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AMELIORATION WITH TITANIUM DIOXIDE NANOPARTICLE FOR REGULATION OF OXIDATIVE STRESS IN MAIZE (*Zea mays* L.)

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ABSTRACT

In the present experiment, *Zea mays* L. was evaluated for its C_4 modality through oxidative stress responses. Moreover, under variable concentrations (0, 50, 100µM) of hydrogen peroxide, the impacts were variable and modulated with titanium dioxide nanoparticle (TiO_2-NP). Plants scored with elevated concentrations of H_2O_2 and superoxide ($O_2^{\cdot-}$) maximum by 1.31 & 1.52-fold respectively over 0µM H_2O_2. TiO_2-NP recovered those maximums with 8.69 & 7.89% as compared to control plants. The effects of H_2O_2 were moderated with malondialdehyde and carbonyl content by peak values 7.45 & 5.91% under TiO_2-NP treatment respectively. A depletion in proline content recorded under H_2O_2 stress but recovered through highest 1.23-fold with TiO_2-NP application. TiO_2-NP recorded NADP-ME activities up-regulated in concentration gradient of H_2O_2. Oxidative exposure was also documented by *in-vivo* detection of H_2O_2 and $O_2^{\cdot-}$ by 3,3′-diaminobenzidine and nitroblue tetrazolium staining. For the anti-oxidative cascades superoxide dismutase activity had induced by 1.42-fold under H_2O_2 when pre-treated with TiO_2-NP. However, glutathione reductase activity was differentially modulated under H_2O_2 through interaction with TiO_2-NP. Activity of guaiacol peroxidase was significantly up-regulated by 1.07-fold when TiO_2-NP applied. The inductions of oxidative stress recorded more evident when a distinct polymorphism for GPX protein resolved variations in band intensities, but not in number. Activity of catalase experience a down-regulated trend all through the H_2O_2 exposure and had significant sensitivity to TiO_2-NP. The results depict the nature and reactivity of C_4 modules under oxidative stress that would be the selection pressures and that might be modulated with TiO_2-NP application

Keywords: TiO_2-nanoparticle; Oxidative stress, Anti-oxidant enzymes, Malic enzyme, Maize

INTRODUCTION

In C_4 system the CO_2 concentration mechanism empowers with an added advantage towards abiotic stress tolerance of plants. The refixation of CO_2 out of cellular respiration including photorespiration is a benefit for C_4 plants through partitioning of carbon more towards the storage compounds **(Bauwe, 2010)**. Still, there recorded a significant variation in loss of carbon through photorespiration in C_3 species which is literally unavoidable. The storage compounds derived from photosynthesis includes structural carbohydrates, compatible solutes, secondary metabolites etc. All these may be well supported with in-built or induced physiological responses under stress. A higher value of water use efficiency could support the osmotic stability of C_4 plants which is perturbed in almost abiotic stresses **(Raven *et al.*, 2004)**. The higher light compensation points of C_4 plants minimizes the photo damages in such a way that photosynthetic photon flux density must not be compromised. Therefore, besides from photo-oxidative stress, C_4 species are expected to be more adaptive against any kind of stress than C_3 **(Yamori *et al.*, 2014)**. With this, C_4 species have been its embracing with xenobiotics and other oxidative stress in plants than C_3. Few of those, however, important industrial pollutants (like H_2O_2) are quite noxious to plants in perturbance of cellular redox. In consequence of the above, oxidative stress is being established. H_2O_2, not a free radical is perceived in plants with dual modes: an inducer of cellular responses as well as a de-generative moiety over the tolerable concentration **(Quan *et al.*, 2008)**. Evidences reveal that enhanced concentration of H_2O_2 disrupts the plants' metabolism in C_3 plant, but may be adjusted for C_4 plants **(Stepien & Klobus, 2005)**. It is the precise concentration of H_2O_2 that may develop an antioxidation cascades through enzymatic as well as non-enzymatic paths **(Ahmad *et al.*, 2008)**. In the present experiment, *Zea mays* L., an established C_4 syndrome practiser has been evaluated from the viewpoints of oxidative stress with inputs of chemical elicitors. The chemical elicitation is a form of allelopathy where any residues either endogenous or exogenous in nature would be supportive in plant responses. These responses may not be necessarily supportive to plants with its normal pace of metabolism but also to develop few other metabolic fluxes in stress tolerance **(Thaler *et al.*, 1999)**. Elicitors may include few growth regulators, simple inorganic salts, secondary metabolites etc. Moderation of plant responses by chemical elicitations is accomplished through two basic paths. Adjustment of osmotic status or cellular hydration, the first and sustenance of biological redox would the second. Thus, bioaccumulations of proline are a reliable index for osmoticum along with few enzymes for C_4 photosynthesis **(Kavi Kishor & Sreenivasulu, 2014)**. NADP-malic enzyme (NADP-ME) is such a C_4 tissue specific protein which concentrates CO_2 from organic acids in anaplerotic reactions. In another mode plants are much revised with different forms of antioxidations by chemical elicitations through both enzymatic and non-enzymatic paths **(Almeselmani *et al.*, 2006)**. To score oxidative exposure plant tissues are often evaluated with their specific reactions mostly the peroxidation types. Lipid peroxidation, protein oxidation, oxidised products of nucleic acids are quite common in plants response to oxidative burst **(Das & Roychoudhury, 2014)**. The antioxidative proteins are mostly induced with their *de-novo* synthesis by few elicitors as already reported in few C_4 species. This was more established with the post translational modifications of those proteins under inductions of few chemicals. The antioxidative enzymes are also evoked with supplement of electron donors from few phenolic residues. Ascorbate, glutathione, guaiacol are most common to induced peroxidase activity in lysis of H_2O_2. Nanomaterials have been another scope for chemical elicitations in plant responses, particularly under abiotic stresses **(Prasad *et al.*, 2017)**. With the modern state-of-art metals and metalloids are extensively implicated with nano forms/structures and proved to be useful. In crop system, engineered nanoparticles (ENPs) from different metals/metalloids verified their efficiency in slow release fertilizer or amendments **(Duhan *et al.*, 2017)**. Likewise, an established and well useful nano-material, like TiO_2 has been implicated to modulate the plant responses, however, varied in crop species **(De *et al.*, 2018)**. As earlier report, maize proved the selectivity for few hazardous metals for hyper-accumulation. This is more illustrated with the tolerance of those by an improved anti-oxidation **(Baig *et al.*, 2010)**. Therefore, the present scope of work, experiments deal with how and whether ENP with TiO_2 has any modulation on

such anti-oxidation in maize would be judicially worth.. With this support we hypothesised that TiO_2-NP would be more realistic to modulate the anti-oxidation potential of maize when faced with oxidative stress inducing agent. H_2O_2 would one of those as documented earlier **(Schützendübel *et al.*, 2002)**. Thus, the present experiment is embodied with the illustration of some physiological responses in maize under varying concentrations of H_2O_2 and to monitor the effects of TiO_2-NP thereon.

MATERIAL AND METHODS

Experimental plant material

The experimental works were conducted in the laboratory of Plant Physiology and Plant Molecular Biology Research Unit, Department of Botany, University of Kalyani, Kalyani-741235, India. The maize seeds (cv. Kaveri 50) were collected from the local farmers. Initially seeds were thoroughly washed to remove all surface bound debris, surface sterilized by 0.01% $HgCl_2$ and finally sun dried. Then seeds were allowed to germinate as described earlier **(Sarkar *et al.*, 2017)** and after sprouting seedlings were allowed to grow for 15 days under control condition of light, moisture content (≥80%) and temperature (30-35°C) in a growth chamber. Seedlings were transferred in nutrient solution of ¼th Murashige & Skoog **(Murashige & Skoog, 1962)** media in two sets and acclimatized for 2 days. This was followed by pre-treatment of 300mg/L TiO_2-NP, pH 7.5 against one set as control (0mg/L) for 3 days. TiO_2-NP (particle size <100nm) with stabilizing agent as cetyl trimethyl ammonium bromide (CTAB) was synthesised in Department of Chemistry, University of Kalyani, properly characterised with opto-physical properties and reported in earlier **(De *et al.*, 2018)**. After 3 days of pre-treatment the sets were further divided into sub-sets with ¼th of the same media & pH in addition of exogenous application of H_2O_2 concentrations: 0, 50 & 100µM. The whole sets were transferred to normal conditions of growth chamber of light 900-1200 $\mu Em^{-2}s^{-1}$, 37-38°C, 80-85% relative humidity. All the treatments were replicated thrice with seedlings in RBD design. On completion of treatment of 3 days plants were recovered, washed with distilled water, separated into root and shoot, preserved under liquid nitrogen and stored in -80°C for further biochemical use.

