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Antioxidant mechanisms to counteract TiO_2-nanoparticles toxicity in wheat leaves and roots are organ dependent

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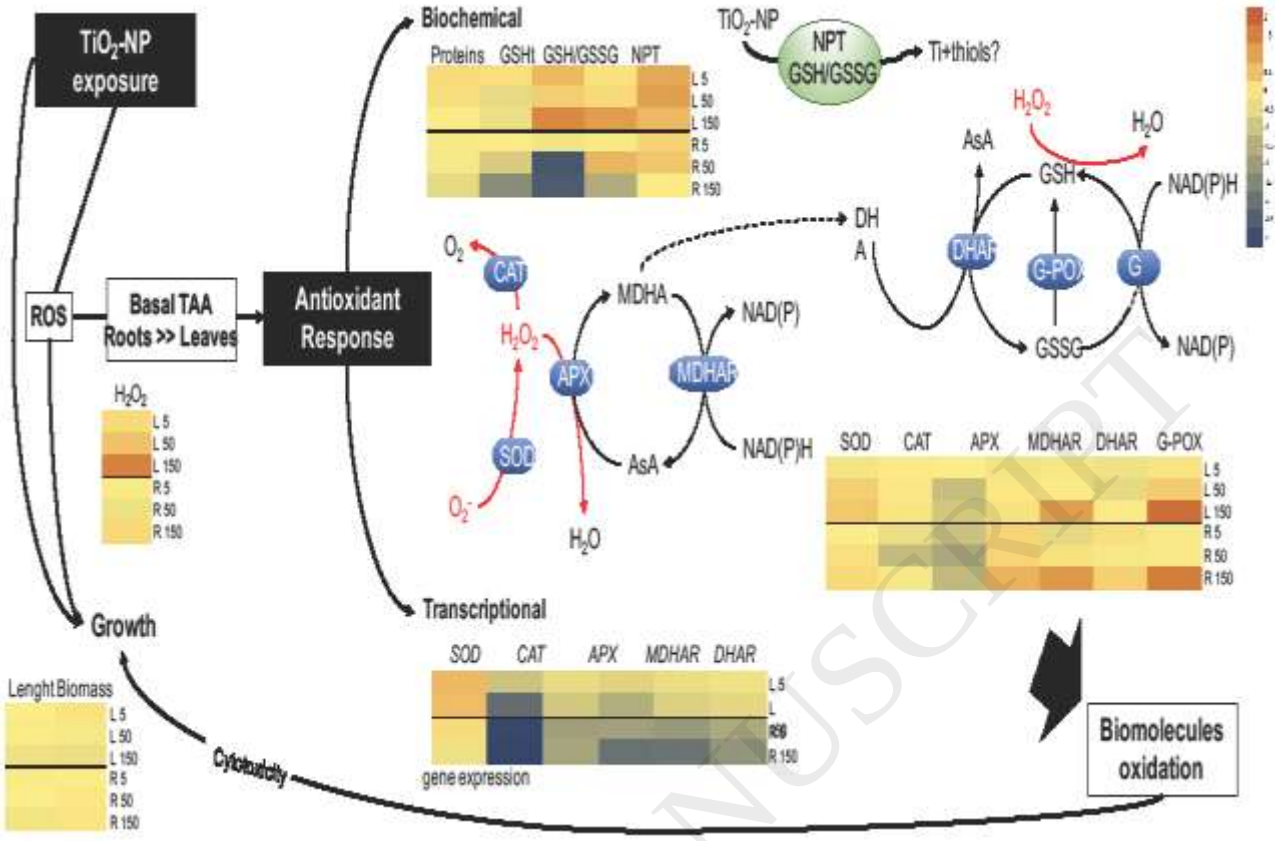
Graphical abstract



Highlights

- TiO_2-NP is toxic above 5 mg L^{-1}, being wheat shoots more sensitive than roots
- Leaves triggered thiol and AsA metabolism to counteract TiO_2-NP toxicity
- Roots preferred antioxidant route involves pre-existing antioxidant capacity
- The induced antioxidant mechanisms did not prevent cellular oxidation
- Biochemical and transcriptional gap responses suggest time-dependent adaptations

Abstract

Nanoparticles (NP) bioactivity is under deep scrutiny. In this work, the antioxidant response to TiO_2-NP in wheat (*Triticum aestivum*) was determined. For that, enzymatic and the non-enzymatic antioxidants were evaluated in plants exposed to the P25 anatase:rutile material composed of TiO_2-NP and under environmentally realistic doses (0; 5; 50; 150 mg/L for 20 days). Shoot but not root growth was reduced. In leaves, thiol metabolism and ascorbate accumulation were the preferred route whereas in roots the pre-existing antioxidant capacity was preferentially

utilized. Both leaves and roots showed increased glutathione reductase and dehydroascorbate reductase activities and decreased ascorbate peroxidase activity. Roots, nevertheless, presented higher enzymatic basal levels than leaves. On the other hand, when examining non-enzymatic antioxidants, the ratio of reduced-to-oxidized glutathione (GSH/GSSG) increased in leaves and decreased in roots. Exposed leaves also presented higher total ascorbate accumulation compared to roots. TiO_2-NP exposure down regulated, with more prominence in roots, antioxidant enzyme genes encoding catalase, ascorbate peroxidase, monodehydroascorbate reductase and dehydroascorbate reductase. In leaves, superoxide dismutase gene expression was increased. All data pinpoint to TiO_2-NP toxicity above 5 mg/L, with aerial parts being more susceptible, which draws concerns on the safety doses for the use of these NPs in agricultural practices.

Abbreviations: Ana: anatase; APX: ascorbate peroxidase; AsA: ascorbate; AsAt: total ascorbate; CAT: catalase; DHAR: dehydroascorbate reductase; DTT: dithiothreitol; G-POX: guaiacol peroxidase; GR: glutathione reductase; GSHt: total glutathiones; GSH/GSSG: reduced glutathione/oxidized glutathione ratio; MDA: malondialdehyde; MDHAR: Monodehydroascorbate reductase; NP: nanoparticles; NPT: non-protein thiols; RMP: relative membrane permeability; Rut: rutile; SOD: superoxide dismutase; TAA: total antioxidant activity; TBARS: thiobarbituric acid reactive substances

Keywords

Antioxidant battery, antioxidant thiols, crops-nanoparticle interaction, nanoparticles toxicity, oxidative stress

1. Introduction

Plants are key organisms for animal and human life and are essential in both terrestrial and aquatic ecosystems. So, it is critical to understand how substances present in the environment interact with plants. In the past few decades, nanotechnology industry developed having become inevitable release of nanomaterials to the environment. Nowadays, numerous studies on manufactured nanoparticles (NP) interaction with organisms are available. Nevertheless, it remains a challenge to predict the concrete consequences of NP exposure on organisms. Concerning plants, several studies show that NP are potentially toxic [1], whereas others highlight

the potential benefits of NP use [2, 3]. So, it remains under discussion the generalized application of nanotechnology on agriculture.

