2013). In the specific case of ACT contamination, Alkimi *et al.* (2019) reported significant decreases in *Lemna gibba* species, in a study where ACT concentrations were lower. One other consequence of anthocyanins increase is the possibility of bounding with GSH (Jozefczak *et al.*, 2012). This may influence the availability of GSH to participate in the ascorbate-glutathione cycle, explaining the lower concentrations of this metabolite in leaves under ACT contamination. The production of anthocyanins is directly related with PAL activity (Figure 4.21b). This is a key enzyme of the phenylpropanoids pathway which will be crucial to anthocyanins production. Therefore, a correlation among these two parameters may be established. The increase pattern of anthocyanins is coincident with higher activity of PAL. Significant differences to control were observed on leaves after 15 days of exposure to 5 mg L⁻¹ of ACT.

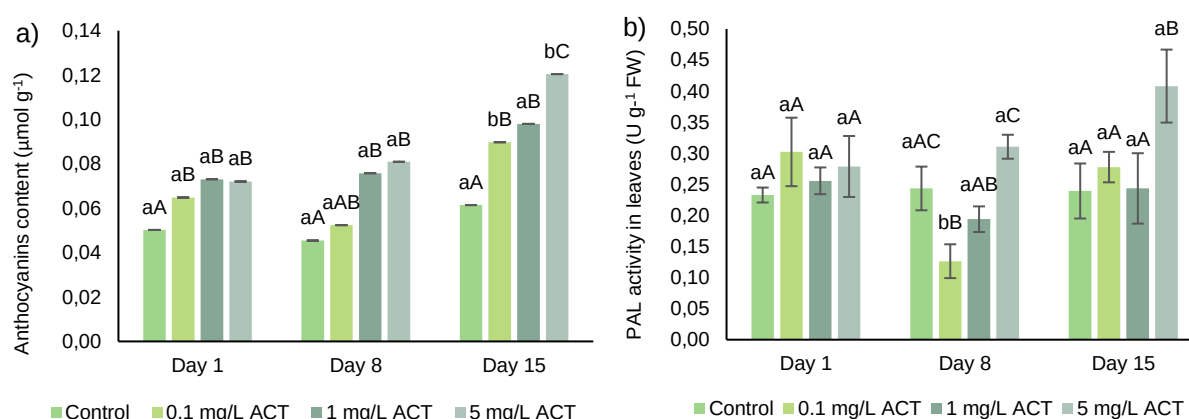


Figure 4.21. - Anthocyanins content ($\mu\text{mol g}^{-1}$ FW) (a) and PAL activity (U g^{-1} FW) (b) in leaves of *Lactuca sativa* under contamination with ACT for up to 15 days. Different uppercase letters represent significant differences between different ACT concentrations at the same time point and different lowercase letters represent significant differences between treatment duration for the same ACT concentration.

A Principal Component Analysis was performed to understand the possibility of grouping among contaminations and enzymatic activities. On Figure 4.22 is possible to observe a clear differentiation between contaminations on both tissues. In roots, control samples remain grouped together with the lower concentration of ACT (0.1 mg L^{-1}), while the most contaminated roots are clearly separated in other class. The same pattern is observed in leaves, however there is an overlap among last day of control and days 1 and 8 of contaminated modalities which indicates some similarity among some individuals.

Phenolic compounds contents (Figure 4.23) were also determined in roots and leaves, however no significant differences were found in contaminated tissues over control. This may be explained by the type of determination, which allows a quantitation of total phenolic compounds. Despite the higher contents of anthocyanins on contaminated tissues, other phenolic compounds may remain similar or lower to control generating similarities or no significant differences to control tissues.

Despite these results of antioxidant species, the global levels of antioxidant capacity in roots and leaves (Figure 4.24) remain statistically similar to the control. Some antioxidative metabolites such as GSH and AsA may contribute to this increase of day 8. In fact, for this day in roots under 5 mg L^{-1} of ACT an increase was registered for both metabolites.

Figure 4.25 shows the main interactions among enzymes and metabolites in roots and leaves under ACT contamination.

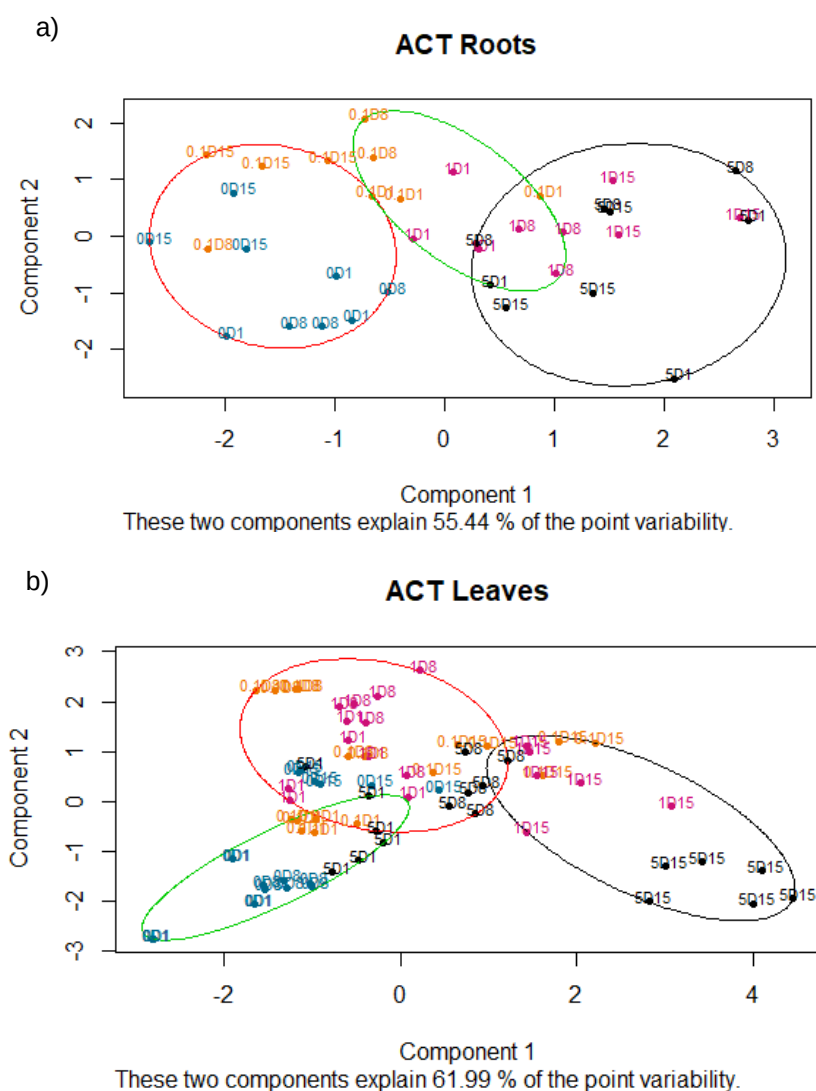


Figure 4.22. - Principal Component Analysis (PCA) of enzymatic activities (CAT, SOD, GPOD, APX, GR, GPX) in roots (a) and leaves (b) of *Lactuca sativa* under contamination with ACT for up to 15 days. Legend: Control – blue dots, 0.1 mg L⁻¹ ACT – orange dots, 1 mg L⁻¹ ACT – pink dots and 5 mg L⁻¹ ACT – black dots.

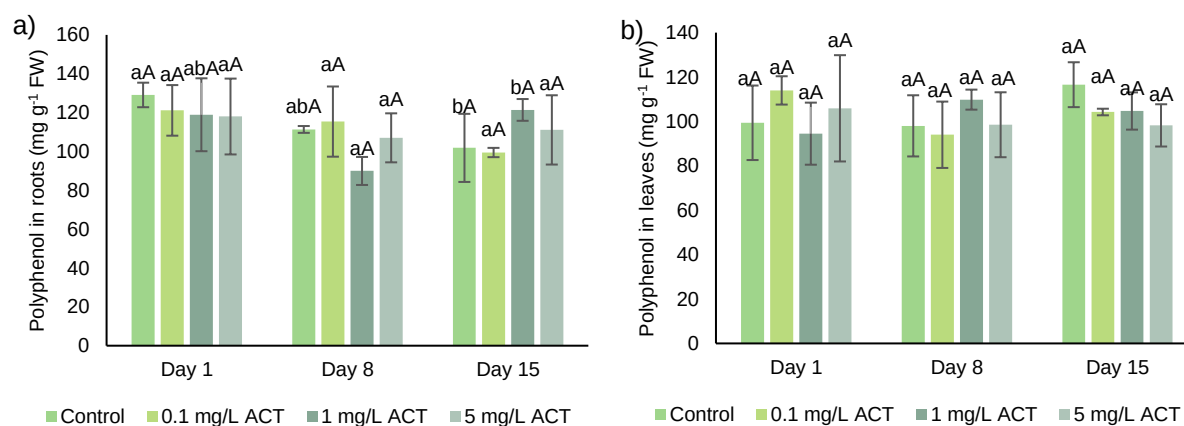


Figure 4.23. - Polyphenols content (mg g⁻¹ FW) in roots (a) and in leaves (b) of *Lactuca sativa* under contamination with ACT for up to 15 days. Different uppercase letters represent significant differences between different ACT concentrations at the same time point and different lowercase letters represent significant differences between treatment duration for the same ACT concentration.

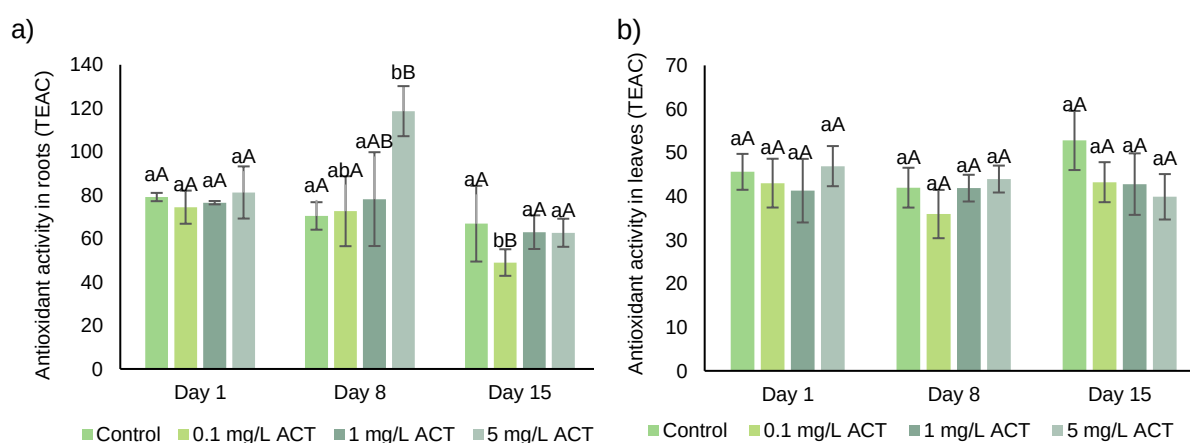


Figure 4.24. - Total antioxidant activity, expressed as TEAC, in roots (a) and leaves (b) of *Lactuca sativa* under contamination with ACT for up to 15 days. Different uppercase letters represent significant differences between different ACT concentrations at the same time point and different lowercase letters represent significant differences between treatment duration for the same ACT concentration.

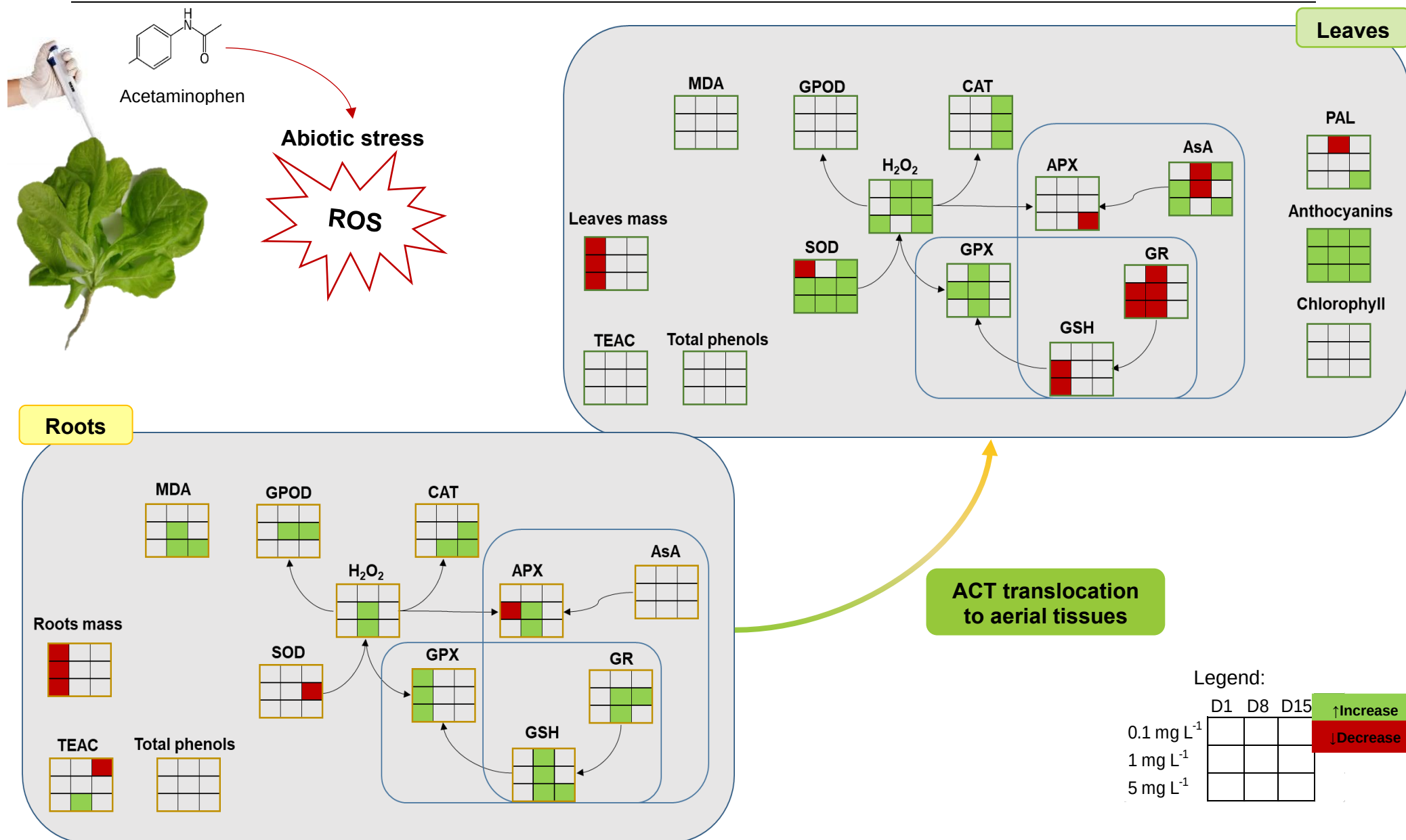


Figure 4.25. – General scheme with significant differences to control over different defence mechanisms against oxidative stress caused by ACT. Significant differences from control are represented in green (increase) and red (decrease) squares.

4.3.5. Mineral elements analysis

The contents of macro (K, Ca, Mg and Na) and micro (Na, Cu, Zn, Fe, Mn) nutrients were evaluated in roots and leaves of lettuce plants under ACT contamination. In Table 4.2 the results in terms of differences in relation to the control (increase or decrease) are presented. The values of the concentrations of each element in each experimental condition are presented in the Appendix (Table A.2). Translocation Factors are also presented in Table 4.3. A relationship with some proteins abundance will be addressed in this discussion, but the complete proteomics approach is presented in Chapter VI.

Under ACT contamination the major differences were found in iron and manganese contents in roots, and in copper in leaves tissue.

On plants contaminated with ACT, in general, no significant differences were found in all macronutrients studied (K, Ca, Mg, P and S). An exception was verified in Ca content on day 8 showing an increase over control in leaves under 1 and 5 mg L⁻¹. It has been previously reported that in animals (mice) acetaminophen increased mitochondrial and cytosolic calcium contents (Boobis *et al.*, 1990; Burcham *et al.*, 1988). In relation to Mg, this is an important element in photosynthetic processes, and these results show that any changes in these processes are not due to Mg deficiency. As for S, although this is an important element for GSH synthesis, there was no increased uptake by lettuce of this element, which agrees with the fact that the changes in GSH contents were also small. Sulphur content in roots under ACT contamination revealed lower values compared to control condition, but once again no significant differences were observed.

Some micronutrients were slightly affected by ACT. Copper in leaves, presented a decrease at day 1 (5 mg L⁻¹) and at day 8 for all concentrations of ACT. However, this trend was not verified on day 15, and this shows that the possible reduction on the translocation of copper between roots and leaves was transient and the plant managed to achieve Cu homeostasis later. The uptake of copper by the roots apparently was not affected as just a slight, non-significant, reduction was detected. Anyway, no negative effects were detected deriving from this Cu decrease in leaves, as they were still above what can be considered a deficient amount for this element.

ACT appeared to not affect Mn contents over time of exposure. Mn translocation to the leaves as, for all times and concentrations of ACT, Mn content in leaves remained similar to control levels.

Regarding Fe contents, a trend to increase Fe uptake was observed, with significant differences over control on day 15, in roots under the higher concentration of ACT. This might be due to a compensation mechanism with the plant absorbing more iron to compensate for the induced toxicity. Although it could be hypothesized that this accumulation of Fe in the roots could be due to a deficient translocation to the leaves this seems not to be the case as the Fe levels in the leaves remain normal levels similar to the control.

Generally, Zn levels were not significantly affected by ACT. Again, a small decrease in Zn leaf levels was detected for 5 mg L⁻¹ ACT, so it seems that at the eighth day of the experiment, for this ACT levels, the translocation of this micronutrient was enhanced but the plant was later able to overcome it as by day 15 they were back to levels similar to the control.

Regarding translocation from roots to leaves, once again macronutrients showed the high ratio of translocation, then ACT did not affect the translocation. Yet, in potassium on day 8 of exposure concentrations in roots remain superior to leaves.

Table 4.2. - Mineral content variations along time and exposure to ACT in roots and leaves of *Lactuca sativa*. Significant differences are represented in red (decreases) and green (increases).

			Cu			Mn			Fe			Zn			Na			K			Ca			Mg			P			S		
			Day 1	Day 8	Day 15	Day 1	Day 8	Day 15	Day 1	Day 8	Day 15	Day 1	Day 8	Day 15	Day 1	Day 8	Day 15	Day 1	Day 8	Day 15	Day 1	Day 8	Day 15	Day 1	Day 8	Day 15	Day 1	Day 8	Day 15	Day 1	Day 8	Day 15
ACT	Leaves	0.1 mg L ⁻¹	—	▼	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	▲	—	—	—	—	—	—	—	—	—	—
		1 mg L ⁻¹	—	▼	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	▲	—	—	—	—	—	—	—	—	—	—
		5 mg L ⁻¹	▼	▼	—	—	—	—	—	—	—	▲	—	—	—	—	—	—	—	—	—	▲	—	—	—	▲	—	—	—	—	—	—
	Roots	0.1 mg L ⁻¹	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
		1 mg L ⁻¹	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
		5 mg L ⁻¹	—	—	—	—	—	—	—	—	▲	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—

Table 4.3. - Translocation factor of mineral content in roots and leaves of *Lactuca sativa* under ACT contamination. Brown represent highest accumulation in roots and green represents highest accumulation in leaves.

ACT		Cu			Mn			Fe			Zn			Na			K			Ca			Mg			P			S		
		Day 1	Day 8	Day 15	Day 1	Day 8	Day 15	Day 1	Day 8	Day 15	Day 1	Day 8	Day 15	Day 1	Day 8	Day 15	Day 1	Day 8	Day 15	Day 1	Day 8	Day 15	Day 1	Day 8	Day 15	Day 1	Day 8	Day 15	Day 1	Day 8	Day 15
TF	Control	0.27	0.35	0.49	0.67	0.57	0.69	0.04	0.02	0.04	0.29	0.23	0.33	0.40	0.50	0.58	1.14	1.06	1.05	1.36	2.07	2.89	2.60	2.88	3.20	0.60	0.58	0.65	0.40	0.40	0.57
	0.1 mg L ⁻¹	0.32	0.28	0.53	0.64	0.65	0.84	0.04	0.02	0.03	0.28	0.27	0.35	0.34	0.37	0.60	1.05	0.98	1.09	1.50	2.03	2.73	2.77	2.96	3.16	0.62	0.59	0.62	0.36	0.43	0.62
	1 mg L ⁻¹	0.31	0.21	0.61	0.66	0.61	0.84	0.03	0.02	0.03	0.30	0.24	0.37	0.48	0.48	0.68	1.10	0.95	1.07	1.41	1.81	2.79	2.54	2.82	3.31	0.67	0.55	0.65	0.42	0.38	0.60
	5 mg L ⁻¹	0.26	0.21	0.53	0.66	0.66	0.87	0.03	0.02	0.03	0.29	0.25	0.36	0.41	0.35	0.69	1.02	0.94	1.10	1.51	1.69	2.40	2.71	2.73	3.05	0.64	0.59	0.61	0.40	0.45	0.59

4.4. Conclusions

The uptake of ACT was detected in roots and leaves even for lower concentrations of this contaminant confirming that this type of pharmaceuticals may reach plants and constitutes a potential contamination hazard in edible vegetables. The fact that, no loss of biomass or visual effects were detected in the plant, make it particularly dangerous, as it maintains a healthy look.

ACT presence affected the normal oxidative status of lettuce plants. The results of the overall antioxidant response confirmed that ACT induced oxidative stress and induced metabolic changes that assured tolerance to phytotoxicity. In roots, CAT and GPOD were the most affected enzymes, increasing its activity for longer periods of exposure to ACT, and exhibiting a positive correlation with H_2O_2 contents. However, in leaves, CAT revealed more significant differences than GPOD when compared to control. SOD activity in leaves was more affected than in roots, revealing significant increases on every day of exposure for concentrations of 1 and 5 mg L⁻¹. In roots, the main differences caused by stress were found in the GSH cycle, with an increase in GR activity and consequently an increase of GSH pools for days 8 and 15 of exposure to ACT. These results may be related with possible detoxification processes involving GSH pathways.

Macronutrients content was not affected by the presence of ACT, revealing some patterns but with no significant differences. Some micronutrients were affected, like Fe in roots for the longer time points, but not enough to cause deficiency symptoms.

Although ACT clearly induced oxidative stress in lettuce plants, the defence mechanisms were adequate to avoid serious deleterious effects, as the plants were able to grow well and with a healthy look.

CHAPTER V

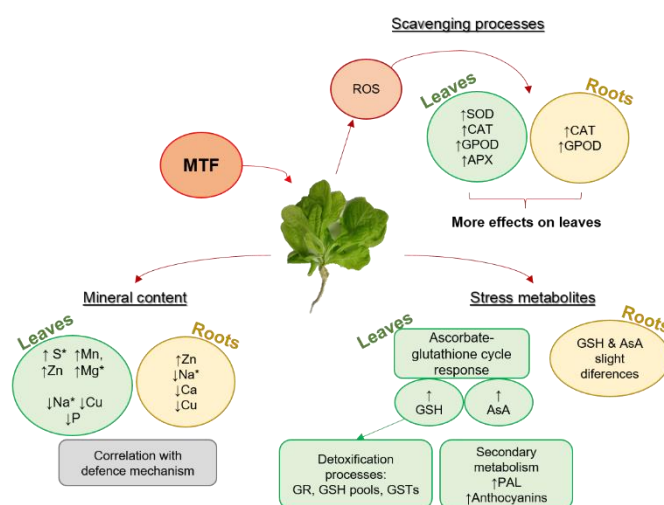
Antioxidative response of lettuce to metformin-induced stress

Leitão I., Mourato, M., Sales, J., Marques, M.M., Oliveira, M.C., Martins, L.L. Effects of metformin on antioxidative response of *Lactuca sativa* plants. Proceedings. 2020. doi:10.3390/IECPS2020-0877.

Chapter V - Antioxidative response of lettuce to metformin-induced stress

Abstract

The occurrence of pharmaceuticals in the ecosystems is considered of growing concern since these compounds may affect different organisms, causing changes on their metabolism and possibly contributing to food chain contamination. Our study aims to understand how lettuce cope with metformin (MTF), evaluating the oxidative stress, and other effects on the plant metabolism. Lettuce was produced in a hydroponic culture contaminated with MTF (0.1, 1 and 5 mg L⁻¹). After 1, 8 and 15 days plants were harvested and analysed. Enzymatic and non-enzymatic parameters were determined following spectrophotometric methods. Concentrations of hydrogen peroxide and malondialdehyde were obtained under MTF contamination, revealing differences on day 15. This study showed that MTF affected plant metabolism inducing oxidative stress, and that different plant parts responded differently to the abiotic stress caused. Antioxidative enzymes results showed different trends in roots and leaves, indicating a specific role in the tolerance mechanism related with plant tissues. Enzymatic response indicated a more intense stress, and a more effective response, in leaves than in roots. The antioxidative protection mechanism in leaves were mainly due to the activity of superoxide dismutase, catalase, guaiacol peroxidase and ascorbate peroxidase, showing that these enzymes have an important role in the defence mechanism against toxic effect of MTF. Other stress related enzymes were also determined. However, their activity was not so affected by the presence of MTF. The uptake of different essential minerals, such as Zn and Mn increased in leaves and roots under MTF exposure, while Ca and Cu showed a trend to decrease.



Graphical Abstract - Main interactions in plants of *Lactuca sativa* under MTF contamination.

Keywords: enzymes, oxidative stress, hydrogen peroxide, defence mechanisms.

5.1. Introduction

Metformin is a widely used drug in type II diabetics patients. Data from 2015 shows that in Portugal around 13.3% of population aged between 20 and 79 years had diabetes, with only 7.5% diagnosed. According to Infarmed (2020) data from April 2020 antidiabetics (metformin included) are on the top 3 of the most consumed drugs in ambulatory mode. Therefore it is possible that as its consumption increases, so will its presence in the environment even after wastewater treatment discharges, due to the lower K_{ow} value (Eggen *et al.*, 2012).

Several authors have already reported the occurrence of metformin in surface and ground waters. In Portugal, metformin was detected in wastewater plant treatment effluent in a concentration range of 0.05 – 58 $\mu\text{g L}^{-1}$ (Gaffney *et al.*, 2017) while in Spain metformin was also detected in surface waters at a maximum concentration of 0.013 $\mu\text{g L}^{-1}$ (Carmona *et al.*, 2017). Metformin was reported in drinking water in Poland (Kot-Wasik *et al.*, 2016), while Huber *et al.* (2016) described its occurrence in sewage sludge in Iceland with a maximum of 7.81 $\mu\text{g g}^{-1}$.

Despite metformin presence in water matrixes, the major threat to the food chain is through the use of treated wastewater in irrigation systems, as well as the use of sewage sludge as fertilizer, which are a common practice among farmers, in order to reduce water consumption as well as to reduce the use of chemical fertilizers.

The bioaccumulation in the environment of metformin and its product guanylylurea² were already described in different crops such as *Solanum tuberosum* (13.27-15.83 $\mu\text{g g}^{-1}$), *Vicia faba* (maximum of 4.85 $\mu\text{g g}^{-1}$) and *Hordeum vulgare* (maximum of 5.0 $\mu\text{g g}^{-1}$) (Eggen *et al.*, 2012). Several authors described different effects on growth of different non-target organisms. For instance, Eggen *et al.* (2011) verified a negative effect on carrots growth, but on the other hand the exposure to metformin did not affect negatively *Lemna minor* and *Desmodesmus* growth (based on EC_{50} parameter) (Briones *et al.*, 2016). Some authors suggest that metformin exposure does not cause acute effects, however the same may not be affirmed to chronic exposure (Al-Odaini *et al.*, 2010).

The uptake of metformin by crops is one of the main concerns in terms of emerging contaminants. Besides, there is still the question if these contaminants are accumulating in plants, and how these contaminants may affect crops productivity and metabolism.

² Guanylylurea ($\text{C}_2\text{H}_6\text{N}_4\text{O}$) – a transformation product of antidiabetic drug metformin (National Center Biotechnology Information, 2021).

In case of metformin, it is still not clear the effect that this pharmaceutical can induce in plants, namely in terms of oxidative stress. Different metabolic pathways are described as the main sources of ROS imbalance and changes in cell homeostasis that causes oxidative stress. Consequently, the ability of plants to cope with stress caused by pharmaceuticals will depend on the efficiency of the induced antioxidant response.

Plant antioxidant system involves several enzymatic and non-enzymatic mechanisms. Enzymes like SOD convert superoxide ions into hydrogen peroxide that can then be degraded into water by enzymes like CAT, GPOD and APX. All these three enzymes use different reductants to eliminate H₂O₂ (Mhamdi *et al.*, 2012; Mittler, 2002).

Metabolites like GSH are very important in detoxification processes and oxidative stress response (Vercampt *et al.*, 2017). The availability of GSH pools mediated by GR in regulation with other cycles as GPX and ascorbate-glutathione cycle are keys to the maintenance of cell redox state and adaptation to abiotic stresses, such as the exposure to different pharmaceuticals. Together, AsA and GSH are important metabolites involved in antioxidative mechanism assuring an effective link to the enzymatic responses as an integrated defence system.

In this study, the main objectives were to analyse the effect on lettuce of the stress caused by exposure to metformin contamination, in order to understand and characterize the induced metabolic response, namely in terms of oxidative stress. Therefore, several stress parameters and mineral composition were analysed, in roots and leaves from lettuce plants.

5.2. Material and Methods

Seeds of the cultivar *Lactuca sativa* L. “Maravilha das Quatro Estações” were germinated in substrate (Bio Siro – peat, humus and fertilizer). During the first 15 days the seedlings were maintained in germination substrate until the development of the first leaf. Afterwards, young lettuce plants were transferred to hydroponic culture with Hoagland solution and developed in this medium for 21 days. On the 36th day of growth, lettuce plants were contaminated with different concentrations of metformin (MTF): 0 (control), 0.1, 1 and 5 mg L⁻¹. These concentrations were based on previous studies from the literature (Cui *et al.*, 2016; Cummings *et al.*, 2018; Eggen *et al.*, 2012) and known to be capable of inducing oxidative stress in different plants. Three pots were used for each concentration, with 12 plants each, for a total of 36 plants per dose.

Growing conditions and more detailed experimental data are described in point 2.1. Lettuce plants production and plants contamination conditions, from Chapter II Material and Methods.

The following procedures were performed, all described in Chapter II:

- Harvesting of plant material (2.2.)
- Determination of mineral elements (2.3.1.)
- Determination of Photosynthetic parameters (2.3.3.)
- Determination of Lipid Peroxidation (2.3.4.1.)
- Determination of Hydrogen Peroxide content (2.3.4.2.)
- Determination of Antioxidant activity (2.3.4.3.)
- Determination of Phenolic Compounds (2.3.4.4.)
- Determination of Ascorbate (2.3.4.5.)
- Determination of Glutathione (2.3.4.6.)
- Determination of Anthocyanins (2.3.4.7.)
- Determination of Enzymatic Activities (2.3.5.)