Biochemical analyses

Biochemical analyses were performed from the shoot portion of the maize plant sample according to standard methods under various treatments condition.

Determination of H_2O_2 & $O_2^{\cdot-}$ content

H_2O_2 content was determined according to **Velikova *et al.* (2000)**. 1.0 gm of fresh tissue was thoroughly crushed under cold condition in 5mL of 1% (w/v) trichloroacetic acid (TCA) solution. The clear supernatant was achieved with centrifugation at 12000 x g for 15 min at 4°C. The supernatant containing the hydrogen peroxide were preserved in cold condition by addition of 10mM phosphate buffer (pH 7.0) and 10mM potassium iodide (KI) solution. The set was incubated in dark for half an hour. The oxidized KI under H_2O_2 presence was measured spectrophotometrically at 390nm. Standard of H_2O_2 was prepared and the content of H_2O_2 was determined as µM/gm f.wt.

For generation of $O_2^{\cdot-}$ content, 1.0 gm fresh tissue was homogenised into fine powder with liquid nitrogen **(Elstner & Heupel, 1976)** and extracted in a buffer of 60mM potassium phosphate buffer (pH 7.5) on centrifugation at 10000 x g for 15 min at 4°C. The supernatant was diluted with 65mM phosphate buffer (pH 7.8) and 10mM hydroxylamine hydrochloride at 25°C and kept for 30 min. The reaction was stopped with 10mM sulphanilamide and 7mM α-naphthyl amine at 25°C for 20 min. The absorbance was taken at 530nm with the blank and the content was derived from a standard of NO_2^-.

Histochemical detection of H_2O_2 & $O_2^{\cdot-}$

In-vivo detection of ROS were done both from leaf and root samples. Leaves and roots were cut into uniform sizes and thoroughly washed with double distilled water and used for infiltration.

Accumulation of H_2O_2 was detected by *in-vivo* infiltration of 3'3'-diaminobenzidine (DAB) as suggested by **Thordal-Christensen *et al.* (1997)**. Freshly collected leaves from the treatment were infiltrated with 5mM freshly prepared DAB solution in phosphate buffer (pH 6.8). The sample in solution was kept for over-night in dark. First the pH of the buffer reduced to acidic condition (pH 3.8) and then adjusted to pH 6.8 towards dissolving the DAB completely. On following day, leaves were maid colourless by removing the chlorophyll using lactic acid-glycerol-ethanol solution (1:1:4-v/v/v) following gentle boiling in water-bath for 10-12 min. The distribution of hydrogen peroxide bound DAB products were visualized as brown patches and were digitally captured (Dewinter camera).

For the detection of superoxide ion ($O_2^{\cdot-}$) within the leaf tissue, an incubation mixture with 50mM phosphate buffer containing 6mM Nitroblue tetrazolium (NBT) salt (pH 4.8) was used **(Fryer *et al.*, 2002)**. On overnight infiltration, the leaves were made transparent as mentioned above. Then the formazan complex with deep blue patches were captured as described above.

Determination of MDA content

For lipid peroxidation, the concentration of MDA was detected as suggested by **Heath & packer (1968)** with slight modification. Under ice cold condition leaf tissues thoroughly homogenised on 80% ethanol. The debris were discarded on centrifugation under ice cold condition at 12000 x g for 15 min and saved the supernatant. An aliquot of 1mL was mixed with 20% TCA containing 0.5% Thiobarbituric acid (TBA). The mixture was reacted in a temperature of 95°C for 30-45 min. The absorbance at 532 and 600nm was recorded against blank in a UV-VIS spectrophotometer. The content of MDA was calculated and expressed as nM/gm f.wt. using $155 mM^{-1}cm^{-1}$ as extinction co-efficient of MDA.

Determination of carbonyl content

The carbonyl content of the leaf extract was done from 1.0 gm of fresh leaf tissue suggested by **Verbeke *et al.* (2000)** and homogenised in 6% sodium dodecyl sulphate (SDS) solution. The homogenate was incubated at 37°C for 30 min with freshly prepared 10mM 2, 4-dinitrophenylhydrazine (DNPH) solution in 1.5mM TCA. The whole mixture was homogenised under constant stirring. By addition of 10% (w/v) TCA solution under cold condition, it was de-proteinated. The coagulated proteins were re-extracted with 20% (w/v) TCA solution. The pellet was washed with 2% (w/v) diethyl ether followed by absolute alcohol. The absorbance was read at 360nm upon resuspend the pellet in 0.2 M phosphate buffer (pH 7.0). Using 530 $M^{-1}cm^{-1}$ as molar extinction co-efficient of DNPH, the carbonyl content was derived.

Determination of Proline content

Accumulation of proline content was analysed with the aqueous extract from 0.1 gm of fresh leaf tissue in 10 ml of 3% sulphosalicylic acid solution. The supernatant was set from centrifugation at 10000 x g at 4°C for 15 min. The supernatant was reacted with ninhydrin solution (dissolved in 0.2M citrate buffer (pH 5.4) with 0.8 gm stannous chloride and 2 gm ninhydrin which was initially dissolved in 100ml 2-methoxy ethanol.) The filtrate within this buffer was diluted by 1ml of glacial acetic acid and boiled for 30 min until the colour develops. The reaction was stopped on ice bath following constant stirring with 4ml 10% toluene. The aqueous toluene layer was aspirated and warmed gently. From the intensity of the colour at 520nm, the content of proline was estimated using a standard of proline according to **Bates *et al.* (1973)**. The concentration was expressed as µM/gm f.wt.

Assay of Nicotinamide adenine dinucleotide phosphate-malic enzyme (NADP-ME) activity

The activity of NADP-malic enzyme (EC 1.1.1.40) (L-malate: NADP-Oxidoreductase) was assayed as suggested by **Murmu *et al.* (2003)** with slight modification. Initially the plant was harvested and fully expanded leaves were excised during day time pre-exposed 4 hours under sun rays. The leaves were kept in darkness for 1 hour. The leaf segments were initially incubated with 2mM bicarbonate solution and illuminated with saturating photosynthetic photon flux density (900-1000). After illumination the enzyme extract was made in 1mL of extraction buffer containing 5mM $MgCl_2$, 10mM EDTA, 5mM DTT, 1µM protease inhibitor Cocktail (Sigma-Aldrich), 1mM PMSF, 1% PVP. The extract was centrifuged at 14000 x g at 4°C for 15 min. the supernatant was used as source of NADP-ME for the assay. The reaction mixture contains 15mM Tris-HCl (pH 8.0), 10mM $MgCl_2$, 0.5 mM NADP and 200µL of supernatant and the reaction was started by adding 5mM malate. The activity was assayed by reduction of NADP reading at 340nm in UV-VIS spectrophotometer. The activity was expressed as nM NADPH produced/min/mg protein.

Assay of anti-oxidative enzymes

For the anti-oxidative enzymes fresh leaf samples were collected from each treatment and frozen with liquid nitrogen to grime properly. The samples were homogenized with ice cold extraction buffer containing 50mM phosphate buffer (pH 7.0), 5mM $MgCl_2$, 1mM PMSF, 10mM EDTA, 1µM protease inhibitor Cocktail (Sigma-Aldrich). The homogenate was separated into supernatant by 14000 x g for 15 min at 4°C. Supernatant containing protein was partially purified with 80% ammonium sulphate ($(NH_4)_2SO_4$) cut. The recovered pellet of purified protein on overnight incubation at 4°C was recovered by centrifugation at 20000 x g for 15 min at 4°C. Finally, the pellet was dissolved in dilution buffer of 25mM phosphate buffer (pH 7.0) with 1.5mM DTT, 2µM BSA and 0.1% SDS. This was used as enzyme extract for following assays **(Sarkar *et al.*, 2017)**.

Guaiacol peroxidase (GPX: 1.11.1.7)

For the activity of GPX, 100µL of protein extract was reacted in a 3mL assay mixture containing 100mM phosphate buffer (pH 6.5), 1.5mM *o*-dianisidine and 20mM H_2O_2. The increase in absorbance at 430nm against a blank was read at 30 sec intervals to measure the changes of the absorbance by 0.1. From this the enzyme activity was calculated and expressed as Unit/mg protein as according to **Ammar *et al*. (2008)**.
For the *in-gel* analysis, partially purified protein was separated in a non-SDS or 10% native PAGE through 10V per lane at 4°C **(Ammar *et al*., 2008)**. After completion of electrophoretic separation, the entire gel was incubated in a solution containing 0.5mM *o*-dianisidine and 0.5% H_2O_2 dissolved in 50mM phosphate buffer (pH 7.0) in dark condition. The densitometric study of the gel was analysed by the Gel Analyzer software (2010a).

