

Universidade do Minho

José Tiago Teixeira de Sousa Moreira

Solanum nigrum L., a fine tool for Phytoremediation of Acetophenone

Master Thesis

Mestrado em Biologia Molecular, Biotecnologia e Bioempreendedorismo em Plantas

Trabalho realizado sob a orientação do Professor Doutor Jorge Teixeira

Outubro de 2013

Acknowledgments

I would like to express my deep gratitude to Professor Doctor Jorge Teixeira, for his patience, support, constant availability and for providing me with useful knowledge, that helped me to develop my scientific knowledge and reasoning.

I also would like to thank Doctor Amalia Calaya, for her help with the quantification of glutathione levels, and to Professor Doctor Manuel Azenha and all his team for their help in the acetophenone quantification. To João Cunha, for his help and contribution to this work.

To my beautiful, loving wife for her patience, help and love, that helped me to surpass every difficulties along the way. Without you, it wouldn´t have been the same. Also to my brother and sister for their fondness throughout this time.

To my mother and father, without whom this work wouldn´t have been possible. Thank you for understanding and supporting my choices.

To Alexandra Sousa and all colleagues in the laboratory for maintaining a good atmosphere in our working place.

In loving memory of my grandma.

Solanum nigrum L., a fine tool for remediation of acetophenone

Abstract

Organic pollutants of several industries constitute an environmental hazard because they may enter food chains, including humans. Phytoremediation is an eco friendly technology for environmental cleanup, and phytodegradtion, a strategy of phytoremediation, consists on the use of plants and associated organisms to render organic pollutants in non-reactive, non-toxic compounds. Acetophenone, a compound used in several industries, is the second most abundant constituent of Ridomil®, which is a systemic fungicide used in horticulture and vineyards and has as its active compound metalaxyl. Moreover, the several industrial uses of acetophenone make it more available and likely to accumulate in the environment. In order to access if acetophenone exerts toxicity towards *Solanum nigrum* L. and to see if this plant species may be used as a phytoremediation tool for acetophenone-contaminated sites, *S. nigrum* plants were exposed for one month to 0.52 and 1.04 ppm of this compound, which was supplied in the nutrient solution. Acetophenone levels in the nutrient medium were quantified along time, and some oxidative stress markers (H_2O_2; Chlorophyll a and b; carotenoids; proline and malondialdehyde levels), as well as some of the plant antioxidant system and detoxification mechanisms (catalase (CAT), guaiacol peroxidase (POD), ascorbate peroxidase (APX), Glutathione-*S*-Transferase (GST), γ-glutamyl-cysteine synthetase (γ-ECS) and glutathione reductase (GR) activities, as well as glutathione- and thiols-related levels) were evaluated at the end of the treatment. Acetophenone exposure impaired plant growth and induced oxidative stress. CAT and APX had enhanced activity in shoots of treated plants, while in roots both POD and APX revealed an increased activity. Increase in non-protein thiol levels revealed the importance of glutathione in acetophenone detoxification, where acetophenone is detoxified partly by conjugation with GSH through GST activity. Changes in GSH/GSSG ratios, alongside with increased γ -ECS activity and absence of statistical differences in GR activity, suggest that *S. nigrum* used *de novo* synthesis of GSH in response to acetophenone exposure. The results presented clearly reveal that *S. nigrum* is an excellent tool for the rapid remediation of contaminated sites with acetophenone, as no acetophenone was detected after 7 days of exposure to the plants.

Solanum nigrum L., uma excelente ferramenta de remediação de Acetofenona

Resumo

Os poluentes orgânicos de várias indústrias constituem um perigo ambiental, podendo ser incorporados em várias cadeias alimentares, incluindo a dos seres humanos. A fitorremediação é uma tecnologia amiga do ambiente de remediação ambiental. A fitodegradação, uma estratégia de fitorremediação, consiste no uso de plantas e organismos associados para tornar poluentes orgânicos em compostos não-reativos e não- tóxicos. Acetofenona, um composto utilizado em diversas indústrias, é o segundo composto mais abundante de Ridomil ®, fungicida sistémico utilizado na horticultura e vinha e tem como composto ativo o metalaxil. Com o objetivos de determinar a toxicidade que acetofenona exerce em *Solanum nigrum* e para averiguar a utilidade desta espécie como ferramenta fitoremediadora de acetofenona, *S. nigrum* foi exposta durante um mês a 0,52 e 1,04 ppm deste composto, fornecido a planta na solução nutritiva. Os níveis de acetofenona no meio nutritivo foram quantificados ao longo do tempo, e alguns marcadores de stress oxidativo (H_2O_2; Clorofila a e b; carotenóides; prolina e níveis de malondialdeído), bem como enzimas do sistema antioxidante e mecanismos de desintoxicação das plantas (catalase (CAT); peroxidase do guaiacol (POD); peroxidase do ascorbato (APX); glutationa-*S*-transferase (GST); γ-glutamil-cisteína sintetase (γ-ECS); glutationa redutase (GR), bem como os níveis de glutationa e tióis) foram avaliadas no final do tratamento. A exposição à acetofenona prejudicou o crescimento da planta e induziu stress oxidativo. A CAT e a APX revelaram maior atividade na parte aérea das plantas tratadas, enquanto que nas raízes a POD e a APX revelaram um aumento da atividade. O aumento dos níveis de tióis não-proteicos realçou a importância da GSH na desintoxicação da acetofenona, onde esta última é, em parte, neutralizada por conjugação com a GSH pela GST. Alterações na proporção de GSH/GSSG, bem como o aumento da atividade γ-ECS e a ausência de diferenças estatisticamente significativas na atividade de GR, sugerem que *S. nigrum* sintetizou *de novo* GSH em resposta à exposição. Os resultados apresentados revelam que *S. nigrum* é uma excelente ferramenta para a rápida recuperação de sítios contaminados com acetofenona, não tendo sido detetada 7 dias após a exposição das plantas.

Index

Acknowledgments ii
Abstract iii
Resumo iv
Index v
Abreviations vii
Introduction 1
1. Phytoremediation of organic pollutants 1
1.1. Phytodegradation of organic pollutants 2
2. Acetophenone 3
3. Glutathione 3
4. Thiol 7
5. Oxidative stress, lipid peroxidation and antioxidant mechanisms 9
5.1 ROS 10
5.2 Lipid Peroxidation 12
5.3 ROS scavenging enzymes 14
5.3.1 Catalase (EC 1.11.1.6) 14
5.3.2 Ascorbate Peroxidase (EC 1.11.1.11) 15
5.3.3 Guaiacol Peroxidase (EC 1.11.1.7) 15
5.3.4 Glutathione Reductase (EC1.8.1.7) 16
6. *Solanum nigrum* L. 16
Materials and Methods 18
1. Seed germination and growth conditions 18
2. Oxidative stress biomarkers 18
2.1 Hydrogen peroxide levels 18
2.2 Photosynthetic pigments quantification 19
2.3 Lipid peroxidation 19
2.4 Proline 19
3. Biochemical determinations 20
3.1 Catalase 20
3.2 Guaiacol Peroxidase 20
3.3 Ascorbate Peroxidase 20
3.4 Thiol levels 21
3.5 Glutathione 21

3.6 Glutathione-S-Transferase 22
3.7 γ -Glutamyl-Cysteine Synthetase (γ -ECS) 23
3.8 Glutathione Reductase 23
4. Statistical analysis 24
Results 25
1. Stress biomarkers 25
1.1 Biometry 25
1.2 Hydrogen peroxide (H_2O_2) 26
1.3 Photosynthetic pigments 26
1.4 Lipid Peroxidation 27
1.5 Oxidative Stress Index (OSI) 27
1.6 Proline contents 28
2. Antioxidant mechanisms 28
2.1 Catalase activity 28
2.2 Guaiacol Peroxidase (POD) 29
2.3 Ascorbate Peroxidase (APX) 29
2.4 Thiol content 30
2.5 Glutathione 31
3. Acetophenone removal by plants 32
4. Glutathione-*S*-Transferase (GST) 32
5. γ-ECS and GR activities 33
Discussion 34
Conclusion 38
Future directions 38
Bibliography 39

Abbreviations

Abs- Absorbance

APX- Ascorbate Peroxidase

AsA- Ascorbate

AsA-GSH- Ascorbate-Glutathione cycle

ATP- Adenosine Triphosphate

CAT- Catalase

CDNB- Chloro-dinitro-benzene

Cys- Cysteine

DHA- Dihydroascorbate

DHAR- Dihydroascrobate reductase

DTNB- 5,5'-dithiobis-2-nitrobenzoic acid

DTT- Dithiotreitol

EDTA- Ethylenediaminetetraacetic Acid

FW- Fresh Weight

GPX- Glutathione Peroxidase

GR- Glutathione Reductase

GR1- Glutathione Reductase gene 1

GRX- Glutaredoxin

GS- Glutathione Synthetase

GSH- Glutathione/Oxidized glutathione

GSH1- γ-Glutamylcysteine Synthetase gene

GSH2- Glutathione Synthetase gene

GSHt- Total Glutathione

GSSG- Reduced Glutathione

GST- Glutathione-*S*-Transferase

IAA- Indoleacetic acid (auxin)

K_m- Michaelis Constant

L•- Lipid Radical

LH- Lipid

LOO•- Lipid Peroxide radical

LOOH- Lipid Hydroperoxide

LOXs- Lipoxygenates

MDA- Malondialdehyde

NADPH- Nicotinamide Adenine Dinucleotide Phosphate

OSI- Oxidative Stress Index

Pi- Inorganic Phosphate

PMSF- Phenylmethylsulfonyl fluoride

POD- Guaiacol Peroxidase

ppm- Parts per milion

PRX- Peroxiredoxin

PSII- Photosystem II

RES- Reactive electrophile Species

ROS- Reactive Oxygen Species

SOD- Superoxide Dismutase

TBA- Thiobarbituric acid

TCA- Trichloroacetic acid

Tris-HCl- Tris(hydroxymethyl)aminomethane hydrochloride

TRX- Thioredoxin

w/v- Weight/Volume

γ-ECS- γ-Glutamyl-Cysteine-Synthetase

Introduction

1. Phytoremediation of organic pollutants

Man is the cause for the accumulation of most of the organic pollutants in the environment, which are xenobiotics to several species. Many of these organic pollutants are carcinogenic and/or constitute an environmental hazard to other species, which may enter food chains, including humans'. These organic pollutants may be released into the environment via spills (fuel, solvents), military activities (explosives, chemical weapons), agriculture (pesticides, herbicides, fungicides), industry (chemical, petrochemical, etc.) and others .

In order to control fungi, weeds and insect pests, the use of pesticides has become commonly used in agricultural regions throughout the world. In the United States there is a generalized concern that pesticides have become major contaminants of the surface and ground water supplies. In fact, United States Geological Survey data indicate that over 90% of streams and 50% of wells sampled are contaminated with one or more pesticides [1].

Due to their chemical structure, many of these organic pollutants are recalcitrant to chemical and biologic oxygenation and hydrolysis, and, therefore, the remediation of water and soils contaminated by such pollutants is difficult [2]. Also, in modern cities, waste water treatments from municipal, agricultural and industrial sources is of primary concern. Such waters can pose a threat to human health, if the pollutants, ranging from raw sewage to chemical contaminants, are not treated before reaching natural water courses [2].

Hence, there is a need of a cheaper and eco friendly technology for environmental cleanup, like phytoremediation. Phytoremediation is an alternative to current methods of remediation that relies on the use of plants and their associated microorganisms, present on the rhizosphere, for environmental cleanup, and can be used in different strategies such as: Rhizofiltration; Phytostabilization; Phytoextraction; Phytoming; Phytostimulation; Phytodegradation and Phytovolatilization [3]. When organic pollutants are considered, phytodegradation is viewed as the more adequate strategy.

1.1. Phytodegradation of organic pollutants

Phytodegradtion consists on the use of plants and associated organisms to render xenobiotics in non-reactive, non-toxic compounds.

Contrasting with inorganic pollutants, organic pollutants can be degraded or converted to stable compounds or even be mineralized into inorganic compounds like water, carbon dioxide or chlorine, by phytodegradation [3]. Another difference between these two types of pollutants is their uptake: as plants have no transporters for organic xenobiotics, their uptake is mainly driven into and within the plants through simple diffusion, depending on their chemical properties, such as hydrophobicity and volatility, whereas metal uptake and movement within the plant relies on protein transporters and a panoply of metal chelators .

Once inside plant cells, and depending on their chemical structure, organic compounds can suffer enzymatic modifications, becoming promptly available for degradation/use by several enzymes, or they can also be conjugated with glucose or glutathione (GSH) and sequestrated.

GSH has an important role in conferring tolerance and in sequestration of the pollutant agent. Glutathione-S-Transferase (GST) catalyses the nucleophilic addition of a thiol group of GSH into the organic compound, making it more hydrophilic and ready to be sequestrated into the vacuole [4, 5].

Depending on its chemical composition, and before the conjugation of the organic compound with GSH occurs; a chemical modification of its radical groups may have to be performed. Several enzymes can oxidise or reduce these radical groups, and these reactions comprehend phase I of xenobiotic processing, the activation. Phase II is its conjugation to biomolecules, like GSH, and phase III is the conjugates' compartmentation, which can then be followed by further enzymatic steps leading to xenobiotic degradation, depending on the conjugate formed.

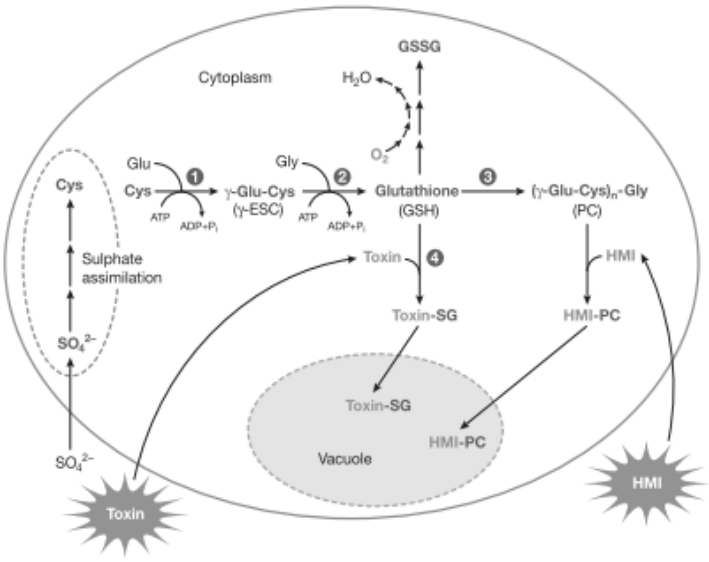


Image 1- Mechanism of detoxification of heavy metals, organic pollutants and oxidative stress in plant cells by glutathione. Cys, cysteine; γ-Glu-Cys, γ-L-glutamyl-L-cysteine; γ-ECS, γ -glutamylcysteine synthetase; GSH, glutathione; GSSG, oxidized glutathione; PC, phytochelatins; HMI, heavy metal ion; HMI-PC, heavy metal–phytochelatin complex; Toxin, xenobiotics; Toxin–SG, toxin–GSH conjugate. (1) γ-Glutamylcysteine synthetase; (2) glutathione synthetase; (3) phytochelatin synthase; (4) glutathione *S*-transferase (GST). Adapted from *[5]*.

