Journal of Molecular Biology Research; Vol. 6, No. 1; 2016
ISSN 1925-430X E-ISSN 1925-4318
Published by Canadian Center of Science and Education

Effect of TiO_2 Nanoparticles on Antioxidant Enzymes Activity and Biochemical Biomarkers in Pinto Bean (*Phaseolus vulgaris* L.)

Ahmad Ebrahimi[1,2], Mohammad Galavi[3], Mahmood Ramroudi[3] & Payam Moaveni[4]

[1] Faculty of Agriculture, Zabol University, Iran

[2] Department of Agriculture, Islamic Azad University, Iranshahr Branch, Iranshahr, Iran

[3] Faculty of Agriculture, Zabol University, Zabol, Iran

[4] Department of Agriculture, Islamic Azad University, Shahr-e-Qods Branch, Tehran, Iran

Correspondence: Ahmad Ebrahimi, Department of Agronomy, Faculty of Agriculture, Zabol University, Iran. E-mail: Ae.iranshahr@gmail.com

Received: July 18, 2016 Accepted: August 27, 2016 Online Published: September 17, 2016

doi:10.5539/jmbr.v6n1p58 URL: http://dx.doi.org/10.5539/jmbr.v6n1p58

Abstract

Tests were done on the effects of titanium dioxide spray on Pinto bean (Phaseolus vulgaris L. c.v 'c.o.s.16'). The study was conducted as a factorial experiment in a randomized complete block design with four replications for two years (2014 - 2015). Treatments consisted of two factors; the first factor was stage of plant growth that spraying was applied (rapid vegetative growth, flowering and pod filling); and the second factor was that of different concentrations of titanium dioxide nanoparticles (TiO_2) that consisted of spray with water (control), nano titanium dioxide at concentrations of 0.01%, 0.02%, 0.03% and 0.05%. Activity of guaiacol peroxidase (GPX), activity of superoxide dismutase (SOD), activity of catalase (CAT), activity of peroxidase (POD), malonyldialdehyde (MDA) Content and 8-deoxy-2-hydroxyguanosine (8-OHDG) content were assayed. Results showed that effect of nano TiO_2 was significant on activity of superoxide dismutase (SOD), activity of catalase (CAT), activity of peroxidase (POD), malonyldialdehyde (MDA) Content and 8-deoxy-2-hydroxyguanosine (8-OHDG) content. Results of combined analysis of variance showed that the effect year significantly affected on SOD and 8-OH-2-DG ($P \leq 0.05$). The effect of different amounts of titanium dioxide nanoparticles (TiO_2) significantly affected ($P \leq 0.05$) on MDA and 8-OH-2-DG. The effects of different amounts of titanium dioxide nanoparticles and year were significant on SOD, POD, MDA and the amount of 8-deoxy-2-hydroxyguanosine in $P \leq 0.05$. None of the physiological traits were affected by spraying of nano titanium dioxide. The effects of TiO_2 nanoparticles times of spraying and year were significant on SOD, CAT and 8-deoxy-2-hydroxyguanosine ($P \leq 0.05$). Interaction effects of nano TiO_2 concentrations × nano TiO_2 spraying times did not have a significant impact on SOD, CAT, POD, GPX, MDA and 8-OH-2-DG. Although, all trait were affected by interaction effects of year × nano TiO_2 concentrations × nano TiO_2 spraying times with the exception of GPX ($P \leq 0.05$).

Keywords: Tio_2 nanoparticles, various growth stages, Antioxidant Enzymes, MDA, 8-OH-2-DG and pinto bean (*Phaseolus vulgaris* L.)

1. Introduction

Common bean (*Phaseolus vulgaris* L.) is the most important food legume worldwide, providing the primary source of protein in human diets, supplying about 20% of the protein intake per person (Broughton et al., 2003). As half the grain legumes consumed worldwide are common beans. Unfortunately, the yields of common beans are low, and the quality of their seed proteins is sub-optimal. Most probably, common bean can be redressed by modern techniques (Broughton et al., 2003).

The use of nanoparticles for the growth of plants and control of plant diseases is a recent practice (Rico et al., 2011). During the last decade, an array of exploratory experiments has been conducted to gauge the potential impact of nanotechnology on crop improvement (Nair et al., 2010). Metal oxide nanoparticles are already being manufactured on a large scale for both industrial and household use. Titanium dioxide (TiO_2) nanoparticles are found in everything from cosmetics to sunscreen, paint, vitamins and water pollution treatment; therefore, TiO_2 Nanoparticles will inevitably reach bodies of water through wastewater and urban runoff (Yang et al., 2007). But, physiological effects, depending on the nanomaterial type, particle size, concentration, and plant species (Rico et al., 2011) For example, it

is reported that TiO_2 nanoparticles in higher concentration had pronounced effects on photosynthetic pigments while lower concentration of Titanium dioxide (TiO_2) nanoparticle had significantly increased root length (Samadi et al., 2014). Nanoparticle-induced toxicity is mainly mediated through the generation of reactive oxygen species (ROS) in cells (Melegari et al., 2013). ROS are partly generated as byproducts of metabolic pathways in chloroplasts and are responsible for chlorophyll deterioration (Rico et al., 2015). Thus, disturbance in plant photosynthetic activity by MONPs can generate ROS and activate the plants' defense mechanisms to combat oxidative stress damage. Enzymes such as (superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), guaiacol peroxidase (GPX), malondialdehyde (MDA) content and 8-deoxy-2-hydroxyguanosine (8-OHDG) are generally altered as a response to the alternation in ROS concentration (Du et al., 2016). In this regard, it is reported that metal oxide nanoparticles exposure can induce the generation of ROS, consequently causing oxidative stress and activating plant responses for detoxification such as enzymatic activity (Ma et al., 2015). Similarly, Servin et al. (2013), Hong et al. (2005) and Song et al. (2013) reported an increase in the activities of SOD, CAT, POD and decreased accumulation of ROS when plants were exposed to TiO_2 nanoparticle.

