Titanium nanoparticles attenuates arsenic toxicity by up-regulating expressions of defensive genes in Vigna radiata L

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abstract
Arsenic (As)-toxicity is recognized as one of the major environmental problems, affecting productivity of crops worldwide, thereby threatening sustainable agriculture and food security. Progression in nanotechnology and its impacts have brought up concerns about the application of engineered nanoparticles (NPs) in various sectors of the economy, including the field of agronomy. Among various NPs, there has been a rising amount of interest regarding the effects of titanium NPs (TiNPs) on plants growth and development, and their fate of abiotic stress tolerance. Hence, the present study was aimed to assess the ameliorative potentialities of chemically and biologically/green synthesized TiNPs to alleviate As-induced toxic responses in Vigna radiata L. The results revealed that exposure to As hindered the growth indices (radicle length and biomass) and membrane integrity, while were improved with the application of chemical and green manufactured TiNPs. In addition, treatment of As provoked the accretion of reactive oxygen species (superoxide and hydrogen peroxide) and malondialdehyde (a lipid peroxidized product), but were diminished by the supplementation of chemical and green synthesized TiNPs. The experimental data also signified that exogenous application of chemical and green synthesized TiNPs conferred tolerance to As-induced oxidative injuries via perking-up the expressions of antioxidant genes and enzyme systems viz; superoxide dismutase and catalase. Therefore, the present study inferred that chemically and green synthesized TiNPs, particularly green manufactured, effectively mitigated the adverse impacts of As by augmenting antioxidant machinery, thereby proving its potentiality in the alleviation of As-toxicity, at least in Vignaradiata L.

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**Introduction**

Arsenic (As) is a perilous metalloid ubiquitously persisting in the natural environments and, highly noxious to all the life forms. Plants accumulate surplus amount of As when cultivated/irrigated with As-contaminated soil/water, leading to oxidative injury inside the cell which ultimately results into reduced growth and biomass accumulation, gaseous exchange from leaves and chlorophyll synthesis, thereby affecting net photosynthesis, nutrient supply, cellular water potential and protein turnover, and altering proper functioning of effective enzymes (Chandrakar et al., 2016).

Plants, when exposed to phytotoxic amounts of As, aggravate generation of reactive oxygen species (ROS) like superoxide anion (O$_2^-$) and hydrogen peroxide (H$_2$O$_2$), hence perturbing the cellular redox homeostasis (Sharma et al., 2012; Chandrakar et al., 2017). This over-production of ROS has been shown to lead membrane disruption via oxidation of lipids (estimated as malondialdehyde (MDA)), proteins and nucleic acids (Xalxo and Keshavkant, 2018; Yadu et al., 2018). To bear As-induced oxidative injury, plants have developed a complex antioxidative protective system which includes enzymatic (superoxide dismutase (SOD), catalase (CAT), etc.) and non-enzymatic components, guarding themselves against excessive ROS (Singh et al., 2009; Chandrakar and Keshavkant, 2018).

One of the advanced approaches that have been implemented to defend plants against various abiotic stresses and maintain long-term sustainability is nanotechnology, which utilizes the concept of ‘minimal usage with maximum effect’ (Yadu et al., 2018). Having an average size of less than 100 nm, nanoparticles (NPs) possess exclusive properties that depend on their phase distribution, size and morphology. In the field of agriculture, nanotechnology currently focuses on objective farming that involves the utilization of NPs that can perk-up the growth and productivity of crops by exerting minimum input and generating lesser wastage than the conventional products and approaches (Batsmanova et al., 2013; Yadu et al., 2018).

