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A STUDY OF PHOTO-BIOLOGICAL REACTIONS UNDER TIO_2 NANOPARTICLE ACCUMULATION IN *SPINACIA OLERACEA* L.

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Abstract

The titanium dioxide nanoparticles (TiO_2 NPs) are widely used in several fields of Science and Technology, trailed to increase concentration in soil with their subsequent accumulation in *Spinacia oleracea*. This article discusses the accumulation of TiO_2 in plants, released in soil resources after its prime use. The pot experiment was conducted in a greenhouse under a natural environmental condition in triplicates where TiO_2 was mixed with the soil for growth monitoring of *S. oleracea* as treated plants. The uptake of NPs from soil to plants was observed through Scan Electron Microscopy (SEM) and Energy Dispersive X-ray (EDX) techniques. The reflection of TiO_2 NPs in the leaves and roots of plants through these techniques provides evidence of the accumulation of NPs for the first time. SEM results showed the translocation of TiO_2 into roots and shoot and their impact on the accumulation of essential micro and micronutrients followed by enhanced photosystem II of plants. The increase in Fe contents and Ca followed by healthy growth of plants reflects the photocatalytic activity of TiO_2. Compared to the control treatment, the concentration of Mn, Cu, Al, and Si significantly increased with the application of TiO_2 nanoparticles. Consequently, TiO_2-NP treatments showed the highest photobiological activity due to increased nutrient uptake by *S. oleracea*. It was established that the accumulation of TiO_2 NPs in the plant was supportive in photosystem II operation due to their photoactive electron at a given dose.

Key words: *Spinacia oleracea,* TiO_2, Photobiological activity, Nutrients elements.

Introduction

The world of nanotechnology comprises several nanomaterials, including Nanorods, Nanostructured, Nano flowers, and nanoparticles. Titanium dioxide Nano particles (TiO_2 NPs) are considered the most eco-friendly and consumable among all these Nanomaterials. This extensive diversity in uses of NPs has directed to the infrequent and prevalent scattering of TiO_2 NPs in numerous ecological areas, with diverse effects on the biological entity (Samadi *et al*., 2014). Nanotechnology allows comprehensive advances in agricultural research, such as reproductive science and technology, transfer of agricultural and food wastes to energy and other valuable by-products through enzymatic Nano bioprocessing, disease prevention, and treatment in plants using various nanocides (Feizi *et al*., 2012; Jaberzadeh *et al*., 2013). The most acute effects of Ti compounds on plants are enhancement of the yield of various crops (about 10–20%); an improvement of some important element contents in plant tissues; an increase in the peroxidase, catalase, and nitrate reductase activities in plant tissues; and an enhancement of the chlorophyll content in paprika (*Capsicum anuum* L.) and green alga (*Chlorella pyrenoidosa*) suggested that the Ti NPs delivered positive effect on these plants including Canola seeds too. Higher concentrations of TiO_2 improve the germination and root growth of the treated seeds (Feizi *et al*., 2012; Kužel *et al*., 2003; Mahmoodzadeh *et al*., 2013; Azmat *et al*., 2020).

Few studies revealed the accumulation of NPs by the plants, from root to shoot, where NPs transferred to leaves, as in the case of *Arabidopsis thaliana*. It was also observed that TiO_2- NP promotes photosynthesis and improves the growth of spinach (Laure *et al*., 2010; Hong *et al*., 2005), while few studies observed that soaking of seeds in the suspension of TiO_2- NP also increased the seed germination and growth (Li *et al*., 2010).

The aims and objective of the current search were to evaluate the impact of TiO_2 NPs on the accumulation of several essential elements through Scan Electron Microscopy and Energy Dispersive X-ray techniques to validate the accumulation of Nanoparticles in plants entity. The photo activity of TiO_2 NPs discussed in relation to the photobiology of plants.