Titanium dioxide NP (TiO_2-NP) are among the most used NP and have many application possibilities in agriculture, such as degradation of pesticides, plant protection, and residue detection [4]. Several reports highlighted the positive effects of Ti application on plant growth and yield [5], but when NP are used negative impacts may be observed [6, 7]. So, it is important to decipher the impact of TiO_2-NP on crop species metabolic processes as well as the targets and mechanisms behind their putative phytotoxicity.

One of the hypotheses stressed out for TiO_2-NP toxicity in both animals and plant species has been related to oxidative stress as a consequence of reactive oxygen species (ROS) increase and of redox status imbalance [8, 9]. In part, this may be justified by the TiO_2-NP capability to generate free radicals, even under no UV radiation [10]. On the other hand, it remains unclear how the direct interaction of these NP with plant biomolecules and tissues ultimately influence the plant oxidative status and antioxidant pathways. When the organism is not able to reduce the excess of ROS, oxidation of biomolecules occurs, impairing several metabolic pathways and eventually compromising plant performance [11].

Alterations of plant redox status induced by TiO_2-NP were found in some species [12, 13], nevertheless the responses were not uniform and depended on the plant species, the NP crystalline phase [anatase (Ana) or rutile (Rut)] and the exposure conditions. Concerning TiO_2-NP phases, most of the studies focused on Ana and less is known about the effects of Rut and the highly-used mixture anatase+rutile (P25), which presents higher photocatalytic capability than the pure phases [14]. Tomato roots and leaves exposed to high doses of Rut (0.5-4.0 g/L) showed an increase in antioxidant enzymes activity and in the expression of glutathione biosynthesis and conjugation genes [13], whereas in wheat plants exposed to moderate doses (up to 0.1g/L) no changes were observed in leaf H_2O_2 nor in thiobarbituric acid reactive substances (TBARS) contents [15].

Concerning Ana, malondialdehyde (MDA) content and the activity of antioxidant enzymes increased in fenugreek stems and leaves [16]. However, several antioxidant enzymes were compromised in rice plants exposed to Ana up to 0.5 g/L [17] and in *Arabidopsis* the antioxidant vitamin E showed an hormetic response [18]. Data on ROS amounts are also controversial with increases in species as cabbage [19], while unchanged in other species like barley [20]. Moreover, the dose and growth system may condition plant responses as observed in wheat, where plants grown on soil (up to 100 mg/kg; [6]) showed a boost in H_2O_2 content while plants grown hydroponically had no changes in ROS [15].

Exposure to P25 induced an antioxidant response in faba bean leaves, which was dependent on particle size [21], whereas in lettuce and oilseed rape no alterations were found in antioxidant enzyme activities [22]. In wheat plants P25 effects were dependent on the plant organ, enhancing total antioxidant activity (TAA) in leaves but decreasing MDA and TAA values in roots [7].

If the TiO_2-NP act mostly at the cytoplasmic biochemical level or if they regulate the gene expression of enzymes involved in the antioxidant battery remains unclear. Metal-based NP exposure affected the regulation of several genes, including some that encode oxidative stress response enzymes [23-26]. Concerning TiO_2-NP effects on gene expression, the limited information available suggests that these NP at high doses may impact gene regulation. In *Arabidopsis* plants exposed to 100 mg/L, P25-NP changed gene expression across a range of metabolic pathways, including genes related to stress response [27, 28]. In tobacco plants, TiO_2-NP up to 10 g/L affected the expression of microRNA and upregulated *ascorbate peroxidase* (*APX*) and *alcohol dehydrogenase* (*ADH*) genes [29], and in tomato (grown on 0.5-4.0 g/L Rut) increased the expression of genes encoding glutathione related biosynthesis and conjugation enzymes [13].

Bearing in mind the importance and dissemination of P25-NP, the scarce information available concerning P25-NP effects on plant oxidative stress and gene expression and the results obtained in previous works of our group [7, 30, 31], we hypothesize here that long-term exposure to P25-NP would differently affect wheat organ oxidative status, mainly due to different detoxification systems. In order to confirm this hypothesis, we comprehensively analysed different antioxidant pathways concerning the ascorbate-glutathione (AsA-GSH) cycle (enzymatic and non-enzymatic antioxidants) and other enzymatic antioxidants, *viz*. catalase (CAT), superoxide dismutase (SOD) and guaiacol peroxidase (G-POX). Non-enzymatic antioxidant levels and enzymatic antioxidant activity were supplemented with growth evaluation and H_2O_2 content, and the expression analysis of antioxidant enzyme encoding genes [*SOD*, *CAT*, *ascorbate peroxidase* (*APX*), *G-POX*, *monodehydroascorbate reductase* (*MDHAR*) and *dehydroascorbate reductase* (*DHAR*)].

2. Material and Methods

2.1 NP dispersion and characterization

TiO_2-NP [Aeroxide® P25: anatase and rutile (80:20); purchased from Sigma Aldrich - St. Louis, MO-USA; purity ≥ 99.5%; 21 nm and 35-65 m^2/g surface area] stock suspensions (1 g/L) were prepared in Milli-Q water, sonicated and used to prepare the final concentrations in Hoagland's nutritive solution (1/4 strength). Based on the literature, predicted environmental concentrations [32] and previous work of this group [7, 30, 31] the following final concentrations were used: 5, 50, and 150 mg/L. NP characterization and dispersion in suspension was already published [7].

2.2 Plant material and growth conditions

Seeds of *Triticum aestivum* L. cv. Arthur were germinated under TiO_2-NP and grown according to Silva et al. [7]. After 3 days germination under 0, 5, 50 or 150 mg/L TiO_2-NP, plantlets were transferred to containers with the same TiO_2-NP concentration prepared in Hoagland's pH 5.8. Plants were grown for 17 days under controlled conditions [7] and the suspensions were continuously aerated. Roots/shoots length and fresh biomass measurements were assessed at the end of the exposure period and roots/leaves samples were frozen in liquid N_2 and stored at -80°C for the biochemical and transcriptional analysis. Ti content in both shoots and roots was reported by Silva et al. [7].

2.3 H_2O_2 quantification

Hydrogen peroxide was quantified according to Silva et al. [33] by incubating the plant extracts with catalase (CAT) and the absorbance was read at 505 nm. The amount of H_2O_2 was obtained against a H_2O_2 standard curve (R^2=0.97). Four leaves/roots pools of 7-10 plants each were used.

2.4 Protein and antioxidant quantifications

Soluble proteins were determined by the Bradford [34] method using the Total Protein Kit, Micro (Sigma-Aldrich, USA). Four leaves/roots pools of 7-10 plants each were used for protein and antioxidant quantifications.

