# 2,4-dichlorophenoxy acetic acid-mediated stress in tomato plants: a biochemical and molecular approach.


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# Resumo

Nos últimos 60 anos, os herbicídas auxínicos como o ácido 2,4-diclorofenoxiacético (2,4-D) têm estado entre os herbicidas mais utilizados na agricultura. O 2,4-D é um herbicida seletivo que mata dicotiledóneas e que atua a nível molecular como a auxina nativa ácido indol-3-acético (IAA). Não obstante, são ainda necessários muitos estudos de forma a desvendar o preciso mecanismo de ação deste herbicida. É sabido que o etileno, o ácido abscísico (ABA) e espécies reativas de oxigénio (ROS) possuem um papel fundamental na toxicidade do 2,4-D, levando a alterações nefastas nos tecidos das plantas. Até ao momento, a forma como as células reagem aos ROS e como estes regulam a expressão de genes relacionados com a defesa e/ou o stress continua por se desvendada. Neste estudo, o tomateiro (*Solanum lycopersicum* L.) foi utilizado para desvendar os efeitos dos ROS induzidos pelo 2,4-D no sistema antioxidante, dando especial atenção à expressão dos genes da classe phi da glutationa S-transferase (GST). Quando as plantas *S. lycopersicum* foram expostas ao herbicida (2,26 mM) durante 48 h, os níveis de $H_2O_2$ e $O_2^{\bullet-}$ nas folhas aumentaram, juntamente com uma redução do fecho dos estomas, da assimilação do $CO_2$ e perda de clorofilas. Contrariamente aos efeitos observados nas folhas, 2,26 mM de 2,4-D não foram suficientes para provocar sintomas claros de stress oxidativo nas raízes. Apesar das diferenças encontradas nos níveis de ROS em ambos os órgãos, a exposição do tomateiro ao 2,4-D levou a um aumento da atividade de enzimas chave do sistema antioxidante, excluindo a superóxido dismutase (SOD) que apenas aumentou nas raízes. As atividades da peroxidase do ascorbato (APX) e da catalase (CAT) aumentaram tanto nas folhas como nas raízes. Mais ainda, os tomateiros expostos a 2,26 mM de 2,4-D responderam ao herbicida aumentando os níveis de ascorbato (AsA) em ambos os órgãos enquanto que um aumento na acumulação de glutationa (GSH) foi apenas observado nas folhas. A exposição ao herbicida levou a um aumento tanto da síntese como da regeneração da GSH, assim como do seu uso para conjugar o 2,4-D, dado que as atividades das enzimas y-glutamil-cisteína-sintetase (γ-ECS), GST e glutationa redutase (GR) aumentaram. A atividade da enzima GST foi aumentada devido a um aumento da expressão dos genes *SlGSTF4* e *SlGSTF5*. No entanto, não foi possível observar o aumento da expressão génica de nenhuma das GST estudadas ao nível das raízes. Este estudo mostra claramente que as folhas e as raízes do tomateiro foram diferencialmente afetadas pela exposição ao 2,4-D na concentração 2,26 mM. Mais ainda, os resultados obtidos sugerem que, no tomateiro, a destoxificação do 2,4-D ocorre principalmente das folhas, com a participação de GST específicas da classe phi, mais concretamente das *SlGSTF4* e *SlGSTF5*.

# Abstract

In the last 60 years, auxinic herbicides like 2,4-dichlorophenoxyacetic acid (2,4-D) have been among the widest and successful herbicides used in agriculture. 2,4-D is a selective herbicide that kills dicots and mimics the natural plant phytohormone indol-3-acetic acid (IAA) at the molecular level. Nevertheless, concerted efforts are still being made to unravel the precise mechanism of action of this herbicide. It is known that ethylene, abscisic acid (ABA) and reactive oxygen species (ROS) play a central role in 2,4-D toxicity, leading to numerous unbeneficial changes in plant tissues. Yet, how ROS are perceived by the cell and how they regulate defense- and/or stress-related genes' expressions remains to be elucidated. In this study, tomato plants (*Solanum lycopersicum* L.) were used in order to unravel the effects of 2,4-D-related ROS in the plant antioxidant system, a special attention being given to the expression of the GST phi class gene family members. When *S. lycopersicum* plants were root-treated with 2.26 mM 2,4-D for 48 h, $H_2O_2$ and $O_2^{\bullet-}$ levels increased in leaves and were accompanied by a reduction in stomatal aperture, $CO_2$ assimilation and chlorophyll loss. Contrary to their effect on the leaves, in roots 2.26 mM 2,4-D did not provoke clear symptoms of oxidative stress, as lipid peroxidation, $H_2O_2$ and $O_2^{\bullet-}$ levels decreased. Despite the difference in ROS levels observed in both organs, the exposure of tomato plants to 2,4-D lead to the activation of key antioxidant enzymes in both organs, apart from superoxide dismutase (SOD) whose activity increased only in roots. Ascorbate peroxidase (APX) and catalase (CAT) activities increased in leaves and in roots. Also, tomato plants responded to 2.26 mM 2,4-D by increasing Ascorbate (AsA) levels in both organs while an increase in Glutathione (GSH) was only observed in leaves. The herbicide increased both the synthesis and the regeneration of GSH, as well as its usage to conjugate 2,4-D, as leaf γ-glutamyl-cysteinyl synthetase (γ-ECS), glutathione reductase (GR) and glutathione S-transferase (GST) activities increased. Leaf GST increased activity was due to an increased expression of *SlGSTF4* and *SlGSTF5*, and none of the *SlGSTF*s increased their expression in roots. This study clearly showed that leaves and roots of tomato plants were differentially affected by the exposure to 2.26 mM 2,4-D for 48 h. Moreover, the obtained results suggest that in tomato plants 2,4-D detoxification occurs mainly in leaves, with the participation of specific glutathione transferase phi class members *SlGSTF4* and *SlGSTF5*.

# Key-words

2,4-dichlorophenoxyacetic acid (2,4-D); *Solanum lycopersicum* L.; reactive oxygen species (ROS); oxidative stress; antioxidant system; stress defense; plant hormones; ethylene; abscisic acid; glutathione S-transferase; plant xenome; detoxification.

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# Abbreviations and Acronyms

**2,4-D** - 2,4-dichlorophenoxyacetic acid;
**$^{\bullet}$OH** - hydroxyl radical
**$^{1}O_2$** - singlet oxygen
**ABA** - abscisic acid
**ACC** - 1-aminocyclopropane-1-carboxylic acid
**ACX** - acyl-CoA oxidase
**APX** - acorbate peroxidase
**ARF** - auxin response factors
**AsA** - ascorbate
***AtGSTF*** - *Arabidopsis thaliana* glutathione S-transferase phi class
**Aux** - auxin
**Carot** - Carotenoids
**CAT** - catalase

**CER** - $CO_2$ exchange rate

**Chl** - Chlorophyll

**Chl a** - Chlorophyll a

**Chl b** - Chlorophyll b

**DHA** - dehydroascorbate

**DHAR** - dehydroascorbate reductase

**ETC** - electron transport chain

**F.W.** - fresh weight

**GR** - glutathione reductase

**GS** - glutathione synthetase

**GSH** - reduced glutathione

**GSSG** - oxidized glutathione

**GST** - glutathione S-transferase

**$H_2O_2$** - hydrogen peroxide

**HPLC–ESI/MS** - high-performance liquid chromatography coupled with electrospray ionization tandem mass spectrometry

**IAA** - Indole-3-acetic acid;

**ICC** - intercellular $CO_2$ concentration

**MDHA** - monodehydroascorbate

**MDHAR** - monodehydroascorbate reductase

**NAA** - 1-Naphthaleneacetic

**NCED** - 9-cis-epoxycarotenoid dioxygenase

**$O_2^{\bullet-}$** - superoxide anion

**P450** - cytochrome P450 monooxygenases

**PM** - plasma membrane

**PSI** - photosystem I

**PSII** - photosystem II

**ROS** - reactive oxygen species

**RT-qPCR** - real-time quantitative PCR

**SAM** - S-adenosyl-L-methionine

**SC** - stomatal conductance

***SlGSTF*** - *Solanum lycopersicum* glutathione S-transferase phi class

**SOD** - Superoxide dismutase

**TR** - transpiration rate

**XOD** - xanthine oxidoreductase

**γ-ECS** - γ-glutamyl-cysteinyl synthetase

# Introduction

## 1. Auxin: Effects and auxin herbicides

In higher plants, metabolic regulation and coordination, as well as, morphogenesis and responses to both biotic and abiotic factors are interceded by signaling molecules, called phytohormones. It is the balance between promoting and inhibiting agents in a network which ultimately governs the normal path of plant growth and development (Vanstraelen and Benkova, 2012). Natural auxins are an important class of phytohormones, consisting of indole-3-acetic acid (IAA), the principal natural auxin in higher plants, and related endogenous molecules such 4-chloroindole-3-acetic acid, phenylacetic acid, and indole-3-butyric acid, which cause the same responses as IAA (Vanneste and Friml, 2009). Auxins play a critical role in plant growth and are involved in many developmental processes, such as cell division and elongation; in developmental processes including vascular tissue and floral meristem differentiation, leaf initiation, phyllotaxy, senescence, apical dominance and root formation. Auxins are also essential components in tropic responses (Taiz and Zeiger, 2010). As a critical plant hormone, auxin modulates plant growth and development from embryogenesis through all stages of development (Sieburth and Lee, 2010). Because IAA influences virtually every aspect of plant growth and development, the development of chemicals that mimic the behavior of natural auxins acquired great importance, not only for their use in *in vitro* systems, but also because of their effects on undesired plants.

It is well known that undesired plants compete with crops for water, carbon dioxide, light, nutrients and space. The discovery of synthetic herbicides in 1945 was a major technical achievement that quickly changed weed management practices. Herbicides are agrochemicals used to control the growth of undesired weeds, and aim to significantly increase crop productivity. Most herbicides are small molecules that inhibit specific molecular target sites within critical plant physiological and/or biochemical pathways, and consequently those inhibitions often have catastrophic and lethal consequences on the affected plants. Target sites of herbicides are usually enzymes involved in primary metabolic pathways (i.e., processes that are necessary for the growth and development of an organism) or proteins carrying out essential physiological functions. For this reason, target sites involved in secondary metabolism are less likely to cause deadly effects on plants (Dayan *et al.*, 2010).

Herbicides are commonly classified as either nonselective or selective. A nonselective herbicide is used to kill or damage all growth and is generally reserved for agricultural use or for clearing large or heavily overgrown areas. In contrast, a selective herbicide controls specific weed species, while leaving the desired crop relatively unharmed, and usually works through some type of hormone disruption. Synthetic auxins opened a new era of weed control in crop production due to their systemic mobility in the plant and by exerting a selective action, primarily against dicot weeds in cereal crops (Grossmann, 2003).

Over the years, several chemical classes of auxin herbicides, with different weed spectra and types of selectivity, have been synthesized and commercially introduced. Auxinic herbicides have an aromatic ring and a carboxylic acid moiety, as does IAA, and contain four major chemical groups, including quinolinecarboxylic acids (e.g., quinmerac and quinclorac), pyridinecarboxylic acids (e.g., picloram, clopyralid, triclopyr), a benzoic acid (e.g., dicamba), and phenoxyalknoic acids (e.g., 2,4-D and MCPA) (Grossmann *et al.*, 2001; Sterling, 1997) (Figure 1). This herbicide family is said to have initiated an agricultural revolution and laid the corner stone of present-day weed science.

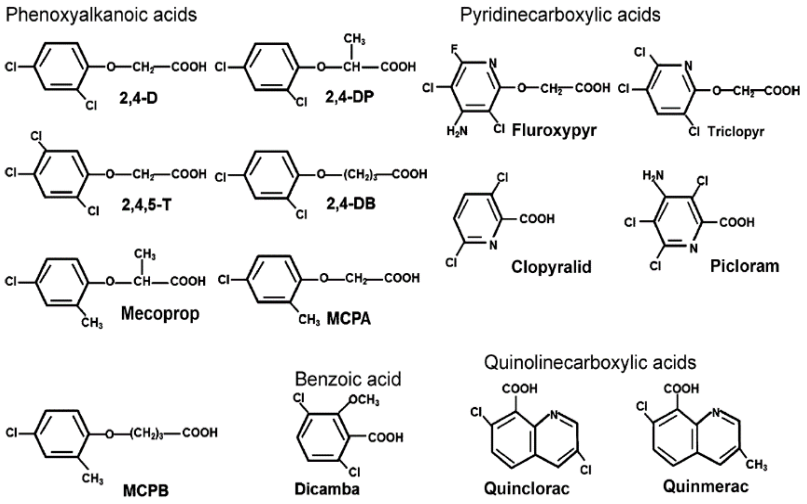



**Figure 1.** Chemical structures of the different groups of auxinic herbicides (Song, 2014).

As one of many so-called phenoxy herbicides, 2,4-dichlorophenoxyacetic acid (2,4-D) was developed during the World War II by aiming to increase crop yields for a nation at war. The development of 2,4-D in the 1940s appears to have occurred through a series of multiple, independent experiments. Although it can be debated to whom should be given credit for the discovery of 2,4-D, its commercialization in 1946 revolutionized weed control. It was the first selective herbicide to be commercially released, which allowed greatly enhanced weed control in rice, maize, wheat, and other similar cereal crops because it specifically targets dicots. 2,4-D's low cost has led to

continued usage today and it remains one of the most commonly used herbicides in the world (Song, 2014).

2,4-D is the active ingredient of the most widely used herbicides in the world, existing over 600 2,4-D-related products currently on the market. However, the acid form of this herbicide is usually not formulated as the end-use product. In its pure form, 2,4-D acid is moderately nonvolatile and it is only slightly soluble in water (44.558 mg $L^{-1}$). The acid form is low in solubility and, for this reason, it has to be modified in other herbicide formulations that consist in more soluble forms. In general, there are two types of formulations with a big acceptance in the marketplace: amine salts and esters. Amine salts are formed when 2,4-D acid reacts with an amine. The amine salt formulations of 2,4-D include, triisopropanolamine salt, isopropylamine and dimethylamine. When in contact with water, these compounds dissociate into the acid part (negative charge) and the amine part (positive charge), being readily soluble in water and forming a true solution. On the other hand, the reaction of 2,4-D acid with an alcohol forms esters (butoxyethylester , ethylhexyl ester, etc.), which are readily dissolved in an organic solvent but insoluble in water. For this reason they are formulated as emulsifiable concentrates for applications in either water or soils (Charles *et al.*, 2001; Peterson *et al.*, 2016).

# 2. Auxin overdose and the deregulation of growth

## 2.1. Mode of action of 2,4-D: metabolic and physiological processes

When applied as herbicides, synthetic auxins mimic the effects of the natural auxin IAA in plants. As shown in IAA-overproducing plants (Romano *et al.*, 1993), high doses of auxin drives plants overgrowth, including stunting and twisting of stems, brittleness and general abnormal growth (Grossmann, 2007; Pazmiño *et al.*, 2012). Although the concentration of natural auxins and its effects are tightly controlled, auxinic herbicides like 2,4-D escape to regulatory mechanisms of sensitive plants and cause an uncontrolled auxin response. Moreover, 2,4-D is long-lasting, particularly due to its higher stability in the plant, and, therefore, more effective than IAA (Song, 2014). This phenomenon has been described as an auxin overdose which leads to an imbalance in auxin homeostasis and interactions with other hormones at the tissue level.

According to several reports, when separating the time course of events, the deregulation of plant growth by 2,4-D (or IAA) at high concentrations can be divided into three phases. The first is the stimulation phase that leads to an induction of 1-aminocyclopropane-1-carboxylic acid synthase (ACCS) resulting in increased ethylene biosynthesis, followed by symptoms such as stem curling and tissue swelling. At this phase, abscisic acid (ABA) also begins to accumulate. In the second phase, which occurs within 24 h, starts abnormal growth and a series of physiological responses such as growth inhibition of root and shoots, decreased internode and leaf area elongation, and intensified green leaf pigmentation. Concomitantly, reductions in stomatal aperture, transpiration, carbon assimilation, starch formation and overproduction of reactive oxygen species (ROS) are observed. The third phase is the phase of tissue decay, which is marked by accelerated chloroplast damage and progressive chlorosis, and by the destruction of membrane and vascular system integrity, leading to cell death. As can be seen in Figure 2, these processes are exemplified for dicot weeds against the background of reported data in the literature (Grossmann, 2004; Grossmann, 2010).

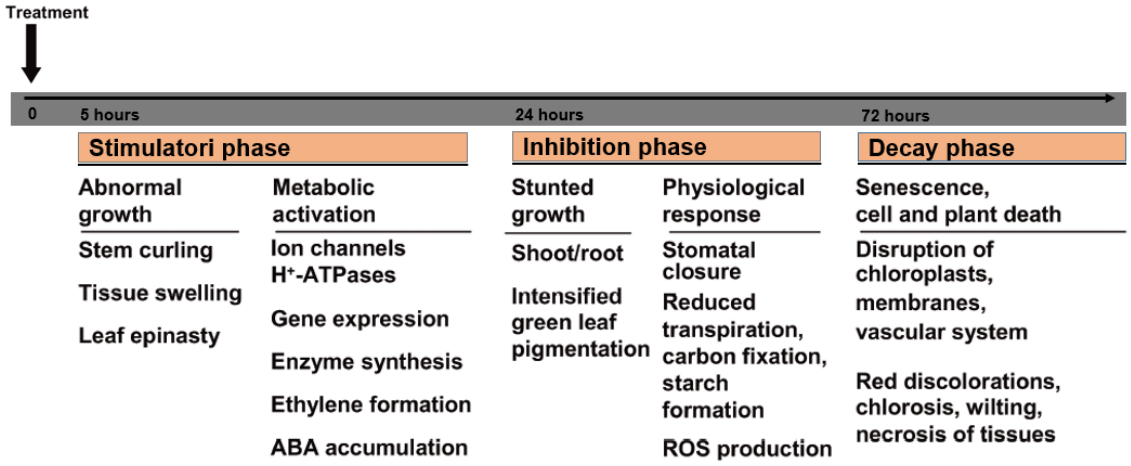



**Figure 2.** Three-phase response in auxin herbicide auxin action for dicot weed plants (modified from (Grossmann, 2010)).

### 2.1.1. 2,4-D action at subcellular level

#### 2.1.1.1. Chloroplasts

In different species it has been demonstrated that 2,4-D affects the development, structure and function of chloroplasts (Pazmiño *et al.*, 2012). While low concentrations of this chemical may increase carbon assimilation and photochemical reactions, higher doses have inhibitory effects on these reactions (Grossmann, 2000). When applied at high concentrations, 2,4-D reduces chlorophyll and carotenoid contents, as well as the

chlorophyll/benzoquinone relation (Saygideger and Okkay, 2008; Wong, 2000). Moreover, by inhibiting the Hill reaction, 2,4-D blocks the electron transport in photosystem II (Wong, 2000). Combined, all these effects result in chloroplast damage and consequent induction of senescence of the photosynthetic apparatus.

#### 2.1.1.2. Mitochondria

One of the main effects of 2,4-D is the increment of the respiratory rate, culminating in an increased $CO_2$ concentration (Kelly and Avery, 1949). Humphreys & Dugger (1957) reported that 2,4-D increases respiration by causing more glucose to be catabolized via the pentose phosphate pathway. On the other hand, they also suggested that an increase in respiration may be due to the induction of cell division by 2,4-D and other auxins (Humphreys and Dugger, 1957).

#### 2.1.1.3. Nucleic acids

The increase in nucleic acids is one of the most characteristic response of plant cells to 2,4-D treatment (Peterson *et al.*, 2016). It has been established that in sensitive tissues (e.g. seedlings), treatment with 2,4-D results in a massive accumulation of DNA and RNA associated with induction of cell division (West *et al.*, 1960). Experiments carried out by Chrispeels and Hanson (1962) showed that soybean seedlings' RNA increased by 175 % after 48 h of exposure to 2,4-D, being that this RNA was mostly ribosomal (Chrispeels and Hanson, 1962).

## 2.2. Auxinic herbicides selectivity

Auxinic herbicides have been widely used to control dicot weeds for several decades in agricultural and in nonagricultural settings. Major sites include pasture and rangeland, commercial golf courses, residential lawns, roadways, and cropland (http://npic.orst.edu/factsheets/archive/2,4-DTech.html). Yet, the underling mechanism of how auxinic herbicides selectively kill dicots and spare monocots is still not understood (McSteen, 2010). Initially, several studies tried to understand the correlation between uptake and tolerance; however, the results showed that there is little correlation between these two factors [See references in (Peterson *et al.*, 2016)]. Early research has proposed that the resistance by weeds includes either altered vascular anatomy (Monaco *et al.*, 2002), altered perception of auxin in monocots (Kelley and Riechers, 2007), limited translocation or rapid degradation of exogenous auxin (Gauvrit and

Gaillardon, 1991; Monaco *et al.*, 2002). In 1982, a study conducted by Hall and collaborators, demonstrated that after exposure to radioactively labeled 2,4-D for 24 h, only 5 % of the total radioactivity moved from treated leafs in tolerant oat compared to 55 % in sensitive soybean (Hall *et al.*, 1982). More recently, it was shown that $^{14}C$-radiolabelled 2,4-D is not effectively transported throughout the resistant plant after uptake into the leaf, leading to localized retention of this herbicide (Goggin *et al.*, 2016). These studies make a strong case for the hypothesis that variations in translocation between tolerant and sensitive species could explain differences in 2,4-D resistance.

## 2.3. Auxin Signaling and Gene Expression

Although 2,4-D has been used in agriculture for several decades, it's molecular mode of action is far from being completely characterized. Due to its similarity to the natural auxin IAA, it is thought that 2,4-D acts like IAA at the molecular level. For this reason, identification of receptors that mediate transcriptional and biochemical responses to auxin may provide basic clues about the molecular mode of action of 2,4-D.

The natural auxin IAA enters the cell through the plasma membrane (PM)-resident auxin transporters like the amino acid permease-like AUXIN RESISTANTS/LIKE AUX (AUX/LAX) proteins (Swarup *et al.*, 2008; Yang *et al.*, 2006). After IAA enters the cell through auxin-influx carriers, it rapidly controls auxin responsive gene expression by regulating the degradation of Aux/IAA repressor proteins, which are negative regulators of auxin-responsive genes (Mockaitis and Estelle, 2008). The main protein responsible for this response is the F-box protein TIR1. Aux/IAA proteins are recruited to TIR1 in an auxin-dependent manner and after binding, the Aux/IAA repressors are degraded by Skp1-cullin-F-box (SCF) E3 ubiquitin ligase ($SCF^{TIR1}$) (Dharmasiri *et al.*, 2005; Hagen and Guilfoyle, 2002; Kepinski and Leyser, 2005). At low concentrations of auxin, Aux/IAA proteins bind to auxin response factors (ARF), repressing the expression of genes controlled by auxins; at high concentrations, IAA functions as a "molecular glue" to enhance TIR1-Aux/IAA protein interaction, mediating the degradation of Aux/IAA proteins. In that way, ARFs are alleviated from AUX/IAA repressors, allowing the homo-dimerization of ARFs and subsequently the expression of auxin-responsive genes (Tan *et al.*, 2007) (Figure 3). Interestingly, crystal structures of TIR1 revealed that, in a slightly weaker manner, 2,4-D also binds at the base of TIR1 acting as a molecular glue to mediate the interaction between Aux/IAA proteins and TIR1 F-box protein (Calderon-Villalobos *et al.*, 2010).