Nanoparticles are often synthesized using different chemical techniques like chemical reduction, solvo-thermal reduction, electrochemical techniques and photochemical reaction in reverse micelles, amongst which chemical reduction is the most commonly applied procedure (Gudikandula and Maringanti, 2016). Studies have illustrated that the use of a chemical reducing agent consumes more energy and generates larger sized particles. These methods have also been reported to have more side products which are non-eco-friendly in nature. Additionally, the chemically synthesized NPs were found to show less stability and more agglomeration (Mukherjee et al., 2001). Hence, alternate eco-friendly protocols should be adopted which can utilize bacteria, fungi and plant extracts as reducing agents and, produce stable and dispersible NPs of desired size by consuming less energy (Kumar et al., 2014). These biological/green synthesis methods are not only environmental friendly but also cost-effective, rapid, less arduous and more proficient than the conventional chemical procedures. As these do not require any toxic chemicals for the synthesis, the manufactured NPs are compatible as well as safer for most of the biomedical and pharmaceutical applications (Willner et al., 2007).

Among various NPs, titanium NP (TiNP) is considered as one of the major engineered NPs which is largely utilized in cosmetics, chemical, paint and food industries (Liu et al., 2014). Also, in the field of agriculture, there has been arising amount of attention regarding the effects of TiNPs on plant growth and yield. These NPs are also recommended as photoprotective elements which guard leaf surfaces against UV-stress and reduce sunburn instances (Gogos et al., 2012). Studies have shown augmentation in the germination rate and radicle length (RL) of Salvia officinalis, Brassica napus and Avenasativa when they were exposed to TiNPs (Andersen et al., 2016; Feizi et al., 2013). Improvement in nitrate reductase activity and stimulation in the antioxidant enzymes was also observed when the mixture of titanium and silicon NPs were applied to Glycine max (Lu et al., 2002). Zheng et al. (2005) have demonstrated enhanced rate of photosynthesis and growth in Spinacia oleracea by the application of TiNP which promote chlorophyll synthesis and increase activity of RubisCo. The positive and negative effects of TiNP depend on the size, shape, concentration, surface coating material, exposure time, and plant species and its developmental stage (Lyu et al., 2017; Moll et al., 2016).

Although, existing reports reveal both promoting and reducing impacts of TiNP on various crops, information about its function under heavy metal stress is still scarce. Studies of Cai et al. (2017) showed that TiNP lowers lead uptake and bioaccumulation in Oryza sativa, thereby reducing the health risk associated with its contamination in the food chain. Likewise, Faraji and Sepehri (2018) demonstrated that co-application of TiNP and sodium nitroprusside could be a promising approach in neutralizing the adverse effects of cadmium on seed germination and early growth of Triticum aestivum. To the best of our knowledge, this is the first report on exploring the promising effects of TiNP dealing with the toxic impacts of As in any of the plant species.

**Vigna radiata** L. was used in this study as a model crop which is one of the important legumes grown globally. It is a protein rich (25%) legume of high digestibility. India is one of its major producers in the world and more than 70% of the world’s production comes from India alone. In India, As-contamination in groundwater was first spotted in the West Bengal in 1983; later several states including Chhattisgarh have been reported to be effected, distressing the agricultural land and the crop productivity in a greater extent (Bhowmick et al., 2018). Vigna radiata L. has been quite extensively studied for As-phytotoxicity and several reports are corroborating its sensitivity towards As-exposure (Upadhayya et al., 2014; Das and Sarkar, 2018). Therefore, the conducted study was aimed to evaluate the ameliorative potentialities of chemically and biologically/green synthesized TiNPs on an important crop by scrutinizing RL, biomass (fresh mass (FM) and dry mass (DM)) and membrane stability index (MSI) towards As-induced toxicity. Additionally, accumulation of As, accretion and detection of ROS, contents of MDA and protein, and expressions of SOD and CAT genes were also monitored in Vigna radiata L. exposed to As alone and its amalgamation with each of the chemically and green manufactured TiNPs.
1. **Materials and methods**