The ultraviolet-B (280–315 nm) light has long been recognized a detrimental factor for plants (Brosche & Strid, 2003; Jenkins, 2009). In addition to being a potential source of oxidative stress, solar UV-B is recognized as a key environmental signal, affecting development and metabolism (Hideg et al., 2013). Responses involve both UV-B-specific signaling and non-specific pathways. Photomorphogenic signaling in response to low intensity UV-B regulates the expression of genes involved in protection against UV (Jenkins, 2009), such as the synthesis of UV-absorbing phenylpropanoids (Brown, et al., 2005). The non-specific pathway involves reactive oxygen species (ROS) such as superoxide radicals ($O_2^{\cdot-}$), singlet oxygen ($\cdot O_2$), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($\cdot OH$) to accumulate in chloroplast during photosynthesis, which caused oxidative damage and photosynthesis reduction of plants (Zheng et al., 2008). One of the consequences of uncontrolled oxidative stress (imbalance between the prooxidant and antioxidant levels in favor of prooxidants) is cells, tissues, and organs injury caused by oxidative damage. It has long been recognized that high levels of free radicals or reactive oxygen species (ROS) can inflict direct damage to lipids. The primary sources of endogenous ROS production are the mitochondria, plasma membrane, endoplasmic reticulum, and peroxisomes (Moldovan and Moldovan, 2004). Lipid peroxidation or reaction of oxygen with unsaturated lipids produces a wide variety of oxidation products. The main primary product of lipid peroxidation is lipid hydroperoxides. Among the many different aldehydes which can be formed as secondary products during lipid peroxidation, including malondialdehyde (MDA), propanal, hexanal, and 4-hydroxynonenal (4-HNE) (Esterbauer et al., 1991), MDA appears to be the most mutagenic product of lipid peroxidation (Esterbauer et al., 1990).

8-deoxy-2-hydroxyguanosine (8-OH-2-DG) is a principal stable marker of hydroxyl radical damage to DNA. It has been related to a wide variety of disorders and environmental insults, and has been proposed as a useful systematic marker of oxidative stress (Bogdanov et al., 1999).

ROS can be activated by higher UV-B (Brosche and Strid, 2003). UV-B induced photomorphological changes in leaves including reduced leaf size, increased leaf thickness and the synthesis of phenolic compounds (Jansen et al., 1998). These changes also affect optical propertiesof leaves and thus may alter the amount of quanta reaching thephotosynthetic apparatus. The main influence of UV-B on photo-synthesis is believed to be more direct. Protein complexes engagedin the light reactions, as well as specific enzymes of the dark reac-tion are functionally impaired by UV-B (Jordan et al., 2016).

It has been reported that UV-B radiation induced oxidative damage of photosystem II and decreased electron transfer rate and thylakoid membrane stability (Renger, 1989; Eva, 1999). Nano-anatase is capable of undergoing electron transfer reactions under ultraviolet light, e.g., the electron was excitated and transferred, then photogenerated electron holes in nano-anatase; the electron holes were reduced when the electron was captured by other molecule, while it was oxidized when self-captured (Crabtree, 1998). It is reported that nano-anatase treatments could markedly promote chlorophyll biosynthesis and the Rubisco activity and the photosynthesis efficiency of spinach (Zheng et al., 2005). Nano-anatase treatment could also activate superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), and guaiacol peroxidase (GPX), and remove ROS in the aged chloroplasts of spinach. Nano-anatase could absorb ultraviolet light and convert light energy to stable chemistry energy finally via electron transport in spinach chloroplasts. Therefore UV-B radiation on choloplasts could be reduced or could avoid the oxidative damage (Zheng et al., 2008).

According to the results obtained, we decided to investigate the effects of TiO_2 nanoparticles on the activities of antioxidant enzymes in pinto bean (*Phaseolus vulgaris* L.) in a two years study.

2. Materials and Methods

This survey was done as a factorial experiment in a complete randomized block design. Treatments consisted of two factors; 1- stage of plant growth that spraying was applied (rapid vegetative growth, flowering and pod filling); and 2- titanium dioxide nanoparticles (TiO_2) concentrations that sprayed; including water (control), 0.01%, 0.02%, 0.03% and 0.05% nano titanium dioxide. Pinto bean seeds were planted in May 2015 and 2016. Fertilization and plant feeding was done according to recommendations from results of a soil test. Spraying treatment was based on growth stages and concentrations of nanoTiO_2. Plants were treated with 240 ml titanium solution per square meter. Control plants were treated with distilled water. Evaluations were made for the parameters of (superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), guaiacol peroxidase (GPX), malondialdehyde (MDA) content and 8-deoxy-2-hydroxyguanosine (8-OH-2-DG).