2. Acetophenone

Acetophenone is the second most abundant constituent in Ridomil® (Syngenta Agro S.A., Portugal), which is a systemic fungicide used in horticulture and vineyards and has as its active compound metalaxyl [6]. Besides, this compound, classified as class D by the United States Environmental Protection Agency (US EPA), is used in a wide range of industries, as a fragrance in perfumes, soaps, detergents, creams, lotions; as a flavouring agent in food, non-alcoholic drinks and tobacco; as a solvent for plastics and resins; as a catalyst for olefin polymerization and in organic synthesis as a photosensitizer[1].

The several uses of this compound make it more available and likely to accumulate in the environment, due to these anthropogenic activities, where consequently it can hazard ecosystems.

3. Glutathione

Glutathione is an essential metabolite with multiple functions in plants. The earliest and fundamental roles recognized are the thiol-dissulphide interactions, in which reduced glutathione (GSH) is continuously oxidized to a disulphide form (GSSG)

[1] http://www.epa.gov/ttnatw01/hlthef/acetophe.html

that is recycled to GSH by NADPH-dependent glutathione reductase (GR), encoded by the gene *GR1* [7]. Its role in defence metabolism was firstly established in animals; in plants, the reductive H_2O_2 metabolism had been link to ascorbate since 1970s, being the role of glutathione as an antioxidant less apparent. However, glutathione depletion in *Arabidopsis* knockouts lacking the fist enzyme of the committed pathway of GSH synthesis causes embryo lethality, revealing that both plant and mammalian cells rely on at least some of the multifunctional properties of glutathione for survival [7, 8].

Glutathione, like other thiols, can undergo numerous redox reactions, and its oxidized form (GSSG) includes the formation of mixed disulphides with proteins and other thiol molecules through their cysteine residues. This oxidized form can form conjugates with a wide array of thiyl radicals, both endogenous or xenobiotic, electrophilic species, and its interaction with the nitric oxide system makes it a reservoir of signalling potential as well as an antioxidant barrier, scavenging reactive oxygen species (ROS) through the GSH-ascorbate cycle, and as an electron donor to glutathione peroxidase (GPX). GSH is also the storage form and the long-distance transport form of reduced sulphur and it is involved in the pathways for the detoxification of heavy metal and xenobiotic as well [7, 8].

Glutathione´s high concentration (it can accumulate to milimolar concentrations), when compared to other thiols, and rapid recycling of its oxidized form by GR, maintaining its high reduction state (in normal conditions GSH:GSSG ratios are at least 20:1) makes it a buffer that maintains the intracellular environment reduced [7, 8], key characteristics that makes it an important antioxidant molecule. Also a marked characteristic of stress-tolerant genotypes is high ratio of GSH:GSSG, when compared to stress-sensitive genotypes. As a result of ROS scavenging, the high GSH:GSSG ratio shifts towards GSSG when plants are facing stress conditions that compromise the reduction of GSSG to GSH. The ratio of GSH:GSSG may also differ along the developmental stage, as for example, cell proliferation stage requires a higher ratio than somatic embryogenesis [8].

Recently, when considered the redox environmental state, low-molecular weight thiols have also been taken into account, to form a thiol-dissulphide redox environment. Overall, during biotic and abiotic stress the thiol-dissulphide redox state

is a marker of stress and cell viability with an important impact upon signalling mechanisms [8].

The amino acids glutamic acid, cysteine and glycine compose glutathione, and these amino acids are put together to form GSH through two ATP-dependent, enzyme-catalysed steps [7, 9]. The gene *GSH1* encodes the enzyme γ -glutamylcysteine synthetase (γ -ECS/GSH1), which catalyses the formation of a covalent bond between γ-glutamate and α-cysteine; afterwards, glycine is added to the dipeptide γ-glutamyl-α-cysteine with the participation of glutathione synthetase (GS), encoded by the gene *GSH2* [9].

Glutathione synthesis is affected by many factors like glycine and ATP, but the most important factors are considered to be γ -ECS activity and cysteine availability, as demonstrated by the overexpression of the first enzyme of glutathione synthesis or of enzymes involved in cysteine synthesis [7]. Despite the well-described increases in glutathione in situations where hydrogen peroxide (H_2O_2) is also increased, neither intracellular generated, nor externally applied H_2O_2 cause marked induction of *GSH1* or *GSH2* in Arabidopsis [10, 11]. γ -ECS can be regulated at translation or post-translationally by redox control. One important factor in the upregulation of glutathione synthesis in response to oxidative stress is the involvement of one of the two disulphide bonds of γ -ECS homodimer in redox regulation [12]. Glutathione homeostasis is also regulated by feedback inhibition of γ -ECS by GSH, which can be alleviated by conditions in which glutathione is being consumed, like in the synthesis of phytochelatins. Nevertheless, both feedback inhibition and thiol/disulphide redox regulation of γ -ECS remains to be elucidated [7].

Glutathione is implied in ROS and ascorbate metabolisms, by reacting chemically with ROS and dehydroascorbate (DHA). Several authors [10, 11, 13-15]**;** conducted studies in which H_2O_2 metabolizing enzymes were genetically or pharmacologically inhibited, revealing a close relationship between augmented H_2O_2 and glutathione status. Queval et al 2009 [11] in their time-coursed analyses with conditional catalase mutants, showed that a several-fold induction of the total glutathione pool is preceded by an initial conversion of GSH to GSSG within the first hours of exposure to H_2O_2. Mhamdi et al 2010 [16], also showed that at moderate rates of endogenous H_2O_2 production, the GSH:GSSG ratio decreased from above 20 to

close to 1, this property making the glutathione status a useful marker for oxidative stress triggered by increased H_2O_2 production [7, 17].

H_2O_2 metabolism may involve several GSH-dependent and independent pathways. There are three peroxidases, besides spontaneous GSH reaction with H_2O_2, that may link peroxide reduction to GSH oxidation: ascorbate peroxidase (APX), certain types of peroxiredoxin (PRX) and Glutathione-S-Transferase (GST) [7]. Haem-based peroxidases found in plants, class III of non-animal peroxidases super-family known as "guaiacol-type" peroxidase (POD), are known to control some biosynthetic processes and/or ROS production [18]. APX reduces H_2O_2, which will donate its electron to NAD(P)H through the ascorbate-glutathione pathway, linking APX activity to the GSH pathway; on the other hand, APX could also be associated with glutathione independent oxidation of NAD(P)H, via monodehydroascorbate reductase (MDHAR) [7].

Unlike guaiacol-type enzymes, thiol peroxidases such as PRXs are less H_2O_2-specific, and can reduce other organic peroxides through thioredoxins (TRX) or similar components. GSH can be used as a reductant power by PRXII via glutaredoxins (GRX) [7]. Another PRX enzyme is GPX, which is implicated in plant stress responses and signalling and, unlike the name indicates, do not peroxidise glutathione, but are thioredoxins (TRX) dependent. This enzyme reduces peroxides involving a formation of a disulphide bridge from the initial sulphenic acid, and the intermediate disulphide is reduced back to the dithiol form by TRX, being therefore not likely to be involved in glutathione mediated peroxide oxidation [7].

On the other hand, GST have GSH peroxidation activity, being, amongst these enzymes, the only ones to appear to act as direct GPX, as all other enzymes require at least one additional protein to link peroxide reduction to GSH oxidation [7].

So, GSH could be linked to H_2O_2 and/or peroxide reduction by, at least, two ascorbate independent routes, as well as the ascorbate-glutathione pathway [7]. Data from gene expression studies evidenced that certain APX, GPX and GST encoding genes are induced in response to oxidative stress [10]. Additionally, transcriptomics and enzyme and metabolite assays of plants deficient in catalase and/or GR suggest that the ascorbate-glutathione cycle, and enzymes such as GST, may act in concert to remove H_2O_2 and/or other peroxides [16].

GST´s (EC 2.5.1.18) are a diverse family of proteins composed by two 25-KDa subunits, with a well defined Glutathione-binding domain at their active site [19]. This family of proteins is divided into four families, Phi, Tau, Zeta and Theta, being Phi and Tau plant specific GST, with major roles in herbicide detoxification. The classical reaction catalysed by GST´s comprehends an addition or substitution, with a covalent bond, between the sulphur atom of GSH and an electrophilic centre of a certain molecule [7, 20]. Besides its major role in detoxifying herbicides, this plant specific GST also have less well characterized functions like acting as GSH peroxidases, counteracting oxidative damage; acting as Flavonoid binding proteins and acting as stress signalling proteins [19, 20].

4. Thiol

Recently, there have been emerging evidences that a network of sulphur-containing molecules and related compounds, together with a number of non-protein (low-molecular weight thiols) and protein thiols (high molecular weight thiols) can contribute to plants stress tolerance [8]. Despite of recent progresses, the knowledge of interactions between thiols and other molecules is yet incomplete. Nevertheless, recent evidences indicate that thiols play a central role in conferring stress tolerance in plants [8].

Throughout *taxa*, from bacteria to higher plants and animals, protein thiols, like thioredoxins (TRX), glutaredoxins (GRX) and glutathionylation of protein sulphydryl groups, are considered most potent protein-based protective and regulatory mechanisms [8]. TRX and GRX are thiol oxireductases that provide reducing power to a variety of stress-related enzymes or exert direct thiol-dissulphide oxireductase activity to various protein targets. Reduction of TRX and GRX depend on different mechanisms: while GRX are directly reduced by 2 GSH molecules to produce GSSG, TRX reduction is dependent on the cellular compartment, requiring different reductases. Cytosolic and mitochondrial TRX require compartment-specific NADPH-dependent TRX reductases and plastid TRX are reduced by ferredoxin/TRX reductase [7, 8].

GRX are also known as thiol transferases and its traditional reaction, as said before, encompasses the reduction of a protein disulphide bond to two thiols oxidizing 2 GSH to GSSG in the process. GRXs comprise a more efficient way in the reduction of

glutathionylated proteins, in contrast to TRXs, that are more involved in the reduction of intra- or inter-molecular disulphide bonds [21]. Some enzymes of this family can also catalyse protein S-glutathionylation or degluthionylation, and are divided into three families that differ on the basis of amino acid motifs in the active site [7].

In proteins, cysteine (Cys) residues are tremendously susceptible to oxidative alteration, such as those associated with ROS production in adverse environmental conditions; these oxidative alterations have also been reported in methionine residues [8]. The three-step oxidation of Cys residues in macromolecules can reach a point of irreversibility, leading to the loss of their native conformation and activity, being further processed to be degraded [8]. The first product of Cys residue oxidation (cysteine sulphenic acid – Cys-SOH) is the only reversible product of the three-step oxidation. Its glutathionylation, a formation of a stable mixed disulphide bond between glutathione and a protein cysteine residue [7, 21], could be produced through a reaction with GSSG or be enzymatically catalysed, leading to a reversible temporary protection of Cys residues [8]. This reaction could modify the conformation, stability or activity of the target protein [7].

The rehabilitation of Cys can be made through reaction of these residues with GRX, which depends on GSH concentration and GR activity [8]. Nonetheless, spontaneous glutathionylation by GSSG is far less likely to occur *in vivo*. However widespread, glutathionylation and the participation of GRX are likely to enhance stress tolerance, but neither the exact mechanism nor the physiological relevance of protein glutathionylation in plants was established to date [8].

Despite major advances in this field, such as the identification of reversible *S*-glutathionylation of glutathione-dependent enzymes such as GR, dehydroascorbate reductases (DHAR), and some peroxiredoxins [19, 21, 22], there are still some blanks regarding quantification, significance and specificity: whether the modified Cys residue is glutathionylated *in vivo*, rather than nitrosylated or a target of TRX, or some combination of these modifications [7].

S-glutathionylation can be mediated through GRX-catalysed thiol-dissulphide exchange between protein thiol groups and GSSG, conversion of thiol groups to thiyl radicals or sulphenic acids, or be mediated through formation of *S*-nitrosoglutathione [7].

Glutathionylation of proteins can be seen as a post-translational regulatory mechanism with an important impact on the activity of various antioxidant enzymes and thiol-peroxidases, regulation of transcription TGA factors (basic/leucine zipper-type) and probably deglutathionylation activity [8, 21].

5. Oxidative stress, lipid peroxidation and antioxidant mechanisms

Production of reactive oxygen species (ROS), like superoxide anion ($O_2^{\bullet -}$), hydroxyl radical ($^{\bullet}OH$), as well as non-radical molecules like hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), and so forth, is an inevitable outcome of a aerobic metabolism [23]. In plants, the electron transport activities from chloroplasts, mitochondria and plasma membrane form ROS by leakage of electrons onto O_2; ROS can also be formed as a by-products of multiple metabolic pathways localized in different cellular compartments [23].

Several biotic and abiotic stresses are able to induce enhanced ROS production, which are known to be extremely harmful to organisms at high concentrations due to the disruption of cellular homeostasis. Hence, the term “oxidative stress” is used to describe when levels of ROS production exceed ROS scavenging mechanisms [23]. However this term still lacks a precise definition, but key marks are: augmented oxidative load (increased ROS production); the possible unrestrained oxidation caused by lower metabolism rates compared to production rates; oxidative damage to cellular components leading to accumulation of damaged cellular components that by some means lead to loss of function and eventual cell death. Enhanced ROS production can pose a hazard to cells by inducing peroxidation of lipids, oxidation of proteins, damage to nucleic acids, enzyme inhibition, activation of programmed cell death (PCD) and eventually lead to death of the cells [23].

On the other hand, ROS can also act as second messengers in a variety of cellular processes, like tolerance towards environmental stresses. It is the fine equilibrium between ROS production and scavenging that defines its damaging or signalling activity [23]. This implicates that ROS levels are tightly controlled by cells, in order to avoid any oxidative injury, rather than complete elimination [23].