1.1. Syntheses and characterization of titanium nanoparticles

For the green synthesis of TiNP, freshly amassed leaves of banana (Musa paradisiaca) were surface cleaned with running tap water followed by milliQ water (MW) (Millipore, Gradient A-10, USA) to remove the surface artifacts. Thereafter, 25 g of leaves were chopped into small pieces and homogenized using 25 mL of MW, followed by heating at 80°C for 5 min. Further, the homogenate was filtered using Whatman filter paper No.1 and then centrifuged at 5000 r/min for 10 min. The supernatant thus obtained was used for NP synthesis, and could be stored in refrigerator for one month (Patel et al., 2015). To 100 mL of 0.5 mol/L titanium oxysulphate solution, 25 mL of filtered plant extract was added drop-wise under stirring condition for 5 hr. For chemical synthesis, a similar procedure was followed, except in place of plant extract, 1 mol/L NaOH was added till the formation of precipitate. The procedure was followed, except in place of plant extract, stirring condition for 5 hr. For chemical synthesis, a similar procedure was followed, except in place of plant extract, 1 mol/L NaOH was added till the formation of precipitate. The precipitate formed of green synthesized TiNP (white colored) and chemically synthesized TiNP (brown colored) were dried, grinded and calcined at 500°C in a muffle furnace for 4 hr (Kumar et al., 2014). The calcinated and powdered TiNPs (both green and chemically synthesized) were characterized following the UV-Vis Spectroscopy (Lambda-25, Perkin Elmer, USA), dynamic light scattering (DLS) (Malvern Zetasizer, Ver 7.01; MAL: 1079370, UK), fourier-transform infrared spectroscopy (FT-IR) (Nicolet iS10, Thermo Fisher Scientific, USA) and X-ray diffraction (XRD) (PANalytical 3 kW X’pert Powder XRD, UK) (Kumar et al., 2014; Khade et al., 2015).

1.2. Collection of seed and growth conditions

Seeds of Vigna radiata L. (var. HUM-16) were procured from the Department of Agronomy, Indira Gandhi Agriculture University, Raipur, and surface sterilized with 0.1% (W/V) mercuric chloride for 3 min followed by repeated washing (3 times) with MW. These seeds were imbibed in 50 mL each of MW, 10 μmol/L of As (sodium arsenate was used as a source of As), chemical TiNP (0.25%), As + chemical TiNP, green TiNP (0.1%), and As + green TiNP, for 2 hr. Thereafter, all the seeds were allowed to germinate by keeping them on double layered filter paper pre-soaked with the respective treatment solutions, in separate boxes at 29 ± 2°C for five consecutive days, under dark conditions. The seeds were irrigated after every 24 hr with 5 mL of respective treatment solutions. After five days of treatments, RL, FM and DM were assessed (Yadu et al., 2018).

1.3. Arsenic content

For determining the content of As, 0.1 g of dried radicles were digested at 80°C using HNO3/H2O2/H2O in a ratio 3/2/10 (W/W/V) to obtain a clear solution and then its volume was maintained up to 15 mL using MW. Further, As content was estimated by using an atomic absorption spectrometer coupled with hydride generation system (Agilent, AA240, USA). For calibration and quality assurance, the standard reference material of As (1.19773.0500, Merck, Darmstadt, Germany) was used (Chandrarak et al., 2017).

1.4. Membrane stability index

Estimation of MSI was performed by applying the procedure of Yuan et al. (2014), and calculated using the formula: MSI % = [(1 − T1/T2)] × 100, where T1: sample containing tube 1 (heated at 40°C for 30 min) and T2: sample containing tube 2 (heated at 100°C for 15 min).

1.5. Reactive oxygen species

To evaluate the levels of O2⁻ and H2O2, the procedures of Schopfer et al. (2001) and Velikova et al. (2000) were respectively followed and the contents were expressed as μmol/min/g FM and μmol/g FM respectively.

1.6. Fluorescence microscopy

Production and localizations of produced O2⁻ and H2O2 were detected using the dye dihydroethidium (DHE) and 2',7'-dichlorodihydrofluorescein diacetate (H2DCFDA) respectively (Kovacik and Babula, 2017). Radicles were stained with respective fluorescent dyes, in the dark, and then thin cross sections were cut from their tips. These cross sections were then focused and imaged using fluorescence microscope (LED 2000; Leica, Germany).