2.1 Enzyme Extraction

A quantity of 0.5 g of fresh foliar tissue from fresh seedlings (uppermost leaves) was harvested, weighed, washed with distilled water and homogenized in ice cold 0.1 M phosphate buffer (pH=7.5) containing 0.5 mM EDTA with prechilled pestle and mortar. Each homogenate was transferred to centrifuge tubes and was centrifuged at 4°C in Beckman refrigerated centrifuge for 15 min at 15000×g. The supernatant was used for enzyme activity assay. (Esfandiari et al., 2007).

2.2 Antioxidant Enzyme Activity Assays

SOD: The activity of superoxide dismutase (SOD) was assayed according to Misra and Fridovich (1972). About 500 mg of leaves were homogenized in 5 mL of 100 mmol L-1 K-phosphate buffer (pH 7.8) containing 0.1 mmol L-1 ethylenediaminetetracetic acid (EDTA), 0.1% (v/v) Triton X-100 and 2% polyvinylpyrrolidone (PVP) (w/v). The extract was filtered and centrifuged at 22,000 g for 10 min at 4ºC, and the supernatant was utilized for assays. The assay mixture consisted of a total volume of 1 mL, containing glycine buffer (pH 10.5), 1 mmol L-1 epinephrine and enzyme material. Epinephrine was the last added component. Adrenochrome formation over the next 4 min was spectrophotometrically recorded at 480 nm. One unit of SOD activity is expressed as the amount of enzyme required to cause 50% inhibition of epinephrine oxidation under the experimental conditions used. This method is based on the ability of SOD to inhibit the autoxidation of epinephrine at an alkaline pH. Since the oxidation of epinephrine leads to the production of a pink adrenochrome, the rate of increase of absorbance at 480 nm, which represents the rate of autoxidation of epinephrine, can be conveniently followed. The enzyme has been found to inhibit this radical-mediated process.

CAT: Catalase activity was measured according to Aebi (1984). About 3 ml reaction mixture containing 1.5 ml of 100 mM potassium phosphate buffer (pH=7), 0.5 ml of 75 mM H2O2, 0.05 ml enzyme extraction and distilled water to make up the volume to 3 ml. Reaction started by adding H2O2 and a decrease in absorbance recorded at 240 nm for 1 min. Enzyme activity was computed by calculating the amount of H2O2 decomposed.

POD: Peroxidase activity was measured using modification of the procedure of McAdam et al. (1992). Guaiacol was used as the substrate. POD activity was measured in a reaction mixture (3 ml) that contained 0.1 ml enzyme extract, 12 mM H_2O_2, and 7.2 mM guaiacol in 50 mM phosphate buffer, pH 5.8. The kinetics of the reaction were followed at 470 nm. Activity was calculated using extinction coefficient (26.6 mM_1 cm_1 at 470 nm) for tetraguaiacol and expressed as units per gram of fresh weight (FW). One unit of POD activity was defined as 1 mmol tetraguaiacol produced per minute. Protein content in enzyme extracts was determined by the method of Bradford.

GPX: Glutathione peroxidase (GPX) activity was measured according to Paglia and Valentine (1987) in which 0.56 M (pH 7) phosphate buffer, 0.5 M EDTA, 1mM NaN3, 0.2 mM NADPH was added to the extracted solution. GPX catalyses the oxidation of glutathione by cumene hydroperoxide in the presence of glutathione reductase and NADPH, the oxidized glutathione is immediately converted to the reduced form with the concomitant oxidation of NADPH to NADP. The decrease in absorbance at 340 nm was measured with a spectrophotometer.

MDA: Malondialdehyde (MDA) was measured by colorimetric method (Stewart and Bewley, 1980). 0.5 g of leaf samples were homogenized in 5ml of distilled water. An equal volume of 0.5% thiobarbituric acid (TBA) in 20% trichloroacetic acid solution was added and the sample incubated at 95°C for 30 min. The reaction stopped by putting the reaction tubes in the ice bath. The samples then centrifuged at 10000×g for 30 min. The supernatant removed, absorption read at 532 nm, and the amount of nonspecific absorption at 600 nm read and subtracted from this value. The amount of MDA present was calculated through the extinction coefficient of 155 $mM^{-1}cm^{-1}$. Enzyme activity and MDA content of samples were recorded with duplication. The following

formula was applied to calculate malondialdehyde (MDA) content using its absorption coefficient (ε) and expressed as nmol malondialdehyde g^{-1} fresh mass, following the formula:

$$MDA\ (nmol\ g\text{-}1\ FM) = [(A532\text{-}A600) \times V \times 1000/\varepsilon] \times W,$$

where ε is the specific extinction coefficient (=155 mM-1 cm-1), V is the volume of crushing medium, W is the fresh weight of leaf, A600 is the absorbance at 600 nm wavelength and A532 is the absorbance at 532 nm wavelength.