Materials and Methods

The Scanning Electron Microscopy (SEM) and Energy Dispersive X-ray Spectroscopy (EDX) techniques were employed on leaves and roots of *Spinacia oleracea* after observing growth in triplicates as reported earlier under used TiO_2 by Azmat *et al*., (2020).

Scanning electron microscopy and Energy dispersive X-ray spectroscopy: The structure morphology, dimensions of sample particles, presence, confirmation, and percentage of elements, and the occurrence of unwanted impurities with side products were determined in the sample of leaves and roots through scanning electron microscopy (SEM) and EDS (Energy Dispersive X-ray Spectroscopy) by ZAF Method (Standard Less Quantitative Analysis). In this technique sample of plants was blanketed with liquid nitrogen, and scanning the sample surface through an electron beam was carried out under a vacuum.

The sample was coated up to 300^{o} A with gold with the sample coater (JFC-1500 JEOL from Japan). With the help of a high-energy electron beam accelerated with 15 kV, the SEM images are magnified up to 15000 times while the current of the probe was maintained at 1.00000 nA. The SEM instrument is JSM-6380A JEOL from Japan (The equipment is placed at Centralized Science Laboratories, University of Karachi). Using the X-ray detector (EX-54175jMU JEOL from Japan), the composition of elements was quantified, limited from 0 to 20 KeV, while the lifetime is 30 sec with 3756 counts per second. Different elements present in the sample were analyzed at less than 1 % by weight detection limit. The percentage of elements was computed with the help of ZAF Method Standard Less Quantitative method.

Results

This article discusses the impact of TiO_2.NPs on photosystem II of *S. oleracea* plant under TiO_2-NPs accumulation which serve as an oxygen-evolving complex. The Scanning Electron Microscopic technique and Electron dispersive spectroscopy were used to monitor the surface of leaves and nutrient ions distribution, respectively, under TiO_2 NPs accumulation. The analysis showed the influence of TiO_2 NPs on light absorption, regulating the distribution of light energy from PS I to PS II and accelerating the alteration from solar energy to chemical energy, water photolysis, and oxygen evolution.

Accumulation studies of TiO_2 through SEM technique: Scanning Electron Microscopy of roots and leaves of control and TiO_2 treated plants was conducted to ascertain the accumulation of TiO_2 NPs in plants (Fig. 1) as roots are directly in contact with soil nutrients or supplied TiO_2 NPs within the soil of the plant. It was observed through Figures 1 & 2 that TiO_2 penetrated the roots and transported to areal parts, i.e., leaves (Fig. 1) which was observed in images of Scanning Electron Microscopy (SEM) of leaves of *S. oleracea* (Fig. 1). SEM analysis of treated plants showed the presence of anatase micro structured TiO_2 in roots and leaves (Fig. 1). It was observed in Figures that the size of the stomatal opening of treated leaves increases as compared to control plants of *S. oleracea* (Fig. 1).

Nutrients elements through EDX: The impact of anatase Nanostructured Ti on essential micronutrients and macronutrients in roots and leaves of *S. oleracea* plants was monitored through Surface Electron Microscopy in conjunction with Energy-dispersive X-ray spectroscopy in comparison to controlled plants. The EDS analysis showed various elemental compositions on the surface of the roots and leaves (Fig. 1). Results showed that Potassium (K) contents in leaves increased from 1.67 % to 4.12 % in plants treated with TiO_2 soil in comparison to control plants (Table 1) while remains constant in the roots (Table 2), the Mg, essential element of chlorophyll is found to be increased from 0.09 % to 0.63 % in leaves whereas 3.89 % to 0.25 % in the root (Tables 1&2), similarly 0.86 % to 11.45 % increase in Ca contents in roots was also observed (Table 2). The iron (Fe) contents in *S. oleracea* increased from 0.06 % to 1.87 % in leaves, and 0.32 % to 24.13 in roots of TiO_2-treated plants where C and O were less on the surface of leaves. Mn is the essential micronutrient of photosystem II found in treated plants over controlled ones. The EDS spectra showed that Ti is detected in roots, whereas in leaves, it is not detected but observed in images (Fig. 2) which showed that only a few particles translocated in the leaves, which was not detectable quantitatively.