1.7. Malondialdehyde

The content of MDA (a lipid peroxidized product) was estimated, following the method of Hodges et al. (1999) and was expressed as mmol/g FM.

1.8. Extraction of protein and enzymes, and their estimations

For the extraction of protein and enzymes, radicles (0.2 g) were macerated with Zivy’s buffer (2 mL) followed by centrifugation (14,000 r/min, 20 min, 4°C). Following the protocol of Bradford (1976), the protein content was estimated, while activities of various antioxidant enzymes were determined as mentioned below:

Procedure of Marklund and Marklund (1974) was applied for determining the SOD (EC: 1.15.1.1) activity and was expressed as Units/min/g FM, while CAT (EC: 1.11.1.6) was evaluated as per the method of Chance and Maehly (1955) and mentioned as μmol/min/g FM.

1.9. Gene expression analysis using reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was isolated using hot phenol method (Verwoerd et al., 1989), and the quality and concentration of it were measured by gel electrophoresis (BioRad, USA) and nanodrop spectrometer (ND1000, Thermo Fisher Scientific, USA) respectively. Further, by using Superscript VILO cDNA synthesis kit, cDNA was synthesized (Invitrogen, Carlsbad, CA, USA) and RT-PCR was performed by using a set of selected primers.
gene specific primers, in a 10 mL reaction volume. The normalization of templates was done with the help of housekeeping gene beta actin. Specific primers for SOD (F- 5’- CGGAAATGGTCCAACACTG-3’; R- 5’-TGTTCCAGTTGAAAGCCAAAC-3’); and CAT (F- 5’- TTCAAGCAGCTGGAGAGAG-3’; R- 5’-CCAGACACTGGGATTTTTACAT-3’) were designed using a web based primer designing tool primer3 (http://frodo.wi.mit.edu), and synthesized by M/s Eurofins Genomics India Pvt. Ltd., Bengaluru, India (Panda and Matsumoto, 2010; Nair and Chung, 2015). The PCR reaction was carried out using the following cycle conditions: an initial denaturation (95°C, 10 min), 35 cycles at 95°C for 60 sec, 57.3°C–59.4°C for 45 sec, 72°C for 60 sec, followed by 10 min extension at 72°C. The amplicons were separated by electrophoresis using 1.5% (W/V) agarose gel in 1 x TAE buffer with a constant voltage of 50 V for 40 min. Gene expression was analyzed and compared by fluorescence intensities of the bands under Gel-Doc system (BioRad, USA).

1.10. Data analysis

The data obtained from different experiments were analyzed following analysis of variance and using SPSS software (Ver 16.0). Duncan’s multiple range tests were performed to determine the effects of different exposures on various parameters as compared to the control. The values of \( P < 0.05 \) was considered significant after analyzing the means ± SD of five separate observations.

2. Results and discussion

2.1. Characterization of titanium nanoparticles

Characterizations of the synthesized TiNPs were done following the UV-Vis spectroscopy, DLS, FT-IR and XRD techniques (Fig. 1a-d). The optical absorption spectrums of chemical and green TiNPs were recorded in the wavelength range of 200 nm–600 nm, and their maximum absorption peaks were obtained at 350 nm and 365 nm respectively.

The DLS is popularly used to determine the size distribution profiles of small particles in suspension or polymers in solution. The DLS histogram revealed the particle size distribution with maximum intensity at 64.28 nm for chemical TiNP and 53.18 nm for green TiNP. Possible reasons for the smaller size of green-synthesized TiNP could be the presence of phytochemicals in the banana extracts (used during synthesis) that served as reducing, stabilizing and capping agent and prevented their aggregation. Similar reduction in size and stability was also observed in bio-synthesized copper and gold NPs as compared to their chemically engineered ones (Saif et al., 2016; Biao et al., 2018). Further, after addition of As in the solution, the particle sizes of chemical and green TiNPs were increased to 105 nm and 115 nm respectively, which might be due to the adsorption of As over the surfaces of TiNPs (Fig. 1b). The reason for more increment in sizes of the green TiNPs could be due to the interaction between the functional

