Ecotoxicology and Environmental Safety 147 (2018) 151–156

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

journal homepage: www.elsevier.com/locate/ecoenv

DDTs-induced antioxidant responses in plants and their influence on phytoremediation process

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ARTICLE INFO

Keywords:
Tomato
Zucchini
Antioxidant enzymes
Phytoremediation

ABSTRACT

Phytoremediation is a low cost technology based on the use of plants to remove a wide range of pollutants from the environment, including the insecticide DDT. However, some pollutants are known to enhance generation of reactive oxygen species (ROS), which can generate toxic effects on plants affecting the phytoremediation efficiency. This study aims to analyze the potential use of antioxidant responses as a measure of tolerance to select plants for phytoremediation purposes. Tomato and zucchini plants were grown for 15 days in soils contaminated with DDTs (DDT + DDE + DDD). Protein content, glutathione-S-transferase (GST), glutathione reductase (GR), glutathione peroxidase (GPx) and catalase (CAT) activities were measured in plant tissues. Exposure to DDTs did not affect protein content or CAT activity in any of the species. GST, GR and GPx activity showed different responses in exposed and control tomato plants. After DDTs exposure, tomato showed increased GR and GPX activity in stems and leaves, respectively, and a decrease in the GST activity in roots. As no effects were observed in zucchini, results suggest different susceptibility and/or defense mechanisms involved after pesticide exposure. Finally, both species differed also in terms of DDTs uptake and translocation. The knowledge about antioxidant responses induced by pesticides exposure could be helpful for planning phytoremediation strategies and for the selection of tolerant species according to particular scenarios.

1. Introduction

The insecticide DDT (1,1,1-trichloro-2,2-bis-(4′-chlorophenyl) ethane) has been widely used for pest control because its low cost, broad spectrum activity and high residual biological activity (Turusov et al., 2002). Although, it's use has been prohibited in most countries because of the negative impact on wildlife and human health in addition to biomagnification process throughout food web, DDT is still being used in some developing countries for essential public health purposes (Foght et al., 2001; Bouwman et al., 2015).

Phytoremediation is defined as the use of green plants to remove pollutants from the environment or to render them harmless (Salt et al., 1998; Campos et al., 2008). Depending on the nature of the contaminant, plant species and soil characteristics, phytoremediation may be achieved in different ways. Phytostimulation or rhizodegradation, occurs when the organic contaminants are degraded in the root zone (rhizosphere), either by exuded plant enzymes or by the associated microbial community (Pilon-Smits, 2009). Pollutants can also be extracted and accumulated into plant tissues, followed by harvesting of the plant material, which is called phytoextraction. Finally, phytodegradation refers to the ability of plants to degrade organic pollutants directly via their own enzymatic activities. After uptake in plant tissue, certain pollutants can leave the plant in volatile form, known as phytovolatilization. These various phytoremediation routes are not mutually exclusive and can occur simultaneously.

The potential of *Cucurbita* species to accumulate significant amounts of Persistent Organic Pollutants (POPs), including dioxins, chlordane, DDT, DDE, DDD, and PCBs has been reported (Hülster et al., 1994; White et al., 2003; White, 2009). Particularly, stems and roots of *Cucurbita pepo* ssp *pepo* accumulate POP concentrations that are 5–30 times greater than present in the soil, often extracting 1–5% of the contamination in a single growing season. On the other hand, other crop species such as tomato plants have a high capacity to increase DDE bioavailability and metabolism in the rhizosphere, as well as to accumulate DDE and other organic persistent contaminants especially in roots (Gonzalez et al., 2003; Mitton et al., 2014, 2016; Mattina et al.,

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http://dx.doi.org/10.1016/j.ecoenv.2017.08.037
Received 18 January 2017; Received in revised form 3 August 2017; Accepted 15 August 2017
Available online 14 September 2017

2003). In this sense, a previous study demonstrated that roots of 15 days tomato plants showed higher DDTs concentration than sunflower, soybean and alfalfa reaching levels of 2075 ng g^{-1}. Particularly, tomato plants presented the highest bioconcentration factor for DDE (2.23) (Mitton et al., 2014).