Data analysis is described in section 2.5, from Chapter II.

5.3. Results and Discussion

5.3.1. Biomass and chlorophyll parameters

Biomass parameters (Figure 5.1 a/b) showed similar trends on both roots and leaves. Significant differences were found mainly on day 15 of exposure. On roots these differences were observed for the concentrations of 1 and 5 mg L⁻¹, while in leaves no significant differences over control were detected.

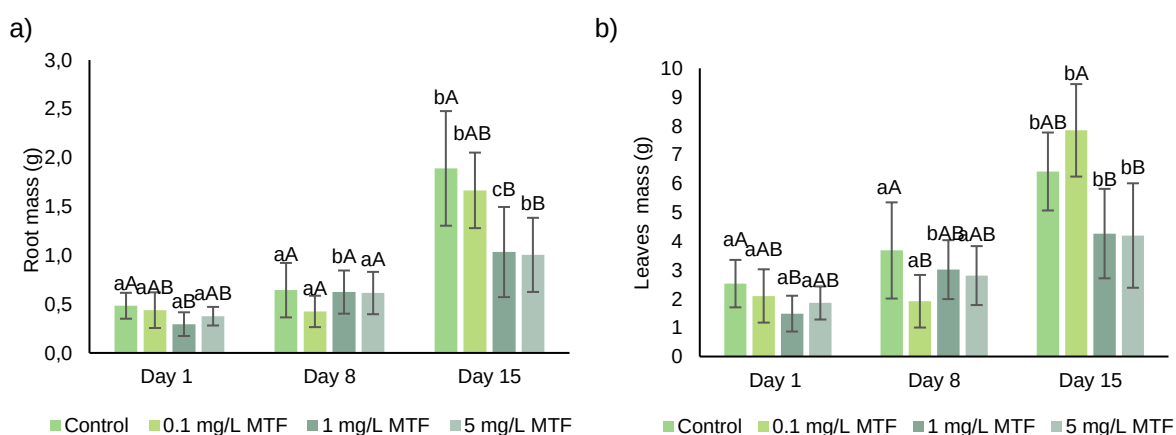


Figure 5.1. - Leaf (a) and root (b) mass of *Lactuca sativa* under contamination with MTF for up to 15 days. Different uppercase letters represent significant differences between different MTF concentrations at the same time point and different lowercase letters represent significant differences between treatment duration for the same MTF concentration.

These results may reflect a higher influence of MTF contamination for longer periods of exposure, showing that significant differences are only revealed on the higher concentrations. Similar results were reported by Eggen *et al.*, (2012) in other crops such as carrot and wheat under MTF contamination.

Figure 5.2 shows the visible aspect of lettuce plants during the experiments for all the MTF concentrations and times studied. It is clear that plant development was affected mainly for the highest concentration of MTF and for longer periods. These results are reflected as well in biomass of leaves and roots observed in Figure 5.1.

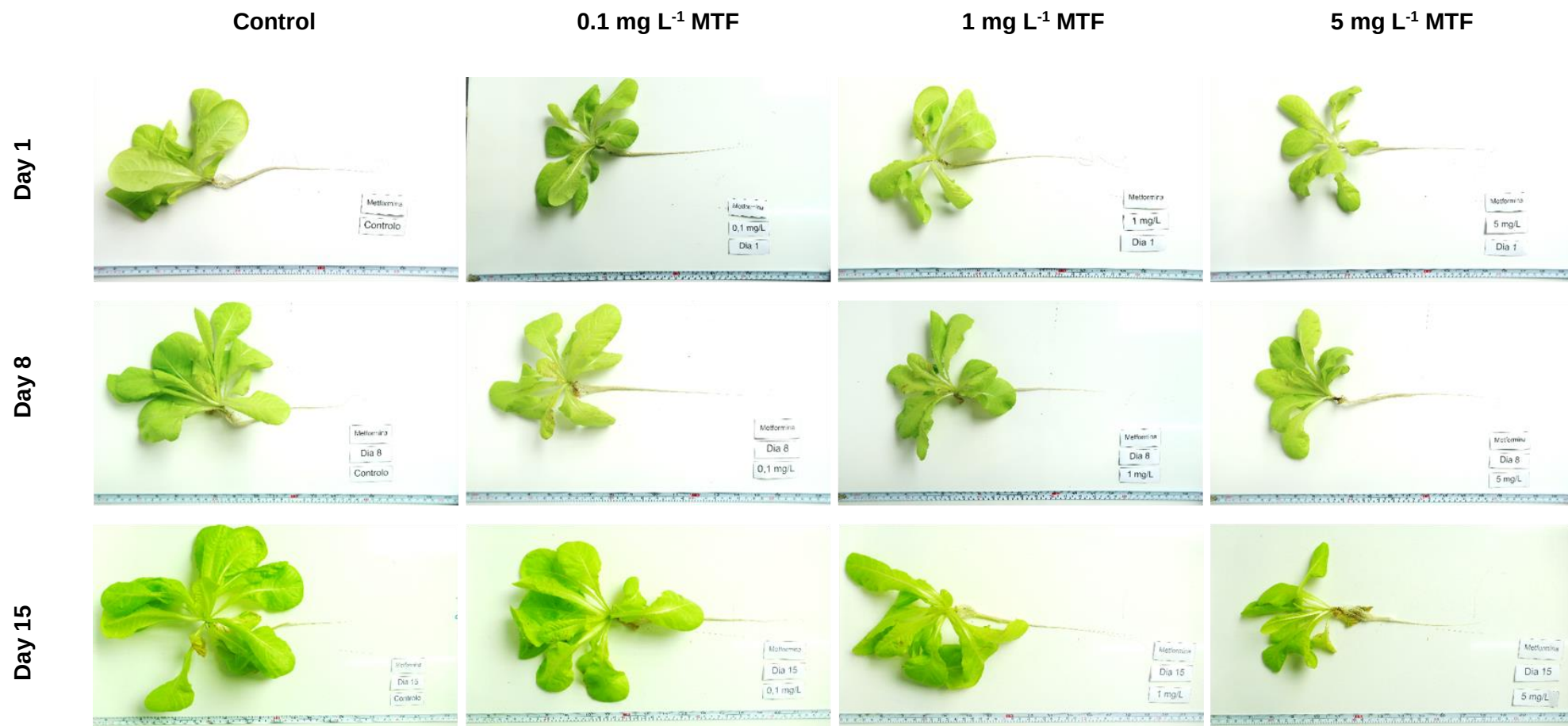


Figure 5.2. - Lettuce plants on days 1, 8 and 15 of contamination with different concentrations of MTF - Control, 0.1 mg L⁻¹, 1 mg L⁻¹ and 5 mg L⁻¹.

Chlorophyll-related parameters (NDVI and PRI – Figure 5.3 a/b) were measured on every harvest day. NDVI remain similar over time of exposure and over control, which may indicate a good regulation of photosynthetic activity, since this parameter measures photosynthetic rates. On the other hand, although there is an apparent PRI increase on day 15 for contaminated leaves with increasing MTF concentrations, these results are not significantly different. This increase on PRI may suggested an efficiency on photosynthetic light use. For leaves under 1 and 5 mg L⁻¹ MTF (day 15), this increase may be related with other pigments presence, such as anthocyanins. PRI is usually known as a pigment content indicator since it measures different regions of the spectrum (Sims *et al.*, 2002). PRI is also addressed as a good parameter for radiation use efficiency in response to stress (Cordon *et al.*, 2016). Therefore, the increase of PRI along the exposure may be associated with an increase of pigments production as response to MTF exposure.

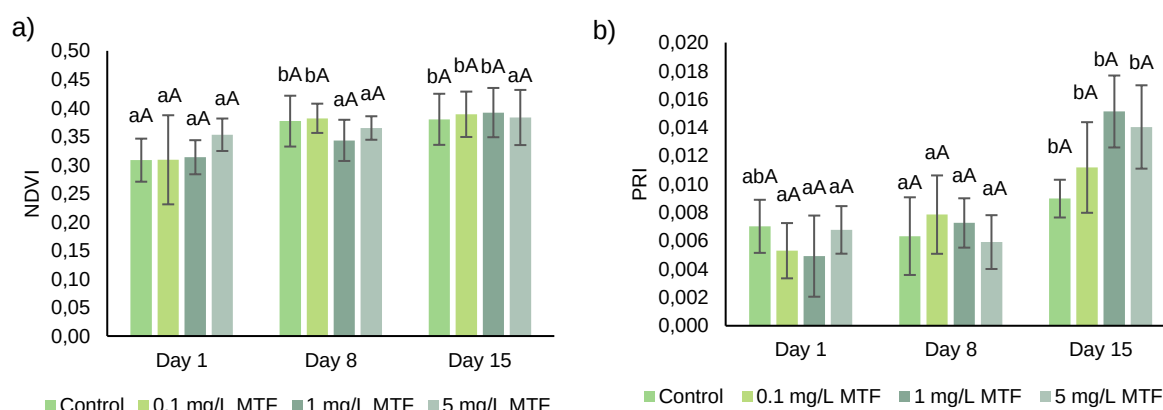


Figure 5.3. - NDVI (a) and PRI (b) of *Lactuca sativa* leaves under contamination with MTF for up to 15 days. Different uppercase letters represent significant differences between different MTF concentrations at the same time point and different lowercase letters represent significant differences between treatment duration for the same MTF concentration.

MTF did not affect total chlorophyll content as can be observed in Figure 5.4, with the exception of day 8 for 1 mg L⁻¹. Despite differences in plant species, Cummings *et al.* (2018) also reported no differences on photosynthesis on *Chlorella vulgaris* microalgae exposed to 1.5 mg L⁻¹ of metformin.

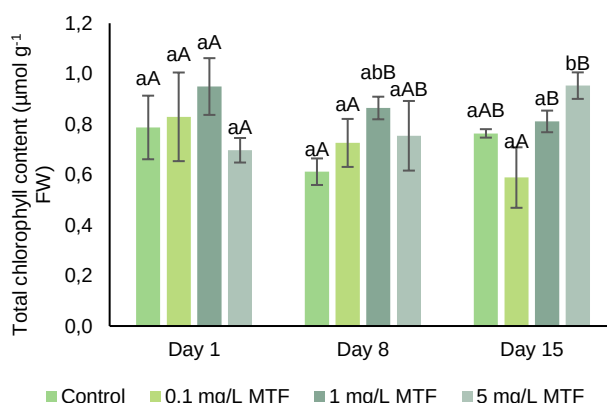


Figure 5.4. - Chlorophyll content of *Lactuca sativa* leaves under contamination with MTF for up to 15 days. Different uppercase letters represent significant differences between different MTF concentrations at the same time point and different lowercase letters represent significant differences between treatment duration for the same MTF concentration.

Despite no significant difference over control, it is possible to observe a slight decrease on chlorophyll content on day 1 for the higher concentration of MTF. This trend is not verified on day 15, where the same concentration reveals higher values of chlorophyll content in comparison to control, still with no significant differences. In any case, chlorophyll contents of leaves under 5 mg L⁻¹ MTF showed a trend to increase along time, since significant differences are observed among days (8 and 15). Similar trend was observed in PRI values for day 15, meaning that chlorophyll content and photosynthetic system are not greatly affected by MTF. Chlorophyll visual effects may also be observed in Figure 5.2 where no differences on pigments coloration on leaves are shown. In fact, perceptible differences occur only in biomass (Figure 5.1).

5.3.2. Stress indicators analysis

The contents of hydrogen peroxide showed different responses for roots and leaves, as it is possible to observe on Figure 5.5 a/b.

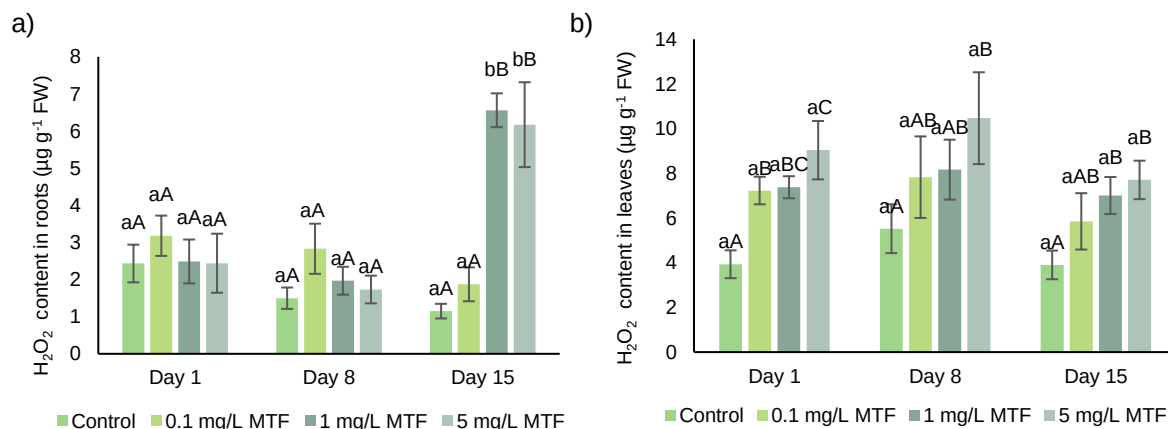


Figure 5.5. - H₂O₂ content (µg g⁻¹ FW) in roots (a) and leaves (b) of *Lactuca sativa* under contamination with MTF for up to 15 days. Different uppercase letters represent significant differences between different MTF concentrations at the same time point and different lowercase letters represent significant differences between treatment duration for the same MTF concentration.

Roots under MTF contamination did not show as many significant differences as in leaves, since in roots significant differences compared to control were only observed on day 15, under 1 and 5 mg L⁻¹. Root growth and development also did not seem to be affected by the presence of MTF, as observed on Figure 5.1. However, H₂O₂ also works as a signalling molecule that will induce other mechanisms of defence. For longer periods of exposure to MTF, it is possible to observe an increasing trend in H₂O₂ contents, indicating the occurrence of oxidative stress. In leaves exposed to MTF contamination, some statistically significant differences were observed on H₂O₂ content considering time and MTF concentration. On day 1, significant differences over control were observed for every concentration of MTF, while on day 8 it was observed a significant increase only on leaves under 5 mg L⁻¹ MTF. On day 15, the H₂O₂ content is significantly different for both 1 and 5 mg L⁻¹ MTF concentrations, reflecting a more intense oxidative stress at longer periods of exposure to MTF. Only at 0.1 mg L⁻¹ MTF, the H₂O₂ content is not significantly different to the control. H₂O₂ contents in leaves were twice the values found in roots, mainly due to the fact that leaves metabolism produces more ROS.

The imbalance of ROS is the main oxidative stress indicator in plants, regarding abiotic stress effects. So, these results suggest that MTF induces oxidative stress in lettuce plant, especially in leaves, and for longer exposition, indicating the possibility of occurrence of oxidative

damage. In general, MTF caused an increase in H_2O_2 contents in both plant parts which is in accordance with other studies regarding exposure to organic compounds (phenanthrene) in tomato (Ahammed *et al.*, 2012) or even more common stresses as salinity or heavy metals presence (Kapoor *et al.*, 2019).

MDA content (Figure 5.6 a) revealed an increase on day 15 in roots under 1 and 5 mg L^{-1} MTF, which is generally in accordance with results for H_2O_2 contents. The highest MDA value corresponds to the highest H_2O_2 obtained and is significantly different compared to control and almost all the other contaminations tested.

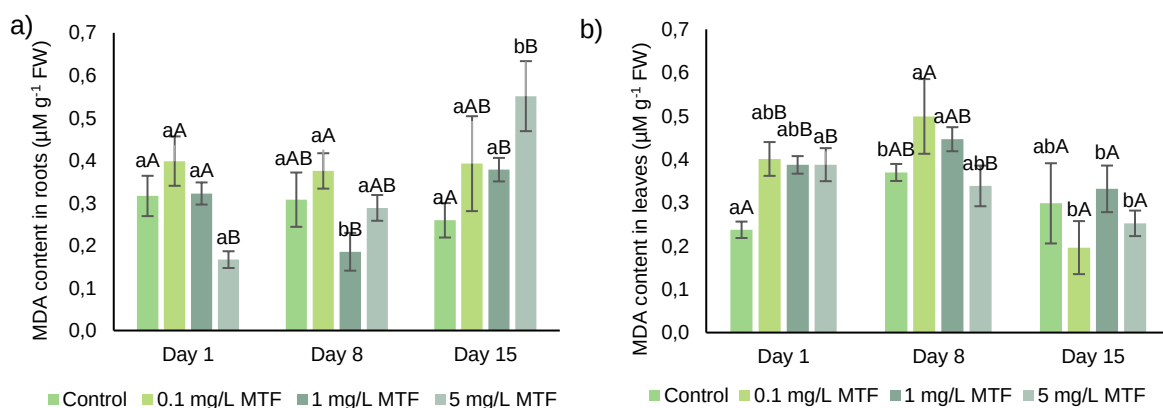


Figure 5.6. - MDA content ($\mu\text{M g}^{-1}\text{ FW}$) in roots (a) and leaves (b) of *Lactuca sativa* under contamination with MTF for up to 15 days. Different uppercase letters represent significant differences between different MTF concentrations at the same time point and different lowercase letters represent significant differences between treatment duration for the same MTF concentration.

In leaves, the main differences on MDA content (Figure 5.6 b) occurred on day 1, where all concentrations of MTF showed significant differences over control. However, for all the other treatments no significant differences were observed. It is important to notice that although leaves present a higher H_2O_2 content this is not reflected in MDA values. In fact, at day 15 MDA content is similar between control and contaminated samples. This shows that leaves probably have efficient defence mechanisms that are avoiding lipid peroxidation. One possible explanation is that plants have other strategies to overcome production of ROS. In roots, it is possible to observe higher contents of MDA and H_2O_2 for longer days of exposure to MTF. This may reflect a low activation of defence mechanisms.

The contents of MDA may vary according to tissues or the applied contaminant. For instance, a study with other organic contaminants – the fungicide chlorothalonil – showed different results on MDA content of *Vitis vinifera* leaves (Wang *et al.*, 2017). These results show similar trends to H_2O_2 in leaves representing a balance among H_2O_2 content and lipid peroxidation.

5.3.3. Enzymatic activities and metabolites related with stress responses

Results regarding SOD, CAT and GPOD activities are presented in Figures 5.7 and 5.8.

SOD activity (Figure 5.7) did not show significant differences in roots, which might suggest that the presence of MTF did not increase $O_2^{\cdot-}$ concentration in cells, although other type of ROS may occur. Results of H_2O_2 contents in roots on days 1 and 8 showed no differences to control, this could be due to the absence of $O_2^{\cdot-}$, since SOD acts on $O_2^{\cdot-}$ to form H_2O_2 . The increase of H_2O_2 on day 15 is not explained by SOD activity since no differences were found over control, indicating that H_2O_2 was formed in another metabolic processes occurring in cells compartments such as vacuoles (acting as a sink) or apoplast and plasma membrane. Different enzymes may produce $O_2^{\cdot-}$ and H_2O_2 , such as NADPH oxidase, copper (Cu) amine oxidases, polyamine oxidases and oxalate oxidase (Smirnoff *et al.*, 2019). Therefore, in case of roots the higher contents of H_2O_2 registered on day 15 may be the result of the activity of some of these enzymes, which will explain the occurrence of no differences in SOD in roots.

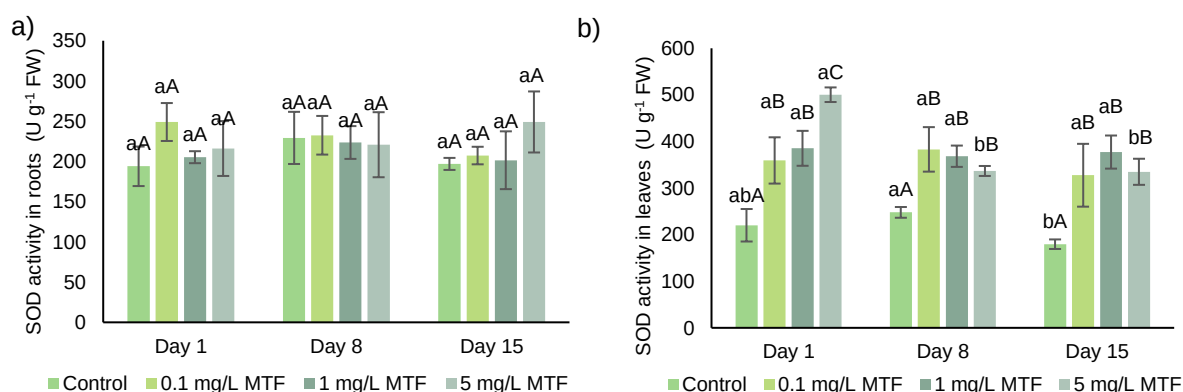


Figure 5.7. - Enzymatic activity (U g⁻¹ FW) of SOD in roots (a) and leaves (b) of *Lactuca sativa* under contamination with MTF for up to 15 days. Different uppercase letters represent significant differences between different MTF concentrations at the same time point and different lowercase letters represent significant differences between treatment duration for the same MTF concentration.

In leaves, Figure 5.7 b, SOD enzyme shows significant increases in all contaminated samples compared to control, considering concentration and time. Unlike roots, this trend in leaves suggest that the increase on H_2O_2 contents may be a result of high activity of SOD due to higher contents of $O_2^{\cdot-}$. Changes in SOD activity are frequently linked to abiotic stress, not only in terms of inorganic contaminants such as heavy metals, but also in case of some organic compounds. Sharma *et al.*, (2013) studied the influence of imidacloprid in a rice variety showing that SOD activity increased with the contamination.

CAT and GPOD are two of the major enzymes responsible for H_2O_2 scavenging and detoxification. Therefore, in parallel with possible production of H_2O_2 by SOD, CAT and GPOD are important to maintain H_2O_2 content under control and contribute for a good regulation of ROS in cells, preventing oxidative damages. Some authors described already some correlations among these enzymes indicating that under abiotic stress (induced by cadmium and imidacloprid) was observed an increase of SOD, CAT, GPOD (Kolahi *et al.*, 2020; Sharma *et al.*, 2013).

In this work some differences regarding CAT and GPOD activities (Figure 5.8) were found when different parts of the plant were directly compared.

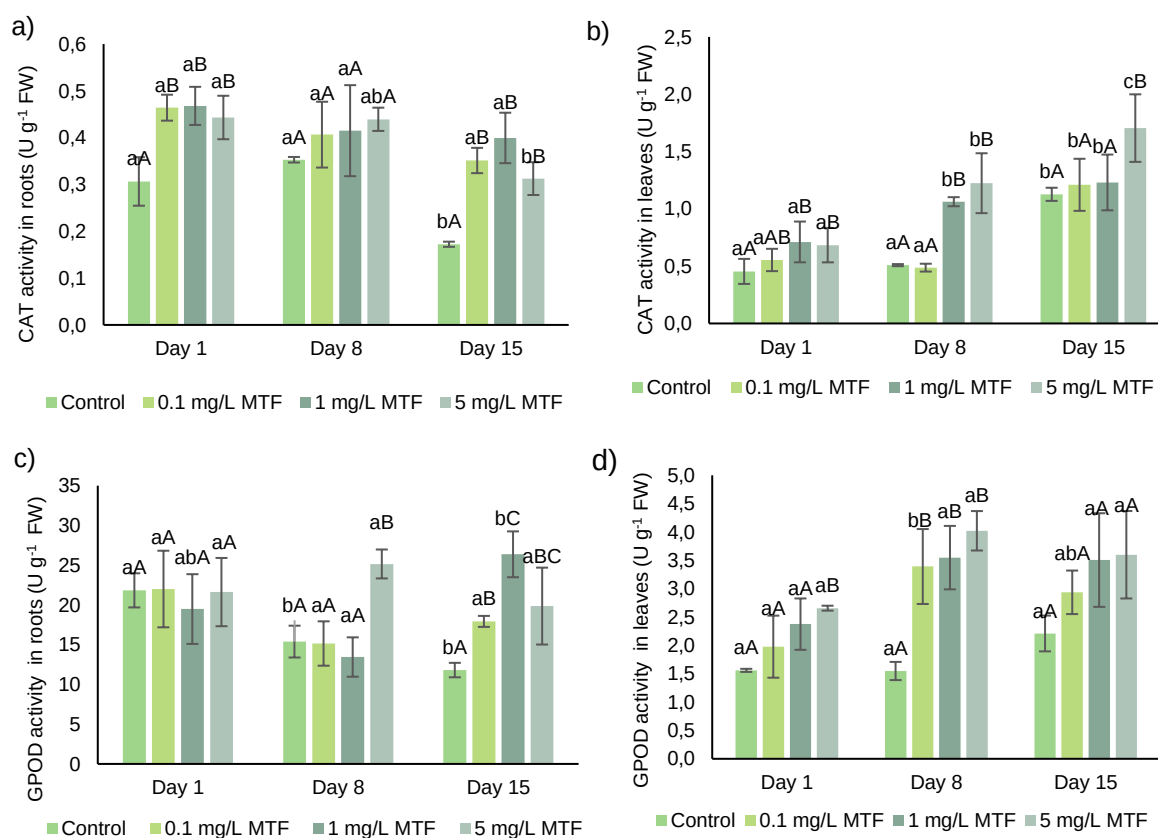


Figure 5.8. - Enzymatic activity (U g⁻¹ FW) of CAT and GPOD in roots (a, c, e) and leaves (b, d, f) of *Lactuca sativa* under contamination with MTF for up to 15 days. Different uppercase letters represent significant differences between different MTF concentrations at the same time point and different lowercase letters represent significant differences between treatment duration for the same MTF concentration.

In roots, the activity of CAT shows a significant increase over control on days 1 and 15 for all MTF concentrations. Previously, results of H_2O_2 in roots revealed no differences on day 1 while on day 15, a highly significant increase was observed. Therefore, CAT might contribute to the

lower contents of H_2O_2 on day 1, while on day 15 CAT was not effective on controlling H_2O_2 concentrations. In any case, for the three concentrations of MTF applied, significant differences were observed when compared to the control, on the same day.

Despite this difference to control, the relation between H_2O_2 and CAT in roots under 1 and 5 mg L^{-1} is not linear, meaning that CAT by itself may not be enough to remove and control the excess of H_2O_2 formed in roots cells (as H_2O_2 content for day 15 are very high).

In leaves, CAT appears to play an important role especially for the highest concentrations and longer times of exposure. CAT activity reveals a clear increasing pattern in leaves exposed to 1 and 5 mg L^{-1} of MTF showing significant differences over control in almost every day. Once again, this trend may be correlated with H_2O_2 contents. On one hand CAT may be activated by the high contents of H_2O_2 in leaf cells, while on the other hand, the decrease of H_2O_2 on day 15 may be a reflection of continuous activity of CAT over time.

GPOD is also responsible for H_2O_2 scavenging in cells and is often associated with a coordinated activity with CAT. GPOD activity in roots revealed differences to control on day 8 in roots under 5 mg L^{-1} MTF and on day 15 for every concentration of MTF. As mentioned before, this may represent a clear mechanism of defence of CAT and GPOD, both responsible for the scavenging of H_2O_2 .

The joint work of CAT and GPOD may still be observed in leaves. Although H_2O_2 levels are much higher in leaves when compared to roots, CAT and GPOD seem to be very efficient to control ROS levels protecting cells against excessive oxidative damage. Considering the relation between ROS and lipid peroxidation, it was also possible to observe a decrease on MDA content. Therefore, in leaves, lipid peroxidation seems to be controlled indicating possible reduction of hydroxyl radical (a result of Fenton reaction with H_2O_2 as product), while in roots lipid peroxidation tend to increase along time of exposure. The relation between CAT and GPOD was established in a correlation analysis (Figure 5.9) showing interesting outputs. For example, it is possible to observe that control values (green dots) remain lower in both enzymes in comparison to other contaminations. Also, the more contaminated leaves (orange and pink dots) show similar trend in CAT and GPOD activities, meaning that both enzymes are in fact working together on stress response, as mentioned before. This means that GPOD and CAT act both on H_2O_2 control reducing risks of oxidative damage on lettuce leaves. This is thus an important part of a well-coordinated defence mechanism to maintain ROS equilibrium in cells.

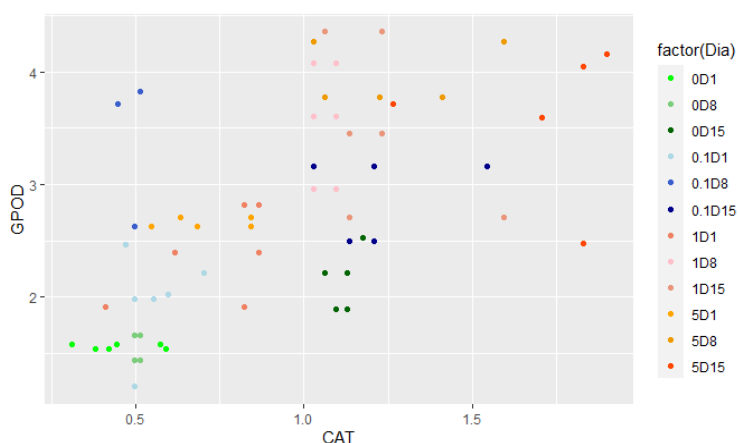


Figure 5.9. - Correlation analysis between GPOD and CAT in leaves of *Lactuca sativa* under contamination with MTF for up to 15 days.

GR and GPX activities were also determined and the results are presented in Figure 5.10. GR plays an important role in response to oxidative stress, given its relationship with chloroplasts and mitochondria. The ascorbate-glutathione pathway is regulated by this enzyme together with APX, DHAR and MDHAR being crucial on ROS detoxification.

In roots, both enzymes showed some significant differences over time and comparing to control condition, but do not show a specific correlation with time of exposure or MTF concentration. For instance, GR activity decreases on day 1 for every MTF concentration but significant differences were only noted in roots under 5 mg L⁻¹, while on day 15 the opposite trend is observed, once again with significant differences on the highest concentration of MTF.

In leaves, some variations in GR activity (increasing trend) occurred progressively on day 8 from 0.1 to 5 mg L⁻¹ MTF. On the last day of exposure, leaves under the highest concentration of MTF showed a decrease in relation to the control. This behaviour suggests that for longer periods of exposure GR activity is affected.

GPX is considered a key component in the ascorbate-glutathione cycle reducing the content of H₂O₂ during the oxidation of GSH to GSSG. Also, this enzyme is capable to catalyse H₂O₂ using thioredoxin as an electron donor instead of GSH.

Regarding GPX activity in roots, it is possible to observe a decrease in day 15 of exposure for all concentrations of MTF. Despite the higher contents of H₂O₂ on last day of exposure, there is no correlation with GPX activity which may indicate that GPX is not responsible for the removal of high contents of H₂O₂. In fact, GPX is described as a key enzyme on low oxidative stress, which is not the case of our results given the high contents of H₂O₂ on day 15, in roots. Also, GPX competes with CAT and GPOD and their higher activity may interfere with the availability of H₂O₂ as substrate for the reaction catalysed by GPX.