Table 1. Elemental study of leaves of *Spinacia oleracea* control and TiO2 treated plant through EDS (ZAF method standard less quantitative analysis).

Elements	keV	Mass %	Error %	At %	K	
C	0.277	30.66	0.92	41.22	13.8816	Control *Spinacia oleracea* plant leaves fitting coefficient:0.7413
O	0.525	45.71	1.95	46.13	53.5515	
Na	1.041	9.03	1.62	6.35	10.2600	
Mg	1.253	0.14	1.43	0.09	0.1366	
Al	1.486	1.71	1.40	1.02	1.9840	
Cl	2.621	6.49	1.59	2.96	10.7597	
K	3.312	4.05	2.41	1.67	6.5349	
Fe	6.398	0.19	8.61	0.06	0.2678	
Cu	8.040	1.20	21.93	0.30	1.5624	
Zn	8.630	0.82	31.21	0.20	1.0616	
Elements	**keV**	**Mass %**	**Error %**	**At %**	**K**	
C	0.277	16.27	0.65	30.54	5.6737	TiO_2 Treated *Spinacia oleracea* plant leaves fitting coefficient: 0.7029
O	0.525	27.19	1.43	38.31	24.6231	
Na	1.041	1.90	1.22	1.86	1.5480	
Mg	1.253	0.68	0.99	0.63	0.5258	
Al	1.486	8.27	0.94	6.91	7.6918	
Si	1.739	1.79	1.01	1.44	1.8757	
Cl	2.621	2.72	1.01	1.73	3.8209	
K	3.312	7.14	1.46	4.12	10.3622	
Ca	3.690	9.14	1.80	5.14	13.5658	
Cr	5.411	2.22	3.78	0.96	2.8069	
Mn	5.894	2.77	4.62	1.14	3.4346	
Fe	6.398	4.64	5.10	1.87	5.8863	
Cu	8.040	9.40	13.01	3.33	11.2020	
Zn	8.630	5.88	18.42	2.03	6.9833	