Superoxide dismutase (SOD: EC 1.15.1.1)

For the activity of SOD, the inhibition of photochemical reduction for NBT was considered. An equivalent volume of 100µg partially purified enzyme extract was incubated in an assay mixture: 100mM phosphate buffer (pH 7.0), 20mM methionine, 75µM NBT, 200mM EDTA and 100µL. By addition of 5mM riboflavin the reaction was initiated under continuous illumination of two 10W fluorescent lamp for 30 min. A blank set was run where illumination was withdrawn. The absorbance of the illuminated set was read at 560nm and activity was calculated with the inhibition of formazan formation through NBT reduction as suggested by **Giannopolitis & Ries (1977); Cakmak & Marschner (1992)**.

Glutathione reductase (GR: EC 1.6.4.2)

The *in-vitro* activity of GR was done as suggested by **Cakmak & Marschner (1992)**. For the enzyme activity, partially purified enzyme extract was reacted in an assay mixture containing 50mM Tris-HCl (pH 7.5), 0.15mM NAD(P)H, 1mM GSSG, 3mM $MgCl_2$ and 100µg equivalent partially purified protein extract. The changes of NAD(P)H oxidation with the decrease in absorbance at 340nm was recorded. Activity was expressed as µM NAD(P)H oxidized/min/mg protein using 6.22$mM^{-1}cm^{-1}$ as extinction co-efficient of NAD(P)H. Protein of the partially purified enzyme extract was determined with Bradford reagent **(Bradford, 1976)**.

Catalase (CAT: EC 1.11.1.6)

The catalase was assayed in an extraction buffer as suggested by **Verma & Dubey (2003)**. With 100µg equivalent protein from partially purified protein extract was reacted in 200mM phosphate buffer (pH 7.0) and 100mM H_2O_2. The incubated mixture at 25°C was immediately recorded for decreasing absorbance at 240nm. The activity of catalase was expressed as µM H_2O_2 oxidized/min/mg protein using the molar extinction co-efficient of 39.4 $M^{-1}cm^{-1}$ for H_2O_2.

Statistical analysis

Recorded data were statistically analyzed with one-way ANOVA using the general linear model. The variations between the means of treatments for three replications were compared with t-tests ($P \leq 0.05$).

RESULTS

Initially plants recorded hardly any significant morphological variations among the treatments. However, the oxidative stress imposed by varied concentration of H_2O_2 recorded with significant variation of the maize plants on cellular activities. Still, pre-treatment with TiO_2-NP satisfied for some responses on any moderation of oxidative stress in maize plants.

Changes of ROS under treatments

Both $O_2^{\cdot-}$ and H_2O_2 was consider to evaluate for the changes of ROS of control and pre-treated maize cultivar. It is clear from the observation that $O_2^{\cdot-}$ accumulation was dose dependent of H_2O_2 treatment. The ranges were 1.20 & 1.52-fold in control plants and 1.12 & 1.45-fold in TiO_2-NP pre-treated plants under 50 and 100µM H_2O_2 treatment as compared to 0µM H_2O_2. Likewise, the application of TiO_2-NP through pre-treatment was significantly in moderation of $O_2^{\cdot-}$generation. Thus, the minimum value was recorded by 10.00 and 7.89% under 50 and 100µM H_2O_2 treatment as compared to control (without treatments) plants (Figure 1). In a similar manner, accumulation of H_2O_2 had the compatible trend through the H_2O_2 treatments in plants. The maximum accumulation was recorded under 50 and 100µM of H_2O_2 treatment by 1.17 & 1.31-fold in control and 1.05 & 1.16-fold in TiO_2-NP pre-treated plants. Interesting to note that, TiO_2-NP pre-treatment on the 0µM H_2O_2 concentration had not any significant changes of H_2O_2 accumulation, whereas, plants were relieved by 7.31 and 8.69% with TiO_2-NP pre-treatment under respective higher oxidative exposure (Figure 2).

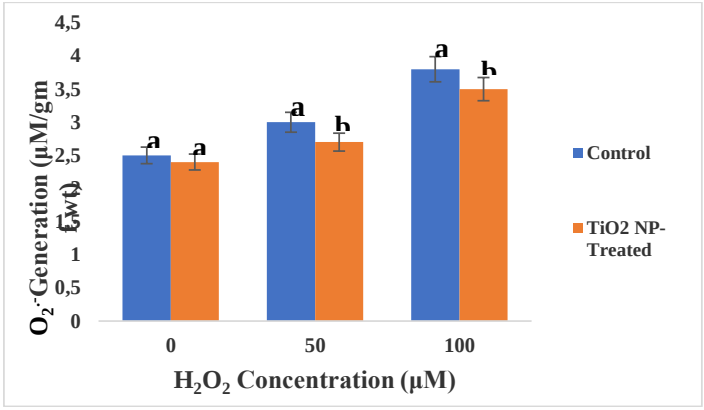


Figure 1 Determination of $O_2^{\cdot-}$ generation under H_2O_2 treatments (0, 50 & 100µM) in control and TiO_2-NP pre-treated maize plants for 3 days. Data represented as mean of three replicates (n=3) ±SE and SE represented as vertical bar on each column. Different letters indicate significant differences through Duncan's t-test at $p \leq 0.05$.

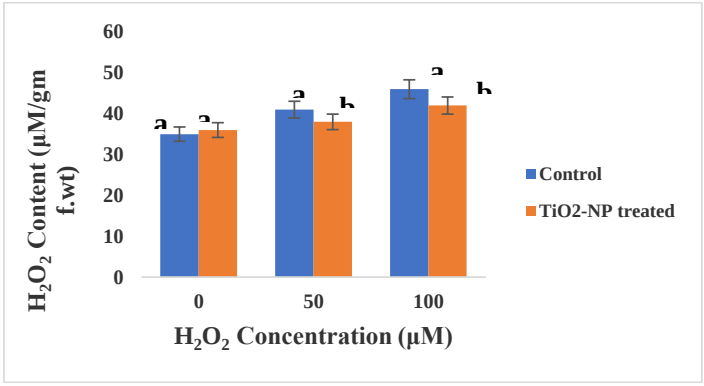
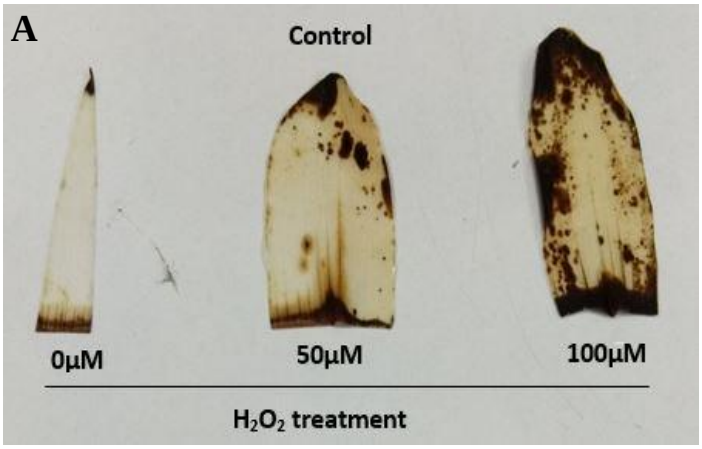


Figure 2 Determination of H_2O_2 content under H_2O_2 treatments (0, 50 & 100µM) in control and TiO_2-NP pre-treated maize plants for 3 days. Data represented as mean of three replicates (n=3) ±SE and SE represented as vertical bar on each column. Different letters indicate significant differences through Duncan's t-test at $p \leq 0.05$.

Tissue specific distribution of H_2O_2 and $O_2^{\cdot-}$

Figure 3C & D shows a distinct demarcation on leaf tissue specific *in-vivo* detection by NBT staining for ROS with regards to $O_2^{\cdot-}$. This is clear from the accumulation of ROS and its complex with tetrazolium through un-uniform patches more toward leaf base and margin. The impact of H_2O_2 is concomitant with the $O_2^{\cdot-}$ accumulation being the maximum patches on 100µM H_2O_2 concentration. Interesting to note that TiO_2-NP observed a significant moderation for $O_2^{\cdot-}$ accumulation through *in-vivo* detection. In comparison to untreated or control, the accumulation of $O_2^{\cdot-}$ recorded more less under TiO_2-NP pre-treatment. Another ROS, H_2O_2 was also more differentially accumulated under control and TiO_2-NP pre-treatment by. From figure 3A & B, it is quite evident that H_2O_2 exposure could also induce *in-vivo* accumulation of H_2O_2 by DAB staining, however, through concentration gradient. Still, on pre-treatment set regardless of H_2O_2 concentration TiO_2-NP undoubtedly registered its quite significant effect by down regulation of ROS accumulation.