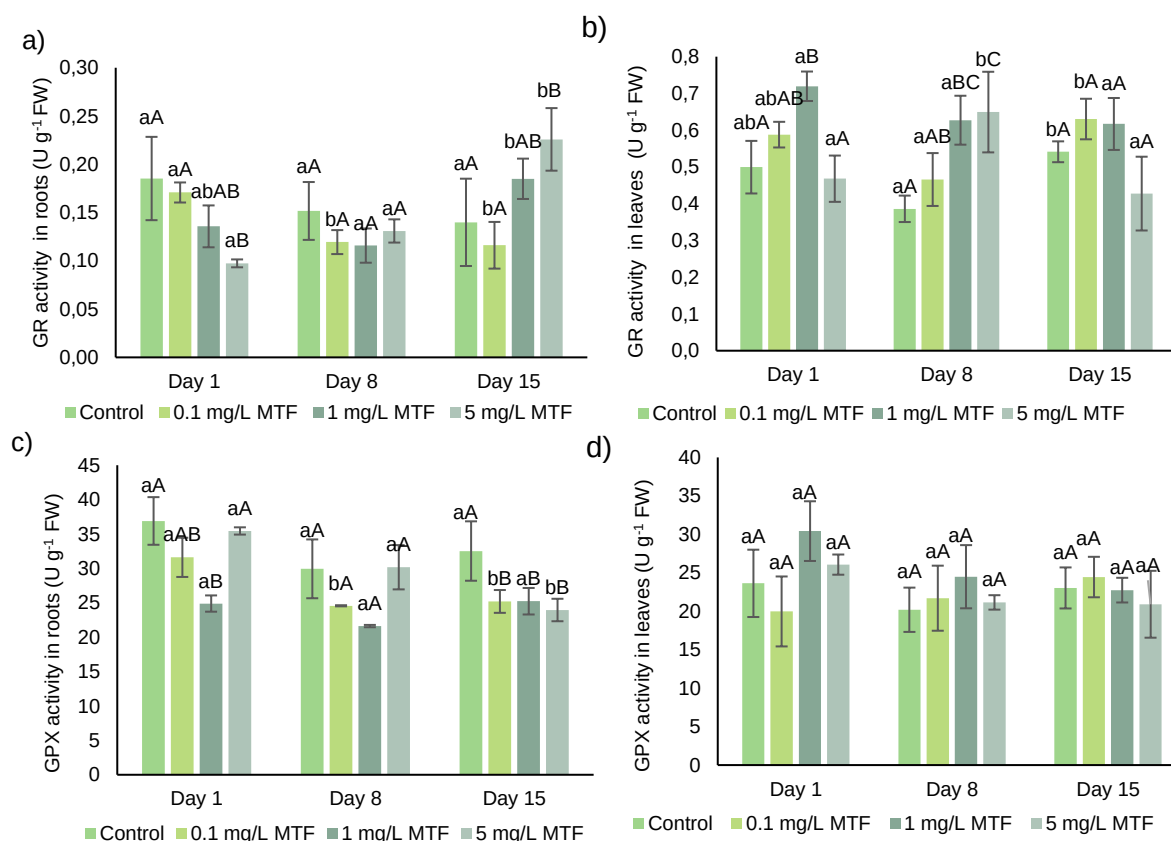


Figure 5.10. - Enzymatic activity (U g⁻¹ FW) of GR and GPX in roots (a, c) and leaves (b, d) of *Lactuca sativa* under contamination with MTF for up to 15 days. Different uppercase letters represent significant differences between different MTF concentrations at the same time point and different lowercase letters represent significant differences between treatment duration for the same MTF concentration.

GPX is not only responsible for H₂O₂ scavenging, but also serves to detoxify products of lipid peroxidation (Islam *et al.*, 2015). In fact, on day 15, a decrease in MDA content is observed while GPX decreased its activity. Then the difference observed on contaminated roots may reflect a possible cell damage due to lipid peroxidation.

This pattern may be observed in Figure 5.11 which shows the correlation between GPX activity and H₂O₂ content in roots. For higher concentrations and longer days of exposure GPX remains lower when compared to days 1 and 8 of contamination. Also, a relation among high contents of H₂O₂ and lower values for GPX may be observed.

In leaves, GPX activity remained similar to control in all MTF concentrations. Even in terms of time of exposure no differences occurred. The similarity among control and contaminated leaves might reflect a good regulation against oxidative stress caused in leaves, as shown by other enzymes responsible for cell redox homeostasis.

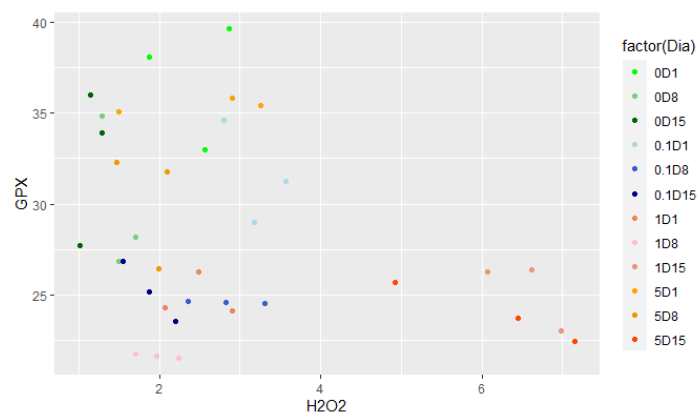


Figure 5.11. - Correlation analysis between GPX and H_2O_2 in roots of *Lactuca sativa* under contamination with MTF for up to 15 days.

GR and GPX enzymes also participate in the regulation of GSH pools in cells. Results regarding GSH contents, in roots and leaves, are shown in Figure 5.12.

GSH is frequently considered a precursor of detoxification process in cells. Wang *et al.* (2017) verified that chlorothalonil treatment affected some enzymes responsible for detoxification processes such as glutathione synthetase, glutathione S-transferase (GST), GR, P450 monooxygenase which increased their gene expression levels, with exception of glutathione synthetase.

In roots, the relationship between GR and GSH contents does not show a clear trend neither with MTF concentrations nor with days, meaning that an increase on GR activity does not always represent an increase on GSH content. It is known that the depletion of GSH may represent a possible use of GSH in other important pathways such as GPX, GSTs or secondary metabolism. For instance, on day 1, GSH content remain similar to control. However, GR, on day 1, showed a significant decrease while on day 15 an increasing trend is observed. Yet GSH remains similar to control. This may reflect different levels of adaptation of roots cells to MTF stress. Meanwhile, GPX shows different variations along time and concentrations, with a significant decrease on the last day of exposure in contaminated roots. The high contents of H_2O_2 did not induce a higher activity of GPX representing a low level of response to ROS by this enzyme in roots. Also, GSH may be used as substrate for other enzymes related with AsA cycle, which will be furthered discussed.

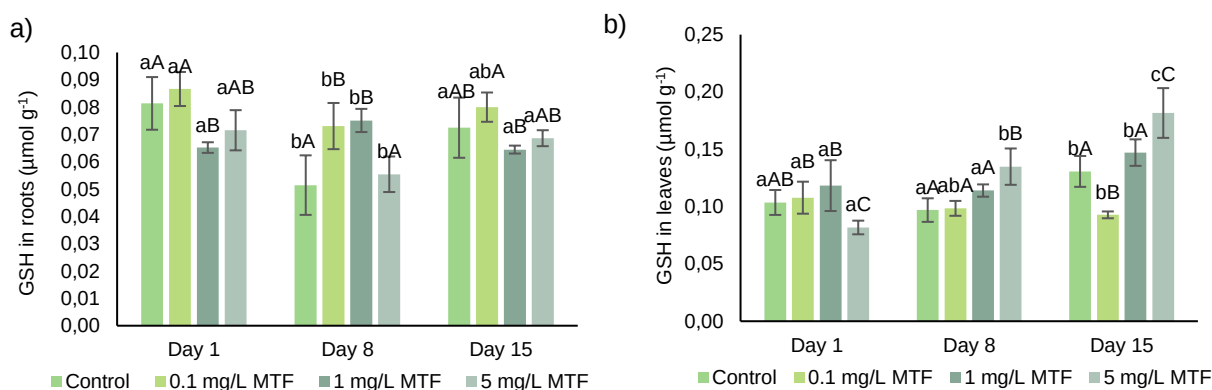


Figure 5.12. - GSH content ($\mu\text{mol g}^{-1}$ FW) in roots (a) and leaves (b) of *Lactuca sativa* under contamination with MTF for up to 15 days. Different uppercase letters represent significant differences between different MTF concentrations at the same time point and different lowercase letters represent significant differences between treatment duration for the same MTF concentration.

In leaves, GPX did not seem to be influenced by the high presence of H_2O_2 contents, and consequently, no depletion of GSH was observed in contaminated leaves. Regarding specific variations on GSH content, on day 1 a decrease was observed on leaves under 5 mg L^{-1} MTF, which may be related with the slight decrease on GR activity for the same period, however in this case the difference was not significant. On day 8, the concentration of GSH in contaminated leaves (for 5 mg L^{-1} MTF) increased, being coincident with a higher activity of GR in relation to the control. On day 15 it is evident a clear decrease in leaves under 0.1 mg L^{-1} MTF, yet with no strict relation with GR and GPX activities. Also, on day 15 an increase in GSH was observed for 5 mg L^{-1} MTF, however this is not directly explained by GR activity. This behaviour may be explained by GPX, which remains similar to control inducing an accumulation of GSH, allowing its availability for other processes.

In some cases (in roots under 1 and 5 mg L^{-1} on day 1, and 1 and 5 mg L^{-1} on day 15, or on leaves of 1, 5 mg L^{-1} on day, and 0.1 mg L^{-1} on day 15) the slight depletion on GSH content may be related with AsA cycle or secondary metabolism response. For instance, the high contents of anthocyanins in leaves, discussed below, may point for a secondary metabolism activation as response to stress and therefore explain some results obtained for GSH contents in leaves.

Besides the described pathways of synthesis and consumption of GSH, certain compounds, including ROS, such as hydroxyl radical or superoxide are able to oxidize GSH which will influence its availability for other processes. The link between AsA and GSH and related enzymes (like APX and DHAR) is extremely important in order to explain a coordinated response to oxidative stress cause by MTF.

APX activities are presented on Figure 5.13. The effects of MTF contamination revealed more differences on aerial part compared to radicular tissues.

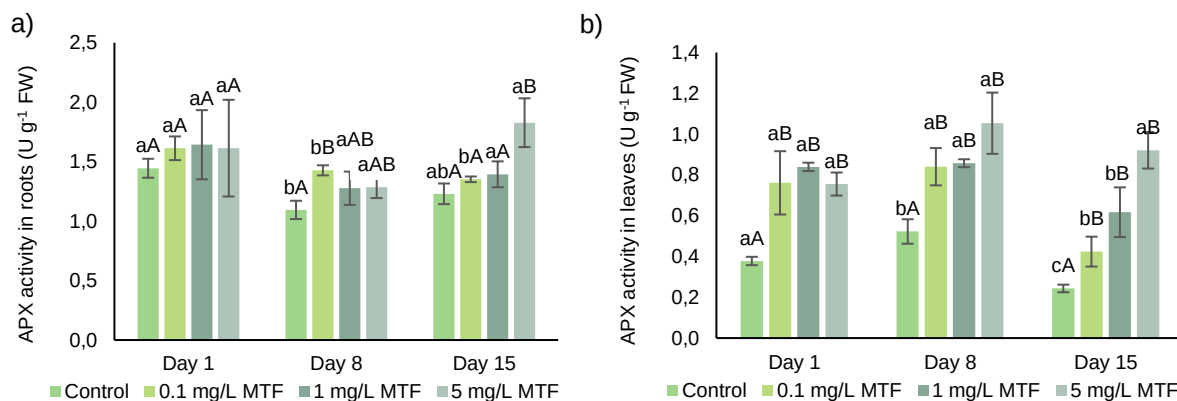


Figure 5.13. - Enzymatic activity (U g⁻¹ FW) of APX in roots (a) and leaves (b) of *Lactuca sativa* under contamination with MTF for up to 15 days. Different uppercase letters represent significant differences between different MTF concentrations at the same time point and different lowercase letters represent significant differences between treatment duration for the same MTF concentration.

In roots, APX showed significant differences on day 15, on 5 mg L⁻¹ MTF contamination. These results correspond to the increase on H₂O₂ concentrations. Therefore, it is possible that APX work on the scavenging of H₂O₂ together with CAT and GPOD. APX is commonly associated with lower concentrations of H₂O₂, yet in this study this enzyme presented a higher activity in a scenario of ROS imbalance. In roots, only at 5 mg L⁻¹ MTF, APX activity presented significant differences over control, indicating that in this part of plants APX is expressed even under high ROS contents.

The activity of APX in leaves revealed an increasing pattern with MTF concentrations. For every day analysed, contaminated leaves showed significant difference over control which means that APX increase its activity probably due to the presence of H₂O₂, working to achieve ROS balance and cell homeostasis. On day 15, for 0.1 and 1 mg L⁻¹ MTF concentrations, a slight decrease in APX activity was detected compared to day 8. These results may be a response to the decrease of H₂O₂ concentration on day 15. Two explanations may be given to this behaviour. First, due to the lower concentration (compared to day 8) of H₂O₂ this enzyme decreases its activity meaning that CAT and GPOD were responsible for the scavenging of H₂O₂ on day 15. Secondly, despite the slight decrease in these two days, it is yet possible to observe the increase pattern among concentrations, which may indicate that MTF influenced the activation of APX in leaves under higher concentrations of MTF for longer times of exposure.

Regarding APX and H₂O₂ contents in roots and leaves Figure 5.14 shows a correlation between these two parameters.

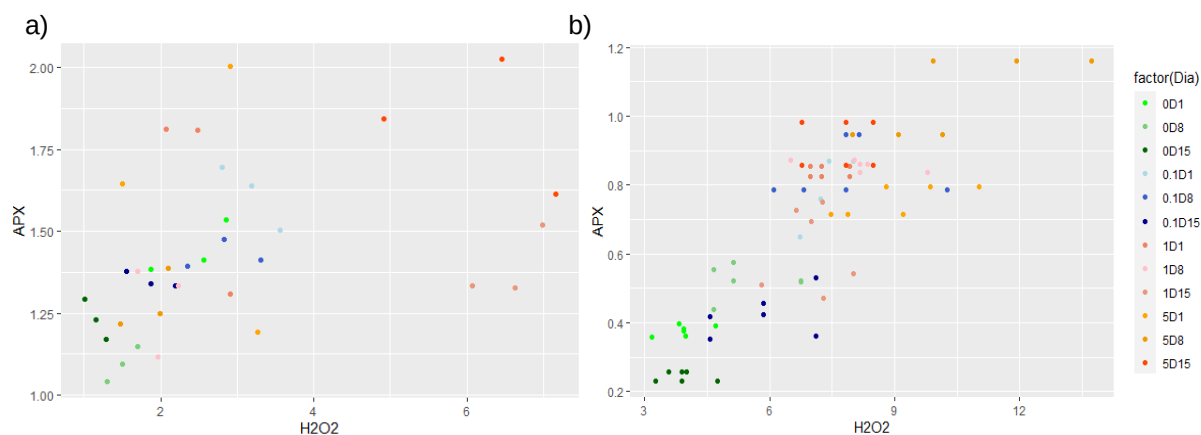


Figure 5.14. - Correlation analysis between APX and H₂O₂ in roots (a) and leaves (b) of *Lactuca sativa* under contamination with MTF for up to 15 days.

Hereby, it is possible to observe that the higher activities of APX occurred in leaves under 5 mg L⁻¹ being coincident with higher contents of H₂O₂. In any case, it is important to highlight that at a medium concentration of H₂O₂ (between 2 and 4 µg g⁻¹ FW in roots, and 6 and 9 µg g⁻¹ FW in leaves), APX suffers a slight increase, which may be representative of its relevance on oxidative response, in a comparison with lower and higher concentrations of H₂O₂.

Previous studies presented similar results regarding APX (increase pattern) in *Oryza sativa* under imidacloprid (Sharma *et al.*, 2013) and in *Vitis vinifera* under chlorothalonil treatment (Wang *et al.*, 2017). In studies with other pharmaceuticals as diclofenac and acetaminophen, APX increased its activity on plants of *Typha latifolia* and *Brassica juncea* (Bartha *et al.*, 2014, 2010).

This activity of APX could be expected to affect the AsA pools, so the contents of AsA in roots and leaves were measured and are presented in Figure 5.15.

In roots, despite the variations on APX activity on day 15, AsA content did not show significant differences, neither in time of exposure nor contaminations, meaning that MTF did not interfere with AsA pools.

Results of AsA content in leaves remained in accordance with the activity of APX, in almost all times of exposure. For instance, high activity of APX on day 8 represent lower concentrations of AsA for the same day. On day 15, leaves under 5 mg L⁻¹ revealed a higher activity on APX while AsA decrease its contents. In the meantime, control showed higher values of AsA contents than in contaminated leaves (and lower APX activity).

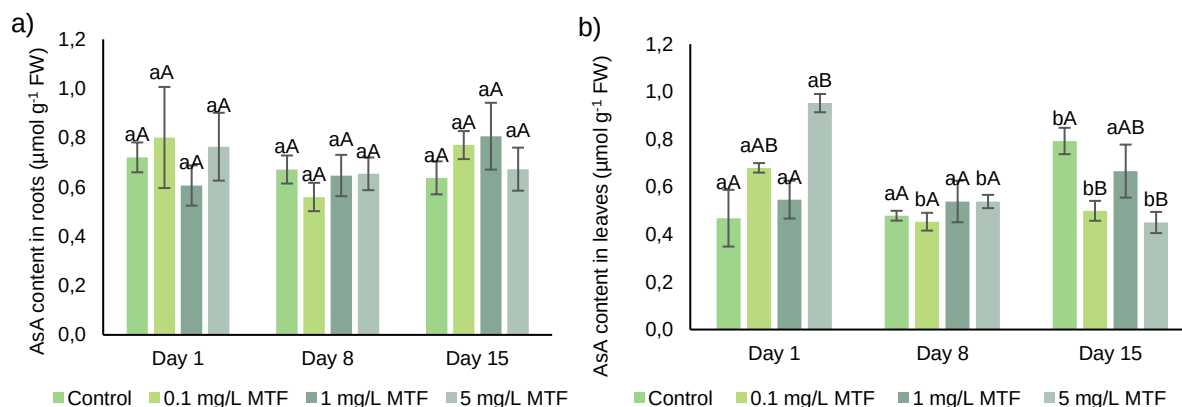


Figure 5.15. - Ascorbate content ($\mu\text{mol g}^{-1}$ FW) in roots (a) and leaves (b) of *Lactuca sativa* under contamination with MTF for up to 15 days. Different uppercase letters represent significant differences between different MTF concentrations at the same time point and different lowercase letters represent significant differences between treatment duration for the same MTF concentration.

AsA levels may be linked with GSH pools throughout ascorbate-glutathione cycle. GSH is used as substrate by DHAR enzyme in order to produce AsA. This equilibrium may explain the lower values of GSH. However, in this study DHAR activity was not measured so specific conclusions regarding this enzyme cannot be given.

Enzymes responsible for H_2O_2 scavenging may be responsible for the decrease on its contents in cells. However, high contents of H_2O_2 may also induce higher activities of enzymes such as CAT, GPOD or APX. In this study, the first option is observed on day 15, while on the other days high contents of H_2O_2 induced higher activities of scavenging enzymes. It is possible to say that different pathways of ROS removal may be activated on different periods of exposure, so for longer days of exposure defence mechanisms are activated by cells in order to adapt and acclimate to the stress caused by MTF presence.

Results reported by Ahammed *et al.* (2012) revealed an increase in SOD activity in leaves of tomato plants under phenanthrene, and a decrease in roots. These authors reported significant differences also in other enzymes such as CAT, GPOD, APX, GR and PPO in roots and leaves of tomato plants. In any case, it is important to highlight that both organic compounds have different structures compared to MTF, however similar trends may be observed in what concerns the stress caused by organic compounds.

Lettuce “Maravilha Quatro Estações” is mainly characterized by its red leaf due to anthocyanins contents. Therefore, determinations regarding secondary metabolism and anthocyanins production were also made in this study.

Anthocyanins are frequently addressed as good indicators of oxidative stress. These pigments play a role in protection against oxidative species, being involved in detoxification of ROS such as $O_2^{\bullet-}$ e H_2O_2 (Gould *et al.*, 2002; Sharma *et al.*, 2019).

Anthocyanin contents were influenced by concentrations of MTF and along time of exposure (Figure 5.16). The concentration of anthocyanins showed different patterns over time but a significant increase was only evident on day 15 on leaves under 5 mg L⁻¹ MTF.

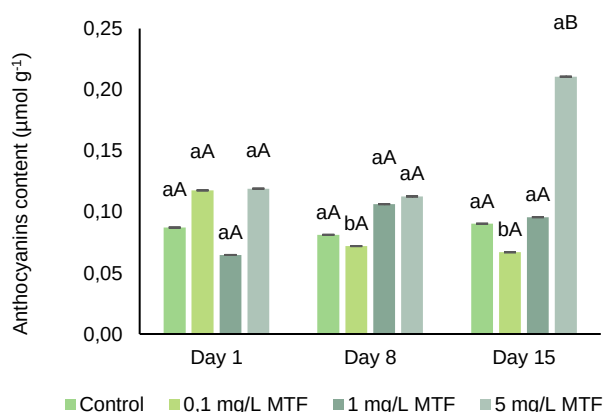


Figure 5.16. - Anthocyanin contents ($\mu\text{mol g}^{-1}$ FW) in leaves of *Lactuca sativa* under contamination with MTF for up to 15 days. Different uppercase letters represent significant differences between different MTF concentrations at the same time point and different lowercase letters represent significant differences between treatment duration for the same MTF concentration.

In fact, despite the observed increase concentration of anthocyanins, only small spots of anthocyanin development were visually detected, with no changes on total leaves coloration (Figure 5.17). This result indicates that for longer periods of exposure to MTF (> 15 days), probably more anthocyanins are produced.

This type of output might be associated with higher concentrations of H_2O_2 in cells, being a later response to the oxidative stress caused by ROS in general. Anthocyanins can also participate in lipid peroxidation inhibition (Mulabagal *et al.*, 2010). Therefore, the increase verified for 5 mg L⁻¹ on day 15 may be a response to the increase of lipid peroxidation. Despite the values of anthocyanins contents on days 1 and 8, on 1 and 5 mg L⁻¹ concentrations, no significant differences were observed.

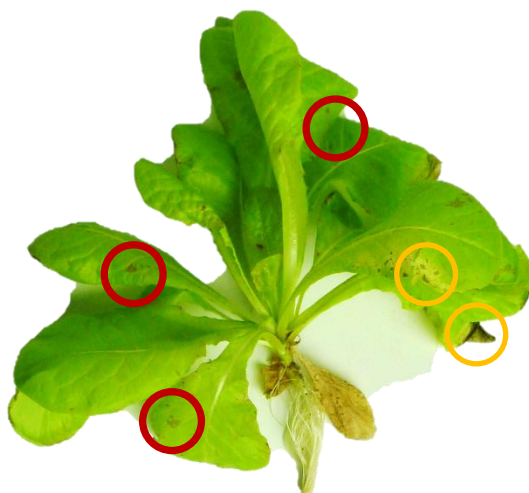


Figure 5.17. – Lettuce plant grown for 15 days under 5 mg L⁻¹ MTF showing anthocyanins spots (red circles) and leaf damage (orange circles).

PAL activity was also determined, as this enzyme is responsible for the production of cinnamic acid, which is part of phenylpropanoid metabolism that is the pathway related to anthocyanins synthesis. Therefore, its activity is intimately correlated with anthocyanins content. Results of PAL activity are shown in Figure 5.18.

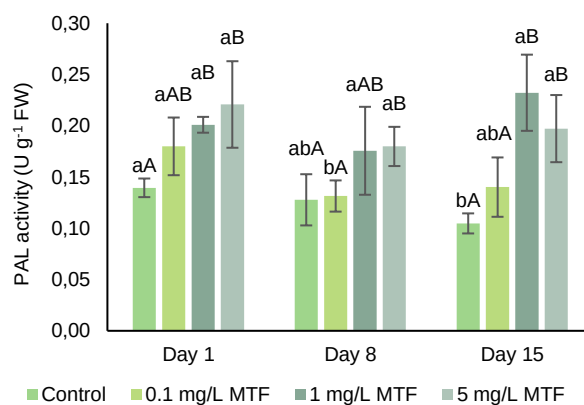


Figure 5.18. - PAL activity (U g⁻¹ FW) (b) in leaves of *Lactuca sativa* under contamination with MTF for up to 15 days. Different uppercase letters represent significant differences between different MTF concentrations at the same time point and different lowercase letters represent significant differences between treatment duration for the same MTF concentration.

PAL showed significant differences on day 1 for 1 and 5 mg L⁻¹ MTF, on day 8 for 5 mg L⁻¹, and on day 15 for 1 and 5 mg L⁻¹ MTF. According to these results, it is possible to say that MTF is inducing the activity of PAL, and it is the highest concentration of MTF that strongly affects the activity of this enzyme, and possibly the production of anthocyanins.

These metabolites are frequently associated with response to abiotic stress since they can act as ROS scavengers, photoprotectants and stress signalling molecules (Kovinich *et al.*, 2015). Besides, other authors reported already some correlation among anthocyanins content and abiotic stresses, caused by pesticides or insecticides. Mahdavi *et al.* (2015) suggest that the increase of phenolic compounds may contribute to a higher tolerance of plants to pesticides toxicity and in the case of insecticides, the increase of anthocyanins content will promote a higher efficiency of photosynthetic activity (Sharma *et al.*, 2016).

Figure 5.19 shows a correlation analysis between PAL activity and anthocyanins contents. It is possible to observe that for the concentration of 5 mg L⁻¹, the activity of PAL shows a trend to increase together with the anthocyanins content, and higher values for both parameters were observed for the 15 days of exposure.

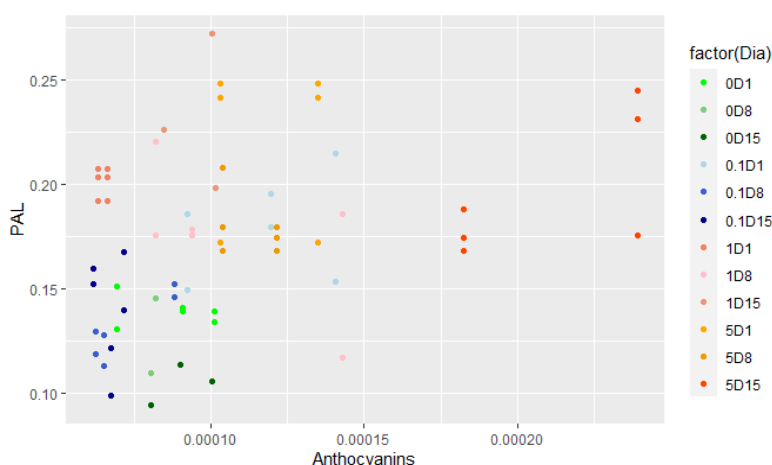


Figure 5.19. - Correlation analysis between anthocyanin content and PAL activity in leaves of *Lactuca sativa* under contamination with CBZ for up to 15 days.

Contents of polyphenol as well as the total antioxidant activity are presented in Figure 5.20. Despite the main changes in the activity of antioxidative enzymes, these two parameters did not show significant differences. Antioxidant activity showed a decrease on last day of exposure, however only in leaves under 1 mg L⁻¹ MTF.

To correlate different response from metabolic functions regarding oxidative stress, enzymes were analysed according to time and concentration of MTF in a Principal Component Analysis (PCA). In both PCA (roots and leaves) results are grouped according to contamination (Figure 5.21). In roots it is possible to observe that the most contaminated roots are grouped in one class (green) showing a clear differentiation to the other conditions, like the control. In leaves,

the same class combination may be observed, and the control samples remain all in one class (green), being possible to observe a clear separation among contaminations. In fact, previous results and analysis revealed that in leaves the oxidative response was more pronounced when compared to roots. This may be explained by the possible translocation and consequently accumulation of MTF to leaves.

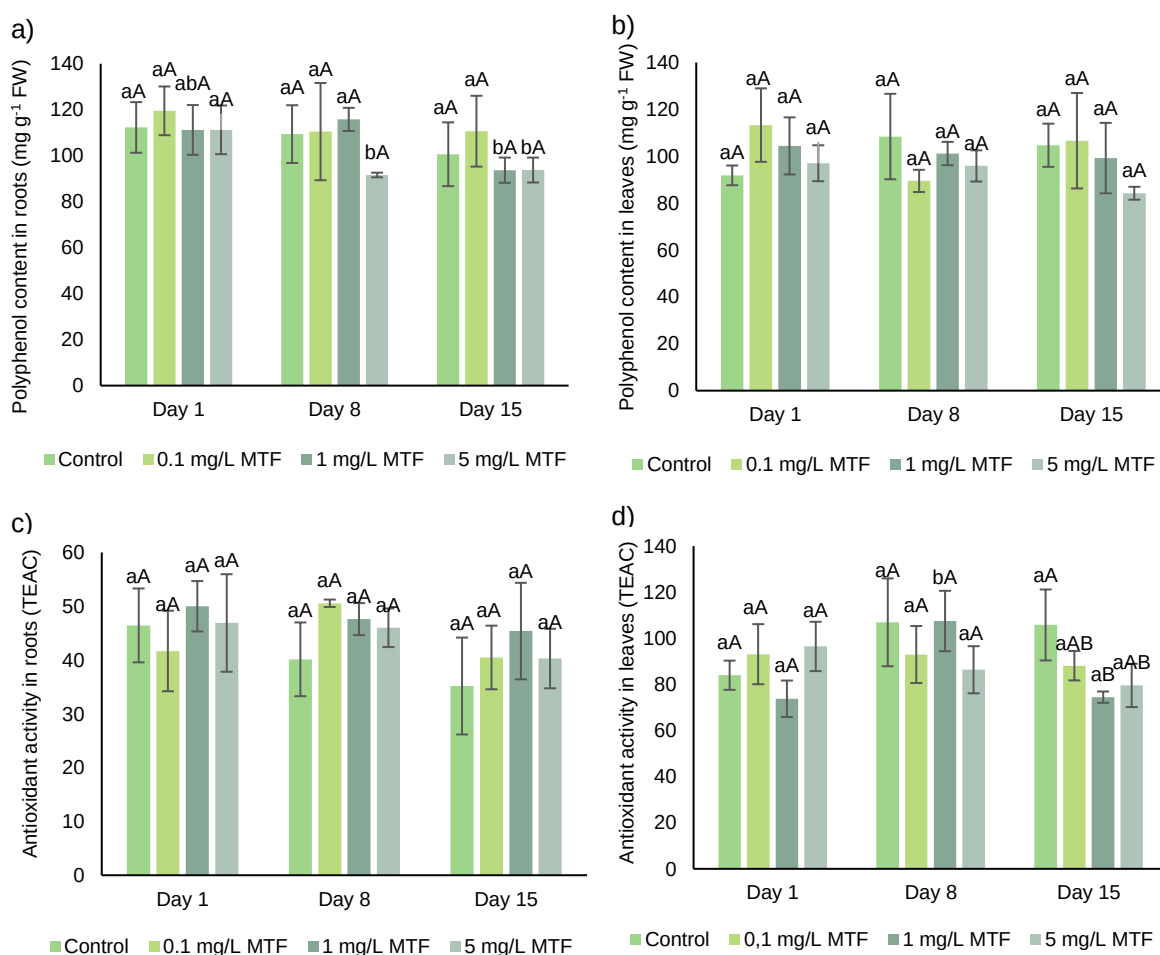


Figure 5.20. - Polyphenols content (mg g⁻¹ FW) in roots (a) and in leaves (b) and total antioxidant activity, expressed as TEAC, in roots (c) and leaves (d) of *Lactuca sativa* under contamination with MTF for up to 15 days. Different uppercase letters represent significant differences between different MTF concentrations at the same time point and different lowercase letters represent significant differences between treatment duration for the same MTF concentration.

