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RESEARCH ARTICLE

Magnetite nanoparticles mitigate arsenic stress by enhancing chlorophyll pigments, photosynthetic efficiency and *psbA* expression in black gram (*Vigna mungo* L.)

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Abstract

The study examines the effects of biosynthesised magnetite nanoparticles (MNPs) on the physiological and molecular properties of arsenic-stressed black gram (*Vigna mungo* L.) varieties, Pratap Urad-1 and Pratap Urad-31. Arsenic treatment (40 and 60 µM) led to differential responses, evaluated through measurements of photosynthetic pigments, photosynthetic efficiency and *psbA* gene expression, a key regulator of Photosystem II. The analyses involved pigments quantification, *psbA* mRNA isolation and real-time PCR to monitor the stress-induced gene expression. The MNPs foliar application, especially at 50 mg L^{-1}, led to a significant rise in chlorophyll content (36, 43 % and 51, 62 % in PU-1 and PU-31, respectively) under arsenic toxicity (40 and 60 µM). It also improved photosynthetic activity through the substantial increase of ΔF/Fm'sat and ETRmax in control and stressed plants. The *psbA* expression was induced by treatment with 50 mg L^{-1} MNPs, with an increase of 72 % and 66 % in PU-1 and 47 % and 41 % in PU-31 under 40 and 60 µM of arsenic, respectively. The enhanced antioxidant activities also contributed to the removal of oxidative damage by free radical scavenging. Overall, PU-1 exposed to arsenic showed more tolerability than PU-31, especially at the higher stress level (60 µM). The 50 mg L^{-1} MNPs was determined to be the most effective in mitigating the toxicity, while the higher concentration (100 mg L^{-1}) caused adverse effects due to stress in both varieties. These findings point out the great potential of MNPs as an eco-friendly and efficient approach to increase the tolerance of black gram cultivated in arsenic-contaminated soils.

Keywords: arsenic; antioxidative enzymes; black gram; magnetite nanoparticles; photosynthetic efficiency; *psbA* gene

Introduction

Contamination of agricultural soil by arsenic (As) is an increasingly serious issue affecting many countries worldwide, particularly those that use As-contaminated groundwater for irrigation. Arsenic accumulation in crops causes chronic exposure to the plant and is considered a serious health threat to the crops as well as to humans who consume it. Inhibition of normal physiological activities in plants such as photosynthesis, metabolic activity and nutrients uptake, which are caused by As toxicity, growth and productivity limits (1). The most damaged system in plants under As stress is photosynthetic machinery, with disturbances in chlorophyll production and photosystem degradation, thus reducing overall plant performance. The most obvious signs of this stress occur in sensitive crop varieties, which are susceptible to excessive As concentrations that may lead to chlorosis, leaf necrosis and even plant death (2).

Black gram (*Vigna mungo* L.) is a legume crop that serves as a rich source of protein. It is commonly consumed as sprouts, whole seeds, or flour (3). From an agricultural perspective, the main issue in areas contaminated with As is the loss of crop yield. In the Indian states where black gram is regularly cultivated as an intercrop, the soil in those areas is found to have As contamination levels that are much higher than the global average of 10 mg/kg (4). Under stress conditions, one of the factors that cause cellular death and the breakdown of metabolic processes in plants is reactive oxygen species (ROS). Previous researchers suggest that As exposure reduces plant growth and productivity because it causes oxidative stress (5). To combat the harmful impacts of ROS, the activity of some antioxidants increased. Therefore, the application of certain eco-friendly approaches to mitigate the effect of As toxicity and control its uptake by black gram plants for food safety is necessary. Through root absorption As first accumulates in plant roots before being transported to other parts (shoots), where it can hinder water and nutrient uptake by competing for the same transporters. The primary As toxicity effect is oxidative stress, which, however, primarily targets cellular membranes. This results in the structural disorganisation of membranes, lipid peroxidation and leakage of electrolytes. In such a way, the decline in membrane quality affects nutrient absorption, stomatal functioning and transpiration. To deal with these consequences, plants bring into action both i.e. enzymatic and nonenzymatic antioxidant defence systems. However, the inhibition of this defence mechanism can occur when As attaches to the thiol clusters of antioxidants thus weakening the