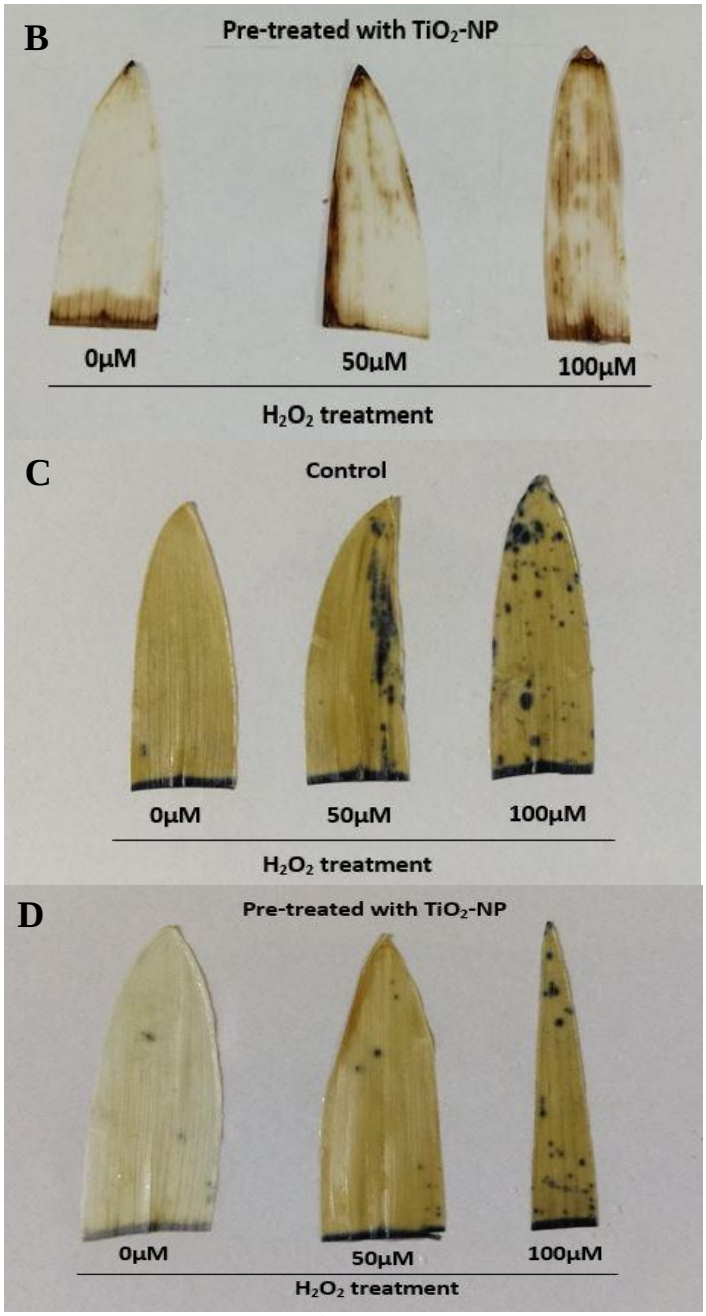
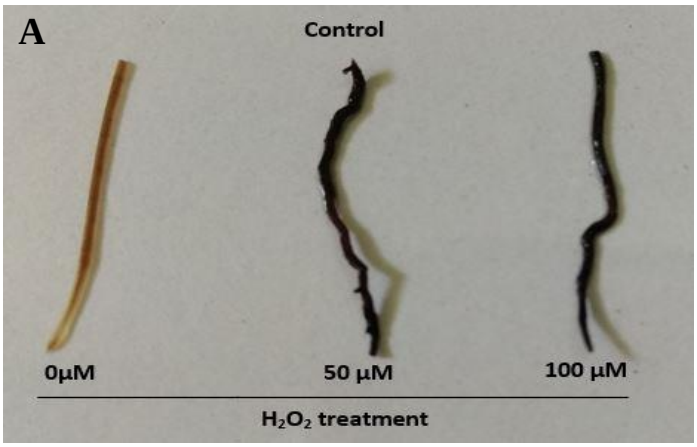
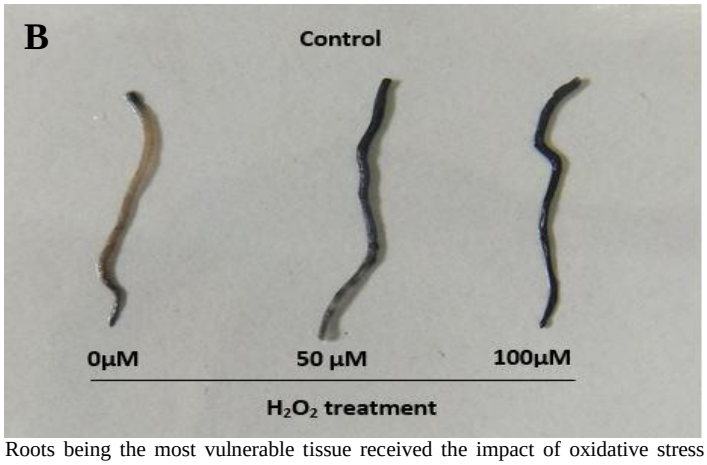


Figure 3 Histochemical detection of H_2O_2 by DAB staining (A and B) and $O_2^{\cdot-}$ by NBT staining (C and D) under 0, 50, 100µM H_2O_2 treatment in control (A & C) and pre-treated TiO_2-NP (B & D) maize plants in leaf.





Roots being the most vulnerable tissue received the impact of oxidative stress were recorded by NBT staining. In comparison to leaf, roots recorded more intense colouration in gradient 0-100µM H_2O_2 concentration under control which under gone more revised with TiO_2-NP pre-treated plants (Figure 4C & D). It is clearly shown that impact of nanoparticle is more pronounced in higher concentration of H_2O_2. The DAB staining of H_2O_2 accumulation had some differential expression where TiO_2-NP appeared as most retrieving at 100µM H_2O_2 (Figure 4A & B). Still, at lower concentration plants were retrieved to reduce the H_2O_2 by TiO_2-NP. Therefore, TiO_2-NP and H_2O_2 is more discriminating in reaction according to external concentration of H_2O_2.

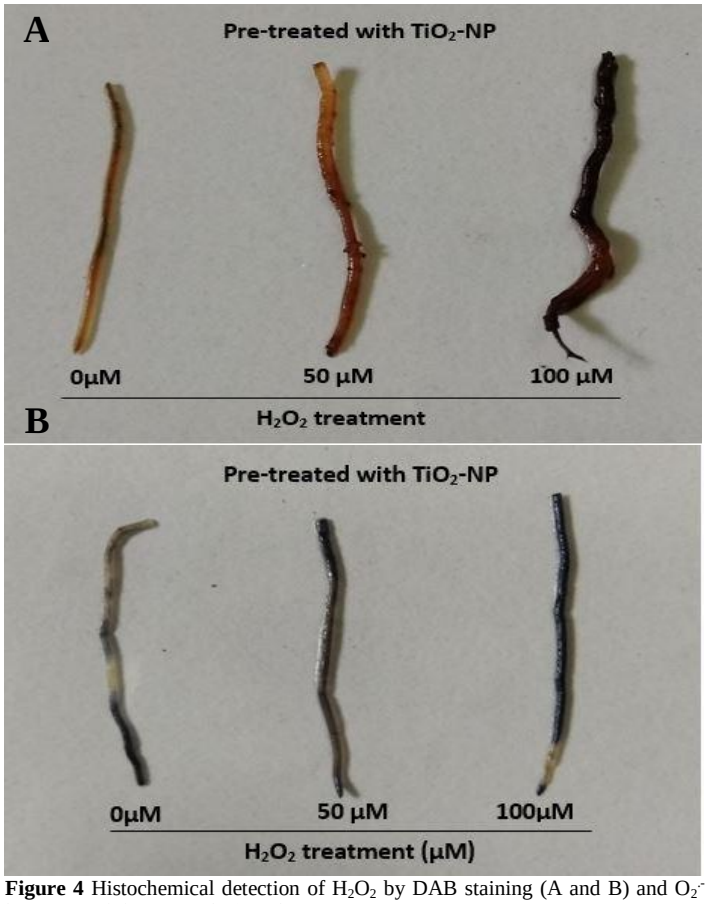


Figure 4 Histochemical detection of H_2O_2 by DAB staining (A and B) and $O_2^{\cdot-}$ by NBT staining (C and D) under 0, 50, 100µM H_2O_2 treatment in control (A & C) and pre-treated TiO_2-NP (B & D) maize plants in roots.