Insecticide-induced oxidative stress was shown to modify the cellular redox balance by altering antioxidant levels or the activity of the cellular defense systems (Bashir et al., 2007). Mishra et al. (2008) reported that the insecticide dimethoate triggered oxidative stress by producing reactive oxygen species (ROS). However, plants have multiple strategies to cope with the insecticide-induced toxicity. Among them, prevention of oxidative damage to cells has been suggested as one of the mechanisms of stress tolerance (Saraf and Sood, 2002; Prasad et al., 2005). Enzymes of the antioxidant system include ROS scavengers like glutathione peroxidase (GPx) and catalase (CAT) (Khan and Kour, 2007). Reduced glutathione (GSH) represents a non-enzymatic defense that protects cells from oxidative stress by scavenging ROS or by reducing oxidized components such as proteins (Pinto et al., 2003; Jan et al., 2012). Besides its antioxidant function, the occurrence and activity of detoxification enzymes is crucial for biotransformation and, eventually, degradation of the contaminants (Schröder, 2006; Schröder and Collins, 2002). In this sense, the conjugation of xenobiotics with GSH, mediated by glutathione-S-transferases (GSTs) is a xenobiotic biotransformation mechanism described in animals and plants (Marrs, 1996; Schröder and Collins, 2002; Kurasvili et al., 2016). This reaction results in both an increase of toxicant solubility that facilitates its excretion and decreases its toxicity (Brentner et al., 2008). In this context, the aim this work was to study the potential use of antioxidant responses as tolerance criteria for selecting plants with phytoremediation capabilities. This work includes the study of two species (tomato and zucchini) with different behavior towards the uptake and translocation of DDT.

2. Material and methods

2.1. Plant growth

Rectangular pots of 6000 cm^3 were filled with 1000 g of dry DDTs polluted soil obtained from an apple and peach production site located in Villa Regina, Rio Negro, Argentina (S 39°04.9′14″, W 67°02.9′59″). DDTs levels ranged between 63.5–101.3 ng g^{-1} dry weight of DDT and 381.4–455.3 ng g^{-1} dry weight of DDE (Gonzalez et al., 2010; Mitton et al., 2012, 2014). Soils are classified as Aridisols order according to Spil Survey Staff, (1999) and had 2.7% of organic carbon, 14.1% of sand, 62.9% of silt and 23% of clay (Gonzalez et al., 2010). Five seeds of *Solanum lycopersicum* "tomato" (cultivar *Platense*) and *Cucurbita pepo* "zucchini" (cultivar *Grey*) were placed in each pot separately. The plants were grown in greenhouse at temperature of 10–26 °C under natural sunlight (light:dark cycle 14:10 h) and five pots were established for each species. Planted control pots were established with non polluted soil (1.9% organic carbon, 60.7% sand, 31.8% silt and 7.3% clay, total organochlorine pesticide levels, including DDTs, lower than 2×10^{-6} mg g^{-1}, Gonzalez et al., 2010). The pots were watered on demand with tap water and weeded.

2.2. Plant sampling

Destructive harvest was done 15 days after germination, obtaining stems, leaves and roots. Attached soil particles were removed from roots by washing with distilled water. Each pot was individually analyzed and the samples were pooled. All samples were kept in ultrafreezer (− 80 °C) until analysis.

2.3. Pesticides analysis

DDTs levels in zucchini tissues were analyzed according to Metcalfe and Metcalfe (1997), as modified by Miglioranza et al. (2003). Briefly, subsamples of wet tissue were homogenized with sodium sulfate and extracted with a mixture of hexane-dichloromethane in a Soxhlet equipment. Lipids were removed by gel permeation chromatography in Bio Beads S-X3 (200–400 mesh size, Bio Rads Laboratory, Hercules, CA, USA) and further purification of the extracts was performed by silica gel chromatography. Samples were concentrated to 1 mL and kept in sealed vials at − 20 °C prior to chromatographic analysis. DDTs (*p,p*-DDE, *p,p*-DDT and *p,p*-DDD) were identified and quantified using a gas chromatograph (with autosampler) Shimadzu 17-A gas equipped with a 63Ni Electron Capture Detector (GC-ECD) and a capillary column coated with SPB-5 [(5-phenyl)-methyl polysiloxane, 30 m × 0.25 mm i.d. × 0.25 μm film thickness; Supelco Inc]. The standard solution used for identification and quantification of single compounds were a Standard Pesticide Mixture of organochlorine pesticides from Ultra Scientific (RI, USA and PCB #103 from Accustandard Absolute Standards, INC, CT, USA). Retention times of each compound were confirmed by running solution of single compound from Dr. Ehrenstorfer, Augsburg, Germany, with purity ≥ 96%. Laboratory and instrumental blanks analyzed through the procedure indicate that there were no contaminants or interference on samples during laboratory handling. Single compounds recoveries, calculated by spiking matrix and surrogate recovery, were greater than 90%. Instrumental detection limits (DL) for DDTs were calculated according to Keith et al. (1983) and were < 0.2 ng mL^{-1}, method detection limits were < 0.033 ng g^{-1}. DDTs data for tomato plants were obtained from Mitton et al. (2014).