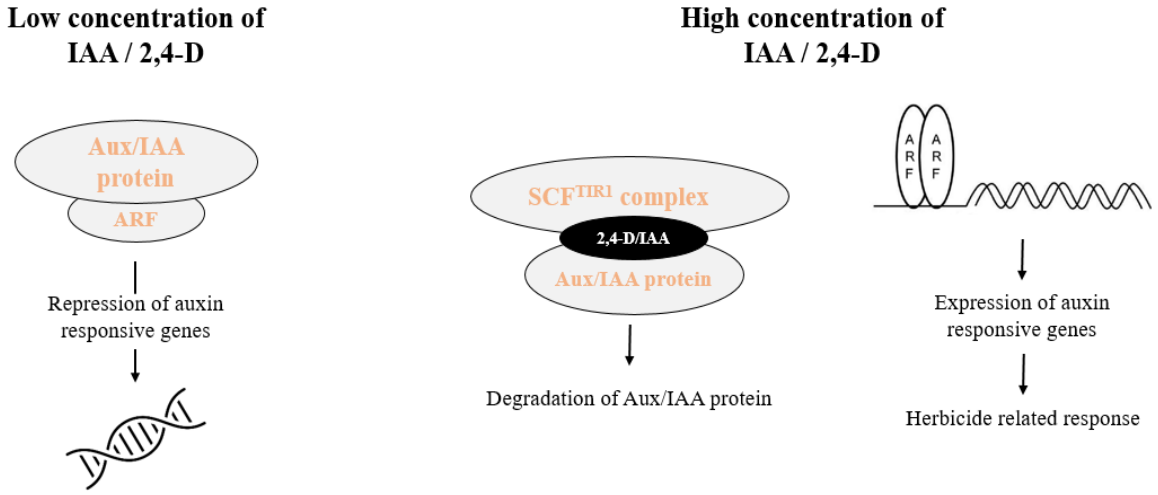



**Figure 3.** A simplified model of the molecular mechanism of IAA/2,4-D (modified from (Song, 2014)).

## 2.4. Hormone interactions and growth response

Hormone interplay is important in the regulation of plant growth and development. IAA or auxinic herbicides at high concentrations are directly related to overexpression of auxin-responsive genes. The induction of ACCS, which is a key enzyme in ethylene biosynthesis, begins a cascade of physiological responses responsible for a sequential hormone interaction, which play a decisive role in the mode of action of 2,4-D on sensitive plants (Pazmiño *et al.*, 2012). Ethylene is a gaseous phytohormone with a very simple structure that plays an important role in a wide range of physiological reactions including plant responses to stress and regulation of senescence and plant growth (Bleecker and Kende, 2000). Ethylene is biosynthesized from the amino acid methionine to S-adenosyl-L-methionine (SAM) by the enzyme SAM synthase. In a further reaction, that is considered to be the rate-limiting step, SAM is then converted to 1-aminocyclopropane-1-carboxylic acid (ACC) by the enzyme ACCS. The final step involves the action of the enzyme ACC-oxidase (ACCO), leading to the production of ethylene (Bleecker and Kende, 2000). Ethylene induces the reorientation of the microtubules of cells, promoting lateral cell expansion and consequent swelling of the stems. Additionally, an increase in the production of this phytohormone causes leaf abscission (Grossmann, 2003). A study conducted by Lin and collaborators showed that both enzymes required for the formation of ethylene can be regulated by several external factors, including 2,4-D (Lin *et al.*, 2009). Moreover, tests with several auxins (IAA, 2,4-D and 1-Naphthaleneacetic acid (NAA)) revealed that 2,4-D and NAA produced more ethylene than IAA at all concentrations tested (Arteca and Arteca, 2008).

Unlike natural auxins that are rapidly eliminated by plants, 2,4-D lasts for a longer time resulting in high levels of ethylene (Song, 2014). Following the ethylene burst in response to induced ACCS activity, huge amounts of ABA were found in roots and even more in shoot tissues. As shown in *Galium aparine,* sensitive plants exposed to auxin treatment had increased levels of ACCS after 2h of treatment followed by increased levels of ABA (70 times more than in control plants) within 4 h (Hansen and Grossmann, 2000; Scheltrup and Grossmann, 1995). Nonetheless, while IAA and different auxin herbicides induce ACCS expression and *de novo* ABA synthesis in sensitive plants, the same was not observed for crop species (Grossmann, 2003; Hansen and Grossmann, 2000). In fact, IAA and different auxin herbicides induce *de novo* ABA synthesis in several sensitive plants while in crop species ACS and ABA levels did not present differences (Grossmann, 2003; Hansen and Grossmann, 2000). The key regulatory enzyme of ABA biosynthesis is the plastid enzyme 9-cis-epoxycarotenoid dioxygenase (NCED), which is encoded by a family of *NCED* genes (Taylor *et al.*, 2005). Whereas increased levels of ethylene appear to stimulate *de novo* ABA biosynthesis, possible increasing synthesis, activity and/or its stability (Tan *et al.*, 2007), IAA and auxin herbicides are also capable to directly trigger gene activation of *NCED* genes. In accordance, transcriptome analysis of 2,4-D-treated *Arabidopsis thaliana* showed increased expression of *NCED1* (Raghavan *et al.*, 2006). ABA is known as a critical phytohormone for plant growth and development and plays an important role in integrating various stress signals and controlling downstream stress responses (Taiz and Zeiger, 2010). While occurrence of leaf abscission and swelling of the steams may be associated to auxin-stimulated ethylene (Grossmann, 2003; Klee and Lanahan, 1995), phenomena like reduction in stomatal aperture with consequent inhibition of transpiration, carbon assimilation, plant growth and progressive foliar tissue damage are correlated with increased levels of ABA. In conclusion, these physiological responses support the hypothesis that ABA, together with ethylene, function as second hormones in auxin signaling (Grossmann *et al.*, 2001).

It is known that a progressive foliar tissue damage is accompanied by an overproduction of ROS such as hydrogen peroxide (Grossmann *et al.*, 2001). This effect appears to be triggered by the failure of photosynthetic activity due to ABA-mediated stomatal closure which leads to higher leakage of electrons from the photosystems to $O_2$ in the chloroplasts (Grossmann, 2010). Moreover, the increase of ROS accumulation induced by 2,4-D is a direct consequence of the activation of specific enzymes such as xanthine oxidoreductase (XOD), involved in ureide metabolism; acyl-CoA oxidase (ACX), involved in fatty acid β-oxidation and jasmonic acid biosynthesis; and lipoxygenase (LOX) (Pazmiño *et al.*, 2011). An accumulation of harmful ROS

concentrations leads to oxidative tissue damage through membrane lipid peroxidation and probable process signaling in senescence (Dat *et al.*, 2000). In addition, 2,4-D can bind to certain phospholipids and alter interactions in membranes, which may increase the availability of lipids for peroxidation (Pogosyan *et al.*, 1984).

Overall, auxin activity alone and auxin-stimulated ethylene and ABA appear to be the main responsible for the symptoms observed for 2,4-D at supraoptimal concentrations. In particular, overproduction of ABA and hydrogen peroxide link the auxin action to the main observed effects in sensitive plants: growth inhibition, senescence and tissue decay. Consequently, using synthetic auxins, new principles have been identified in auxin perception and hormone interactions of signaling.

## 3. Reactive Oxygen Species, Sites of Production and Their Effects

Earth's atmosphere contains 21 % of molecular oxygen ($O_2$). The first trace of $O_2$ appeared approximately 2.7 billion years ago due to photosynthetic organisms (Halliwell, 2006) and its introduction into the atmosphere enabled respiratory metabolism and a more efficient generation of energy, with $O_2$ being the final electron acceptor. An unavoidable consequence of aerobic metabolism is the production of ROS (Temple *et al.*, 2005).

Molecular oxygen itself is not a harmful molecule, which makes it unlikely to participate in reactions with biomolecules unless it is activated (Apel and Hirt, 2004). However, $O_2$ can be converted in to reactive ROS forms either by electron-transfer reactions, leading to production of free radicals such as superoxide anion ($O_2^{\bullet -}$), hydroxyl radical ($^{\bullet}OH$) and non-radical molecules like hydrogen peroxide ($H_2O_2$); or by high-energy exposure leading to the production of the non-radical molecule singlet oxygen ($^1O_2$) (Figure 4) (Gill and Tuteja, 2010). In plants, it has been estimated that 1-2 % of $O_2$ consumption is diverted to produce ROS in several subcellular organelles such as chloroplasts, mitochondria and peroxisomes (Asada, 2006). Under normal physiological conditions these molecules are scavenged by the antioxidant system components. However, when the equilibrium between ROS production and scavenging change, these unwelcome companions of aerobic life damage the biomolecules of plant' cells (Gill and Tuteja, 2010). This equilibrium may be perturbed by different developmental signals or by various environmental factors such as drought, salinity, chilling, metal toxicity, and UV-B radiation as well as exposure to herbicides and pesticides (Mittler, 2002; Sharma *et al.*, 2012).

Despite their ability to damage plant cells, localized and temporal production of ROS is likely to be extremely important in the cellular and intracellular transduction of ROS signals. To date, it is known that ROS play key functions in the control and regulation of several biological processes such as growth, development, and responses to biotic and/or abiotic stresses in plants. Whether ROS will act as damaging or signaling molecules depends on the equilibrium between ROS production and scavenging (Mittler, 2002; Mittler *et al.*, 2004).

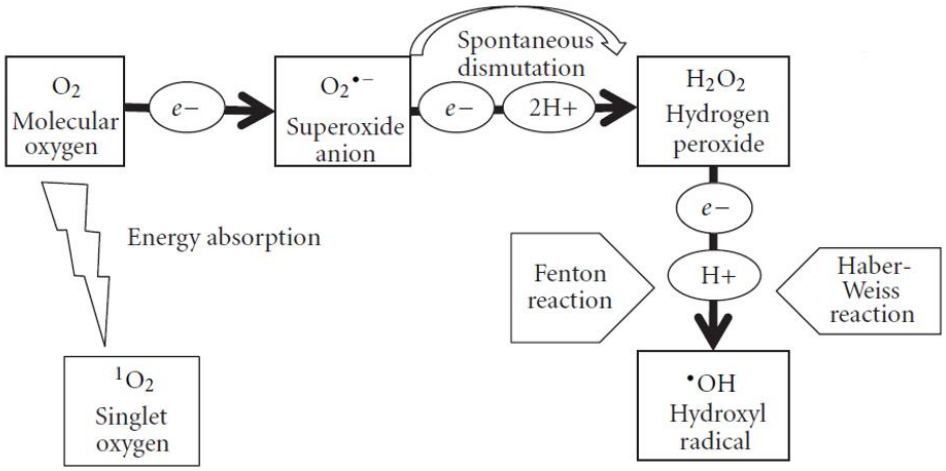



**Figure 4.** Schematic representation of generation of ROS in plants. Reduction of $O_2$ can occur by two different mechanisms. Sequential monovalent reduction of $O_2$ leads to formation of $O_2^{\bullet-}$, $H_2O_2$ and $^{\bullet}OH$. On the other hand, energy transfer to $O_2$ leads to formation of $^1O_2$. Adapted from (Sharma et al., 2012).

## 3.1. Types of Reactive Oxygen Species

The most common ROS are $O_2^{\bullet-}$, $H_2O_2$, $^{\bullet}OH$ and $^1O_2$. As mentioned before, molecular oxygen itself is not a harmless molecule. However, activation of $O_2$ may occur by two different mechanisms: absorption of sufficient energy to reverse the spin on one of the unpaired electrons and stepwise monovalent reduction (Figure 4).

The single electron reduction of $O_2$ results in the generation of $O_2^{\bullet-}$. This free radical is mainly produced in the primary electron acceptor of photosystem I (PSI) in thylakoid membranes (Gill and Tuteja, 2010; Moller, 2001). However, superoxide is also produced in the apoplast via the function of respiratory burst oxidase homologues (RBOHs), named NADPH oxidases (Wi *et al.*, 2012). $O_2^{\bullet-}$ is a moderately reactive, short-lived ROS with approximately 2 - 4 µs of half-life. It is the primary ROS to be formed, which triggers a cascade of reactions that produce other ROS (Valko *et al.*, 2005). $O_2^{\bullet-}$ has been shown to reduce cytochrome C and oxidize enzymes that contain the [4Fe-4S] clusters (Imlay, 2003; Sharma *et al.*, 2012). Furthermore, it can also give an electron to iron ($Fe^{3+}$)

resulting in a reduced form of iron ($Fe^{2+}$) which can then reduce $H_2O_2$ to $^{\bullet}OH$ (Gill and Tuteja, 2010). At low pH, the dismutation of this free radical is inevitable and its added electron is given to other $O_2^{\bullet-}$. Additionally, $O_2^{\bullet-}$ can also accept two more protons to form $H_2O_2$. The formation of $H_2O_2$ from $O_2^{\bullet-}$ can easily occur either spontaneously (1) or catalytically by the action of superoxide dismutase (SOD) (2).

$$2O_2^{\bullet-} + 2H^+ \rightarrow H_2O_2 + O_2, \quad (1)$$

$$2O_2^{\bullet-} + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2, \quad (2)$$

$H_2O_2$ is formed in the cells under normal conditions as well as in a wide variety of stressful conditions such as drought, exposure to intense light and UV radiation, chilling, as well as wounding and intrusion by pathogens (Sharma *et al.*, 2012). Organelles with intense rate of electron flow such as electron transport chains (ETC) of chloroplasts, mitochondria and others are good sites of $H_2O_2$ production (Mittler, 2002). The production of $H_2O_2$ during various stressful conditions results from pathways such as photorespiration, in which the transformation of glycolate to glyoxylate by the enzyme glycolate oxidase leads to increased levels of this ROS (Fahnenstich *et al.*, 2008). Furthermore, production of $H_2O_2$ can also occur in microbodies during *β*-oxidation of fatty acids (Mittler, 2002; Pazmiño *et al.*, 2011). $H_2O_2$ is moderately reactive, being able to oxidize the cysteine (–SH) or methionine residues ($–SCH_3$), and to inactivate enzymes by oxidizing their thiol groups (Gill and Tuteja, 2010; Gutteridge and Halliwell, 1992). Moreover, it has a relatively long half-life (1 ms) (Bhattacharjee, 2005). Unlike other ROS, $H_2O_2$ has no unpaired electrons, a condition that allows it to cross biological membranes and consequently cause oxidative stress far from the site of its formation (Gill and Tuteja, 2010). Because of its relatively long life and its ability to cross biological membranes, $H_2O_2$ is accepted as a cell-to-cell signaling molecule involved in the regulation of specific biological processes (Desikan *et al.*, 2007; Wi *et al.*, 2012).

Both $H_2O_2$ and $O_2^{\bullet-}$ are only moderately reactive. The cellular damage observed in plant cells appears to be due to their conversion into the more reactive specie $^{\bullet}OH$. The formation of $^{\bullet}OH$ is dependent on both $O_2^{\bullet-}$ and $H_2O_2$. In the presence of metals such as Fe, the reaction through $O_2^{\bullet-}$ and $H_2O_2$ generate $^{\bullet}OH$ is called Fenton's reaction (3) (Desikan *et al.*, 2007).

$$H_2O_2 + O_2^{\bullet-} \xrightarrow{Fe^{2+},\, Fe^{3+}} OH^- + O_2 + {}^{\bullet}OH, \quad (3)$$

$^{\bullet}OH$ is the most reactive ROS. $^{\bullet}OH$ interacts with all molecules, being the principal responsible for oxygen toxicity in plants. Due to its high reactivity, $^{\bullet}OH$ radicals will react with all molecules they encounter, whether they are proteins, lipid or nucleic acid, causing cellular damage such as lipid peroxidation and membrane destruction (Foyer *et*

*al.*, 1997; Sharma *et al.*, 2012). Since cells have no enzymatic pathways to eliminate $^{\bullet}OH$, its action can lead to cell dead (Pinto *et al.*, 2003).

$^1O_2$ is another form of ROS. However, contrarily to $O_2^{\bullet-}$, $H_2O_2$ and $^{\bullet}OH$ that suffer addition of extra electrons to $O_2$, $^1O_2$ is formed when one electron of $O_2$ is elevated to a higher energy orbital (Asada, 2006). When energy from photosynthesis is not dissipated a chlorophyll (Chl) triplet state is formed ($^3$Chl) (4) (Krieger-Liszkay, 2005), which can transfer its electrons to molecular $O_2$, resulting in the production of $^1O_2$ (5) (Asada, 2006).

$$\mathrm{Chl} \xrightarrow{\text{Light}} {}^3\mathrm{Chl}, \quad (4)$$

$$^3\mathrm{Chl} + {}^3O_2 \rightarrow \mathrm{Chl} + {}^1O_2, \quad (5)$$

Several environmental stresses can lead to closure of stomata resulting in limited $CO_2$, a factor that favors the formation of $^1O_2$ (Sharma *et al.*, 2012). Moreover, formation of $^1O_2$ during photosynthesis damages the photosynthetic machinery (Gill and Tuteja, 2010). This ROS has a limited live time in water, about 3 µs, and is capable to react with most of biological molecules such as proteins, unsaturated fatty acids, and DNA (Wagner *et al.*, 2004). $^1O_2$ can be efficiently quenched by *β*-carotene, plastoquinone and tocopherol, as well as to activate genes involved in the photooxidative stress response (Asada, 2006; Gill and Tuteja, 2010).

## 3.2. Effects of Reactive Oxygen Species

Lipid peroxidation is perhaps the primary cytotoxic effect of ROS. It triggers a series of changes in the cell, which makes it commonly used to assess the degree of oxidative stress (Gill and Tuteja, 2010). ROS react with the fatty acids of biological membranes leading to their gradual destruction and loss of integrity, which results in increased permeability and consequent loss of selectivity for the ion input and/or output, nutrients and toxic substances to the cell that may even lead to cell death (Sharma *et al.*, 2012).

As a consequence of excessive ROS production, site-specific amino acid modification, fragmentation of the peptide chains and increased susceptibility of proteins to proteolysis occur (Moller and Kristensen, 2004). Different amino acids have different susceptibilities to ROS, being the amino acids that contain thiol groups and sulphur the most susceptible (Sharma *et al.*, 2012). ROS can also cause oxidative damage to nuclear, mitochondrial, and chloroplast DNA. It has been reported that $^{\bullet}OH$ is the most reactive ROS towards DNA, damaging both pyrimidines and purines. DNA damage by ROS include: base deletions, pyrimidine dimers, cross-links, strand breaks and base modifications (Desikan *et al.*, 2007; Halliwell, 2006).

# 4. Elimination of Reactive Oxygen Species

All ROS can be toxic when accumulated in excess. In plants, scavenging or detoxification of excess ROS is counteracted by antioxidant systems that include a variety of scavengers and non-enzymatic low molecular metabolites. It was the evolution of highly efficient scavenging mechanisms that enabled plant cells to overcome ROS toxicity and led to the use of several of these ephemeral reactive molecules as signal transducers (Gill and Tuteja, 2010).

## 4.1. Non-enzymatic components of the antioxidant system

### 4.1.1. Ascorbate

Ascorbate (AsA) is generally the most abundant and powerful antioxidant that acts in preventing or reducing the damage caused by ROS in plants (Gill and Tuteja, 2010). It is present in almost all plants tissues. However, it tends to be more concentrated in photosynthetic cells and meristems (Smirnoff, 2007). In plants, AsA is synthesized in the mitochondria and then transported to other cell components through a proton-electrochemical gradient or through facilitated diffusion (Shao *et al.*, 2008). A study conducted by Wheeler and collaborators (1998) showed that *vtc-1* mutants (deficient in the activity of a key enzyme of AsA biosynthetic pathway) were found to be more sensitive to UV-B treatment than wild type plants (Wheeler et al., 1998). Moreover, AsA levels have been reported to alter in response to several stresses (Hernández *et al.*, 2001; Mishra *et al.*, 2011; Sharma and Dubey, 2005). This metabolite protects biological membranes by directly reacting with $O_2^{\bullet -}$ and $H_2O_2$ and regenerating α-tocopherol due to its ability to donate electrons in a large number of enzymatic and non-enzymatic reactions (Gill and Tuteja, 2010; Sharma *et al.*, 2012). After removal of $H_2O_2$ by the ascorbate-glutathione (AsA-GSH) cycle, AsA suffers two sequential oxidations, first producing monodehydroascorbate (MDHA) and subsequently dehydroascorbate (DHA) (Figure 5). For this reason, the regeneration of AsA is extremely important and counts with the activity of several enzymes involved in AsA-GSH cycle.

### 4.1.2. Glutathione

The tripeptide glutathione (GSH; γglu-cys-gly) is a crucial metabolite in plants. Besides its importance in the defense against ROS oxidative stress, GSH is also important in several physiological processes, including signal transduction, regulation of sulfate transport, conjugation of metabolites, detoxification of xenobiotics (Xiang *et al.*, 2001) and the expression of stress-responsive genes (Mullineaux and Rausch, 2005). GSH has been detected in all cellular compartments. However, its biosynthesis occurs in the cytosol and chloroplasts of plant cells by two specific enzymes: γ-glutamyl-cysteinyl synthetase (γ-ECS) and glutathione synthetase (GS). First, γ-ECS catalyzes formation of γ-glutamylcysteine from Cys and Glu, which is the rate limiting step of the pathway. In a second step, Gly is added by GS. Both steps are ATP-dependent (Mullineaux and Rausch, 2005). As synthesized, GSH can protect cells, either as proton donor in the presence of ROS (acting as a potential scavenger) or by the formation of adducts directly with reactive electrophiles (glutathiolation). Moreover, GSH also plays a key role in plant defense by regenerating AsA, by the ASH-GSH cycle (Gill and Tuteja, 2010; Xiang *et al.*, 2001). The maintenance of reduced GSH by *de novo* synthesis or *via* recycling by Gluthatione Reductase (GR) is very important for the maintenance of cells' redox homeostasis (Sharma *et al.*, 2012) (Figure 5).

### 4.1.3. Others

Proline was initially known for its action as an osmoprotectant and osmoregulator. However, recent studies showed that proline is an important protein stabilizer by acting as a scavenger of $^{\bullet}OH$ and $^{1}O_2$, also protecting biological membranes from lipid peroxidation (Ashraf and Foolad, 2007; Trovato *et al.*, 2008).

In all photosynthetic organisms, carotenoids, like β-carotene and tocopherols, have protective roles in the photosynthetic apparatus. Besides acting like accessory pigments (Siefermann-Harms, 1987) carotenoids are also important in the photo-oxidative stress, either by dissipating the excess of energy or by scavenging ROS and suppressing lipid peroxidation (Gill and Tuteja, 2010).