Generation of MDA and carbonyl content

On account of ROS induced oxidative damages, plants recorded a significant variation with regards to lipid peroxidation and protein oxidation. A quantification of lipid peroxide as MDA has synergistic trend according to control and TiO_2-NP pre-treatment was recorded. Thus, the peak accumulation of MDA at maximum H_2O_2 concentration was 1.23-fold as compared to 0µM H_2O_2 concentration in control plants. This was quite significant for the effect of pre-treatment with TiO_2-NP that show a moderation with 7.45 & 7.06% depletion at 50 and 100µM H_2O_2 concentration respectively and also 5.24% decline recorded even in 0µM H_2O_2 concentration (Figure 5). However, MDA content increases through H_2O_2 concentrations in both control and pre-treated plants. This was

quite inconsistent level of protein and its epoxide product as carbonyl content had an almost compatible trend. The moderation of carbonyl content of protein oxidation with TiO_2-NP pre-treatment was maximum at 50 & 100µM H_2O_2 concentration by lowering the value 5.91 and 5.74% as compared to control plants (Figure 6). But, at 0µM H_2O_2 concentration TiO_2-NP had not any impacts. Therefore, TiO_2-NP behaved as a reliever only when it encountered the oxidative stress mediated by varying concentration of H_2O_2.

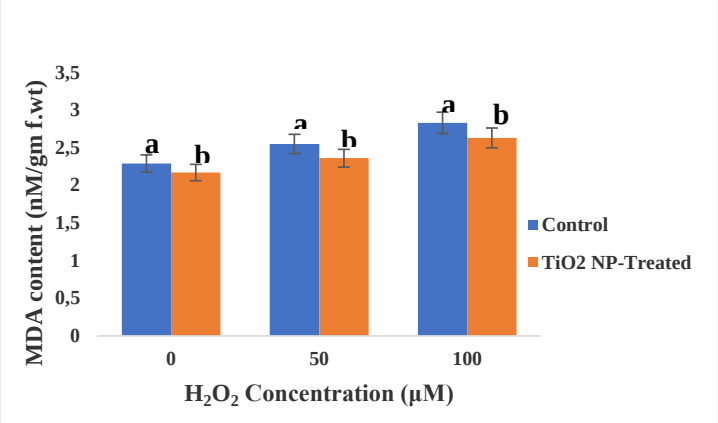


Figure 5 Determination of MDA content under H_2O_2 treatments (0, 50 & 100µM) in control and TiO_2-NP pre-treated maize plants for 3 days. Data represented as mean of three replicates (n=3) ±SE and SE represented as vertical bar on each column. Different letters indicate significant differences through Duncan's t-test at p≤0.05.

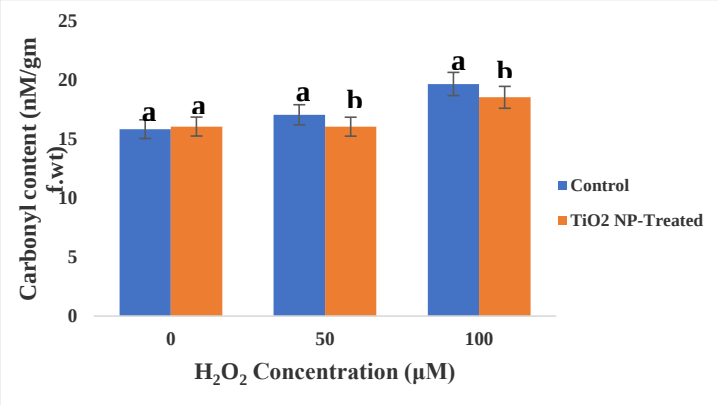


Figure 6 Determination of carbonyl content under H_2O_2 treatments (0, 50 & 100µM) in control and TiO_2-NP pre-treated maize plants for 3 days. Data represented as mean of three replicates (n=3) ±SE and SE represented as vertical bar on each column. Different letters indicate significant differences through Duncan's t-test at p≤0.05.

Changes in proline content

In the present experiment, the sensitivity of plants to H_2O_2 was also recorded otherwise through compatible solute accumulation. The activity of proline was granted as compatible solutes developed in the tissues as the plant progressed through 50, 100µM H_2O_2 concentration. Under control condition they are recorded a subdued accumulation of proline in a range of 11.6 & 26.6% (Figure 7). TiO_2-NP as pre-treatment was quite interesting to relieve the water stress as mediated by H_2O_2 through up-regulated values. Notably, the trend of proline accumulation under TiO_2-NP pre-treatment was in downhill order, however, significantly (P≤0.05) higher against control plants for each H_2O_2 concentration as exogenous exposure. Thus, the values recorded to retrieve the proline content by 1.17-fold & 1.23-fold (Figure 7) under 50 & 100µM H_2O_2 concentration established a good source of proline induction.

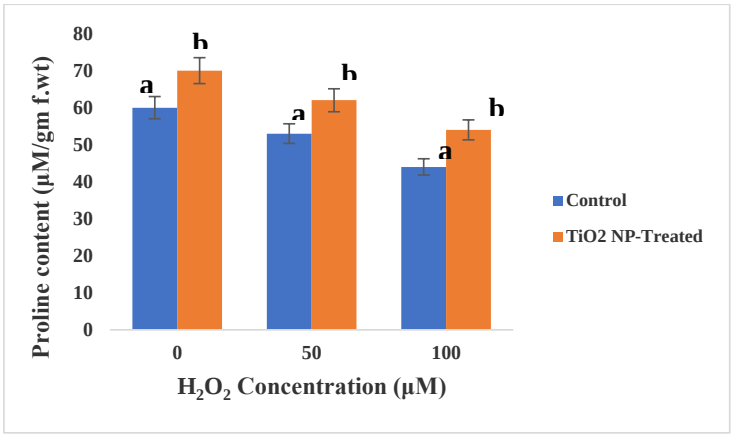


Figure 7 Determination of proline content under H_2O_2 treatments (0, 50 & 100µM) in control and TiO_2-NP pre-treated maize plants for 3 days. Data represented as mean of three replicates (n=3) ±SE and SE represented as vertical bar on each column. Different letters indicate significant differences through Duncan's t-test at p≤0.05.

Changes in NADP-ME activity

In an attempt to analyse the photosynthetic mode of plant's response through H_2O_2 toxicity NADP-ME activity was assayed. Interesting to note that, this C_4 enzyme, basically an oxidative decarboxylase has its discriminatory trend as a function of H_2O_2 concentration as compared to 0µM H_2O_2 concentration. The activity got significantly subdued under 50µM H_2O_2 treatment. This again retrieved the activity by 1.25 & 1.22-fold in control and TiO_2-NP pre-treated plants under highest concentration of H_2O_2, i.e. 100µM (Figure 8). The activity of TiO_2-NP pre-treated plants had the concomitant response following the H_2O_2 doses. Therefore, the TiO_2-NP as pre-treatment appeared to retrieve the decarboxylase activity with a complex manner with interaction of H_2O_2. The inducing activity of TiO_2-NP was evident both under 50 & 100µM H_2O_2 concentration which recorded 1.23 & 1.20-fold over expression as compare to control plants respectively.

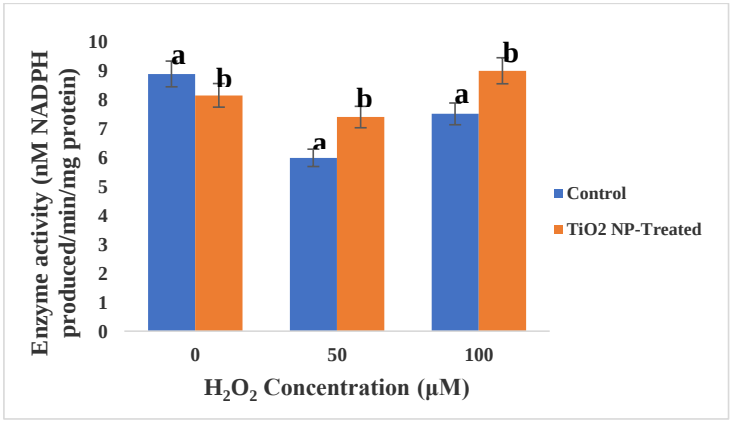


Figure 8 Assay of NADP-ME activity under H_2O_2 treatments (0, 50 & 100µM) in control and TiO_2-NP pre-treated maize plants for 3 days. Data represented as mean of three replicates (n=3) ±SE and SE represented as vertical bar on each column. Different letters indicate significant differences through Duncan's t-test at p≤0.05.