An overall analysis of enzymes activity and time of exposure is presented on Figure 5.22.

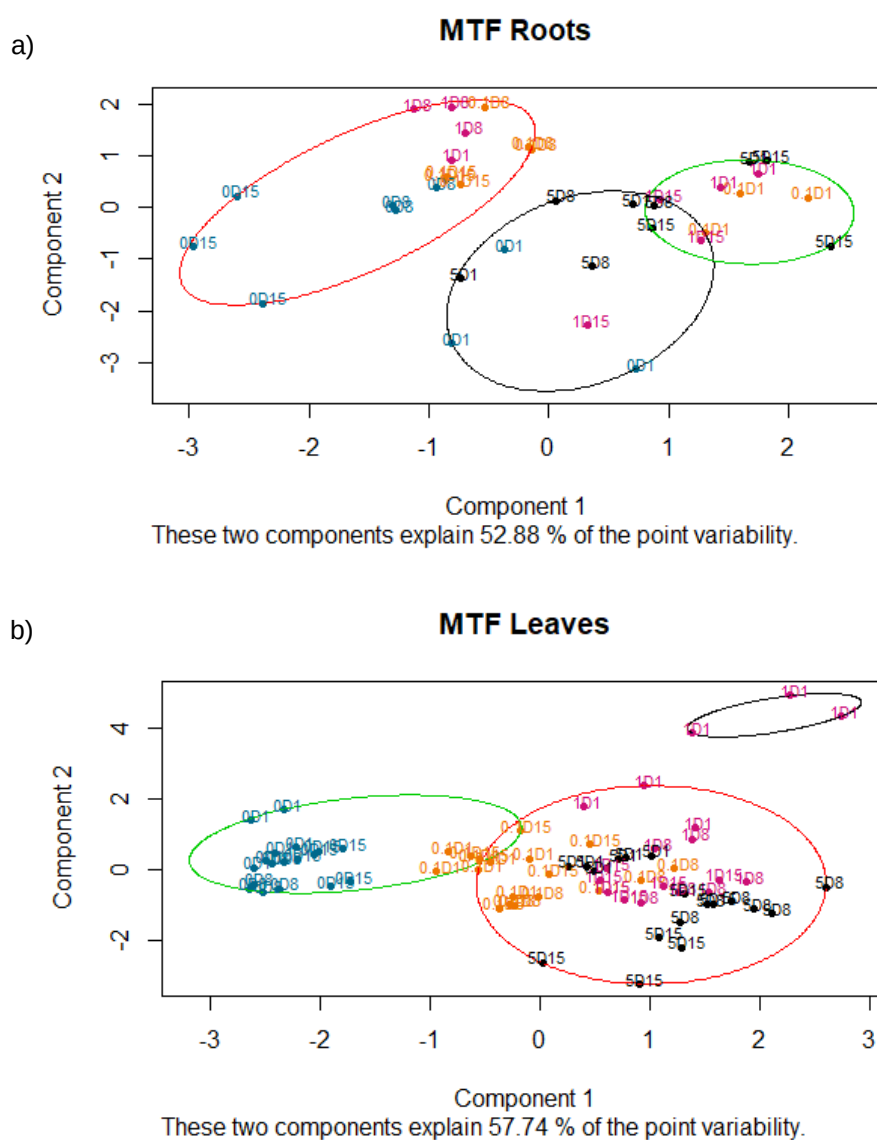


Figure 5.21. - Principal Component Analysis (PCA) of enzymatic activities (CAT, SOD, GPOD, APX, GR, GPX) in roots (a) and leaves (b) of *Lactuca sativa* under contamination with CBZ for up to 15 days. Legend: Control – blue dots, 0.1 mg L⁻¹ CBZ – orange dots, 1 mg L⁻¹ CBZ – pink dots and 5 mg L⁻¹ CBZ – black dots.

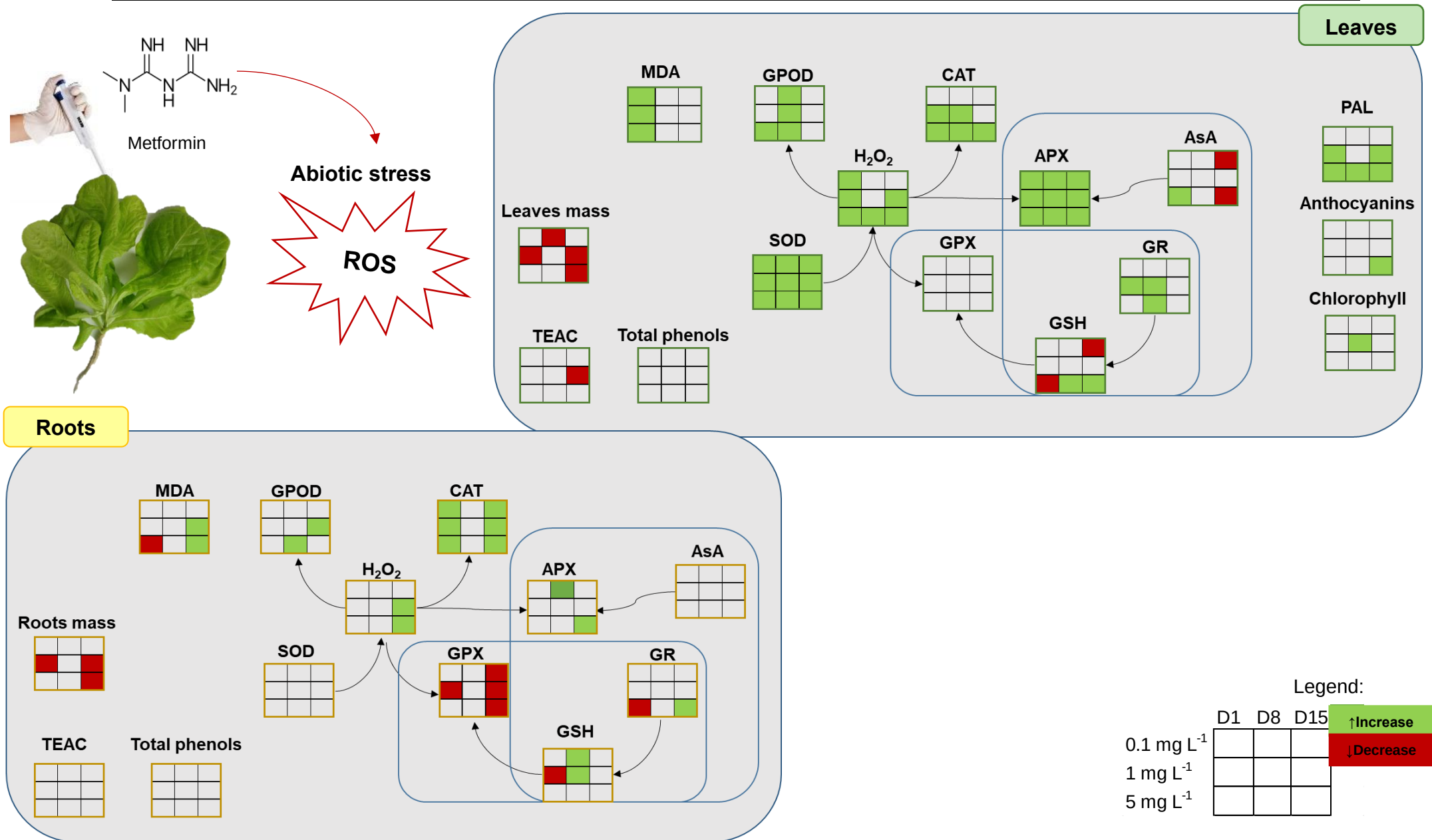


Figure 5.22. - Final scheme with significant differences to control over different defence mechanisms against oxidative stress caused by MTF. Significant differences from control are represented in green (increase) and red (decrease) squares.

5.3.4. Mineral elements analysis

The changes in the contents of mineral elements may influence different cell metabolism processes, growth, and normal development of lettuce. MTF affected the concentrations of some macro and micronutrients as can be seen in Table 5.1. Copper, zinc and calcium showed significant variations in radicular tissue compared to control, mainly on day 8 of exposure. On the other hand, in leaves these differences were found in manganese, zinc, magnesium and sulphur. The specific values of the measured concentrations of each element are presented in the Appendix (Table A.3).

In general, MTF contamination did not affect K contents, showing no significant differences along time and in relation to control roots and leaves. Potassium performs several biological functions in cells, enhancing damage repair, regulating protein synthesis, promoting the synthesis of secondary metabolites, among other functions (Wang *et al.*, 2013). Several enzymes are activated by this element like the ones related with C-metabolism such as pyruvate kinase or ADP-glucose starch synthase (Marschner, 1995). Also, K homeostasis will ensure transport of anions such as NO_3^- for shoots (Maathuis, 2009). Along time, K tends to increase on control leaves and roots. For longer periods of exposure to MTF (1 and 5 mg L^{-1}) a slight, non-significant, decrease is observed.

Under MTF contamination, a decreasing pattern was observed on day 1 in both tissues on Cu contents for 1 and 5 mg L^{-1} MTF revealing significant differences in some concentrations and days of exposure (5 mg L^{-1} in leaves on day 1, 1 and 5 mg L^{-1} in roots on day 8). This element is one of the cofactors responsible for SOD activity. In leaves, this enzyme increased its activity for all applied concentrations of MTF. Therefore, the decrease on Cu contents did not directly affect the availability of Cu as SOD cofactor. Some authors have suggested an affinity of MTF to Cu ions, which may influence the normal uptake of Cu (Logie *et al.*, 2012). On the other hand, Cu transporters, such as natural resistance - associated macrophage proteins (NRAMP) are also shared with other elements (Zn and Fe), which may reduce the entrance of copper in root cells.

MTF presence seems to improve the uptake of Mn in roots, and its translocation to leaves. An increase was observed in both tissues, but it was only statistically significant in leaves. Manganese is required for several cell processes such as photosynthesis, respiration, and ROS scavenging. Oxygen-evolving in photosystem II, MnSOD and oxalate oxidase are exclusively dependent of Mn availability (Alejandro *et al.*, 2020). Once again, the high activity of SOD may be related with the availability of Mn as a cofactor. On the other hand, oxalate oxidase catalyses the oxidation of oxalate to CO_2 reducing O_2 to H_2O_2 , contributing to the

increase of this ROS in cells. In fact, on day 8 an increase of H_2O_2 was observed in leaves under 0.1, 1 and 5 mg L^{-1} , agreeing with significant increases observed in Mn contents. Manganese also plays an important role as cofactor of PAL, which may be related with higher activity of this enzyme on contaminated leaves.

No significant differences to control were observed in Fe concentration in roots. Therefore, it is possible to state that MTF did not interfere with Fe uptake. In leaves, a slight decrease was observed on day 15 on 1 mg L^{-1} concentration, considered statistically significant.

Concentrations of Zn increased in roots and leaves under MTF contamination. Significant differences were observed on day 8 for all MTF concentrations in roots, while in leaves were observed on days 1 and 8, but only at 1 and 5 mg L^{-1} . Zn is a micronutrient, which is absorbed by roots in the Zn^{2+} form or in complexes. Zinc regulated transporter (ZIP) family proteins are the main responsible for Zn transport, together with Heavy Metal Transporting ATPases (HMA) family, all these proteins are common to other elements such as Cu (Milner *et al.*, 2013; Sturikova *et al.*, 2018). Therefore, a competition among these two elements may occur affecting Cu uptake in both tissues. Zinc is essential for the activation of different enzymes responsible for the response to toxic effects, such as RNA polymerase, SOD, alcohol dehydrogenase and carbonic anhydrase (Palmer *et al.*, 2009). The increase in the expression of SOD could lead to increased uptake of Zn, to activate the relevant isoforms of this enzyme.

Sodium is absorbed by roots throughout mediation of different transporters such as non-selective cation channels (NSCCs), NHX, salt overly sensitive (SOS) and plant high-affinity potassium transporters (HKT) (Yamaguchi *et al.*, 2013). Due to its chemical similarity to K, it is common to assume that these two elements compete in roots uptake (Subbarao *et al.*, 2003). Most plants (C_4 and CAM) use Na on carbon fixation processes, and Na stimulates growth and enhances cell expansion (Taiz *et al.*, 2014). MTF appeared to affect the contents of Na in both tissues, with more expression in roots. Significant differences were observed on days 8 and 15 in roots under 0.1, 1 and 5 mg L^{-1} . In leaves, significant decreases were observed in 1 and 5 mg L^{-1} conditions in day 8.

MTF showed some influence on the decrease of Ca contents in roots and an increase in leaves. Calcium is a macronutrient, being a constituent of cell wall contributing to membrane stability (Thor, 2019). Some authors described that Ca may alleviate the effects on lipid peroxidation caused by abiotic stresses, such as heavy metal exposure (Wang *et al.*, 2009). Regarding roots, the low Ca concentrations could influence lipid peroxidation, which showed higher values for longer periods of exposure to MTF. In leaves under MTF exposure, an increase of calcium content occurred on days 1 and 8 for 5 mg L^{-1} MTF. This could be due to some defence mechanism or to a disturbance of the uptake processes. These values are

followed by the high translocation factor observed in these days for Ca uptake. However, at the 15th day, significant differences on Ca content were not visible and presumably an equilibrium was reached.

Magnesium is involved in different cell processes such as photosynthesis, enzymes activity as a cofactor, respiration, protein synthesis and stabilisation of acid nucleic and nucleotides (Maathuis, 2009). The contamination with MTF did not show significant differences on Mg concentrations in roots. In leaves, significant differences were observed on days 1 and 8 with an increase of Mg concentrations in leaves under 1 and 5 mg L⁻¹. The increase in Mg translocation may be due to a defence mechanism to protect the photosynthetic apparatus (which indeed was not negatively affected as seen above).

Phosphorous content was only slightly affected by MTF, with a decrease in leaves for day 1 (5 mg L⁻¹) and day 8 (1 mg L⁻¹). Phosphorus is absorbed by roots as inorganic form (PO₄³⁻) throughout high affinity with H⁺ transporters. Phosphorus transporters are mainly represented by phosphate transporter (PHT) family, subdivided in five cluster (Zhang *et al.*, 2016). These transporters were apparently not affected by MTF as P content in the roots did not change in relation to the control (proteomic analysis). Phosphorous is an important constituent of lipid membrane and plays a key role on photosynthesis. Besides, P is an important element on the coordination of some kinases and phosphatases proteins (Maathuis, 2009). In leaves, several proteins related with phosphorylation processes were identified as less abundant in leaves under MTF contamination, such as GTP-binding nuclear protein, which may reflect P lower concentrations on day 8 and 1 mg L⁻¹ (see Chapter VI).

Regarding S contents, MTF did not affect the concentrations of S in roots, meaning that S uptake from nutrient solution was not compromised. Interestingly, in leaves an increase S contents occurred in certain treatments, mainly for the higher MTF concentrations. According to proteomic analysis (Chapter VI), a specific protein sulfurtransferase showed higher abundance in MTF condition over control. This protein is associated with molecular function of thiosulfate sulfurtransferase activity and biological processes of response to oxidative stress, which may be a mechanism of response to MTF exposure. Sulphur is also an essential element in the synthesis of GSH, and the increase in S uptake by lettuce for higher MTF concentrations may be explained by the need to synthesize more GSH as a response mechanism (Maathuis, 2009). It is interesting to highlight that GSH contents in leaves presented an increasing tendency which may be related with S increase found in leaves.

In Table 5.2 the translocation factor for each element is presented. Potassium, Ca and Mg were detected in higher contents in leaves, while all the other elements content remain superior in radicular tissue.

Table 5.1- Mineral element content variations in comparison to control for every concentration of MTF in roots and leaves of *Lactuca sativa* up to 15 days of exposure. Significant differences are represented in red (decreases) and green (increases) marks.

		Cu			Mn			Fe			Zn			Na			K			Ca			Mg			P			S		
		Day 1	Day 8	Day 15	Day 1	Day 8	Day 15	Day 1	Day 8	Day 15	Day 1	Day 8	Day 15	Day 1	Day 8	Day 15	Day 1	Day 8	Day 15	Day 1	Day 8	Day 15	Day 1	Day 8	Day 15	Day 1	Day 8	Day 15	Day 1	Day 8	Day 15
MTF	Leaves	0.1 mg L ⁻¹	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
		1 mg L ⁻¹	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
		5 mg L ⁻¹	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	Roots	0.1 mg L ⁻¹	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
		1 mg L ⁻¹	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
		5 mg L ⁻¹	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—

Table 5.2 - Translocation factor (TF) of mineral content in roots and leaves of *Lactuca sativa* under MTF contamination up to 15 days of exposure. Brown represent highest accumulation in roots and green represents highest accumulation in leaves.

MTF		Cu			Mn			Fe			Zn			Na			K			Ca			Mg			P			S		
		Day 1	Day 8	Day 15	Day 1	Day 8	Day 15	Day 1	Day 8	Day 15	Day 1	Day 8	Day 15	Day 1	Day 8	Day 15	Day 1	Day 8	Day 15	Day 1	Day 8	Day 15	Day 1	Day 8	Day 15	Day 1	Day 8	Day 15	Day 1	Day 8	Day 15
TF	Control	0.45	0.36	0.67	0.45	0.52	0.79	0.03	0.04	0.03	0.24	0.28	0.34	0.47	0.42	0.53	1.11	1.04	1.14	2.27	1.73	3.07	2.43	2.29	3.09	0.65	0.68	0.66	0.35	0.36	0.47
	0.1 mg L ⁻¹	0.41	0.33	0.55	0.45	0.60	0.66	0.04	0.04	0.03	0.24	0.25	0.32	0.47	0.44	0.60	1.03	0.93	1.15	2.24	2.31	3.20	2.73	2.55	2.99	0.61	0.57	0.60	0.34	0.36	0.44
	1 mg L ⁻¹	0.43	0.40	0.69	0.48	0.67	0.59	0.04	0.03	0.02	0.27	0.28	0.32	0.50	0.43	0.71	1.05	1.00	1.24	2.39	2.43	3.24	2.86	2.86	2.89	0.62	0.58	0.59	0.39	0.42	0.45
	5 mg L ⁻¹	0.35	0.40	0.70	0.49	0.71	0.79	0.05	0.04	0.03	0.28	0.29	0.35	0.53	0.47	0.90	0.98	1.05	1.19	2.78	3.15	3.34	2.84	3.44	3.56	0.61	0.64	0.68	0.38	0.45	0.55

5.4. Conclusions

The study of the influence of MTF in lettuce involved a complete analysis of oxidative stress. MTF is frequently found in rivers and streams, which may consist in a possible pathway of contamination into soil and food crops.

Stress indicators as H_2O_2 content and MDA revealed more differences for longer periods of exposure. Despite this, no changes in leaf biomass were observed, suggesting that the possible uptake of metformin does not significantly affect leaf development, however in roots some differences were observed for the longest day of exposure.

The overall antioxidative response to MTF exposure revealed a joint action of ROS scavenging enzymes. CAT and GPOD worked together in H_2O_2 scavenging, over time in both tissues. SOD revealed higher activity in leaves, suggesting the removal of superoxide radical. GSH enzymes exhibited more differences in roots tissues, suggesting that the direct contact with the contaminant may impact the GSH cycle. APX and AsA pools presented significant differences, more enhanced in leaves than in roots. These results also suggest an involvement in ROS detoxification, trying to maintain cell redox state.

MTF did affect the contents of some minerals, in leaves and roots. In leaves, Mn, Zn, Mg and Ca were present in higher contents than control, in certain times of exposure. In roots, decreases in Cu and Ca contents were observed. In general, it is possible to say that the uptake of MTF did not affect the translocation of essential elements in lettuce plants.

CHAPTER VI

Stress response of lettuce to environmental contamination with selected pharmaceuticals: A Proteomics study

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Chapter VI: Stress response of lettuce to environmental contamination with selected pharmaceuticals: A Proteomics study.

Abstract

Pharmaceutical compounds have been found in rivers and in treated wastewaters. They often pass to irrigation waters and consequently edible vegetables, causing changes in plants metabolism.

The main objective of this work is to understand how lettuce plants cope with the contamination of three selected pharmaceuticals using a label free proteomic analysis.

A lettuce hydroponic culture, grown for 36 days, was exposed to metformin, acetaminophen and carbamazepine (at 1 mg L⁻¹), for 8 days, after which roots and leaves were sampled and analysed using a LC-MS/MS proteomic approach.

In roots, a total of 612 proteins were differentially expressed while in leaves 237 proteins were identified with significant differences over controls. Carbamazepine was the contaminant that most affected proteins abundance in roots, while in leaves the highest number of differentially accumulated proteins was observed for acetaminophen. In roots under carbamazepine, stress related proteins such as catalase, superoxide dismutase and peroxidases presented higher abundance. Ascorbate peroxidase increased in roots under metformin. Contaminants influenced the abundance of aquaporins which may be related with possible H₂O₂ transports or detoxification. The results achieved with this study contribute to an overall interpretation of detoxification and induced oxidative stress pathways in lettuce plants under pharmaceuticals contamination.

Keywords: Lettuce, proteomics, stress proteins, pharmaceuticals.

6.1. Introduction

Proteomics is considered one part of the omics approach (together with genomics, transcriptomics, proteomics and metabolomics) and allows a full characterization of proteins providing not only a qualitative approach but also quantitative results. These outputs provide knowledge regarding different processes involved in DNA, RNA, proteins and metabolites levels and how they can be affected by different stressors (Chen *et al.*, 2006; Witzel *et al.*, 2015).

The introduction of omics in plants contamination and stress response analysis is presently an extensively used tool (Chen *et al.*, 2006). This technology provides new dimensions about proteins types and expression on different stages of plants development and under different environmental pressures. Plants are constantly subjected to environmental conditions which sometimes cause a negative impact on its metabolism. A lot of work has already been performed in relation to crops adaptation to drought, flood, extreme temperatures or exposure to contaminants (Foyer *et al.*, 2016; Mittler, 2006; Soares *et al.*, 2019). Therefore, the knowledge of proteins that have a role on stress response and plants acclimation is essential to evolve and improve practices in agriculture and food production (Agrawal *et al.*, 2012).

Sample preparation is one of the major obstacles of proteomic analysis. The quality, the type of tissue and the interference of other compounds such as polyphenols, carbohydrates, starch, pigments and polysaccharides have a high impact on proteomics procedure and results (Chen *et al.*, 2006). Protein extraction from plants tissue is frequently divided into three steps: precipitation (remove the major part of interferences), solubilisation (extract the major number of proteins from tissue) and protein rehydration (improve protein quality for the analysis) (Timperio *et al.*, 2008). Figure 6.1 shows a schematic overview of proteomic analysis and its main areas of action.

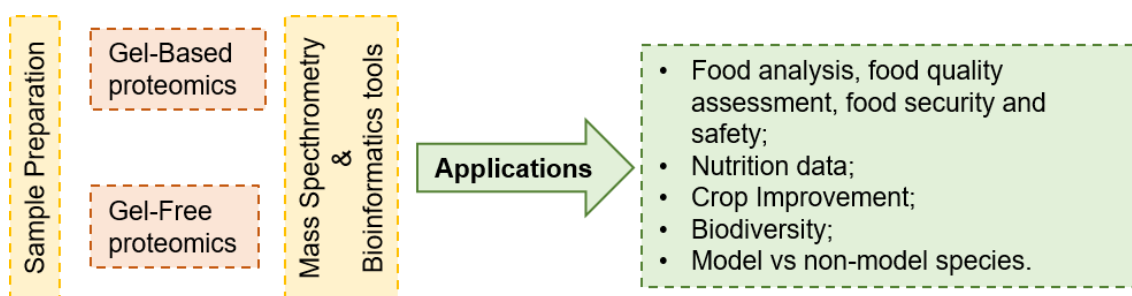


Figure 6.1. - Proteomics workflow and applications. Adapted from Agrawal *et al.* (2012).

Lettuce (*Lactuca sativa*) is one of the most consumed leafy vegetables worldwide and can easily be subjected to different abiotic stresses like water deficit, pathogens, heavy metals, salinity and pharmaceutical products, among other factors (Galieni *et al.*, 2015; Jiménez-Arias *et al.*, 2019; Kalhor *et al.*, 2018; Kolahi *et al.*, 2020). It is also considered a model plant and has thus been used as a bioindicator or sentinel species in studies concerning the effect of different contaminants (Carvalho *et al.*, 2019; Charles *et al.*, 2011; dos Santos *et al.*, 2019).

Although several references may be found in the literature regarding oxidative stress induced by contaminants in lettuce (Kolahi *et al.*, 2020; Martins *et al.*, 2011; Sofo *et al.*, 2017), there is still a generalized lack of information concerning the detoxification response of plants to specific pharmaceutical products. The mechanisms related to this metabolic response are in fact very diverse and depend on different factors, such as plant species, tolerance to contaminant, developmental stage, type of contaminant and its persistence (Kolahi *et al.*, 2020), among many others.

MTF is used as therapeutic treatment for type 2 diabetes adult patients. The occurrence of this pharmaceutical in the environment has already been reported, for instance, in Canada (Blair *et al.*, 2013) and in Germany (Trautwein *et al.*, 2014), where MTF was detected in surface and drinking water. The uptake of this pharmaceutical by plants was addressed by Eggen *et al.* (2012) who described the translocation of MTF to the edible part of different cultures (beans, potatoes, tomato and carrots). ACT is an extensively used analgesic and antipyretic. Regarding plant uptake, literature described the translocation of ACT from roots to shoots of radish (Li *et al.*, 2019). Additionally, Chuang *et al.* (2019) described the accumulation of this pharmaceutical in lettuce tissues. CBZ is an anticonvulsant reported already as a pharmaceutical with high occurrence in environment and Chuang *et al.* (2019) showed the effects of CBZ uptake in lettuce plants.

These compounds have been reported as being present in different ecosystems (rivers, soils, groundwater) in different European countries such as Portugal, Germany, France, Luxembourg, Spain and Poland (Carvalho *et al.*, 2016; Wu *et al.*, 2015). As such, the exposure of edible vegetables to this type of contaminants will likely become a problem, not only to the food chain but also to the environment.

Numerous tools have been developed regarding the study of abiotic stresses, particularly oxidative stress in plants as a consequence of the imbalance of ROS and the resulting oxidative damage. The main regulators of oxidative stress are antioxidant enzymes such as CAT, SOD, peroxidases (POX) and GSH and AsA as non-enzymatic antioxidants (Mourato *et al.*, 2012; Noctor *et al.*, 2015; Pinto *et al.*, 2017). In fact, plant stress is a dynamic process with

different phases according to the adaptation level of the plant: first an alarm phase occur, followed by acclimation, maintenance and exhaustion phases (Kosová *et al.*, 2011).

To study plants stress, different methods have been recently addressed, mainly using omics-based approaches. In particular, a proteomics-based approach with liquid chromatography coupled to mass spectrometry (LC-MS/MS) may provide detailed information about abundance and distribution of a plethora of proteins. As such, the identification and quantification of proteins involved in different metabolic pathways will provide detailed information about the changes during the response to environmental factors, such as abiotic or biotic stresses (Chen *et al.*, 2006), being able to highlight the major metabolic pathways triggered by such response and therefore contributing to understand the toxic effects of these compounds.

Under exposure to contaminants, plants activate their defence mechanisms against oxidative stress which will result in the production of signalling molecules and stress related proteins, namely heat shock proteins (HSP) and other proteins responsive to ROS. Proteome analysis of plants (e.g. tomato, maize, poplar, salicornia, grapevine) under mineral stress, water deficit, heavy metals content, salinity, herbicides contamination have already been reported by several authors using different proteomics approaches (Brumbarova *et al.*, 2008; Castro *et al.*, 2005; Kieffer *et al.*, 2008; Wang *et al.*, 2009; Zhu *et al.*, 2007).

This work aims to study the main metabolic pathways in lettuce tissues contaminated with three different pharmaceuticals (metformin, carbamazepine and acetaminophen) using a proteomic analysis. The main focus of this analysis will be the relation among stress related proteins and specific metabolic pathways regarding defence mechanisms. This work will contribute to improve current knowledge regarding the effect of lettuce contamination with these pharmaceutical compounds and for a better understanding of plant defence mechanisms against oxidative stress.

6.2. Material and Methods

Lettuce plants were germinated and grown as previously described (Sections 2.1 and 2.2), in grown media contaminated with 1 mg L⁻¹ of each pharmaceutical under study, carbamazepine (CBZ), acetaminophen (ACT) and metformin (MET). Samples were collected for proteomic analysis 8 days after contamination. Three pots were used for each concentration, with 12 plants each, for a total of 36 plants per pharmaceutical.

The following procedures were performed, all described in Chapter II Material and Methods:

- Lettuce production and sample collection (2.1. and 2.2.)
- Protein extraction and sample preparation (2.4.1.)
- LC-MS/MS (2.4.2.)
- Protein Identification (2.4.3.)
- Data analysis and interpretation (2.4.4.)

Data analysis is described in section 2.5 from Chapter II.

6.3. Results

Regarding lettuce growth and development, no statistical differences among control and MTF, CBZ or ACT contaminations were found in plant biomass (as can be seen in the previous three chapters). Some changes in the development of both the leaves and the roots were detected (Figure 6.2). With CBZ contamination, plants revealed darker and smaller roots as well as etiolated leaves. Plants exposed to ACT were more similar to control (leaves and roots), while plants with MTF showed less leaves, compared to control.