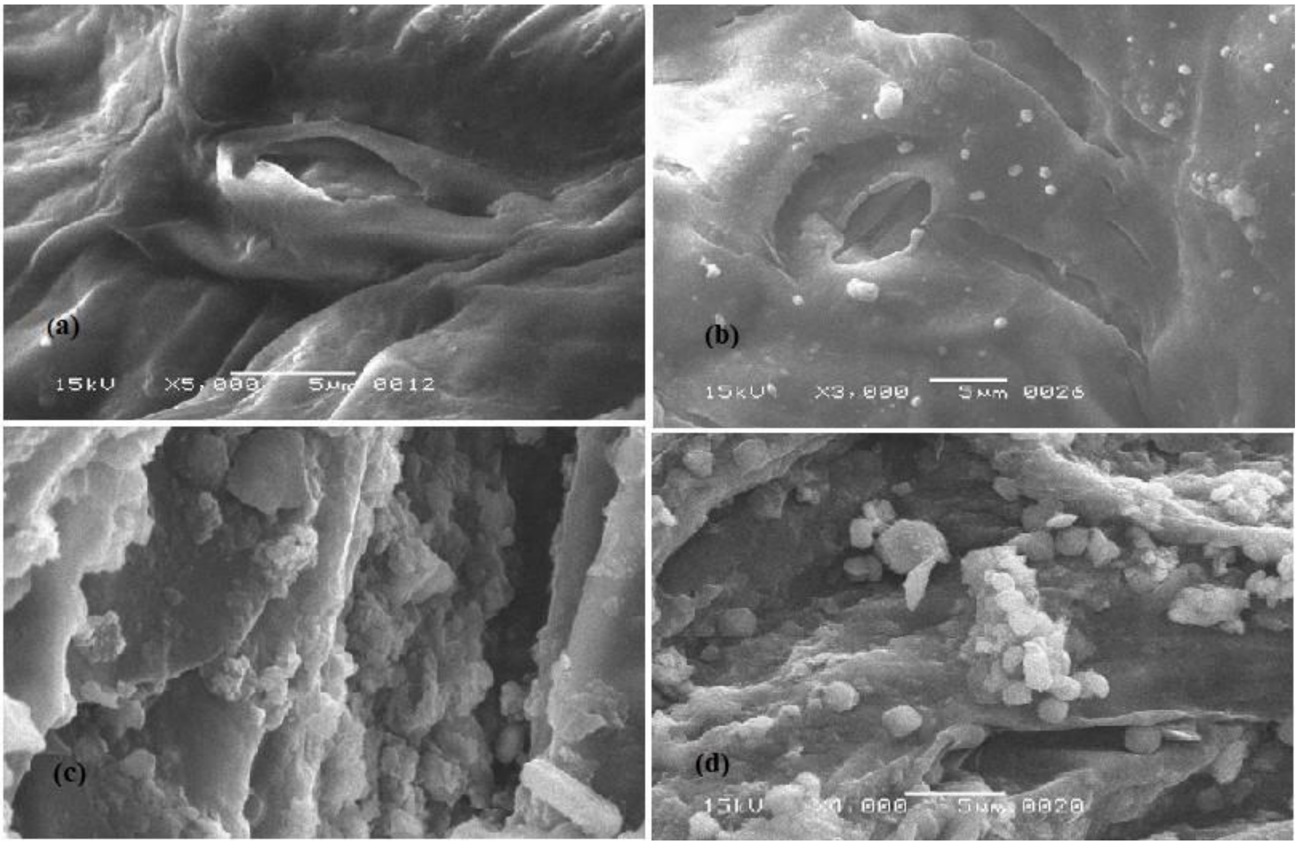

Fig.1. (a) Scan electron microscopy of stomatal opening of Control Leaves (b) Scan electron microscopy of stomatal opening of TiO2 treated leaves (c) Scan electron microscopy of root of control plant (d) Scan electron microscopy of root of TiO2 treated plant.