Thus, cells have a well-organized antioxidant system, formed of non-enzymatic as well as enzymatic antioxidants, capable of scavenging or detoxifying excess ROS.

Superoxide dismutase (SOD), catalase (CAT), guaiacol peroxidase (POD), as well as ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR) and glutathione reductase, enzymes of the antioxidant ascorbate-glutathione cycle (AsA-GSH), are part of the enzymatic antioxidant system. Molecules such as ascorbate (AsA), glutathione (GSH), carotenoids, tocopherols and phenolics are part of non-enzymatic antioxidants of the cell [23].

Several researchers have stated that increased activity of several enzymes of the antioxidant defence system, as well as maintenance of a high capacity to scavenge ROS in plants, are linked to increased tolerance of plants to several environmental stresses [23]. In scope of this work, hydrogen peroxide (H_2O_2) and lipid peroxidation; the antioxidant enzymes APX, CAT, POD, GR, as well as the roles of GSH and carotenoids in the antioxidant defence, will be further discussed.

5.1 ROS

ROS are a group of free radicals, reactive molecules and ions that are derived from O_2 (molecular oxygen). ROS can act as both deleterious and beneficial, depending on their concentration, as discussed before.

Oxygen is a completely non-hazardous molecule, and, due to its two unpaired electrons with parallel spins, it is unlikely to take part in reactions with organic molecules, unless it is activated. O_2 can be activated through absorption of enough energy to reverse the spin on one of the unpaired electrons or by stepwise monovalent reduction. Oxygen singlet ($^1O_2$) is formed through energy absorption, while stepwise monovalent reduction can reduce O_2 into superoxide anion ($O_2^{\bullet-}$), H_2O_2 and hydroxyl radical (•OH) [23, 24].

The oxygen singlet has the ability to cause divalent reduction. In other words, it is able to transfer two electrons, due to the absorbance of energy that reverses the spin of one of its electron. It can perform direct oxidation of proteins, unsaturated fatty acids and DNA [23, 24].

Electron spin restriction prevents O_2 from receiving four electrons at a time to produce water; it accepts one electron at a time, enabling the formation of stable intermediates during its stepwise reduction. In this stepwise reduction, $O_2^{\bullet-}$ is the primary ROS formed in the cell, which begins multiple reactions to generate other ROS. $O_2^{\bullet-}$ is a nucleophilic reactant with oxidizing and reducing properties [23]. $O_2^{\bullet-}$ has an

anionic charge that enables the transfer of its electrophilic activity onto electron-rich molecules [23]. $O_2^{\bullet-}$ oxidizes enzymes containing the [4Fe-4S] cluster and can reduce cytochrome C.

H_2O_2 is formed by the acceptance of one electron and two protons by $O_2^{\bullet-}$, which is easily dismutated either non-enzymatically or by SOD-catalysed reactions:

$$2O_2^{\bullet-} + 2H^+ \longrightarrow H_2O_2 + O_2$$

$$2O_2^{\bullet-} + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2$$

A wide range of stressful conditions can induce H_2O_2 generation. Due to not having unpaired electrons, it can cross the membrane, thus causing oxidative damage far from the site of its formation. Being the only ROS that can pass through aquaporins in the membrane and due to its relatively stability compared to other ROS, it has been a target of attention as a signal molecule involved in the regulation of specific biological processes, and to prompt tolerance to environmental stresses at low concentrations. On the other hand, when present in high concentrations, it can oxidize sulfur-containing amino acid residues (cysteine –SH and methionine $–SCH_3$), and inactivate enzymes and transcription factors by oxidizing their thiol groups [23, 24]. Also it orchestrates programmed cell death.

However, both $O_2^{\bullet-}$ and H_2O_2 are only moderately reactive, and the cellular damage induced by ROS appears to be as a result of their conversion into more reactive species [23]. Thus, the hydroxyl radical ($^{\bullet}OH$) is reliant on $O_2^{\bullet-}$ and H_2O_2, and its formation is dependent on inhibition of SOD and CAT [23].

The Haber-Weiss reaction generates ($^{\bullet}OH$) from H_2O_2 and $O_2^{\bullet-}$, and consists of the following reactions:

$$Fe^{3+} + O_2^{\bullet-} \longrightarrow Fe^{2+} + O_2,$$

In this reaction $O_2^{\bullet-}$ reduces Fe(III), and is followed by dihydrogen peroxide (Fenton reaction):

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^- + {}^{\bullet}OH,$$

And:

$$O_2^{\bullet -} + H_2O_2 \rightarrow {}^{\bullet}OH + OH^- + O_2.$$

$^{\bullet}OH$ is the most reactive ROS, its single unpaired electron makes it available for interaction with all biological molecules and induces cellular damage, like protein damage, lipid peroxidation and membrane destruction [23].

Oxidation of organic substrates can happen through two reactions, either due to abstraction of hydrogen atoms or through addition of hydroxyl radicals to the substrate [23].

5.2 Lipid Peroxidation

Both enzymatic and non-enzymatic processes can oxidize lipids. A small number of lipidic regulators, such as jasmonates, can be generated by the enzymatic process and are seen as beneficial for survival for their role in defence and reproduction [25]. These enzymatic processes, like enzymatic oxylipin production, lipases and lipoxygenase (LOX) will not be discussed in this work. Instead, non-enzymatic reactions that generate lipid peroxides and their products are going to be subject of description, despite the difficulty to separate both processes as both processes numerous times occur simultaneously and may influence each other.

Innumerous oxygenated lipids are generated through lipid peroxidation, these products of lipid peroxidation, when not dealt with correctly, may interfere with protein function through uncontrolled hydrophobic interactions; also, many of the resulting lipids are reactive electrophile species (RES) and can covalently modify macromolecules [23, 25]. Oxidized lipids are being continuously produced even under optimal conditions, and the machinery that has evolved towards dealing with several lipids produced by non-enzymatic reactions could also be used by plants to handle xenobiotic compounds, such as herbicides and safeners [25].

Lipid peroxidation is widely used as a marker of ROS-mediated injury to cell membranes under stressful conditions, as augmented peroxidation has been reported in plants growing under several environmental stresses. Increase in lipid peroxidation parallels with increased production of ROS [23]. Lipid peroxidation follows three steps: initiation, progression and termination.

The non-enzymatic processes of lipid peroxidation are ROS-related, in which the extent of peroxidation reactions differ quantitatively between shoots and roots, due to the nature of ROS produced in these organs [25]. The first step of lipid peroxidation includes the activation of oxygen, and is rate limiting [23].

In photosynthetic tissues, lipid peroxidation reaction occurring in healthy tissues are initialize by singlet oxygen ($^1O_2$), a by-product of light capture at photosystem II (PSII), formed by the transfer of energy from excited chlorophyll to O_2 [25]. $^1O_2$ is mainly quenched by reaction center carotenoids to unreactive O_2; nevertheless, a part of $^1O_2$ is incorporated into plastid membranes, which occurs alongside O_2 insertion catalyzed by LOXs. Oxygenation by $^1O_2$ is non-catalytic, as each $^1O_2$ only produces one lipid hydroperoxide (LOOH), unlike the LOX mediated oxygenation [25]. High levels of LOOH present in membranes makes them more vulnerable to fragmentation and this process generates new radicals. Contrasting to $^1O_2$-related lipid peroxidation, free radical peroxidation of lipids is a catalytic process that may quickly extend through membranes. When the radicals scavengers are over challenged, this process may harshly damage membranes [25].

On the other hand, in underground tissues, there is little $^1O_2$ generation and basal non-enzymatic peroxidation, dominated by radical reactions [25]. Another potential radical source is hydrogen peroxide (H_2O_2), which is prone to iron-catalysed degradation to produce hydroxyl radicals ($^{\bullet}OH$) and superoxide anion radicals ($O_2^{\bullet-}$) [25]. Hydroxyl radical, unlike superoxide anion radicals, can remove hydrogen from fatty acids, turning lipids (LH) to lipid radicals ($L^{\bullet}$), that once are generated are the basis of a diffusion-limited catalytic cycle that consumes O_2 and other lipids to produce yet more lipid peroxides [25].

The second step of lipid peroxidation (progression) consists of lipid fragmentation, which is closely linked to radical amplification and supplementary enhancement of the peroxide pool [25]. In this step, a wide array of chemical structures is formed. Single or serial insertion of O_2 may take place, before hydrogen abstraction, and thus secondary oxidation events form a large variety of alcohols, acids and reactive epoxides, aldehydes (malonyldialdehyde, acrolein and crotonaldehyde), ketones and cyclopentenones in membrane lipids [23, 25].

The LOOHs formed are more likely to radical-catalysed oxidation than non-oxidized lipids (LH) [25]. This phase of membrane peroxidation is known by the reaction of LOOH with lipid peroxide radicals ($LOO^{\bullet}$) and O_2, after a hydrogen abstraction by LH in membranes occurred. LOOH pool decreases, as membranes become more peroxidised, in favour of $L(OOH)_2$ and higher oxidation products. Lipid peroxide expansion involves the formation of non-radical glycerolipids dimers, which can spontaneously decompose into four fragments, among them there are glycerolipids with an oxidized acyl chain and a hydroxyl radical, which in turn can abstract hydrogens from LH, producing new $L^{\bullet}$ radicals [25].

5.3 ROS scavenging enzymes

The antioxidant system not only allows plants to face stress conditions, it maintains an appropriate balance between ROS production and scavenging under normal conditions, as well. Specific ROS producing and scavenging mechanisms are present in different organelles of plant cells, like chloroplasts, mitochondria and peroxisomes, and these different ROS scavenging pathways are coordinated [23]. ROS production exceeding ROS quenching activity, in stressful conditions, leads to injury in different macromolecules in the cell. To avoid such harmful events, plants raise the levels of the endogenous antioxidant defences. In the scope of this work, only catalase (CAT), ascorbate peroxidase (APX), guaiacol peroxidase (POD) and glutathione reductase (GR) will be referred.

5.3.1 *Catalase (EC 1.11.1.6)*

This enzyme was the first to be discovered and characterized among the antioxidant enzymes present in plants. This ubiquitous tetrameric heme-containing enzyme, catalyses the dismutation of H_2O_2, for which has high affinity, into water and oxygen, requiring no cellular reducing equivalents. Despite its affinity for hydrogen peroxide it has poor activity against organic peroxides [23, 24, 26, 27].

When compared to APX, CAT have a much lower affinity to H_2O_2, but a very fast turnover rate. Based on Willekens et al. 1997 proposed classification, there are three CAT genes. Class I genes are expressed in photosynthetic tissues and are regulated by light, class II have high levels of expression in vascular tissues and class III are abundant in seeds and young seedlings [10].

As it has been discussed, H_2O_2 is implicated in many stress conditions, and when cells are stressed for energy rapidly generate H_2O_2 through catabolic processes, which in turn is degraded in an energy efficient manner by CAT [23]. These stresses cause either enhancement or depletion of CAT activity, depending on intensity, duration and type of stress; overall, stresses that reduce protein turnover also reduce CAT activity. Enhanced CAT activity showed accumulation of GSSG and decrease in AsA, demonstrating that CAT is decisive for the redox balance during oxidative stress [10].

5.3.2 *Ascorbate Peroxidase (EC 1.11.1.11)*

APX participates in the ascorbate-glutathione cycle, and plays a central role in the control of intracellular ROS levels. It uses two molecules of AsA reducing H_2O_2 to water, generating as well two molecules of monodehydroascorbate [23]. APX is regulated by H_2O_2 and by redox signals. APX forms are found in cytosol, plastidial stroma and thylakoids, mitochondria and peroxisomes. Whereas the APX found in the organelles scavenges H_2O_2 found in those organelles, cytosolic APX eliminates H_2O_2 produced in the cytosol, apoplast or that diffused from organelles [23, 24, 26, 27]. Chloroplastidial and cytosolic APX forms are specific for AsA as electron donor, but the Cytosolic form is less sensitive to AsA depletion than the chloroplastidial isoenzyme [23].

APX correspond to one of the most broadly distributed antioxidant enzymes, and APX forms have much higher affinity for H_2O_2 than CAT, being thus more efficient in scavenging this ROS under stressful conditions [23].

5.3.3 *Guaiacol Peroxidase (EC 1.11.1.7)*

POD is implied in many important biosynthetic processes, such as IAA degradation, cell wall lignification, ethylene biosynthesis, wound healing and defence against biotic and abiotic stress [23]. It is widely accepted as a stress enzyme, and can function as quencher of reactive intermediary forms of O_2 and peroxy radicals [23]. In addition to scavenge H_2O_2, POD also serves to detoxify products of lipid peroxidation. Is the major form of protection against low levels of oxidative stress, and, while decomposing peroxides to water or alcohol it simultaneously oxidizes GSH [27].

5.3.4 *Glutathione Reductase (EC1.8.1.7)*

GSH is oxidized to GSSG through enzymatic and non-enzymatic oxidation-reduction cycles, when acting as an antioxidant. In the AsA-GSH cycle, GSH is oxidized by DHAR, and reduced by GR, a NAD(P)H-dependent enzyme, maintaining high GSH/GSSG ratios [23]. GR belongs to a group of flavoenzymes and contains an important disulphide group [28]. The reduction of GSSG into GSH involves two steps: First, the flavin moiety is reduced by NAD(P)H, the flavin is oxidized and a redox active disulphide bridge is reduced in order to produce a thiolated anion and a cysteine[28]. Afterwards, GSSG is reduced by a thioldisulphide interchange reaction [28]. A reversible inactivation may happen if the enzyme is not deoxidized by GSSG [23].

Most of GR activity in photosynthetic tissues is achieved by chloroplastidial forms, where GSH and GR are involved in detoxification of H_2O_2 generated by Mehler reaction, but some are located in the cytosol, mitochondria and peroxisomes [23]. Augmented GR activity results in increased GSH levels and ultimately leads to plant tolerance [27].

Nevertheless, because of the complexity of ROS detoxification system, there is no certainty whether or not overexpressing one component of such system will change the ability of the pathway as a whole [23].

6. *Solanum nigrum* L.

Solanum nigrum L. has good key features to be used as a phytoremediation tool, such as: wide range global distribution (cosmopolitan), good adaptation to several climacteric conditions, fast growth and great shoot biomass, annual or biannual, and has been demonstrated as being a hyperaccumulator of both organic and non organic pollutants [6, 29-34]. Black nightshade, as it is commonly known, belongs to the family Solanaceae, such as other economically important species: potato (*Solanum tuberosum* L.); tomato (*Solanum lycopersicum* L.); eggplant (*Solanum melongena* L.); pepper (*Capsicum* sp.) [35].