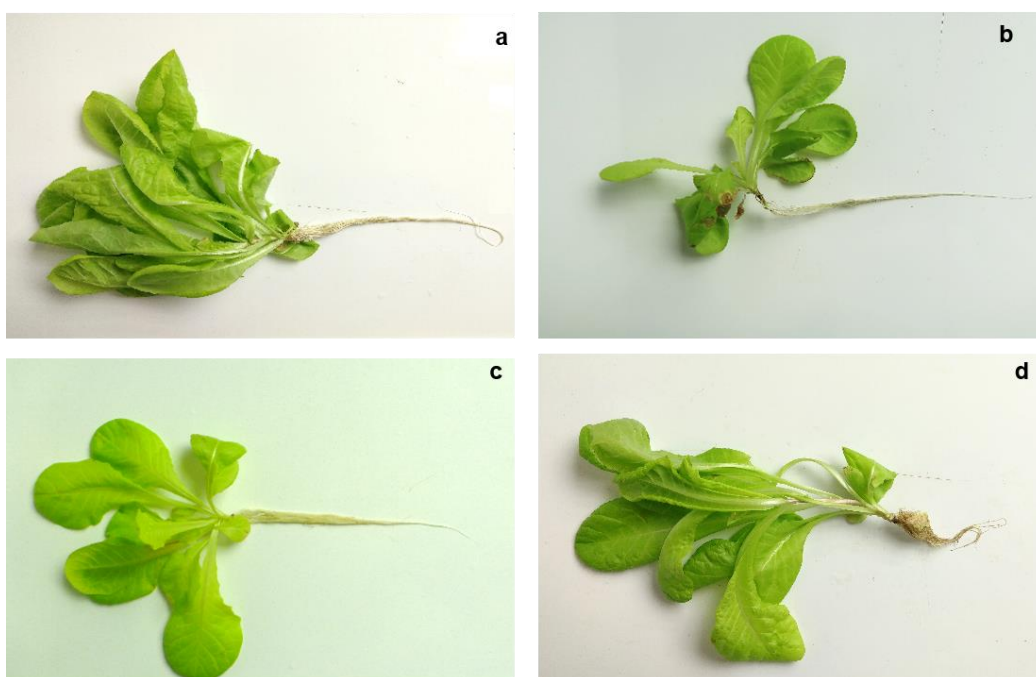


Figure 6.2. - Lettuce after 8 days of experiment: Control (a), 1 mg L⁻¹ MTF (b), 1 mg L⁻¹ ACT (c) and 1 mg L⁻¹ CBZ (d).

6.3.1. Differential protein expression

The list of selected proteins that were differentially expressed in contaminated plants are presented in Tables 6.1 to 6.6.

Contamination with Metformin: When comparing roots from control and MTF contaminated plants, 8 proteins were identified as significantly different while in leaves a total of 32 significant proteins showed differential abundance. In Tables 6.1 and 6.2, the most relevant proteins in relation to our study are presented. The protein identified as more expressed in roots for MTF

over control was Ras-related protein Rab11C. Additionally, other proteins remained more expressed in control than in MTF roots, with special focus on isocitrate dehydrogenase (IDH) and heat shock 70 kDa protein 3 (HSPs).

In leaves histones H2A and H2B, and photosystems II protein D1 presented the highest abundance in the control group. Among the proteins differentially expressed in the leaves of MTF group, sulfurtransferase, aquaporin PIP1, pectinesterase, ATP synthase subunit beta and rbsL (RuBisCo) revealed higher abundance in comparison to control.

Contamination with Acetaminophen: A total of 47 proteins were identified with significant differences concerning the roots of control and ACT conditions: 38 proteins with higher expression in control roots, and 9 proteins in the ACT contamination. In Tables 6.3 and 6.4, proteins of interest were summarized according to the main goal of our study. In the case of control, differentially expressed proteins include proteasome subunit beta, GLOBIN domain-containing protein and other proteins such as aquaporin PIP1-2, ribosomal_S13_N domain-containing protein, cytochrome c domain-containing protein, -succinate-CoA ligase and UDP-glucose 6-dehydrogenase. BZIP domain-containing protein, ABC transporter C family member 5, DUF3591 domain-containing protein and Hsc70 protein prevail in ACT roots over controls.

In leaves, plasma membrane ATPase, aconitase_C domain-containing protein, PKS_KS domain-containing protein, RuBisCO_large domain-containing protein and DEAD-box ATP-dependent RNA helicase 56 showed values of abundance at least 5 times higher in the controls (Table 6.4). Sulfite reductase 1 and MADS-box protein 2 reached at least 5 times more abundance in ACT contaminated leaves. Among those 17 proteins (Table 6.4), some proteins of interest such as malate dehydrogenase, diverse cytochrome P450 proteins, HSPs, aquaporin PIP2-2 or thioredoxin reductase showed statistically higher abundance in ACT than control.

Contamination with Carbamazepine: In roots, a total of 181 proteins revealed significant differences between the two conditions, control and CBZ. Taking into account the high number of identified proteins, Tables 6.5 and 6.6 indicate the most important to our study. Some of these enzymes are linked to oxidative stress and are important for our analysis. CBZ was the contamination that most influenced the expression of these proteins.

Among most upregulated proteins with differential expression in roots of CBZ contamination (Table 6.5) several HSPs and peroxidases, as well as IDH [NAD] subunit and SOD were identified.

A total of 43 proteins showed significant differences between control and CBZ leaves, and the most relevant ones are indicated in Table 6.6. Among them it is possible to find ribosomal

protein L19, pyruvate dehydrogenase E1 component subunit beta, DNA helicase, as well as thioredoxin domain-containing protein and malate dehydrogenase which remained more expressed in control leaves. Several proteins revealed more abundance in leaves under CBZ contamination within a range of max fold change (FC) between 1.5 and 36.3. Chlorophyll a-b binding proteins, RuBisCo large chain, GR and ascorbate L-oxidase were more expressed in contaminated leaves.

6.1.1. Protein profile characterization

The proteomic characterization and identification of specific proteins in plant samples allows a better understanding of the metabolic processes according to applied contaminants. In our study the number of identified proteins varied among tissues and among conditions. In detail, a total of 1240 and 919 proteins were identified for roots and leaves, respectively. Of these, 639 proteins for roots and 237 proteins for leaves were found to have differential accumulation. To perform a comparative analysis, the three contaminants were compared to the control taking into account the normalized abundance values.

These comparisons were established in order to characterize the contamination influence on the proteome. Figure 6.3 a/b shows that some proteins were expressed only in certain conditions in both tissues, and it is possible to observe that for each contaminant studied (MTF, ACT, CBZ) a different number of proteins were obtained, suggesting that different contaminations affect tissues possibly in different pathways. Regarding the influence of the contaminant on lettuce tissues, Figure 6.3 c/d shows the number of proteins up- and down-regulated when compared to control. Overall, Figure 6.3 shows that CBZ was the pharmaceutical that most influenced proteins expression and abundance in roots, while in leaves ACT revealed higher number of upregulated proteins, both in relation to control.

Considering the main objective of our study, some individual proteins related with stress response were selected. The mean abundance among conditions, in both tissues, is presented in Figures 6.4 (roots) and 6.5 (leaves).

Table 6.1 – Selected proteins upregulated (green) and downregulated (red) in roots contaminated with MTF. Legend: Max Fold Change reveals the trend among contamination and control leaves and roots for each protein.

Metformin: proteins expression in Roots						
Accession number	Protein name	Anova (p)	Max Fold Change (FC)	Control Mean	MTF Mean	
A0A2J6KXJ0_LACSA	Ras-related protein Rab11C*	0.0113	1.8	87.38	154.32	
A0A2J6KT66_LACSA	Isocitrate dehydrogenase [NADP]	0.0026	10.4	607.83	58.56	
A0A2J6LQ77_LACSA	Heat shock 70 kDa protein 3*	0.0269	3.6	3323.49	933.15	
A0A2J6LYZ4_LACSA	Leucine--tRNA ligase, cytoplasmic*	0.0267	37.5	224.87	6.00	
A0A2J6MG26_LACSA	Coatomer subunit beta'	0.0019	175.8	166.23	0.95	

Table 6.2 – Selected proteins upregulated (green) and downregulated (red) in leaves contaminated with MTF. Legend: Max Fold Change reveals the trend among contamination and control leaves and roots for each protein.

Metformin: proteins expression in Leaves						
Accession number	Protein name	Anova (p)	Max Fold Change (FC)	Control Mean	MTF Mean	
A0A2J6K5S3_LACSA	Aquaporin TIP1-1*	0.0190	2.5	3365.70	8459.03	
A0A2J6K8E6_LACSA	Sulfurtransferase	0.0007	11.7	1.55	18.16	
A0A2J6KJN7_LACSA;A0A0K0M2A2_9ASTR;	ATP synthase subunit beta	0.0236	2.6	139.23	364.88	
A0A2J6LPS1_LACSA	UDP-glucose 6-dehydrogenase	0.0371	1.5	721.22	1095.65	
A0A2J6KL66_LACSA	Pectinesterase	0.0172	4.0	19.33	76.71	
A0A2J6LII2_LACSA	Ribulose biphosphate carboxylase large chain (Fragment)	0.0009	3.2	454.67	1445.50	
S5RCK4_9CHLO	Histone H2B	0.0177	122.1	1388.14	11.37	
A0A2J6L886_LACSA	Histone H2A	0.0013	3.7	5809.62	1574.77	
A0A2J6LX58_LACSA	Photosystem II protein D1	0.0027	179.3	546.53	3.05	
A0A4D5XX70_ULVLA						

Table 6.3 – Selected proteins upregulated (green) and downregulated (red) in roots contaminated with ACT. Legend: Max Fold Change reveals the trend among contamination and control leaves and roots for each protein.

Acetaminophen: proteins expression in Roots					
Accession number	Protein name	Anova (p)	Max Fold Change (FC)	Control Mean	ACT Mean
A0A2J6JN51_LACSA	BZIP domain-containing protein	0.0282	2.3	343.56	776.88
A0A2J6JQH6_LACSA	Heat shock cognate 70 kDa protein	0.0168	1.5	253.00	390.14
A0A2J6LDA2_LACSA	ABC transporter C family member 5*	0.0043	2.8	97.91	272.18
A0A2J6LKE3_LACSA	DUF3591 domain-containing protein	0.0216	5.1	18.64	95.54
A0A2J6JUJ9_LACSA	Peroxidase	0.0011	2.0	859.65	437.83
A0A2J6JWK1_LACSA	Succinate--CoA ligase	0.0290	42.9	720.72	16.80
A0A2J6K261_LACSA	Peroxidase	0.0322	2.6	321.03	123.60
A0A2J6K8N7_LACSA	UDP-glucose 6-dehydrogenase	0.0455	10.7	189.40	17.77
A0A2J6KFP0_LACSA	Proteasome subunit beta	0.0036	489.4	219.90	0.45
A0A2J6KNL8_LACSA	L-ascorbate oxidase*	0.0032	1.6	768.99	490.50
A0A2J6KQP9_LACSA	Ribosomal_S13_N domain-containing protein	0.0432	20.1	611.04	30.36
A0A2J6KTF4_LACSA	GLOBIN domain-containing protein	0.0018	1487.2	1130.48	0.76
A0A2J6LK16_LACSA	ML domain-containing protein	0.0083	233.9	122.52	0.52
A0A2J6LSU9_LACSA	Sod_Fe_C domain-containing protein	0.0363	5.1	105.37	20.53
A0A2J6L4F3_LACSA	Iso_dh domain-containing protein	0.0094	8.5	578.34	68.33
A0A2J6M212_LACSA	Aquaporin PIP1-2*	0.0424	21.7	1397.11	64.37
A0A2J6MB56_LACSA	Cytochrome c domain-containing protein	0.0000	40.7	79.59	1.96

Table 6.4. – Selected proteins upregulated (green) and downregulated (red) in leaves contaminated with ACT. Legend: Max Fold Change reveals the trend among contamination and control leaves and roots for each protein.

Acetaminophen: proteins expression in Leaves						
Accession number	Protein name	Anova (p)	Max Fold Change (FC)	Control Mean	ACT Mean	
A0A2J6JJY7_LACSA	Malate dehydrogenase	0.0383	4.6	18.65	85.43	
A0A2J6JKZ8_LACSA	HATPase_c domain-containing protein	0.0412	3.9	252.71	990.80	
A0A2J6JRK6_LACSA	Heat shock 70 kDa protein. mitochondrial*	0.0465	2.7	35.47	96.28	
A0A2J6JSS3_LACSA	ATP synthase subunit beta	0.0170	3.4	375.91	1295.04	
A0A2J6K617_LACSA;Q2PBP2_LACSA	Aquaporin PIP2-2*	0.0496	1.8	1308.76	2307.24	
A0A2J6L1W8_LACSA	Cytochrome P450 89A9*	0.0217	4.1	136.80	555.26	
A0A2J6L8C5_LACSA	Chlorophyll a-b binding protein, chloroplastic	0.0386	1.8	3624.23	6579.81	
A0A2J6L8J8_LACSA	Pyruvate dehydrogenase E1 component subunit beta	0.0257	2.1	21.19	44.12	
A0A2J6LBI7_LACSA	Fructose-bisphosphate aldolase	0.0320	1.6	23455.71	38659.06	
A0A2J6LKG3_LACSA	Sulfite reductase 1*	0.0003	6.0	7426.61	44463.03	
A0A2J6LRA7_LACSA	Mitochondrial outer membrane protein porin 2*	0.0227	5.0	9.22	46.02	
A0A2J6LWI3_LACSA	Cytochrome P450 704C1*	0.0379	1.7	294.03	493.88	
A0A2J6M9H3_LACSA	Thioredoxin reductase	0.0131	1.6	2970.47	4758.43	
A0A2J6MB66_LACSA	Cytochrome c domain-containing protein	0.0172	2.0	38.62	75.68	
A0A2J6MBG4_LACSA	MADS-box transcription factor 18*	0.0365	14.5	143.60	2080.32	
Q9SEC1_LACSA	Cytosolic fructose-1,6-bisphosphate (Fragment)	0.0041	1.6	641.15	1055.44	
A0A2J6JWH7_LACSA	FBPase domain-containing protein	0.0146	1.6	1395.41	2212.37	
A0A2J6JT18_LACSA;A0A2J6JSV4_LACSA	Plasma membrane ATPase	0.0003	5.1	17394.51	3412.09	
A0A2J6JUD5_LACSA	Aconitase_C domain-containing protein	0.0040	14.3	68.94	4.83	
A0A2J6KBC0_LACSA	PKS_KS domain-containing protein	0.0013	6.9	11010.62	1585.68	
A0A2J6M8C0_LACSA	DEAD-box ATP-dependent RNA helicase 56*	0.0230	7.0	524.49	74.87	
A0A2J6MFR3_LACSA	RuBisCO_large domain-containing protein	0.0101	8.2	3807.87	465.52	

Table 6.5.– Selected proteins upregulated (green) and downregulated (red) in roots contaminated with CBZ. Legend: Max Fold Change reveals the trend among contamination and control roots for each protein.

Carbamazepine: proteins expression in Roots					
Accession number	Protein name	Anova (p)	Max Fold Change (FC)	Control Mean	CBZ Mean
A0A0K0M234_9ASTR;A0A2J6LNH2_LACSA	ATP synthase subunit alpha	0.0000	21.4	187.21	4009.43
A0A2J6JJ32_LACSA	HATPase_c domain-containing protein	0.0000	7.6	735.73	5621.28
A0A2J6JJ68_LACSA;A0A2J6JJ90_LACSA	Peroxidase	0.0168	1.5	1554.12	2367.78
A0A2J6JSR2_LACSA	Peroxidase	0.0000	7.6	193.69	1474.29
A0A2J6JVK2_LACSA	Heat shock 70 kDa protein 16*	0.0224	2.6	1370.78	3503.50
A0A2J6JY36_LACSA	Glucose-6-phosphate 1-dehydrogenase	0.0002	1.8	371.12	665.97
A0A2J6JZN8_LACSA	Cytochrome P450 81Q32*	0.0000	2.6	1536.44	3974.35
A0A2J6JZS6_LACSA	Beta-galactosidase	0.0181	12.3	41.76	512.42
A0A2J6K6E5_LACSA	PPM-type phosphatase domain-containing protein	0.0001	47.3	6.09	287.96
A0A2J6K758_LACSA	Xanthotoxin 5-hydroxylase CYP82C4*	0.0000	3.9	192.59	757.55
A0A2J6K7D2_LACSA	Alpha-galactosidase	0.0000	48.6	148.51	7218.26
A0A2J6KAN3_LACSA	Superoxide dismutase	0.0085	1.8	1491.84	2752.89
A0A2J6KD90_LACSA	Heat shock 70 kDa protein 1	0.0006	1.7	372.15	643.13
A0A2J6KP89_LACSA	Ultraviolet-B receptor UVR8*	0.0009	33.8	5.58	188.95
A0A2J6KV41_LACSA	Probable serine/threonine protein kinase IREH1*	0.0063	1.6	883.85	1426.96
A0A2J6L7K5_LACSA	Isocitrate dehydrogenase [NAD] subunit, mitochondrial	0.0000	2.5	2112.44	5199.63
A0A2J6L8E9_LACSA	ATPase_AAA_core domain-containing protein	0.0025	35.1	47.94	1684.04
A0A2J6LNF5_LACSA	Serine/threonine-protein phosphatase	0.0005	2.4	1560.34	3708.91
A0A2J6MA69_LACSA	Serine/threonine-protein phosphatase	0.0000	17.4	242.98	4224.18
A0A2J6MBU6_LACSA	Protein disulfide isomerase-like 1-6	0.0000	10.3	52.62	541.74
ACCD_LACSA	Acetyl-coenzyme A carboxylase carboxyl transferase subunit beta, chloroplastic	0.0054	3.0	388.60	128.04
A0A1V0JFJ8_9ASTR;B3IXP5_LACSA	Glutamine synthetase	0.0140	2.4	4209.42	1758.38
A0A2J6JKN6_LACSA	Ferredoxin--nitrite reductase*	0.0160	1.9	4474.49	2384.09
A0A2J6JNJ9_LACSA	Serine/threonine-protein phosphatase 2A 65 kDa regulatory subunit A beta isoform	0.0033	221.1	198.85	0.90
A0A2J6JWG1_LACSA	FAD-binding FR-type domain-containing protein	0.0063	8.3	31.37	3.77
A0A2J6KGI4_LACSA	NOT2_3_5 domain-containing protein	0.0169	18.2	22.93	1.26
A0A2J6KWR2_LACSA	TITAN-like protein	0.0000	10.7	165.12	15.38
A0A2J6L401_LACSA	Phytoeyanin domain-containing protein	0.0118	10.3	90.80	8.85
A0A2J6L551_LACSA	Probable aquaporin PIP2-2*	0.0007	2.7	2493.33	921.46
A0A2J6L5S6_LACSA	Epimerase domain-containing protein	0.0119	26.6	20.76	0.78
A0A2J6LR00_LACSA	FAD-binding PCMH-type domain-containing protein	0.0406	33.0	994.01	30.15
A0A2J6M2H0_LACSA	Aquaporin PIP1-2*	0.0015	3.9	2610.86	662.84

Table 6.6 – Selected proteins upregulated (green) and downregulated (red) in leaves contaminated with CBZ. Legend: Max Fold Change reveals the trend among contamination and control leaves for each protein.

Carbamazepine: proteins expression in Leaves					
Accession number	Protein name	Anova (p)	Max Fold Change (FC)	Control Mean	CBZ Mean
A0A0F6VYH0_ULVLA	Ribulose biphosphate carboxylase large chain (Fragment)	0.0215	3.5	198.80	690.09
A0A2J6JGI3_LACSA	Helicase ATP-binding domain-containing protein	0.0064	16.8	2.72	45.52
A0A2J6JKZ1_LACSA	Chlorophyll a-b binding protein, chloroplastic	0.0005	2.3	2288.55	5323.18
A0A2J6JVT9_LACSA	L-ascorbate oxidase homolog	0.0055	1.6	1110.73	1733.09
A0A2J6JXU8_LACSA	PKS_ER domain-containing protein	0.0373	25.6	7.97	204.18
A0A2J6K3X3_LACSA	SCP domain-containing protein	0.0066	36.3	6.44	233.84
A0A2J6KG99_LACSA	Glutathione reductase	0.0028	1.5	1051.30	1586.49
A0A2J6KYJ5_LACSA	Chlorophyll a-b binding protein, chloroplastic	0.0087	2.3	223.54	521.29
A0A2J6L8H4_LACSA;A0A2J6K212_LACSA	Chlorophyll a-b binding protein, chloroplastic	0.0161	2.0	2810.82	5533.39
A0A2J6JKN6_LACSA;A0A2J6JKU0_LACSA	Ferredoxin--nitrite reductase. chloroplastic*	0.0207	1.7	2890.17	1748.00
A0A2J6JXX4_LACSA	Pyruvate dehydrogenase E1 component subunit beta	0.0178	4.3	169.95	39.79
A0A2J6JZV2_LACSA	Ribosomal protein L19	0.0485	40.4	698.94	17.28
A0A2J6K7W1_LACSA	Heat shock cognate 70 kDa protein 2*	0.0331	2.2	10210.12	4627.58
A0A2J6KH56_LACSA	Malate dehydrogenase	0.0486	2.0	1450.58	730.37
A0A2J6LDE7_LACSA	DNA helicase	0.0145	4.9	1786.17	363.28
A0A2J6LLM6_LACSA	Pyruvate dehydrogenase E1 component subunit alpha-3. chloroplastic*	0.0040	1.8	1004.41	553.58

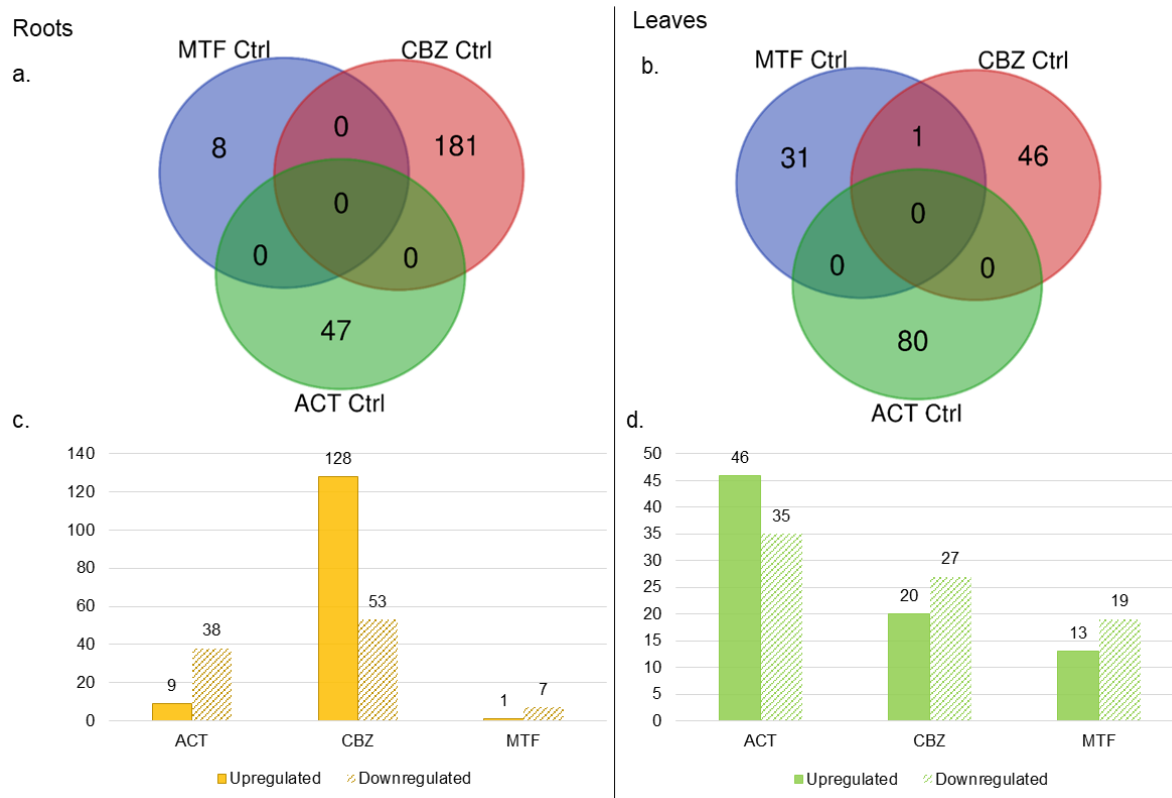


Figure 6.3. - Venn diagram (top) for roots (a) and leaves (b) of differentially expressed significant proteins for each contamination VS compared to Control, obtained from Bioinformatic and Evolutionary Genomics tool (<http://bioinformatics.psb.ugent.be/webtools/Venn/>). Bar figure (down) with up and downregulated proteins in roots (c) and leaves (d) contrasting each contamination with the control condition.

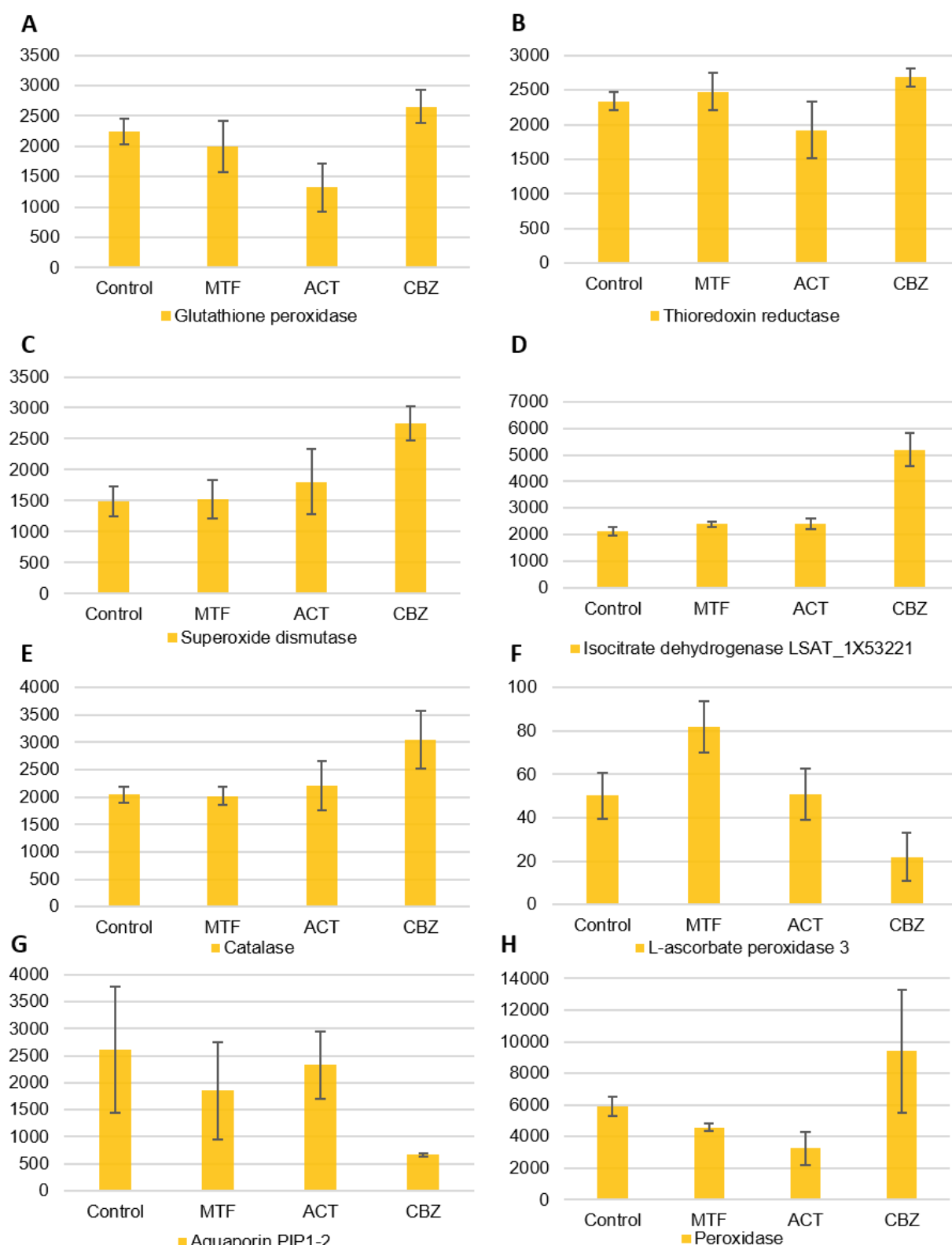


Figure 6.4. - Differential abundance (y-value) of some proteins (potential stress-related) identified as proteins of interest, in roots of different conditions. Legend: A – Glutathione peroxidase, B – Thioredoxin reductase, C – Superoxide dismutase, D – Isocitrate dehydrogenase, E – Catalase, F – L-ascorbate peroxidase 3, G – Aquaporin PIP1-2, H – Peroxidase.

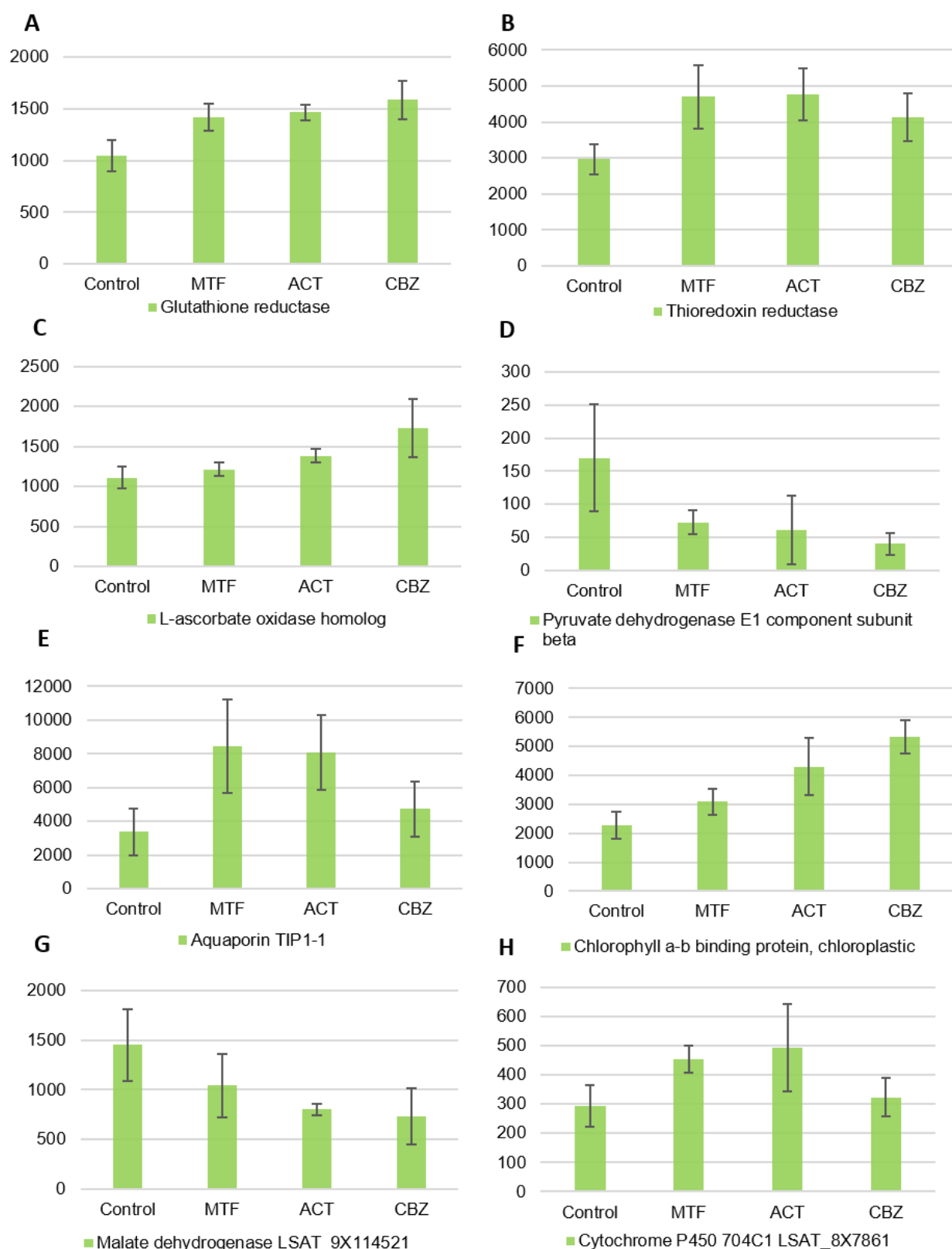


Figure 6.5. - Differential abundance (y-value) of some proteins (potential stress-related) identified as proteins of interest, in leaves under different conditions. Legend: A – Glutathione reductase abundance, B – Thioredoxin reductase, C – L-ascorbate oxidase, D – Pyruvate dehydrogenase E1 componente, E- Aquaporin TIP1-1, F – Chlorophyll a-b binding protein, G – Malate dehydrogenase, H – Cytochrome P450.