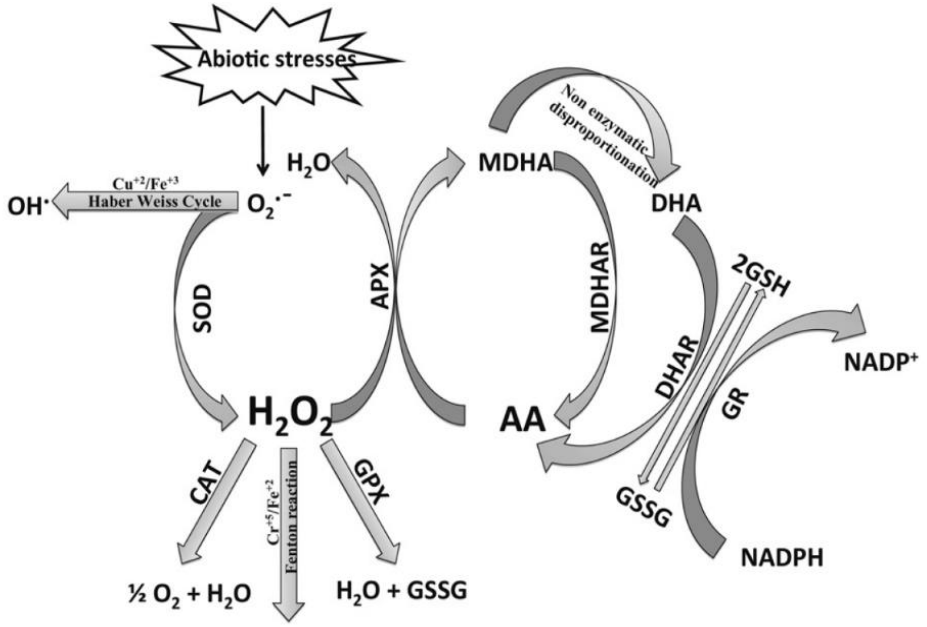



**Figure 5.** Enzymatic and non-enzymatic antioxidant defense pathways and ROS homeostasis in plant cells (Gill and Tuteja, 2010).

## 4.2. Enzymatic components of antioxidant system

### 4.2.1. Superoxide dismutase (SOD; EC 1.15.1.1)

SOD is a metalloenzyme that acts as the first line of defense against ROS by catalyzing $O_2^{\bullet-}$ dismutation in $H_2O_2$ and $O_2$ thereby decreasing the risk of $^{\bullet}OH$ production (Figure 5). $O_2^{\bullet-}$ dismutation by SOD is 10,000 fold faster than $O_2^{\bullet-}$ spontaneous dismutation (Gill and Tuteja, 2010). It is ubiquitous in most of the subcellular compartments that generate $O_2^{\bullet-}$. Depending on the metal present in the active center of this enzyme, there are three types of SODs: Cu/ZnSOD, when they contain copper and zinc; MnSOD, if the metal is manganese; or FeSOD, if the metal is iron (Sharma *et al.*, 2012). Among the three metalloproteins, Cu/ZnSOD is the most abundant in plants, being distributed throughout the cytosol, chloroplasts, peroxisomes and apoplast (Gómez *et al.*, 2004). In general, total SOD activity is increased in response to unfavorable environmental conditions, confirming their importance in plant defense against oxidative stress caused by such situations (Arbona *et al.*, 2008; Bhargava *et al.*, 2007; Kochhar and Kochhar, 2005).

### 4.2.2. Catalase (CAT; EC 1.11.1.6)

CAT enzyme as an important role in the protection against the harmful effects of $H_2O_2$, since it is one of the enzymes responsible for the control of the concentration of this ROS inside plant cells (Figure 5). In the presence of high concentrations of $H_2O_2$, CAT breaks down this molecule in water and molecular oxygen, without using reducing power (Scandalios, 2005). CAT has one of the highest turnover rates: CAT molecules can eliminate 6 million molecules of $H_2O_2$ per minute. Yet, this enzyme has a low affinity for $H_2O_2$, being more relevant when high concentrations of this ROS are present in the cell (Willekens *et al.*, 1997). In a study conducted by Polidoros and Scandalios (1999), it was demonstrated that high levels of $H_2O_2$ induced the expression of *CAT* genes, while low concentrations seem to inhibit it (Polidoros and Scandalios, 1999). CAT is found mainly in peroxisomes, which participates in the removal of the $H_2O_2$ generated during photorespiration and *β*-oxidation of fatty acids (Gill and Tuteja, 2010). There are also reports that suggest the presence of this enzyme in the cytosol, chloroplast, and mitochondria, although at a much lower level that the ones found in peroxisomes (Sharma *et al.*, 2012).

### 4.2.3. Enzymes of the Ascorbate-Glutathione cycle

The change in the ratio of AsA to DHA and GSH to oxidized glutathione (GSSG) is crucial for the cell to sense oxidative stress and respond accordingly. The AsA-GSH cycle is the recycling pathway of AsA and GSH that involves consecutive oxidation and reduction of AsA, GSH and NADPH, with the participation of ascorbate peroxidase (APX; EC 1.1.11.1), monodehydroascorbate reductase (MDHAR; EC 1.6.5.6), dehydroascorbate reductase (DHAR; EC 1.8.5.1) and glutathione reductase (GR; EC 1.6.4.2) (Figure 5).

APX, one of the key enzymes involved in $H_2O_2$ scavenging, uses two molecules of AsA as electron donors to reduce $H_2O_2$ to two molecules of MDHA and water. APX enzyme has a great affinity to $H_2O_2$, even at low concentrations, and is present in all organelles that produce this ROS (chloroplasts, mitochondria and peroxisomes) (Mittler, 2002). As observed for SOD and CAT, the literature reported enhanced activity of APX in response to abiotic stresses such as drought, salinity, chilling and herbicides, among others (Chool Boo and Jung, 1999; Hefny and Abdel-Kader, 2009; Sharma and Dubey, 2005).

The reduction of AsA is essential to cell redox homeostasis and the maintenance of its reduced levels can be assured by two different ways. First, MDHAR catalyzes the regeneration of AsA from MDHA using NADPH as the electron donor. Second, MDHA

produced by APX has a short lifetime and if not rapidly reduced suffers spontaneous dismutation in DHA. In this case, the DHA formed is then reduced to AsA by the action of DHAR, which uses GSH (Miller *et al.*, 2010). The resulting GSSG is reduced to GSH by GR (Figure 5). This enzyme also needs NADPH as a reductant for this reaction (Sharma *et al.*, 2012).

GR is mainly found in chloroplasts, whereas a small amount of this enzyme isozymes are also found in mitochondria, cytosol and peroxisomes. In higher plants, different GR isozymes are differentially stimulated depending of the environmental signals and plant stress (Yousuf *et al.*, 2012). It was found that transgenic *Nicotiana tabacum* with decreased GR activity (30-70 %) showed enhanced sensitivity to oxidative stress, suggesting that GR has a key role in the response to oxidative stress. GR plays an important role in plant homeostasis both by regeneration of GSH and also by maintaining the ASH pool (Ding *et al.*, 2008).

### 4.2.4. Glutathione *S*-Transferase

GSTs play a key role in cell detoxification because they catalyze a wide range of reactions involving the conjugation of GSH to electrophilic compounds to form more soluble peptide derivatives. Usually, GSTs are responsible for the transference of GSH to a substrate (R-X) that contains a reactive electrophilic center, to form a polar S-glutathionylated reaction product (R-SG) (Edwards and Dixon, 2005).

To the ensemble of all GSTs expressed by an organism and their collective roles in it is called GSTome (Mannervik, 2012). The GSTome comprises three distinct families: cytosolic, mitochondrial and microsomal. The last two are membrane-associated proteins that are likely to be involved in eicosanoid and glutathione metabolism, not being described to have a role in detoxification reactions (Labrou *et al.*, 2015). An extensive analysis by Liu and collaborators (2013) revealed that plant cytosolic GSTs are grouped into ten different classes: GSTU (tau), GSTF (phi), GSTL (lambda), GSTT (theta), GSTZ (zeta), DHAR, TCHQD, EF1Bg, hemerythrin and Iota. However, only phi, tau, DHAR, and lambda GSTs are specific to plants (Labrou *et al.*, 2015). For example, it was found that *A. thaliana* contains 55 *GST*-encoding genes, distributed in 8 classes, with 28 belonging to tau, 13 belonging to phi and the rest distributed to the theta, zeta, lambda, DHAR, TCHQD and microsomal GSTs (Dixon *et al.*, 2002; Wagner *et al.*, 2002). In rice genome, a total of 79 *GST*-encoding genes were found, with the phi and tau classes been the largest ones comprising 17 and 52 genes, respectively (Jain *et al.*, 2010). A similar picture arises in barley, in which tau and phi classes were the dominant of the 84 *GST*s sequences found (Rezaei *et al.*, 2013). In tomato, a total of 81 *GST* gene

sequences divided in 9 classes were found. Among these, the tau class was the most heterologous comprising 56 members, while in the phi class only 5 gene sequences were represented (Csiszar *et al.*, 2014). Despite the large number of *GST* gene sequences found in several species, especially for the major GSTs classes' tau and phi, they share a relatively conserved gene structure, with some studies revealing that they usually are grouped in the same chromosome as tandem duplications (Lan *et al.*, 2009; Rezaei *et al.*, 2013).

Plant GSTs have two domains: a N-terminal domain and a C-terminal domain (Figure 6). The N-terminal domain contains both α helices and β strands. The C-terminal domain is all α-helical and is connected to the N-terminal domain by a short linker sequence of ~ 10 residues. While the N-terminal domain is conserved, the C-terminal domain is variable, being responsible for the distinction of the several hydrophobic substrate specificities of the different plant GSTs. The active site is formed by two subsites: a glutathione-specific site (G-site) and a substrate binding site (H-site) (Cummins *et al.*, 2011).

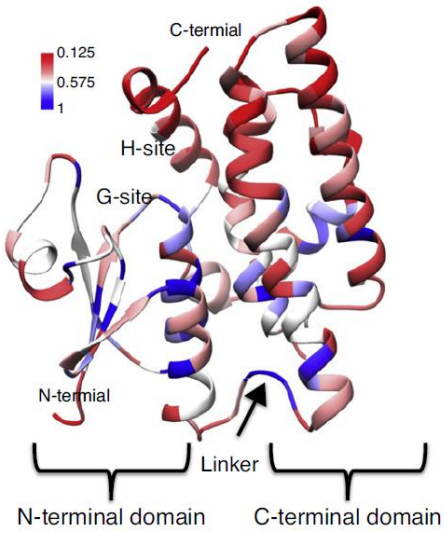



**Figure 6**. Sequence conservation of plant GSTs depicted in the crystal structure of *Glycine max* GST (PDB id 2vo4). The color bar shows the level of conservation from low (red) to high (blue) (Labrou et al., 2015).

Whereas the functional genomics of the GST superfamily is still in the beginning, it is clear that tau and phi classes are primarily responsible for herbicide detoxification in plants, showing class specificity in substrate preference (Edwards and Dixon, 2005). Moreover, GSTs from tau, phi and theta classes also exhibit GSH-dependent peroxidase activity (GPx; EC1.11.1.9). In this case, GSH is not used to form stable conjugates with

substrates but instead undergoes oxidation to form the GSSG disulfide, which is subsequently reduced by GR (Cummins *et al.*, 2011).

Recent studies reported that alterations in expression levels of specific GSTs appear to be associated with plant adaptations to non-optimal environmental conditions such as exposure to pathogens, high doses of chemicals, and UV-inducible signal transduction, as well as to hormone homeostasis through binding of auxins and cytokinin and transportation of endogenous substrates (Csiszar *et al.*, 2014; Cummins *et al.*, 2011; Dixon *et al.*, 2010; Edwards and Dixon, 2005; Wagner *et al.*, 2002). For these reasons, the elevated *GST* expression as a marker for plant response to stress is gaining attention. Additionally, the interest is being focused on the selection of *GST* that allows the genetically engineered plants to be resistant to abiotic and abiotic stresses. In particularly, the detoxification properties of tau and phi classes have been used to develop herbicide tolerance in some crops (Benekos *et al.*, 2010; Yu and Powles, 2014). For instance, overexpression of a rice tau class in *A. thaliana* resulted in both reduced plant sensitivity and oxidative stress (Sharma *et al.*, 2014). Although plants overexpressing *GST*s have not been commercially used for increased tolerance to environmental stresses, much of the current understanding of GSTs resulted from these initiatives, in special of their genetic diversity and functional roles in endogenous metabolism and xenobiotic detoxification.

## 5. 2,4-D detoxification by plants

Herbicides are chemicals that are not the natural substrate for transporters or enzymes of plant's cells. Even so, these compounds are capable to inhibit specific target sites leading to catastrophic consequences. In response to the constantly changing environment, plants evolved a sophisticate detoxification system against several xenobiotics (Shimabukuro *et al.*, 1971). Plants usually metabolize herbicides via a four-phase schema that converts the parent molecule into a more polar and insoluble product (Hatzios *et al.*, 2005). Phase I is activation, in which herbicide molecules are modified, via hydrolysis or oxidation, so they can be exposed to phase II enzymes. In phase II, detoxification, plants generally are able to conjugate a diverse range of hydrophilic molecules to the activated agrochemical, which enables the recognition by the phase III transporters. Phase III involves further transportation of the conjugates into the vacuole or deposited as bound residues into biopolymers found in the cell walls of plant cells (Coleman *et al.*, 1997; Schroder and Collins, 2002). ABC transporters are the most common group involved in this phase (Yuan *et al.*, 2007). In phase IV, after transportation

into the vacuole or extracellular spaces, conjugated molecules are further degraded (Cobb and Reade, 2010b) (Figure 7). Several plant detoxification pathways are involved in the metabolism of xenobiotics. However, to date, participation in 2,4-D detoxification has been established for: direct conjugation with glucose and amino acids, cytochrome P450 monooxygenases (P450s) activation, conjugation by GSTs and ABC transporters involvement.

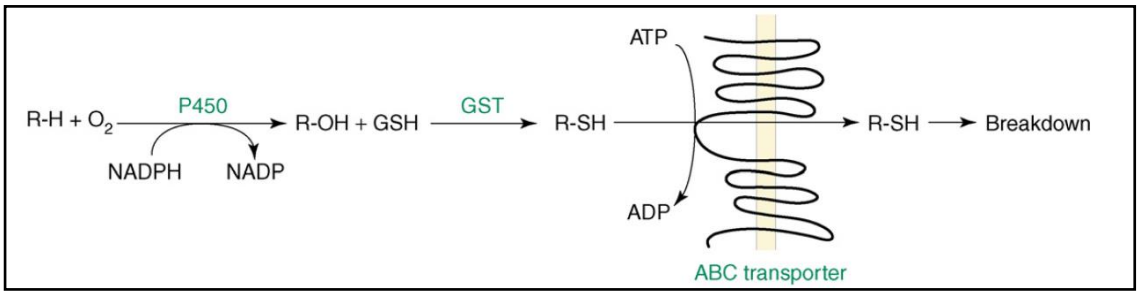



**Figure 7.** Schema of P450, GSTs and ABC transporter into a four-step detoxification process. Adapted from (Yuan et al., 2007).

## 5.1. Cytochrome P450 monooxygenases

In recent years, genome sequencing revealed that the *P450* gene family has a lot of diversity in both monocots and dicots, comprising 246 P450 genes and 26 pseudogenes in *A. thaliana* (Nelson et al., 2004). P450s are monooxygenases involved in the phase I that insert one atom of oxygen into inert hydrophobic molecules to make them more reactive and hydrosoluble (Figure 7) (Urlacher and Girhard, 2012). The function of P450s has been well stablished through the correlation of its activity with herbicide weed resistance (Kemp *et al.*, 1990). In fact, ring hydroxylation of 2,4-D by P450 is more readily observed in tolerant monocots than in dicots, suggesting differences in 2,4-D metabolism between tolerant and sensitive species (Hatzios *et al.*, 2005; Peterson *et al.*, 2016).

## 5.2. Sugar and amino acid conjugation

The conjugation of endogenous or xenobiotic toxicants with sugars is commonly described as glycosylation (Roberts, 2000). Herbicides like 2,4-D that have hydroxylated aromatic rings are mainly conjugated with glucose, forming *O*-glucosides. In fact, *O*-glycosylation of herbicides that were hydroxylated during the phase I has been shown to play an important role in the plant metabolism (Hatzios *et al.*, 2005). On the hand,

phenoxyacetic herbicides like 2,4-D also form amino acid conjugates through peptide bonding (mainly aspartate and glutamate). However, conjugation with amino acids are more prevalent in dicots plants (Cobb and Reade, 2010a; Hatzios *et al.*, 2005; Peterson *et al.*, 2016).

## 5.3. Glutathione S-transferase

GSTs are considered key enzymes in the detoxification of hydrophobic and electrophilic toxic chemicals, and consequently an important component involved in phase II detoxification.

In the 1970s, for the first time GSTs were implied in herbicide resistance, when the relationship between a GSH conjugate and atrazine resistance was discovered (Jensen *et al.*, 1977). In the next years, further evidence that GSTs are involved in herbicide resistance came from GST activity assays and their relationship with herbicide-resistant weeds. Correlations between herbicide resistance in a weed and increased GST activity were established in velvetleaf (*Abutilon theophrasti*), in which increased glutathione conjugation of atrazine was observed in the resistant biotype (Anderson and Gronwald, 1991). This increase in GST activity is often a result of increased gene expression (Basantani and Srivastava, 2007). Besides genes like *ACCS* and *NCED,* early reports also revealed that *GSTs* genes are induced by the herbicide 2,4-D (Abel and Theologis, 1996; Hagen and Guilfoyle, 1985). It is known that tau and phi classes are primarily responsible for herbicide detoxification in plant cells (Chronopoulou *et al.*, 2012; Chronopoulou *et al.*, 2014). However, it is still not known which specific *GST*s genes are involved in the detoxification of this herbicide.

## 5.4. ABC transporters

ABC transporters are driven by ATP hydrolysis that energize the transport of solutes across membranes and can act as exporters as well as importers, independent of concentration gradient (Kang *et al.*, 2011). ABC transporters were initially identified as transporters with an important role in herbicide detoxification through compartmenting metabolites in phase III (Figure 7) (Martinoia *et al.*, 1993). Since this finding, their functions extend far beyond detoxification, being frequently involved in plant nutrition, plant development, response to abiotic stresses, transport of the phytohormones auxin and abscisic acid, among many other key functions (Kang *et al.*, 2011; Kretzschmar *et*

*al.*, 2011). Microarray analysis of yeast treated with 2,4-D revealed the up-regulation of 11 genes encoding transporters of the ABC superfamily (Teixeira *et al.*, 2006). As mentioned before (Chapter 2.2), Goggin and collaborators (2016) make a strong case for their hypothesis of transport inhibition as the cause of 2,4-D resistance. To support their hypothesis application of ABCB-type transporter inhibitors to 2,4-D-sensitive plants caused a mimicking of the reduced-translocation resistance phenotype. These results suggested that 2,4-D resistance could mainly be due to an alteration in the activity of a ABCB-type PM auxin transporter rather than to differential uptake (Goggin *et al.*, 2016).

# 6. *Solanum lycopersicum* L. as a fine tool for biochemical and molecular studies

*Solanum* plants belong to the Solanaceae family, which groups many important plant species to humans with high agronomic importance such as tobacco (*N. tabacum*), tomato (*Solanum lycopersicum* L.), potato (*Solanum tuberosum* L.) and eggplant (*Solanum melongena* L.).

Native to South America, the tomato plant was spread around the world following the Spanish colonization of the Americas and is now considered as one of the most important agricultural products in the world. Beyond being a very important food source, *S. lycopersicum* also possesses several characteristics which make it a perfect model species in plant science. For example, the dwarf cultivar of tomato – Micro-Tom, has been proposed as a convenient model system for research because of its small size, rapid growth, and easy transformation (Martí *et al.*, 2006). Moreover, in 2012, the total genome sequence of tomato was published (Tomato Genome, 2012), opening new doors for the study of this species and enabling several molecular, proteomics and metabolomics approaches.

# Objectives

Tomato sensitivity to auxinic herbicides has been the subject of several published studies (Bennet, 1989; Breeze and West, 1987; Fagliari *et al.*, 2005; Hemphill and Montgomery, 1981). Plant development and morphology of several crops and native vegetation have been adversely affected by herbicides such as 2,4-D. Although the most common damage is not lethal, injured plants can be more susceptible to insect attack and diseases [see references in (Freemark and Boutin, 1995)]. Moreover, even at recommended rates of use in agriculture, 2,4-D can persist in soils for 1 month at optimal conditions and its persistence can be extended in soils at high pH and low soil moisture (Freemark and Boutin, 1995). The high sensitivity of tomato plants to 2,4-D has led to some restrictions of its usage in countries like Brazil because of 2,4-D-based products drifting (Fagliari *et al.*, 2005).

Besides 2,4-D has been used in agriculture for several decades, it's mode of action is far from being completely characterized. It is known that 2,4-D leads to increased levels of ROS, which play a central role in its toxicity. However, how 2,4-D is perceived by the cell and how it activates defense-, detoxification-, stress-related genes and proteins remains to be elucidated. Knowing that the antioxidant system plays an important role in cell homeostasis, it is hypothesized in this work that tomato plant cells may activate different pathways in order to cope with 2,4-D-induced stress: either by the accumulation of non-enzymatic metabolites, the induction of a more efficient antioxidant response or through increased 2,4-D conjugation with GSH. For that reason, one of the objectives of this study was to understand the effects of this herbicide on *S. lycopersicum*'s antioxidant system. On the basis of the obtained results, a possible enzymatic and non-enzymatic mechanism involved in the ROS-mediated effect of 2,4-D will be proposed.

For the first time, Csiszar and collaborators (2014) unraveled the GST superfamily in tomato (Csiszar *et al.*, 2014). A total of 81 *GST* gene sequences were found, with 56 sequences belonging to tau and 5 sequences to phi family, the main groups responsible for herbicide detoxification (Labrou *et al.*, 2015). Being tomato one of the most important vegetable plants in the world, the study of *S. lycopersicum* GST (SlGST) superfamily may shed some lights on its involvement in herbicide detoxification, in particular to 2,4-D detoxification. For that, the expression of all five *SlGSTF* genes will be evaluated and compared, and the obtained results will provide an essential clue for the understanding the detoxification metabolism of 2,4-D and related herbicides.

# Material and Methods

## 1. Plant material and growth conditions

*Solanum lycopersicum* L. cv. Micro-Tom (Tomato Genetics Resource Center (TGRC); germplast LA3911) seeds used in this study were surface-sterilized with 70 % ethanol for 10 min, followed by 20 % commercial bleach containing 0.02% tween-20 for 5 min, in constant agitation. Then, the seeds were washed several times with sterilized double-distilled water and placed in sterile Petri dishes (10 cm diameter) with 50 % (w/v) of Murashige and Skoog medium (Duchefa Biochemie, Haarlem, the Netherlands) solidified with agar 0,625% (w/v). Seeds were maintained for one week in a growth chamber (16 h light/8 h dark, at 25 °C), with a photosynthetically active radiation (PAR) of 60 µmol $m^{-2}$ $s^{-1}$. After this period, seedlings were cultivated in individual pots (35 mL) with a mixture of vermiculite:perlite (2:1) and maintained in a growth chamber under the same conditions described above for 21 days, watered with Hoagland Solution (HS) (Taiz and Zeiger, 2010). After this period, selected 2,4-D concentrations (see as follows) were applied once to the nutrient solution and plants were harvested after 48h of exposure.