This work follows a previous report by Teixeira et al 2011 [6], in which it was demonstrated that *Solanum nigrum* L. plants were a good tool for phytoremediation of metalaxyl-contaminated sites. In that work, two concentrations of Ridomil (a fungicide with metalaxyl as its active compound) were used.

Because acetophenone is the second most abundant component in Ridomil, and an organic compound with an extended use in several industries, it was of interest to see how *Solanum nigrum* L. plants would cope with such compound to further discriminate if the observed toxicity to Ridomil [6] was due solely to metalaxyl, or if it was the result of the combination of metalaxyl plus acetophenone.

So, in order to access if acetophenone exerts toxicity towards *Solanum nigrum* L. and to see if this plant species may be used as a phytoremediation tool to remediate and detoxify acetophenone, acetophenone levels in the nutrient medium were quantified along time, and some oxidative stress markers as well as some of the plant antioxidant system and detoxification mechanisms were evaluated after one month of exposure. For comparison purposes, the concentrations of acetophenone used were equal to those present when Ridomil was tested in a previous work [6].

Materials and Methods

1. Seed germination and growth conditions

S. nigrum L. seeds were harvested from black nightshade plants in Francos, Porto, Portugal (1º9'55 79''N 8º38'19 8''O). They were sterilized in 70% ethanol for three minutes and dried in a laminar flux chamber. Afterwards, they were uniformly laid in Petri dishes with paper embedded in sterile Hoagland solution. No acetophenone was present during seed germination. Two days after incubation at 4 °C in the dark, they were placed in a growth chamber at 23 °C with a 16 h light/8 h dark photoperiod.

Seedlings were transferred to pots, with 2:1 vermiculite:perlite as substrate, 4 weeks after incubation. Plants were fed with a Hoagland solution, to which was added 0.0 (control), 0.52 and 1.04 ppm of acetophenone. Each pot had 2 plants; 6 pots were used for each experimental condition.

Samples of the nutrient solution were taken on a weekly basis, in order to evaluate its acetophenone content. One month after exposure plants were removed from the pots and biomass and length of each organ (roots and shoots) was evaluated. Vegetal material (n≥3 plant organs) was ground to dust with liquid nitrogen, and aliquots of each growth situation were made and stored at -80 °C until needed.

2. Oxidative stress biomarkers

2.1 Hydrogen peroxide levels

Shoot aliquots were homogenized with 0.1% (w/v) TCA on ice, and the homogenate was centrifuged at 15 000 x g for 15 minutes. To 0.5 ml of each supernatant, 0.5 ml of 10 mM phosphate buffer (pH 7.0) and 1.0 ml of 1 M KI were added and mixed gently. The blank consisted of 0.5 ml of 0.1% (w/v) TCA instead of extract. Absorbances of these mixtures were read at 390 nm and H_2O_2 was quantified by using its extinction coefficient (ε_{390nm}=0.28 $\mu M^{-1}cm^{-1}$). Results were expressed in nmol H_2O_2 g^{-1} fresh weight [36].

2.2 Photosynthetic pigments quantification

Chlorophyll a, b and carotenoids content was measured according to Lichtenthaler 1987 [37], and contents were calculated according to the following formulas:

Chlorophyll a= 12.25 × Abs663-2.79 × Abs647 ;

Chlorophyll b =21.50 x Abs647 − 5.10 x Abs663 ;

Carotenoids =(1000 x Abs470 − 1.82 x Clor a − 85.02 x Clor b)/198

Results were expressed in mg g^{-1} fresh weight [37].

2.3 Lipid peroxidation

For each 0.2 g of vegetal material 1 ml of TCA 1% (w/v) was added, and the homogenates were agitated with a vortex for 90 seconds. Afterwards, the homogenates were centrifuged for 5 minutes at 10 000 x g, and the supernatant was recovered. To 250 µl of supernatant, 1 ml of TBA 0.5% (w/v) in TCA 20% (w/v) was added, in triplicates. Blanks were made in double with 1 ml of TBA 0.5% (w/v) in TCA 20% (w/v) and 250 µl of TCA 0.1% (w/v), and were given the same treatment as samples. Microtubes were pierced and incubated at 95 °C for 30 min, being cooled down for 10 min in ice before centrifugation at 10 000 x g for 15 minutes. Supernatants were recovered and their absorbance at 532 nm and 600 nm measured. Malondialdehyde (MDA) levels were calculated by subtracting absorbance at 600 nm to that at 532 nm, considering ε to be 155 $mM^{-1}.cm^{-1}$, results were expressed in nmol MDA.g^{-1} FW [38].

2.4 Proline

Aliquots of 0.5 g were homogenised in 10 ml of 3% (w/v) sulfosalisylic acid and centrifuged at 500 x g for 10 minutes. The supernatant was recovered and 200 µl of it were mixed with 200 µl of glacial acetic acid and 200 µl of acid nyhydrin. This mixture was incubated at 96 °C, cooled down in ice and 1 ml of toluene was added. Resulting tubes were vigorously agitated and kept at room temperature in order to separate the solution into two phases, the upper phase with reddish colour (organic phase) was used for recording its absorbance at 520 nm, using toluene as a blank. The levels of

proline were determined using a linear pattern with known proline concentrations, and expressed in µg g^{-1} fresh weight [39] .

3. Biochemical determinations

3.1 Catalase

Tissue samples were homogenised with an extraction buffer containing 100 mM phosphate buffer (pH 7.3), 8% glycerol, 1 mM PMSF and 1 mM EDTA. After homogenization, the extract was centrifuged at 26 000 g, at 4 °C for 18 minutes. The resulting supernatant was recovered and kept on ice, for protein quantification by the Bradford method [40].

The activity of catalase was determined by reacting 8 to 12 µg of protein with 4 µl of 30% H_2O_2 and 100 mM phosphate buffer (pH 7.0) until the final volume was 1 ml. The reaction was followed in a spectrophotometer at 240 nm, for 60 seconds. The activity was calculated by using the coefficient of molar extinction of H_2O_2, 39.4 $mM^{-1}cm^{-1}$ and was expressed as nkat mg^{-1} of protein.

3.2 Guaiacol Peroxidase

Plant tissue aliquots were homogenised with extraction buffer (100 mM phosphate buffer (pH=7.3); 1 mM EDTA; 1 mM PMSF; 8% glycerol and 5 mM L-ascorbic acid), on a proportion of 1 g: 2.5 ml and centrifuged at 10 000 x g for 10 minutes at 4 °C. Protein content in the supernatant was quantified by the Bradford method [40]. 250 µl of 200 mM phosphate buffer (pH=7.3), 333 µl of 60 mM guaiacol, 197 µl of water 20 µl of extract and 200 µl of 1 mM H_2O_2 were pipetted into a cuvette and the increase in absorbance at 470 nm was monitored for 1 minute. The activity of POD was determined using ε=26.6 $mM^{-1}cm^{-1}$, and results were expressed as nkat mg^{-1} of protein [41].

3.3 Ascorbate Peroxidase

Tissue samples were homogenised and centrifuged in similar conditions to catalase and protein content was determined by the Bradford method [40]. The oxidation of ascorbate by APX was determine by spectrophotometry, at 300 nm for 30 seconds, by pipetting into a cuvette 100 mM phosphate buffer (pH 7.3) 0.5 mM ascorbate, 0.1 mM H_2O_2 and 12 µg of protein extract, with a final volume of 1 ml. The

reaction begun after the addition of H_2O_2, and the activity of the enzyme was determined using ε=0.49 $mM^{-1}cm^{-1}$ and expressed as nkat mg^{-1} of protein [41].

3.4 Thiol levels

Tissue aliquots were homogenized in 3 ml (shoots) or 2.5 ml (roots) of extraction buffer (20 mM EDTA and 20 mM of ascorbate), in ice and were centrifuged at 12 000 x g for 20 minutes at 4 °C.

The supernatant was recovered and to 200 µl of it, 960 µl of 200 mM Tris-HCl (pH=8.2) and 40 µl of 10 mM 5,5´-dithiobis(2-nitrobenzoic acid) were added, in triplicates. After 15 minutes at room temperature colour developed and absorbance at 412 nm was measured. Using ε_{412}=13600 $M^{-1}cm^{-1}$ the total thiols groups were calculated and expressed in nmol g^{-1} fresh weight.

To determine the non-protein thiol groups, 500 µl of supernatant was mixed with 500 µl of 10% (w/v) sulfosalicylic acid, in duplicates. The solution was incubated at room temperature for 15 minutes and centrifuged for 15 minutes at 3 000 x g. The supernatant of both tubes were recovered and mixed together. To 500 µl of this supernatant 475 µl of 400 mM Tris-HCl (pH=8.9) and 25 µl of 5,5´-dithiobis(2-nitrobenzoic acid) were added, in triplicates. Colour development was allowed for 5 minutes at room temperature and absorbance was read at 412 nm. Using ε_{412}=13600 $M^{-1}cm^{-1}$ the total thiols groups were calculated and expressed in $nmol^{-1}g$ fresh weight.

Proteins thiol levels were calculated by subtracting the non-protein thiol content to the total thiol levels [42].

3.5 Glutathione

The tissue samples were homogenised in a proportion of 1:9 with a homogenization buffer consisting of 10 mM of HCl and 1.3% (w/v) 5-sulfosalicylic acid, and centrifuged at 13 000 x g for 10 minutes at 4 °C.

After centrifugation the supernatant were recovered, and total glutathione (GSHt) levels were determined. For that, 20 µl of supernatant, 20 µl of 63.5 mM phosphate buffer (pH=7.4) and 200 µl of reaction buffer (containing 5 ml of 10 mM 5,5'-dithiobis-2-nitrobenzoic acid (DTNB); 8.5 ml 2 mM NADPH; and 36.5 ml of phosphate buffer pH=7.4, on a final volume of 50 ml) and 40 µl of glutathione reductase (GR) enzyme were pipetted. In this reaction, the GSH is oxidized by the

presence of DTNB, and the all GSSG is reduced to GSH, by GR action. This reaction was measured at 415 nm in a spectrophotometer, and levels of glutathione were calculated with the help of a linear pattern of known GSH concentrations (Attachment 13). Levels were expressed in nmol g^{-1} fresh weight.

As for oxidized glutathione (GSSG) levels, 100 µl of the supernatant were pipetted together with 6 µl of trietanolamine (TEA) and 2 µl of 2-vinilpiridine and were incubated with agitation at 25 °C for 1 hour. In this reaction, 2-vinilpiridine will form a conjugate with GSH, allowing the measurement of the remaining GSSG. The GSSG is measured by reacting 40 µl of the former solution with 200 µl of reaction buffer and 40 µl of GR. The reaction of GR is measured in a spectrophotometer for 3 minutes at 415 nm. Levels of GSSG were calculated with the help of a linear pattern of known GSSG concentrations (Attachment 13), and were expressed in nmol g^{-1} fresh weight.

In order to determine the levels of reduced glutathione (GSH), the levels of GSSG were subtracted to those of GSHt [43].

The Oxidative Stress Index was calculated according to the formula:

OSI=(2×GSSG /GSHt)×100

3.6 Glutathione-*S*-Transferase

Aliquots were homogenised with an extraction buffer containing 100 mM phosphate buffer (pH=7.3); 1 mM EDTA; 1 mM PMSF; 8% glycerol and 5 mM L-ascorbic acid, on a proportion of 1 g: 2.5 ml. The homogenised samples were centrifuge at 10 000 x g for 10 minutes at 4 °C and the protein content was quantified using the Bradford method [40]. Seven hundred µl of 71.43 mM of phosphate buffer (pH=7.5); 100 µl of 1.25 mM chlorodinitrobenzene – CDNB; 100 µl of extract and 100 µl of 10 mM GSH (initiates the reaction) were pipetted into a cuvette and the variation in the absorbance (ΔAbs) was read at 340 nm for 2 minutes. In order to determine the non-enzymatic conjugation of CDNB to GSH, 100 µl of extract were substituted by 100 µl of extraction buffer, and this ΔAbs was subtracted to the ΔAbs read with the extract.

The determination of GST activity was made according to the coefficient of extinction of CDNB, 9.6 $mM^{-1}cm^{-1}$, and results were expressed in nkat mg^{-1} protein [44].

3.7 γ -Glutamyl-Cysteine Synthetase (γ -ECS)

Aliquots were homogenized with extraction buffer (1 g roots – 2 ml extraction buffer; 1 g shoots – 2.5 ml extraction buffer) containing 100 mM Tris-HCl (pH=8.1); 10 mM $MgCl_2$ and 1 mM EDTA, in ice, and centrifuged for 15 minutes at 10 000 x g (6 °C).

After centrifugation, the supernatant was recovered. Protein content was determined according to the Bradford assay [40]. To a 1.5 ml tube were added 350 µl of reaction mixture, containing: 143 mM Hepes pH=8.7; 1.43 mM $MgCl_2$; 28.57 mM glutamate; 1.43 mM cysteine; 7.14 mM ATP, 7.14 mM phosphoenolpyruvate and 7.14 mM DTT, 10 µl of pyruvate kinase and 140 µl of protein extract (initiates the reaction). For the blank extraction buffer substituted the protein extract. This solution was incubated at 37 °C for 45 minutes, allowing the reaction, which was stopped by the addition of 100 µl of 50% (w/v) TCA. This mixture was centrifuge for 15 minutes at 10 000 x g at 6 °C, and the supernatant was recovered. To 50 µl of this new supernatant 150 µl of colour solution (equal volumes of 12% (w/v) ascorbic acid in 1 M HCl and 2% (w/v) ammonium molybdate tetrahydrate ($(NH_4)_6Mo_7O_{24}.4H_2O$) were mixed) were added in triplicates. The triplicates were incubated for 20 minutes at room temperature, in order to develop a bluish colour (if phosphate is present). At the end of the 20 minutes 0.5 ml of the stop solution, composed by 2% acetic acid and 2% (w/v) sodium citrate tribasic dehydrate, were added. The absorbance at 240 nm was read, and the free phosphate content resulting of the enzyme activity was calculated according to a linear pattern using known phosphate concentrations (Attachment 16).

In order to determine the γ -ECS activity the amount of inorganic phosphate (Pi) determined in the blank was subtracted to that quantified with the samples. Such difference corresponds to the amount of ATP used by γ-ECS during the 45 minutes for 50 µL of reaction. Results were expressed in nkat mg^{-1} protein [45].