Principal Component Analysis (PCA) is represented for both roots and leaves in Figure 6.6 including all the experimental conditions and different patterns were observed among tissues. In roots, CBZ group showed a clear differentiation regarding other contaminations and control, which can be also observed in case of up and downregulated proteins (Figure. 6.3 c/d). MTF contamination was the one that showed more similarity to control even in PCA, which may indicate that MTF does not influence as much the protein abundance when compared to control. On the other hand, in the leaves, PCA indicates an evident differentiation between protein abundances in control and contaminated plants.

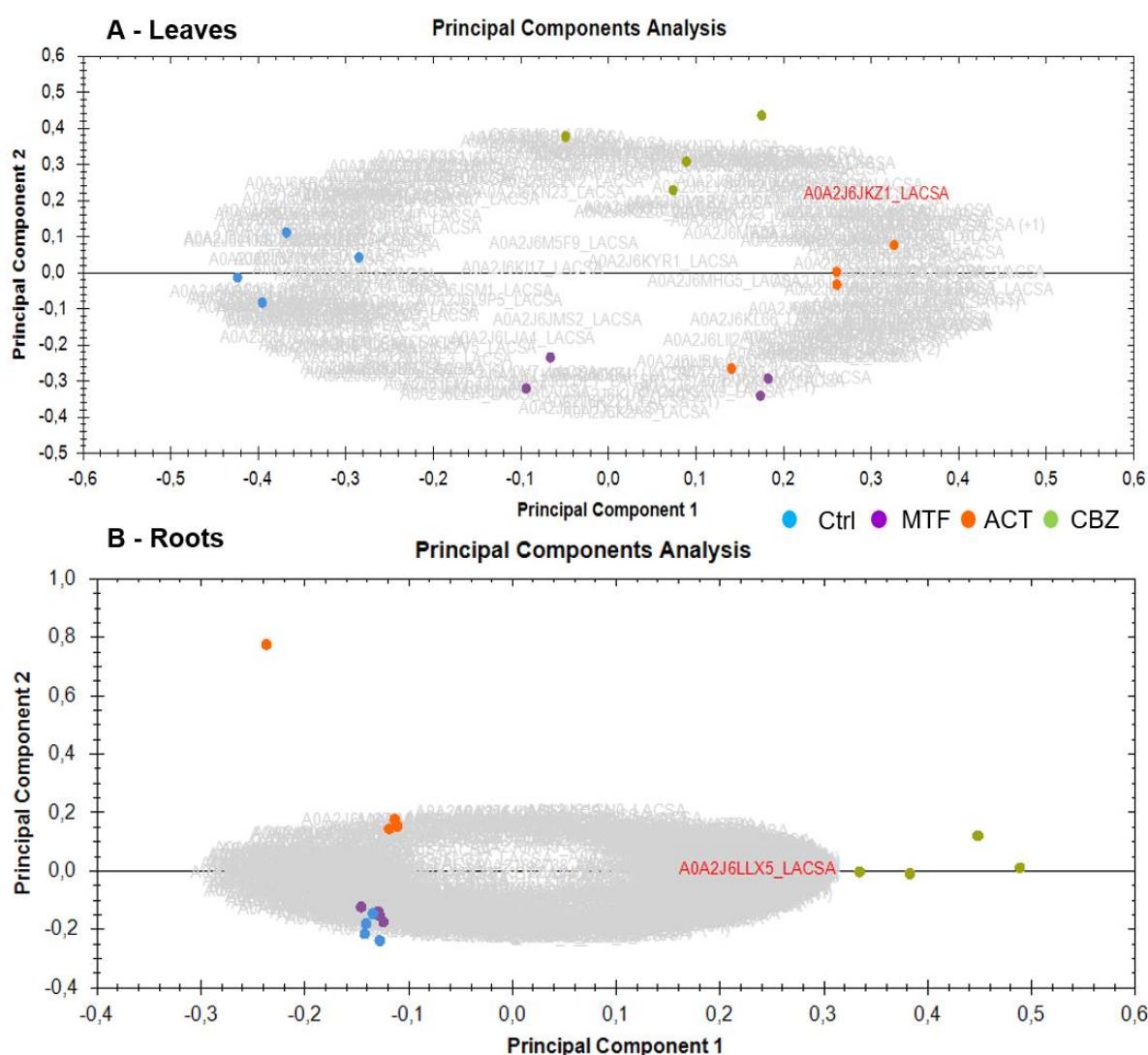


Figure 6.6. - Principal Component Analysis carried out with Progenesis QIP for the lettuce leaves (A) and roots (B) for Ctrl, MTF, ACT and CBZ conditions, with the filters: ANOVA P-value<.05, at least 1 unique peptide, at least 2 peptides identified per protein and fold change higher than 1.5.

In order to provide an overview of protein abundance in several conditions Figure 6.6 a/b shows a hierarchical cluster analysis and heatmap of the proteins with differences in abundance in studied conditions, in both leaves and roots, in direct comparison to control.

In roots, it is possible to observe a clear differentiation among CBZ contamination and other contaminants. In leaves, this type of differentiation occurs among control and contaminated conditions in general. The difference among contaminants is not as evident in leaves as it is in roots.

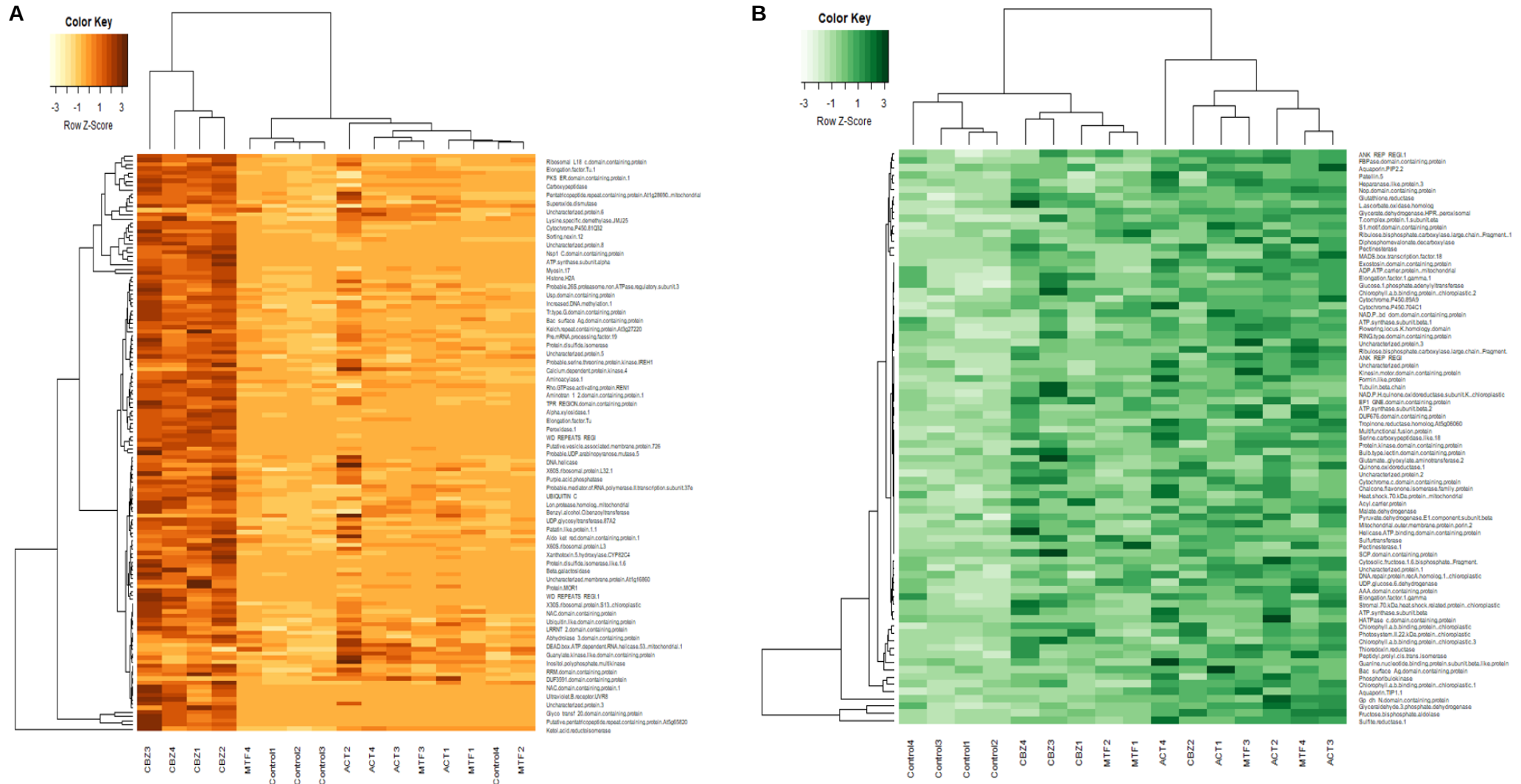


Figure 6.7. - Heatmaps obtained for the normalized differentially abundant proteins identified. A – roots, B – leaves. Lines represent different proteins and columns are different replicates. For roots (A) dark orange indicates higher abundance, whereas light orange indicates lower abundance. For leaves (B) dark green indicates higher abundance, whereas light green indicates lower abundance.

6.1.2. Molecular and biological functions analysis

Figure 6.8 shows the different molecular functions of the differentially expressed proteins among roots and leaves for each condition against control. The different pharmaceuticals showed different patterns. ACT showed binding (35%), catalytic activity (46%) and transporter activity (12%) in roots while in leaves the main molecular functions were binding (38%), catalytic activity (26%) and translation regulator activity (34%). In CBZ contaminated plants the main molecular functions were, in roots, binding (14%), catalytic activity (80%) and transporter activity (1%) while in leaves were binding (39%), catalytic activity (42%) and structural molecule activity (12%). MTF showed only binding (20%) and catalytic activity (80%) as molecular functions, mainly due to the low number of differentially expressed proteins in roots. However, in leaves, the classification was wider for molecular function with binding (43%), catalytic activity (38%) and structural molecule activity, translation regulator activity, transporter activity and molecular function activity all with 4.8%.

In Figure 6.9, the proteins differentially expressed in relation to the control are classified according to the biological processes (BP), both in leaves and in roots. ACT in roots had two major BP, cellular processes (30%) and metabolic processes (30%), and the same pattern was observed in leaves with cellular processes (42%) and metabolic processes (42%). In case of CBZ, besides cellular processes and metabolic processes, also biogenesis exhibited an increment in roots with 16% while in leaves the third BP was response to stimulus (13%). With MTF, in roots, cellular processes (40%), metabolic processes (30%) and response to stimulus (20%) were the three main BP. In leaves, cellular processes (34%), biogenesis (22%) and metabolic processes (25%) presented the main BP.

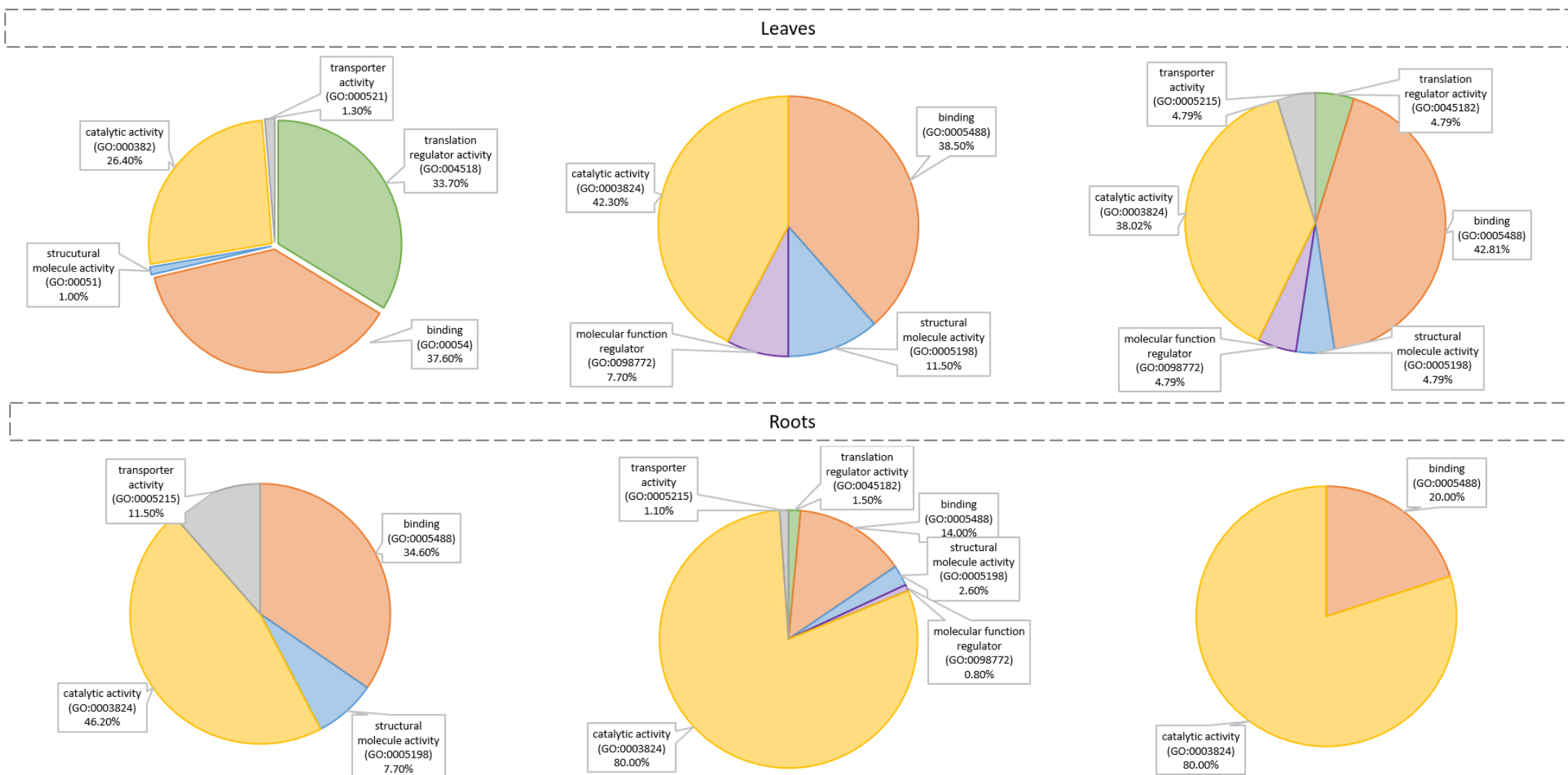


Figure 6.8. - Molecular Functions analysis of three contaminations vs control for Leaves (top) and Roots (down), by performing a search in the PANTHER tool. ACT vs Control (left), CBZ vs Control (middle) and MTF vs Control (right).

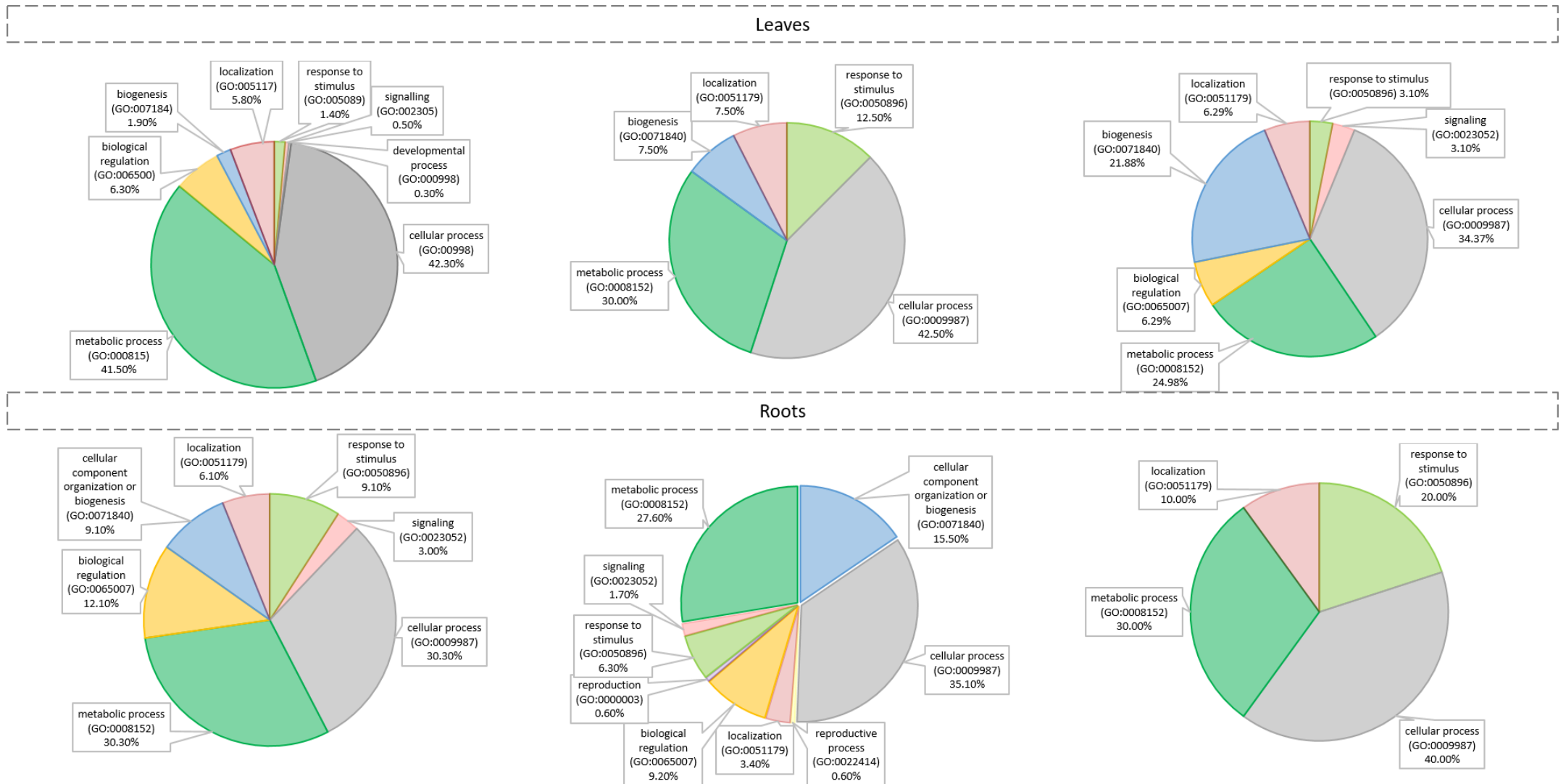


Figure 6.9. - Biological processes analysis of three contaminations vs control for Leaves (top) and Roots (down), by performing a search in the PANTHER tool. ACT vs Control (left), CBZ vs Control (middle) and MTF vs Control (right).

6.2. Discussion

This proteomic approach contributes to an overall understanding of the MTF, ACT and CBZ effect in the metabolism of lettuce helping to identify the biological processes that are more affected by the induced stress. Therefore, a special emphasis is placed on stress related proteins in order to ascertain the toxic effects of the different compounds. A summary of the main alterations in lettuce cells is present in Figure 6.10, according to tissue and contaminant in lettuce cells. As stress related proteins are the focus of our study, proteins related with ROS scavenging and cell oxidative stress were preferentially selected according to abundance in different contaminations.

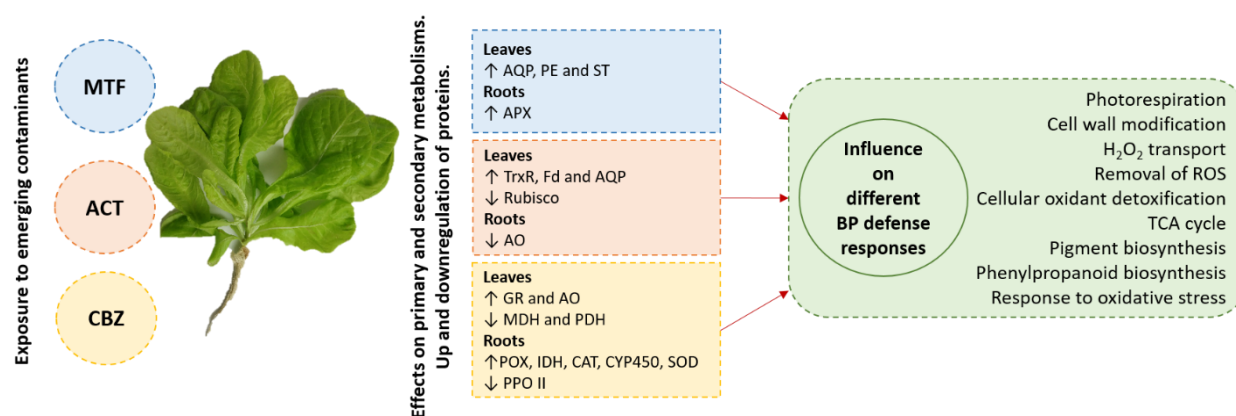


Figure 6.10. - Proteins of interest and main biological processes associated to the overall response of lettuce to pharmaceuticals (MTF, ACT and CBZ). Legend: AQP – Aquaporin, PE – Pectinesterase, ST – Sulfotransferase, APX – Ascorbate Peroxidase, TrxR – Thioredoxin Reductase, Fd – Ferredoxin (or Sulfite Reductase), AO – Ascorbate oxidase, GR – Glutathione Reductase, MDH – Malate Dehydrogenase, PDH – Pyruvate Dehydrogenase, POX – Peroxidase, IDH – Isocitrate Dehydrogenase, CAT – Catalase, CYP450 – Cytochrome P450, SOD – Superoxide Dismutase, PPO II – Polyphenol oxidase II.

6.2.1. Effects on roots

6.2.1.1. Stress response proteins

In roots of plants exposed to MTF contamination several proteins revealed higher abundance compared to control, in a pattern that was also observed for the CBZ contamination. One example is ascorbate peroxidase, an enzyme that is involved in the ascorbate-glutathione cycle and has a crucial role under oxidative stress conditions. Focusing on the proteins of interest mentioned in Figure 6.4, only L-ascorbate peroxidase (APX) showed higher

abundance by comparison to control, whereas the other proteins of interest remained at similar levels. This may indicate an influence on maintaining the ascorbate pools when roots are subjected to MTF contamination, as an antioxidative response. This result may influence the activity of IDH and consequently the reason for its lower abundance in roots under MTF contamination.

Regarding ACT contamination, BZIP domain-containing protein was upregulated when compared to control. Some authors addressed its relation with responses to abiotic stress (Corrêa *et al.*, 2008; Llorca *et al.*, 2014), and our study show an increase in the expression of this protein when plants were subjected to the exposure to the three pharmaceuticals. ACT is the contamination that most affected the expression of this protein. For instance, the abundance on MTF leaves was 2 times lower than those of leaves contaminated with ACT.

Glutamate decarboxylase (GAD) was detected in higher abundance in ACT roots and revealed a downregulation over control roots in the case of CBZ contamination. This enzyme transforms glutamate into gamma-aminobutyric acid (GABA). GABA shunt provide NADH and succinate to the Krebs cycle (Kinnersley *et al.*, 2000). Some authors suggest that the degradation of GABA will contribute to the scavenging of ROS, preventing oxidative stress damage in cells (Bouché *et al.*, 2004). In our study, the higher abundance of GAD will likely increase GABA contents as response to stress caused by MTF exposure. GAD is modulated by Ca²⁺-calmodulin (CaM) complex, therefore calcium pools will influence GAD abundance or activity. Compared to other contaminants, ACT was the one that most influenced the abundance of this protein. CBZ showed a reduction of GAD production rate in cells leading to a lower expression of GAD. This result may influence the pools of NADH and succinate available for the Krebs cycle. Indeed, succinate dependent proteins were also downregulated under CBZ contamination a possible consequence of alterations in GAD expression.

Peroxidases and superoxide dismutase increased their abundance in CBZ roots when compared to control conditions. These are two of the most important proteins related to the enzymatic response to oxidative stress, controlling ROS imbalance. The increase of ROS in contaminated cells, are usually the main cause for this upregulation.

An overall analysis of differentially expressed stress proteins in roots, for the three contaminants highlights that the major effects are due to CBZ contamination. In terms of proteins of interest (Figure 6.4) roots under CBZ showed a clear trend to increase the expression of SOD, CAT, glutathione peroxidase (GPX) and other peroxidases, while MTF revealed more effects on APX abundance. The high contents of ROS are known to be related with SOD, CAT, POX, thioredoxin reductase (TrxR) and thioredoxin peroxidase (TrxP) so the higher abundance of these proteins in this contamination may be related to the activation of

some ROS scavenging enzymes. However, in case of APX, a decrease in abundance was observed. It is thus clear that these contaminants act differently on plant metabolism as they induced a response involving different stress proteins.

6.2.1.2. Heat shock proteins

HSP 70 protein showed higher abundance in the ACT contaminated roots. This protein is frequently associated to ROS and stress response (Suzuki *et al.*, 2006) and is mainly present in nucleus, cytosol and mitochondria (Taiz *et al.*, 2014). HSPs are indeed induced by a wide range of stresses such as drought, temperature or osmotic stress, and even heavy metals or other chemicals stresses (Timperio *et al.*, 2008).

In roots under CBZ contamination, the abundance of heat shock proteins remains higher than those of controls. Two different HSP were identified: HPS70 and HSP90 (HATPase_c domain-containing protein). In fact, the two HPS revealed higher expression in all contaminant exposure, being CBZ the contaminant where the expression was higher. Some authors studied the effects of phytohormones and heavy metals in HSP90 revealing that this group of proteins may respond to this type of stress (Krishna *et al.*, 2001). Therefore, it is possible to assume that CBZ may have induced the overexpression of HSPs, similarly to other contaminants.

6.2.1.3. Aquaporins

Aquaporins are integral membrane proteins responsible for the formation of water-selective channels across the membrane. However, some of these proteins may act as H₂O₂ transporters and can be affected by different types of abiotic stresses.

The higher abundance of these proteins in roots of control plants indicates a good regulation of water transport through the membrane in comparison to roots exposed to contaminants. These proteins are considered to be crucial for the water permeability of the plasma membrane and for the root system morphology (Bellati *et al.*, 2016). Aquaporins were found to be more abundant in roots of control group by comparison to all the other groups and similar roles can be inferred. In the case of ACT and CBZ contamination, a downregulation was observed for Aquaporins PIP1-1 (ACT and CBZ) and PIP1-2 (CBZ), latter shown in Figure 6.4. These results may be related with possible protein denaturation in contaminated roots. In general, all aquaporins identified in our study were downregulated in contaminated roots suggesting there is interference disruption of the different pharmaceuticals on water transport process. This outcome will induce negative effects on water transport into cells and possibly affect or

denature important proteins, mainly related with osmotic state, composition and integrity of cell walls or lignin biosynthesis catalysed by peroxidases.

6.2.1.4. Detoxification proteins

In roots under CBZ contamination, cytochrome P450 81Q32 and cytochrome P450 82C4 showed significant upregulation in comparison to control. Proteins from cytochrome P450 complex are frequently associated with xenobiotics detoxification or transport (Xu *et al.*, 2015). Therefore, CBZ influenced the abundance of cytochrome P450 complex in roots.

ABC transporters were identified in higher abundance on ACT contaminated roots. These transporters are present in tonoplast, plasma membrane and mitochondria and are mainly responsible for the transport of different molecules across biological membranes. These proteins were identified as transporters involved in detoxification processes and are required for plant development and response to abiotic stresses (Kang *et al.*, 2011). Also, ABC transporter C family member 5 participated in ATPase-coupled xenobiotic transmembrane transporter activity as molecular function and xenobiotic detoxification by transmembrane export across the plasma membrane and response to stress as biological processes. Conversely, the other contaminants had accumulation patterns for this protein that were similar to those found on ACT roots with higher abundances values than those of control roots. Thus, the abundance of ABC transporters may be related to xenobiotic transport into vacuole.

6.2.1.5. Secondary metabolism related proteins

Cinnamyl alcohol dehydrogenase was upregulated in CBZ roots when compared to control. This protein is responsible for the conversion of cinnamyl alcohol into cinnamaldehyde and it is involved in the phenylpropanoids metabolism. Usually phenylpropanoids are associated with stress conditions as previously reported (Dixon *et al.*, 1995; Sharma *et al.*, 2019), suggesting that CBZ may interfere with the plant's secondary metabolism.

4-coumarate-CoA ligase protein was also upregulated in CBZ roots, revealing lower abundance on control and other contaminated roots. So, a closer relation among CBZ and secondary metabolism may be established. This protein characterized by phenylpropanoid and auxin biosynthetic biological processes will induce a different response from roots under CBZ contamination. Moreover, phenylalanine ammonia-lyase (PAL) showed higher abundance in roots under CBZ contamination over control. The activity of PAL is known to increase according to environmental factors such as oxidative stress (Sharma *et al.*, 2019). Therefore, abiotic stress caused by CBZ can induce changes on phenylpropanoids biosynthesis. Additionally,

the lettuce used is a red leaf variety which may present higher antioxidative response involving secondary metabolism compounds.

6.2.1.6. Cell respiration proteins

Different proteins from mitochondrial electron transport such as cytochrome c biological process were identified as differentially expressed. Cytochrome b-c1 complex subunit Rieske-2, mitochondrial (complex III) was identified in higher abundance in ACT and in lower abundance in CBZ (Supplementary material 1). These results may suggest that CBZ may interfere with redox reaction on ubiquinone, since it is frequently addressed as a marker for oxidative stress. This may result in some negative impact on electron transport chain, affecting mitochondrial metabolism. However, results regarding proteins from cytochrome complexes are different according to the b-type, as in the case of NADH-cytochrome b5 reductase 1 (complex III as well), the lower abundances were found for CBZ group, following the same trend as described above and also for the ACT group.