For the selection of the suitable herbicide concentration, 28-days-old plants were divided into four different groups: watered only with HS, without the addition of 2,4-D (control group) or supplemented with 2.26, 4.52 or 9.04 mM of 2,4-D. After 48 h, leaves and roots of *S. lycopersicum* plants were separated. At least 10 plants of each group were used for root and leaves fresh weight determination. Because the treated plants did not produce enough root biomass for the expected assays, only the leaves of all groups were frozen and grounded to a fine powder in liquid nitrogen and subsequently stored at – 80 ºC to be used for stress biomarkers and enzyme activity analysis.

After the selection of the definite working concentration, 28-days-old *S. lycopersicum* plants (as above) were divided in two distinct experimental conditions: control plants, only watered with HS; and 2,4-D-treated plants, watered with HS supplemented with 2.26 mM 2,4-D. After 48 h, plants were collected and separated in roots and leaves. Part of the material was immediately used for biochemical analysis while the remainder was frozen in liquid $N_2$ and aliquoted for future molecular and biochemical analysis.

# 2. Biochemical determinations

## 2.1. Photosynthetic pigments determination

The extraction and quantification of photosynthetic pigments were achieved according to Lichtenthaler (1987). The entire procedure was performed under the lowest light intensity possible.

For each situation, pigments from 150-200 mg of frozen leaves were extracted with 80 % acetone with quartz sand. After homogenization, the extracts were centrifuged at 2,000 *g* for 10 min. All supernatants were transferred to 15 mL conic tubes and the volume was completed to 10 mL with 80 % acetone. Then, the absorbance was measured at 470, 647 and 663 nm, using 80 % acetone as blank, and the content of chlorophyll a (Chl a), chlorophyll b (Chl b) and carotenoids (Carot) were calculated, based on the following formulas (Lichtenthaler, 1987):

$$\text{Chl a}\left(\frac{\text{mg}}{\text{L}}\right) = 12.25 \times \text{Abs}\,(663\text{ nm}) - 2.79 \times \text{Abs}\,(647\text{ nm})$$

$$\text{Chl a}\left(\frac{\text{mg}}{\text{L}}\right) = 12.25 \times \text{Abs}\,(663\text{ nm}) - 2.79 \times \text{Abs}\,(647\text{ nm})$$

$$\text{Carot}\left(\frac{\text{mg}}{\text{L}}\right) = 1000 \times \text{Abs}\,(470\text{ nm}) - 1.82 \times \text{Chl a} - 85.02 \times \text{Chl b}$$

Chlorophyll and carotenoid content were expressed in mg $g^{-1}$ of fresh weight (f.w.).

## 2.2. Determination of lipid peroxidation

In the present study, thiobarbituric acid-reactive substances (TBARS) were used as an indicator of membrane lipid peroxidation level and were measured in terms of malondialdehyde (MDA) content, according to Heath and Packer (1968).

For that, frozen 200 mg samples of leaves and roots were grounded in 1.2 mL of 0.1 % (w/v) trichloroacetic acid (TCA) and centrifuged at 10,000 *g* for 5 min. To each 250 µL of supernatant 1 mL of 0.5 % (w/v) thiobarbituric acid (TBA) in 20 % (w/v) TCA were added. For each experimental condition 3 repetitions were prepared. In parallel, one blank tube was prepared, in which the supernatant was substituted by 0.1 % (w/v) TCA. Then, all tubes were incubated at 95 ºC for 30 min. In order to stop the reaction, tubes were cooled on ice for 15 min. After a new centrifugation (10,000 *g* for 15 min), the

absorbance of each sample was read at 532 and 600 nm (non-specific turbidity). The MDA content was calculated using the extinction coefficient of 155 $mM^{-1}$ $cm^{-1}$ (Heath and Packer, 1968) and expressed as nmol $g^{-1}$ of f.w..

## 2.3. Determination of $H_2O_2$

### 2.3.1. Colorimetrical measurement of $H_2O_2$

The production of $H_2O_2$ was colorimetrically measured as described by Jena and Choudhuri (1981).

$H_2O_2$ was extracted from frozen leaves and roots (300 mg) using liquid $N_2$ and macerated in mortars with 1.2 mL of phosphate buffer (50 mM, pH 6.5). The homogenate was centrifuged at 6,000 *g* for 25 min. To determine $H_2O_2$ levels, 0.1 mL of supernatant was mixed with 1 mL of 0.1 % (w/v) titanium sulfate in 20 % $H_2SO_4$, and the mixture was then centrifuged at 6,000 *g* for 15 min. In parallel, one blank was prepared, containing 1 mL of the mixture and 0.1 mL of 50 mM phosphate buffer (pH 6.5) instead of the sample. The intensity of the yellow color of the supernatant was measured at 410 nm. $H_2O_2$ levels were calculated using a molar extinction coefficient of 0.28 $\mu M^{-1}$ $cm^{-1}$ (Jana and Choudhuri, 1982) and expressed as nmol $g^{-1}$ of f.w..

### 2.3.2. Histochemical visualization of $H_2O_2$

$H_2O_2$ *in situ* localization was performed according to Romero-Portas et al. (2014). Leaves from control and 2,4-D-treated plants were immersed in a 1 % (w/v) solution of 3,3′-Diaminobenzidine (DAB) in 10 mM MES buffer (pH 6.5) at room temperature for 8 h in the absence of light. Then, leaves were bleached by immersing in boiling 96 % ethanol and placed in absorbent paper in order to visualize the brown spots corresponding to the sites where $H_2O_2$ was produced (sites of polymerized DAB). These brown areas were immediately observed and recorded (Romero-Puertas *et al.*, 2004).

## 2.4. Determination of $O_2^{\cdot -}$

The levels of $O_2^{\cdot -}$ were assayed spectrophotometrically by measuring the reduction of exogenously supplied nitroblue tetrazolium (NBT) according to Gajewska & Sklodowska, (2007). For each assay, samples of fresh material (about 150 mg) were cut in small pieces (1 mm width) and incubated in 1.5 mL of a mixture containing 0.01 M

sodium phosphate (pH 7.8), 0.05 % (w/v) NBT and 10 mM azide ($NaN_3$), for 60 min in constant agitation. The reaction solution (1 mL) was transferred to a new tube and incubated once again at 85 ºC for 15 min. At the end of this incubation period, samples were cooled in ice and then centrifuged at 12,000 *g* for 1 min. Their absorbance was read at 580 nm (Gajewska and Sklodowska, 2007) and expressed as *Abs* 580 per hour per gram fresh weight ($A_{580}$ $h^{-1}$ $g^{-1}$ f.w.).

## 2.5. Gas exchange (IRGA)

Gas exchange characteristics were measured using an open system LCA-4 ADC portable infrared gas analyzer (Analytical Development Company, Hoddeson, England). These measurements were carried out from 12:00 to 13.00 hours with the following specifications/adjustments: leaf surface area 6.25 $cm^2$, temperature of leaf chamber between 25-26 ºC, leaf chamber volume gas flow rate (v) 222 mL $min^{-1}$ and ambient pressure (P) 97.95. Measurement of $CO_2$ exchange rate (CER), transpiration rate (TR), stomatal conductance (SC), and intercellular $CO_2$ concentration (ICC) were made in the terminal leaflet on of the sixth leaf (counting from the apex) of each plant.

## 2.6. Quantification of non-enzymatic antioxidants

### 2.6.1. Free proline

For each assay, plant material (200 mg) was homogenized in 2.5 mL of 3 % (w/v) sulfosalicylic acid with quartz sand at 4 ºC. After centrifugation at 720 *g* for 10 min, 200 µL from the supernatant were added to 200 µL of glacial acetic acid and 200 µL of acidic ninhydrin. After incubation of the mixture at 96 ºC for 60 min, the reaction was stopped by incubating on ice. The extraction of free proline was accomplish by adding 1 mL of toluene to the reaction mixture followed by a vigorous agitation in order to allow a fine emulsion. After a complete separation of the organic and water phases, the organic (upper) phase was removed to new tubes and the absorbance was read at 520 nm, using toluene as blank. The free proline was determined using a standard curve constructed with the same procedure using proline and the results were expressed in mg $g^{-1}$ f.w. (Bates *et al.*, 1973).

### 2.6.2. Reduced, oxidized and total ascorbate

Plant material (300 mg) was homogenized in 1 mL 6% (w/v) TCA and then centrifuged at 13,000 *g* for 5 min at 4 ºC.

For the ascorbate assay triplicates for blanks, standards and samples for both reduced and total AsA were prepared. The ascorbate assay was started by adding 100 µL of 75 mM phosphate buffer and 200 µL of either 6% (w/v) TCA (blank), AsA standards (0.15–10 mM) or sample extract to a 2 mL tube. Then, to reduce the pool of oxidized AsA, 100 µL of 10 mM dithiothreitol (DTT) were added to the total AsA tubes and incubated at room temperature for 10 min. After 10 min, 100 µL of 0.5% (w/v) N-ethylmaleimide (NEM) was added to remove the excess of DTT. In order to account for the volume of DTT and NEM added to the total AsA assay tubes, 200 µL of water were added to the tubes of reduced AsA. To all assay tubes 500 µL of 10% (w/v) TCA, 400 µL of 43% $H_3PO_4$, 400 µL of 4% (w/v) α-α'-bipyridyl and 200 µL of 3% (w/v) $FeCl_3$ were added, followed by vigorous shaking to avoid the formation of a precipitate. After incubation of the mixture at 37 ºC for 60 min, a standard curve from the blank-corrected $A_{525nm}$ of the reduced and total AsA was obtained. All the samples were read at 525 nm and the reduced and total AsA obtained were calculated using the respective standard curve. Oxidized AsA (DHA) was calculated as the difference between the total pools and the reduced pools (Gillespie and Ainsworth, 2007).

### 2.6.3. Reduced (GSH) and oxidized (GSSG) glutathione

For each assay, leaves and roots (500 mg) were homogenized at 4 ºC in 2 mL of 3 % (w/v) metaphosphoric acid supplemented with 1% (w/v) Polyvinylpyrrolidone (PVPP) and quartz sand just before homogenization. After centrifugation at 19,000 *g* for 15 min, the supernatant was filtered through a 0.2 µm nylon membrane filter (Schleicher & Schuell, Microscience) and used for glutathione quantification.

The concentrations of GSH and GSSG in tomato plants were directly determined by high-performance liquid chromatography coupled with electrospray ionization tandem mass spectrometry (HPLC–ESI/MS). Analysis was carried out by injecting 25 µL aliquots of the filtered sample in a reverse phase $C_{18}$ column (125 × 4.6 mm, 3 µm; Phenomenex Gemini) with the following linear gradient program from solvent A (water containing 0.1% formic acid) to B (acetonitrile): from 0 to 10% B over 25 min for the separation of the compound, followed by 100% B for 15 min to wash the column, at a flow rate of 0.3 mL $min^{-1}$. An ion-trap mass spectrometer (Finnigan LCQ Deca XP Plus, San Jose, CA, USA) equipped with an electrospray ionization (ESI) source in the positive ion mode and Xcalibur software version 2.2 (Finnigan, San Jose, USA) were used for data acquisition

and processing. To optimize the MS signal, direct injection of standards solutions prepared in 0.1 % (w/v) metaphosphoric acid were carried out using a Finnigan syringe pump operated at 0.3 mL $min^{-1}$. GSH and GSSG content were identified by comparing their retention times and mass spectra with the reference time obtained for standard solutions (0.008-0.8 µM) of GSH and GSSG. Quantitation was performed using calibration curves established from standard solutions based on the peak area obtained for the two forms of glutathione (GSH and GSSG) present in the samples. The concentrations of GSH and GSSG were expressed in µM per gram of f.w..

## 2.7. Quantification of soluble proteins

For the extraction of soluble proteins, 300 mg samples of plant material were homogenized, on ice, in 2 mL of 100 mM potassium phosphate buffer (pH 7.3) containing 1 mM Phenylmethylsulfonyl fluoride (PMSF), 1 mM Ethylenediaminetetraacetic acid (EDTA), protease inhibitor cocktail (CompleteTM, Mini, EDTA-free – Protease Inhibitor Cocktail Tablets (1.4 tablets/10 mL extraction medium)), 8 % glycerol, 5 mM L-ascorbic acid and 1 % (w/v) PVPP. The extracts were centrifuged at 16,000 *g* for 25 min at 4 ºC and the supernatants were collected and maintained on ice. After centrifugation, the supernatants were used for the quantification of soluble proteins according to the Bradford method, using BSA as standard (Bradford, 1976). The reaction mixtures were incubated at room temperature for 15 min and their absorbances were read at 595 nm. The results were expressed as mg $g^{-1}$ f.w..

## 2.8. Enzymes of the Antioxidant System

### 2.8.1. SOD (EC 1.15.1.1)

SOD activity was assayed by the inhibition of the photochemical reduction of nitroblue tetrazolium (NBT), as described by Donahue *et al*. (1997).

Leaf and root material (400 mg) were grounded in a mortar on ice with a buffer containing 100 mM potassium phosphate (pH 7.3), 8 % glycerol, Complete$^{TM}$, Mini, EDTA-free – Protease Inhibitor Cocktail Tablets (1.4 tablets/10 mL extraction medium), 1 mM PMSF, 1 mM EDTA, 5 mM L-ascorbic acid and 1 % (w/v) PVPP. After centrifugation at 16,000 *g* for 25 min at 4 ºC, aliquots were collected from the supernatant for protein quantification (Bradford, 1976) and enzyme activity assays.

For the spectrophotometric assay, 3 mL reaction mixture was prepared containing 100 mM phosphate buffer (pH 7.8), 0.093 mM EDTA, 12.05 mM L-methionine, 0.0695 mM NBT, 0.0067 mM riboflavin and the appropriate volume of substrate (corresponding to 35 µg of protein). The reaction was started by adding riboflavin and placing the tubes under six 8 W lamps in a gyratory support for 10 min. A complete reaction mixture without enzyme served as control. Reaction products were measured at 560 nm. The enzyme activity was expressed as units $mg^{-1}$ protein and one SOD unit was defined as the amount of enzyme that inhibited 50 % of NBT reduction (Donahue *et al.*, 1997).

### 2.8.2. CAT (EC 1.11.1.6)

CAT activity was determined spectrophotometrically based on the method described by Aebi (1984) with some modifications.

Extraction of CAT was accomplish as described for SOD. After extraction and protein quantification the activity of CAT was assayed.

In a microplate, 160 µL of 50 mM potassium buffer (pH 7.0) and 20 µL of extract were mixed. The reaction was initiated by the addition of 20 µL of 100 mM $H_2O_2$ and the activity of CAT determined by monitoring the degradation of $H_2O_2$ at 240 nm over 1 min. The total activity of CAT was calculated using the extinction coefficient 39.4 $mM^{-1}$ $cm^{-1}$ (Aebi, 1984) and expressed as µmol $H_2O_2$ decomposed $min^{-1}$ $mg^{-1}$ protein.

### 2.8.3. APX (EC 1.11.1.11)

APX activity was determined by the method of Murshed et al. (2008). Again, the extraction of APX was accomplish as described for SOD.

In a microplate, 190 µL of 50 mM potassium buffer (pH 7.0) containing 0.6 mM ascorbic acid and 20 µL of extract were mixed. The reaction was started by the addition of 10 µL of 254 mM $H_2O_2$ and the activity determined by monitoring the oxidation of ascorbate at 300 nm over 1 min. The total activity of APX was calculated using the extinction coefficient 0.49 $mM^{-1}$ $cm^{-1}$ (Murshed et al., 2008) and expressed as µmol $min^{-1}$ $mg^{-1}$ protein.

### 2.8.4. GR (EC 1.8.1.7)

For the determination of GR activity, the method described by Murshed *et al.* (2008) was used.

Plant tissues (400 mg) were homogenized into 2 mL of 50 mM MES/KOH buffer (pH 6.0), containing 40 mM KCl, 2 mM $CaCl_2$ and 1 mM L-ascorbic acid (AsA). The homogenate was centrifuged at 14,000 *g* for 10 min at 4 ºC, and the supernatant was analyzed for protein quantification using the Bradford method (Bradford, 1976) and for enzyme activity.

In the microplate assay the 200 µL reaction mixture contained 160 µL of the reaction buffer (50 mM HEPES (pH 8) containing 0.5 mM EDTA and 0.25 mM NADPH), 30 µL of extract and 10 µL 20 mM GSSG. The reaction was initiated by the addition of protein and the activity of GR was determined by monitoring the oxidation of NADPH at 340 nm for 1 min. The activity was calculated using the extinction coefficient 6.22 $mM^{-1}$ $cm^{-1}$ and expressed as µmol $min^{-1}$ $mg^{-1}$ protein.

### 2.8.5. γECS (EC 6.3.2.2)

Samples of leaves and roots of 400 mg were homogenized on ice with 2 mL of 50 mM potassium phosphate (pH 8) containing 1 mM PMSF and 1 % (w/v) PVPP. The extracts were centrifuged at 13,000 *g* for 10 min at 4 ºC and the supernatants were collected and maintained on ice for protein quantification (Bradford, 1976) and posterior enzyme quantification.

The activity of γECS was assayed according to Rüeggseggerand Brunold (1992) with some alterations. The reaction was started by addition of the enzyme extract (50 µL) to give 450 µL assay mix containing 100 mM Hepes (pH 8.0), 50 mM $MgCl_2$, 20 mM glutamate, 1 mM cysteine, 5 mM ATP, 5 mM phosphoenolpyruvate, 5 mM DTT and 10 U $mL^{-1}$ pyruvate kinase. The reaction mixture was incubated at 37°C for 45 min, and the reaction was stopped by addition of 200 µL of 50% (w/v) TCA (Ruegsegger and Brunold, 1992). The mixture was centrifuged, and the supernatant was used for estimation of phosphate content by the phosphomolybdate method (Sengupta *et al.*, 2012). For that, 50 µL of supernatant were added to 750 µL of 1.4 % (w/v) of ascorbic acid containing 0.36 % (w/v) ammonium molybdate and 2.5 % of $H_2SO_4$. After 20 min incubation for complete color development, the absorbance was determined at 660 nm. The results were compared to a standard curve with a range of 50 to 500 ppm $PO_4^{3-}$ ($KH_2PO_4$) and expressed as mg $PO_4^{3-}$ $min^{-1}$ $mg^{-1}$ protein.

### 2.8.6. GST (EC 2.5.1.13)

Plant tissues (400 mg) were homogenized in 2 mL of 50 mM Tris-HCl (pH 7,5); 1 mM EDTA; 1 mM PMSF and 1 mM 1,4-Dithiothreitol (DTT). The homogenized samples

were centrifuge at 20,000 *g* for 25 minutes at 4 °C and the protein content was quantified using the Bradford method (Bradford, 1976).

The activity of GST was assayed according to Teixeira *et al*. (2011). In order to determine the activity of GST, 700 µL of 50 mM of phosphate buffer (pH 7.5), 100 µL of 1 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. The determination of GST activity was made according to the coefficient of extinction of CDNB, 9.6 $mM^{-1}$ $cm^{-1}$ and expressed as µmol $min^{-1}$ $mg^{-1}$ protein (Teixeira *et al.*, 2011).

## 3. Bioinformatics characterization of *Solanum lycopersicum* GSTFs

For the study of *SlGSTFs* relative expressions, Tomato eFP browser (bar.utoronto.ca.) was used.

Alignment of all SlGSTFs sequences was performed using MEGA 7 (Molecular Evolutionary Genetics Analysis) software, that permits to infer overtime the molecular evolutionary relationships between genes, genomes and species (Kumar *et al.*, 2016). The construction of the phylogenetic tree was performed using the Neighbor-Joining method (Saitou and Nei, 1987) and the percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). The evolutionary distances were computed using the p-distance method (Nei and Kumar, 2000) and are in the units of the number of amino acid differences per site.

## 4. Evaluation of *SlGSTF* family expression by real-time RT-PCR

### 4.1. Primer design

Based in tomato glutathione transferase (*SlGST*) sequences identified at the SOL Genomics Network (SGN) database (https://solgenomics.net/), Csiszár and collaborators (2014) generated a functional family tree of tomato GST based on their homology to known *A. thaliana* GSTs. Among these putative GST-encoding gene

sequences, phi (GSTF) family is represented by 5 sequences: SlGSTF1, SlGSTF2, SlGSTF3, SlGSTF4, SlGSTF5 (Csiszar *et al.*, 2014) (Appendix 1).

In order to evaluate the response of the different *SlGSTF* genes to 2,4-D exposure, primers for all GSTF sequences were used. Gene-specific primers for SlGSTF4 and SlGSTF5 were already described (Csiszar *et al.*, 2014). However, no primers were described for *SlGSTF1*, *SlGSTF2* and *SlGSTF3*. For that reason, in this work such primers were designed using *QuantPrime* (http://www.quantprime.de) to specifically anneal to each *SlGSTF*-coding-gene. All primers were synthetized at STAB VIDA (Portugal). 18S and Ubiquitin genes (Leclercq *et al.*, 2002; Lovdal and Lillo, 2009) were used as internal control for normalization of GST gene expression. All forward (F) and reverse (R) primers for *SlGSTFs* are listed in Table 1.

**Table 1.** Gene-specific primers used in real-time RT-PCR analysis.

| Gene name | Accession number | Primers sequences | Tm ºC | Amplicon |
|---|---|---|---|---|
| **SlGSTF1** | Solyc02g081340 | F: 5' TTACAGCCATTTGGACAGGTTCC 3'<br>R: 5' GTCGTTCCGGTTAGTTTCTTTCCC 3' | 57.5<br>57.8 | 125 bp |
| **SlGSTF2** | Solyc06g009020 | F: 5' GTCTGTATGGATGGAAGTAG 3'<br>R: 5' GAAGTTTCCCGAGTTTCTC 3' | 49.4<br>50.6 | 143 bp |
| **SlGSTF3** | Solyc06g009040 | F:5' ACATGGTGACTGATGATGCAATC 3'<br>R: 5' GCGTGGTTCAAATCAGCTAGGG 3' | 57.6<br>58.4 | 136 bp |
| **SlGSTF4** | Solyc09g074850 | F: 5' CGTGTGAGTGTATGGTGTGCT 3'<br>R: 5' CATCTTCTCCAACCCCTTCA 3' | 57.3<br>54.3 | 66 bp |
| **SlGSTF5** | Solyc12g094430 | F: 5' CCGATCTCTCTCACCTTCCA 3'<br>R: 5' TGCTCTGTGTGTCCCGTTC 3' | 55.7<br>57 | 56 bp |

In a series of initial experiments, real-time PCR product sizes were checked on 0.8% (w/v) agarose gels electrophoresis performed according to standard molecular biology procedures. All the obtained results were captured with a ChemiDoc™ MP System (Bio-Rad Laboratories, USA).