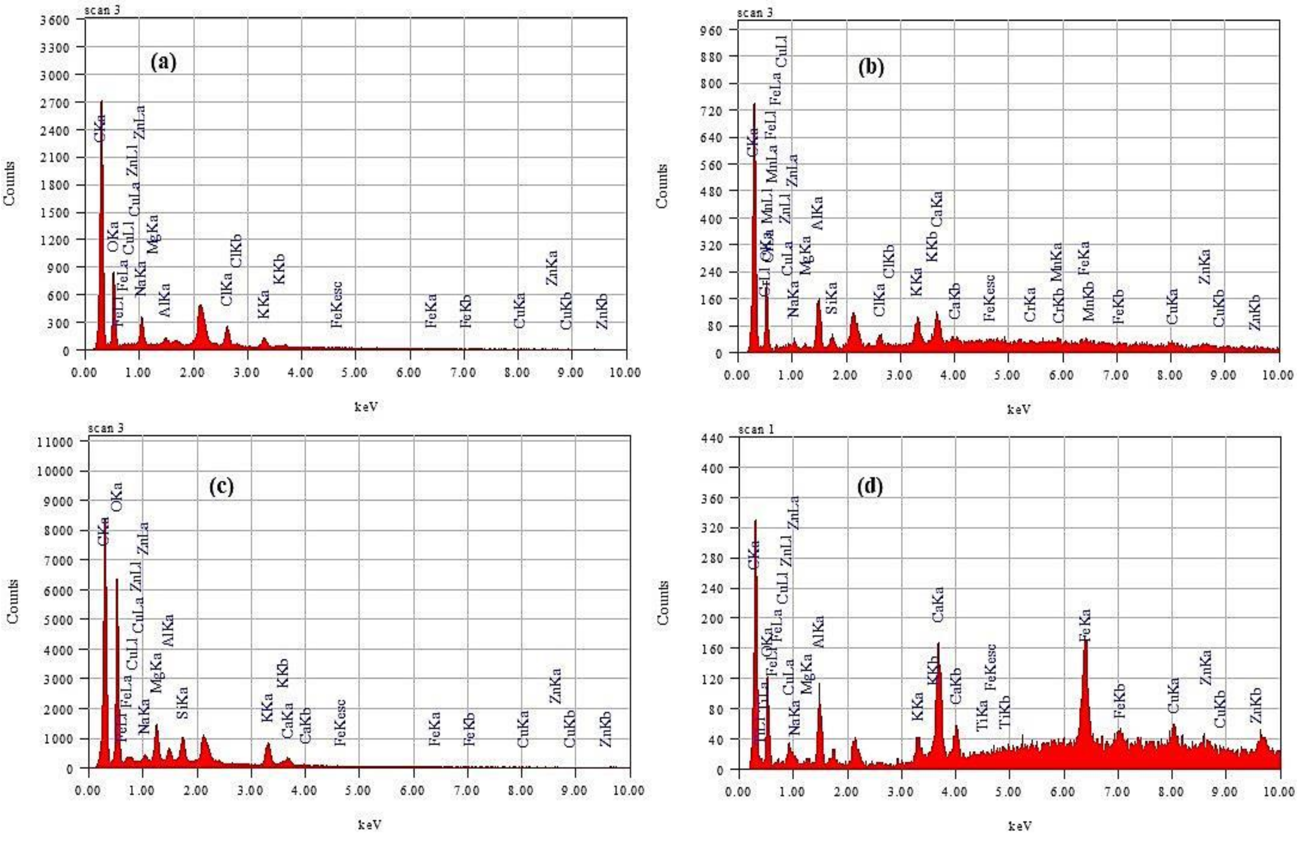

Fig. 2. (a) Energy Dispersive X-Ray Spectroscopy of leaves of control *Spinacia oleracea* plant (b) Energy Dispersive X-Ray Spectroscopy of leaves of TiO2 treated *Spinacia oleracea* plant (c) Energy Dispersive X-Ray Spectroscopy of root of control *Spinacia oleracea* plant (d) Energy Dispersive X-Ray Spectroscopy of root of TiO2 treated *Spinacia oleracea* plant.

Table 2. Elemental study of roots of *Spinacia oleracea* control and TiO2 treated plant through EDS (ZAF method standard less quantitative analysis).

Elements	keV	Mass %	Error %	At %	K	
C	0.277	20.00	0.43	27.77	9.8209	Control *Spinacia oleracea* plant roots fitting coefficient:0.5901
O	0.525	57.80	1.03	60.25	65.3140	
Na	1.041	1.25	1.19	0.90	1.1084	
Mg	1.253	5.67	0.83	3.89	4.9089	
Al	1.486	1.82	0.87	1.13	1.7236	
Si	1.739	3.41	0.90	2.02	3.7385	
K	3.312	6.38	1.45	2.72	8.7043	
Ca	3.690	2.07	1.81	0.86	2.8798	
Fe	6.398	1.08	5.30	0.32	1.2367	
Cu	8.040	0.11	13.43	0.03	0.1206	
Zn	8.630	0.41	19.11	0.11	0.4443	
Elements	**keV**	**Mass %**	**Error %**	**At %**	**K**	
C	0.277	6.28	0.21	18.31	1.9119	TiO_2 Treated *Spinacia oleracea* plant roots fitting coefficient: 0.5684
O	0.525	10.90	0.40	23.85	9.7361	
Na	1.041	0.46	0.62	0.69	0.2367	
Mg	1.253	0.18	0.42	0.25	0.0911	
Al	1.486	4.81	0.39	6.24	3.1397	
K	3.312	2.56	0.50	2.29	3.1129	
Ca	3.690	13.11	0.60	11.45	16.6327	
Ti	4.508	0.41	0.80	0.30	0.4377	
Fe	6.398	38.50	1.71	24.13	41.7542	
Cu	8.040	16.85	4.41	9.28	16.9481	
Zn	8.630	5.95	6.21	3.18	5.9988	