3.8 Glutathione Reductase

0.5 g of plant tissue were homogenized using 1.5 ml of extraction buffer, composed of 100 mM potassium phosphate buffer (pH 7.5); 1 mM EDTA; PMSF 1 mM and 10 mM dithiothreitol (DTT) and centrifuged at 20 000 x g for 30 minutes at 4 °C. The supernatant was recovered, and GR activity was determined by pipetting 500 µl of 200 mM potassium phosphate buffer (pH=7.8); 100 µl of 20 mM EDTA; 100 µl of 2 mM NADPH; 100 µl of extract and 100 µl of water into a cuvette and reading its absorbance

at 340 nm. Blanks were determined by substituting extract by extraction buffer. The activity of the enzyme was determined using the NADPH extinction coefficient ε= 6.22 $mM^{-1}cm^{-1}$. Results were expressed as nkat mg^{-1} protein [46].

4. Statistical analysis

All assays and measurements were performed at least in triplicate (n≥3). Variance analysis was performed by Fisher test and the means were statistically analysed using a two-sided t-test. Statistical significance is assumed at $P < 0.05$.

Results

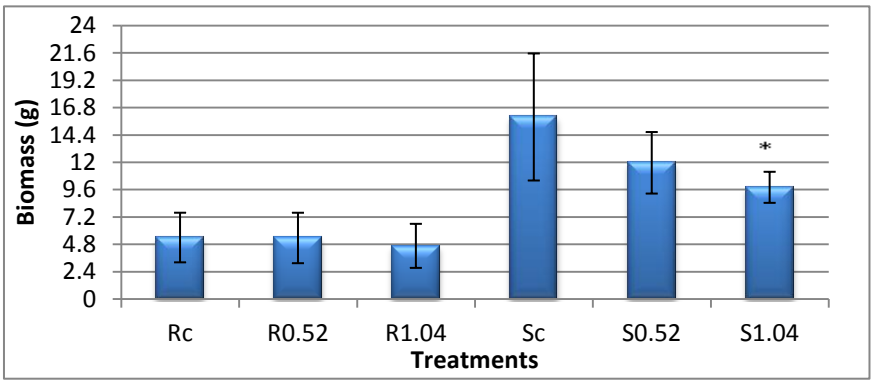
1. Stress biomarkers

1.1 Biometry

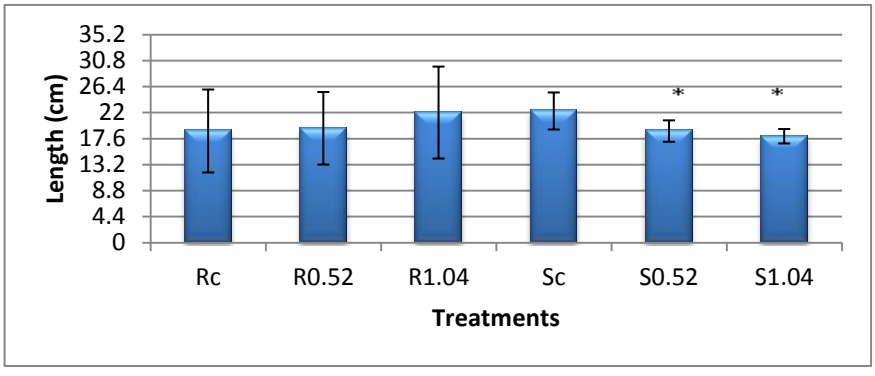
The biomass and length of shoots and roots of plants exposed to acetophenone treatments were analysed in order to determine if plant growth was affected by the used treatments.

As it can be seen in Graphic 1, there was a 1.63 fold reduced shoot biomass of plants exposed to 1.04 ppm of acetophenone, and these were the only organs affected.

Considering the length of roots and shoots (Graphic 2), the exposure to acetophenone lead to a significant 1.18 and 1.24 fold reduction in shoots, in the 0.52 ppm and 1.04 ppm treatments, respectively, with no changes in roots length being recorded.



Graphic 1- Biomass of roots and aerial organs, expressed in grams. Data presented are mean ± SE (n≥3). Rc- roots of control plants; R0.52- Roots of plants exposed to 0.52 ppm of acetophenone; R1.04- Roots of plants exposed to 1.04 ppm of acetophenone; Sc- Shoots of control plants; S0.52- Shoots of plants exposed to 0.52 ppm of acetophenone; S1.04- Shoots of plants exposed to 1.04 ppm of acetophenone. The asterisk stands for significant statistical difference from control at P = 0.05. Attachment 1

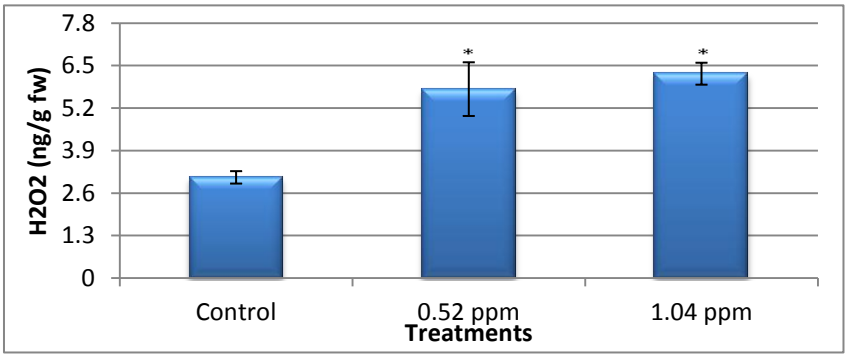


Graphic 2- Length of roots and aerial organs, expressed in centimetres. Data presented are mean ± SE (n≥3). Rc- roots of control plants; R0.52- Roots of plants exposed to 0.52 ppm of acetophenone; R1.04- Roots of plants exposed to 1.04 ppm of acetophenone; Sc- Shoots of control plants; S0.52- Shoots of plants exposed to 0.52 ppm of acetophenone; S1.04- Shoots of plants exposed to 1.04 ppm of acetophenone. The asterisk stands for significant statistical difference from control at P = 0.05. Attachment 2

1.2 Hydrogen peroxide (H_2O_2)

There was a significant increase of this ROS in shoots from both treatments, which followed the increase in acetophenone concentration, being 1.7 fold higher in plants exposed to 0.52 ppm and 2.2 times in plants exposed to 1.04 ppm of acetophenone (Graphic 3).

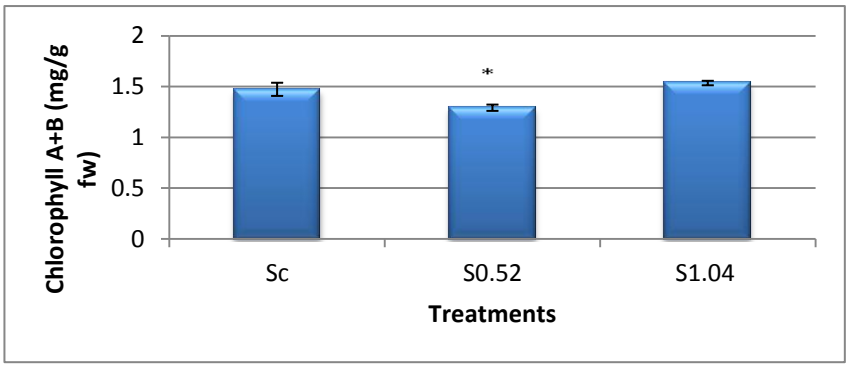
.



Graphic 3- Hydrogen peroxide (H_2O_2) contents in shoots, expressed as ng/g of fresh weight. Data presented are mean ± SE (n≥3). 0,52- Plants exposed to 0.52 ppm of acetophenone; 1.04- Plants exposed to 1.04 ppm of acetophenone. The asterisk stands for significant statistical difference from control at P = 0.05. Attachment 3

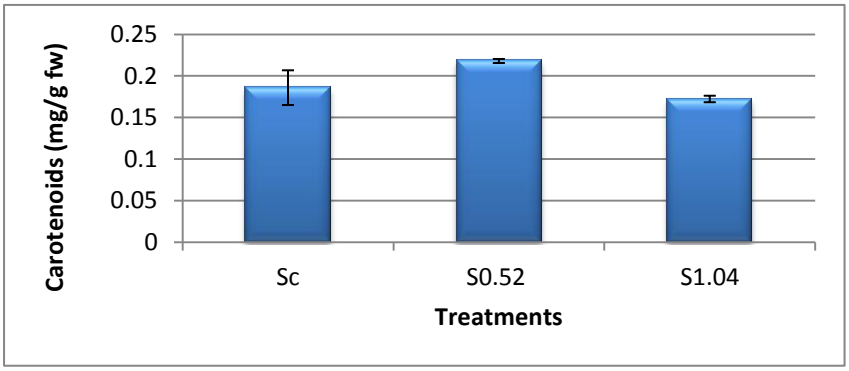
1.3 Photosynthetic pigments

The contents of chlorophyll A and B of plants exposed to the different acetophenone concentrations used significantly decreased by 0.9 fold only with the 0.52 ppm treatment (Graphic 4).



Graphic 4 - Chlorophyll A and B content in shoots, expressed in mg/g of fresh weight. Data presented are mean ± SE (n≥3). Sc- Shoots of control plants; S0.52- Shoots of plants exposed to 0.52 ppm of acetophenone; S1.04- Shoots of plants exposed to 1.04 ppm of acetophenone. The asterisk stands for significant statistical difference from control at P = 0.05. Attachment 4

There were no differences for carotenoids contents among the different acetophenone concentrations used (Graphic 5).

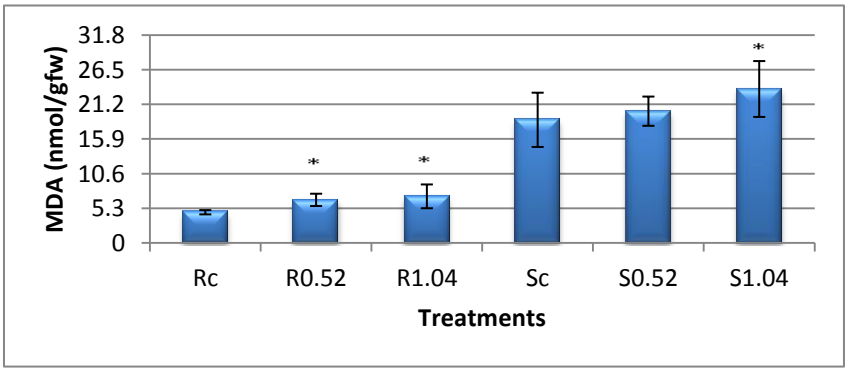


Graphic 5 - Carotenoids content in shoots, expressed in mg/g of fresh weight. Data presented are mean ± SE (n≥3). Sc- Shoots of control plants; S0.52- Shoots of plants exposed to 0.52 ppm of acetophenone; S1.04- Shoots of plants exposed to 1.04 ppm of acetophenone. The asterisk stands for significant statistical difference from control at P = 0.05. Attachment 5

1.4 Lipid Peroxidation

The content of malondialdehyde (MDA) was used as an indicator to access membrane damage by lipid peroxidation (Graphic 6). MDA levels in roots clearly increased alongside the increasing acetophenone concentrations used. Thus, in roots of the 0.52 ppm situation it significantly increased 1.4 fold and in the 1.04 ppm situation this increase was 1.5 fold.

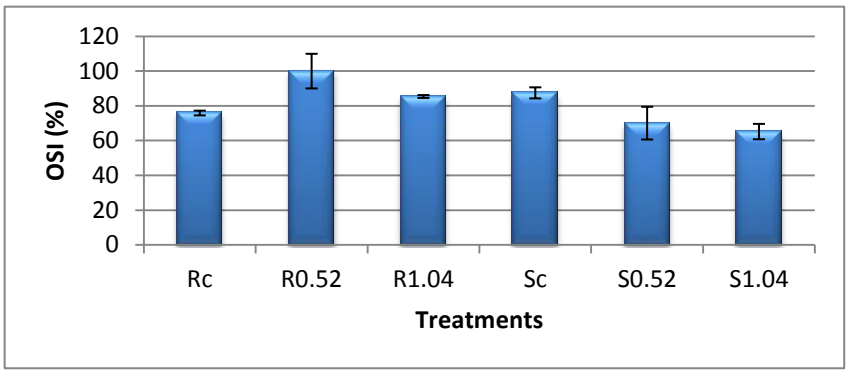
In shoots, MDA levels appear to increment, but only in with of 1.04 ppm this increase was statistical significant (1.25 fold).



Graphic 6 - Malondialdehyde content in roots and aerial organs, expressed in nmol/g of fresh weight. Data presented are mean ± SE (n≥3). Rc- roots of control plants; R0,52- Roots of plants exposed to 0.52 ppm of acetophenone; R1.04- Roots of plants exposed to 1.04 ppm of acetophenone; Sc- Shoots of control plants; S0.52- Shoots of plants exposed to 0.52 ppm of acetophenone; S1.04- Shoots of plants exposed to 1.04 ppm of acetophenone. The asterisk stands for significant statistical difference from control at P = 0.05. Attachment 6

1.5 Oxidative Stress Index (OSI)

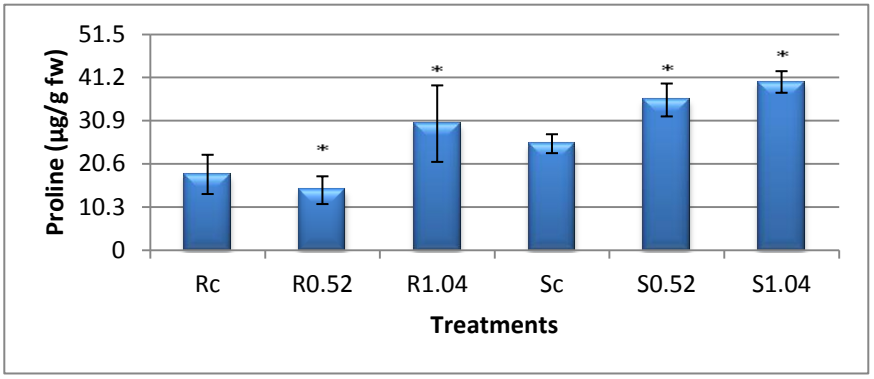
The Oxidative stress index revealed no statistical difference when compared to control situation, nevertheless, it is clear that Roots showed higher stress than Shoots (Graphic 7).