More detailed information about the antioxidant quantifications (2.4.1 and 2.4.2) is found in supplementary data.

2.4.1 Enzymatic antioxidants

Two different enzyme extracts were used: *A*) catalase (CAT), guaiacol peroxidase (G-POX) and superoxide dismutase (SOD); *B*) L-ascorbate peroxidase (APX), glutathione reductase (GR), dehydroascorbate reductase (DHAR), and monodehydroascorbate reductase (MDHAR). The extract for *A)* was prepared by grinding frozen samples with 0.1M potassium phosphate buffer containing 0.5 mM EDTA. The extract for *B)* was prepared according to Murshed et al. [35]. The homogenates were centrifuged and the activities were determined.

CAT activity was assayed by estimating the decrease of H_2O_2 at 240 nm [36] and using the 39.4 L/mol/cm molar extinction coefficient. G-POX specific activity was estimated at 470 nm [37] and using the 26.6 L/mmol/cm molar extinction coefficient. SOD activity was determined by indirect measurement of formazone as product of nitro-blue tetrazolium reduction with superoxide radicals at 560 nm [33, 38].

The APX activity was assayed by recording the decrease in AsA content at 290 nm [39] and using the 2.8 L/mmol/cm molar extinction coefficient. GR, MDHAR and DHAR activity were determined according to Murshed et al. [35]. For GR and MDHAR the absorbance was recorded at 340 nm and the specific activity was calculated using the 6.22 L/mmol/cm molar extinction

coefficient. DHAR activity was determined by measuring the increase in the reaction rate at 265 nm and using the 14 L/mmol/cm molar extinction coefficient.

2.4.2 Non-enzymatic antioxidants

Total AsA (AsAt), total glutathione (GSHt), GSSG and total non-protein thiols (NPT) concentrations were determined following according to Queval and Noctor [40]. Briefly, frozen samples were extracted with HCl, centrifuged and the supernatants were neutralized with NaOH. For AsAt the increase in absorbance at 265 nm was read, after incubation with dithiothreitol (DTT) and in the presence of ascorbate oxidase, and the 14 L/mmol/cm molar extinction coefficient was used. GSHt and GSSG were measured by the GR-dependent reduction of 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) at 412 nm. For GSSG, neutralized extracts were incubated with 2-vinylpyridine. GSH was estimated from the difference between total glutathione and GSSG.

2.5 Gene Expression: RNA extraction, primer design and qPCR

The method of Le Provost et al. [41] was used for RNA extraction, with modifications (see detailed description in supplementary data). RNA was extracted from ground material by adding cetyltrimethylammonium bromide based extraction buffer heated at 65ºC. After 65ºC incubation, chloroform:isoamyl alcohol (CIA) mixture was added and samples were centrifuged 16,000xg. The upper phase was mixed with CIA, centrifuged and this process was repeated twice. The upper phase was mixed with sodium acetate:absolute ethanol mixture and incubated at -20°C. Samples were centrifuged (16,000xg), the pellets were then washed with ethanol and centrifuged. After this, pellets were left to dry, resuspended in diethyl pyrocarbonate (DEPC)-treated water and stored at -80°C. For cDNA synthesis, 2 µg total RNA were incubated with DNase I (amplification grade, Life Technologies, Carlsbad, CA-USA) according to manufacturer's instructions. RNA was then reverse-transcribed with 1 µM oligo$(dT)_{18}$ using the Omniscript RT kit (Qiagen, Hilden, Germany). After reverse transcription, cDNA solutions were diluted in ultrapure water and qPCR reactions were performed with 150 nM each gene-specific primer, iTaq SYBR Green Supermix (BioRad, Hercules, CA-USA) and 1:4 (v/v) prediluted cDNA.

The cDNA-solutions were subject to denaturation (see detailed description in supplementary data), and a melting program was performed at the end of qPCR. Average PCR efficiency values and cycle thresholds (CTs) were estimated from the fluorescence data using the algorithm Real-Time PCR Miner [42]. At least 3 qPCR technical replicates were performed per sample from each of 2 independent leaves/roots pools of 7-10 plants.

Gene-specific primers (Table 1) were designed from published *T. aestivum* coding sequences using the Primer3 design tool according to [43].

2.6 Statistical analyses

Values are given as mean ± standard deviation. The comparison between treatments was made using One Way ANOVA test, followed by a Holm Sidak Comparison Test when data was statistically different ($p < 0.05$) or using t-test. All analyses were performed using Sigma Plot (Systat Software, San Jose, CA). Principal component analysis (PCA) was performed using the "CANOCO for Windows" programme v4.02. The heat map analysis was performed using the Microsoft Excel for Mac (version 15.32).

3. Results

3.1 Growth analysis and H_2O_2 content

Wheat length and biomass were only affected in shoots and at the higher concentrations, with a decrease of 22 and 25% in length and biomass, respectively (Table 2).

Concerning H_2O_2 content was only observed a trend to increase at higher concentrations in leaves ($p>0.05$) (Table 2).

3.2 Protein content and antioxidants

Concerning total protein content, changes were detected only in roots exposed to 150 mg/L TiO_2-NP, decreasing 31.1% (Fig. 1A).

3.2.1 Enzymatic antioxidants

TiO_2-NP exposure induced significant alterations only in the activity of GR (Fig. 1C) and APX (Fig. 1F): GR increased under 150 mg/L in both organs; APX diminished in leaves under 50 mg/L, nevertheless a trend to decrease was also detected in roots.

No significant changes were detected in MDHAR activity, but roots exposed to the highest concentration presented 67% higher activity than the control ones (Fig. 2A). In both roots and leaves an augment in DHAR activity was observed: 120% and 102% in roots and leaves, respectively (Fig. 2B).

3.2.2 Non-enzymatic

The exposure to TiO_2-NP induced imbalances in the glutathione and ascorbate pools as well as in NPT contents (Fig. 3). Furthermore, the response profile differed between roots and leaves.

GSHt decreased in 50 and 150 mg/L exposed roots (44 and 81%, respectively) and in leaves treated with 50 mg/L (Fig. 3A). In roots, GSSG increased with exposure (50 mg/L) whereas the GSH values decreased more than 80% under 50 and 150 mg/L. These results lead to a decline in

GSH/GSSG values below 0.5 in 50 and 150 mg/L exposed roots, corresponding to a decrease of > 90% of the control values.

In leaves, GSH augmented under the lowest concentration whereas decreased at 50 mg/L. GSSG decreased with TiO_2-NP treatments and GSH/GSSG values increased at the highest concentration (Fig. 3 B, D, E).

NPT augmented in roots treated with 50 mg/L but were impaired under 150 mg/L. In leaves, NPT increased in plants treated with 150 mg/L (Fig. 3C).

Concerning AsAt changes were detected only in leaves, with an increase in all treatments (86, 109 and 56% higher than control in 5, 50 and 150 mg/L, respectively) (Fig. 3F).