Discussion

The surface of leaves in plants acts as a device for light capturing, while stomata serve as an exchanger of water vapors with carbon dioxide and oxygen gases. These tiny pores are located on the epidermal section of land plants and can adjust their pores on the surface of tissues of the above-ground plant. This adjustment in size bounds the loss of water and aids in the absorption of carbon dioxide gas. Scanning Electron Microscopy images of leaves of *Spinacia oleracea* are shown in Figure 1. It was observed by emphasizing the magnification parameter of both Figures (1&2) that the stomatal opening of TiO_2 treated plant is enhanced, resulting in increased exchange for vital gases, i.e., carbon dioxide and oxygen. The impact of enhanced growth is due to the presence of TiO_2 on the surface of leaves and near the stomata (Fig. 1), suggesting that it enhanced CO_2 absorption and conversion into monomer and polymer, which results in good growth of the plants. It was related to the photocatalytic activity of TiO_2, which helps to capture more light in photosystem I & II and activates the rate of photosynthesis that results in increased biomass of *Spinacia oleracea* (Azmat *et al*., 2020). Water and light are critical for plant survival, whereas guard cells are present near the surroundings of stomata and help in determining the amount of CO_2 that should be accumulated in the plant and the quantity of water must retain in the plant. In water-rich condition, guard cells absorb water that becomes stretched, which cause the widening of the stomatal opening, and if water is lost, the guard cells contract, which results in the closing of the surrounding pores. This regulation of intake of carbon dioxide gas and the cycle of water through stomata play a vital role in the use of plants to assess global warming. Stomatal conductance was also investigated by Gonzalez-Mendoza *et al*., 2013 who concluded that reduction in exchangeability through stomata due to copper (II), results in a considerable decrease in photosynthetic activity in *Avicennia germinans*. While current investigation under TiO_2 NPs proves that the photocatalytic activity of NPs helps to guard cell functioning and assimilation of CO_2, which results in increased biomass, pigments, biochemical parameters, better photosynthetic activity, and stomatal conductance, larger leaf area, and growth rate. These are the direct consequences of TiO_2 NPs activity on *S. oleracea,* which are prominent even at shallow concentrations in soil (0.1 g/kg).

Impact of NPs on the root: The underground part of the plant's i-e, roots, is in direct contact with the living and nonliving material; therefore, it is always the first main targeted area of any biotic and abiotic stress. It was observed that TiO_2 penetrated the roots and can be seen in Figure 1, which then transported to the upper parts of the plant, i.e., leaves (Fig. 1). It was observed and reported that the growth parameter of roots, like length, fresh weight, dry weight, moisture content, carbohydrate, reducing sugar and protein was enhanced in TiO_2 treated plant as compared to the control (Azmat *et al*., 2020). Scanning Electron Microscopy of roots of control and TiO_2-treated plants was carried out to assess the fluctuation in the morphology presented in Figure (1) when NPs of concentration 0.1 g/kg were used in soil. Therefore, roots were directly in contact and the main target of Ti NPs within the plant. Sheldon & Menzies (2005) reported that Cu enrichment was destructive to plant roots, due to which cuticle of roots were damaged and decreased in root hairs propagation and distorted root structure observed, but in the current study, slight enrichment of TiO_2 in soil was found positive to the structure, functions, and mass of roots with increased biomass of the overall plant. Constructive effects on *S. oleracea* are in accordance with the work of Zheng *et al*., 2005, where an increase in biomass is observed with the seedlings of *S. oleracea* when the concentration of TiO_2 NPs was high, i.e., up to 4%. It was established that the altered morphology of roots of *S. oleracea* had an influence on the transportation of water molecules from soil to plants, due to which moisture content was increased (Table 2).