Fig. 1 – UV-Vis absorption spectra of chemical and green synthesized titanium nanoparticles (TiNPs) (a), DLS of A) chemical, B) arsenic with chemical (As + chemical), C) green, and D) arsenic with green (As + green) synthesized TiNPs (b), FTIR of chemical and green synthesized TiNPs (c) and, XRD of A) chemical B) green synthesized TiNPs.
groups of phytochemicals present in the banana extract and As, which then resulted in sequestration of As.

In FTIR spectrum, a broad band of green synthesized TiNP was observed in between 3500 to 3000 cm\(^{-1}\) which was due to the hydroxyl (O–H) stretch, representing the presence of water as moisture. The peak at 1603 cm\(^{-1}\) explained the stretching of C–O and C=O bonds of carboxylate group which might be present in the plant extract. The intense peak between 800 and 450 cm\(^{-1}\) described the Ti–O stretching bands. However, in chemically synthesized TiNP, an absorption peak around 3288 cm\(^{-1}\) was obtained which might be due to the stretching vibration of −OH groups on the TiO\(_2\) surface. The peak at 1641 cm\(^{-1}\) confirmed the O–H bending of dissociated or molecularly adsorbed water molecules, respectively. The slight peak formed between 400 and 800 cm\(^{-1}\) was ascribed to be the strong stretching vibrations of Ti–O–Ti bonds. Shifts in the bands were associated with the binding of functional groups, contained in plant aqueous extract, to surfaces of NPs.

The XRD profiles of chemically and green synthesized TiNPs are shown in Fig. 1d. In this figure, five diffraction peaks were observed that were approximately similar and consistent with the standard data of anatase form of TiNP (JCPDS 21–1272) revealing that the manufactured TiNP was in crystalline anatase form (Kumar et al., 2014). Sharp peaks were recognized in chemically synthesized TiNPs, whereas the peaks in green manufactured TiNP were found to be blunt with little broadening, suggesting the reduction in size of green TiNP. The peak intensities of green TiNP were less than the chemical TiNP which might be due to the presence of phytochemicals of the banana extract. The coating of NPs surface with the functional groups might have decreased the signal: noise ratio because of internal strain in the particles, surface with the functional groups might have decreased the phytochemicals of the banana extract. The coating of NPs the chemical TiNP which might be due to the presence of green TiNP. The peak intensities of green TiNP were less than with little broadening, suggesting the reduction in size of peaks in green manufactured TiNP were found to be blunt recognized in chemically synthesized TiNPs, whereas the talline anatase form (Kumar et al., 2014). Sharp peaks were observed that were approximately similar and consistent with the standard data of anatase form of TiNP (JCPDS 21–1272) revealing that the manufactured TiNP was in crystalline anatase form (Kumar et al., 2014). Sharp peaks were recognized in chemically synthesized TiNPs, whereas the peaks in green manufactured TiNP were found to be blunt with little broadening, suggesting the reduction in size of green TiNP. The peak intensities of green TiNP were less than the chemical TiNP which might be due to the presence of phytochemicals of the banana extract. The coating of NPs surface with the functional groups might have decreased the signal: noise ratio because of internal strain in the particles, due to which intensities decreased (Kannan and John, 2008). Hence, the overall patterns of XRD analysis suggested that the size of chemical TiNP was comparatively more with sharp peaks and high intensities than that of green synthesized TiNP. The XRD data also provided evidence that the protocol developed using banana extract is convincing for the synthesis of bio-functionalized and bio-stabilized TiNPs.