Graphic 7 – Oxidative stress index in roots and aerial organs, expressed in percentage. Data presented are mean ± SE (n≥3). Rc- roots of control plants; R0,52- Roots of plants exposed to 0.52 ppm of acetophenone; R1.04- Roots of plants exposed to 1.04 ppm of acetophenone; Sc- Shoots of control plants; S0.52- Shoots of plants exposed to 0.52 ppm of acetophenone; S1.04- Shoots of plants exposed to 1.04 ppm of acetophenone. Attchment 7

1.6 Proline contents

Plants exposed to acetophenone suffered significant changes in their proline content, as shown in Graphic 8. In roots, proline levels decreased 1.3 fold when plants were exposed to 0.52 ppm of acetophenone, but increased 2.7 fold with the 1.04 treatment. Considering the shoots, proline levels revealed a dose-dependent increase with the acetophenone concentrations used. Thus, in shoots of plants exposed to 0.52 ppm there was an increase of 1.4 fold and of 1.6 fold with the 1.04 ppm treatment.



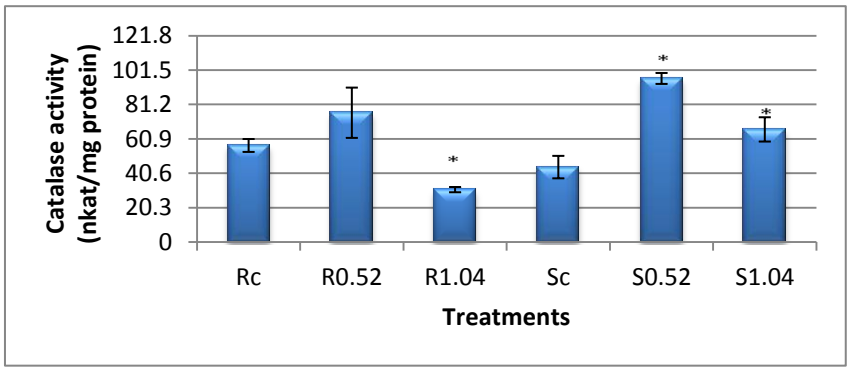
Graphic 8 - Proline content in roots and aerial organs, expressed in µg/g of fresh weight. Data presented are mean ± SE (n≥3). Rc- roots of control plants; R0.52- Roots of plants exposed to 0.52 ppm of acetophenone; R1.04- Roots of plants exposed to 1.04 ppm of acetophenone; Sc- Shoots of control plants; S0.52- Shoots of plants exposed to 0.52 ppm of acetophenone; S1.04- Shoots of plants exposed to 1.04 ppm of acetophenone. The asterisk stands for significant statistical difference from control at P = 0.05. Attachment 8

2. Antioxidant mechanisms

2.1 Catalase activity

As Graphic 9 depicts, there was a significant decrease of 1.84 fold in CAT activity in roots exposed to 1.04 ppm, while there was no statistical difference in activity in roots exposed to 0.52 ppm. The catalase activity significantly increased in shoots, by 2 fold in plants exposed to 0.52 ppm and a 1.5 fold in plants exposed to 1.04

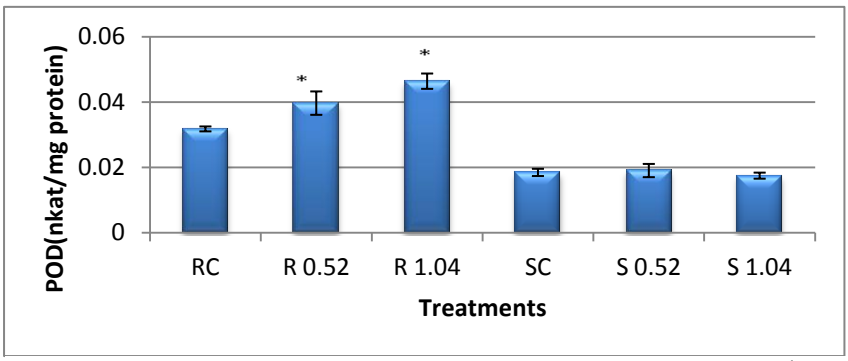
ppm. In this latter situation, there was a significant decrease of 1.45 fold when compared to that of 0.52 ppm of acetophenone.



Graphic 9 - Catalase activity in roots and aerial organs, expressed in nkat/mg of protein. Data presented are mean ± SE (n≥3). Rc- roots of control plants; R0.52- Roots of plants exposed to 0.52 ppm of acetophenone; R1.04- Roots of plants exposed to 1.04 ppm of acetophenone; Sc- Shoots of control plants; S0.52- Shoots of plants exposed to 0.52 ppm of acetophenone; S1.04- Shoots of plants exposed to 1.04 ppm of acetophenone. The asterisk stands for significant statistical difference from control at P = 0.05. Attachment 9

2.2 Guaiacol Peroxidase (POD)

Graphic 10 depicts the activity of POD determined in roots and shoots of plants from the three growth conditions used. POD activity was always higher in roots than in shoots. The activity was significantly higher only in roots of plants exposed to 0.52 ppm and 1.04 ppm, by 1.2 and 1.5 fold, respectively.



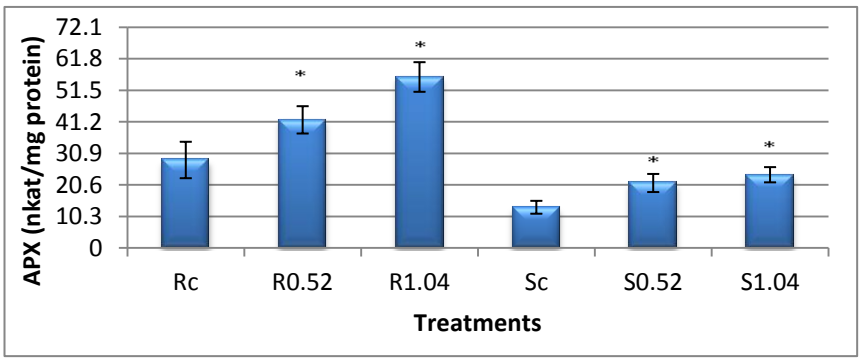
Graphic 10 - Guaiacol peroxidase activity in roots and aerial organs, expressed in nkat/mg of protein. Data presented are mean ± SE (n≥3). Rc- roots of control plants; R0.52- Roots of plants exposed to 0.52 ppm of acetophenone; R1.04- Roots of plants exposed to 1.04 ppm of acetophenone; Sc- Shoots of control plants; S0.52- Shoots of plants exposed to 0.52 ppm of acetophenone; S1.04- Shoots of plants exposed to 1.04 ppm of acetophenone. The asterisk stands for significant statistical difference from control at P = 0.05. Attachment 10

2.3 Ascorbate Peroxidase (APX)

Ascorbate peroxidase activity was higher in roots than in shoots of plants exposed to both treatments, and significantly increased with the increasing acetophenone concentrations used in both plant parts analysed (Graphic 11).

In roots, the increases were about 1.4 and 1.9 fold in the 0.52 ppm and 1.04 ppm treatments, respectively, where that of the 1.04 ppm-derived roots was 1.33

times higher than of the 0.52 ppm. In shoots, APX activity augmented 1.6 (0.52 ppm plants) and 1.8 (1.04 ppm plants) fold, with no statistical difference between the two treatments.



Graphic 11 - Ascorbate peroxidase (APX) activity in roots and aerial organs, expressed in nkat/mg of protein. Data presented are mean ± SE (n≥3). Rc- roots of control plants; R0.52- Roots of plants exposed to 0.52 ppm of acetophenone; R1.04- Roots of plants exposed to 1.04 ppm of acetophenone; Sc- Shoots of control plants; S0.52- Shoots of plants exposed to 0.52 ppm of acetophenone; S1.04- Shoots of plants exposed to 1.04 ppm of acetophenone. The asterisk stands for significant statistical difference from control at P = 0.05. Attachment 11

2.4 Thiol content

The total, non-protein and protein thiols were determined in order to evaluate changes in thiol content in the different organs resulting from the different treatments performed. The results are depicted in Graphic 12.

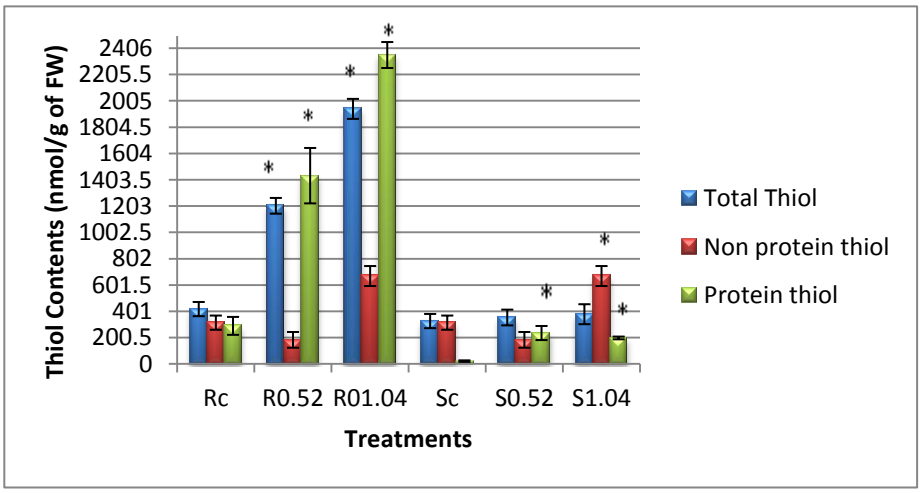
The total thiol content in roots significantly increased when plants were exposed to acetophenone, unlike the contents in shoots, which remained at the same level after acetophenone exposure.

The augmented content of thiol in roots was of 2.9 and 4.6 fold in the 0.52 ppm and 1.04 ppm treatments, respectively, and the levels of total thiol content in roots exposed to 1.04 ppm of acetophenone was 1.6 fold higher than that of the 0.52 ppm.

As for non-protein thiols contents, these were significant different in shoots of plants exposed to 1.04 ppm of acetophenone, increasing by 2.0 fold (Graphic 12).

The contents of thiolated proteins in plants exposed to the different concentrations of acetophenone under study significantly increase in both organs analysed (Graphic 12). This increase was particularly higher in roots, where the contents of thiolated proteins increased along the increasing concentrations of acetophenone experimented, being 5 fold in plants exposed to 0.52 ppm and 8.1 fold in those exposed to 1.04 ppm. The difference between the different concentrations used was also statistical significant, resulting in a 1.6 fold increase in the roots of the plants exposed to 1.04 ppm of acetophenone relatively to those exposed to 0.52 ppm.

The contents of thiolated proteins in shoots also statistically increased accordingly to the increase of acetophenone concentrations used, being of 11 and 9 fold in the 0.52 ppm and 1.04 ppm treatments, respectively.



Graphic 12 - Total thiol, non-protein thiol and protein thiol contents in roots and aerial organs, expressed in nmol/g of fresh weight. Data presented are mean ± SE (n≥3). Rc- roots of control plants; R0,52- Roots of plants exposed to 0.52 ppm of acetophenone; R1.04- Roots of plants exposed to 1.04 ppm of acetophenone; Sc- Shoots of control plants; S0.52- Shoots of plants exposed to 0.52 ppm of acetophenone; S1.04- Shoots of plants exposed to 1.04 ppm of acetophenone. The asterisk stands for significant statistical difference from control at P = 0.05. Attachment 12

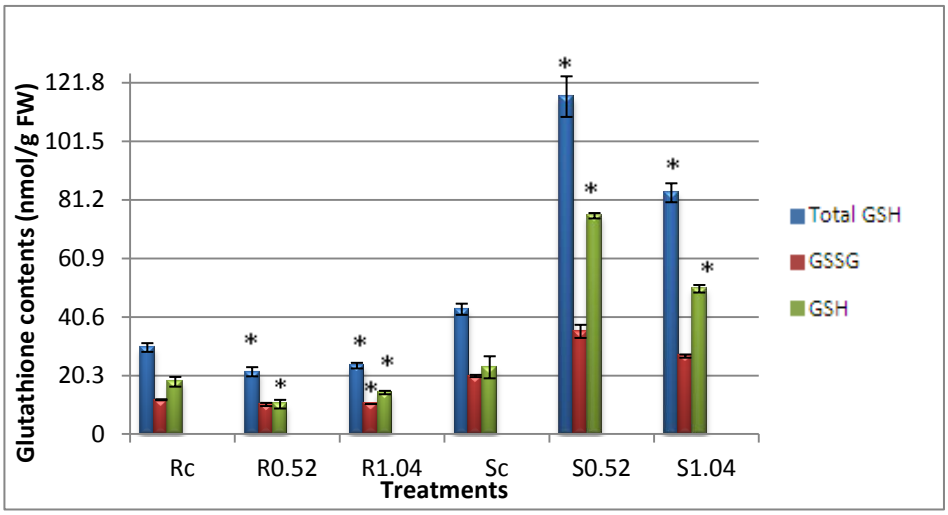
2.5 Glutathione

As Graphic 13 shows, the contents of GSHt in roots significantly decrease about 1.4 fold in plants exposed to 0.52 ppm of acetophenone and 1.3 fold in roots of plants exposed to 1.04 ppm of acetophenone. As for aerial organs, the total content of glutathione significantly increased 2.6 and 1.8 fold, in plants exposed to 0.52 ppm of acetophenone and 1.04 ppm of acetophenone, respectively. The pool of total GSHt was higher in shoots than in roots and increased in response to the exposure to acetophenone.

Reduced glutathione contents in roots significantly decreased 1.7 and 1.3 fold in plants exposed to 0.52 ppm of acetophenone and 1.04 ppm of acetophenone, respectively. On the other hand, in shoots of plants exposed to 0.52 ppm of acetophenone and 1.04 ppm of acetophenone, the contents of GSH significantly increased about 3.3 and 2.2 fold, respectively. Similarly, to GSHt, there was more GSH in shoots than in roots and shoots' contents also increased in response to both acetophenone treatments.

As for GSSG, its contents did not show an evident increase or decrease in any situation, statistically speaking. Except for a slight decrease (1.1 fold) of its contents in

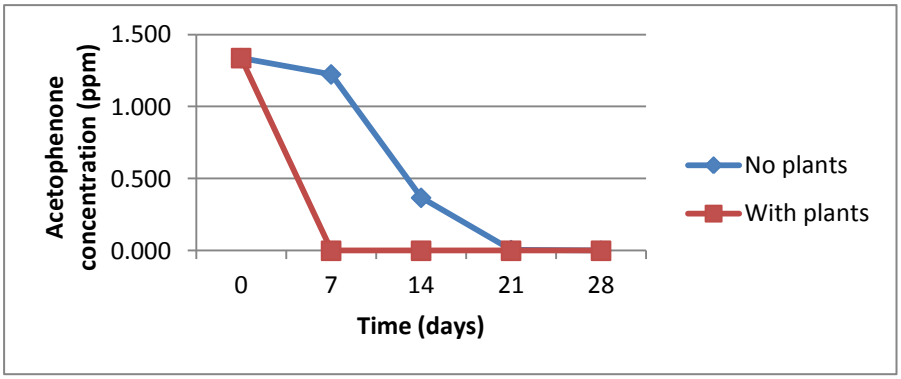
roots of plants exposed to 1.04 ppm, and a slight increase (1.3 fold) in shoots of the same plants (Graphic 13).