3.3 Gene Expression

Exposure of TiO_2-NP led to a generalized decrease of the relative expression of antioxidant enzymes encoding genes in both wheat roots and leaves, excluding *SOD* and *GR* in leaves (Fig. 4). In wheat roots, both 5 and 150 mg/L TiO_2-NP treatments down regulated the expression of *CAT*, *APX*, *GR*, *MDHAR* and *DHAR* genes in more than 50%. In leaves, significant down regulation was only obtained under 150 mg/L, nevertheless a decreasing trend was also observed under 5 mg/L. *CAT* was the gene more affected with an expression decrease of 88.9% whereas *SOD* was the only gene that was up regulated, increasing 56.5 and 50.7% at 5 and 150 mg/L, respectively.

3.4 PCA approach

The biochemical data obtained in this work were subjected to principal component analysis (PCA) in order to comprehend the mechanisms triggered by TiO_2-NP exposure. The PCA in leaves (Fig. 5) explained 79.6% of variance between the treatments: the first principal component (PC1) (X-axis), was conditioned by DHAR, NPT, GR, H_2O_2, SOD and GSH/GSSG; the PC2 (Y-axis) was defined only by AsAt, G-POX and total proteins. In roots, the PCA explained 96.4% of the variance, the PC1 was conditioned by MDHAR, DHAR, GSHt, GR, SOD, length and total proteins and the PC2 was defined by CAT, APX, AsAt, GSH/GSSG and H_2O_2.

In both leaves and roots the first PC (PC1-PC2) isolated 150 mg/L from the other treatments and with the PC2 it was possible to separate control and 50 mg/L. Concerning the lowest concentration, in leaves it was closer to 50 mg/L whereas in roots was closer to the control.

The highest concentration in leaves was positively associated with DHAR, NPT, GR, H_2O_2, GSH/GSSG and SOD but unrelated to length and biomass, whereas in roots 150 mg/L exposure was associated with GR, SOD, MDHAR and DHAR and unrelated with length, proteins and GSHt. In leaves, 50 mg/L treatment was correlated with AsAt whereas in roots was related with APX and GSH/GSSG. Control was associated with APX and GSHt in leaves and AsAt and NPT in roots.

4. Discussion

Reactive oxygen species are produced at unstressed conditions during normal development transitions and mostly as a product of photosynthesis and respiratory metabolism [11]. Under stressful conditions the equilibrium between ROS production and scavenging can be disturbed and plants may have to face oxidative stress, with leaves being especially prone to it due to photosynthetic processes. To counteract the elevated ROS production and maintain the redox status plants use the antioxidant machinery and ROS themselves act as signalling molecules inducing differential gene expression [44].

In this work, shoots showed higher susceptibility to TiO_2-NP exposure than roots, showing decreased fresh and dry weight [7] and length simultaneously with a trend to increase H_2O_2 content, as can be seen in PCA and heat map results (Fig. 5 and 6). This behaviour was paralleled by increases in membrane permeability and TAA at the highest concentration and Ti translocation to leaves, as reported previously [7]. Contrarily, TiO_2-NP decreased root TAA and MDA content [7] and root length and biomass were not affected. Additionally, control roots exhibited higher enzymatic antioxidant pool (SOD, CAT, G-POX, GR, APX, MDHAR) than leaves, which is in line with the superior values of TAA observed by Silva et al. [7] and shows that roots have naturally higher capacity to detoxify ROS boost than leaves. Since Ti presence was detected in both organs, the observed impairments may be a consequence of direct interaction of Ti and/or TiO_2-NP with tissues/molecules, nevertheless water or nutrient supply limitations should also be considered due to TiO_2-NP deposition at roots surface [7]. An overview of the organ differential response and direct *vs* indirect action of TiO_2-NP exposure are represented in Fig. 6.

With TiO_2-NP treatment no significant changes were detected in the first line defence enzymes SOD, CAT and G-POX activities, but a trend to decreased activity was observed in APX (close to control score in PCA) with no effects on AsAt content. Similarly, red bean roots and leaves exposed to similar doses of Ana showed no changes in CAT, SOD and APX activities [12]. It seems that the preferential route for ROS detoxification in TiO_2-NP exposed roots was the already existing enzymatic antioxidant capacity (Fig. 6). Nevertheless, roots showed TiO_2-NP susceptibility by decreasing in TAA, total protein content and oxidising the GSH pool (Fig. 6) with the decrease of GSH (50 mg/L) and increase of GSSG (50 and 150 mg/L). The higher decrease of total protein content in roots, comparatively to leaves, may be a consequence of accentuated down regulation of protein synthesis in this organ, as it was found a more accentuated underexpression of genes encoding for antioxidant enzymes in roots than in leaves. The alteration in the GSH pool and in the GSH/GSSG ratio seems to be a consequence of an increase of the DHAR activity not accompanied by the needed increment of GR activity. Changes in GSH *de*

novo synthesis and/or phytochelatins (PCs) production should also be considered once GSHt levels decreased, but as NPT also decreased this last hypothesis seems less plausible. Similarly to wheat roots, in a macrophyte exposed to TiO_2-NP (P25) GSH/GSSG ratio decreased together with GR increase. GSH is a key component of metal scavenging due to its thiol group, as precursor of PCs, as redox buffer and as signal transducer [45]. The signalling pathway generated by GSH is dependent on the dose and duration of the disturbance [45]. In both roots and leaves it is clear that TiO_2-NP dose influences the GSH redox status with higher doses inducing higher effects. Other factor that affected GSH pool in wheat and turned evident in the PCA (Fig. 5) was the plant organ, where leaves and roots showed opposite behaviour: in roots there was an oxidation of GSH pool whereas in leaves the GSH/GSSG ratio increased. This distinct behaviour may induce different signalling routes with divergent outcomes (damage vs acclimation) [45] and ultimately may limit plant growth and survival. Organ dependent antioxidant responses were also triggered to restore redox homeostasis in plants under other metals such as Pb [46] and Cd [47].

As observed in roots, leaf GR and DHAR activities increased (Fig. 6), but in this organ GSSG recycling to GSH by GR counterbalanced its increased generation by DHAR. In tomato roots and leaves exposed to Rut, it was found an overexpression of GST genes of other glutathione-S-transferase (GST), but not of DHAR [13], which unlike in wheat was not responsive.