plant's protective barrier. Therefore, the toxicity of As impairs photosynthesis, a phenomenon that is corroborated by several research studies. For instance, it was found that in tomato plants treated with As, showed a marked reduction in photosynthetic pigments (6), which was also the case in *Glycine max* (L.) Merr. (7). In similar way, the stress negatively impacted the protein content in maize and bean plants (8, 9). In addition, high levels of As would result in the generation of ROS, which is the primary cause of protein and nucleic acid degradation within plant cells (10). To be more precise, arsenite [As (III)] is known to bind to sulfhydryl clusters in proteins and therefore disable their biological actions. Similarly, the damage caused by this process is even more extensive when it comes to photosynthetic activity, as it leads to both physical and functional alteration of the structure where chlorophyll is synthesized, through the slow-down of the chloroplasts and disturbance of the PSII (photosystem II) activity (11).

The *psbA* gene is the one that tells the cells to produce the D1 protein which forms an integral part of PSII that is engaged in many reactions that are necessary for light energy being transformed into chemical energy during photosynthesis and hence, the cycle of production going on. The *psbA* gene is not only sensitive to oxidative stress but also its downregulation under As exposure prevents the repair of the D1 protein, a situation that leads to reduced PSII and overall photosynthetic efficiency (12). Environmental stress in the form of heavy metal poisoning makes the D1 protein an important area of destruction. Consequently, the electron transport gets blocked within PSII and, hence, photosynthetic efficiency is further reduced (13). Understanding how As stress affects *psbA* and identifying methods to mitigate its impact on crop yield are critical areas of research.

The application of nanomaterials, mainly magnetite nanoparticles (MNPs), has been shown to ease the above-mentioned stress by eliminating ROS, thereby safeguarding the cellular components and maintaining the integrity of the photosynthetic machinery (6). Nanotechnology has opened up new horizons for using nanoparticles (NPs) to improve plant tolerance to environmental stresses. Moreover, the focus on the remediation of soil polluted with toxic metals has shifted toward approaches that are not only cost-effective but also environmentally sustainable. Out of all these, the application of nanotechnology for heavy metal pollution treatment has become more prominent than traditional methods (14). The combination of nanomaterials with agriculture, especially through the application of nano-fertilisers, promises to increase crop yield (15). Nano-fertilisers can considerably reduce nutrient losses, thus becoming more effective (16). The role of metal-based nanomaterials, especially iron (Fe), in agriculture has been highlighted in recent studies as a major factor that could lead to better plant responses and more tolerant plants during stressful environments (17, 18). It has already been shown that the application of zinc oxide and FeNPs externally not only improves the balance of these two metals but also prevents cadmium (Cd) accumulation, thereby supporting wheat growth in a Cd environment (17). Plants treated with FeNPs, for instance, showed the least Cd accumulation and better growth as compared to the untreated controls (19). In a similar way, FeNPs enhanced the growth and yield of the rice cultivar under As stress by the mechanism of lowering As levels and facilitating Fe absorption. Iron is considered a vital micronutrient for plants due to its primary roles in photosynthesis, respiration, DNA synthesis and being a cofactor in many enzymatic reactions (20). Among NPs, magnetite NPs (Fe_3O_4) have been found promising in improving plant health under a variety of abiotic stresses like heavy metal toxicity (6). These NPs stimulate nutrient uptake, induce root development and improve the defence response in plants as they scavenge ROS. Eco-friendly production methods have also generated MNPs in the laboratory to make this source of NPs inexpensive and environment friendly in comparison with chemically prepared NPs. These NPs have shown oxidative stress relieving ability and improved chlorophyll content along with stabilisation of PSII activity in different species of plants (11). Despite these findings, it is still unclear how MNPs affect the expression of important photosynthetic genes like *psbA*, particularly in stressful situations (12).