Changes in anti-oxidant enzymes activities

The activities of peroxidase when consider taking guaiacol as electron donor, it recorded an inconsistent manner as a function of H_2O_2 concentration. Thus, even under 0µM H_2O_2 concentration TiO_2-NP recorded as most inducing to raise the activity at the peak by 1.07-fold. This trend was maintained in compatible manner by 1.069-fold increase for 50µM H_2O_2 treatment over control plants. But the activity of enzyme subdued by 20.04 & 20.14% for control and TiO_2-NP pre-treated plants respectively at 50µM H_2O_2 concentration over the 0µM H_2O_2 concentration. However, the activity has significantly recovered with the induction of 100µM H_2O_2 by 1.23 & 1.11-fold increase in control and TiO_2-NP pre-treated plants respectively as compare to 50µM H_2O_2 concentration (Figure 9).

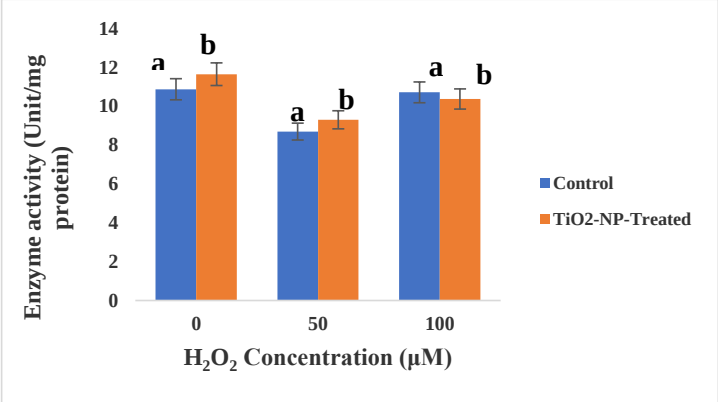


Figure 9 Assay of GPX activity under H_2O_2 treatments (0, 50 & 100µM) in control and TiO_2-NP pre-treated maize plants for 3 days. Data represented as mean of three replicates (n=3) ±SE and SE represented as vertical bar on each column. Different letters indicate significant differences through Duncan's t-test at $p \leq 0.05$.

From the zymographic studies with GPX as revealed by *o*-dianisidine reactions, the variations for polypeptides were quite significant (Figure 10). As a function of H_2O_2 concentration, maize plants recorded maximum over expression at 0µM H_2O_2 regardless of control and TiO_2-NP pre-treatment. Though there are no variations in number, however, their intensities varied all through the treatments. This was more clarified with densitometric analysis for individual polypeptides as represented in Figure 11. Interestingly, this trend of TiO_2-NP induced over expression was carried forward through H_2O_2 stress more towards lower concentration.

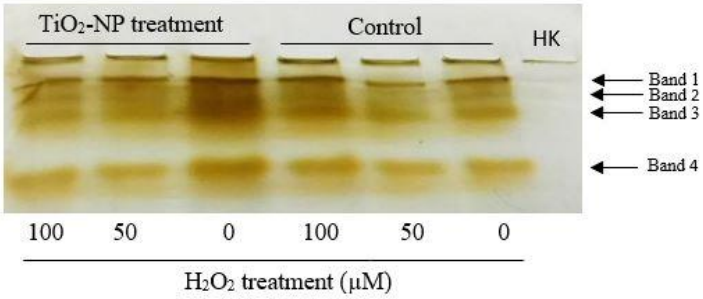


Figure 10 Resolving of polymorphism of peroxidase (GPX) through various H_2O_2 treatments (0, 50, 100µM) when interacted with TiO_2-NP. 'HK' denotes Heat killed enzyme protein.

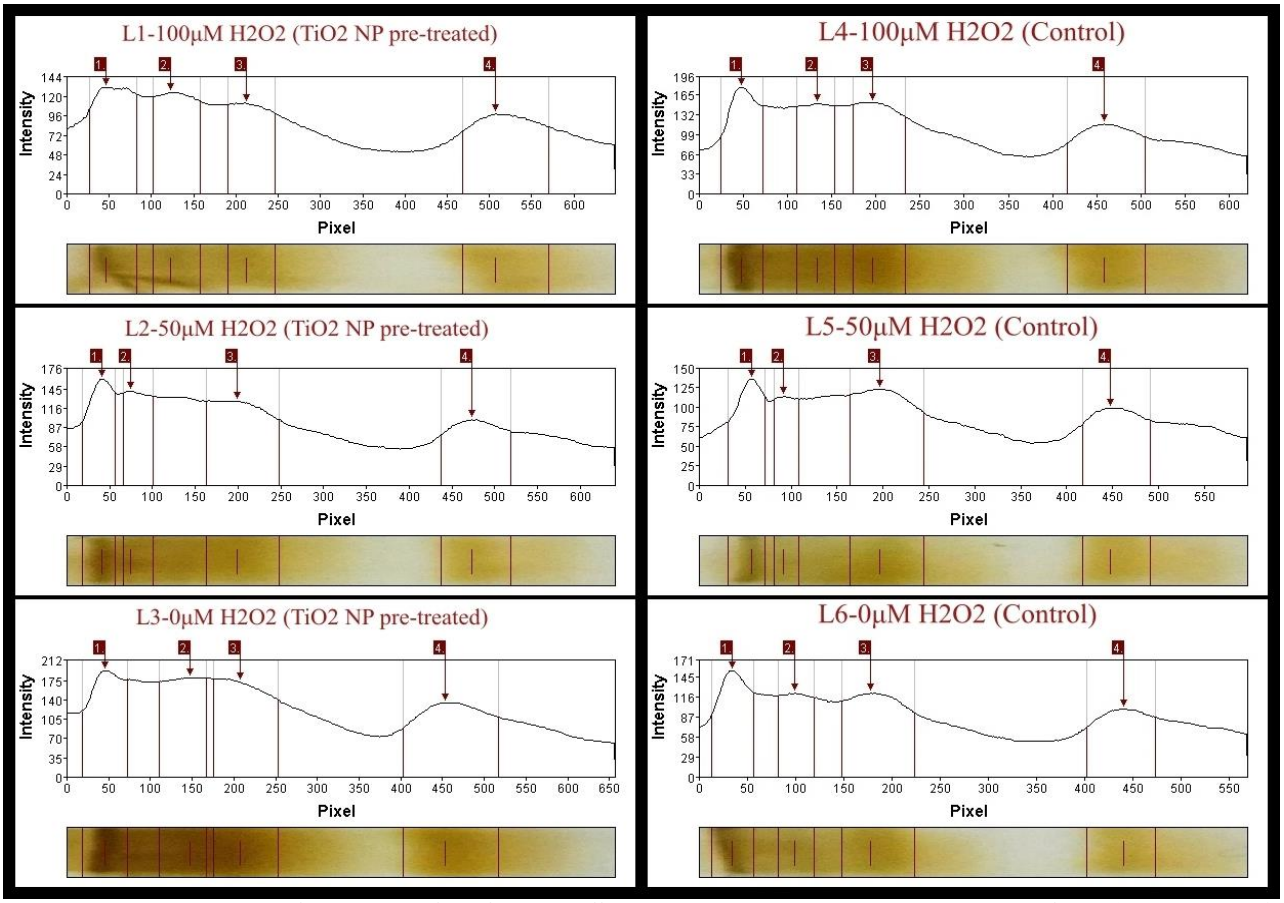


Figure 11 Densitometric analysis for intensity profiles of GPX on different polymorphic bands under varying treatments of H_2O_2 (0, 50 & 100µM) in control and TiO_2-NP pre-treated plants. L1, L2, L3, L4, L5 & L6 denotes the different lanes on native gel run with partially purified protein

In continuation with peroxidase reaction, the maize plants have also shown their potential to minimize the $O_2^{\cdot-}$ content by SOD activity. Interesting to note that in control plants the variations of H_2O_2 concentration was not significantly demarked the SOD activity. Still, TiO_2-NP becomes an inducer to over-express the SOD activities in a linear order by 1.27, 1.36 & 1.42-fold through H_2O_2 concentrations (Figure 12). Thus, in comparison to 0µM H_2O_2 concentration the variations in enzyme activities under TiO_2-NP pre-treatment were 1.18 and 1.15-fold through H_2O_2 concentrations.

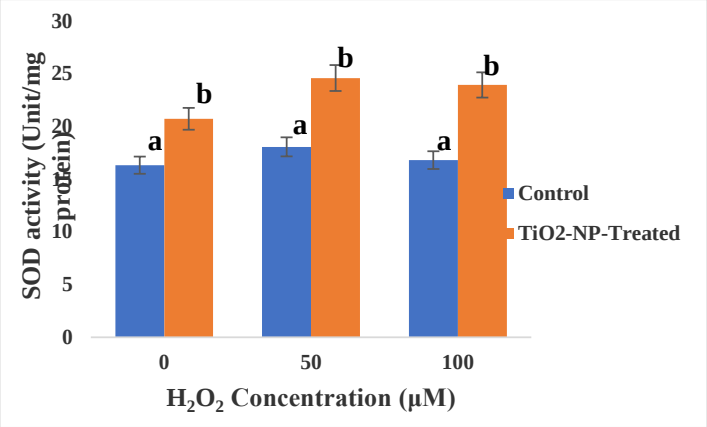


Figure 12 Assay of SOD activity under H_2O_2 treatments (0, 50 & 100µM) in control and TiO_2-NP pre-treated maize plants for 3 days. Data represented as mean of three replicates (n=3) ±SE and SE represented as vertical bar on each column. Different letters indicate significant differences through Duncan's t-test at p≤0.05.