### 2.2. Growth traits

Significant declines in RL (45%), FM (53%) and DM (53%) of the radicles exposed to As were observed as compared to their respective controls (Table 1). These reductions are probably due to the increased permeability of cell membranes; thereby loss of constituents and improper nutrients uptake that are the basic requirements of any tissue or organ to grow and develop optimally (Yadu et al., 2019). In addition, exposure of As leads to reduced rate of cell elongation that can also contributed to inhibition of RL. Diminution in biomass accumulation was the result of loss of water, and also lesser uptake of water by the root cells during As-stress (Chandrarak et al., 2017). In contrast, exogenous application of chemically and green manufactured TiNPs, green TiNP enhanced all the growth indices more effectively, which might be due to the growth enhancing properties of it that includes high surface reactivity and enlargement of root pores, and in turn, enhanced water absorption and nutrients availability to the growing plants (Feizi et al., 2013). Additionally, NPs augment the activity of nitrate reductase which promotes plant growth (Alumutairi and Alharbi, 2015). Similar results have also been observed in Triticum aestivum and Vicia faba (Hamed et al., 2018; Jiang et al., 2017). However, increasing concentration of TiNP results in its accumulation which might decrease the water availability due to blockage of root pores (Movafeghi et al., 2018). Previous reports indicated that TiNP have a positive effect at a suitable concentration, while posses adverse impacts when applied at higher concentrations (Hamed et al., 2018). Hence, the above data illustrates that green synthesized TiNP has better efficiency than the chemical ones in ameliorating As-toxicity.

#### 2.3. Arsenic content

Radicles of Vigna radiata L. exposed alone to As accumulated 45 µg As/g DM, while the tissues treated with chemical and green TiNPs amended As solutions accrued only 40 and 31 µg As/g DM respectively, signifying the importance of TiNP in reduced uptake of As in Vigna radiata L. (Table 1). Also, least accumulation was observed in green synthesized TiNP treated radicles, suggesting that it has better capacity in lowering the accretion of As than the chemical ones. The possible reason behind the lesser uptake and accrual of As might be the increased adsorption of As on TiNPs from the solution leading to increased particle size (Fig. 1b). Thus, diffusion of As across the plasma membrane is inhibited as it may not be able to cross the cell wall. Pena et al. (2006) explained this adsorption mechanism of As on TiNPs by using a combination of macroscopic and microscopic techniques, including extended

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>As</th>
<th>Chemical TiNP</th>
<th>Chemical TiNP + As</th>
<th>Green TiNP</th>
<th>Green TiNP + As</th>
</tr>
</thead>
<tbody>
<tr>
<td>Radicle length (cm)</td>
<td>5.86 ± 0.23</td>
<td>3.22 ± 0.22</td>
<td>6.26 ± 0.18</td>
<td>4.5 ± 0.14</td>
<td>7.24 ± 0.2</td>
<td>5.22 ± 0.25</td>
</tr>
<tr>
<td>Fresh mass (g)</td>
<td>0.73 ± 0.008</td>
<td>0.34 ± 0.01</td>
<td>0.74 ± 0.01</td>
<td>0.57 ± 0.01</td>
<td>0.8 ± 0.02</td>
<td>0.62 ± 0.01</td>
</tr>
<tr>
<td>Dry mass (mg)</td>
<td>68 ± 2.7</td>
<td>31.8 ± 2.5</td>
<td>73 ± 1.5</td>
<td>52.8 ± 1.4</td>
<td>75.4 ± 1.6</td>
<td>54.6 ± 1.6</td>
</tr>
<tr>
<td>Arsenic content (µg/g DM)</td>
<td>Not done</td>
<td>45 ± 0.5</td>
<td>Not done</td>
<td>40 ± 0.4</td>
<td>Not done</td>
<td>31 ± 0.3</td>
</tr>
</tbody>
</table>

Values presented are mean ± SD of five sampling replicates. Different superscript alphabets cited in the table indicate significant differences (P < 0.05) among six treatments.
X-ray absorption fine structure, FT-IR, and electrophoretic mobility measurements, which showed that the As formed a bidentate binuclear inner-sphere complex on the surface of TiNPs. Moreover, Praveen et al. (2018) also observed that particle size of Fe3O4 NPs had increased after the addition of As in solution, resulting its lesser uptake in Brassica juncea.