2.4. Tissue homogenization

Enzyme extraction from tomato and zucchini tissues was done following the method described by Martínez-Domínguez et al. (2008), as modified by Mitton et al. (2014). After grinding in liquid nitrogen, tissues were homogenized (1:2 w/v) in ice-cold buffer [0.1 M sodium phosphate buffer (pH 6.5), 20% glycerol, 14 mM dithiothreitol (DTE), 1 mM phenylmethylsulfonylfluoride (PMSF) and 1 mM (ethane-1,2-diyldinitrilo) tetra acetic acid (EDTA)]. Homogenates were centrifuged at 15,000 × *g* for 20 min (4 °C). Supernatants were collected and stored at − 80 °C for further analysis of protein determination and enzymatic activity using a microplate spectrophotometer (Epoch Bio Tek). All reagents were supplied by Sigma-Aldrich.

2.5. Protein determination

Protein concentration was determined by the Bradford (1976) method with bovine serum albumin (BSA) as standard protein.

2.6. Measurement of glutathione-S-transferase (GST) activity

GST activity was measured using the Habig and Jakoby (1981) methodology. The absorbance at 340 nm generated by the conjugation of 1 mM glutathione (GSH) with 1 mM of 1-chloro-2,4-dinitrobenzene (CDNB) was monitored during 5 min at 25 °C.

2.7. Measurement of glutathione reductase (GR) activity

GR activity was analyzed by the methodology described by Gallagher et al. (1992) using sodium phosphate buffer (200 mM, pH 7.5), oxidized glutathione (GSSG, 10 mM) and NADPH (1 mM). The oxidation of NADPH was monitored at 340 nm during 5 min at 25 °C.

2.8. Measurement of glutathione peroxidase (GPx) activity

GPx activity was based on methodology described by Arun et al. (1999) where the decrease of NADPH at 340 nm was monitored at 25 °C during 5 min. The reaction buffer contained reduced glutathione (GSH, 2 mM), NADPH (0.12 mM). H_2O_2 (2 mM), sodium azide (20 mM) and

glutathione reductase (0.1 U mL^{-1}) in sodium phosphate (100 mM, pH 7.5).

2.9. Measurement of catalase (CAT) activity

CAT activity was analyzed following Rao et al. (1997) determining the initial of H_2O_2 decomposition at 240 nm at 25 °C during 5 min. The reaction buffer contained EDTA (5 mM) and H_2O_2 (10 mM) in Tris-HCl (1 M, pH 8.0).

2.10. Statistical analysis

Results of protein content, GST, GR, GPX and CAT activity represent the mean of five independent experiments. Normality and variance homogeneity were verified in all variables and mathematical transformation applied if at lest one assumption was violated. Parametric two-way ANOVA was applied to assess differences among species and plant tissues. The significance level was set at 0.05 (Zar, 1984).

3. Results

Tomato and zucchini plants showed different phytoremediation strategies, as reflected by the DDTs accumulation of both plant species. Zucchini plants contained 559 and 141 ng g^{-1} dw in roots and aerial tissues, respectively (Fig. 1). Tomato showed DDT levels of 2075 and 30.85 ng g^{-1} dw for roots and aerial, respectively (Fig. 1), previously reported in Mitton et al. (2014).