## 4.2. Extraction, quantification and assessment of the state of purity of total RNA

Total RNA from plant tissues was extracted using NZYol (Nzytech®, Portugal), according to the supplier's instruction. Leaves and roots (100 mg) were homogenized in 1 mL of NZYol followed by a centrifugation at 12,000 *g* for 10 min at 6 ºC. Samples were incubated for 5 min at room temperature and then 0.2 mL of chloroform was added. After a vigorous shake for 15 sec, samples were incubated for 3 min at room temperature. All tubes were centrifuged at 12,000 *g* for 15 min at 6 ºC. At the end of the centrifugation, samples were separated into a pale green, phenol-chloroform phase, an interphase, and

a colorless upper aqueous phase that contains the RNA. The aqueous phase of each assay was transferred to a new tube and the RNA was precipitated by mixing it with 0.5 mL of cold isopropyl alcohol. After an incubation of 10 min at room temperature samples were centrifuged at 12,000 *g* for 10 min at 6 ºC. The pellet was washed with 75 % ethanol and then centrifuged at 7,500 *g* for 5 min at 6 ºC. After ethanol evaporation, the pellet was dissolved in nuclease-free water immediately prior to determining RNA concentrations and quality. RNA concentration was spectrophotometrically assessed at 260 nm and its quality/purity confirmed by the ratio $A_{260}/A_{280}$. Only RNA samples with $A_{260}/A_{280}$ greater than 1.8 were used. In order to eliminate a possible contamination with DNA, NZY DNase I (Nzytech, Portugal) was used according to the instructions supplied. RNA preparations were stored at - 80 °C until for future use.

## 4.3. Reverse Transcription (RT - cDNA Synthesis)

The RT reactions for each treatment/organ were performed using Reverase™ (M-MuLV RT) (Bioron, Alemanha) according to the instructions supplied. The RT procedure used 2.5 µg of total RNA as starting template, 0.5 µg of the primer R9, that contains the poli-T region: 5' CCA GTG AGC AGA GTG ACG AGG ACT CGA GCT CAA GCT TTT TTT TTT TTT TTT 3' and $H_2O$ to a final volume of 8 µL. The mixture was incubated 10 min at 70 ºC and placed on ice. After 15 min, the remaining reaction components were added: 4 µL of 5x RT buffer reaction, 1 µL of dNTP mix (10 mM of each dNTP), 0.5 µL RNAsin (30 units), 1 µL Reverase™ (200 units) and $H_2O$ up to 20 µL. The reaction tubes were incubated at 45 ºC for 2 h and then for 10 min at 65 ºC in order to inactivate the Reverase™. At the end of the procedure, cDNAs were stored at -20 °C for future processing.

## 4.4. Expression of *SlGSTF* genes by Real-Time PCR

For both control and 2,4-D treatment, reactions were carried out in two replicates using cDNA synthesized from independently extracted RNAs and the experiments were repeated twice. The 18S ribosomal RNA and ubiquitin (UBI) genes were used as internal controls (Leclercq *et al.*, 2002; Lovdal and Lillo, 2009). Because the 18S exhibited constant expression in the experiments it was used for data normalization.

All assays were tested using SsoAdvanced™ SYBR® Green supermix (Bio-Rad Laboratories, Inc.) and 500 µM final primer concentration, and were run in a CFX384

Touch™ real-time PCR detection system (Bio-Rad) using standard cycling parameters: 2 min at 98 ºC and 40 cycles alternating between 2 s at 98 ºC and 5 s at 58 º C. After efficient amplification of cDNA, melting curve analysis (60 - 95 ºC, increment 0.2 ºC) was routinely performed to verify primer specificity. Melting curves showed a single amplified product for all genes (Appendix 2). Data analysis was performed using CFX Manager™ software (Bio-Rad) with auto calculated baseline and fixed threshold settings (300 relative fluorescence units [RFU]). Expression levels for each sample were calculated on three analytical replicates and recorded as $C_T$ (threshold cycle) at the default threshold (0.2).

## 5. Statistics

All experiments were performed in four biological replicates (n=4).

Dunnett's test is a multiple comparison procedure to compare each of a number of treatments with a single control. For this reason, the selection of the suitable herbicide concentration data were checked for normal distribution and homogeneity of variances and normalized when appropriate. Significant differences among means ($P < 0.05$) were determined using a single factor ANOVA test followed by Dunnett's test using IBM SPSS Statistica 23 software package (SPSS® Inc., Chicago, IL, USA) for windows.

The results of the remaining experiments are expressed as mean ± standard error of mean (SEM) and the significance of differences between mean values was analyzed by the Student t-test using Prism® 6 (GraphPad Software, Inc.). Differences at $P < 0.05$ were considered significant.

Considering the RT-qPCR data analysis, to determine the relative fold differences for each sample in each experiment, the Ct value for the five *SlGSTF* was normalized to the Ct value for 18S using the formula $2^{(-\Delta\Delta Ct)}$ (Livak and Schmittgen, 2001).

# Results

## 1. Effects of increasing 2,4-D concentrations on several physiological parameters of *S. lycopersicum*

In order to get a greater insight into the mechanisms of action of 2,4-D, different levels of 2,4-D (0 (control), 2.26, 4.52 and 9.04 mM) were applied to the nutrient solution of tomato plants for 48 h, and the effect of the herbicide on different plant physiological parameters was studied (Figure 8, 9, 10 and Table 2). At the end of this preliminary study, the definite herbicide concentration for further experiments was ascertained.

### 1.1. Effect of increasing 2,4-D concentrations on visible symptoms of toxicity

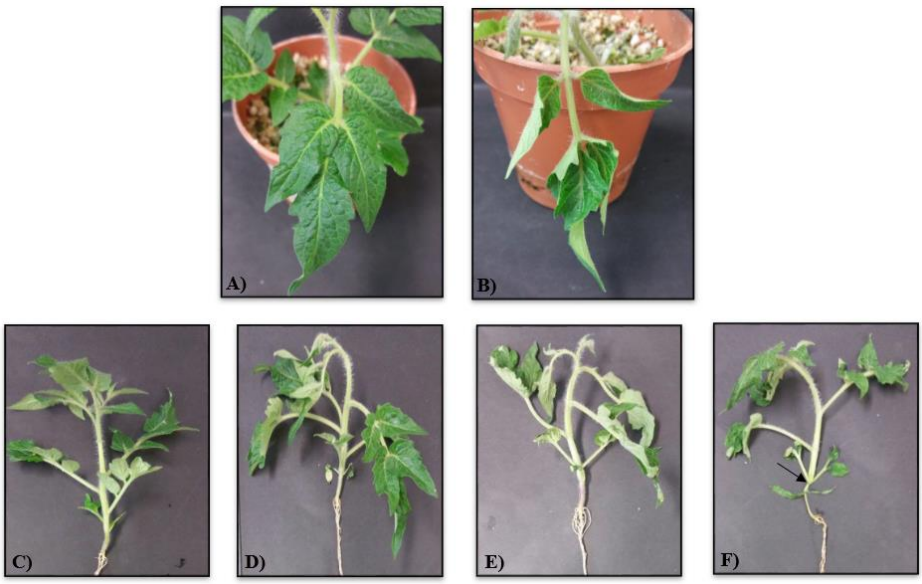



**Figure 8.** Effect of different concentrations of 2,4-D on 28-d tomato plants. Plants were treated once with 0 (control), 2.26, 4.52 and 9.04 mM 2,4-D and then were grown for 48 h in a greenhouse. (A) Leaves of control plants (0 mM). (B) Leaves of plants treated with 2.26 mM 2,4-D. (C) Aerial organs of control plants. (D) Aerial organs of plants treated with 2.26 mM 2,4-D. (E) Aerial organs of plants treated with 4.52 mM 2,4-D. (F) Aerial organs of plants treated with 9.04 mM 2,4-D. The arrow indicates a constriction from the hypocotyl above cotyledons.

Comparatively to leaves of the control group (Figure 8A and C), the supply of 2.26 mM 2,4-D was enough to produce a severe curling of tomato leaves (Figure 8B and D), a common visual effect observed in plants exposed to this herbicide. As it can be seen

in Figure 8D, E and F, all the concentrations tested produced a loss of leaf turgidity and curling of the stem, reaching a maximum effect in the plants treated with 9.04 mM 2,4-D, where is possible to observe severe symptoms of toxicity, including a constriction from the hypocotyl above the cotyledons (Figure 8F, arrow).

## 1.2. Effect of increasing 2,4-D concentrations in leaves and roots fresh weights

As observed in Figure 9A, leaf fresh weight of plants exposed to 2,4-D were found to be similar to those of control plants. However, the treatment with 2,4-D produced a rapid and significant decrease in root fresh weight, even at the lowest 2,4-D concentration (2.26 mM) (Figure 9B). Because plants treated with the lowest concentration of 2,4-D showed a major reduction in roots' fresh weight (about 60 %), and because treated plants did not produce sufficient root biomass for all expected assays ($H_2O_2$, $O_2^{\cdot-}$ and lipid peroxidation), only the shoots from control and 2,4-D-treated plants were assayed for the following stress biomarkers.

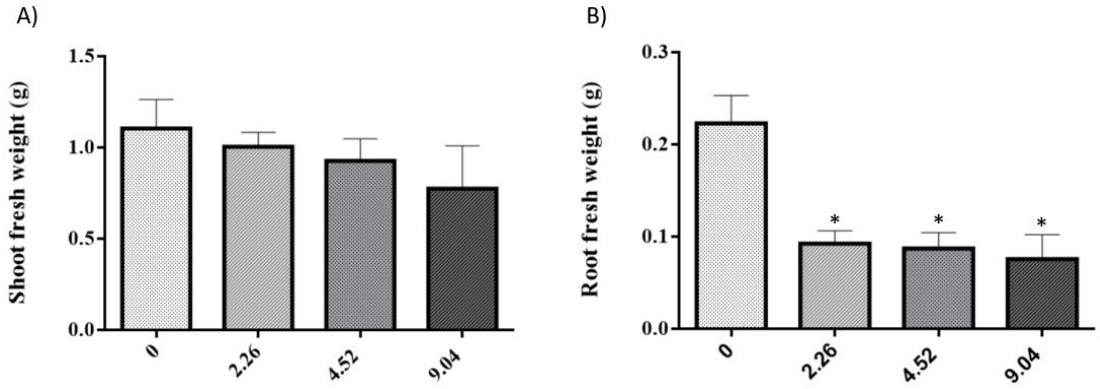



**Figure 9.** Shoot (A) and root (B) biomass of S. lycopersicum plants grown in nutrient medium supplemented with different concentrations of 2,4-D. *above bar indicates significant statistical differences from control at $P < 0.05$.

## 1.3. Effect of 2,4-D in total chlorophyll and carotenoid content

As shown in Table 2, the presence of 2.26 mM 2,4-D in the growth medium affected the levels of total chlorophyll (a+b) and carotenoid by about 18.7 and 14.6 %, respectively. In plants treated with 4.52 mM 2,4-D, total chlorophyll decreased 22.81 % while carotenoids content decreased 21.51 %. The biggest alteration in total chlorophyll,

as well as carotenoid content appeared in plants treated with 9.02 mM 2,4-D, where total chlorophyll decreased 39.59 % and carotenoids 28.81 %.

## 1.4. 2,4-D-induced oxidative stress in *S. lycopersicum* leaves

Comparatively to the control, the analysis of $H_2O_2$ levels in *S. lycopersicum* leaves' extracts after the treatment with 2.26 mM 2,4-D showed a significant 40 % increase (Table 2). More pronounced and significant increases were observed in the 4.52 and 9.04 mM 2,4-D-treated plants, where $H_2O_2$ levels rose 1.9 and 2.6-fold, respectively. By using the histochemistry DAB staining method in whole leaves, a correlation with the spectrophotometric data was obtained, denoting a strong increase of $H_2O_2$ in the central and peripheral areas of 2,4-D-treated plants (Figure 10). In tomato plants treated with 2.26 mM 2,4-D, an increase in $H_2O_2$ was observed only in the peripheral areas, while with the higher concentrations of the herbicide, $H_2O_2$ accumulation occurred also in the central areas of the leaves.

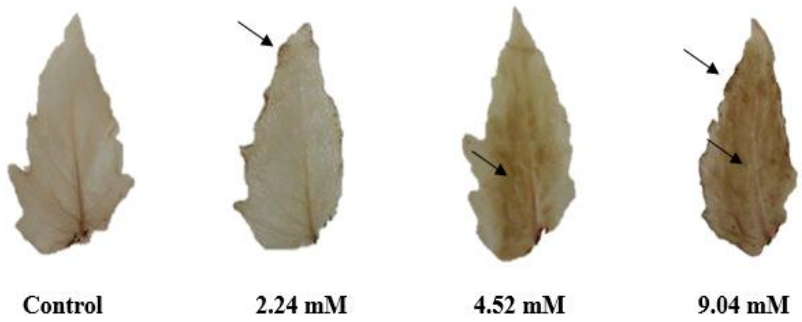



**Figure 10.** Histochemical localization of $H_2O_2$ in terminal leaflets of plants treated with different concentrations of 2,4-D and then grown for 48 h in a greenhouse. $H_2O_2$ labelling was mainly detected as brown spots (arrows) in the central vein and in peripheral zones of 2,4-D treated plants.

Considering the $O_2^{\bullet-}$ content, exposure of *S. lycopersicum* plants to 2.26 mM 2,4-D for 48 h lead to a 30 % an increase in its accumulation. Treatment of plants with 4.52 and 9.04 mM 2,4-D resulted in significantly increased values of $O_2^{\bullet-}$ (2 and 3.2-fold, respectively) when compared to the control (Table 2).

MDA content was used as an indicator of membrane lipid peroxidation. The results showed that 2.26 mM 2,4-D treatment did not induce any changes in MDA content. However, lipid peroxidation suffered a significant increase in tomato plants treated with 4.52 and 9.04 mM 2,4-D (30 and 62 %, respectively) (Table 2).

**Table 2.** Effect of different 2,4-D concentrations on several physiological parameters of tomato plant leaves.

| 2.4 D (mM) | Chl a + b (mg $g^{-1}$ prot) | Carot (mg $g^{-1}$ prot) | $H_2O_2$ (nmol $g^{-1}$ f.w.) | $O_2^{\cdot-}$ (Abs $g^{-1}$ f.w.) | MDA (nmol $g^{-1}$ f.w.) |
|---|---|---|---|---|---|
| 0 | 0.465 ± 0.02 | 0.053 ± 0.005 | 117.9 ± 5.69 | 0.215 ± 0.024 | 30.80 ± 0.44 |
| 2.26 | 0.378 ± 0.011* | 0.046 ± 0.002* | 168.8 ± 3.37* | 0.278 ± 0.010* | 31.72 ± 0.24 |
| 4.52 | 0.36 ± 0.01* | 0.044 ± 0.001* | 224.3 ± 8.96* | 0.424 ± 0.029* | 40.11 ± 0.74* |
| 9.04 | 0.280 ± 0.01* | 0.038 ± 0.001* | 301.3 ± 8.55* | 0.685 ± 0.056* | 49.95 ± 3.97* |

Plants were treated once with different concentrations of 2,4-D and then grown for 48 h in a greenhouse. After this time the depicted physiological parameters were determined. Chl a+b, Chlorophyll a +b; Carot, Carotenoids. Differences were significant at $P < 0.05$ (*).

On the basis of these physiological results (Figure 8, 9, 10 and Table 2), 2.26 mM 2,4-D was selected to be applied to the nutrient solution of tomato plants in consecutive studies.

## 2. 2.26 mM 2,4-D treatment-induced responses of the antioxidant system

With the concentration of 2.26 mM 2,4-D being selected, it was important to study the responses of the antioxidant system when tomato plants were exposed to the herbicide for 48h.

### 2.1. 2.26 mM 2,4-D-induced oxidative stress in *S. lycopersicum* roots

To gain a greater insight in the responses of the antioxidant system at the whole plant level, several physiological parameters were also obtained for roots of tomato plants. Again, levels of MDA, one of the final products of polyunsaturated fatty acids peroxidation in cells, were used as an indicator of membrane lipid peroxidation. While leaves of treated tomato plants' MDA levels did not present differences, a significant decrease was found in roots of treated *S. lycopersicum* plants (19%). For this concentration of the herbicide, leaves presented a general and significant increase for the already mentioned ROS species studied (Table 2). However, in roots of the treated plants, $H_2O_2$ and $O_2^{\cdot-}$ levels presented a significant reduction of 39.6% and 34.1%, respectively (Table 3).

**Table 3.** Effect of 2.26 mM 2,4-D on several physiological parameters of tomato plant roots.

| 2.4 D (mM) | $H_2O_2$ (nmol $g^{-1}$ f.w.) | $O_2^{\cdot-}$ (Abs $g^{-1}$ f.w.) | MDA (nmol $g^{-1}$ f.w.) |
|---|---|---|---|
| 0 | 88.66 ± 3.26 | 1.39 ± 0.07 | 5.40 ± 0.10 |
| 2.26 | 71.76 ± 4.02* | 0.84 ± 0.07* | 3.56 ± 0.16* |

Plants were treated once with 2.26 mM 2,4-D and then grown for 48 h in a greenhouse. After this time several physiological parameters were determined. Differences were significant at $P < 0.05$ (*).

## 2.2. Effect of 2.26 mM 2,4-D on several physiological parameters of tomato leaves

Treatment with 2.24 mM 2,4-D produced a higher inhibition in stomatal conductance (SC) and $CO_2$ exchange rate (CER) (19.5 % and 30 %, respectively) (Table 4). Transpiration rate (TR) was also inhibited, suffering a decrease of 26 % in the presence of 2,4-D. On the other hand, intercellular $CO_2$ concentration of treated plants increased by 1.48 fold compared to those without treatment (Table 4).

**Table 4.** Effect of 2.26 mM 2,4-D on physiological parameters of tomato leaves

| 2.4 D (mM) | TR (M $H_2O$ $m^{-2}$ $s^{-1}$) | SC (M $m^{-2}$ $s^{-1}$) | CER (µM $CO_2$ $m^{-2}$ $s^{-1}$) | ICC (mM $M^{-1}$) |
|---|---|---|---|---|
| 0 | 0.442 ± 0.056 | 0.236 ± 0.034 | 16.34 ± 0.36 | 211 ± 19.24 |
| 2.26 | 0.325±0.014* | 0.19 ±0.01* | 11.43 ± 0.59* | 313 ± 23.64* |

Plants were treated once with 2.26 mM 2,4-D and them grown for 48 h in a greenhouse. After this period several physiological parameters were determined. TR, transpiration rate; SC, stomatal conductance; CER, $CO_2$ exchange rate; ICC, intercellular $CO_2$ concentration. Differences were significant at $P < 0.05$ (*).

## 2.3. Effect of 2.26 mM 2,4-D on soluble protein content

The content of soluble proteins decreased significantly in both leaves and roots in the treated plants. The leaf material of the plants treated with 2,4-D exhibit a decrease of 15.5%. A more pronounced decrease was observed for roots of the treated plants, with the content of soluble protein decreasing by about 54.8% (Figure 11).

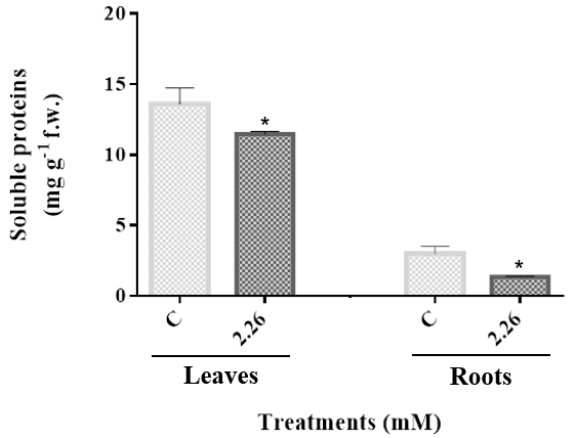



**Figure 11.** Total soluble proteins content in leaves and roots of 4-weeks-old tomato plants treated with 2.26 mM 2,4-D for 48 h. *above bar indicates significant statistical differences from control at $P < 0.05$.

## 2.4. Effects of 2.26 mM 2,4-D in the enzymatic component of antioxidant system

Hydrogen peroxide and superoxide radicals' levels are controlled by the activity of several enzymes. First, enzymes of the ROS scavenging antioxidant defense mechanism were studied in detail. As can be seen in Figure 12A, SOD activity did not change with the 2,4-D treatment in leaves. In roots, 2,4-D treatment significantly improved SOD activity, which exhibited a higher value (1.4-fold) than that of the plants without treatment (Figure 12B). When CAT activity is concerned, this enzyme had a maximum in roots of tomato plants treated with 2.26 mM of the herbicide. The activity of this enzyme significantly increased by 2.46-fold in roots, whereas in leaves this treatment lead to a significant increase of 2.04-fold (Figure 12C and D). Enzymatic activity analysis showed that APX had a significant increase in leaves and roots of tomato plants in response to 2,4-D treatment. As previously observed for CAT activity, the increase in APX activity continued to be significantly higher in roots of treated plants. This increase in roots was about 83 % while in leaves APX activity increased by 58 % (Figure 12E, F).

In addition to the importance of several ROS-scavenging enzymes, some enzymes like γ-ECS and GR have essential roles in the defense system against ROS by sustaining a reduced status of GSH. Furthermore, others enzymes like GSTs, play a major role in defense by catalyzing the conjugation of several potentially cytotoxic substrates to GSH. Tomato plants exposed to 2.26 mM 2,4-D presented improved γ-ECS activity both for leaves and roots. After treatment for 48 h, the increase of this enzyme activity was very similar for both organs, increasing significantly by 51 and 63 % in shoots and roots, respectively (Figure 13A and B).