8-OH-2-DG: 8-deoxy-2-hydroxyguanosine (8-OH-2-DG) was measured according to Bogdanov et al. (1999). In this case, extract from the carbon column 8 (C8) is passed, this column is for the absorption of purines. After reaching equilibrium, passing all the solvent in the extraction, then column by passing the new mobile phase containing Tris Hcl with pH 8-deoxy-2-hydroxyguanosine of this column is removed and transferred to C8 new column. After equilibration column was washed with the mobile phase containing adenosine concentrations of 0.65 M. This has led to proprietary separation of the desired material as a private peak, so that the peak detector devices of the type Colometric were identified and transferred. The amount of 8-deoxy-2-hydroxyguanosine for a certain proportion of total purine peak will be assessed. To separate the extract, the leaves of beans are weighed and then determine the total protein ratio, a part of the leaf tissue in buffer solution of phosphate bicarbonate (mono sodium) 1.6 M (PH 7.4), crushed and then quickly the presence of ice and cold conditions were homogenized. Dimethyl sulfoxide at a concentration of 0.4 M was added to the solution. After adding the new buffer (pH 5.6) acetate monosodic, it is in the dialysis against distilled water for 6 hours and then the remaining content was centrifuged at 1500×g for 10 minutes. The supernatant in a spectrophotometer at wavelengths of 280 and 260 nm, respectively, were absorbed. Then, by adding trichloroacetic acid was free 35/0 mole of protein. This solution is then centrifuged at 300×g for 15 minutes was centrifuged and the supernatant is used for sensing of guanosine hydrate.

2.3 Characterization Analysis of TiO_2 NPs

The anatase TiO_2 NPs was purchased from Nano Pars Lima Company. The TiO_2 NPs had a purity of greater than 99.5%, average of particle diameter of 21 nm, and a surface area of 60 m2/g. Then, in order to prepare concentrations of nano TiO_2, 20 g nano TiO_2 was dissolved into water and then 0.01 ml of solution was filled up to 1000 ml. Thus, different concentrations of titanium dioxide (0.01%, 0.02%, 0.03% and 0.05%) were prepared. An ultrasound instrument was used to homogenize the solution. Titanium dioxide nanoparticles were sprayed on plants using a calibrated pressurized backpack sprayer (capacity 20 l).

2.4 Statistical Calculations

Data were subjected to analysis of variance (ANOVA) using software Statistical Analysis System 9.0 (SAS Institute 1988) and followed by Duncan's multiple range tests. Terms were considered significant at $P \leqslant 0.05$.

3. Results

Results of combined analysis of variance showed that the effect year significantly affected on SOD and 8-OH-2-DG ($P \leqslant 0.05$, Table 1). The effect of different amounts of titanium dioxide nanoparticles (TiO_2) significantly affected ($P \leqslant 0.05$, Table 1) on MDA and 8-OH-2-DG. The effects of different amounts of titanium dioxide nanoparticles and year were significant on SOD, POD, MDA and the amount of 8-deoxy-2-hydroxyguanosine in $P \leqslant 0.05$ (Table 1). None of the physiological traits were not affected by spraying of nano titanium dioxide. In the event that, the effects of TiO_2 nanoparticles times of spraying and year were significant on SOD, CAT and 8-deoxy-2-hydroxyguanosine ($P \leqslant 0.05$, Table 1). Interaction effects of nano TiO_2 concentrations × nano TiO_2 spraying times did not have a significant impact on SOD, CAT, POD, GPX, MDA and 8-OH-2-DG (Table 1). Although, all traits were affected by interaction effects of year × nano TiO_2 concentrations × nano TiO_2 spraying times with the exception of GPX ($P \leqslant 0.05$, Table 1).

Comparison of means demonstrated that nano TiO_2 at concentrations of 0.01% and 0.03% increased activity rate of SOD in leaves compared with control, although there was no significant difference between the nano TiO_2 concentrations regarding catalase enzyme and nano TiO_2 at concentrations of 0.02%, 0.03% and 0.05% boosted activity rate of CAT in leaf tissue (Table 2). In the case of nano titanium application, the highest amount of POD was obtained at concentrations of 0.01%, 0.03% and 0.05%, whereas the lowest amount of POD was found at concentrations of 0.02%. In the case of TiO_2 usage, the highest amount of GPX enzyme has been showed at concentrations of 0.03% and 0.05%, whereas the highest content of MDA was found in those plots which were treated with titanium dioxide 0.02% (Table 2). Finnaly, the highest content of 8-OH-2-DG (12.821 nm/ mg Protein) was found at concentration of 0.03% (Table 2).

According to Table 2, the highest amount of SOD, CAT, GPX and MDA were found in rapid vegetative growth stage in pinto bean leaves, whereas the highest content of POD was seen in pod filling stage and for 8-OH-2-DG, the highest content was found in flowering stage.