Roots of both species accumulated higher DDTs levels than aerial tissues, although differences between species were observed. Tomato roots accumulated DDTs residues 4 times higher than zucchini roots, while the translocation of DDTs to aerial tissues was higher in zucchini plants (16 times more than tomato).

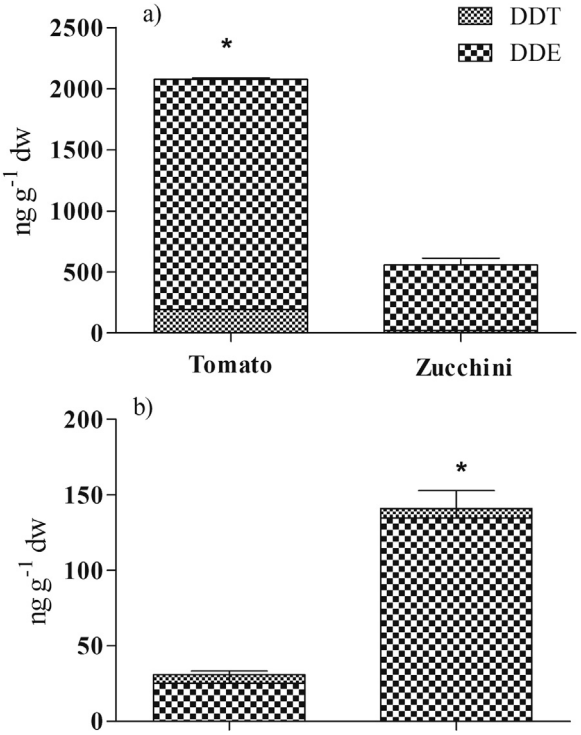

Fig. 1. DDTs levels in roots (a) and aerial organs (b) of 15-days old tomato and zucchini plants grown in polluted (Ex) and unpolluted (Un) soil. * indicate significant differences ($p \leq 0.05$). The results of tomato plants were reported in Mitton et al. (2014).

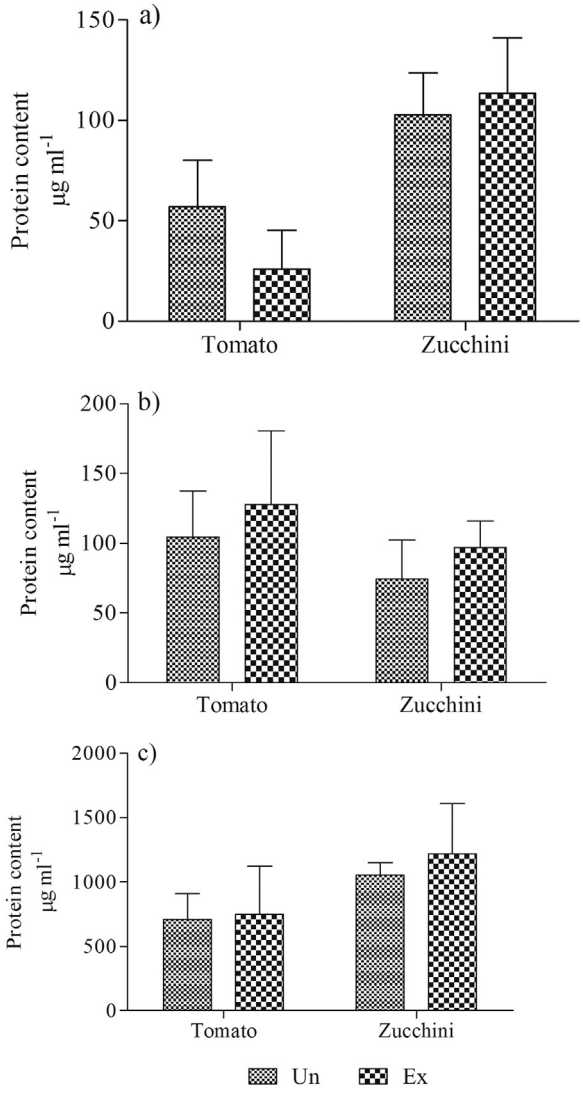

Fig. 2. Protein content in roots (a), stems (b) and leaves (c) of 15-days old tomato and zucchini plants grown in polluted (Ex) and unpolluted (Un) soil. * indicate significant differences ($p \leq 0.05$).