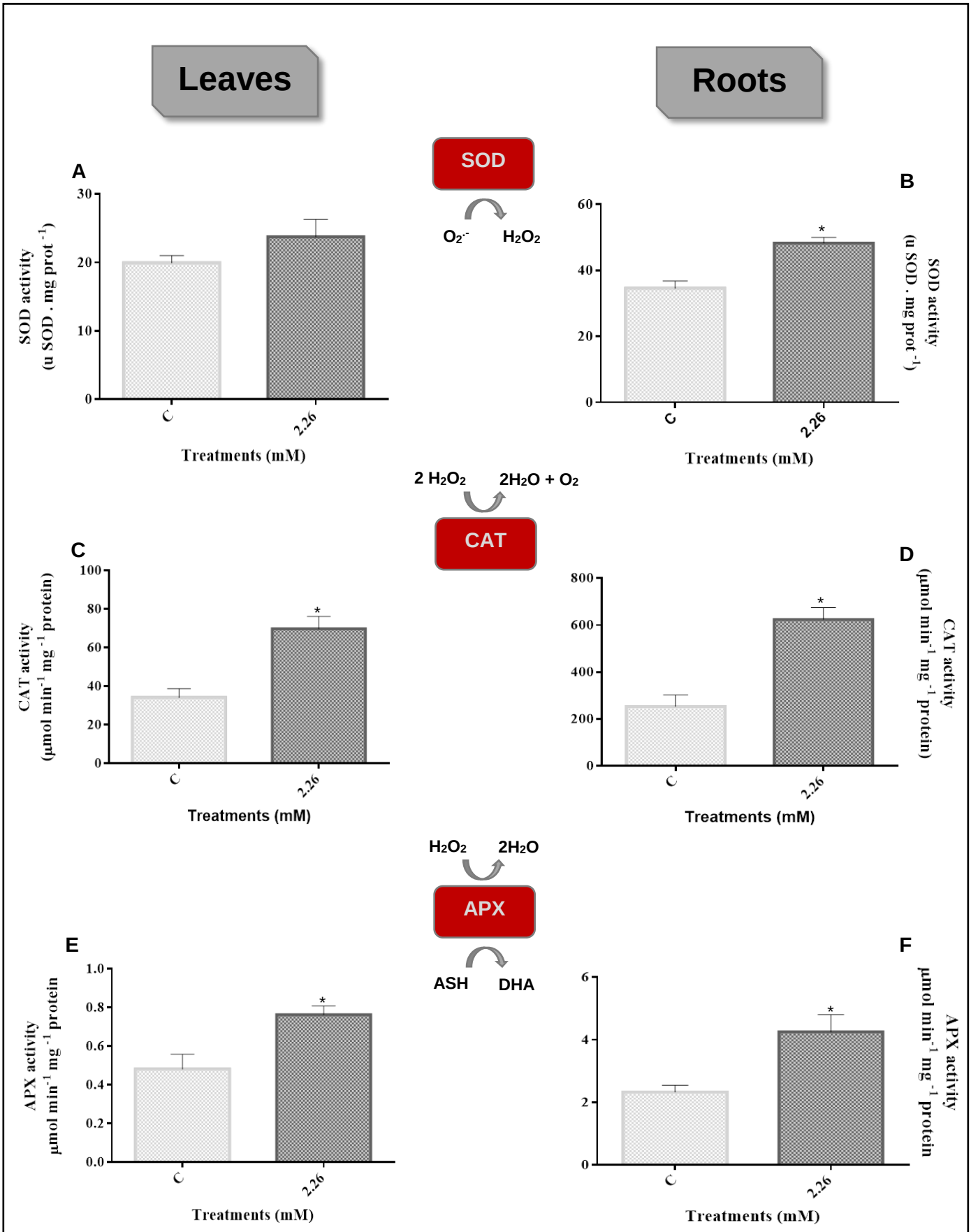



**Figure 12.** Response of ROS scavenging enzymes in 4-weeks-old tomato plants exposed to 2.26 mM 2,4-D for 48 h. Panels represent SOD activity (A,B), CAT activity (C,D) and APX activity (E,F), in leaves and roots, respectively. *above bar indicates significant statistical differences from control at $P < 0.05$.

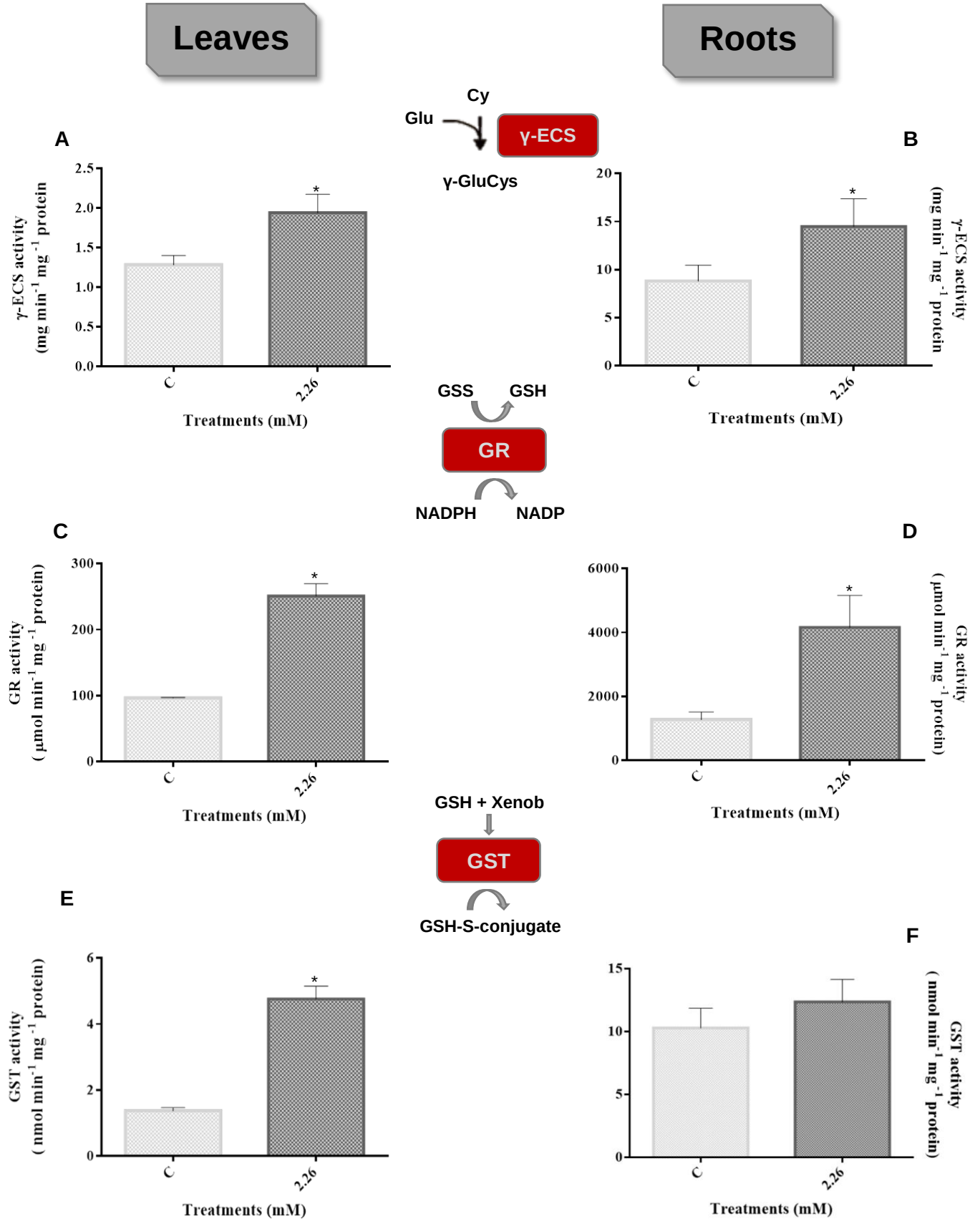



**Figure 13.** Response of GSH-related enzymes in 4-weeks-old tomato plants exposed to 2.26 mM 2,4-D for 48 h. Panels represent γ-ECS activity (A,B), GST activity (C,D) and GR activity (E,F), in leaves and roots, respectively. *above bar indicates significant statistical differences from control at $P < 0.05$.

As shown in Figure 13C and D, GR activity significantly increased with 2.26 mM 2,4-D exposure in both organs. The treatment lead to an increase of about 2.6-fold in its activity for leaves, whereas for roots GR activity augmented by 3.26-fold. When GST activity is concerned, a major and significant increase was observed in leaves of 2,4-D treated-plants (3.46-fold) (Figure 13E). For roots, GST activity did not present any changes (Figure 13F).

## 2.5. Effects of 2.26 mM 2,4-D in the non-enzymatic components of the antioxidant system

Concerning the non-enzymatic antioxidants after the exposure to 2,4-D, reduced ascorbate (AsA) content in leaves of tomato plants was significantly higher (33 %) than those observed for leaves of control plants (Table 5). The same behavior was observed in roots; however, in this organ, AsA levels only increased 21 %. Oxidized ascorbate (DHA) levels did not present the same behavior in both organs, since its levels did not change in leaves and significantly increased in roots by 19 % (Table 5). For this reason, total ascorbate (AsA+DHA) levels only changed significantly in roots of *S. lycopersicum* treated plants. Differences in the ratio AsA/DHA were significantly higher in leaves of treated plants (43 %); whereas for roots of tomato plants the ratio AsA/DHA did not present any changes compared to untreated plants (Table 5).

**Table 5.** Ascorbate content in leaves (A) and roots (B) of 4-weeks-old tomato plants exposed to 2.26 mM 2,4-D for 48 h. * above bar indicates significant statistical differences from control at $P < 0.05$. Values represent mean ± SEM (n = 4).

| 2.4 D (mM) | AsA (µmol $g^{-1}$ f.w.) | DHA (µmol $g^{-1}$ f.w.) | AsA+DHA (µmol $g^{-1}$ f.w.) | AsA/DHA |
|---|---|---|---|---|
| **Leaves** | | | | |
| 0 | 0.24 ± 0.02 | 0.30 ± 0.03 | 0.54 ± 0.03 | 0.90 ± 0.05 |
| 2.26 | 0.32 ± 0.01* | 0.26 ± 0.004 | 0.58 ± 0.01 | 1.29 ± 0.04* |
| **Roots** | | | | |
| 0 | 0.28 ± 0.01 | 0.21 ± 0.01 | 0.49 ± 0.01 | 1.39 ± 0.08 |
| 2.26 | 0.34 ± 0.003* | 0.25 ± 0.006* | 0.62 ± 0.03* | 1.29 ± 0.11 |

Plants were treated once with 2.26mM 2,4-D and then grown for 2 d in a greenhouse. After this time Ascorbate contents in leaves and roots were determined. Differences were significant at $P < 0.05$ (*).

Treatment with 2.26 mM 2,4-D resulted in a significant increase in GSH and GSSG levels in leaves of tomato plants by about 27.2-fold and 2.4-fold, respectively, whereas in roots GSH and GSSH levels did not presented any changes (Table 6). As a result of increased levels of both GSH and GSSG in leaves, total glutathione increased 2.7-fold in this organ (Table 6).

**Table 6.** Glutathione content in leaves (A) and roots (B) of 4-weeks-old tomato plants exposed to 2.26 mM 2,4-D for 48 h. * above bar indicates significant statistical differences from control at P < 0.05.

| 2.4 D (mM) | GSH (µmol $g^{-1}$ f.w.) | GSSG (µmol $g^{-1}$ f.w.) | GSH+GSSG (µmol $g^{-1}$ f.w.) | GSH/GSSG |
|---|---|---|---|---|
| **Leaves** | | | | |
| 0 | 4.63 ± 1.8 | 371.9 ± 10.98 | 376.5 ± 5.39 | 0.012 ± 0.03 |
| 2.26 | 126 ± 4.78* | 898.5 ± 87.59* | 1025 ± 53.9* | 0.14 ± 0.06* |
| **Roots** | | | | |
| 0 | 4.038 ± 0.46 | 126.2 ± 28.05 | 130.2 ± 27.60 | 0.037 ± 0.01 |
| 2.26 | 5.65 ± 1.21 | 119.4 ± 12.32 | 127.0 ± 22.26 | 0.046 ± 0.001 |

Plants were treated once with 2.26mM 2,4-D and then grown for 48 h in a greenhouse. After this time ascorbate contents in leaves and roots were determined. Differences were significant at P < 0.05 (*).

As can be seen in Figure 14, proline levels significantly increased in leaves and decreased in roots of plants exposed to 2.26 mM 2,4-D. The levels of soluble proline were increased 30 % in leaves, while in roots its levels decreased by 31 %.

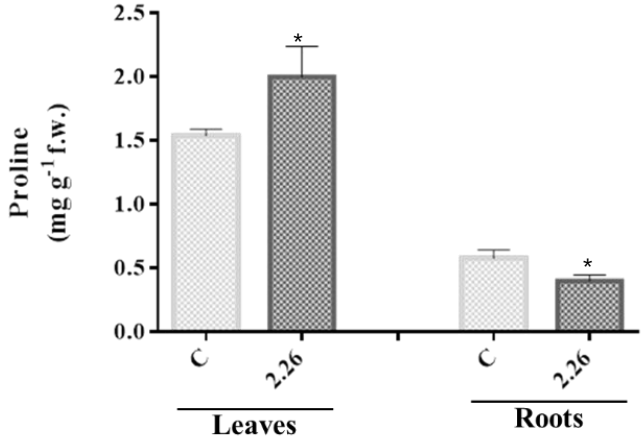



**Figure 14.** Free proline content in leaves and roots of 4-weeks-old tomato plants exposed to 2.26 mM 2,4-D for 48 h. *above bar indicates significant statistical differences from control at P < 0.05.

# 3. Bioinformatics characterization of *S. lycopersicum GSTFs*

In this study, the candidate genes *SlGSTF1*, *SlGSTF2*, *SlGSTF3*, *SlGSTF4* and *SlGSTF5* were selected based on a functional family of tomato GST described by Csiszár and collaborators (2014) and the sequences of the selected GSTFs were identified at SOL Genomics Network (SGN) database (https://solgenomics.net/).

## 3.1. Phylogenetic analysis of *S. lycopersicum* GSTFs

In order to understand their association with each other, a phylogenetic analysis was performed to study the evolutionary relationships of the different SlGSTs proteins

using Molecular Evolutionary Genetics Analysis (MEGA 7). MEGA 7 is a program widely used for multiple sequence alignments, which allows inferring statistical analysis of molecular evolution and the construction of phylogenetic trees. The alignment was performed using protein sequences of all *SlGSTFs* genes in study (Appendix 1) and the phylogeny was performed using the bootstrap test for a neighbor-joining tree. As can be seen in Figure 15, the analysis revealed that *SlGSTF2* and *SlGSTF3* from *S. lycopersicum* are closely related to each other. *SlGSTF4* and *SlGSTF5* are phylogenetically close to *SlGSTF2* and *SlGSTF3*, while *SlGSTF1* seems to be the farthest phylogenetically SlGSTF of all (Figure 15).

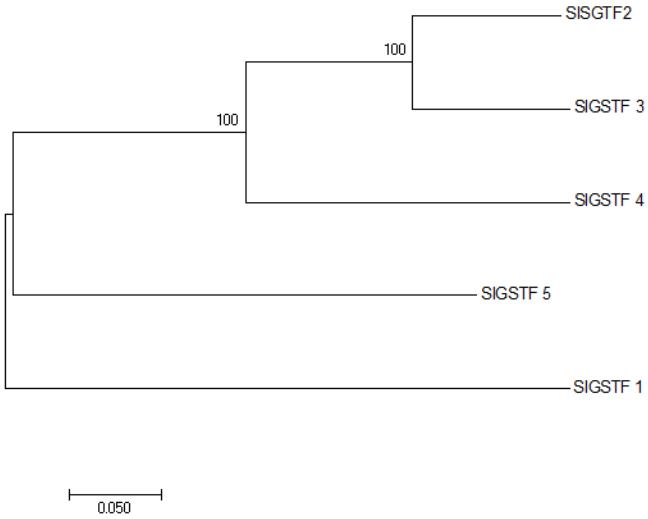
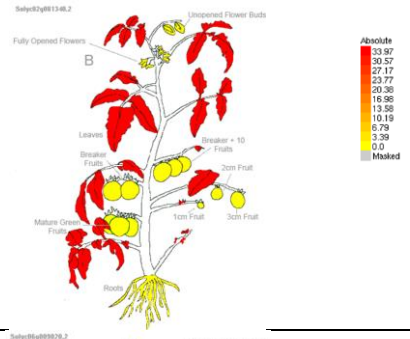
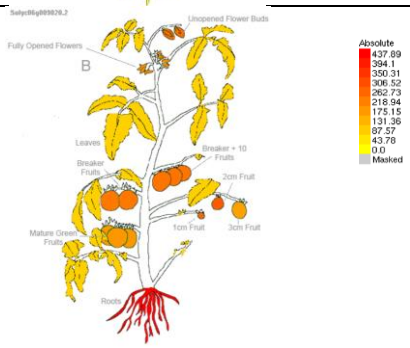
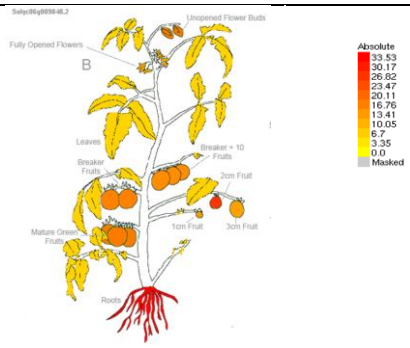
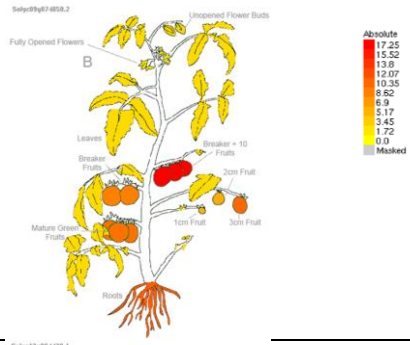
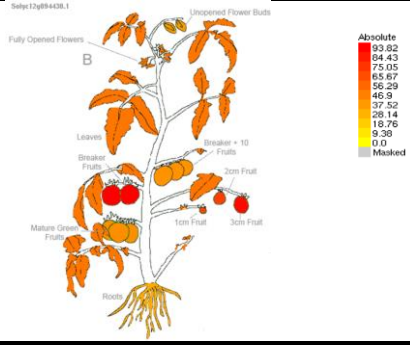



**Figure 15.** Evolutionary relationships of SlGSTFs. The evolutionary history was inferred using the Neighbor-Joining method and the percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. Evolutionary analyses were conducted in MEGA7.

## 3.2. Analysis of *SlGSTF*s relative expression using eFP browser

The tomato eFP Browser 2.0 tool is a suite of interactive tools that allows the visualization of gene expression data from tomato gene expression databases. These data visualization tool enables to explore which genes are expressed in which parts of the plant, and at what levels.

**Table 7.** Relative expression data of the 5 GST phy-encoding genes from tomato.

| Gene | Accession number | Relative expression<br>Scale |
|---|---|---|
| ***SlGSTF1*** | Solyc02g081340.2 |  |
| ***SlGSTF2*** | Solyc06g009020.2 |  |
| ***SlGSTF3*** | Solyc06g009040.2 |  |
| ***SlGSTF4*** | Solyc09g074850.2 |  |
| ***SlGSTF5*** | Solyc12g094430.1 |  |

| Gene | Accession number | Relative expression | |
|---|---|---|---|
| | | Leaves | Roots |
| *SlGSTF1* | Solyc02g081340.2 | 33.97 | 0.27 |
| *SlGSTF2* | Solyc06g009020.2 | 80.61 | 437.89 |
| *SlGSTF3* | Solyc06g009040.2 | 5.76 | 33.53 |
| *SlGSTF4* | Solyc09g074850.2 | 2.37 | 11.01 |
| *SlGSTF5* | Solyc12g094430.1 | 50.46 | 30.9 |

According to eFP Browser tool, the *GSTFs* genes in study have different levels of expression in *S. lycopersicum*. As can be seen in table 7, *SlGST1* and *5* present higher values of expression in the leaves, compared to roots. Particularly, the expression levels of *SlGSTF1* in roots are almost 0 (0.27). In *SlGSTF5*, despite its levels are higher in leaves (50.46), in roots this gene reaches values up to 30.9 (Table 7). In contrast to these genes, *SlGSTF2*, *3* and *4* present higher levels of expression in roots. *SlGST2* is the gene with more expression in roots, with values that reaches up to 437.89. Nevertheless, the basal expression of this gene in leaves is also relatively high (80.61) (Table 7).

## 3.3. Changes in transcript levels of selected *SlGSTF* genes in tomato plants under 2,4-D stress

To investigate the transcript amount of specific tomato GSTFs after the exposure to 2,4-D, information found in literature and databases was taken into consideration. Five different tomato *GST*-coding sequences belonging to the phi class were selected in the present experiments (Appendix 1). Their transcripts' amounts were investigated by RT-qPCR in 4-week-old tomato plants after 48 h of 2,4-D treatment.

For RT-qPCR reactions, total RNA extracted from leaves and roots of the two treatments was quantified and its quality was assessed in agarose gel electrophoresis (Figure 16).

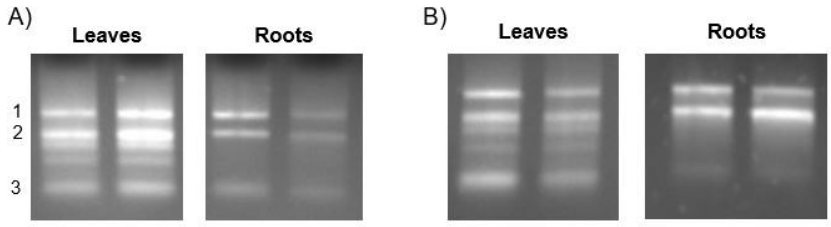



**Figure 16.** Total RNA extracted from control (A) and from plants exposed to 2.26 mM 2,4-D (B). For quality assessment of total RNA, it was separated on agarose gel at 0.8 % (w/v). 1, 28 S rRNA; 2, 18 S rRNA; 3, 5S + tRNA.

In a series of initial experiments, the product sizes of the designed primers were checked. As seen in Figure 17, all the real-time PCR products had the expected size (Table 1). *SlGSTF2* is the one that has the biggest amplicon (143 bp), while the amplicon of *SlGSTF3* is slightly smaller (136 bp). The primers for *SlGSTF1* produced a band relatively smaller than that of *SlGSTF2* (125 bp). On the other hand, primers for *SlGSTF5* amplified the smallest fragment of all (56 bp) (Figure 17). In addition to all fragments corresponding to the expected size, all primers produced a melting curve with a single peak (Appendix 2), therefore being suitable for the RT-qPCR studies.

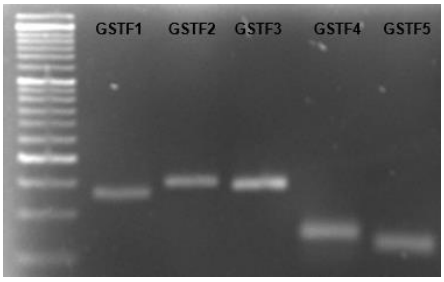



**Figure 17.** Agarose gel (0.8 % (w/v)) electrophoresis evidencing the RT-qPCR products of GSTF1, GSTF2, GSTF3, GSTF4 and GSTF5 of S. lycopersicum. The used Ladder was NZYDNA Ladder VI (Nzytech®, Portugal).

Since this is the first study describing the expression of all *GSTF* genes in tomato in response to 2,4-D exposure, first it was very important to understand and verify which *SlGSTF* genes are expressed in which parts of the plant, and at what levels, without any treatment. For that, the relative transcript level of each *SlGSTF* in leaves was compared to the relative transcript level in roots, being the samples of the leaves equaled to one for each gene. As it can be seen in Figure 18, despite a slight decrease in *SlGSTF1* expression levels, no significant alterations were observed for *SlGSTF1* and *SlGSTF5* expression levels in roots, compared to leaves. However, the same cannot be observed for *SlGSTF2, SlGSTF3* and *SlGSTF4*. In fact, it was possible to observe that *SlGSTF2*, *SlGSTF3* and *SlGSTF4* were more expressed in roots compared to leaves of tomato plants. For *SlGSTF2* it was possible to observe up to a 363-fold higher transcript amount in roots, while the expression levels of *SlGSTF3* and *SlGSTF4* were 137 and 27-fold higher in roots, compared to their expression levels in leaves.

In order to compare the changes in transcription of individual *SlGSTF*s after 48h of treatment with 2.26 mM 2,4-D, the relative transcript level measured in leaves and roots of control samples was equaled to one for each gene. The 2,4-D applied for 48h to tomato plants induced a very different pattern of expression between the five *SlGSTF* studied in leaves (Figure 19).