When it comes to the combined effects, results show that the maximum of the activity measure of the SOD enzyme (208.13 U mg $^{-1}$ Protein) in leaves was observed by the application of 0.02% nano titanium dioxide at the rapid vegetative growth stage and the minimum of the activity mount of the SOD enzyme (95.50 U mg $^{-1}$ Protein) in leaf tissue were found by the foliar of 0.05% nano titanium dioxide at the rapid vegetative growth stage (Table 3). Interaction effects of nano TiO_2 concentrations × nano TiO_2 spraying times on CAT showed the maximum of the activity measure of the catalase enzyme (232.75 U mg $^{-1}$ Protein) in leaves were observed by the application of 0.03% nano titanium dioxide at the rapid vegetative growth stage. By contrast, when nano titanium dioxide (0.03%) was applied on plants during the flowering stage, the lowest of CAT enzyme (111.50 U mg $^{-1}$ Protein) was produced (Table 3). The maximum and minimum POD enzyme were obtained when plants were treated with titanium dioxide nanoparticles (0.05%) at the pod filling stage and titanium dioxide nanoparticles (0.02%) at the flowering stage, respectively (Table 3). The combined effects among growing stages and nano TiO_2 concentrations indicate that the most GPX enzym were produced when titanium dioxide nanoparticles (0.05%) were applied at the rapid vegetative growth stage, whereas the least GPX enzyme was obtained from nano titanium application at the flowering stage in pinto bean (Table 3). When the effects of tow experimental factors were combined, the highest MDA amount was obtained when titanium dioxide nanoparticles (0.02%) at the pod filling and the lowest MDA content was obtained when titanium dioxide nanoparticles (0.05%) at the pod filling stage (Table 3). The highest 8-OH-2-DG amount was observed when plants were treated with titanium dioxide nanoparticles (0.05%) at the flowering stage; on the other hand, the lowest 8-OH-2-DG amount was obtained from those plants which were not treated with nano TiO_2 at the flowering stage (Table 3). A significant positive correlation was found between GPX enzyme amount and CAT enzyme amount under experimental treatments of pinto bean ($r_{0.01} = 0.266$) (Table 4). Also, there was a significant positive correlation between GPX enzyme amount and 8-OH-2-DG content in leaves of pinto bean under experimental treatments ($r_{0.05} = 0.202$) (Table 4).

4. Discussion

In this two-year study, we found that foliar of TiO_2 nanoparticles at different growth stages induce some physiological and biochemical responses that can promote tolerance levels in pinto bean.

The nano TiO_2, attributed to their photocatalytic property and thermal conductivity, enhanced water absorption, improved light absorption in chlorophyll a, and induced oxygen evolution rate, consequently showed beneficial effects on photosynthesis (Rezaei et al., 2015; Rico et al., 2015b).

Our results showed that nano TiO_2 sprayed on pinto bean leaves inhibited ROS accumulation and increased antioxidant enzymes activity and also, showed a significant increase in the contents of each biomarkers MDA and 8-OH-2-DG.

Similarly, nano CuO caused significant oxidative stress with higher ROS, MDA content, but increased activities of antioxdative enzymes in rice (Da Costa & Sharma, 2016; Shaw & Hossain, 2013; Wang et al., 2015).

In the event of oxidative stress in plant, the leaves damaged by the collapse of chlorophyll, is reduced the rate of photosynthesis and eventually decline yields crops.

In this case, by increasing the amount and activity of antioxidant enzymes in plant leaf tissue, can be reduced the amount of damage and prevent yield losses. In this regard, it was reported that internal O2 concentration is high during photosynthesis, and chloroplasts are especially prone to generate activated oxygen species; therefore, these cytotoxic active oxygen species can seriously disrupt normal metabolism through oxidative damage of lipids, nucleic acids, and proteins. Deleterious effects of ROS and lipid peroxidation products are counteracted by an antioxidant defense system (Pejic et al., 2009). From one aspect, it can be expressed that probably with the application of nano TiO_2 in the plant and then with the entry of it into the leaf tissue, causing phytotoxicity in plants, at the same time increases the amount of oxidants in plants, finally, increase the amount and activity of antioxidants (including antioxidant enzymes and biomarkers MDA and 8-OH-2-DG) to counteract the disadvantageous effects of ROS.

Malondialdehyde is the decomposition product of polyunsaturated fatty acids of bio membranes and its increase is the result of greater accumulation under high antioxidant stress. Malondialdehyde content serves as an indicator of the extent of lipid peroxidation and an indirect reflection of the extent of cell damage (Wang et al., 2011).

Servin et al. (2013) reported an increase in CAT activity (250-750 mg/kg) but a decrease in APX (500 mg/kg) when cucumber plants were exposed to nano TiO_2. Also, Laware and Raskar (2014) reported that CAT and GPX activities can be enhanced in the presence of 10-30 μgml^{-1} TiO_2 nanoparticles, but their activities decrease by higher concentrations of TiO_2 nanoparticles. Also, Lei et al. (2008) showed that nano-anatase treatment could activate SOD, CAT, APX, and GPX of spinach chloroplasts. SOD can convert $O_{2^{\cdot}}^{-}$ into H2O2 and O2; moreover, CAT, APX, and GPX can reduce H2O2 into H2O and O_2. Therefore, SOD, CAT, APX, and GPX can maintain a low level of ROS and prevent ROS toxicity and protect cells.

From other aspect, it can be declared that with the foliar application of titanium dioxide nanoparticles in the plant, nano TiO_2 can reduce the effects of photo oxidative stress and prevent the chloroplasts destruction and chlorophyll degradation of leaf tissue. About this issue, Lei et al. (2008) announced that nano-anatase could absorb ultraviolet (UV) light and convert light energy to steady chemistry energy finally via electron transport in spinach chloroplasts, so that UV-B radiation on choloplasts could be reduced or could avoid the oxidative damage. In other words, Zheng et al. (2007) reported that enriched energy electron from nano-anatase, which entered chloroplast under ultraviolet light, was transfered in photosynthetic electron transport chain and made $NADP^+$ reduced into NADPH, coupled to photophosphorylation, and made electron energy be transformed to ATP, and nano-anatase h^+, photogenerating electron holes, captured an electron from water that accelerated water photolysis and O_2 evolution.