Graphic 13 - Total glutathione, oxidized (GSSG) and reduced (GSH) glutathione contents present in roots and shoots, expressed in nmol/g of fresh weight. Data presented are mean ± SE (n≥3). Rc- roots of control plants; R0.52- Roots of plants exposed to 0.52 ppm of acetophenone; R1.04- Roots of plants exposed to 1.04 ppm of acetophenone; Sc- Shoots of control plants; S0.52- Shoots of plants exposed to 0.52 ppm of acetophenone; S1.04- Shoots of plants exposed to 1.04 ppm of acetophenone. The asterisk stands for significant statistical difference from control at P = 0.05. Attachment 13

3. Acetophenone removal by plants

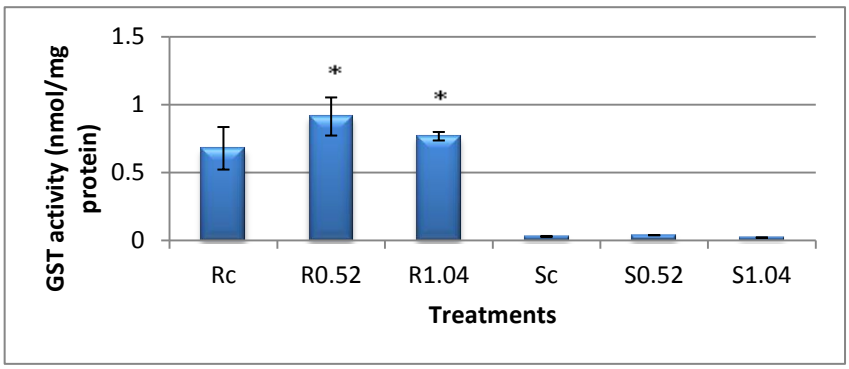
Acetophenone levels decreased with time, in the presence or absence of *S. nigrum* plants (Graphic 14), and were not detected in the nutrient solution after seven days of plant exposure and after 21 days in the absence of plants (Graphic 14).



Graphic 14 - Acetophenone levels quantified in the nutrient solution (1.04 ppm treatment) during 28 days, on a weekly basis, in the presence or absence of S. nigrum plants. Attachment 14

4. Glutathione-*S*-Transferase (GST)

Determined GST activities were always higher in roots than in shoots, and there were no significant differences amongst the aerial organs of the different treatments. The activity was 1.3 and 1.1 fold significantly higher in roots of plants exposed to 0.52 ppm and 1.04 ppm respectively (Graphic 15).

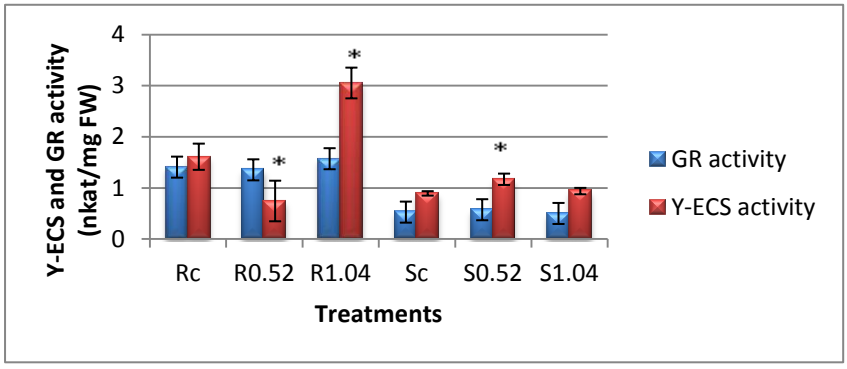


Graphic 15 - Glutathione-*S*-Transferase in roots and shoots, expressed in nmol/mg protein. Data presented are mean ± SE (n≥3). Rc- roots of control plants; R0,52- Roots of plants exposed to 0.52 ppm of acetophenone; R1.04- Roots of plants exposed to 1.04 ppm of acetophenone; Sc- Shoots of control plants; S0.52- Shoots of plants exposed to 0.52 ppm of acetophenone; S1.04- Shoots of plants exposed to 1.04 ppm of acetophenone. The asterisk stands for significant statistical difference from control at P = 0.05. Attachment 15

5. γ-ECS and GR activities

γ -ECS is the key enzyme for the formation of the tripeptide glutathione. As Graphic 16 shows, γ -ECS activity in roots of plants exposed to 1.04 ppm of acetophenone significantly increased 0.52 fold, and 4 fold when compared to roots of plants exposed to 0.52 ppm of acetophenone. These latter treatment lead to a significant decreased in activity of 2.2 fold in roots. In shoots of plants exposed to 0.52 ppm there was a significant increase of 1.3 fold.

Although GR activity showed no statistical difference between treatments, it was possible to verify that its activity was higher in roots than in shoots (Graphic 16).



Graphic 16 - Y-ECS and GR activity in roots and shoots, expressed in nkat/mg of protein. Data presented are mean ± SE (n≥3). Rc- roots of control plants; R0.52- Roots of plants exposed to 0.52 ppm of acetophenone; R 1.04- Roots of plants exposed to 1.04 ppm of acetophenone; Sc- Shoots of control plants; S0.52- Shoots of plants exposed to 0.52 ppm of acetophenone; S1.04- Shoots of plants exposed to 1.04 ppm of acetophenone. The asterisk stands for significant statistical difference from control at P = 0.05. Attachment 16

Discussion

The reduction in plant shoot and root biomass and length clearly demonstrates that acetophenone impaired plant growth, indicating that treated plants suffered some stress. Nevertheless, plants were able to complete their life cycle, as floral buds were seen at the date of harvest.

In fact, shoot H_2O_2 levels increased alongside acetophenone concentrations, this way supporting the observed biometric changes previously mentioned and leading to the reduction in chlorophyll levels, as well as an increase in membrane damage. Hence, the increase observed in the oxidative stress biomarker for membrane damage – MDA content – revealed that roots and shoots of plants exposed to both acetophenone concentrations suffered oxidative stress. MDA is a product of the second step of lipid oxidation, which is closely related to radical amplification and expansion of the peroxide pool, making it a marker for free radical-catalysed lipid peroxidation [25]. Thus, exposure to acetophenone caused lipid fragmentation in roots of plants from both treatments, as well as in shoots of plants exposed to the higher concentration of acetophenone, which are in accordance with the hydrogen peroxide contents determined. Increased hydrogen peroxide levels have already been described in response to oxidative stress in *Solanum nigrum* L. caused by organic pollutants [6]. Nevertheless, in shoots of plants exposed to 0.52 ppm of acetophenone no lipid peroxidation was detected, but a decrease in total chlorophyll occurred, indicating that the plant antioxidant defence mechanisms are acting differentially in shoots from plants exposed to 0.52 ppm and 1.04 ppm acetophenone, to counteract the high H_2O_2 levels present therein. In spite of this results, the OSI, revealed no difference amongst the different situation.

Proline is an amino acid known to accumulate under biotic and abiotic stresses [6]. Its increased levels in both organs after the exposure to acetophenone indicate that proline may be protecting these organs from the rise in Reactive Oxygen Species (ROS) formed in consequence of those treatments. However, roots from plants exposed to 0.52 ppm suffered a decrease in this amino acid content. This reduction may be due to the fact that proline produced in roots is being channelled towards the shoots, as its concentration increased there. In addition, other protective mechanisms,

like catalase, POD and APX, were put into action in order to fight this oxidative burst, thus contributing to the reduction in proline levels.

In fact, CAT and APX had enhanced activity in shoots of plants exposed to acetophenone, while in roots both POD and APX revealed an increased activity in treated plants. These results indicate that these enzymes are differentially protecting these organs: both peroxidases were more actively removing H_2O_2 from roots, while CAT and APX acted more strongly it in shoots. Furthermore, due to enhanced activity of both peroxidases in roots, which have a lower K_m for H_2O_2 then catalase, the latter enzyme could not participate in the removal of this ROS, because of its relative higher K_m, making it less competitive towards H_2O_2 removal in relation to root POD and APX. These results are in accordance with those obtained in a previous study performed with *S. nigrum* plants exposed to the fungicide Ridomil, which has as it second main component acetophenone, where root and shoot POD also showed increased activities in the exposed plants [6].

Curiously, in a recent study, the exposure of *Arabidopsis thaliana* to polycyclic aromatic hydrocarbons (PAHs) lead to an increase in shoot POD and APX activities, and to no changes in the CAT pattern of activity [47]. Such differences with the results herein described may be due to the different xenobiotic used and/or to different responses of *Arabidopsis thaliana* compared to *S. nigrum*. In fact, in a previous report by Ahammed et al 2012, CAT and POD were increased in different species (viz. pakchoi, flowering chinese cabbage, lettuce, cucumber and tomato) following exposure to phenanthrene [48], thus reinforcing the interpretation that xenobiotic composition can influence plant defence and detoxification responses.

Furthermore, it is very possible that increased POD activity may be also due to the fact that it used some acetophenone at the root level as substrate, as this xenobiotic has a phenolic ring in its chemical structure[2], leading to its rapid incorporation in root cell walls and thus contributing to acetophenone detoxification and tolerance in root plant cells .

Total thiol levels were always higher in roots than in shoots. Interestingly, root total thiol levels are a consequence of a higher protein thiol content, while in shoots non-protein thiols also contributed to its increase. Consequently, the increasing levels

[2] http://www.epa.gov/ttnatw01/hlthef/acetophe.html

for root total thiols alongside the increasing acetophenone levels added are the reflection of the increasing protein thiol levels. In contrast, the non-protein thiols, which consist of glutathione and its conjugates and some other conjugates [9], increased in shoots of plants exposed to acetophenone but did not contribute to the increase in total thiol content, neither did the increased protein thiol content, because, when added together, the total thiol content did not differ from the control situation.

Hence, this increase in non-protein thiol levels reveals the importance of glutathione in acetophenone detoxification, where acetophenone is detoxified partly by conjugation with GSH, as already described for tomato [9]. Moreover, the acetophenone levels measured in the plant nutrient solution revealed a rapid decrease, reaching zero just after 7 days of plant exposure, contrasting with the 21 days that took acetophenone to disappear without any plant action.

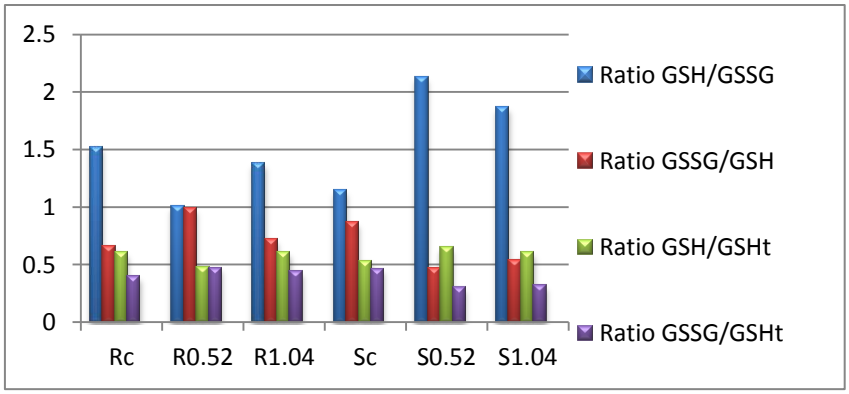
Also, taking into account GST activities, the thiol-related results evidence that the glutathione conjugates produced by the enhanced activity of GST in roots of plants exposed to 1.04 ppm were translocated to shoots. Additionally, the unaltered activity of GST in shoots and the enhanced contents of protein thiols, suggest that other mechanisms were used to protect shoot proteins against the oxidative stress [8], e.g. thioredoxins and/or glutaredoxins. However, GST activity as long been described as having an important role in the strategy of plants coping with several xenobiotics, by conjugating them to glutathione, rendering them more polar and less toxic [6, 49, 50]. This would explain why thiolated proteins increased in roots alongside with GSSG contents, because glutaredoxins are directly reduced by GSH, while thioredoxins require different reductases [7, 8].

Considering the levels of GSHt, GSH and GSSG, GSH/GSSG ratios decreased in roots of plants exposed to both acetophenone concentrations tested (Graphic 13 and Graphic 17), by 1.5 and 1.1 fold, respectively. Unlike roots, in shoots there was an increment of 1.8 and 1.6 of these ratios in 0.52 ppm and 1.04 ppm, respectively. When analysing the GSH content in relation to the total glutathione pool, in roots, there was a decrease of 1.3 fold in plants exposed to 0.52 ppm acetophenone, while with 1.04 pm of acetophenone plant roots revealed an increase of 1.1 fold. As for shoots, both plants depicted an increase of 1.2 and 1.1 fold, in the 0.52 and 1.04 ppm treatments, respectively. These changes in GSH/GSSG ratios suggest that *S. nigrum* used *de novo*

synthesis of GSH instead of recycling GSSG into GSH. In addition, considering GSH and GSSG contents in the total glutathione pool, it is clear that neosynthesis of GSH is much higher than GSSG formation, meaning that GSH is being synthesised in order to fight the deleterious effect of oxidative stress caused by the acetophenone treatments, or to be used for root acetophenone conjugation and further translocation to shoots. The turnover of GSH as well as the GSH content in both organs of plants exposed to the xenobiotic, in absence of statistical differences in GR activity, is consistent with the statistical differences obtained for the increased γ-ECS activity, revealing that GSH pools are indeed under the direct control of γ-ECS, i.e., its synthesis.

The reduction of the GSSG/GSH ratios in response to the exposure to acetophenone also indicates that GSH production is channelled to shoots, resulting in a higher turnover of GSH into GSSG in roots than in shoots (Graphic 17).

The content of GSSG in the total pool of glutathione, revealed an increase of 1.2 and 1.1 fold in roots of plants exposed to both treatments, 0.52 ppm and 1.04 ppm of acetophenone respectively, while a 1.5 and 1.4 fold decrease was showed by shoots, in the same plants.