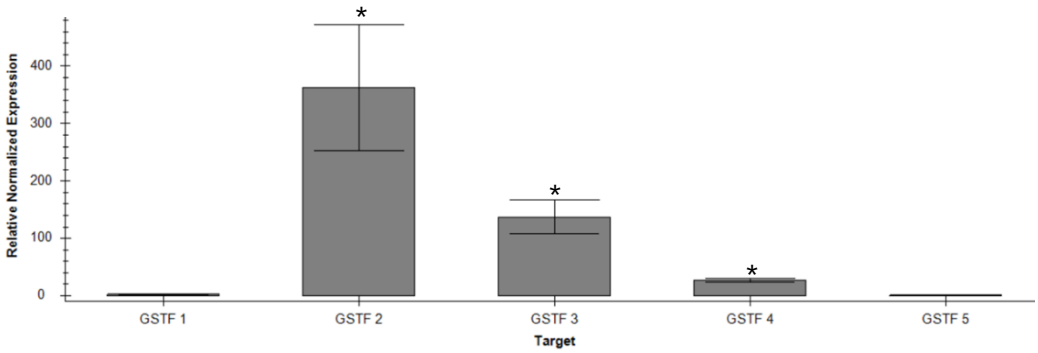



**Figure 18.** Comparison between the transcript levels of selected tomato GSTF genes in leaves and roots of 4-week-old tomato plants without any treatment. Data were normalized using the tomato 18S gene as internal control and the relative transcript level of leaves control sample was arbitrarily considered as 1 for each gene. *above bar indicates significant statistical differences from control at $P < 0.05$.

The expression of *SlGSTF1* and *SlGSTF2* was severely affected by 2,4-D since no transcript amounts were observed in leaves (Figure 19A). In the presence of 2,4-D, a 29-fold increase in transcript amount of *SlGSTF4* in the leaves was detected, while the expression level of *SlGSTF5* increased 6-fold. No changes were observed in the expression of *SlGSTF3* compared to control samples in leaves (Figure 19A.). Exposure of tomato to 2,4-D lead to a general decrease in the abundance of *SlGSTF2*, *SlGSTF3*, *SlGSTF4* and *SlGSTF*5 transcripts in roots (Figure 19B). In Figure 19B, it can be observed that it was *SlGSTF2* which exhibited a more significant down-regulation (by about 3.19-fold) compared to control of tomato roots. Nevertheless, the abundance of *SlGSTF3*, *SlGSTF4* and *SlGSTF5* decreased by 2.77, 1.31 and 1.42-fold respectively. For *SlGSTF1*, no transcript amount was observed (Figure 19B).

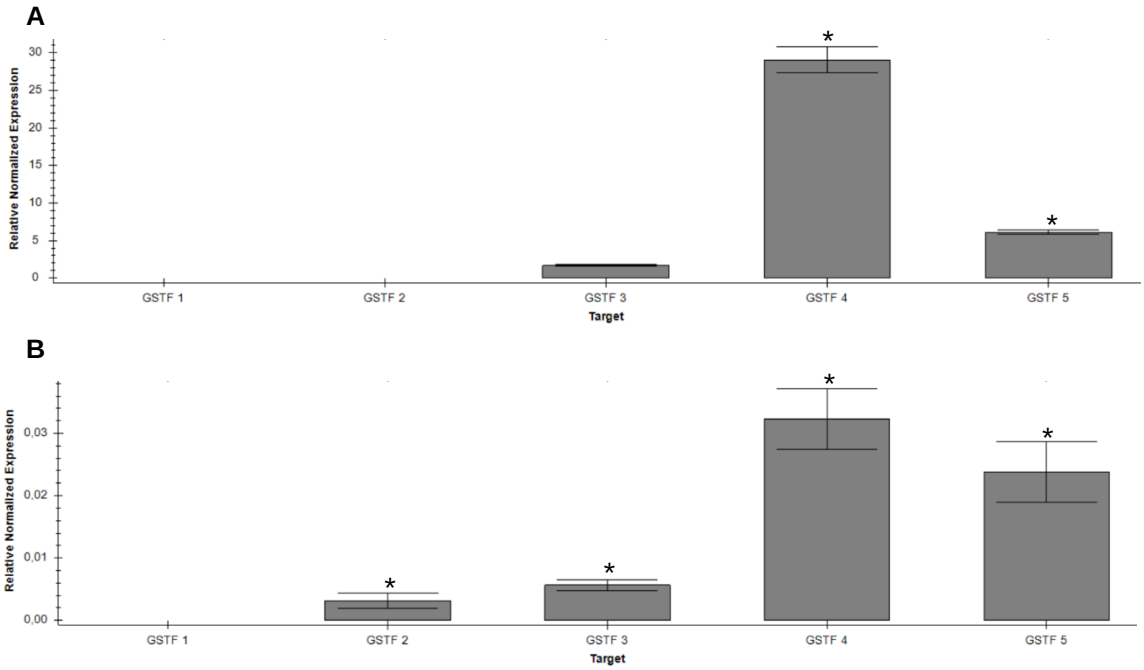



**Figure 19.** Effect of 2.26 mM 2,4-D treatment on the transcript levels of selected tomato *GSTF* genes in leaves (A) and roots (B) of 4-week-old tomato plants after 48 h of 2,4-D treatment. Data were normalized using the tomato *18S* gene as internal control and the relative transcript level in the control samples was arbitrarily considered as one for each gene. *above bar indicates significant statistical differences from control at $P < 0.05$.

# Discussion

Since its discovery in the 1940s, 2,4-D has been one of the most widely used herbicide in the world. Its excessive use in agriculture resulted in toxicological and environmental problems, eventually being put under restricted control by US Environmental Protection Agency (EPA) (Bradberry *et al.*, 2000; Chu *et al.*, 2004). Despite being used for several decades, the cellular and molecular mechanisms that govern its toxicity on sensitive plants are not well understood. It is known that 2,4-D and similar auxins interact with other hormones and can disrupt their balance. An overdose of 2,4-D results in increased expression of ACCS, which is a key enzyme in ethylene biosynthesis. In response to an ethylene burst, the levels of ABA increase causing stomatal closure, with consequent inhibition of transpiration, carbon assimilation, plant growth and progressive foliar tissue damage, and overproduction of ROS (Grossmann, 2010; Song, 2014).

The present study integrates physiological, biochemical and molecular data in an effort to characterize the response of *S. lycopersicum* to 2,4-D, further unravelling a possible enzymatic and non-enzymatic mechanisms involved in the ROS-mediated effect of 2,4-D.

## 2,4-D increased ROS production and resulted in toxic visual effects even at the lowest concentration tested.

Since this is the first study that focuses on the response of *S. lycopersicum* to a ROS burst caused by 2,4-D, it was necessary to initially test several concentrations of the herbicide in order to proceed with further studies on antioxidant system.

The results presented in this study clearly showed that even at the lowest concentration treated tomato plants were affected by 2,4-D, since after 48 h of treatment they presented the typical visual symptoms induced by auxins (Figure 8). Auxin herbicides, such as 2,4-D, cause different effects on plants depending on the concentration applied (Peterson *et al.*, 2016). One of the most distinctive visual effects of 2,4-D on sensitive plants is the development of epinasty and stem curvature, as well as reduction of shoot and root growth (Grossmann, 2010; Pazmiño *et al.*, 2012). A structural analysis by light and electron microscopy showed that the epinasty observed in leaves of pea plants may be associated with an increased volume of epidermis and mesophyll cells. Also, it was suggested that proliferation of the vascular tissue from

midribs and secondary veins can contribute to the leaf curling (Pazmiño *et al.*, 2011). Since tomato adult leaves should have lost their ability to grow, it was expected that those leaves would not show any signs of epinasty (Keller and Van Volkenburgh, 1997). Yet, differences in epinasty between young and older leaves were not clear, perhaps because 28 days were not enough for a complete cessation of the older leaves' growth.

In the present study, $O_2^{\bullet-}$ and $H_2O_2$ levels for tomato leaves increase in response to 2,4-D in a dose-dependent manner (Table 2). It has been reported that ROS overproduction is a key point in the effects of 2,4-D (Pazmiño *et al.*, 2011), which in turn can lead to harmful effects on proteins and nucleic acids and to lipid peroxidation. It is widely accepted that lipid peroxidation occurs as a consequence of oxidative stress, being one of the most damaging process to all organisms (Sharma *et al.*, 2012). Analysis of ROS-generating enzymes in *Pisum sativum* showed a pro-oxidant action of 2,4-D. Evaluation of different sources of ROS under 2,4-D toxicity pointed to XOD/XDH and ACX as the main agents responsible for ROS production (Pazmiño *et al.*, 2011). Nevertheless, enzymes like LOX and NAPDH oxidases also seem to have a role in ROS generation (Pazmiño *et al.*, 2014; Pazmiño *et al.*, 2012). Increased activity of ACX and XOD, that are mainly responsible for $H_2O_2$ and $O_2^{\bullet-}$ production, respectively (McCarthy-Suárez *et al.*, 2011; Palma *et al.*, 2002), could be in the basis of the increased levels of both ROS found in tomato plants exposed to the different concentrations of 2,4-D (Table 2). Furthermore, $O_2^{\bullet-}$ formed by XOD can lead to the formation of $H_2O_2$, resulting in higher levels of this ROS (Gill and Tuteja, 2010). Although the increased activity of these enzymes by 2,4-D is only described for pea plants, the same mechanism may be present in tomato plants, resulting in the increased levels of ROS observed in this study.

Under 2,4-D toxicity *A. thaliana* mutants deficient in XOD showed a significant reduction of epinasty (Pazmiño *et al.*, 2014). A more recent study proved that ROS incite post-translational changes in actin, causing disturbances in the cytoskeleton that appear to be responsible for the characteristic epinastic deformation in leaves (Rodríguez-Serrano *et al.*, 2014). In this study, tomato plants exposed to higher concentrations of 2,4-D, and consequently higher levels of ROS (Table 2), also presented a higher degree of epinasty, which is in accordance with the hypothesis presented by Rodríguez-Serrano and collaborators (2014) (Figure 8C, D, E and F).

In 2,4-D-treated plants chlorophyll contents were also reduced (Table 2). In this study, the changes produced by the ROS metabolism may be responsible for the enhanced chlorophyll degradation, since it was found that synthetic auxins stimulate $H_2O_2$ production, which results in tissue damage and cell death (Grossmann *et al.*, 2001). Also, high concentrations of 2,4-D in two different species of algae (*Chlorella vulgaris* and *Spirulina platensis*) had inhibitory effects not only on growth but also in pigment

levels (Saygideger and Okkay, 2008). On the other hand, Wong (2000) suggested that several herbicides, including 2,4-D, lead to reduced levels of Chl-a, not only by its degradation, but also by inhibition of its biosynthesis (Wong, 2000). So, the decrease in chlorophyll observed for the different concentrations of 2,4-D used in this study might be partly due to 2,4-D-induced degradation of chlorophyll and also by inhibition of its biosynthesis.

In this study, lipid peroxidation was determined in terms of thiobarbituric reactive substances (TBARS), such as malondialdehyde (MDA) (Gill and Tuteja, 2010). For the obtained results, overproduction of ROS were coincident with increased lipid peroxidation (Table 2). Lipid peroxidation takes place when high ROS levels are reached, affecting not only normal cellular function but also aggravating the oxidative stress through production of lipid-derived radicals (Sharma *et al.*, 2012). However, despite the increase observed in $H_2O_2$ and $O_2^{\bullet-}$ for the first concentration of 2,4-D tested (2.26 mM), lipid peroxidation did not presented differences comparatively to control, suggesting a positive response of the antioxidant system in order to protect tomato plants from the oxidative stress. For these reason, this concentration was chosen to understand the role of the enzymatic and non-enzymatic systems in the protection of plants against 2,4-D.

## The onset of 2,4-D-induced oxidative stress is marked by a decrease in stomatal closure and $CO_2$ assimilation

Photosynthesis is sensitive to disturbances in gas exchange through the stomata and, as shown by the values of stomatal conductance obtained, 2,4-D stimulated stomata to close, also resulting in decreased transpiration rates (Table 4). In fact, quinmerac (synthetic auxin) stimulates $H_2O_2$ production in shoots of cleavers as well as ABA-mediated stomatal closure. Restriction in $CO_2$ diffusion through stomatal closure appears to be responsible for a decline in $CO_2$ uptake and assimilation (Grossmann *et al.*, 2001), which is in accordance with the obtained results.

The decrease of chlorophyll content in plants treated with 2,4-D has been proposed as being responsible for a reduction in photosynthesis rates (Pazmiño *et al.*, 2011). A previous study showed that the chloroplasts of 2,4-D-treated cotyledons presented changes in the organization of the grana thylakoids, resulting in a decreased size of the photosynthetic unit. These ultrastructural changes suggest important disturbances in the metabolic functions of these organelles (Nadakavukaren and McCracken, 1977). 2,4-D-treated plants exhibited lower levels of $CO_2$ assimilation rates suggesting that the photosynthetic apparatus could have been be damaged, directly or

indirectly, by 2,4-D. Furthermore, the overproduction of ROS found in the present study might also have inhibited enzymes of the carbon reduction cycle, such as fructose-1,6-bisphosphatase and sedoheptulose-1,7-bisphosphatase, resulting in lower photosynthesis rates (Halliwell and Gutteridge, 2007).

## Roots of 2.26 mM 2,4-D- treated plants did not show clear evidences of oxidative stress

Roots of plants treated with 2.26 mM 2,4-D did not showed clear symptoms of oxidative stress, considering the $O_2^{\bullet-}$ and $H_2O_2$ levels and MDA content (Table 3). The lack of clear evidences of oxidative stress in roots and the ROS overproduction in the leaves treated with 2.26 mM 2,4-D suggest that, in tomato plants, the effect of 2,4-D in inducing oxidative metabolism might be shoot-specific. Analyses of ABA biosynthesis showed that ABA is exclusively induced in shoot tissues by increasing xanthophyll cleavage, resulting in increased amounts of the ABA precursor xanthoxin (Hansen and Grossmann, 2000). Although ABA is distributed within the plant, in shoots ABA mediates a range of physiological responses that are accompanied with an overproduction of ROS. ABA, together with ethylene, as already mentioned above, promotes foliar senescence with chloroplast damage (Grossmann, 2010). Considering the obtained results and all the above mentioned reasons, it is clear that the differences observed between both organs are related to the fact that upon 2,4-D exposure leaves become more prone to oxidative stress than roots. Nevertheless, it cannot be ruled out that the observed decrease in ROS and MDA levels might also be due to a positive response of the antioxidant system at the root level.

Another important distinctive effect of exposure to 2,4-D is growth inhibition (Hansen and Grossmann, 2000). In this study, tomato roots fresh weight was greatly affected by 2,4-D (Figure 9), but by mechanisms not related to PM modifications or oxidative stress, as similar results were observed in *Phaseolus vulgaris* cultured cells when exposed quinclorac, an auxinic herbicide (Largo-Gosens *et al.*, 2016). An early work with susceptible oat also showed that application of different auxin herbicides severely inhibited root growth (Jacobson *et al.*, 1985). In plants, there are conclusive evidences showing that auxin treatment correlates positively with the stimulation of ACCS activity which consequently increases the levels of endogenous ACC and ethylene (Grossmann, 2010; Peterson *et al.*, 2016). In fact, between other effects, shortening of root is an unmistakable hallmark of ethylene accumulation (Wang *et al.*, 2002). Moreover, the obtained results in this study show that tomato plants exposed to

2.26 mM 2,4-D had altered stomatal function that limits transpiration and consequently water balance in tomato plants. So, the marked decrease observed in root fresh weight may be due to the ethylene effect and/or related to the water content of tomato plants exposed to 2.26 mM 2,4-D.

## Total protein content was reduced in both leaves and roots of tomato plants exposed to 2.26 mM 2,4-D

Protein degradation in plants is a very complex process that involves many proteolytic pathways that can occur in several cell compartments. It is evident from this study that the total protein content was reduced in response to 2.26 mM 2,4-D exposure, both in leaves and roots (Figure 11), behavior that may be a consequence of the oxidative alteration of proteins by ROS, as demonstrated recently (Rodríguez-Serrano *et al.*, 2014). In plants, cleavage of oxidatively modified proteins is usually linked to oxidative stress situations induced by biotic and abiotic stresses and senescence (Gill and Tuteja, 2010). It is known that proteins that suffer oxidation are usually more prone to proteolysis (Juszczuk *et al.*, 2008). A study conducted by Teixeira and collaborators (2005) revealed that following aggression by 2,4-D, increased concentrations of proteins involved in protein degradation were found.

## The antioxidant system of *S. lycopersicum* improved performance with 2.26 mM 2,4-D and GSH played a major role in defense

A balance between generation and degradation of ROS under stressful conditions is required in order to maintain a normal cell metabolism. Plants developed a powerful and complex antioxidant network of both non-enzymatic and enzymatic constituents (Foyer and Noctor, 2005), with SOD, CAT, and APX representing the major ROS-scavenging enzymes controlling the basal levels of $O_2^{\bullet-}$ and $H_2O_2$ (Sharma *et al.*, 2012). The responses obtained for these enzymes showed a higher level of antioxidant response in roots of 2.26 mM 2,4-D-treated plants (Figure 12B, D and F; Figure 13B, D and F), indicating that the ROS enzymatic scavenging mechanism in tomato plants was more effective in roots than in leaves. Even so, in both leaves and roots, 2,4-D treatment triggered a higher activity of these ROS-scavenging enzymes (Figure 12). The only exception was observed for SOD in tomato leaves where there were no changes (Figure 12A). Considering that $O_2^{\bullet-}$ radicals are the substrate of SOD, with production of $H_2O_2$,

it can be assumed that SOD enzymes did not contribute for the increased levels of $H_2O_2$ found in leaves of 2.26 mM 2,4-D treated-tomato plants. Moreover, the no increase in SOD activity is in accordance with the observed accumulation of $O_2^{\bullet-}$ in leaves of 2.26 mM 2,4-D-treated plants (Table 2).

APX and CAT are the major hydrogen peroxide-detoxifying enzymes. In this study, CAT activity increased in both organs of tomato plants exposed to 2.26 mM 2,4-D (Figure 12C and D), which suggests that its activity is essential for the cellular homeostasis maintenance in tomato plants. Some authors have reported both an increase (Peixoto *et al.*, 2008) and decrease (Pasternak *et al.*, 2007) in CAT activity under the influence of toxic compounds. It is known that CAT is sensitive to $H_2O_2$ radicals and thus a high content of this ROS may result in the reduction of CAT activity, as already reported (Pasternak *et al.*, 2007). Besides its essential role in the control of intracellular ROS, APX is a central component of AsA-GSH cycle (Sharma *et al.*, 2012). The AsA-GSH cycle, one of the most important metabolic pathway for $H_2O_2$ scavenging in plants cells (Foyer and Noctor, 2011), was clearly influenced by the 2,4-D treatment. The activity of two enzymes of this cycle, APX and GR, were higher in 2,4-D-treated plants (Figure 12E and F; Figure 13C and D), which indicates that $H_2O_2$ is not being only removed by CAT, but also by APX. Similar to these observations, in alfalfa protoplasts the application of growing concentrations of 2,4-D produced an increase of APX activity (Pasternak *et al.*, 2007), {Peixoto, 2008 #316} (Peixoto *et al.*, 2008). The habituation of bean calluses to high concentrations of the auxinic herbicide quinclorac was directly associated with increased levels of peroxidases, GR, and SOD. The data correlated with a reduction in the lipid peroxidation levels in habituated cell lines (Largo-Gosens *et al.*, 2016). Since there were no changes in MDA levels in tomato plants exposed to 2.26 mM 2,4-D, these results suggest that high activities of enzymes like SOD, CAT, APX and GR are important factors for an adaptive antioxidant response to auxinic herbicides. Taking into account that auxinic compounds undergo polar transport within the plant (Jones, 1998; Muday and DeLong, 2001) and that 2,4-D triggered the same response in both leaves and roots for CAT, APX and GR enzymes, this work allows to propose that the responsive mechanism of tomato plants appears to be directly of the responsibility of 2,4-D, and not an indirect consequence of this herbicide.

Soluble proline was quantified in leaves and roots of tomato plants exposed to 2.26 mM of 2,4-D (Figure 14). To date there are no reports on the influence of proline under 2,4-D toxicity. Tomato plants responded to 2.26 mM 2,4-D treatment with an accumulation of proline in leaves and a reduction of its accumulation in roots, showing that the levels of proline may be differentially regulated in tomato through an organ-specific manner in response to 2,4-D. This proteinogenic amino can act as

osmoprotectant, membrane stabilizer and ROS scavenger, reducing oxidative stress under several unfavorable situations (Gill and Tuteja, 2010). Proline can also act as a signal molecule, which can be essential for plant recovery after exposure to environmental stress conditions (Szabados and Savouré, 2010). In leaves, it can be assumed that the higher proline content might be related to the unchanged contents observed in lipid peroxidation, where it played a protective role. Yet, in cases where protein degradation takes place, proline acts as a sensitive molecule instead of assuming a protective role (Cia *et al.*, 2012). In tomato plants exposed to 2,4-D, the data for soluble protein content suggests that higher proline levels could have been interpreted as signal molecule. Together with 2,4-D-induced $H_2O_2$, proline may act as a signal molecule in *S. lycopersicum'*s roots, being responsible for the regulation of defense-related genes and, consequently, increased activities of SOD, CAT and APX (Figure 12B, D and F)) and low levels of ROS and lipid peroxidation (Table 3).

AsA and GSH are the most abundant low molecular weight non-enzymatic antioxidants in plant cells, participating in ROS scavenging through the AsA-GSH cycle (Sharma *et al.*, 2012). Results obtained in this study showed a significant increase in AsA levels in both organs of tomato plants exposed to the 2,4-D treatment (Table 5). As increased levels of DHA were only observed for roots, consequently a significant activity of enzymes like DHAR or MDHAR could be correlated with the increase in AsA/DHA ratio observed for leaves of 2.26 mM 2,4-D-treated plants. The oxidation of AsA by APX produces the short-lived MDHA, which could disproportionate non-enzymatically to DHA. DHA is then recycled into AsA by DHAR (Sharma *et al.*, 2012). A significant increase in DHAR and MDHAR activity was observed in mung bean exposed to 2,4-D (Karuppanapandian *et al.*, 2011). It is known that in plants the activities of DHAR and MDHAR increase along with the activity of other antioxidant enzymes in stressful conditions, and were already observed for a range of different species (Karuppanapandian and Manoharan, 2008; Kukavica and Jovanovic, 2004; Prochazkova *et al.*, 2001; Song *et al.*, 2005). These data suggest that enzymes like DHAR and MDHAR could also be targets of different types of stresses or changing physiological situations in plants. Because the increase in AsA/DHA ratio was only observed for leaves of tomato plants, it strongly suggests that DHAR and MDHAR also played an important role in AsA homeostasis, which is evidenced from the increased AsA content even though there was a higher APX activity.