The slight decrease on GSHt content in wheat leaves may be justified by the increase of NPT (NPT close to 150 mg/L score in PCA; Fig. 5) indicating that PCs production at the expense of GSH may have occurred. NPT increase in leaves together with the increase of S in xylem sap in these plants [7] shows that plants augmented the need for S, probably to produce thiols (eg. GSH and PCs) as a leaf mechanism to sequester and detoxify ROS and Ti translocated to leaves [7]. This hypothesis is exposed in Fig. 6 and should be further analysed. Changes in GSH (increase) and GR (decrease) induced by TiO_2-NP (Ana) were detected in lettuce leaves [48]. It seems that GSH-related metabolism may be a crucial mechanism triggered to detoxify TiO_2-NP induced ROS augment [49, 50]. GSH can react directly with ROS or act as an indirect antioxidant as reducing agent of DHAR to produce AsA in the AsA-GSH cycle, an essential cycle in plant defence against oxidative stress [51]. This cycle was of particular importance in leaves, where was prompted to face TiO_2-NP toxicity, contrarily to roots (Fig. 5 and 6). Ascorbate is widely spread in the cell compartments, acting as a direct and indirect antioxidant, and also in hormone signalling [52] and gene expression regulation. In chloroplasts, AsA participates in the Mehler peroxidase reaction to control the cellular redox state [52] and prevent the oxidative damage of the photosynthetic apparatus and other molecules. In this study, leaves exposed to 50 mg/L presented APX activity decrease (APX was unrelated to TiO_2-NP treatments in PCA, Fig. 5) and *APX* gene expression was down regulated, compromising H_2O_2 detoxification. On the other hand, AsAt content in leaves increased (positively associated with 50 mg/L in PCA, Fig. 5) in all concentrations and Cu/Zn-*SOD* was overexpressed (5 and 150 mg/L). This, together with other

cytostatic and physiological impairments reported here and by Silva et al. [7] and Dias et al. [31] namely in the photosynthesis, suggest that TiO_2-NP induced ROS burst and that leaves triggered the Mehler reaction and AsA-GSH cycle to detoxify it in detriment to the first line enzymatic antioxidants. On the other hand, AsAt increase may be a consequence of *de novo* synthesis to be also incorporated in other antioxidant routes such as in the xanthophyll cycle, required for the thermal dissipation, and in the regeneration of the antioxidant tocopherol [53]. Nevertheless, AsA augment together with GSH/GSSG ratio (150 mg/L) and NPT (150 mg/L) increments were incapable to scavenge ROS and did not prevent oxidative damages of chlorophyll *a* and impairments on electron transport chain and ultimately of efficiency of PSII and in net photosynthetic rate already reported for these wheat plants [31] (Fig. 6).

Interestingly, the expression of genes encoding antioxidant enzymes (Cu/Zn*SOD*, *CAT*, *APX*, *GR*, *MDHAR* and *DHAR*), except for *SOD* in leaves, were underexpressed in roots and/or leaves and, mostly for the highest concentration (150 mg/L), which did not parallel the estimated enzyme activity as can be observed in Fig. 6. This may reflect a time lag between cytoplastic-biochemical responses (including the fast activation of antioxidant enzymes) and the regulation of genes expression in the nucleus. The gene expression analyses, as all the antioxidant evaluations in this study, were made after 20 d exposure. This time lag observed between biochemical and transcriptional endpoints evidences the complex relationships often observed when these two approaches are combined to assess the same response [54]. We propose here that the biochemical changes observed may evidence transient cytosolic short-term responses to the stress, while the triggered transcriptional changes may represent more definitive adaptations occurring in the cell transcriptome towards a longer adaptation to these nanoparticles. These transcriptional changes may thus involve numerous pathways of adaptation, which include profound biochemical, cellular, and physiological changes, as stressed by Morari et al. [54] for drought and salt stress. However, other explanations like the interference of regulatory feedback mechanisms exerted by eg. ROS or by protein/intermediates should also be considered. Independently of the mechanism, the observed underexpression in roots together with decreased TAA and antioxidant enzymes activity indicates that longer exposure periods will lead to oxidative damage and root growth impairments, as those detected in leaves. Moreover, once at 150 mg/L TAA values in roots and leaves become similar [7] it seems that above this concentration root cellular metabolism and development will be compromised.

Due to their photocatalytic property, TiO_2-NP are prone to produce ROS under light [10]. Chloroplasts are major sites of ROS production, being particularly sensitive to excessive ROS generation. Considering that Ti/ TiO_2-NP translocation to leaves was already demonstrated in these wheat leaves [7] and in other wheat plants [15], we hypothesize that direct light exposure promoted higher TiO_2-NP photocatalytic activity, which induced superior ROS burst and higher oxidation in leaves at the higher doses. Once roots naturally produce lower levels of ROS than

leaves and are not directly exposed to light, we also propose that the NPs photocatalytic activity was inferior thus leading to lower increases of ROS compared to leaves. So, in roots the higher content of TiO_2-NP but not the higher photocatalytic activity was responsible to the superior decrease of gene expression and enzymatic antioxidant activity observed.

4.1 Conclusions

The discussed above together with the fact that in all treatments leaves showed higher contents of non-enzymatic antioxidants such as AsA, GSH and NPT than roots suggest that leaves and roots use distinct antioxidant main routes to face TiO_2-NP toxicity: in roots the preferred path is related with the antioxidant enzymatic machinery and the pre-existing antioxidant capacity whereas in leaves the thiols, AsA and AsA-GSH cycle are favoured (Fig. 6). Nevertheless, the thiol-related antioxidant machinery had an important role in detoxification against TiO_2-NP toxicity in both organs, which is in line with other authors [49].

Despite TAA increment [7] and induction of antioxidant machinery under the highest concentration, leaves were not able to face cellular oxidation (Fig. 6). Considering also the photosynthesis impairment in these plants, it remains to clarify if this oxidative unbalance is mostly due photosynthetic processes or have their origin in non-photosynthetic related sources. Our results also point out that the use of these NP above 5 ppm in agriculture may hinder crop performance and, under these doses, NP effects should be further analysed.

5. Acknowledgements

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Tables

Table 1. Gene-specific primers

Gene	Description	Oligonucleotide primers
SOD	Cu/Zn superoxide dismutase, plastidial	L: GAAGATGACTTGGGAAAAGGTG R: GATGCAAAACCAGAGATGGAA
CAT	catalase 1	L: CGCATCCTCGACTTCTTCTC R: TTGACGAGCGTGTAGGTGTT
APX	ascorbate peroxidase 2, cytosol	L: TTTGTGGGGAGAAGGAAGG R: GCCTCAGCGTAGTCAGCAA
GR	glutathione reductase, cytosol	L: GCGTCATACGTGGCTGTGT R: CTTTTTCCAGTTGTAGTTGATGTCC
MDHAR	monodehydroascorbate reductase 6, plastidial	L: AGGCTGTTCCACCATACGAG R: CCACCAGATCCAACACAGG
DHAR	dehydroascorbate reductase	L: CTGGAAGGTCCCCGAAAC R: GTTCTCCTTGGTCGCCTTG
UBC21	ubiquitin-conjugating enzyme 21	L: GCGACTCAGGCAATCTTCTC R: GCAACCCTGCAAATCACTCT

Table 2. *Triticum aestivum* length and fresh biomass (roots and shoots) and H_2O_2 content (roots and leaves) exposed to 0, 5, 50 and 150 mg/L TiO_2-NP (P25). Values are mean ± SD. Different letters indicate statistical differences between treatments ($p \leq 0.05$).