3.1. Protein content

The results from Fig. 2 indicate that DDTs exposure did not alter protein content ($p < 0.05$). Moreover, protein content was species-specific, being "roots < stems < leaves" in tomato and "stems < roots < leaves" in zucchini plants.

3.2. Detoxification/ROS scavenging

3.2.1. GST-activity

Glutation-S-transferase is a key enzyme under stress conditions, particularly during the conjugation step in the pollutants bio-transformation process. Unexposed plants from both species showed the general pattern of GST activity roots > stems > leaves (Fig. 3). Upon DDTs exposure, tomato roots showed decreased enzyme activity ($p < 0.05$), while no significant differences were found for aerial tissues ($p > 0.05$), except for tomato leaves that GST activity increased in polluted plants ($p < 0.05$).

3.2.2. GR-activity

The general pattern of GR activity differed between tomato and zucchini for unexposed plants, being roots= stems > leaves in tomato

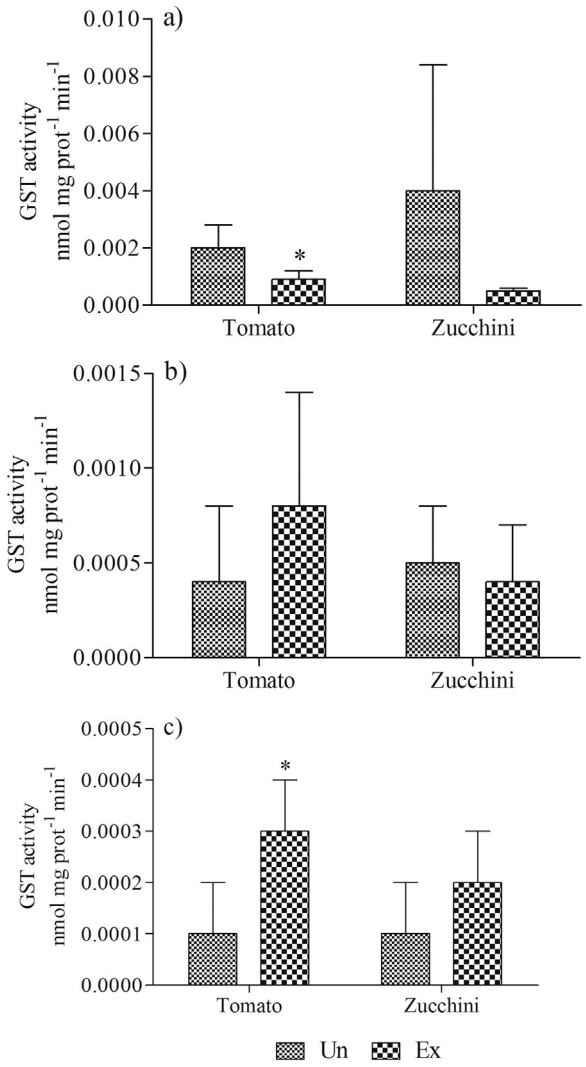

Fig. 3. GST-activity in roots (a), stems (b) and leaves (c) of 15-days old tomato and zucchini plants grown in polluted (Ex) and unpolluted (Un) soil. * indicate significant differences ($p \leq 0.05$).