In response to depleted redox through H_2O_2 concentrations the maize plants recorded a significant (P≤0.05) as well as discriminating variations in glutathione metabolism in the present experiment. Interesting to note that TiO_2-NP was in command to induce the GR activity over control plants but in downhill order through H_2O_2 concentrations (Figure 13). A record of 1.30-fold increase of GR activity with TiO_2-NP pre-treatment even under 0µM H_2O_2 concentration was found. It is also interesting to note that the changes in GR activities through 50 and 100µM H_2O_2 concentrations were discriminatory between control and TiO_2-NP pre-treatment. However, H_2O_2 and TiO_2-NP interactions had the maximum value with 1.42-fold increase under 50µM H_2O_2 concentration. This got reversed at 100µM H_2O_2 concentration where TiO_2-NP had suppressed the by 13.4% over 0µM H_2O_2 concentration.

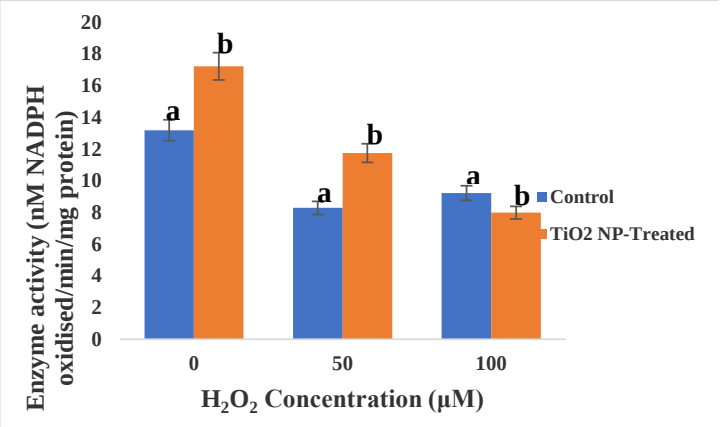


Figure 13 Assay of GR activity under H_2O_2 treatments (0, 50 & 100µM) in control and TiO_2-NP pre-treated maize plants for 3 days. Data represented as mean of three replicates (n=3) ±SE and SE represented as vertical bar on each column. Different letters indicate significant differences through Duncan's t-test at p≤0.05

In accompanying with anti-oxidative enzymes, CAT is the one which doesn't require any phenolic residues as electron donor to reduce the ROS. Therefore, in present case TiO_2-NP pre-treated plants are found with discriminating trends in activities passing through varying concentration of H_2O_2. Notably, the plants recorded the maximum CAT activity under 0µM H_2O_2 dose, more with TiO_2-NP treated plants by 1.17-fold over expression over control plants. Thereafter, a significant downhill activity through 50 and 100µM H_2O_2 concentration were recorded by 33 & 53% and 37 & 57% in control and TiO_2-NP pre-treated plants respectively as compared to 0µM H_2O_2 concentration (Figure 14). However, at intermediate H_2O_2 concentration (50µM) both control and TiO_2-NP treatment were significantly varied showing 1.10-fold expression under nanoparticle treatment. Unexpectedly at maximum H_2O_2 concentration had no such significant (P≤0.05) variation between control and TiO_2-NP pre-treated plants.

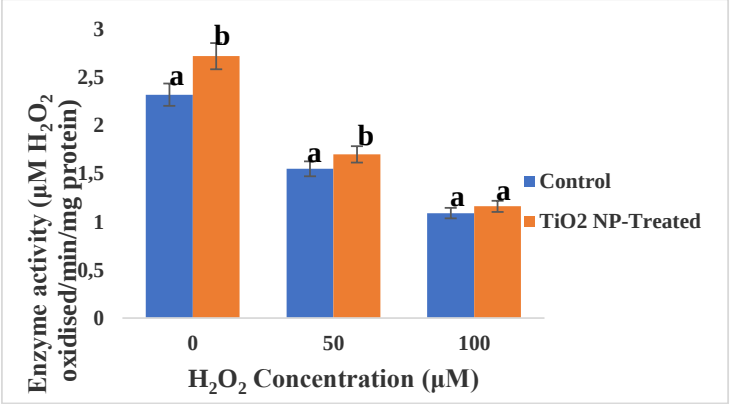


Figure 14 Assay of CAT activity under H_2O_2 treatments (0, 50 & 100µM) in control and TiO_2-NP pre-treated maize plants for 3 days. Data represented as mean of three replicates (n=3) ±SE and SE represented as vertical bar on each column. Different letters indicate significant differences through Duncan's t-test at p≤0.05

DISCUSSION

The impacts of nano particle mediated plant responses are complex as well as inconsistent in nature for genotypes and types of nano materials. Oxidative burst is common consequence for induced ROS accumulation in plant tissues. H_2O_2 has already been implicated with dual functions: as ROS itself and an inducer of biochemical paths as secondary messenger **(Petrov & Van Breusegem, 2012)**. Therefore, TiO_2-NP would be interesting to note the variations in ROS under the influence of oxidative exposure in maize. On initial observation, maize plants appeared to be more sensitive to accumulate a significant amount of H_2O_2 and $O_2^{\cdot-}$ over 0µM H_2O_2 dose. On the same time TiO_2-NP had been able to register its impact in down-regulation of ROS. TiO_2-NP has already been referred with some inconsistent manner to the plant physiological responses. In the cereal crops this particle has its implication to induce the germination, seedling growth but not to any changes in apoplastic ROS **(Goodarzi *et al.*, 2017)**. However, in the present experiment TiO_2-NP might be speculated with an effector to ROS mitigation. Gene activation either to induce the transcripts of secondary metabolites to scavenge the H_2O_2 or TiO_2-NP itself to interact the apoplastic ROS would be the possibilities **(Liu *et al.*, 2017; Zahra *et al.*, 2017)**. ROS being the primary inducer and inevitable fate of the molecular oxygen through it's over reduction is related to every kind of cellular process. With adequate osmotic potential, primarily, maize plants sustained the osmotic turgidity under H_2O_2 stress. This is evident from the down-regulation of proline in dose dependent manner. It is the fact that any abiotic factors influencing water stress may necessarily be linked with the changes of redox. In *vis-a-vis* a deficit of saturating moisture level induces the over-reduction of electron transport chain generating ROS. Maize, an established C_4 species is more tolerant to water stress and thereby, photo-oxidation and its down-stream effects of ROS generation thought to be related. Therefore, plant cellular redox and osmotic homeostasis are complementary to each through the involvement of proline metabolism **(Gupta *et al.*, 2016)**. Thereby, TiO_2-NP might have another role through maintenance of tissue hydration that undergoes perturbed with concentration of oxidizing agent, H_2O_2 as herein.

There is a clear-cut evidence about the interaction of H_2O_2 when *in-vivo* histochemical detection of H_2O_2 and $O_2^{\cdot-}$ were recorded. This is more pronounced with the midrib portion for $O_2^{\cdot-}$ accumulation by NBT staining within the tissues. This suggests initially the oxidative burst in development through the conducting tissues and its water stream movement by basipetally order. An excess amount of moisture deficit may initially be developed into tissue dehydration along the midribs **(Lu & Neumann, 1998)**. This might later be distributed through the mesophyll cells which are in-contiguous to xylem ducts and thus, a radical dispersion of the $O_2^{\cdot-}$ the results. This is quite in agreement that in the present study the distribution of $O_2^{\cdot-}$ in the leaves is in order of marginal or central midrib traversing to the mesophyll tissues. However, the amelioration of $O_2^{\cdot-}$ by TiO_2-NP pre-treatment would either be justified to block the conversion of oxygen into $O_2^{\cdot-}$ by single electron reduction **(Karuppanapandian *et al.*, 2011)** or/and a consistent lysis by SOD activities. Still, later is well established with proportional activities of SOD as a function of TiO_2-NP pre-treatment. Similar pattern was also recorded for DAB reaction mediated H_2O_2 accumulation *in-vivo*. In few citations it strengthens the activities of H_2O_2 lysing enzymes like peroxidase, catalase over-expressed with concomitant lysis for H_2O_2 **(Zhao *et al.*, 2012; Mandal *et al.*, 2016)**. TiO_2-NP mediated regulation of H_2O_2 production under consideration of oxidizing agents might not demand any accessory paths to check the generations of ROS. For the root segments H_2O_2 and $O_2^{\cdot-}$ appear to be most sensitive at the root tip region as it shown in the present experiment. Thus, the activity of TiO_2-NP for nano-treatment is also likely to concentrate on those regions. TiO_2-NP with its semi-conductor in nature has been more align with membrane potential of the root membrane. Thus, any changes regarding the

electron transfer through respiratory chain and its over reduction to generate ROS is likely to be erased by TiO_2-NP. Moreover, for collaborative assessment of $O_2^{\cdot-}$ and H_2O_2 lysis, SOD and GPX would be efficient instrumentals. The present investigation is more likely to accept this finding with earlier instances in maize treated with TiO_2-NP under different abiotic stress **(Yaqoob *et al*., 2018; Morteza *et al*., 2013)**.