	Length (cm)		**Biomass (mg)**		**H_2O_2 (nmol mg/FW)**	
TiO_2-NP (mg/L)	Roots	Shoots	Roots	Shoots	Roots	Leaves
0	32 ± 4.0 [a]	38.0 ± 1.5 [a]	270 ± 86.4 [a]	609 ± 72.5 [a]	0.40 ± 0.152 [a]	0.19 ± 0.038 [a]
5	29 ± 4.0 [a]	35.3 ± 4.1 [a]	249 ± 66.5 [a]	631 ± 109.6 [a]	0.39 ± 0.049 [a]	0.21 ± 0.055 [a]
50	30 ± 5.5 [a]	34.5 ± 2.0 [ab]	265 ± 63.2 [a]	529 ± 55.2 [ab]	0.31 ± 0.064 [a]	0.27 ± 0.193 [a]
150	26 ± 5.4 [a]	29.7 ± 4.2 [b]	270 ± 63.6 [a]	451 ± 66.2 [b]	0.45 ± 0.026 [a]	0.54 ± 0.368 [a]

Figures

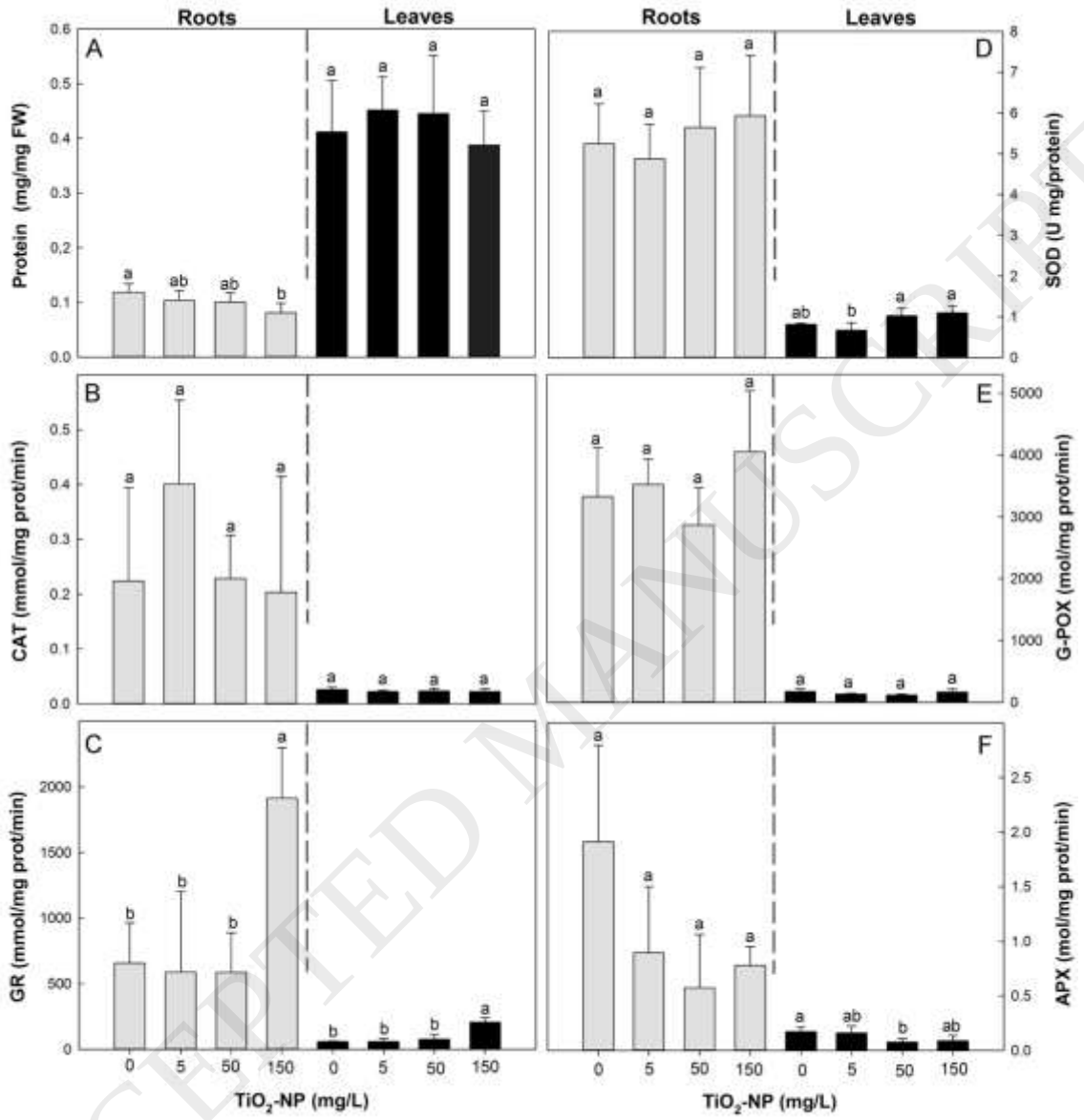

Figure 1. Variation in total protein content (A) and enzymatic activity of catalase (CAT) (B), glutathione reductase (GR) (C), superoxide dismutase (SOD) (D), guaiacol peroxidase (G-POX) (E) and ascorbate peroxidase (APX) (F) in *Triticum aestivum* roots and leaves exposed to 0, 5, 50 and 150 mg/L TiO_2-NP (P25). Values are mean ± SD. Different letters indicate statistical differences between treatments ($p \leq 0.05$).

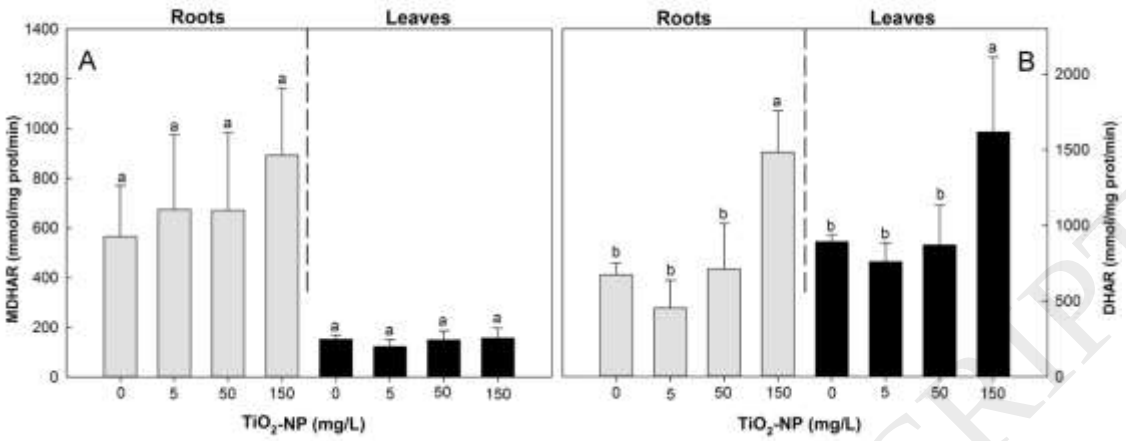

Figure 2. Variation in monodehydroascorbate reductase (MDHAR) (A) and dehydroascorbate reductase (DHAR) (B) activities in *Triticum aestivum* roots and leaves exposed to 0, 5, 50 and 150 mg/L TiO_2-NP (P25). Values are mean ± SD. Different letters indicate statistical differences between treatments ($p \leq 0.05$).