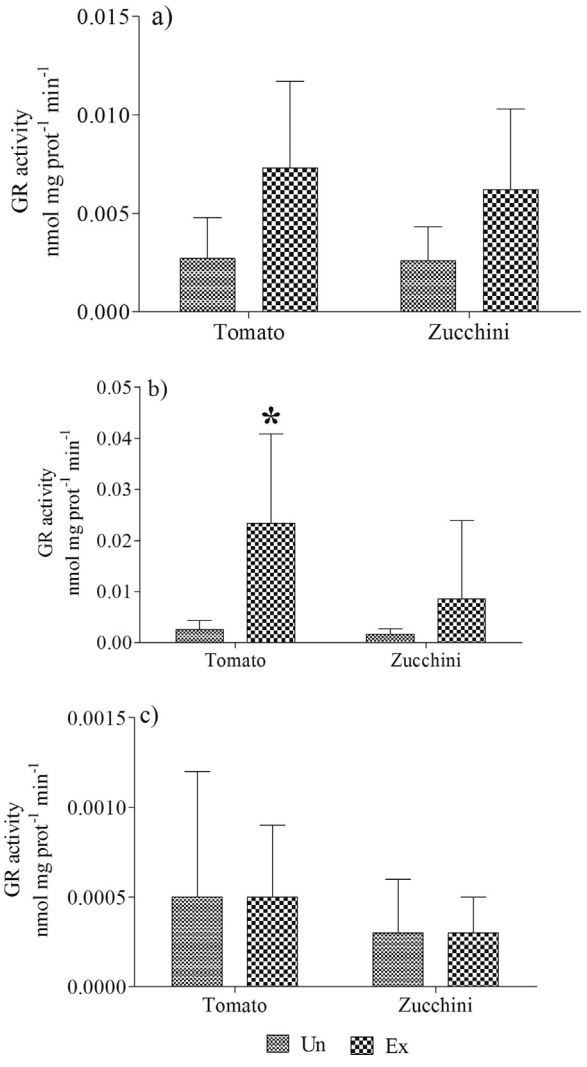

Fig. 4. GR-activity in roots (a), stems (b) and leaves (c) of 15-days old tomato and zucchini plants grown in polluted (Ex) and unpolluted (Un) soil. * indicate significant differences ($p \leq 0.05$).

and roots > stems > leaves in zucchini (Fig. 4). DDT exposure led to an increased GR activity ($p < 0.05$) in stems of tomato plants (Fig. 4b).

3.2.3. GPX-activity

The general tissue pattern was roots = stems > leaves and roots > stems > leaves for tomato and zucchini, respectively. Only the leaves of exposed tomato plants presented differences with non-exposed plants showing an increased GPX activity (Fig. 5c; $p < 0.05$).

3.3. CAT-activity

No significant differences on CAT activity were observed after DDT exposure in any of both species, (Fig. 5; $p > 0.05$). The general pattern of CAT activity was root > stems = leaves for unexposed tomato plants while zucchini did not show differences among tissues ($p < 0.05$) (Fig. 6).

4. Discussion

It is known that vegetable species vary in the accumulation of weathered DDTs and that zucchini plants can accumulate significant amounts of weathered POPs, including chlordane, DDTs and PCBs (White et al., 2003; Mattina et al., 2006; White, 2009). In this work, results showed the high translocation capacity of zucchini plants as well as the high root bioconcentration of tomato plants. Differences in the physiological and biochemical processes including the antioxidant defenses may lead to differential pesticide uptake, translocation, degradation or subsequent conjugation. Several authors showed that the contaminant exposure reduced growth, metabolic activity (carbohydrates, proteins, etc.) and altered enzymatic activities in different organisms (Bartha et al., 2010; Chauhan et al., 2011).

In this study, the results of the DDTs content and antioxidant responses in zucchini plants come from the same experiment, while for tomato, the results of DDTs effects on plants come from the present study and are interpreted comparing them with the corresponding content of DDTs previously reported in Mitton et al. (2014).

The present study showed that DDTs exposure and uptake did not induce any changes in protein content neither in tomato nor in zucchini plants. Similarly, previous studies demonstrated that DDT exposure did not affect root protein content on alfalfa and soybean plants after 60 days of growth. However, alfalfa plants showed an increase in the total protein content in aerial tissues (Mitton et al., 2016). The results observed in tomato and zucchini plants may indicate that protein synthesis and/or degradation were not affected by DDT exposure.

Pollutant biotransformation can be catalyzed by different enzymes depending on the organism, tissues and compound. The tripeptide