Graphic 17 - Ratios of reduced glutathione turnover, oxidized glutathione turnover, reduced and oxidized glutathione pool in total glutathione pool. Rc-roots of control plants; R0.52-Roots of plants exposed to 0.52 ppm of acetophenone; R 1.04- Roots of plants exposed to 1.04 ppm of acetophenone Sc- Shoots of control plants; S0.52- Shoots of plants exposed to 0.52 ppm of acetophenone; S1.04- Shoots of plants exposed to 1.04 ppm of acetophenone.

Considering GSSG/GSH ratios, the results suggest a 34% and 6% increase in roots of both treated plants, with the 0.52 and 1.04 ppm treatments, respectively, whilst in shoots there was a 40% and 33% significant reduction of GSSG turnover with the 0.52 and 1.04 ppm treatments, respectively. This evidence is in consonance with the fact that GSH production is being channel towards shoots, making the turnover of GSH into GSSG higher in roots rather than in shoots, as already described.

Conclusion

Overall, the results presented clearly point out that acetophenone provoked deleterious effects in *S. nigrum*, and that these plants responded to this xenobiotic with a burst of newly synthesised glutathione and thiols, thus busting the redox potential of the plant, alongside with a differential participation of antioxidant enzymes (APX, POD and CAT) in roots and shoots.

These results also demonstrate that *S. nigrum* were able to rapidly uptake acetophenone by their roots and process it either by using it as substrate for root POD, incorporating it in the cell wall; or conjugating it to GSH via root GST, rendering it into a less toxic compound that was translocated to the shoots.

In this line of thought, this work demonstrates that *S. nigrum* plants can be used as an excellent tool for the rapid remediation of contaminated sites with acetophenone, as no acetophenone was detected after 7 days of exposure to the plants.

Future directions

It would be interesting to see how the genes that encode the enzymes that were biochemically studied in this work responded to acetophenone exposure and to which extent the changes in the redox buffer of the cell can induce post-translational changes performed by proteins like TRX and GRX, to better acknowledge the role of glutathione. Such information would provide hints for future genetic manipulations of this plant species in order to increase even more its phytoremediation potential towards organic pollutants.

Bibliography

[1] J.B.B. Keri L.D. Henderson, Shaohan Zhao, and Joel R. Coats, Phytoremediation of Pesticide Wastes in Soil, (2005).
[2] K. Panz, K. Miksch, Phytoremediation of explosives (TNT, RDX, HMX) by wild-type and transgenic plants, Journal of environmental management, 113 (2012) 85-92.
[3] J.H. Lee, An overview of phytoremediation as a potentially promising technology for environmental pollution control, Biotechnology and Bioprocess Engineering, 18 (2013) 431-439.
[4] E. Pilon-Smits, Phytoremediation, Annual Review Plant Biology, (2005).
[5] A.D.P.a.H. Rennenberg, Phytoremediation, EMBO, 6 (2005) 497-501.
[6] J.S. Teixeira, Ad Azenha, M. Moreira, J. T. Fidalgo, F. Fernando Silva, A. Faria, J. L. Silva, A. M., *Solanum nigrum* L. weed plants as a remediation tool for metalaxyl-polluted effluents and soils, Chemosphere, 85 (2011) 744-750.
[7] G.M. Noctor, A. Chaouch, S. Han, Y. Neukermans, J. Marquez-Garcia, B. Queval, G. Foyer, C. H., Glutathione in plants: an integrated overview, Plant, cell & environment, 35 (2012) 454-484.
[8] L. Zagorchev, C.E. Seal, I. Kranner, M. Odjakova, A central role for thiols in plant tolerance to abiotic stress, International journal of molecular sciences, 14 (2013) 7405-7432.
[9] G.B.Z. Yu, Y. Ahammed, G. J. Xia, X. J., Mao, W. H. Shi, K. Zhou, Y. H. Yu, J. Q., Glutathione biosynthesis and regeneration play an important role in the metabolism of chlorothalonil in tomato, Chemosphere, 90 (2012) 2563-2570.
[10] S.C. Hilde Willekens, Mark Davey, Martina Schraudner, Christian Langebartels, Marc Van Montagu, Dirk Inze and Wim Van Camp, Catalase is a sink for H_2O_2 and is indispensable for stress defence in c3 plants, The EMBO journal, 16 (1997) 4806-4816.
[11] G. Queval, D. Thominet, H. Vanacker, M. Miginiac-Maslow, B. Gakiere, G. Noctor, H_2O_2-activated up-regulation of glutathione in Arabidopsis involves induction of genes encoding enzymes involved in cysteine synthesis in the chloroplast, Molecular plant, 2 (2009) 344-356.
[12] A. Michael Hothorn, RolandGromes, Tobias Stuwe, Thomas Rausch and Klaus Scheffzek, Structural Basis for the Redox Control of Plant Glutamate Cysteine Ligase, The Journal of Biological Chemestry, 281 (2006) 27557-27565.
[13] S.V.-J. Graham Noctor, Simon Driscoll, Larissa Novistkaya and H. Christine Foyer, Drought and Oxidative Load in the Leaves of C3 Plants: a Predominant Role for photorespiration?, Annals of biology, 89 (2002) 841-850.
[14] E.H.-H. Ludmila Rizhsky, Frank Van Breusegem, Shimon Rachmilevitch, Jason E. Barr, Steven Rodermel,, D.I.a.R. Mittler, Double antisense plants lacking ascorbate peroxidase and catalase are less sensitive to oxidative stress than single antisense plants lacking ascorbate peroxidase or catalase, The Plant Juornal, 32 (2002) 329-342.
[15] G.Q. Sejir Chaouch, Sandy Vanderauwera, Amna Mhamdi, Michaël Vandorpe, Mathilde Langlois-Meurinne, Frank Van Breusegem , Patrick Saindrenan and Graham Noctor, Peroxisomal Hydrogen Peroxide Is Coupled to Biotic Defense Responses by ISOCHORISMATE SYNTHASE1 in a Daylength-Related Manner, Plant Physiology, 153 (2010) 1692-1705.
[16] J.H. Amna Mhamdi, Sejir Chaouc h, Guillaume Quev al, Yi Han, Ludivine Taconnat, Patrick Saindrenan, Houda Gouia, Emmanuelle Issakidis-Bourguet, Jean-Pierre Renou

and Graham Noctor, Arabidopsis Glutathione Reductase1 Plays a Crucial Role in Leaf Responses to Intracellular Hydrogen Peroxide and in Ensuring Appropriate Gene Expression through Both Salicylic Acid and Jasmonic Acid Signaling Pathways, Plant Physiology, 153 (2010) 1144-1160.
[17] G.Q. Amna Mhamdi, Sejir Chaouch, Sandy Vanderauwera, Frank Van Breusegem and Graham Noctor, Catalase function in plants: a focus on Arabidopsis mutants as stress-mimic models, Journal of Experimental Botany, 61 (2010) 4197-4220.
[18] J.D. Laurence V. Bindschedler, Kris A. Blee, Julie M. Stone , Tsuneaki Asai, Julia Plotnikov, Carine, T.H. Denoux, Chris Gerrish, Dewi R. Davies, Frederick M. Ausubel and G. Paul Bolwell, Peroxidase-dependent apoplastic oxidative burst in Arabidopsis required for pathogen resistance, The Plant Journal, 47 (2006) 851-863.
[19] B.G.D.a.R.E. David P. Dixon, Functional Divergence in the Glutathione Transferase Superfamily in Plants, The Journal of Biological Chemestry, 277 (2002) 330859-330869.
[20] D.P.D.a.R. Edwards, Glutathione Transferases, American Society of Plant Biologists, The Ar (2010).
[21] N. Rouhier, Plant glutaredoxins: pivotal players in redox biology and iron–sulphur centre assembly, New phytologist, 186 (2010) 365-372.
[22] E.L. Lionel Tarrago, Mirko Zaffagnini, Christophe Marchand, Pierre Le Maréchal, Nicolas Rouhier, Stéphane D. Lemaire and Pascal Rey, Regeneration Mechanisms of *Arabidopsis thaliana* Methionine Sulfoxide Reductases B by Glutaredoxins and Thioredoxins, Journal Of Biological Chemestry, Volume 284 (2009) 18963-18971.
[23] P. Sharma, A.B. Jha, R.S. Dubey, M. Pessarakli, Reactive Oxygen Species, Oxidative Damage, and Antioxidative Defense Mechanism in Plants under Stressful Conditions, Journal of Botany, (2012) 1-26.
[24] J.A. Imlay, Pathways of oxidative damage, Annual Review of Microbiology, 57 (2003) 395-418.
[25] E.E.F.a.M.J. Mueller, ROS-Mediated Lipid Peroxidation and RES-Activated Signaling, Annual Review Plant Biology, 64 (2013) 429–450.
[26] R. Mittler, Oxidative stress, antioxidants and stress tolerance, Tends in Plant Science, 7 (2002) 405-410.
[27] M.S. Parvaiz Ahmad, and Satyawati Sharma Reactive Oxygen Species, Antioxidants and Signaling in Plants, Journal of Plant Biology, 51 (2008) 167-173.
[28] S.C.a.V. Massey, Mechanisms of flavoprotein-catalyzed reactions, Eur. J. Biochem., (1989) 1-17.
[29] X. Deng, Y. Xia, W. Hu, H. Zhang, Z. Shen, Cadmium-induced oxidative damage and protective effects of N-acetyl-L-cysteine against cadmium toxicity in *Solanum nigrum* L, Journal of hazardous materials, 180 (2010) 722-729.
[30] F. Fidalgo, R. Freitas, R. Ferreira, A.M. Pessoa, J. Teixeira, *Solanum nigrum* L. antioxidant defence system isozymes are regulated transcriptionally and posttranslationally in Cd-induced stress, Environmental and Experimental Botany, 72 (2011) 312-319.
[31] G. Qu, Y. Tong, P. Gao, Z. Zhao, X. Song, P. Ji, Phytoremediation Potential of *Solanum nigrum* L. Under Different Cultivation Protocols, Bulletin of environmental contamination and toxicology, 91 (2013) 306-309.
[32] L.Z. Wang, Q. Ding, L. Sun, Y., Effect of cadmium toxicity on nitrogen metabolism in leaves of *Solanum nigrum* L. as a newly found cadmium hyperaccumulator, Journal of hazardous materials, 154 (2008) 818-825.

[33] Y. Sun, Q. Zhou, C. Diao, Effects of cadmium and arsenic on growth and metal accumulation of Cd-hyperaccumulator *Solanum nigrum* L, Bioresource Technology, 99 (2008) 1103-1110.
[34] M.M. Petra Kuverová, Ludmila Chromá, Jiri Burkhard, Jan Tríska, Katerina Demnerová and Tomáš Macek, Metabolism of polychlorinated biphenyls by *Solanum nigrum* hairy root clone SNC-90 and analysis of transformation products, Plant and Soil, (2000) 109-115.
[35] J. Edmonds, Chweya, J., Black nightshades-*Solanum nigrum* and related species, ed. International Plant Genetic Resource Institute, (1997) 56-59.
[36] D.R.B.a.R.K.K. Gurpreet Kaur, Growth, photosynthetic activity and oxidative stress in wheat (*Triticum Aestivum*) after exposure to soil., Chemosphere, 91 (2012) 265-269.
[37] H.K. Lichtenthaler, Chlorophylls and carotenoids: Pigments of photosynthetic biomembranes, in: R.D. Lester Packer (Ed.) Methods in Enzymology, Academic Press, 1987, pp. 350-382.
[38] R. Heath, L., L. Packer, Photoperoxidation in isolated chloroplasts, Archives of Biochemistry and Biophysics, 125 (1968) 189-198.
[39] L.S. Bates, R.P. Waldren, I.D. Teare, Rapid determination of free proline for water-stress studies, Plant and Soil, 39 (1973) 205-207.
[40] M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Annals of Biochemistry, 72 (1976) 248-254.
[41] K. Amako, G.-X. Chen, K. Asada, Separate Assays Specific for Ascorbate Peroxidase and Guaiacol Peroxidase and for the Chloroplastic and Cytosolic Isozymes of Ascorbate Peroxidase in Plants, Plant and Cell Physiology, 35 (1994) 497-504.
[42] H.L. Zhang, C., Shen, Z., Proteomic identification of small, copper-responsive proteins in germinating embryos of *Oryza sativa*, Annals of Botany, 103 (2009) 923-930.
[43] I.G. C. Vandeputte, I. Genestie-Denis, B. Vannier and G. Lorenzon, A microtiter plate assay for total glutathione and glutathione disulfide contents in cultured/isolated cells: performance study of a new miniaturized protocol, Cell Biology and Toxicology, 10 (1994) 415-421.
[44] Z.S. Li, R.G. Zhen, P.A. Rea, 1-Chloro-2,4-Dinitrobenzene-Elicited Increase in Vacuolar Glutathione-*S*-Conjugate Transport Activity, Plant Physiol., 109 (1995) 177-185.
[45] M.R. De Groeve, G.H. Tran, A. Van Hoorebeke, J. Stout, T. Desmet, S.N. Savvides, W. Soetaert, Development and application of a screening assay for glycoside phosphorylases, Analytical Biochemistry, 401 (2010) 162-167.
[46] G.G. Yannarelli, A.J. Fernandez-Alvarez, D.M. Santa-Cruz, M.L. Tomaro, Glutathione reductase activity and isoforms in leaves and roots of wheat plants subjected to cadmium stress, Phytochemistry, 68 (2007) 505-512.
[47] H. Liu, D. Weisman, Y.-b. Ye, B. Cui, Y.-h. Huang, A. Colón-Carmona, Z.-h. Wang, An oxidative stress response to polycyclic aromatic hydrocarbon exposure is rapid and complex in *Arabidopsis thaliana*, Plant Science, 176 (2009) 375-382.
[48] G.J. Ahammed, M.M. Wang, Y.H. Zhou, X.J. Xia, W.H. Mao, K. Shi, J.Q. Yu, The growth, photosynthesis and antioxidant defense responses of five vegetable crops to phenanthrene stress, Ecotoxicology and Environmental safety, 80 (2012) 132-139.

[49] A. Amaya-Chavez, L. Martinez-Tabche, E. Lopez-Lopez, M. Galar-Martinez, Methyl parathion toxicity to and removal efficiency by *Typha latifolia* in water and artificial sediments, Chemosphere, 63 (2006) 1124-1129.
[50] V.L. Taylor, I. Cummins, M. Brazier-Hicks, R. Edwards, Protective responses induced by herbicide safeners in wheat, Environ Exp Bot, 88 (2013) 93-99.