Although several studies have reported the role of metal-based NPs in alleviating heavy metal stress in plants, most of these investigations have primarily focused on physiological and biochemical parameters, with limited attention to underlying molecular mechanisms. In particular, the regulation of photosynthesis -related genes, such as *psbA*, under As stress remains poorly understood, especially in black gram.

Therefore, the present study aims to investigate the role of biosynthesised MNPs in modulating *psbA* gene expression along with photosynthetic performance under As stress, thereby providing novel insights into nanoparticle-mediated stress tolerance mechanisms. In this research, MNPs were biosynthesised using *Cymbopogon citratus* (DC.) Stapf leaf extract and characterised through FTIR, Zeta sizer, UV-VIS spectrophotometry, SEM, EDX and XRD analyses. It was determined that the NPs had an average size of 6–7 nm and cuboidal shape. The objective of this study was to ascertain if these biosynthesised MNPs can mitigate As-induced stress in black gram varieties Pratap Urad-1 (PU-1) and Pratap Urad-31 (PU-31). To evaluate the impact of MNPs on photosynthetic pigment content, protein, polyphenols, flavonoids, photosynthetic efficiency and *psbA* gene expression to increase photosynthesis and As tolerance, foliar application of MNPs was carried out under As stress. This work will help in developing sustainable farming methods using nanotechnology to increase plant health in contaminated regions.

Materials and Methods

The seeds of black gram varieties PU-1 (As resistant) and PU-31 (As sensitive), based on primarily study, were collected from Krishi Vigyan Kendra, Kota and Banasthali Vidyapith, Rajasthan. The chosen seeds were sterilised using 0.05 % $HgCl_2$, followed by a thorough rinse in distilled water before being sown in pots, measuring 12 by 15 cm and filled with a 2:1 w/w combination of soil and soilrite. The experimental soil was sandy loam, with a bulk density of 1400 kg m^{-3}, pH of 7.56±0.86, water retention capacity of 12 % and electrical conductivity of 0.61 dS/m. Nutrient composition included N:P:K at 196:16:282 kg ha^{-1}, zinc 0.71 ppm, manganese 2.82 ppm, sulphur 13 kg ha^{-1}, calcium 0.71 kg ha^{-1} and background As level was below detection level. The soil was taken from the garden of the Biotechnology department. The study was conducted on plants grown in the pots, which were kept in growth chamber at 14 hr photoperiod with a photon density of 160 µM quanta $m^{-2}s^{-1}$ and a relative humidity of 60–65 % at 26 ± 2 °C to assess the toxicity of As stress on black gram varieties. A range of concentrations of As (0, 40 and 60 µM sodium arsenate) was applied to each variety at the time

of sowing. Ten black gram seeds were planted in pre-prepared plastic pots, which were later thinned to five seedlings per pot and ten days after sowing (DAS), foliar spray with MNPs at concentrations of 0, 25, 50 and 100 mg L^{-1} (based on preliminary experiments) was done on black gram varieties with 3 replicates for each treatment. To determine biochemical characteristics, completely formed leaves in the upper third part of the plants were taken. Leaf samples in triplicates of each treatment were taken for gene expression studies. Samples were collected and quickly frozen at -80 °C to maintain RNA integrity for subsequent analysis. All the experiments were conducted using analytical-grade chemicals. All parameters were examined twenty DAS. The leaves of *C. citratus* were used for the biosynthesis of MNPs. The leaf extract worked as a reducing and stabilising agent. The freshly prepared plant extract was mixed with an aqueous solution of iron salt in a 1 : 1 ratio (v/v). The reaction mixture was maintained at pH ~13 and a temperature of 60 °C under constant stirring for 1 hr. The formation of MNPs was indicated by a visible dark-brown color change, after which the NPs were collected, washed and dried for further characterisation by FTIR, Zeta sizer and UV-VIS spectrophotometer, SEM, EDX and XRD. In our previous study, we explained the biosynthesis and characterisation of MNPs.