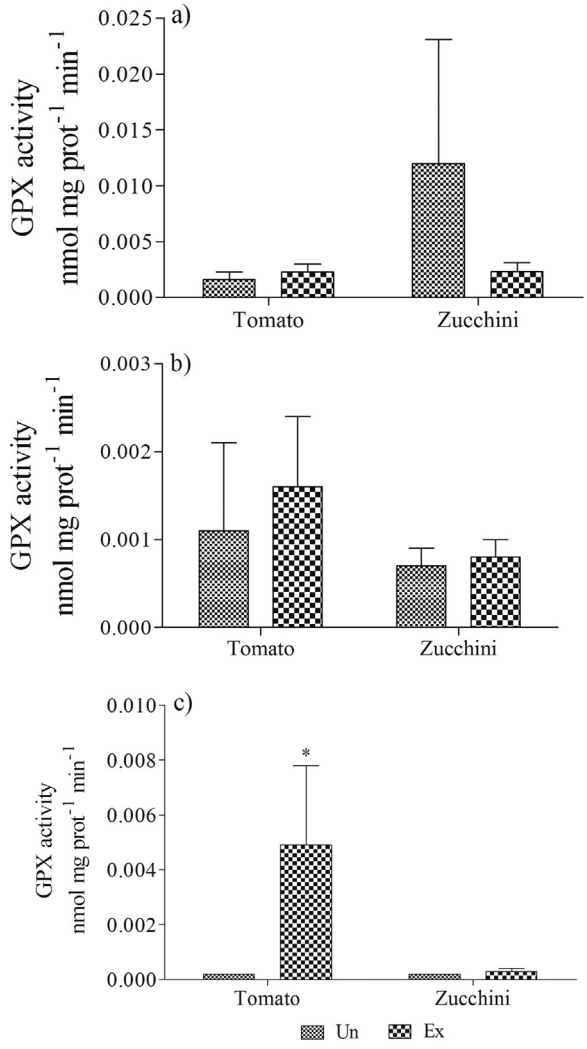

Fig. 5. GPX-activity in roots (a), stems (b) and leaves (c) of 15-days old tomato and zucchini plants grown in polluted (Ex) and unpolluted (Un) soil. * indicate significant differences ($p \leq 0.05$).

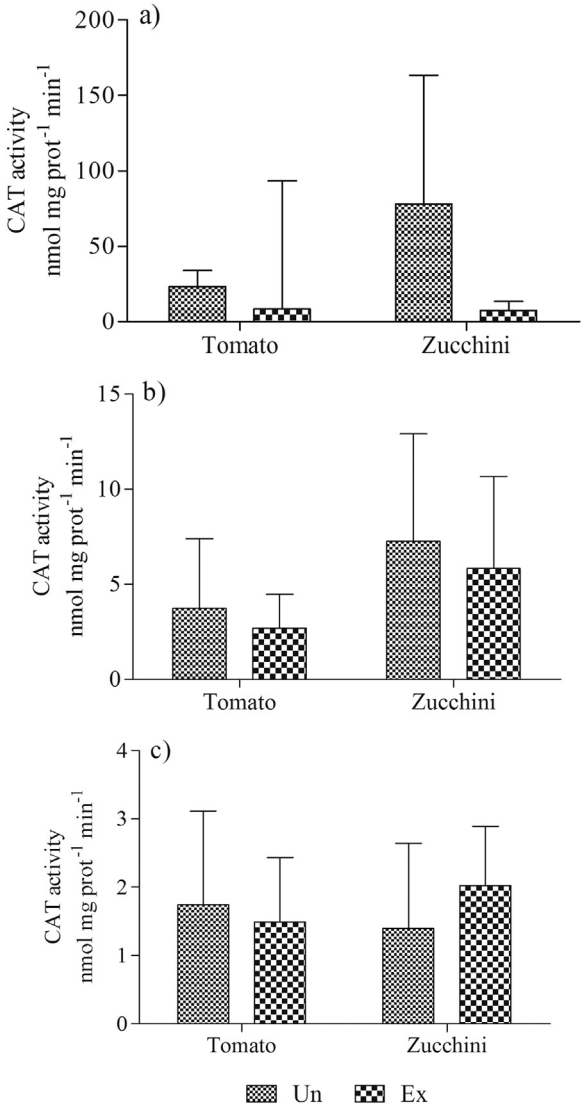

Fig. 6. CAT-activity in roots (a), stems (b) and leaves (c) of 15-days old tomato and zucchini plants grown in polluted (Ex) and unpolluted (Un) soil. * indicate significant differences ($p \leq 0.05$).