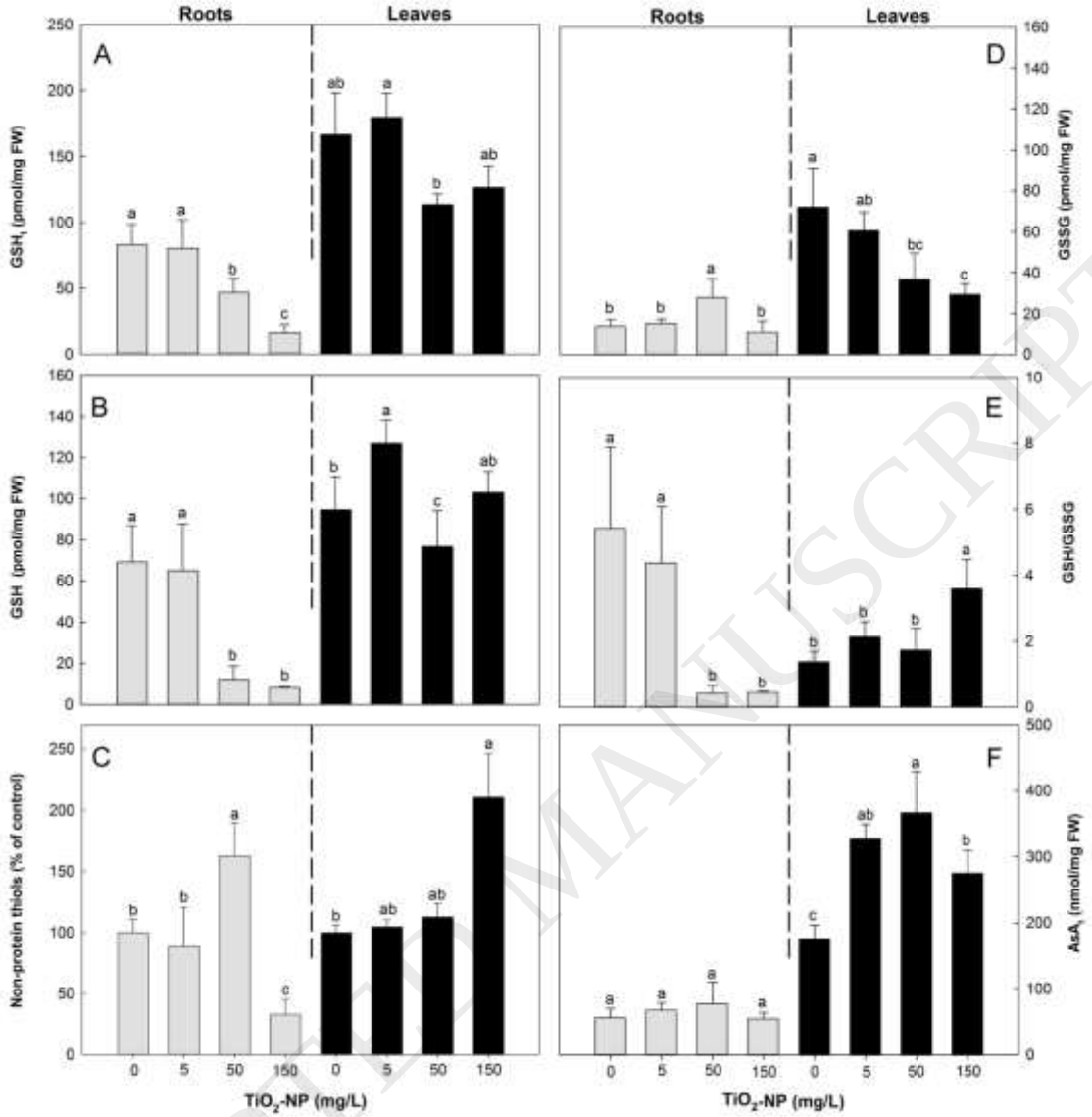

Figure 3. Variation in total glutathiones (GSH_t) (A), reduced glutathione (GSH) (B), non-protein thiols (C) and oxidized glutathione (GSSG) (D) contents, GSH/GSSG ratio (E) and total ascorbate (AsA_t) contents (F) in *Triticum aestivum* roots and leaves exposed to 0, 5, 50 and 150 mg/L TiO_2-NP (P25). Values are mean ± SD. Different letters indicate statistical differences between treatments ($p \leq 0.05$).

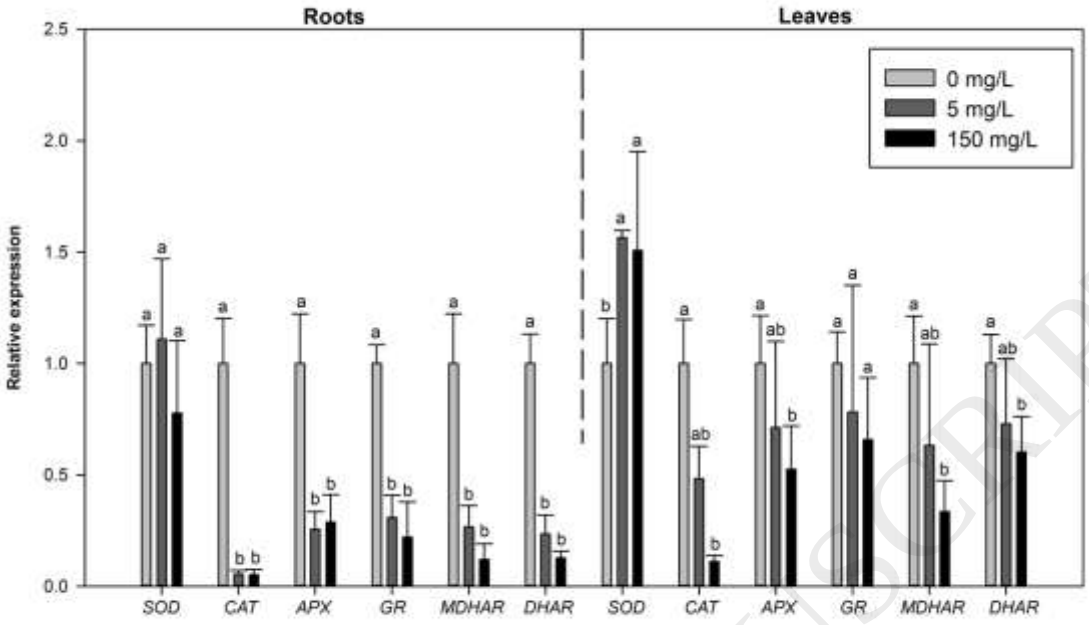

Figure 4. Variation in relative expression of genes encoding antioxidant enzymes in *Triticum aestivum* roots and leaves exposed to 0, 5, 50 and 150 mg/L TiO_2-NP (P25). Values are mean ± SD. Different letters indicate statistical differences between treatments ($p \leq 0.05$).