Table 1. Combined analysis of variance on SOD, CAT, POD, GPX, MDA and 8-OH-2-DG of pinto bean affected by nano TiO_2 concentrations and different growth stages (2014 - 2015)

Means square							
8-OH-2-DG	**MDA**	**GPX**	**POD**	**CAT**	**SOD**	**df**	**Sources of variation**
1.79*	0.02 n.s	0.67 n.s	0.92 n.s	1.41 n.s	1.89*	1	Y (Year)
0.90	0.40	0.16	0.77	0.70	0.37	6	Error
0.85*	0.70*	0.31 n.s	0.44 n.s	0.08 n.s	0.30 n.s	4	(A) Concentrations of nano TiO_2
0.79*	0.31*	0.25 n.s	0.72*	0.12 n.s	1.13*	4	Y × A
0.22 n.s	0.05 n.s	0.08 n.s	0.03 n.s	1.01 n.s	0.47 n.s	2	(B) Times of spraying
1.02*	0.32 n.s	0.06 n.s	0.15 n.s	0.00*	0.27*	2	Y × B
0.41 n.s	0.42 n.s	0.12 n.s	0.42 n.s	0.71 n.s	0.58 n.s	8	A × B
0.41*	0.82*	0.52 n.s	0.46*	0.27*	0.25*	8	Y × A × B
0.41	0.39	0.62	0.64	0.66	0.49	84	Error
30.41	17.45	17.74	24.22	16.98	14.73		C.V (%)

Note: Ns, Non Significant, * and **, Significant at 5% and 1% levels respectively.

Table 2. Mean comparison of physiological traits of pinto bean in nano TiO_2 concentrations and nano TiO_2 spraying times (2014 - 2015)

8-OH-2-DG (nm/ mg Protein)	MDA (nm/ mg Protein)	GPX (U mg^{-1} Protein)	POD (U mg^{-1} Protein)	CAT (U mg^{-1} Protein)	SOD (U mg^{-1} Protein)	Treatments
						Concentrations of nano TiO_2
8.317b	48.967ab	105.25a	0.040958a	149.92a	135.42a	(Distilled water) Control
9.738ab	38.200b	97.38a	0.042500a	141.38a	151.88a	(0.01%) Nano TiO_2
9.388ab	51.300a	97.50a	0.040417a	150.83a	134.75a	(0.02%) Nano TiO_2
12.821a	39.054ab	115.92a	0.051500a	157.22a	152.71a	(0.03%) Nano TiO_2
12.454ab	37.471ab	111.83a	0.064042a	160.71a	130.92a	(0.05%) Nano TiO_2
						Times of spraying of nano TiO_2
10.403a	44.360a	107.33a	0.045522a	171.35a	157.50a	Rapid vegetative growth
11.025a	42.113a	102.55a	0.045400a	140.35a	135.88a	Flowering
10.203a	42.523a	106.85a	0.053025a	144.33a	130.03a	Pod filling

Means in each column followed by similar letter(s) are not significantly different using Duncan's Multiple Range Test.

Table 3. Interaction effects of nano TiO_2 concentrations × nano TiO_2 spraying times on physiological traits of pinto bean (2014 - 2015)

8-OH-2-DG (nm/ mg Protein)	MDA (nm/ mg Protein)	GPX (U mg $^{-1}$ Protein)	POD (U mg $^{-1}$ Protein)	CAT (U mg $^{-1}$ Protein)	SOD (U mg $^{-1}$ Protein)	Times of spraying of nano TiO_2	Concentrations of nano TiO_2
8.688ab	50.605abc	104.13a	0.04150a	145.38ab	162.13ab	Rapid vegetative growth	Control
9.750ab	44.93abc	95.38a	0.04150a	132.00ab	153.13ab	Rapid vegetative growth	(0.01%)
9.888ab	45.28abc	90.38a	0.03425a	182.75ab	208.13a	Rapid vegetative growth	(0.02%)
11.250ab	40.55abc	114.00a	0.05713a	232.75a	168.63ab	Rapid vegetative growth	(0.03%)
12.438a	40.55abc	132.75a	0.05175a	163.88ab	95.50b	Rapid vegetative growth	(0.05%)
6.138b	51.665ab	95.00a	0.03938a	169.50ab	113.25ab	Flowering	Control
7.425ab	28.655c	92.13a	0.04113a	144.88ab	141.63ab	Flowering	(0.01%)
9.963ab	46.015abc	99.25a	0.02838a	144.25ab	98.13b	Flowering	(0.02%)
15.675a	38.365abc	119.88a	0.05200a	111.50b	154.25ab	Flowering	(0.03%)
15.925a	45.875abc	106.50a	0.06613a	131.63b	172.13ab	Flowering	(0.05%)
10.125ab	44.64abc	116.63a	0.04200a	134.88ab	130.88ab	Pod filling	Control
12.038ab	41.03abc	104.63a	0.04488a	147.25ab	160.88ab	Pod filling	(0.01%)
8.313ab	62.615a	102.88a	0.05863a	125.50b	98.00b	Pod filling	(0.02%)
11.538ab	38.25bc	113.88a	0.04538a	127.41b	135.25ab	Pod filling	(0.03%)
9.000ab	26.09c	96.25a	0.07425a	186.63ab	125.13ab	Pod filling	(0.05%)

Means in each column followed by similar letter(s) are not significantly different using Duncan's Multiple Range Test.