Impact on the elemental composition of *Spinacia oleracea* leaves and root: It was observed that TiO_2-NP applications positively affected macro (N, P, K) and micronutrient (Zn, Mn, Cu) concentration, including Fe, in comparison to the control treatment, the concentration of Zn, Mn, Cu, N, and P significantly increased with the application of TiO_2 nanoparticles. The vital macronutrient elements like K, its intensification linked with the stomatal opening of stomata where better exchange of gases was observed due to greater leaf area (Table 1) followed by better photosystem I observed (Azmat *et al*., 2020). Water contents in leaves are also controlled by K because the stretching of the stomata pore is correspondingly controlled by the potassium ion pump (Lequeux *et al*., 2010). It was concluded that the increase in growth and biomass is related to the increased K contents. Mg a chief macronutrient, concentration in plants increased from 0.09 % to 0.63% in leaves whereas 3.89% to 0.25% in the root (Tables 1&2). Mg is the key element of chlorophyll and a vital nutrient for the plant, as well as acts as a catalyst for the activation of various enzymes to overcome any stress (Sonmez, 2007; Lequeux, *et al*., 2010). The drastic increase in Calcium (Ca) was also noted in roots from 0.86 % to 11.45 % (Tables 1&2), which assists in the transportation of nutrients and increases resistance to stress with the synthesis of tissues of the plant. The presence of the Ca and Mn over controlled plants also supports the activity of oxygen-evolving complex or photobiological reactions in plants where oxidation of water to diatomic oxygen and H ions takes place, due to which good growth of the plant is observed.

$$2H_2O + hv = O_2 + 4e + 4H^+ \qquad (1)$$

According to the equation, oxygen released through stomata (Fig. 1) and H used in ATP.

Mechanism operating under Ti accumulation: As reported earlier by our group Azmat *et al*., (2020), an increase in the chlorophyll contents with Mg, K Fe, and Mn regulate the release of oxygen through photosystem II, therefore, inhibiting the formation of reactive oxygen species and helping in removing oxygen from plants through the stomatal opening. Lower values of oxygen (O) in the leaves and roots of plants indicate that the incidence of oxidative stress and generation of reactive oxygen species (ROS) is controlled under Ti-NPs. Carbon (C) is the fundamental element of plants that is the foundation of different C-containing compounds like protein, amino acid glucose, etc. The less C contents in treated plants in EDX scanned images over control prove that due to photoactivity of TiO_2 and elevated concentration of essential pigments like Chlorophyll "a" and "b" increased the conversion rate of C into glucose and other polymers. This is a positive sign for plants under low concentrations of TiO_2 NPs; consequently, good growth of plants was observed. The current results are in accordance with earlier reports of Lyu *et al*., (2017), who observed the constructive roles of Ti NPs in the growth of plants which was related to its interaction with the other essential nutrient elements like iron (Fe), where the synergistic and antagonistic relationships of Fe and Ti is important. It was established that the photocatalytic properties of TiO_2 NPs induce an oxidation-reduction reaction that markedly promotes growth rates and chlorophyll synthesis and stimulates several enzymes, thereby enhancing the activities of photosystems I and II (Yang *et al*., 2006). TiO_2NPs increase light absorbance, hasten the transport and conversion of light energy, protect chloroplasts from aging, and prolong the photosynthetic time of the chloroplasts (Yang *et al*., 2006). It may be due to TiO_2 NPs protecting the chloroplast from excessive light by augmenting the activity of antioxidant enzymes, such as catalase, peroxidase, and superoxide dismutase as reported by Hong *et al*., (2005).

Conclusion

It was concluded that the presence of TiO_2 in the soil had favorable impacts on *S. oleracea* at a low concentration which provides help in photosystem II in the release of diatomic oxygen and proton to activate the ATP in plants, thereby regulating the plant metabolic pathway in an activated state. It was also concluded that elemental composition on the surface of leaves was found to be enhanced in comparison to the control, which was linked with enhanced membrane pores due to the accumulation of TiO_2 NPs.

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