The cytochrome c domain-containing protein called complex IV, also known as cytochrome c oxidase, is a protein involved in the latter stages of the electron transport chain. In our study, this protein showed lower abundance in roots contaminated with ACT in comparison to controls. Cytochrome-c is a peripheral protein involved in the transfer of electrons among complexes III and IV in the mitochondrial electron transport chain (Taiz *et al.*, 2014). ACT was the contaminant that most affected the expression of cytochrome c domain-containing protein followed by CBZ. However, CBZ revealed similar values to control in this specific protein. Recently, Racca *et al.* (2018) studied the effect of reducing the levels of the mitochondrial electron carrier cytochrome c in *Arabidopsis thaliana* treated with gibberellins studying also the interplay with cytochrome c. The results showed that plants with cytochrome c deficiency have delayed growth and development and tend to accumulate starch and glucose. This outcome may influence pentose phosphate pathway and consequently the Krebs cycle. Roots contaminated with ACT did not reveal damages or changes in development in our experiment. Indeed, Complex I, II, III and IV are often mentioned as being inhibited by several compounds (Taiz *et al.*, 2014). As plants have an alternative pathway for oxygen reduction, involving alternative oxidases, changes in this complex may not be directly related to root cell damage.

Glucose-6-phosphate 1-dehydrogenase (G6PDH) is an enzyme related with glucose metabolism and it is reported that some G6PDH isoforms play a role in redox balance and oxidative stress producing cytosolic NADPH (Long *et al.*, 2016). It was found that G6PDH was overexpressed in roots under CBZ contamination. The increase in G6PDH abundance is related with a response to oxidative stress acting on control of excess ROS. Several authors

described already similar patterns in other cultures such as rice under salt stress (Zhang *et al.*, 2013), soybeans subject to drought stress (Liu *et al.*, 2013) and sugarcane under salt, drought, low temperature and heavy metal stresses (Yang *et al.*, 2014). In all of them, a correlation among cell redox balance and ROS metabolism was highlighted. Moreover, Liu *et al.* (2013), reported a role of G6PDH in maintaining GSH and AsA pools levels regulating H₂O₂ contents in cells.

IDH [NADP] and Iso dh domain-containing protein had lower abundance in MTF and ACT respectively, by comparison to controls. It is known that these proteins play an important role in the regulation of the Krebs cycle. Such results may indicate a downregulation of these proteins in contaminated roots, clearly showing that Krebs cycle and consequently the primary metabolism of the contaminated roots present significant changes in this metabolic pathway as a whole. This downregulation was also detected in CBZ in both proteins but to a lesser extent. Some authors also reported the inhibition of some metabolites from the first phases of Krebs cycle, in roots exposed to stress conditions (Dumont *et al.*, 2019). For instance Lehmann *et al.* (2009) showed a decrease in α -ketoglutarate, which may be a consequence of low activity of IDH, in *Arabidopsis* roots under menadione stress. This enzyme is downregulated in MTF and ACT contamination and is correlated with the cellular ratio NADH/NADPH, which in turn affects the regulation of other metabolites such as GSH/GSSG pools and ascorbate pathway (Araújo *et al.*, 2012; Popova *et al.*, 1998). Thus, a possible negative effect of these contaminations can be the regulation of other metabolites responsible for the response to stress and redox homeostasis.

Under CBZ contamination lettuce roots showed an upregulation of IDH in comparison to controls. This upregulation was also reported by Leterrier *et al.* (2012) in *Arabidopsis Thaliana* subjected to salinity stress. Therefore, alterations in the Krebs cycle caused by CBZ may also induce some changes on oxidative phosphorylation, glycolysis, and secondary metabolism processes directly dependent of Krebs cycle sub products. This trend was only detected in CBZ contaminated roots indicating a different effect on cell respiration processes compared to ACT and MTF.

Another Krebs cycle enzyme, Succinate-CoA ligase, was also detected in lower abundance in ACT roots. Maršálová *et al.* (2016) conducted a proteomic analysis to study the influence of salinity stress in glycophytes and halophyte plants and showed that Krebs cycle enzymes, including succinate-CoA ligase, enhanced their abundance in control plants, reinforcing that under stress conditions succinate-CoA ligase is downregulated. A similar trend was also shown in MTF and CBZ contaminations, albeit to a lower extent, indicating that the three contaminants have a similar effect on the Krebs pathway, as suggested by those authors.

Despite the presence of contaminants, roots are able to maintain a good regulation of Krebs cycle, ensuring that the primary metabolism is not completely affected. This is probably what is supporting a certain tolerance of the plant to these pharmaceuticals.

6.2.1.7. Cell wall proteins

UDP-glucose 6-dehydrogenase (UGHD) is described as a key enzyme involved in the biosynthesis of the plant cell wall. In this experiment, it showed a lower abundance in ACT contaminated roots. This result may indicate an impact of the pharmaceutical on cell wall biosynthesis or even on glycosylation of proteins and lipids as suggested by Kleczkowski *et al.* (2010).

The previously mentioned downregulation of several aquaporins in contaminated roots, may also have some impact on cell wall processes due to the imbalance on water transport into the cells causing changes on cell wall integrity.

α -galactosidase and β -galactosidase were differentially expressed in CBZ roots, presenting higher values of abundance in comparison to control. These proteins are involved in cell wall modifications and carbohydrate metabolic process, being responsible for changes in pectin network and structural modifications as a response to stress. In the case of α -galactosidase higher abundance in CBZ roots, other authors suggested its upregulation when plants were subjected to abiotic stress in different tissues (Hara *et al.*, 2008). Similar results were reported by Gutsch *et al.* (2018) in *Medicago sativa* L. subjected to heavy metal stress. Differences among contaminants (CBZ, MTF and ACT) were also detected in alpha-galactosidase expression since CBZ remain at least 40 times higher than the abundances presented in roots under ACT and MTF. CBZ contamination will induce alterations in cell wall proteins promoting structural degradation and deficient root development. Indeed, Figure 6.2 shows a clear differentiation among root length under CBZ contamination (d) when compared to control plants (a).

In the roots of MTF plants, Ras-related protein Rab11C was identified as upregulated in comparison to control. This protein is considered one of the most complex proteins among Rab families. In plants, Rab11 proteins were detected in Golgi bodies (pea) and endosomes (tomato) (De Graaf *et al.*, 2005). Also, Rab11 homologs have been implicated in the secretion of cell wall enzymes, in ripening tomato (Lu *et al.*, 2001). Moreover, taking into account the relationship between GTP activity and Rab11C, it is possible to assume that Rab11C is involved in catalytic activity and binding, a likely consequence of the toxic effects of MTF contamination.

6.2.1.8. Nutrients assimilation and nitrogen metabolism proteins

Enzymes such as glutamine synthase and ferredoxin-nitrite reductase were more abundant in control roots in comparison to CBZ. In general, MTF and ACT also affected the expression of these proteins, however CBZ was the contamination that affected most the expression levels (lower mean abundance). These two proteins are present in roots plastids and play different roles in cells. Glutamine synthase produces glutamine using glutamate as substrate. This reaction involves not only ammonium but also ATP and Mg^{2+} , Mn^{2+} or Co^{2+} as cofactors. The lower contents found in this protein may indicate a poorer regulation in contaminated roots. It is however important to state that the presence of proteins is not always necessarily linked to higher activities. Other studies have reported a decrease in glutamine synthase activity when rice was subjected to cadmium contamination (Lee *et al.*, 2013). Ferredoxins have been reported to be downregulated in plants when environmental stress occurs (Lodeyro *et al.*, 2012). In our case, the same trend was showed, with lower abundance of Ferredoxin-nitrite reductase in CBZ roots. Lodeyro *et al.* (2012) observed that in wild type tobacco plants under stress conditions ferredoxin levels decreased and NADPH accumulated in cells leading to changes in ROS. A possible interference on ammonium assimilation may be correlated with the presence of pharmaceuticals. Consequently, for longer periods of exposure to pharmaceuticals, NH_4^+ and NO_2^- pools may suffer alterations leading to changes on plant growth and development.

6.2.1.9. Other proteins

ATPase_AAA proteins are responsible for protein homeostasis as defined by Yedidi *et al.* (2017) and an upregulation was observed in roots under CBZ contamination. As mentioned previously, an upregulation of these proteins in rice cells under salinity stress, was described by Shen *et al.* (2011). As for those authors, our results suggest a possible interference of CBZ with protein homeostasis which induced the increase of ATPase_AAA proteins. CBZ was the contamination with which the expression of this protein increased most, suggesting a clear influence on protein homeostasis.

Some DEAD-box proteins showed high differential expression in roots of CBZ condition, being upregulated in comparison to control. These proteins were described by Tujeda *et al.* (2001) as playing a role in stabilizing growth in plants under stress conditions throughout the regulation of some stress-induced pathways.

6.2.2. Effects on leaves

6.2.2.1. Stress response proteins

Different stress response proteins were identified as more abundant in contaminated leaves. It was noticed that the expression of sulfurtransferase in leaves contaminated with ACT and CBZ was higher when compared to control leaves, suggesting a biochemical response to this type of abiotic stress. Previous studies suggest that sulfurtransferase is often involved with detoxification of ROS, cyanide and heavy metals (Most *et al.*, 2015; Papenbrock *et al.*, 2000).

Additionally, thioredoxin reductase increased its abundance on leaves contaminated with ACT, CBZ and MTF (Figure 6.5). Thioredoxins are present in different organelles (cytosol, mitochondria and chloroplast) using different substrates and playing a role in defence mechanisms against oxidative stress (Michelet *et al.*, 2009). This protein can be affected by the presence of ROS in cells, which in turn may be related to the higher abundance of thioredoxin, often linked to role in ROS scavenging (Cha *et al.*, 2015). Indeed, GOs identified for this protein revealed removal of superoxide radicals as well as cell redox homeostasis. Additionally, this protein will act on NADPH pools, using it as a substrate, available to the glutathione cycle and thus alterations in thioredoxin reductase may induce changes in this cycle. However, the increase of glutathione reductase expression in contaminated leaves allows us to conclude that the consumption of NADPH triggered by thioredoxin reductase did not interfere on NADPH pools available for GR activity. Indeed, NADPH-Thioredoxin and glutathione systems are known to play overlapping roles in physiological development, however the degree of interplay is not fully described (Noctor *et al.*, 2012). Therefore NADPH-dependent thioredoxins may not reflect a direct downregulation of glutathione cycle proteins.

As mentioned before, proteins such as GR and ascorbate L-oxidase were more expressed in contaminated leaves, not only with CBZ but also with ACT contamination. The contribution of these proteins to defence mechanisms has already been described by other authors (Akram *et al.*, 2017; Venkatesh *et al.*, 2014). The activity of these proteins may interfere with ascorbate pools available for the scavenging of ROS by ascorbate peroxidase (Bath *et al.*, 2017). The regeneration of the reduced form of ascorbate may occur throughout ascorbate-glutathione cycle, since glutathione is partially responsible for the production of ascorbate through dehydroascorbate reductase and GSH/GSSG conversion (Foyer *et al.*, 2011). In fact, the higher content of GR reflects this mechanism in lettuce leaves under CBZ, ACT and MTF contamination. An upregulation of proteins related with sulphur assimilation was observed which can have some impact in glutathione cycle regulation. In case of L-ascorbate oxidase

the major differences to control were observed in CBZ contamination. This reinforces the possibility of a CBZ effect on the ascorbate pools in leaves, as a response to stress.

6.2.2.2. Heat shock proteins

Similarly to roots, two different HSPs (HATPase_c domain-containing protein, Heat shock 70 kDa protein, mitochondrial) were identified as more abundant in leaves under ACT. This type of response in ACT leaves may translate an influence of this contaminant in both tissues. The linking of HSP to ROS and stress response is known and these proteins are considered as potential biomarkers for this stress (Ireland *et al.*, 2004).

Also the synthesis of HSP in plants under stress will improve the protection of proteins in stress conditions (Timperio *et al.*, 2008). Despite the abundance of this protein obtained with the other contaminants did not differ as much from control leaves, other HSP were identified in this study. Stromal 70 kDa heat shock-related (chloroplastic) protein was identified as differentially expressed in CBZ leaves, as well as HSP90. The latter had higher abundance in leaves under CBZ and ACT. Among all the contaminants studied, MTF was the only one that did not show so many differences in HSPs expression in leaves. This pattern was also observed in roots suggesting a similar response to MTF exposure in both tissues.

6.2.2.3. Aquaporins

Aquaporins contribute not only to intracellular H₂O₂ fluxes but also to intercellular fluxes, being of major importance to signalling pathways (Bienert *et al.*, 2014). The higher abundance of Aquaporin TIP1-1 in the MTF leaves over control condition is likely related to a putative higher content of H₂O₂ in MTF exposed leaves. Similarly, to the MTF condition, ACT showed a higher abundance when compared to control leaves, as presented in Figure 6.5.

A similar trend was observed in aquaporin PIP2-2 which showed higher abundance in leaves contaminated with ACT when compared to controls. Once again, a possible explanation for the upregulation of this aquaporin in leaves under stress can be due to the stress-induced increase in H₂O₂. In leaves under CBZ contamination, aquaporin PIP2-1 was differentially expressed, revealing an upregulation. Unlike roots, where aquaporin proteins were downregulated under contamination, the three pharmaceuticals used here revealed a high impact on different aquaporin expression. These results may point out to differences between leaf and root cell metabolism, as leaves reflect a higher level of response to this type of abiotic stress.

6.2.2.4. Detoxification proteins

Proteins from cytochrome P450 complex (Cytochrome P450 89A9 and Cytochrome P450 704C1) revealed also higher abundance in ACT contaminated leaves, a trend followed also by MTF and CBZ. These proteins are frequently associated with biosynthesis of endogenous molecules and xenobiotics detoxification (Schückel, 2012) often showing an increased activity under stress conditions (Pandian *et al.*, 2020) Thus, the increase in abundance of these proteins is correlated with the presence of the contaminants.

Cytochromes P450 (CYP450) in lettuce leaves follow a similar trend in every contamination which seems to be a response to the presence of different xenobiotics. The direct involvement of CYP450 in the secondary metabolism facilitates detoxification processes as well as the activation of different scavenging by-products as a response to the induced stress. The increased expression of CYP450 was identified by other authors carrying out studies of different abiotic stresses. For instance, the upregulation of CYPs have been identified in wheat and rice under heavy metal stress and in *Arabidopsis thaliana* under salinity stress (Mao *et al.*, 2013; Pandian *et al.*, 2020).

6.2.2.5. Cell respiration proteins

Cytochrome c domain-containing protein (complex IV) revealed a higher abundance in ACT-contaminated leaves, a different trend when compared to roots under ACT contamination as well. This result confirms that different tissues may reveal differences among metabolic pathways.

FBPase domain-containing protein, cytosolic fructose1,6-bisphosphate (fragment) and fructose-bisphosphate aldolase are three proteins related with glycolysis processes and highly expressed in ACT contaminated leaves. Fructose 1,6-bisphosphatase is responsible for the allocation between glycolysis and sucrose synthesis. Fructose-bisphosphate aldolase is involved in glycolysis and gluconeogenesis, in cytosol, and in Calvin cycle at plastids level. Indeed, several proteins related to the glycolysis process are often addressed as moonlighting proteins. Taking into account its abundance in ACT leaves, it is possible to link this protein as a response to abiotic stress. Lu *et al.* (2012) focus their research in fructose 1,6-bisphosphate aldolase genes in *Arabidopsis Thaliana* and showed a positive response not only in terms of plant growth and development, but also in stress response. Similar results were reported by Lv *et al.* (2017) in wheat under different abiotic stresses (drought, cold, heat, ABA, salt). Previous studies indicated that oxidative stress modifies functions of cytosolic aldolase (Dumont *et al.*, 2019). This type of alteration will promote C-flux into the oxidative pentose-

phosphate pathway to regenerate NADPH necessary to ROS detoxification processes. Moreover, aldolase was addressed as a moonlighting protein that display redox-dependent changes according to the subcellular localization and biological functions (O'Leary *et al.*, 2020; van der Linde *et al.*, 2011).

Malate and pyruvate dehydrogenases were also two proteins considered to be of interest to our study and both are involved in the cell respiration process. Malate dehydrogenase is part of the Krebs cycle, while pyruvate dehydrogenase contributes to the acetyl-coA formation (by oxidative decarboxylation) that will be used in Krebs cycle. Under ACT contamination, leaves showed higher abundance of these two proteins over control leaves. The detected high abundances of Krebs cycle proteins may be representative of a strategy for defence mechanisms in order to withstand stress conditions. Other authors suggested that enzymes such as malate and pyruvate dehydrogenases may comprise potential targets for redox regulation (Balmer *et al.*, 2004). Indeed, these two proteins are closely related since malate dehydrogenase will produce pyruvate as a substrate to pyruvate dehydrogenase. Therefore, the higher expression of the first is consequently linked to the upregulation of the latter.

On the other hand, aconitase_C domain-containing protein showed significantly lower expression in leaves under ACT exposure, and also with CBZ and MTF contaminated leaves but to a lower extent. Aconitases are responsible for the first step of Krebs cycle (Wang *et al.*, 2016). Several authors reported on the importance of plant aconitases in mediation of oxidative stress and cell death. Lehmann *et al.* (2009) carried out a study with *Arabidopsis* roots where a concentration of 60 μ M menadione caused a decrease of aconitase activity, and a similar trend was observed by Obata *et al.* (2011) in *Arabidopsis* seedling during oxidative stress. This type of fluctuation on aconitase levels, was also reported by Moeder *et al.* (2007) in *Arabidopsis* and *Nicotiana benthamiana* plants, where reduced levels of aconitase displayed enhanced resistance to oxidative stress. We can therefore infer that a similar situation happened in our experiment, supporting the idea that aconitase plays a role in ROS homeostasis.

On the other hand, homologues malate and pyruvate dehydrogenases proteins revealed distinct behaviour in leaves under CBZ contamination. Pyruvate dehydrogenase E1 component subunit beta and malate dehydrogenase suffered a downregulation when under CBZ contamination. This result does not follow the same trend as observed for other contaminations and tissues, for instance MTF did not affect malate dehydrogenase expression. ACT revealed different trends in these proteins, as previously mentioned. Thus, of the three pharmaceuticals studied, CBZ is the one that affects the most enzymes from Krebs cycle and consequently the energy processes. Kumar and co-workers (Kumar *et al.*, 2000) observed also a decrease in total activities of mitochondrial and chloroplast malate dehydrogenase for rice

seedlings under high salinity levels on less tolerant cultivars. So, it is possible to state that this type of expression may be a consequence of different abiotic stresses.

This may consist in one important outcome of our study, suggesting that the exposure to different pharmaceuticals will induce a different response and affect pathways differently.

6.2.2.6. Cell Wall proteins

UGHD is responsible for the synthesis of UDP-alpha-D-glucuronate, a key sugar nucleotide involved in the biosynthesis of the plant cell wall. Similarly, pectinesterase is also related with cell wall modifications, catalysing pectin deesterification. In both cases, it was possible to find an upregulation in MTF contaminated leaves. Indeed, Vítámvás *et al.* (2015) reported an increase of UGHD, under drought stress conditions, which may indicate an enhanced synthesis of pectins and hemicelluloses. Le Gall *et al.* (2015) pointed out some correlation among different types of stresses and pectinesterase activity, showing that in some abiotic stresses the regulation of this protein, or even pectin content itself, increased. Therefore, both proteins may lead to changes on cell walls as a response to the induced stress. The relation among these two proteins and abiotic stress also happened for ACT and CBZ contaminations, yet with minor differences to control. This result for UGDH expression in leaves revealed an opposite pattern from the one observed in roots. This points to a dissimilar pattern of response among tissues.

6.2.2.7. Photosynthesis and Calvin cycle

Photosynthesis was not so affected by the presence of MTF, ACT and CBZ. During the experiments no visual effects were observed in terms of green leaf coloration on day 8, as it is possible to observe in Figure 6.2. However, some changes were observed in chlorophyll a-b binding protein which revealed higher expression in CBZ leaves in comparison to control. MTF and ACT also influenced the pools of chlorophyll a-b binding protein, however this difference was not as high as the one with CBZ contamination.

Ribulose biphosphate carboxylase large chain (fragment, rbcL) (Table 1b) was upregulated in contaminated leaves, mostly with MTF contamination. On the other hand, RuBisCO_large domain-containing protein (Table 2b) was identified in lower abundance in contaminated leaves when compared to controls, revealing the lower expression in ACT leaves. The proportion of fold change (8.2) is higher than the max fold change for the first one (3.2). A downregulation of RubisCO has been reported in different stress conditions as in soybean subjected to drought stress (Das *et al.*, 2011). Such results are likely related with a shutdown

of photosynthetic activity as reported by Vu *et al.* (1999) and by Freitas *et al.* (2015). However, in our study, no alterations on photosynthesis were shown during experiments and the abundance of chlorophyll a-b binding protein reveals that alteration in RuBisCO did not impact the plant photosynthetic ability.

6.2.2.8. Nutrients assimilation proteins

The plasma membrane ATPase generates the gradient of electrochemical potentials of H⁺ across the plasma membrane. This protein plays a crucial role in the regulation of intracellular pH and proton export across plasma membrane as well as proton transmembrane transport. ATPase plays a fundamental role in the resistance of plants to abiotic and biotic stress (Janicka *et al.*, 2018). Results of plasma membrane ATPase may fluctuate according to tissue and types of abiotic stress, however the lower abundance in contaminated leaves in the present study may reflect a pattern for downregulation of this protein because of the exposure to these pharmaceuticals. Thus, the lower expression of plasma membrane ATPase may cause a negative impact on solute transport into and across membrane. Shen *et al.* (2011) reported an increase in plasma membrane ATPase activity when rice was exposed to salinity stress. On the other hand, Wakeel *et al.* (2010) reported a decrease on plasma membrane ATPase activity in sugar cane under salt stress.

Sulphite reductase is a key enzyme in the assimilatory sulphate reduction, being responsible for the reduction of sulphite to sulphide. The maintenance of sulphur levels is crucial for a good regulation of S assimilation pathway. In fact, leaves are much more active than roots in what concerns S assimilation. This protein was accumulated in contaminated leaves, particularly in ACT contamination. According to Brychkova *et al.* (2015), an increase in sulphite content was reported under dark stress in tomato leaves. The presence of sulphite reductase may also result from an accumulation of sulphite in cells. Hänsch *et al.* (2007) indicated that when the cell shows a higher content of sulphite, it may be oxidized by hydrogen peroxide in a non-enzymatic reaction occurring in the peroxisomes. This reaction will promote ROS detoxification in the cells. Therefore, the expression of sulphite reductase may be an indicator to high accumulation of sulphite in cells as a consequence of a response to oxidative stress. Also, according to our results, the abundance of sulphite may be a precursor of other important metabolites such as glutathione.

6.3. Conclusions

Several protein groups were differentially accumulated in our study. Herein we have highlighted the main proteins related to the stress response, glycolysis, cell respiration and cell wall processes, among others. Additionally, specific proteins of significance to ROS scavenging, cell homeostasis and photosynthesis were identified in order to form a complete picture of cell regulation processes.

An overall analysis of differentially expressed stress proteins, for the three contaminants, highlights that the major effects are due to CBZ contamination. However, the three contaminants revealed different patterns regarding stress proteins. It is therefore possible to state that different organic compounds will influence the regulation and possible detoxification pathways in lettuce cells. In general cytochrome P450 proteins revealed higher abundance under contamination in both tissues showing a clear activation of detoxification pathways. Besides pharmaceuticals contamination also interfered on cell respiration proteins suggesting a correlation among oxidative stress and dysregulation on Krebs cycle together with glycolysis pathways. Unlike stress proteins, where CBZ was the contaminant that most influence the redox state of cells, ACT was the one that most influence respiration metabolism, mainly in leaves. MTF was clearly the contaminant that less affected metabolic pathways, in the set of roots and leaves, revealing the lower number of abundant and differentially expressed proteins. Lettuce plants revealed a higher tolerance to MTF and to be able to cope with CBZ and ACT with a different response to each of them, in a short period of exposure. Longer periods of exposure to these pharmaceuticals, even in lower concentrations, may lead to more damages in cells and possible alterations in biomass parameters affecting productivity standards, and even consumers safety.

Future proteomics approach together with metabolomics will improve data regarding emerging contaminants and their interference in metabolic pathways. Besides, the interaction among pharmaceuticals and essential minerals will be an asset in future research in order to provide a complete analysis not only in terms of minerals uptake but also correlating this with up and downregulation of different proteins.

CHAPTER VII

Final Conclusions and Future Perspectives

7.1. Final Conclusions

The large-scale use of pharmaceuticals by modern societies have led to its dissemination in different environmental compartments, potentially affecting both plants and animals. The knowledge about uptake, accumulation and the effect on plants is still scarce, then more scientific studies are essential to completely understand these processes.

Different pharmaceutical compounds have been studied which, due to their different chemical nature, may manifest effects of different intensities and with different consequences for the plant, not only from a biological point of view but also as contaminants with impact on food systems.

The present work represents an integrative analysis concerning emergent contaminants and their effects on plants metabolism, including a proteomic analysis that was a useful tool to that purpose.

Carbamazepine (CBZ), acetaminophen (ACT) and metformin (MTF) were the three pharmaceuticals that were analysed, and they manifested different uptake characteristics and affected plant metabolism differently.

Although some metabolic responses were similar (like ROS production, scavenger mechanisms and secondary metabolism response), CBZ clearly induced the strongest toxicity effects evidenced by the activation of more defence mechanisms.

CBZ was detected in both roots and leaves, even at the lowest concentration tested. The effects of this contaminant resulted in the decrease of the total biomass of the plant, as well as in the decrease of the root length. Lettuce leaves showed high levels of reddish colour due to the increase in the content of anthocyanins as a result of PAL activity induced by stress. Stress indicators showed that oxidative stress was more intense in leaves than in roots, and this agrees with the overall defence mechanisms detected. The antioxidant response in leaves had, as main defence mechanism, the action of enzymes directly involved in the ROS scavenging, such as SOD, CAT, GPOD and APX. However, in roots only CAT, GPOD and GR activities increased. In addition, metabolites such as AsA and GSH have also been shown to play an important role as antioxidants in response to increased concentrations of CBZ. Despite the roots being in direct and continuous contact with the contaminant, it was the leaves that showed the greatest effects in terms of response to oxidative stress. The uptake of some essential elements like Cu, Mn, Zn, Mg, K and P in leaves decreased under the presence of CBZ, while in roots the main differences were observed in K and Cu. Indeed, changes in mineral contents were reflected by the proteomic analysis. For instance, S content in both

tissues decreased significantly in certain times of exposure. According to proteomic analysis, proteins related with sulphur activity were identified as less abundant in case of CBZ contamination, which may be a consequence of lower S contents in leaves and roots. In general, proteins related to the stress response (as SOD and peroxidases) revealed a higher abundance in plants contaminated with CBZ.

Lettuce plants contaminated with ACT did not show any visual signs of damage during the experimental period and the chlorophyll content did not change in relation to the control. Regarding the oxidative stress indicators and antioxidant mechanisms, the main differences were observed in leaves. Hydrogen peroxide levels increased significantly on day 15 of exposure, accompanied by an increase in CAT activity. SOD also showed an increase over the exposure time. In general, the roots were, again, less affected, regarding ROS scavenging enzymes. Even so, the quantification of ACT showed similar levels in leaves and in roots. On the other hand, the possible translocation of ACT to the aerial part can condition the response observed in the roots. ACT did not significantly affect the absorption of essential mineral elements. Regarding proteomics analysis, ACT contamination was the one that revealed the largest number of proteins with the most significant differences in relation to the control in leaves, as for example, the increase in the abundance of proteins such as TRx and GR. The global response of lettuce plants showed considerable tolerance to the presence of ACT in the studied concentrations and up to 15 days of contamination.

Exposure to MTF showed some tolerance by lettuce plants. Visually, no root or leaf damages were observed. Even so, some significant differences were detected in the mass of leaves and roots on the last day of contamination (day 15). Although the quantification of MTF was not performed in this work, the absorption of this drug has already been evidenced in other studies. This contaminant had an impact in terms of stress indicators, namely in the concentration of H₂O₂ in leaves. This increase in hydrogen peroxide is related to SOD activity, which also increased with exposure to MTF. The scavenging of ROS in leaves was more evident with the increase in the activity of enzymes such as CAT and GPOD, but it was APX that revealed the most significant differences. The roots were the least affected by the exposure to this contaminant and they showed to be more affected for longer periods of exposure to MTF. Lipid peroxidation showed to be higher in roots under MTF for 15 days. Enzymes such as CAT, GPOD, APX and GR showed the main differences after 15 days of exposure, as well as H₂O₂ concentration. In terms of the content of mineral elements, MTF contributed to increase the absorption of Zn and Mn in leaves on certain days of exposure while the absorption of Ca, Cu and K was lower than the control. Proteomic analysis allowed to correlate some of these results with proteins abundance. For instance, the increase in Zn content in both parts of lettuce may

be a reflection of higher content for cofactor of SOD enzyme. Besides, Ca pools may also reflect lipid peroxidation of cells. Proteomic analysis allowed a broader approach to the significant differences in root and leaf proteins. For instance, MTF affected the abundance of the protein APX, in roots.

Proteomics studies allowed an integration of all the obtained results as it was possible to relate defence mechanisms with proteins abundance. Although significant differences have not been detected in some enzymatic mechanisms, their abundance reflects a response at the cellular level according to the abundance obtained for some proteins. The changes in proteins abundance were detected, in some cases, for the three contaminants, remaining lower in relation to the control. The increase in the abundance of proteins involved in the response to oxidative stress, found in leaves, addresses the importance of this approach for a better understanding of the overall plant tolerance process. CBZ was the pharmaceutical that most influenced proteins expression and abundance in roots, while in leaves ACT revealed higher number of upregulated proteins, both in relation to control.

The present work was focused on the quantification of the studied pharmaceuticals in different plant parts and in the induced phytotoxicity and response mechanism involved in plant defence, mainly related to oxidative stress. Therefore, we consider that this work represents an important contribution to the existing knowledge in this area of this relatively new contaminants in the environment.

7.2. Future Perspectives

In the present study, a characterization of antioxidant defence mechanisms as well as the biochemical response of lettuce to abiotic stress was addressed and the uptake of pharmaceuticals was quantified (with exception of metformin). However, some questions regarding pharmaceutical contamination are still open. Therefore, future work perspectives may pass by the following suggestions:

- A full characterization of pharmaceuticals allocation and metabolization within plant cells, relating with plants detoxification systems.
- A study conducted in soil, in order to study the main changes in compounds structure and bioavailability for roots uptake.
- Studies with other crops, such as tomato and aromatic species in order to study how pharmaceuticals may reach fruits or interfere with aromatic characteristics of these plants.

- Crops highly dependent of water availability such as rice or watercress. This would make possible to understand if these crops are more susceptible to pharmaceuticals.
- A case study of irrigation with treated wastewater and/or application of manure into soil, screening the main pharmaceuticals found.
- The application of soil additives such as cork or forest biomass sub products, with the objective to understand if the application of organic sub products can influence the availability of pharmaceuticals.