Table 4. Pearson's correlation coefficients among GPX, SOD, CAT, POD, MDA and 8-OH-2-DG of pinto bean affected by nano TiO_2 concentrations and different growth stages (2014 - 2015)

Trait	GPX	SOD	CAT	POD	MDA	DHG
GPX	1	-0.002	0.266**	0.155	-0.019	0.202*
SOD	-0.002	1	-0.002	-0.169	0.061	-0.137
CAT	0.266**	-0.002	1	-0.004	-0.059	0.042
POD	0.155	-0.169	-0.004	1	-0.068	0.057
MDA	-0.019	0.061	-0.059	-0.068	1	0.004
DHG	0.202*	-0.137	0.042	0.057	0.004	1

* and **, significant difference at 5 and 1%, respectively.

References

Aebi, H. (1984). In S. P. Colowick, & N. O. Kaplan (Eds.), *Catalase in Vitro. Methods in Enzymology* (Vol. 105, pp. 114–121). Florida: Acad. Press.

Bogdanov, M. B., Beal, M. F., Meccabe, D. R., Griffin, R. M., &Matson, W. R. (1999). A carbon column based LCEC approach to routine 8-hydroxy-2- deoxyguanosine measurements in urine and other biological matrices. *Free Rad Biol Med., 27*, 647-666.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem, May 7*(72), 248–254.

Brosche, M., & Strid, A. (2003). Molecular events following perception of ultraviolet-Bradiation by plants. *Physiol. Plant, 117*, 1–10.

Broughton, W. J., Hernandez, G., Blair, M., Beebe, S., Gepts, P., & Vanderleyden, J. (2003). Beans (Phaseolus spp.)—model food legumes. *Plant Soil, 252*, 55–128.

Brown, B. A., Cloix, C., & Jiang, G. H. (2005). A UV-B-specific signaling componentorchestrates plant UV protection. *Proc. Natl. Acad. Sci. U. S. A., 102*, 18225–18230.

Crabtree, R. H. (1998). A new type of hydrogen bond. *Science, 282*, 2000 – 2001.

Da Costa, M. V. J., & Sharma, P. K. (2016). Effect of copper oxide nanoparticles on growth, morphology, photosynthesis, and antioxidant response in Oryza sativa. *Photosynthetica, 54*(1), 110-119.

Du, W., Tan, W., Peralta-Videa, J. R., Gardea-Torresdey, J. L., Ji, R., … Guo, H. (2016). Interaction of metal oxide nanoparticles with higher terrestrial plants: Physiological and biochemical aspects. *Plant Physiology and Biochemistry,* 1-16.

Esfandiari, E., Shakiba, M. R., Mahboob, S., Alyari, H., & Toorchi, M. (2007). Water stress, antioxidant enzyme activity and lipid peroxidation in wheat seedling. *Journal of Food, Agriculture & Environment, 5*, 149-153.

Esterbauer, H., Eckl, P., & Ortner, A. (1990). Possible mutagens derived from lipids and lipid precursors. *Mutation Research, 238*(3), 223–233.

Esterbauer, H., Schaur, R. J., & Zollner, H. (1991). Chemistry and Biochemistry of 4 hydroxynonenal, malonaldehyde and related aldehydes. *Free Radical Biology and Medicine, 11*(1), 81–128.

Eva, H. (1999). Utilizing new adamantly spin traps in studying UV-B-induced oxidative damage of photosystem II. *J Photochem Photobiol B Biol, 48*, 174-179.

Hideg, E., Jansen, M. A. K., & Strid, A. (2013). UV-B radiation ROS and stress; inseparablecompanions or loosely linked associates? *Trends Plant Sci., 18*, 107–115.

Hong, F. S., Yang, F., Liu, C., Gao, Q., Wan, Z. G., Gu, F. G., … Yang, P. (2005). Influences of nano-TiO_2 on the chloroplast aging of spinach under light. *Biol. Trace Elem. Res., 104*(3), 249-260.

Jansen, M. A. K., Gaba, V., & Greenberg, B. M. (1998). Higher plants and UV-B radiation:balancing damage, repair and acclimation. *Trends Plant Sci., 3*, 131–135.

Jenkins, G. I. (2009). Signal transduction in responses to UV-B radiation. *Annu. Rev. Plant Biol., 60*, 407–431.

Jordan, B. R., Strid, A., & Wargent, J. J. (2016). What role does UVB play in determiningphotosynthesis? In M. Pessarakli (Ed.), *Handbook of Photosynthesis* (pp. 275–286). CRCPress, Boca Raton.

Laware, S. L., & Raskar, S. (2014). Effect of titanium dioxide nanoparticles on hydrolytic and antioxidant enzymes during seed germination in onion. *Int J Curr Microbiol App Sci., 3*(7), 749-760.

Lei, Z., Mingyu, S., Xiao, W., Chao, L., Chunxiang, Q., Liang, C., ... & Fashui, H. (2008). Antioxidant stress is promoted by nano-anatase in spinach chloroplasts under UV-B radiation. *Biological Trace Element Research*, *121*(1), 69-79.

Ma, C. X., White, J. C., Dhankher, O. P., & Xing, B. (2015. Metal-based nanotoxicity and detoxification pathways in higher plants. *Environ. Sci. Technol., 49*(12), 7109-7122.