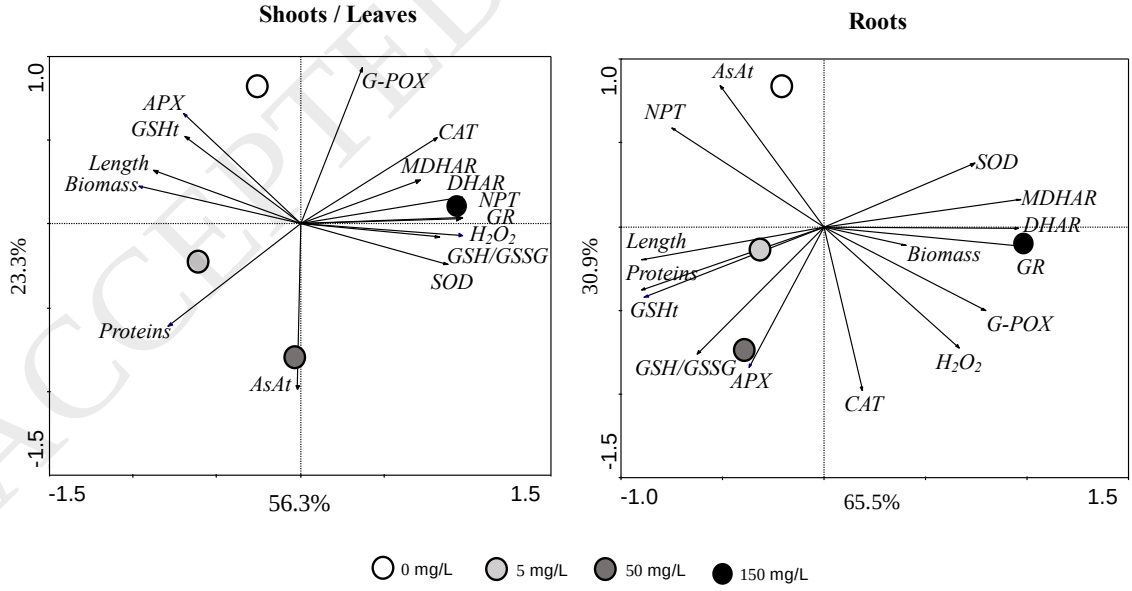

Figure 5. PCA analyses of biochemical responses of *Triticum aestivum* shoots/leaves and roots exposed to 0, 5, 50 and 150 mg/L TiO_2-NP (P25). For shoots/leaves, variance is explained by the two first components (C1 and C2) was: X-axes 56.3% (C1); Y-axes 23.3% (C2). For roots,

variance explained by the two first components (C1 and C2) was: X-axes 65.5% (C1); Y-axes 30.9% (C2).

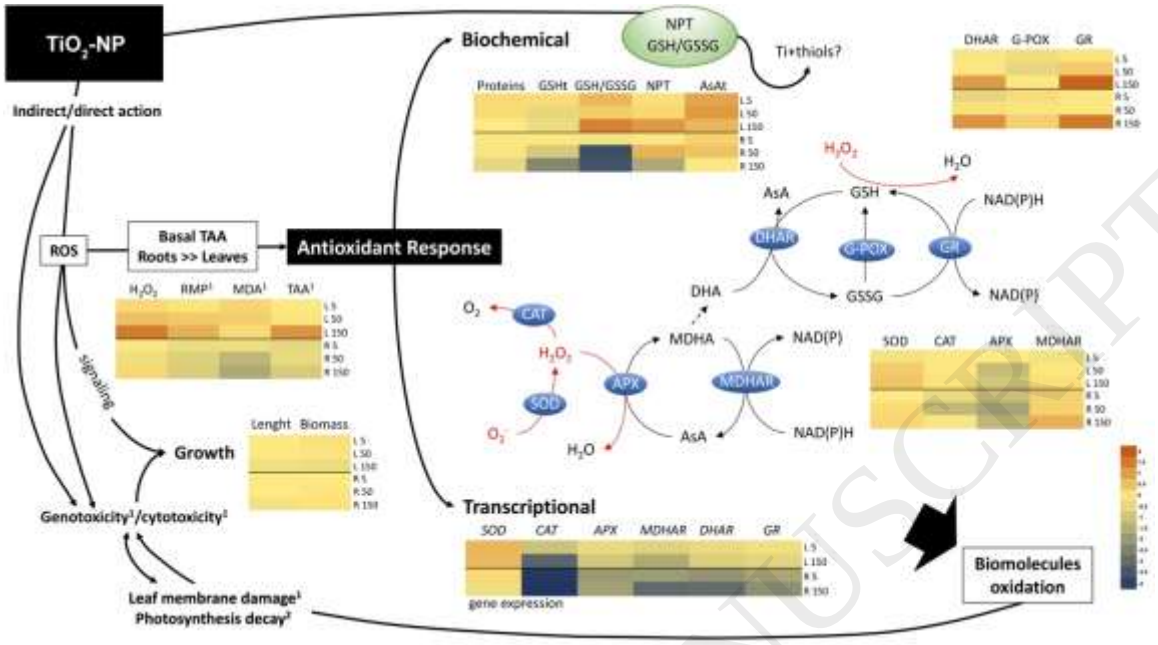

Figure 6. Squematic representation of principal effects and antioxidant routes triggered by roots and leaves to detoxify ROS formation induced by TiO_2-NP (P25) exposure. Relative levels are expressed as log2 (5/0 mg/L) or (50/0 mg/L) or (150/0 mg/L), and given besides each parameter as a heat-map. L: leaf; R: roots; 5 (5 mg/L); 50 (50 mg/L); 150 (150 mg/L). [1]Genotoxicity/cytotoxicity effects and RMP, MDA and TAA data were already described by Silva et al. [7], from which they were adapted. [2]Effects on photosynthesis were reported by Dias et al. [31].