glutathione (γ-glutamyl-cysteinyl-glycine) plays a key role in detoxifying reactive low molecular weight organic compounds of endogenous or xenobiotic origin. Under normal redox conditions, glutathione is predominantly found in its reduced form (GSH), with only a small proportion available in the fully oxidized state (GSSG; Dixon et al., 2002). Glutathione-S-transferases (GST) catalyze the conjugation of electrophilic and frequently hydrophobic toxic compounds with GSH to form non-toxic peptide derivatives. Complementary to this detoxification function throughout conjugation, glutathione also has a protective role reducing cytotoxic hydroperoxides of fatty acids and nucleic acids to the corresponding monohydroxyalcohols. This reduction plays a pivotal role in preventing the degradation of organic hydroperoxides to cytotoxic aldehyde derivatives, which arise because of oxidative stress, to the respective alcohols (Dixon et al., 2002; Rahman, 2007). These reductions are catalyzed by glutathione peroxidases (GPXs) among other enzymes. Our results showed a clear tissue-dependent pattern of the enzymatic activity. Roots, as the main uptake pathway of pollutants in plants present the highest pesticide levels and it is expectable to find biochemical responses to DDT accumulation in this tissue. The GST activity pattern (roots > stems > leaves) in both species was in line with the DDTs accumulation, as was previously observed on soybean and alfalfa grown for 60 days in DDTs polluted soils (Mitton et al., 2016). Additionally, the results showed that GST activity decreased in roots of exposed tomato plants. Since total protein levels did not change and GSTs represent more than 1% of soluble proteins in plants cells (Edwards et al., 2005) this reduction may be related to enzyme inhibition linked with the high DDTs root concentration. On the other hand, leaves with lower DDTs levels presented an inverse response.

Therefore, the changes in GST, GR and GPX activities observed in this work could indicate the role of glutathione in the oxidative stress prompt in tomato plants by DDTs exposure. Conversely, for zucchini plants, the lower DDTs levels in roots might be related to the absence of difference in antioxidant enzymes activity. In case of zucchini leaves, with higher contaminants levels than tomato, the results could indicate a lower susceptibility for the contaminant. The results seem to indicate that zucchini antioxidant responses are not affected by DDTs uptake and translocation.

The trend to a diminished CAT activity after DDT exposure in tomato and zucchini plants agrees to the behavior of this enzyme in other plant species exposed to other pollutants. In this sense, Liu et al. (2009) described reduction in the activity of this enzyme in *Arabidopsis thaliana* exposed to phenanthrene and Bashir et al. (2007) showed a decrease in the CAT activity in soybean leaves, exposed to deltamethrin for 10, 45 and 70 days. This inhibition of CAT activity by pesticide presence was associated to the ROS formation, especially superoxide radical which is a CAT inhibitor (Kono and Fridovich, 1982; Farrington et al., 2007).

5. Conclusions

The species reacted differently to DDTs exposure, suggesting diversity in susceptibility and/or mechanisms involved in pesticide response. The findings of this study indicate that DDTs uptake trigger antioxidant responses in tomato plant tissues and it is directly related to DDT levels. For zucchini plants the results might indicate that an acclimation steady in the antioxidant response was reached.

The response of GSH-dependent antioxidant enzymes showed by roots of tomato plants could be due to the high DDTs levels found for this species. On the other hand, the results registered in the leaves might indicate higher susceptibility to DDT translocation by tomato than zucchini plants considering the lower pesticide levels found in tomato leaves.

Finally, the results of this study show that the antioxidant response may be used as a criteria of tolerance of pesticides in plants with phytoremediation purposes.

Tomato and zucchini plants present diverse antioxidant responses linked with the different DDTs levels in plants, indicating that these species have different strategies for pesticide uptake and translocation. Tomato plants would be better in phytoremediation processes than zucchini plants due to the existence of antioxidant responses against DDT uptake that could be the responsible for the higher pesticide levels showed into the roots.

Acknowledgements

We gratefully acknowledge to Dr. Daniela Sueldo from the University of Oxford for her helpful in check and revise the Eglish grammar, spelling and sentences construction which highly improved this manuscript. This work was funded by grants from Universidad Nacional de Mar del Plata (EXA 703/14) and ANPCyT (PICT-07/410 and PICT-12/2239). This work is part of the Ph.D. Thesis of the first author (Mitton, F. M).

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