Photosynthetic pigments analysis

Fresh leaves were taken from black gram plants subjected to As stress (0, 40 and 60 µM) and treated with biosynthesised MNPs (0, 25, 50 and 100 mg L^{-1}) 14 DAS. The pigment extraction was conducted using 80 % acetone and pigment content was measured spectrophotometrically and calculated by the formula of Lichtenthaler and Wellburn (21).

Chlorophyll a

Chl a (µg/mL) = 12.25 x A663 - 2.79 x A646

Chlorophyll b

Chl b (µg/mL)=21.50 x A646-5.10 x A663

Total chlorophyll (a + b)

Chl a + b = 7.15 x A663 + 18.71 x A646

Carotenoids

Carotenoids (µg/mL)= 198 (1000 x A470 - 1.82 x Chl a-85.02 x Chl b)

*A663, A646 and A470 are absorbance values at 663 nm, 646 nm and 470 nm, respectively

Bioactive compounds

The protein content was calculated using methodology described in a previous study (22). The protein content was ascertained by homogenising 100 mg of the leaf in 5 mL of trichloroacetic acid, centrifuging the mixture for approximately 10 min at 10000 rpm, added 40 mL of reagent. A and 1 mL of reagent B to the filtrate and then adding 0.5 mL of Folin reagent. After 30 min of dark incubation, the mixture's absorbance at 650 nm was calculated. A BSA calibration curve was used to calculate the protein content.

To determine the polyphenol content from a leaf sample, the method established by McDonald et al. (23) was used. First, 100 mg of leaf sample was grounded finely and placed in 1 mL methanol. After fully vortexing the mixture, it was kept at ambient temperature or in a water bath for 30 min. Following the completion of the extraction period, the obtained samples were centrifuged at a high speed and the polyphenol analysis was conducted using the centrifugation supernatants. A gallic acid calibration curve was used to measure the polyphenol content.

The method from Sheng et al. was used to determine the leaf sample's flavonoid content (24). Consistent with McDonald et al. (23), 80 % methanol was used to grind and extract 400 mg of the leaf sample. To determine the flavonoid content, distilled water and a 0.1 mL extract were mixed. The mixture was added to 5 % sodium nitrite solution and waited for 5 min. The solution was incubated for 6 min after adding 10 % aluminium chloride. After the incubation time, sodium hydroxide (1 M) was mixed to the mixture and diluted with distilled water. The absorbance was measured at a wavelength of 510 nm. The flavonoid content was determined using a calibration curve of quercetin.

Photosynthetic efficiency analysis

The DUAL-PAM-100 system (Heinz Walz, Effeltrich, Germany), was used for Chl fluorescence measurement following the protocol described by Pfundel (25). Measurements were conducted on 25-day-old plants at the vegetative stage. During the assessment, both the effective quantum yield (ΔF/Fm′) of PSII and the electron transport rate (ETR) through PSII were evaluated simultaneously. Fm′ displays the maximum fluorescence of photo-adapted leaves under 800 ms saturating light pulse whereas F stands for steady-state fluorescence. The DUAL-PAM system's light response curve software was used to assess photosynthetic performance, with increased photosynthetic photon flux density (PPFD) across 10 steps, each lasting 30 sec, over a total period of 5 min. The PPFD levels ranged from 0 to 1500 µmol/ m^2 s (e.g., 0, 500, 1000, up to 1953 µmol m^{-2} s^{-1}). After every light interval, a saturating light pulse was administered to capture the corresponding chlorophyll fluorescence parameters. The maximum observed electron transport rate (ETR_{max}) and effective quantum yield at $PPFD_{sat}$ ($\Delta F/Fm'_{sat}$) were estimated using regression analyses of the light response curves.