2.4. Membrane stability index

A remarkable reduction in the MSI (52%) of Vigna radiata L. radicles was observed under As-toxicity (Table 1). The probable reason for reduction in MSI might be that during As-toxicity, production of ROS was exaggerated leading to oxidative stress inside the cells; consequently peroxidation of membrane lipid moieties due to which leakage of cellular constituents takes place. In the current study, an inverse correlation between MSI and ROS production ($O_2^- : r = -0.98$ and $H_2O_2 : r = -0.98$) was observed under As-stress. Similar result was also observed by Chandrakar et al. (2017) in Glycine max radicles. Contrarily, external application of chemical and green synthesized TiNPs increased the integrity of membranes revealing their membrane stabilizing property.

2.5. Reactive oxygen species

Plants on exposure to As produces excessive production of ROS, hence disturbing the cellular redox homeostasis. In our study, As caused high accrual of the ROS ($O_2^- : 114%$ and $H_2O_2 : 191%$) as compared to that determined in the MW grown radicles. However, reduced level of ROS was recorded in tissues grown into exogenously applied chemical ($O_2^- : 20%$, $H_2O_2 : 35%$) and green manufactured ($O_2^- : 29%$, $H_2O_2 : 43%$) TiNPs with As solution (Table 2). Data specified that the green TiNP recompensed the harmful impacts of As more proficiently than the chemical ones. This might be due to the presence of phytochemicals in green synthesized TiNPs that augmented the antioxidant defense system, and thus lowered the level of ROS. Similar properties of titanium and silver (Ag) NPs were also reported by Hamed et al. (2018) and Yadu et al. (2018). Overall, green TiNP exhibited foremost role in the prevention of As-driven lipid peroxidation reaction in comparison to chemical ones.

2.6. Malondialdehyde

During severe stress condition like As exposure, level of ROS increases resulting into the oxidative injury inside the cells and enhancing lipid peroxidation reaction (Yadu et al., 2018). Considerable elevation in the MDA (90%) content was found in the As-exposed Vigna radiate L. radicles as compared to control (Table 2). On the contrary, the application of chemical and green TiNPs along with As significantly lowered the level of MDA by 39% and 44% respectively. The results suggest that TiNP might have the capacity to guard the membranes against As-induced oxidative damage. Similar findings were also observed in Leucaena leucocephala and Cajanus cajan revealing the protective functions of zinc oxide NPs and AgNPs against heavy metal and F-induced toxicity (Venkatachalam et al., 2017; Yadu et al., 2018). Overall, green TiNP exhibited foremost role in the prevention of As-driven lipid peroxidation reaction in comparison to chemical ones.

2.7. Protein content

Due to high affinity towards –SH groups, As readily combines with proteins affecting their structures and functions (Chandrakar et al., 2016). The data revealed that protein content considerably (41%) decreased when radicles were exposed to As singly (Table 2). Remarkable fall in the protein content was also documented in response to As-toxicity in Oryza sativa (Singh et al., 2009) and Vigna radiata L. (Ismail, 2012). However, after the application of chemically and green synthesized TiNPs, protein content significantly increased by 34% and 59% respectively, even in tissues exposed to As, proving that green TiNP were more proficient than the chemical ones. Earlier studies also confirmed an increase in the protein content in Trigonella foenum-graecum and Fism sativum on application of AgNPs (Mahmood and Murtaza, 2017; Sadak, 2019).

2.8. Activities and gene expressions of antioxidants

Data of the present study revealed that exposure to As significantly decreased the activities of SOD (59%) and CAT treated radicles suggesting that it has better potential to compensate the oxidative injury caused by As treatment (Fig. 2a and b).

### Table 2 – Fluctuations in the contents of superoxide, hydrogen peroxide, malondialdehyde and protein in radicles of Vigna radiata L. in response to As alone and in combination with titanium nanoparticles (chemical and green synthesized).