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Appendix

Table A.1. – Mineral content in roots and leaves under CBZ contamination for 15 days. Significant differences are expressed in blue cells.

[mg kg ⁻¹]		Leaves						Roots					
		Day 1		Day 8		Day 15		Day 1		Day 8		Day 15	
Cu	Control	8.07 ± 0.92	aA	6.65 ± 0.32	aA	7.78 ± 0.49	aA	10.83 ± 2.63	aA	10.05 ± 1.86	aA	12.61 ± 0.83	aA
	0.1 mg L ⁻¹ CBZ	7.70 ± 0.97	aA	4.26 ± 0.88	bA	3.65 ± 0.10	bB	11.63 ± 1.57	aA	9.35 ± 1.10	aA	10.35 ± 2.26	aA
	1 mg L ⁻¹ CBZ	8.32 ± 1.24	aA	3.43 ± 0.25	bB	2.97 ± 0.14	bC	13.14 ± 1.26	aA	9.90 ± 1.05	bA	6.66 ± 1.01	cB
	5 mg L ⁻¹ CBZ	8.73 ± 1.28	aA	5.89 ± 0.55	bB	6.36 ± 0.35	bC	11.56 ± 2.35	aA	9.11 ± 2.80	aA	5.67 ± 0.47	bB
Mn	Control	559.81 ± 14.12	aA	523.88 ± 17.56	abA	502.52 ± 18.35	bA	439.37 ± 33.42	aA	575.24 ± 64.36	aA	613.42 ± 106.69	aA
	0.1 mg L ⁻¹ CBZ	585.57 ± 31.98	aA	562.39 ± 41.37	aA	510.65 ± 46.87	aA	524.72 ± 75.19	aA	531.62 ± 67.31	bA	1035.94 ± 133.85	bB
	1 mg L ⁻¹ CBZ	331.68 ± 8.94	aB	220.55 ± 9.14	bB	241.15 ± 10.37	cB	634.03 ± 111.62	aA	1068.32 ± 161.85	bB	1456.79 ± 115.15	cC
	5 mg L ⁻¹ CBZ	460.20 ± 10.35	aC	366.36 ± 6.06	bC	267.50 ± 22.84	bB	588.89 ± 121.83	aA	1171.90 ± 54.58	bB	1516.86 ± 51.93	cC
Fe	Control	87.06 ± 1.29	aA	92.10 ± 6.84	aA	80.41 ± 6.82	bA	2087.63 ± 401.13	aA	1989.56 ± 232.26	aA	2409.01 ± 281.98	aA
	0.1 mg L ⁻¹ CBZ	105.23 ± 4.46	aB	102.42 ± 4.87	aA	74.36 ± 9.62	aA	2106.54 ± 526.22	aA	1595.86 ± 404.62	aA	2406.23 ± 443.03	cA
	1 mg L ⁻¹ CBZ	91.84 ± 4.01	abB	97.53 ± 5.90	aA	74.13 ± 12.14	bA	2089.81 ± 353.10	aA	1906.80 ± 480.13	aA	1676.99 ± 176.92	abB
	5 mg L ⁻¹ CBZ	69.08 ± 3.07	aC	93.02 ± 3.07	bA	84.10 ± 13.48	abA	2246.22 ± 403.31	aA	1330.23 ± 375.48	bA	1491.86 ± 38.05	abB
Zn	Control	149.16 ± 1.94	aA	144.36 ± 1.63	bA	152.85 ± 1.37	aA	507.89 ± 18.77	aA	494.20 ± 50.19	aA	551.99 ± 49.35	aA
	0.1 mg L ⁻¹ CBZ	97.79 ± 3.31	aA	79.61 ± 2.64	aB	82.03 ± 3.19	aB	483.30 ± 58.23	aA	443.63 ± 55.40	aA	594.81 ± 100.64	aA
	1 mg L ⁻¹ CBZ	118.90 ± 2.35	aB	102.38 ± 0.45	bC	96.85 ± 6.34	bC	536.84 ± 42.09	aA	538.32 ± 78.60	aA	449.38 ± 67.50	aA
	5 mg L ⁻¹ CBZ	141.69 ± 14.61	aB	125.69 ± 3.16	bD	128.63 ± 8.95	bC	492.78 ± 84.78	aA	491.81 ± 70.57	aA	481.60 ± 11.58	aA
Na	Control	823.72 ± 95.79	aA	831.58 ± 85.45	aA	904.59 ± 80.09	aA	1220.65 ± 148.54	aA	1263.04 ± 130.54	aA	706.29 ± 230.96	bA
	0.1 mg L ⁻¹ CBZ	858.62 ± 111.24	aA	822.05 ± 107.46	aA	835.10 ± 84.94	aA	1310.16 ± 231.60	aA	1139.82 ± 75.00	aA	873.68 ± 179.04	aA
	1 mg L ⁻¹ CBZ	879.54 ± 57.92	aA	947.94 ± 44.63	aA	917.41 ± 125.69	aA	1018.07 ± 198.52	aA	1273.19 ± 173.66	aA	852.46 ± 137.86	aA
	5 mg L ⁻¹ CBZ	805.78 ± 46.87	aA	988.46 ± 87.43	aA	1018.09 ± 140.76	aA	1028.43 ± 228.79	aA	1159.16 ± 170.36	aA	764.58 ± 130.37	aA
K	Control	96186.09 ± 1577.62	aA	82881.57 ± 1782.36	bA	78398.34 ± 3691.96	bA	67958.97 ± 5963.98	aA	71130.54 ± 4180.47	aA	73477.77 ± 4400.48	aA
	0.1 mg L ⁻¹ CBZ	90711.32 ± 2427.24	aAB	77764.54 ± 4045.68	bAB	71016.88 ± 5436.49	bAB	68082.73 ± 4978.92	aA	63679.71 ± 2340.59	aA	67772.47 ± 1351.97	aA
	1 mg L ⁻¹ CBZ	81594.07 ± 646.74	aBC	64289.13 ± 1605.35	bBC	66584.34 ± 1108.49	bB	71224.28 ± 453.30	aA	51372.29 ± 1305.15	bB	53295.03 ± 5318.98	bB
	5 mg L ⁻¹ CBZ	87301.79 ± 5436.63	aC	70952.39 ± 4734.98	bC	68309.95 ± 1529.12	bB	65710.57 ± 1074.93	aA	44376.59 ± 6717.24	bB	47714.98 ± 2343.78	bB
Ca	Control	13773.40 ± 211.35	aA	13018.89 ± 395.01	abA	12186.18 ± 599.34	bA	6609.06 ± 854.72	aA	7025.83 ± 1190.49	aA	6885.74 ± 1083.32	aA
	0.1 mg L ⁻¹ CBZ	14492.05 ± 637.11	aA	13951.82 ± 636.66	aA	13473.29 ± 664.85	aA	7661.39 ± 978.86	aA	7486.62 ± 834.16	aA	7563.66 ± 921.43	aAB
	1 mg L ⁻¹ CBZ	14698.56 ± 658.18	aA	13003.73 ± 483.44	aA	12755.17 ± 1170.85	aAB	7905.04 ± 605.13	aA	8814.84 ± 1289.71	aA	9083.49 ± 785.79	aBC
	5 mg L ⁻¹ CBZ	14559.71 ± 334.55	aA	14241.97 ± 617.99	aA	15157.29 ± 271.31	aB	7319.45 ± 1238.12	aA	9132.70 ± 998.15	abA	11040.74 ± 179.48	bC
Mg	Control	2524.42 ± 69.92	aA	2752.49 ± 133.99	abA	2924.89 ± 119.55	bA	970.69 ± 32.50	aA	845.33 ± 192.12	aA	772.95 ± 116.96	aA
	0.1 mg L ⁻¹ CBZ	2525.41 ± 56.40	aA	2467.47 ± 31.37	aB	2619.37 ± 169.32	aA	911.68 ± 82.28	aAB	980.65 ± 147.76	aA	915.69 ± 175.67	aA
	1 mg L ⁻¹ CBZ	2253.15 ± 123.45	aAB	2283.59 ± 65.95	aB	1971.39 ± 213.39	aB	872.24 ± 111.12	aAB	1036.96 ± 119.13	abA	1319.59 ± 108.75	bB
	5 mg L ⁻¹ CBZ	2301.54 ± 96.44	aB	2258.64 ± 82.93	aB	2132.49 ± 56.61	aB	732.03 ± 107.82	aB	1251.22 ± 215.49	bA	1858.04 ± 61.47	cC
P	Control	6882.10 ± 101.44	aA	7270.84 ± 38.87	bA	7969.55 ± 49.17	cA	20268.22 ± 496.47	aA	21362.30 ± 588.09	aA	21921.70 ± 1728.36	aAB
	0.1 mg L ⁻¹ CBZ	6738.83 ± 106.11	aA	4811.68 ± 37.90	bB	4504.34 ± 216.51	aB	18870.28 ± 1715.55	aA	21200.15 ± 1105.69	abA	23535.74 ± 1112.23	bA
	1 mg L ⁻¹ CBZ	6665.93 ± 69.38	aA	5743.29 ± 176.61	bC	6443.80 ± 439.46	bC	18705.31 ± 968.07	aA	20993.85 ± 1737.07	aA	20643.81 ± 1958.11	aAB
	5 mg L ⁻¹ CBZ	6811.79 ± 231.39	aA	4082.77 ± 143.47	bD	3726.32 ± 82.19	bD	19742.86 ± 365.53	aA	22527.83 ± 1080.00	bA	18348.97 ± 461.79	aB
S	Control	3402.01 ± 11.44	aA	3782.56 ± 103.42	bA	4372.10 ± 59.69	aA	6243.75 ± 875.74	aA	5950.73 ± 201.64	aA	7238.69 ± 662.63	aA
	0.1 mg L ⁻¹ CBZ	3469.91 ± 190.06	aA	3739.86 ± 80.04	aA	3878.47 ± 300.99	aB	6080.54 ± 684.15	aA	5829.95 ± 456.22	aA	6809.59 ± 493.37	aAB
	1 mg L ⁻¹ CBZ	3216.63 ± 191.60	aA	3852.34 ± 41.82	bA	4029.22 ± 130.66	bB	5342.86 ± 340.25	aA	5434.55 ± 695.46	aA	6003.18 ± 569.71	aAB
	5 mg L ⁻¹ CBZ	3349.40 ± 115.94	aA	3702.58 ± 129.48	bA	3824.76 ± 160.23	bAB	5861.32 ± 356.06	aA	5475.06 ± 311.57	aA	5829.88 ± 165.98	aB

Table A.2 - Mineral content in roots and leaves under ACT contamination for 15 days. Significant differences are expressed in blue cells.

[mg kg ⁻¹]		Leaves						Roots					
		Day 1		Day 8		Day 15		Day 1		Day 8		Day 15	
Cu	Control	13.19 ± 0.87	aA	13.57 ± 0.37	aA	10.89 ± 0.70	bA	49.26 ± 10.00	aA	39.13 ± 12.54	abA	22.30 ± 3.06	bA
	0.1 mg L ⁻¹ ACT	13.39 ± 0.84	aA	11.92 ± 0.43	abB	10.63 ± 0.35	bA	41.30 ± 6.21	aA	43.19 ± 7.17	aA	20.11 ± 6.95	bA
	1 mg L ⁻¹ ACT	12.31 ± 0.44	aAB	11.19 ± 0.14	aB	11.29 ± 0.90	aA	39.98 ± 6.76	aA	52.49 ± 12.61	aA	18.64 ± 2.62	bA
	5 mg L ⁻¹ ACT	10.94 ± 0.21	aB	11.07 ± 0.71	aB	11.36 ± 0.27	aA	42.57 ± 8.68	abA	52.09 ± 15.07	aA	21.59 ± 1.19	bA
Mn	Control	528.53 ± 16.33	aA	532.59 ± 17.48	aA	563.84 ± 32.79	aA	793.75 ± 108.63	aA	929.75 ± 11.04	aA	821.29 ± 40.77	aA
	0.1 mg L ⁻¹ ACT	549.27 ± 40.44	aA	544.53 ± 4.92	aA	560.36 ± 21.97	aA	861.83 ± 70.47	aA	833.03 ± 94.58	aA	670.94 ± 78.00	aA
	1 mg L ⁻¹ ACT	488.81 ± 9.80	aA	518.00 ± 8.00	abA	566.21 ± 31.83	bA	735.19 ± 105.37	abA	855.86 ± 39.48	aA	672.44 ± 46.60	bA
	5 mg L ⁻¹ ACT	534.93 ± 12.10	aA	538.27 ± 40.46	aA	568.79 ± 29.16	aA	805.10 ± 73.44	aA	811.31 ± 76.15	aA	652.96 ± 97.75	aA
Fe	Control	170.96 ± 10.08	aA	127.47 ± 14.87	bA	136.99 ± 15.57	abA	3991.48 ± 547.39	aA	6577.34 ± 292.44	bA	3244.16 ± 232.53	aA
	0.1 mg L ⁻¹ ACT	173.64 ± 23.05	aA	108.63 ± 4.48	bA	115.85 ± 16.81	bA	4373.21 ± 455.99	aA	6516.70 ± 848.30	bA	4348.61 ± 355.58	aAB
	1 mg L ⁻¹ ACT	135.03 ± 3.56	aA	126.03 ± 18.46	aA	125.20 ± 6.64	aA	4077.06 ± 687.16	aA	7115.50 ± 793.98	bA	4340.88 ± 212.41	aAB
	5 mg L ⁻¹ ACT	142.04 ± 18.18	aA	115.65 ± 10.47	aA	127.58 ± 12.23	aA	4344.15 ± 480.45	aA	7239.72 ± 778.84	bA	5020.64 ± 726.70	aB
Zn	Control	181.67 ± 6.50	abA	177.26 ± 6.37	aA	194.30 ± 3.39	bAB	625.59 ± 77.66	aA	772.97 ± 12.64	bA	587.09 ± 26.15	aA
	0.1 mg L ⁻¹ ACT	184.73 ± 11.95	aA	186.67 ± 3.49	aAB	189.69 ± 4.73	aA	650.55 ± 49.61	abA	701.14 ± 75.56	aA	547.07 ± 36.71	bA
	1 mg L ⁻¹ ACT	169.63 ± 3.10	aA	182.62 ± 3.86	bAB	204.77 ± 0.29	cB	559.58 ± 75.85	aA	763.56 ± 45.38	bA	560.42 ± 28.02	aA
	5 mg L ⁻¹ ACT	174.38 ± 1.06	aA	190.75 ± 5.94	bB	201.59 ± 5.75	bB	609.46 ± 57.18	abA	759.28 ± 69.83	aA	563.53 ± 60.01	bA
Na	Control	1096.58 ± 80.99	aA	838.46 ± 113.80	bA	542.06 ± 22.81	cA	2741.67 ± 499.08	aA	1661.08 ± 345.24	bA	941.15 ± 115.24	bA
	0.1 mg L ⁻¹ ACT	965.07 ± 82.04	aA	790.93 ± 58.04	bA	554.76 ± 54.57	cA	2801.84 ± 596.68	aA	2130.11 ± 455.74	aA	924.25 ± 200.55	bA
	1 mg L ⁻¹ ACT	1082.69 ± 56.73	aA	846.66 ± 102.40	bA	503.56 ± 31.32	cA	2262.42 ± 428.61	aA	1770.41 ± 345.81	aA	736.72 ± 55.69	bA
	5 mg L ⁻¹ ACT	975.25 ± 77.44	aA	754.76 ± 148.03	abA	615.05 ± 91.05	bA	2366.17 ± 485.46	aA	2186.25 ± 505.57	aA	885.64 ± 68.28	bA
K	Control	73439.89 ± 3381.20	aA	73384.78 ± 2713.92	aA	69675.00 ± 2339.40	aA	64452.03 ± 2802.88	aA	69538.30 ± 3139.61	aA	66191.83 ± 5699.58	aA
	0.1 mg L ⁻¹ ACT	71512.63 ± 3228.67	aA	71756.42 ± 1142.30	aA	69324.31 ± 2019.52	aA	67838.69 ± 5507.16	aA	72997.20 ± 5471.19	aA	63583.65 ± 5416.94	aA
	1 mg L ⁻¹ ACT	71798.47 ± 832.46	aA	71798.81 ± 2110.39	aA	68806.39 ± 4718.48	aA	65248.71 ± 3195.63	aA	75724.97 ± 7864.46	aA	64425.91 ± 2606.76	aA
	5 mg L ⁻¹ ACT	70605.54 ± 1876.87	aA	70908.54 ± 3653.58	aA	68516.61 ± 2133.90	aA	69328.12 ± 732.54	abB	75754.18 ± 3228.31	bA	62556.63 ± 1383.02	cA
Ca	Control	16109.03 ± 881.20	aA	15773.88 ± 377.29	aA	16204.35 ± 965.10	aA	11849.74 ± 709.60	aA	7607.64 ± 852.14	bA	5616.45 ± 318.73	cA
	0.1 mg L ⁻¹ ACT	17004.38 ± 1772.38	aA	16164.16 ± 139.53	aAC	16760.87 ± 290.55	aA	11328.39 ± 1232.12	aA	7962.54 ± 848.44	bA	6150.05 ± 645.44	bA
	1 mg L ⁻¹ ACT	15760.15 ± 339.57	aA	17409.76 ± 115.26	bBC	16067.16 ± 896.39	abA	11206.55 ± 1570.74	aA	9617.89 ± 1325.50	aA	5762.71 ± 176.98	bA
	5 mg L ⁻¹ ACT	15941.97 ± 537.14	aA	16684.94 ± 444.93	aC	16898.52 ± 124.83	aA	10548.24 ± 1557.06	aA	9847.86 ± 1251.84	abA	7036.86 ± 1069.15	bA
Mg	Control	3988.66 ± 238.53	aA	3597.60 ± 236.09	aA	3786.84 ± 321.10	aA	1531.82 ± 157.64	aA	1251.27 ± 87.45	bA	1184.32 ± 61.08	bA
	0.1 mg L ⁻¹ ACT	3859.32 ± 184.82	abA	3609.49 ± 51.27	aA	3935.36 ± 73.87	bA	1392.80 ± 35.70	aA	1217.56 ± 106.46	aA	1245.75 ± 63.08	aA
	1 mg L ⁻¹ ACT	3711.39 ± 89.13	aA	3651.69 ± 131.57	aA	3901.31 ± 255.51	aA	1462.95 ± 113.54	aA	1295.59 ± 158.06	aA	1179.64 ± 50.03	aA
	5 mg L ⁻¹ ACT	3784.36 ± 10.09	aA	3652.31 ± 87.86	aA	3866.42 ± 275.70	aA	1396.28 ± 42.48	aA	1336.71 ± 91.97	aA	1267.88 ± 108.53	aA
P	Control	6825.88 ± 229.78	aA	7006.07 ± 299.70	aA	7543.65 ± 641.63	aA	11341.15 ± 595.32	aA	12158.44 ± 272.60	aA	11630.10 ± 784.16	aA
	0.1 mg L ⁻¹ ACT	7176.20 ± 132.07	aAB	7415.86 ± 147.00	abA	7576.37 ± 140.33	bA	11510.24 ± 1739.62	aA	12662.13 ± 844.29	aA	12286.67 ± 849.38	aA
	1 mg L ⁻¹ ACT	7316.83 ± 237.57	aB	7318.67 ± 192.00	aA	7942.33 ± 476.92	aA	10869.56 ± 44.79	aA	13316.22 ± 1259.30	bA	12301.25 ± 825.28	abA
	5 mg L ⁻¹ ACT	7094.99 ± 28.25	aAB	7208.64 ± 140.14	aA	7535.87 ± 383.49	aA	11140.24 ± 141.85	aA	12139.22 ± 323.25	bA	12434.42 ± 379.69	bA
S	Control	3974.15 ± 114.06	aA	3918.68 ± 124.23	aA	4210.45 ± 292.52	aA	10020.30 ± 1061.97	aA	9689.80 ± 831.48	abA	7412.74 ± 922.17	bA
	0.1 mg L ⁻¹ ACT	4063.52 ± 162.01	aA	4021.60 ± 66.96	aA	4107.43 ± 45.33	aA	11371.60 ± 1691.52	aA	9417.56 ± 1591.53	abA	6629.01 ± 756.36	bA
	1 mg L ⁻¹ ACT	4109.92 ± 42.95	aA	3905.06 ± 84.39	aA	4153.96 ± 162.77	aA	9778.30 ± 1362.02	aA	10201.53 ± 1016.49	aA	6977.05 ± 579.22	bA
	5 mg L ⁻¹ ACT	3964.68 ± 90.44	aA	4098.88 ± 91.44	aA	4288.57 ± 272.36	aA	9863.46 ± 498.44	aA	9176.89 ± 1273.93	abA	7317.13 ± 498.98	bA

Table A.3 — Mineral content in roots and leaves under MTF contamination for 15 days. Significant differences are expressed in blue cells.

[mg kg ⁻¹]		Leaves						Roots					
		Day 1		Day 8		Day 15		Day 1		Day 8		Day 15	
Cu	Control	12.15 ± 1.32	aA	12.03 ± 0.55	aA	10.46 ± 0.74	aA	27.04 ± 3.44	aA	33.63 ± 4.00	aA	15.62 ± 1.28	bAB
	0.1 mg L ⁻¹ MTF	12.00 ± 0.99	abA	10.61 ± 0.36	aA	9.91 ± 0.56	bA	29.45 ± 3.48	aA	31.93 ± 3.49	aAB	17.92 ± 1.24	bA
	1 mg L ⁻¹ MTF	10.29 ± 0.59	aAB	10.24 ± 1.33	aA	9.60 ± 0.30	aA	24.18 ± 1.74	aA	25.56 ± 0.44	aB	13.95 ± 1.68	bB
	5 mg L ⁻¹ MTF	8.54 ± 0.33	aB	10.00 ± 0.46	aA	9.46 ± 0.93	aA	24.21 ± 2.72	aA	24.96 ± 1.91	aB	13.53 ± 0.90	bB
Mn	Control	446.76 ± 1.97	aA	388.05 ± 9.12	bA	614.68 ± 1.70	cA	1003.70 ± 108.96	aA	746.00 ± 76.40	aA	774.54 ± 81.95	aA
	0.1 mg L ⁻¹ MTF	462.57 ± 61.03	aAB	514.96 ± 26.02	abB	628.43 ± 46.54	bAB	1037.67 ± 171.43	aA	853.76 ± 132.74	aA	948.95 ± 108.38	aAB
	1 mg L ⁻¹ MTF	529.46 ± 31.01	aAB	579.55 ± 59.01	aBC	619.21 ± 14.89	aAB	1113.29 ± 50.45	aA	865.19 ± 34.45	aA	1054.49 ± 140.42	abB
	5 mg L ⁻¹ MTF	539.81 ± 15.46	aB	658.96 ± 55.08	bC	685.13 ± 13.94	bB	1102.62 ± 108.34	aA	926.31 ± 80.31	abA	870.82 ± 72.36	bAB
Fe	Control	112.05 ± 9.34	aA	140.99 ± 20.31	aA	137.73 ± 17.30	aA	3393.91 ± 395.60	aA	3673.21 ± 155.33	aA	4119.24 ± 428.12	aA
	0.1 mg L ⁻¹ MTF	125.82 ± 22.46	aA	169.94 ± 7.54	bA	141.04 ± 9.46	abA	3348.78 ± 422.23	aA	4094.33 ± 470.67	abA	4501.42 ± 317.42	aA
	1 mg L ⁻¹ MTF	145.77 ± 19.17	aA	148.13 ± 24.85	aA	103.07 ± 4.40	aB	3795.01 ± 236.09	aA	4499.46 ± 471.96	aA	4212.79 ± 265.77	aA
	5 mg L ⁻¹ MTF	147.70 ± 7.43	aA	147.14 ± 12.89	aA	113.67 ± 10.00	bAB	3059.09 ± 299.52	aA	4019.27 ± 81.48	bA	4075.38 ± 364.08	bA
Zn	Control	140.10 ± 4.49	aA	167.79 ± 3.24	bA	193.70 ± 2.39	cA	587.69 ± 76.65	aA	589.75 ± 33.67	aA	566.25 ± 56.33	aA
	0.1 mg L ⁻¹ MTF	146.85 ± 8.47	aA	169.73 ± 4.36	bAB	206.24 ± 5.98	cB	623.86 ± 86.56	aA	680.86 ± 25.40	aB	642.28 ± 56.16	aA
	1 mg L ⁻¹ MTF	172.82 ± 4.87	aB	192.44 ± 16.23	aBC	191.77 ± 3.26	aA	635.32 ± 16.28	aA	677.17 ± 26.27	aB	602.87 ± 55.11	aA
	5 mg L ⁻¹ MTF	167.20 ± 3.94	aB	197.09 ± 7.28	bC	197.38 ± 2.89	bAB	601.91 ± 42.71	abA	685.50 ± 38.60	aB	565.34 ± 40.54	bA
Na	Control	757.34 ± 62.13	aA	751.68 ± 17.98	aA	386.87 ± 17.72	bAB	1596.09 ± 232.33	aA	1773.38 ± 41.12	aA	731.13 ± 32.63	bA
	0.1 mg L ⁻¹ MTF	794.78 ± 92.45	aA	738.11 ± 14.21	aA	423.87 ± 21.48	bA	1692.00 ± 146.82	aA	1660.29 ± 85.16	aB	709.01 ± 116.56	bA
	1 mg L ⁻¹ MTF	840.93 ± 101.57	aA	542.64 ± 42.83	bB	338.68 ± 24.08	cB	1671.65 ± 75.96	aA	1262.32 ± 206.46	bBC	477.22 ± 51.79	cB
	5 mg L ⁻¹ MTF	803.03 ± 60.51	aA	545.81 ± 30.45	bB	358.75 ± 7.73	cB	1513.77 ± 258.93	aA	1159.74 ± 215.13	bB	399.89 ± 46.48	bB
K	Control	63858.41 ± 2662.11	aA	62057.10 ± 1748.88	aA	71479.85 ± 3401.27	bA	57706.08 ± 1758.45	aA	59874.80 ± 4887.99	aA	62802.93 ± 8327.85	aA
	0.1 mg L ⁻¹ MTF	65853.38 ± 2178.98	aA	59800.94 ± 483.40	bA	71412.65 ± 3166.04	aA	63827.06 ± 1956.61	aA	64318.83 ± 4952.48	aA	62277.62 ± 6125.13	aA
	1 mg L ⁻¹ MTF	65922.49 ± 1482.36	aA	62103.64 ± 7403.28	aA	68798.87 ± 4109.50	aA	63064.95 ± 5955.06	aA	61946.39 ± 5558.63	aA	55572.43 ± 7698.83	aA
	5 mg L ⁻¹ MTF	61776.45 ± 1208.90	aA	64602.08 ± 1560.40	abA	67493.16 ± 540.23	bA	62846.82 ± 3113.76	aA	61476.92 ± 4829.71	aA	56481.59 ± 3250.43	aA
Ca	Control	11065.46 ± 417.77	aA	11059.72 ± 226.68	aA	15469.25 ± 362.89	bA	4869.46 ± 272.14	aA	6396.83 ± 474.69	bA	5040.34 ± 379.57	aA
	0.1 mg L ⁻¹ MTF	11065.74 ± 928.55	aA	12643.15 ± 700.28	aAB	15188.38 ± 335.34	bA	4934.03 ± 245.44	aA	5482.39 ± 650.25	aAB	4747.58 ± 246.18	aA
	1 mg L ⁻¹ MTF	11996.85 ± 275.41	aAB	12737.77 ± 1069.15	abAB	14479.02 ± 803.11	aA	5019.49 ± 110.88	aA	5245.97 ± 260.88	aB	4465.17 ± 485.75	aA
	5 mg L ⁻¹ MTF	12570.47 ± 206.67	aB	13790.10 ± 658.33	bB	14873.55 ± 471.61	bA	4516.76 ± 218.26	aA	4375.24 ± 106.40	aB	4446.54 ± 231.92	aA
Mg	Control	2717.06 ± 32.48	aA	2706.33 ± 34.24	aA	3696.72 ± 143.86	bA	1116.03 ± 70.20	aA	1181.74 ± 141.50	aA	1197.75 ± 159.74	aA
	0.1 mg L ⁻¹ MTF	2852.32 ± 277.62	aA	3192.59 ± 310.03	abAB	3817.20 ± 160.22	bA	1045.78 ± 95.80	aA	1249.97 ± 90.51	aA	1275.53 ± 147.34	aA
	1 mg L ⁻¹ MTF	3397.44 ± 81.28	aB	3484.34 ± 295.59	aBC	3620.07 ± 118.58	aA	1188.41 ± 70.33	aA	1219.66 ± 124.74	aA	1250.57 ± 71.26	aA
	5 mg L ⁻¹ MTF	3379.35 ± 66.97	aB	3840.97 ± 242.25	bC	3821.07 ± 109.56	bA	1191.53 ± 124.41	aA	1115.42 ± 61.18	aA	1073.42 ± 67.92	aA
P	Control	6321.02 ± 211.28	aAB	7264.54 ± 34.27	bA	7341.28 ± 278.31	bA	9702.19 ± 761.39	aA	10688.75 ± 848.68	aA	11166.73 ± 1057.22	aA
	0.1 mg L ⁻¹ MTF	6550.24 ± 89.89	aA	6508.73 ± 415.35	aAB	7143.82 ± 256.40	aA	10679.92 ± 443.38	aA	11474.64 ± 731.32	aA	11836.61 ± 569.34	aA
	1 mg L ⁻¹ MTF	6108.93 ± 176.15	aBC	6414.84 ± 436.71	aB	6574.03 ± 215.95	aA	9831.15 ± 451.55	aA	11047.78 ± 942.64	aA	11051.86 ± 513.14	aA
	5 mg L ⁻¹ MTF	5916.67 ± 52.19	aC	6875.05 ± 179.18	bAB	6911.16 ± 514.11	bA	9686.43 ± 491.02	aA	10823.15 ± 252.90	bA	10125.33 ± 537.39	abA
S	Control	2698.03 ± 31.93	aA	2901.53 ± 51.47	bA	3394.20 ± 24.34	cA	7604.04 ± 956.42	aA	8028.03 ± 613.80	aA	7285.55 ± 1175.92	aA
	0.1 mg L ⁻¹ MTF	2987.49 ± 192.70	aAC	3070.20 ± 181.07	aA	3546.26 ± 65.23	bA	8715.02 ± 784.13	aA	8557.06 ± 1078.57	aA	8139.66 ± 1234.08	aA
	1 mg L ⁻¹ MTF	3341.48 ± 149.85	aBC	3286.06 ± 218.46	aAB	3616.53 ± 180.32	aAB	8492.08 ± 895.64	aA	7844.57 ± 578.44	aA	7954.02 ± 843.81	aA
	5 mg L ⁻¹ MTF	3317.86 ± 62.86	aC	3564.92 ± 242.67	abB	3880.33 ± 111.06	bB	8716.38 ± 183.42	aA	7923.55 ± 760.51	aA	6992.97 ± 283.43	aA