As a consequence of oxidative stress as ensured by ROS ($O_2^{\cdot-}$ and H_2O_2) maize plants were synchronised with tolerance reactions to oxidative stress. Lipid peroxidation and protein oxidation are such those of ROS impacts where a sequential degradation of lipid residues are the features **(Catala, 2009; Mano, 2012)**. In plant system, the development of lipid peroxides, hydroperoxides and carbonyl contents are produced in nucleophilic reactions by ROS on unsaturated residues of the membrane. In the present experiment, TiO_2-NP was not appeared in a consistent manner but significant ($P \leq 0.05$) for moderation of ROS impacts. Thus, for both lipid and protein oxidation, the TiO_2-NP appeared as an ameliorator against H_2O_2 exposure. This obviously arises the possibilities for finer tuning but complex interaction of TiO_2-NP with H_2O_2. Possibilities may exist either to down-regulate H_2O_2 generations from reduction of $O_2^{\cdot-}$ and/or lysis of H_2O_2 by any nucleophilic reactions with TiO_2-NP. This was supported by other NP mediated direct oxidation of H_2O_2 following relieve the oxidative stress in crop species **(Sharma *et al*., 2012)**.

Maize being a typical C_4 species is worth of justification with carbon metabolism through minimized photo-respiratory loss of CO_2. The elevation of carbon loss through photo-respiration under abiotic stresses is compensated by few reactions for re-fixation of CO_2. NADP-ME is the key regulatory enzyme in oxidative decarboxylation of malate to release CO_2 **(Doubnerova & Ryslava, 2011)**. Undoubtedly, oxidative stress would be more prone to damage the cellular membrane by ROS that may hamper the chloroplastic reactions mechanisms also. In C_4 plants the high light compensation point would be more attributing to tolerate ROS either by development of anti-oxidation cascades or minimization of energy absorption **(Yamori *et al*., 2014)**. Whatever the cases replenish more CO_2 through oxidative decarboxylase like NADP-ME would be justified to study, particularly, with TiO_2-NP interaction. Through the gradient of H_2O_2 concentration, maize plants in the present experiment has been able to sustain the activity of NADP-ME more with TiO_2-NP application. Development of ROS and perturbed cellular redox are overcome by the C_4 traits as observed in other C_4 species **(Keech *et al*., 2017)**. This agrees with the present experiment that by TiO_2-NP moderation to circumvent re-generation of CO_2 by NADP-ME reactions. TiO_2-NP documented to induce the release of CO_2 from organic acids pool. This otherwise supports the anaplerotic reactions leading to more acquisition of reduced carbon. The later is required for allocation of carbon in other residues like anti-oxidants, compatible solutes, storage compounds, signalling molecules etc to support tolerance **(Gomez-Merino & Trejo-Tellez, 2018)**.

Tolerance to oxidative stress is much ahead for a C_4 species with demarcation of tissues where photorespiratory loss are minimized. Still, NP would be more interesting as revealed from the present experiment where a significant modulation of anti-oxidation was the results. A significant accumulation of H_2O_2 and $O_2^{\cdot-}$ undoubtedly proved the genotoxicity and opens the probability for TiO_2-NP interaction. TiO_2-NP mediated stress response is not much frequent except in few cases. However, in cotton the peroxidase activity was markedly induced under salinity stress **(Meloni *et al.*, 2003)**. The signal for salinity happens to be amplified by TiO_2 as a semi-conductor through the cellular system. The interactions of TiO_2-NP either to *de-novo* synthesis of anti-oxidative proteins or to induce a hyper-activity that favoured to maintain an adequate redox. Therefore, significant changes of GPX activity would be more contributing to lysis of H_2O_2 of the bio-molecules before it is converted to other radicals. Thus, the activity of GPX through on-going H_2O_2 concentration and more with TiO_2-NP induction is hopeful to support for tolerance. This could be more prudent where polymorphisms of GPX was recorded varying band intensities in the present case. The polymorphic band intensities adhered to their expression tissue specifically have also mentioned in many crops as well as non-crops plant species against xenobiotic toxicity **(Rout *et al*., 2019)**. Moreover, these variations in protein content from different isozymes is expected to set more complexity for TiO_2-NP induction under H_2O_2 treatment. The over expression of peroxidase with nano-material in plants is supposed to have two consequences. In general, nano-material itself as a xenobiotic can induce oxidative stress and thus the expression would be marked tolerant strategies. In addition, the interaction with TiO_2-NP as a semiconductor might have changed the redox potential of the tissues following membrane functions. Electrons carrier proteins on membrane are quite sensitive to any changes of redox and thereby ensures the dismutations of oxygen into superoxide, peroxides etc. For mitigation of superoxide plant potential to induce few variants of enzyme activity has been supportive in selection of genotypes **(Kubis, 2008)**. Moreover, the modulation of such an expression under any chemical elicitations would be expecting its steering role both at gene transcription and translational level. The present study where an induction with TiO_2 NP consistently improvised through H_2O_2 stress is quite interesting. On the other hand, the initiation of ROS through over reduction of molecular O_2 by electron is regulated by SOD activity. It is interesting to note that maize plants in the present experiment were more lenient to exercise the TiO_2-NP in a dose dependent manner. Up-regulation of SOD kinetics in support of cellular defense has also been documented in few nano-materials like cadmium sulphide etc **(Tang *et al*., 2015)**. This is a good exercise that nano-material would be more adjusted on the onset of oxidative stress by over-expression of SOD before it is converted into others like OH^-, $OH^{\cdot}$ etc. A depletion of redox and its retrieval through glutathione is common regardless of crop species **(Jozefczak *et al*., 2012)**. Therefore, glutathione metabolising enzymes like GR would be important. Through the course of H_2O_2, TiO_2-NP has been up-regulatory except under 100µM H_2O_2. This may limit a threshold value for induction of glutathione with its turn-over in its reduced form. Therefore, nanoparticle is also evident with its discretion to favour anti-oxidation in differential modes **(Rico *et al*., 2013)**. It would also be speculated at higher concentration of H_2O_2 or other related effectors, nano materials would also be itself restraining to moderate the oxidative stress. On the other hand, a discriminatory role of CAT is played under same condition where a linear fall of activity through H_2O_2 treatment were recorded. But the activity was up-regulated when TiO_2-NP was applied as pre-treatment. The discriminating behaviour of CAT activity either function of concentration of stressors has well been justified in cereal like rice **(Nounjan *et al*., 2012)**. It is the concentration dependence for any gene to adapt any inducer as promotive or repressive in nature was nicely justified with CAT. A prediction for down regulation of CAT activity would be referred both by inhibited *de-novo* synthesis of the enzyme protein or/and a static binding with any inhibitors **(Michelet *et al*., 2013)**. TiO_2-NP might have equal possibility either to induce the transcripts for CAT or erasing the inhibition from CAT regulatory sites **(Rico *et al*., 2013)**. Thus, TiO_2-NP with its best possibilities would be resourceful to ameliorate the impact of oxidative stress in C_4 plants as established with the maize system in the present case.

CONCLUSION

The present paper represents the documentation of C_4 module in response to oxidative stress through H_2O_2 exposure with maize species. The responses of plants to H_2O_2 induced oxidative stress is dose dependent. C_4 module has an in-built improved anti-oxidation strategy which goes over expressed with TiO_2-NP application for both ROS and anti-oxidation cascades. It is evident that TiO_2-NP is most bio-compatible, at least in maize in the present experiment. Therefore, TiO_2-NP itself being a xenobiotic could also interact with C_4 system to ameliorate the oxidative stress. So, any formulation with metal nanoparticle-based system is expected to be lenient in recovery of stress tolerance through C_4 module. This paper is expected to deliver two lines of expectations: the use of TiO_2-NP through C_4 modules for evoking the anti-oxidation potential and that could be exercise by others C_4 crop species to mitigate related abiotic stresses.

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