MacAdam, J. W., Nelson, C. J., Sharp, R. E. (1992). Peroxidase activity in the leaf elongation zone of tall fescue. *Plant Physiol., 99*, 872-878.

Melegari, S. P., Perreault, F., Costa, R. H. R., Popovic, R., & Matias, W. G. (2013). Evaluation of toxicity and oxidative stress induced by copper oxide nanoparticles in the green alga Chlamydomonas reinhardtii. *Aquat. Toxicol., 142*, 431e440.

Misra, H. P., & Fridovich, I. (1972). The generation of superoxide radical during auto oxidation. *J. Biol. Chem., 247*, 6960-6966.

Moldovan, L., & Moldovan, N. I. (2004). Oxygen free radicals and redox biology of organelles. *Histochemistry and cell biology*, *122*(4), 395-412.

Nair, R., Varghese, S. H., Nair, B. G., Maekawa, T., Yoshida, Y., & Kumar, D. S. (2010). Nanoparticulate material delivery to plants. *Plant Sci, 179*, 154–163.

Paglia, D. E., & Valentine, W. N. (1987). Studies on the quantitative and qualitative characterization of glutation proxidase. *J. Lab. Med., 70*, 158-165. Methods of soil analysis, 2. Chemical and Agronomy and Soil Science Society of America.

Pejic, S., Todorovic, A., Stojiljkovic, V., Kasapovic, J., & Pajovic, S. B. (2009). Antioxidant enzymes and lipid peroxidation in endometrium of patients with polyps, myoma, hyperplasia and adenocarcinoma. *Reprod Biol Endocrinol, 7*, 149.

Renger, G. (1989). On the mechanism of photosystem II deterioration by UV-Birradiation. *Photochem. Photobiol., 49*, 97–105,

Rezaei, F., Moaveni, P., & Mozafari, H. (2015). Effect of different concentrations and time of nano TiO_2 spraying on quantitative and qualitative yield of soybean (Glycine max L.) at Shahr-e-Qods. *Iran. Biol. Forum, 7*(1), 957-964.

Rico, C. M., Hong, J., Morales, M. I., Zhao, L. J., Barrios, A. C., Zhang, J. Y., … Gardea-Torresdey, J. L. (2013b). Effect of cerium oxide nanoparticles on rice: a study involving the antioxidant defense system and in vivo fluorescence imaging. *Environ. Sci. Technol., 47*(11), 5635-5642.

Rico, C. M., Majumdar, S., & Duarte-Gardea, M. (2011). Interaction of nanoparticles with edible plants and their possible implications in the food chain. *J Agr Food Chem, 59*, 3485-98.

Rico, C. M., Peralta-Videa, J. R., & Gardea-Torresdey, J. L. (2015b). Chemistry, Biochemistry of Nanoparticles, and Their Role in Antioxidant Defense System in Plants. In *Nanotechnology and Plant Sciences* (pp. 1-17). Springer International Publishing.

Samadi, N., Yahyaabadi, S., & Rezayatmand, Z. (2014). Effect of TiO_2 and TiO_2 Nanoparticle on Germination, Root and Shoot Length and Photosynthetic Pigments of Mentha Piperita. *International Journal of Plant & Soil Science, 3*(4), 408-418.

Servin, A. D., Morales, M. I., Castillo-Michel, H., Hernandez-Viezcas, J. A., Munoz, B., Zhao, L. J., … Gardea-Torresdey, J. L. (2013). Synchrotron verification of TiO_2 accumulation in cucumber fruit: a possible pathway of TiO_2 nanoparticle transfer from soil into the food chain. *Environ. Sci. Technol., 47*(20), 11592-11598

Shaw, A. K., & Hossain, Z. (2013). Impact of nano-CuO stress on rice (Oryza sativa L.) seedlings. *Chemosphere, 93*(6), 906-915.

Song, U., Jun, H., Waldman, B., Roh, J., Kim, Y., Yi, J., & Lee, E. J. (2013). Functional analyses of nanoparticle toxicity: a comparative study of the effects of TiO_2 and Ag on tomatoes (Lycopersicon esculentum). *Ecotoxicol. Environ. Safe, 93*, 60-67.

Stewart, R. R. C., & Bewley, J. D. (1980). Lipid peroxidation associated aging of soybean axes. *Plant Physiology, 65*, 245-248.

Wang, H. F., Zhong, X. H., Shi, W. Y., & Guo, B. (2011). Study of malondialdehyde (MDA) content, superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) activities in chickens infected with avian infectious bronchitis virus. *Afr J Biotechnol, 10*, 9213 – 9217.

Wang, S. L., Liu, H. Z., Zhang, Y. X., & Xin, H. (2015). The effect of CuO NPs on reactive oxygen species and cell cycle gene expression in roots of rice. *Environ. Toxicol. Chem., 34*(3), 554-561.

Yang, F., Liu, C., Gao, F., Su, M., Wu, X., Zheng, L., … Yang, P. (2007). The importance of spinach growth by nano-anatase TiO_2 treatment is relate to nitrogen photoreduction. *Biol Trace Elem Res, 119*, 77 – 88.

Zheng, L., Su, M. Y., Liu, C., Chen, L., Huang, H., Wu, X., … Hong, F. S. (2007). Effects of nano-anatase TiO_2 on photosynthesis of spinach chloroplasts under different light illumination. *Biol Trace Elem Res*.