GSH, another important metabolite in plant homeostasis, not only is responsible for the removal of $H_2O_2$ (Foyer and Noctor, 2011), but it also acts as a substrate for the detoxification of peroxides produced when plants are subjected to oxidative stress (Li *et al.*, 2010). Regarding GSH levels, a clear differential response was observed among the

two organs of the treated tomato plants (Table 6). In *S. lycopersicum* exposed to 2.26 mM of the herbicide GSH accumulation was greatly increased in leaves, but the levels were not significantly affected in roots. So, it appears that this metabolite could be extremely important in leaf homeostasis, being directly responsible for scavenging ROS and/or in the detoxification of radical products derived from the lipid peroxidation, which resulted in lower MDA levels in this organ, even though high values of $O_2^{\bullet -}$ and $H_2O_2$ were registered. GSH is particularly important in plant chloroplasts because it helps to protect the photosynthetic machinery from oxidative stress (Gill and Tuteja, 2010). Nevertheless, this metabolite may be translocated from one organ to another, specifically from leaves to roots, being able to perform and strengthen its functions in roots. The rate-limiting enzyme of GSH biosynthesis is considered to be γ-ECS (Mullineaux and Rausch, 2005). The obtained data for γ-ECS activity (Figure 13) suggests that both organs of tomato plants responded to the 2,4-D treatment by increasing GSH synthesis. In accordance, a study which evaluates transgenic *N. tabacum*, overexpressing *S. lycopersicum* γ-ECS (*LeECS*) gene, reported enhanced levels of GSH in comparison with wild-type plants exhibiting higher tolerance to biotic stress (Ghanta *et al.*, 2011). Moreover, increased GR activity observed in leaves and roots of the treated tomato plants (Figure 13) could be an attempt to convert GSSG into GSH for maintaining the high GSH pool in the cell. However, while in roots this attempt seems to be sufficient to maintain the levels of GSH, in leaves this seemed to be insufficient for returning all GSSG to its pre-stress level.

GST is another antioxidant enzyme that removes xenobiotics, including herbicides as well as toxic endogenous products like membrane lipid peroxides by conjugating then with GSH, thus decreasing the levels of oxidative stress in plants (Edwards and Dixon, 2005). Increased GST activity in leaves of tomato plants exposed to 2.26 mM 2,4-D (Figure 13) could be correlated with its possible involvement in the removal of highly toxic and reactive intermediate products of lipid and protein breakdown and/or in the conjugation of 2,4-D with GSH, decreasing its levels in plant cells. In accordance, some studies suggest that several GSTs can be strongly induced during cell division or by the treatment with different herbicide classes such chlorotriazine, chloroacetanilide, and thiocarbamate (Cummins *et al.*, 2011; Dixon *et al.*, 2010; Moons, 2005). Since GST activity did not increase in roots of tomato plants exposed to the herbicide it is suggested that 2,4-D is mainly metabolized in the leaves.

Comparing and relating the results obtained for GSH, GSSG, γ-ECS, GR and GST in leaves it is possible to postulate that plants responded to 2,4-D by increasing both the synthesis and the regeneration of GSH, as well as its usage to conjugate 2,4-D

and other metabolites derived from the exposure to the herbicide, principally in the leaves.

## Changes in transcript levels of selected *SlGSTF* genes in tomato plants under 2,4-D toxicity

Specific GSTs have been found to bind to phytohormones (i.e., auxins or cytokinins), affecting their distribution within the plants (Moons, 2005). A study developed in 1994, found that *A. thaliana* GST phi class (AtGSTF2) were able to bind to the auxin IAA and that was also implicated in auxin transport (Zettl *et al.*, 1994).

Five putative *S. lycopersicum* GSTF are described (Csiszar *et al.*, 2014). However, their functional characterization is still missing, and the full characterization of GSTs classes in tomato plants waits for more studies.

Since this is the first study focusing on SlGSTFs, the transcript amounts of selected GSTFs in both organs were investigated in 4-week-old tomato plants after 48 h without any treatment in order to understand which genes are expressed in which part of the plant and at what levels. For that, the relative transcript level of each *SlGSTF* in leaves was compared to the relative transcript level in roots, being the samples of the leaves equaled to one for each gene (Figure 18). The present study revealed that *SlGSTF2*, *SlGST3* and *SlGSTF4* transcript amounts were mainly found in roots of tomato plants when compared to leaves, which is in accordance with the obtained results with the tomato eFP Browser 2.0 tool (bar.utoronto.ca.) (Table 7). Moreover, it was first revealed that *SlGSTF2* transcripts had a production peak in roots without any treatment, which suggest that this gene may have an important role in basal biological processes of roots. Despite their well-known role in detoxification of several xenobiotics, plant GSTs fulfill diverse functions in numerous cellular processes that have in common the recognition and transport of a broad spectrum of reactive electrophilic endogenous compounds (Marrs, 1996).

In order to understand the involvement of GST phi class in herbicide detoxification, in particular of 2,4-D, the expression of all *SlGSTF* genes of plants exposed to 2.26 mM 2,4-D for 48 h was evaluated. For that, the changes in transcription of individual *GSTF*s after the 2.26 mM 2,4-D treatment was accessed and the relative transcript level measured in both organs of control samples was equaled to one for each gene (Figure 19A and B). The expressions of *SlGSTF4* and *SlGSTF5* were highly enhanced in the leaves but not in the root tissues of tomato plants by 2.26 mM 2,4-D.

In *A. thaliana*, *AtGSTF8* is a major phi-type GST (Thatcher *et al.*, 2007). According to the SGN database, two homologues of *AtGSTF8* have been found in tomato: *SlGSTF4* and *SlGSTF5.* However, *SlGSTF4* is the one which has a closer relationship to *AtGSTF8* (Csiszar *et al.*, 2014). It is known that the expression of *AtGSTF8* can be induced by $H_2O_2$, salicylic acid (SA) and herbicides, being used as a marker for early stress/defense responses (Thatcher *et al.*, 2007). The close phylogenetic relationship of *SlGSTF4* and *SlGSTF5* to *AtGSTF8*, and the increased transcriptional regulation by 2,4-D (Figure 19A), make a strong point that both genes may have a pivotal role in 2,4-D detoxification in leaves of tomato plants, decreasing the levels of 2,4-D in plant cells.

There were striking differences in mRNA abundance of the *SlGSTF* genes in roots when compared to the results observed for the leaves. In fact, exposure of tomato plants to 2.26 mM 2,4-D lead to a general decrease in the abundance of *SlGSTF2, SlGSTF3, SlGSTF4* and *SlGSTF5* transcripts in roots (Figure 19B). These results suggest that *SlGSTF4* and *SlGSTF5* might be differentially regulated in tomato in an organ-specific manner. Moreover, the obtained results strongly indicate that in tomato plants root GST is mainly regulated at the post-transcription level, considering that results GST activity in roots did not change despite the decrease in the expression of the *SlGSTF* genes (Figure 13), while it is regulated at the transcriptional level at the leaves. Moreover, this reduced transcript accumulation of all *SlGSTF* in roots and the specific increase of *SlGSTF4* and *SlGSTF5*, together with an increased GST activity in leaves, also suggests that 2,4-D detoxification occured mainly in the aerial part of the plant of tomato plants.

# Conclusion

The obtained results show that exposure of tomato plants to the auxinic herbicide 2,4-D lead to oxidative stress in leaves, characterized by an increase in $O_2^{\bullet-}$ and $H_2O_2$ levels as a possible consequence of activation of enzymes like XOD/XDH and ACX. Even though it was observed a high degree of epinasty and a significant damage in the photosynthetic apparatus plus protein degradation, the present results suggests that exposure of *S. lycopersicum* for 48 h to high levels of 2,4-D did not induce a severe oxidative stress condition. Such phenomena can be partially related to *S. lycopersicum* ability to increase SOD, CAT and APX activities, major ROS scavenging enzymes, suggesting that increasing activities of these enzymes is an important factor for an adaptive antioxidant response to auxinic herbicides.

Plus, these results suggest that auxin herbicides do not have a whole-plant toxicity mechanism involving oxidative stress in tomato plants. Nevertheless, proline, as well as 2,4-D-induced $H_2O_2$, may act as potential intermediates in signal transduction pathways involved in defense-related gene expression regulation, being responsible for high activities of the major ROS scavenging enzymes, leading to the decreased levels of ROS observed in roots.

This is the first report of a study regarding the gene expression pattern of specific GST-encoding genes of *S. lycopersicum* in response to the auxinic herbicide 2,4-D at a herbicidal concentration and it was shown that *SlGST* genes are important participants in the tomato defense against 2,4-D. The altered expression levels of *SlGSTF4* and *SlGSTF5*, and the increased GST activity in leaves strongly suggest that these two genes play a major role in 2,4-D stress response detoxification, which occurred in the aerial part of the plants. Also, they strongly suggest that leaf phi class GSTs are regulated at the transcription level.

The results obtained for GSH, GSSG, γ-ECS, GR and GST in leaves allow to postulate that plants responded to 2,4-D by increasing both the synthesis and the regeneration of GSH, as well as its usage to conjugate 2,4-D and other metabolites derived from the exposure to 2,4-D. On the basis of the obtained results, a possible enzymatic and non-enzymatic mechanism involved in the toxicity of the herbicide 2,4-D is proposed.

# Future perspectives

- Study different sources of ROS, namely XOD/XDH and ACX, and their involvement in 2,4-D-induced stress;

- Determination of the levels of 2,4-D in both leaves and roots, and evaluation of the fruit nutritional quality under 2,4-D toxicity;

- Characterization of other *SlGSTs*, namely genes of the GST tau family, since this may be another important family in herbicide detoxification;

- Further studies using *S. lycopersicum* plants deficient in *SlGSTF4* and *SlGSTF5* genes will provide a better understanding of the detoxification mechanism of auxinic herbicides and will allow to ascertain the importance of these specific genes in the response to 2,4-D.

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# Appendixes

**Appendix 1.** Genome loci of tomato GST coding sequences, the new and the former (if any) names of the proteins, the fully deduced coding and protein sequences.

- Solyc02g081340.2.1 (*SlGSTF1*)

Nucleotide sequence:

ATGGTAGTGAAAGTGTATGGTTCAGCAATGGCTGCATGTCCACAAAGGGTCATGGTTTGTCTTATAGA
ATTGGGAGTCGATTATGAACTTATACATGTTGATCTTGATTCTCTCCAGCAGAAAAAACCTGATTTCTT
GCTTTTACAGCCATTTGGACAGGTTCCTGTCATTGAAGAGGGCGATTTCAGGCTTTTCGAATCTAGAG
CAATAATAAGGTACTATGCAGCAAAATATGAAGACAAGGGAAAGAAACTAACCGGAACGACATTGGAA
GAAAAAGCTCTAGTAGATCAATGGCTAGAAGTGGAATCCAACAACTACAATGACTTGGTATACAACAT
GGTACTCCAACTCCTCGTATTCCCTAAAATGGGACACAAAAGTGACTTGATCGTCGTACAAAAATGTG
CCAACAATTTAGAGAAAGTGTTCGATATCTATGAACAAAGGTTGTCCAAGAGTAAATACTTAGCAGGA
GAT
TTTTTCTCCTTAGCTGATCTAAGCCACCTCCCTAGCCTTAGATTTTTGATGAATGAAGGTGGCTTTGCA
CATTTGGTGACTCAAAGGAAGTATTTGCATGATTGGTATTTGGATATTTCAAGTAGGCCTTCTTGGAGC
AAAGTGTTGGACTTCATGAATTTGAAGAAATTAGAGATGTTACCCGGCCCACCTAAAGAAGAAGTAAA
AGTTTAACAAACACTACAACGCCATGATTATTCTGTTACGAGTGGCATGGATACTGAGAATTCATATTG
CCAACTCTGTCTATCTAATCAGTTAGGTTTTGAAATTGAAGCGTTTTGTGTTTCGTTGTGTTGTAATCAC
CAAAAATAAAATAAAATTGAACTATGGGTTTACCATCAAGACATGTAACATATTACATATCCGCAATAAC
CAATACCTTAATGACAAATATGATCAAATTATATGGAAAACAGAATAAATAATACTTGTTGATA

Protein sequence:

MVVKVYGSAMAACPQRVMVCLIELGVDYELIHVDLDSLQQKKPDFLLLQPFGQVPVIEEGDFRLFESRAIIR
YYAAKYEDKGKKLTGTTLEEKALVDQWLEVESNNYNDLVYNMVLQLLVFPKMGHKSDLIVVQKCANNLE
KVFDIYEQRLSKSKYLAGDFFSLADLSHLPSLRFLMNEGGFAHLVTQRKYLHDWYLDISSRPSWSKVLDFM
NLKKLEMLPGPPKEEVKV

- Solyc06g009020.2.1 (*SlGSTF2*)

Nucleotide sequence:

TTCAACTCATTATTCCACGTTTTCTCACATCTCACACACAAATTTCATTTTCTACTTTCACTTTCTCTCTC
TAGAAAACAAAAATGGCGATCAAGGTTCATGGCCCTATGATGTCCCCTGCTGTTATGAGAGTCGTAGC
TACACTCAAAGAGAAAGATCTTGATTTTGAACTTGTTCCTGTTAATATGCAAGCTGGTGATCACAAAAA
GGAACCATTCATTTCTCTAAATCCGTTTGGTCAAGTTCCAGCTTTTGAAGATGGAGATTTAAAGCTTTT
TGAGTCAAGAGCTATTACACAATACATAGCTCACACATATGCAGACAAAGGGAACCAACTTTTACCCA
ATGACCCAAAGAAAATGGCAGTCATGTCTGTATGGATGGAAGTAGAAGCCCAGAAATTCGACCCCATT
GGTTCAAAACTAGGGTTTGAGATTGTCATTAAGCCAATGTTGGGCATGGTGACTGATGATGCAGTCGT
GGCAGAGAACGAAGAGAAACTCGGGAAACTTCTTGATGTGTATGAATCTAGACTCAAGGAATCGAAAT
ATTTGGGTGGTGAGAGTTTCACCCTAGCTGATTTGCACCACGCCCCGTCTTTGCACTACTTGTCGGG

GAGTAAAGTGAAGAGCTTGTTCGATGCTAGGCCTCATGTTAGCGCTTGGGTTGCTGATATTTTGGCTA
GGCCAGCTTGGTCTAAGACAATTGAGTTGTCAAAACAGTAAGTTTAGTGGCGGATGGGTGAGTATCG
CGTTGAAAGGAAGGGGGAACAATAGTTGGAAATGGCTGCTAATACCTCGTAGACTGAGGAGCAAAAT
ATGGCCTATGTATTACTAGTAGGTTAATAAATATAGGCCACAAGTAACACTCCTTTTGTAACCTTTTTGC
TTTGTGGTGTGCTCTTTTTTTTCCTTCCTATCCTTCATCCTTGTCTGTGAGTCAAGTGTTTGTTGTATCG
TAAGACGTTCTCTTTTTATTGAATTTTTTGTGTGCGCGTATGCGTAAGCCGACTTGTACTTTTTTGAACC
TACATTGCACTT
TCATTTTGTGCCTTGTATAGGTCATAAAATAAGTGTTTACCATTAACAAATTCTGGACATTGCAACAATC
AATCACATTTGAAATATTTTACTCAATTTAATGTTGGGTATTCAAAGTTAACAAGCATTGTCCAAATTCA
AAAAGGTGAAAATCTTTATGAATTACAGACTAAAAGCATAAAATTTGTCTCGGCAGCAAAATTCGCTTT
AATATTATAATTTTATAGGTAATTATTTGCTCCTTAATTTTTTTTCAAGTGAATTAAATTGCATCTTATCTA
TACAATATCAGTTTCATATTATGGGTGAAGTGCACGCGCTCTACTAAGTAAAATCTCAGTTTCATATTG
ATGACATGTCATA

Protein sequence:

MAIKVHGPMMSPAVMRVVATLKEKDLDFELVPVNMQAGDHKKEPFISLNPFGQVPAFEDGDLKLFESRAIT
QYIAHTYADKGNQLLPNDPKKMAVMSVWMEVEAQKFDPIGSKLGFEIVIKPMLGMVTDDAVVAENEEKLG
KLLDVYESRLKESKYLGGESFTLADLHHAPSLHYLSGSKVKSLFDARPHVSAWVADILARPAWSKTIELSK
Q

- Solyc06g009040.2.1 (*SlGSTF 3*)

Nucleotide sequence

ATGGCAATCAAAGTTCATGGCCCTATGTTGTCACCTGCTGTTGTGAGAGTTGTAGCTATGCTCAAAGA
GAAAAATCTTGATTTTGAACTTGTTCATGTTGATTTGCAAAATGGTGATCAAAAGAAGGAACCATTCATT
TCCCTGAATCCATTTGGTCAAGTTCCTGCTTTTGAAGATGGAGATCTCAAGCTTTTTGAGTCAAGAGCT
ATTACACAATACATAGCTCACACATATGCTGACAAGGGGAACCAACTCTTACCAAATGACCCAAAGAA
AATGGCAATCATGTATGTATGGATTGAAGTTGAAGCCCAAAGATTTGAACCTGTTGTTTCAAAACTATG
CTATGAGATTGTCATCAAGCCATTGTTGGACATGGTGACTGATGATGCAATCGTGGCGGAGAACGAA
GAAAAACTTAGCAAACTTCTTGACGTTTATGAATCTAGACTCAAGGATTCGAAATATTTGGGTGGTGAT
AGTTTTACCCTAGCTGATTTGAACCACGCCCCGGCTTTGCACTACTTGATGGGGACGAAAGTGAAGA
GCTTGTTCAATGCTAGGCCTCATGTTGGTGCTTGGGTTGCTAATATCTTGGCTAGGCCAGCTTGGGCT
AAGTCACTTGAGTTGACTAAATAGTAAGATTTAAGAACAATAGCTGAAAACGGCTGCTAATAGCTCGTA
GGCTGAGGAGCAAAAGTATGGTTTATGTATTACTAGTAGGTTTAATAAAATATAGGCCATGGTAACCTT
ATTTTATAATTTGTTTGCTTTTTGGTGCAACTCTTTTTACTTATCATCTCTATGATTAGAGTGTTTGTTAT
ATCGTAAGATGTTTTTTTTATTAAA

Protein sequence:

MAIKVHGPMLSPAVVRVVAMLKEKNLDFELVHVDLQNGDQKKEPFISLNPFGQVPAFEDGDLKLFESRAI
TQYIAHTYADKGNQLLPNDPKKMAIMYVWIEVEAQRFEPVVSKLCYEIVIKPLLDMVTDDAIVAENEEKLSKL
LDVYESRLKDSKYLGGDSFTLADLNHAPALHYLMGTKVKSLFNARPHVGAWVANILARPAWAKSLEL
TK

- Solyc09g074850.2.1 (*SlGSTF4*)

Nucleotide sequence:

TCAACTTGGAGCAATCTATCATAATCCTAATTTATTATTCTTGAAATAATGGCAATCAAAGTCCATGGTA
TCCCCTTGTCAACTGCAACCATGAGAGTTATTTCTTGCCTTATTGAGAAGGATTTGGATTTTGAGTTTG
TCTTTGTTGATATGGCCAAAGAAGAACACAAGAGGCACCCTTTCCTCTCACTCAATCCTTTTGCTCAAG
TACCAGCATTTGAAGATGGAGACTTGAAGCTCTTTGAATCAAGGGCAATCACTCAATACATTGCTCAG
GTTTATGCTAGCAATGGCATTCAACTAATACTCCAAGATCCAATGAAAATGGCCATTATGTCAGTATGG
ATGGAAGTAGAAGGCCAAAAATTTGAACCACCAGCTTCAAAATTAACATGGGAGCTAGTCATAAAACC
AATGATTGGCTTGGGCAGTACCGATGATGTTATTGTGAAGGAAAGTGAAGAACAATTGTCTAAGGTTC
TTGACATCTACGAAACTCGATTGACAGAGTCAAAATACTTGGGTGGCGACTCCTTTACACTTGTTGATT
TGCATCATATACCAAATATATACCATCTGATGAATACAAAAGCTAAGGCACTGTTTGATTCGCGCCCTC
GTGTGAGTGTATGGTGTGCTGATATATTGGCTAGGCCAGCTTGGGTGAAGGGGTTGGAGAAGATGCA
AAAATGAAAAAAAGTCGTGAATTAATGGATGATCATAATTCATATATATGTTTTTGTTTTGAAGCATTTG
TGTCTTAATATGTTGTGTTTCTTGTCTGAAGATGTTTGTCTTGCAATACAATAAACAGTGATCTATATCT
ATGTGATTTTACTAATTGTACTGATGTAAAATATGCTATGTTCCGGTCATTTATAAAATAATTGCGCGCT
ATATTTTTGTG

Protein sequence:

MAIKVHGIPLSTATMRVISCLIEKDLDFEFVFVDMAKEEHKRHPFLSLNPFAQVPAFEDGDLKLFESRAITQY
IAQVYASNGIQLILQDPMKMAIMSVWMEVEGQKFEPPASKLTWELVIKPMIGLGSTDDVIVKESEEQLSKVL
DIYETRLTESKYLGGDSFTLVDLHHIPNIYHLMNTKAKALFDSRPRVSVWCADILARPAWVKGLEKMQK

- Solyc12g094430.1.1 (*SlGSTF5*)

Nucleotide sequence:

ATGGCTACTCCGGTGAAAGTGTACGGACCAACTTTATCAACAGCAGTGTCAAGAGTTTTAGCTTGTCT
TCTTGAAAAAAATGTTCAATTTCACCTCATCCCTGTTAATATGGCAAAAGGGGAACACAAAAAACCTGC
CTATCTCAAAATTCAGCCTTTTGGTCAAGTTCCAGCTTATCAAGATGAGGATATCACTTTGTTTGAATC
CAGATCTATAAATAGGTACATATGTGACAAATATGGAAGTCAAGGTAACAAGGGATTATATGGAACGA
ATCCGTTAGAGAAAGCGTCTATAGATCAATGGATAGAGGCAGAAGGACAAAGCTTCAATCCACCAAGT
TCAGTTCTTGTATTCCAGCTGGCTTTTGCACCGCGAATGAAGCTCAAACAAGACGAGAACTTGATCAG
ACAGAACGAAGAGAAGCTCAAAAAAGTACTTGATGTGTATGAAAAGAGGCTCGGAGATAGTCAGTACT
TGGCTGGAGATGAATTCACATTGGCCGATCTCTCTCACCTTCCAAACATCCAATACTTGGTGAACGGG
ACAGACAGAGCAGAGCTCATCACTTCTCGAGAGAACGTGGGGAGGTGGTGGGGTGAGATATCCAAC
CGAGAGTCATGGAAGAAGGTAGTTGAAATGCAGACCTCACCCCCTCCTTCCTAG

Protein sequence:

MATPVKVYGPTLSTAVSRVLACLLEKNVQFHLIPVNMAKGEHKKPAYLKIQPFGQVPAYQDEDITLFESRSI
NRYICDKYGSQGNKGLYGTNPLEKASIDQWIEAEGQSFNPPSSVLVFQLAFAPRMKLKQDENLIRQNEEKL
KKVLDVYEKRLGDSQYLAGDEFTLADLSHLPNIQYLVNGTDRAELITSRENVGRWWGEISNRESWKKVVE
MQTSPPPS

**Appendix 2.** Melting curves for all *SlGSTF* genes assayed. Red curve - *SlGSTF1*; Green curve - *SlGSTF2*; Black curve - *SlGSTF3*; Pink curve - *SlGSTF4*; Blue curve - *SlGSTF5*.