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>As</th>
<th>Chemical TiNP</th>
<th>Chemical TiNP + As</th>
<th>Green TiNP</th>
<th>Green TiNP + As</th>
</tr>
</thead>
<tbody>
<tr>
<td>Superoxide (μmol min/g FM)</td>
<td>75.7±6.7</td>
<td>162±5</td>
<td>68.3±7.8</td>
<td>129.7±6.8</td>
<td>62.3±5</td>
<td>115±3.1</td>
</tr>
<tr>
<td>Hydrogen peroxide (μmol g/FM)</td>
<td>2.56±0.2</td>
<td>7.45±0.3</td>
<td>2.19±0.08</td>
<td>4.8±0.1</td>
<td>2.12±0.07</td>
<td>4.2±0.03</td>
</tr>
<tr>
<td>Malondialdehyde (mmol g/FM)</td>
<td>21.7±2</td>
<td>41.3±1.4</td>
<td>18.7±1.16</td>
<td>25.3±1.5</td>
<td>17.8±1.17</td>
<td>23±1.5</td>
</tr>
<tr>
<td>Protein content (mg g/FM)</td>
<td>317.1±8</td>
<td>188±13.2</td>
<td>345.2±11.1</td>
<td>252.1±10.5</td>
<td>379.4±5.5</td>
<td>298.5±5.2</td>
</tr>
</tbody>
</table>

Values presented are mean ± SD of five sampling replicates. Different superscript alphabets cited in the table indicate significant differences ($P < 0.05$) among six treatments.
(65%) in the As-treated radicles of Vigna radiata L. On the other hand, in As-stressed tissues treated with chemical and green synthesized TiNPs, augmentation in the activities of both SOD and CAT were observed (Fig. 3a and b). These results are in agreement with the findings of Hamed et al. (2018) and Movafeghi et al. (2018) revealing the antioxidant boosting property of TiNPs in Spirodela polyrrhiza and Vicia faba respectively. A negative correlation between SOD and O$_2^-$ ($r = -0.92$) and, CAT and H$_2$O$_2$ ($r = -0.96$) were also depicted in this study. The highest activities of these enzymes were determined in the green TiNP treated tissues, in which amount of accrued ROS was least. Hence, it could be assumed that TiNP application improved the enzymatic antioxidant defense system which would help plants to achieve a better management of increased ROS levels generated under As-stress.

For further confirmation, SOD and CAT-specific gene analysis was performed following RT-PCR. Expressions of both the SOD and CAT genes were found to be down-regulated in As-exposed radicles. In contrast, their expressions were up-regulated when chemically and green synthesized TiNPs were added along with As (Fig. 3a and b). Also, among these two, green TiNP proved to be more proficient in boosting the gene expressions of antioxidant enzymes than the chemically manufactured ones. The results were in agreement with their spectrophotometric data.

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Fig. 2 – Fluorescence visualization of superoxide (a) and hydrogen peroxide (b) in Vigna radiata L. radicles treated with arsenic and titanium nanoparticles (TiNPs) (chemical and green synthesized).
3. Conclusions

The results of the present study illustrated the ameliorative impacts of TiNPs in the growing tissues of Vigna radiata L. towards As-toxicity via enhancing the growth traits, MSI and protein content. Additionally, the chemically and green synthesized TiNPs reduced the levels of ROS and MDA via increasing the activities/gene expressions of antioxidant enzymes (SOD and CAT) thus, directly or indirectly hindering the generation of ROS. The plant mediated synthesis method employed in this study was simple, cost-effective and eco-friendly, and performed far better than the chemical NPs with most of the parameters, amending the toxic impacts of As treatment. Hence, it can be inferred that the use of TiNPs synthesized by green method, using plant extract, may provide a better alternative to chemically synthesized NPs which are not only expensive but also toxic when applied in higher concentration, and possess potential danger to the environment and food security.

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