Isolation of mRNA for the *psbA* gene quantification

Leaf tissues were extracted with the TRIzol reagent to get total RNA. Agarose gel electrophoresis and spectrophotometer measurement (A260/A280 ratio) were used to assess the purity and integrity of RNA. Only samples with ratios between 1.8 and 2.0 were approved for a further assessment. Following RNA isolation, complementary DNA (cDNA) was produced using a reverse transcription kit. Using *psbA* gene-specific primers and SYBR Green chemistry, the RT-PCR was performed. The reference gene, *Actin*, was used to standardise the relative expression of *psbA*. We used the $2^{\wedge-\Delta\Delta Ct}$ approach to assess relative expression levels. RT-PCR tests were carried out using a thermocycler with fluorescence detection (Rotor-Gene Q). The reaction conditions were optimised to enable selective amplification of the *psbA* gene. The accuracy of the PCR products was confirmed by melting curve analysis and negative controls were included in the experiment to assess the impurities. The RT-qPCR amplification of cDNA was conducted by the Schmittgen and Livak method (26), employing oligonucleotide primers (Table 1) designed using the NCBI Primer-BLAST tool.

Statistical analysis

All the tests were performed in triplicate. The mean and standard deviation (SD) of every test were analysed. Results are presented as mean ± standard error of the mean. One-way and Two-way ANOVA were performed using the software SPSS (version 20.0). To ascertain significant variances among all categories, post-hoc mean comparisons were done using Tukey's multiple range test with a statistical significance threshold of $p < 0.05$. Plots were created using Origin Pro software version 8.5. Sigma Plot 12.0 software was used to plot DUAL-PAM graph.

Table 1. Primers for this study were sourced from the NCBI database

Gene	Primer sequence
psbA (forward primer)	5′-AGCACTAAATAGGGAGCCGC-3′
psbA (reverse primer)	5′-GAAAGCCTATGGGGTCGCTT-3′
Actin (forward primer)	5′-GTTCTGTTCCAGCCATCCAT-3′
Actin (reverse primer)	5′-GTGGTGCGACAACCTTGATT-3′

Results

Impact of As and magnetite nanoparticles on photosynthetic pigments

The main pigment in chloroplasts that facilitates photosynthesis, i.e. chlorophyll (Chl), is extremely susceptible to environmental stress. The leaf Chl content is commonly used as a sign of photosynthetic efficiency. In the present research, MNPs application enhanced the level of photosynthetic pigments in both black gram varieties PU-1 and PU-31, particularly under increasing As stress concentrations. The most significant enhancement was recorded at a NPs concentration of 50 mg L^{-1}, as shown in Fig. 1. With rising MNPs concentrations (25–50 mg L^{-1}), PU-1 exhibited an increase in Chl-a by 25–43 %, Chl-b by 30–42 %, total Chl by 27–43 % and carotenoids by 21–38 % in Fig. 1. Similarly, PU-31 showed increase of 30–50 % in Chl-a, 25–66 % in Chl-b, 17–50 % in total Chl and 13–38 % in carotenoids under As treatments of 40 and 60 µM, relative to the control. These enhancements in pigment concentration suggest a significant improvement in photosynthetic performance, as reflected in the overall rise in total Chl content. The influence of As and NPs treatments on Chl-a, Chl-b total Chl and carotenoid content of black gram leaf samples was depicted in Fig. 1. In all cases, Chl and carotenoid content decreased as As concentration increased, with PU-31 samples exhibiting larger reductions than PU-1. For those samples exposed to As, applications of 25–50 mg L^{-1} NPs enhanced relative pigment levels. However, 100 mg L^{-1} NPs applications tended to reduce pigment content as well, suggesting that 100 mg L^{-1} NPs may be detrimental to the system as well. PU-1 samples consistently had higher relative levels of Chl and carotenoids compared with PU-31, which suggests better stress tolerance. Under conditions of As stress, PU-1 had higher photosynthetic pigment content than PU-31 at 50 mg/kg NPs concentration for 40 µM and 60 µM As treatments. Statistical considerations of all supported the differences detected, with PU-1 C (control) having the highest pigment content in every case.

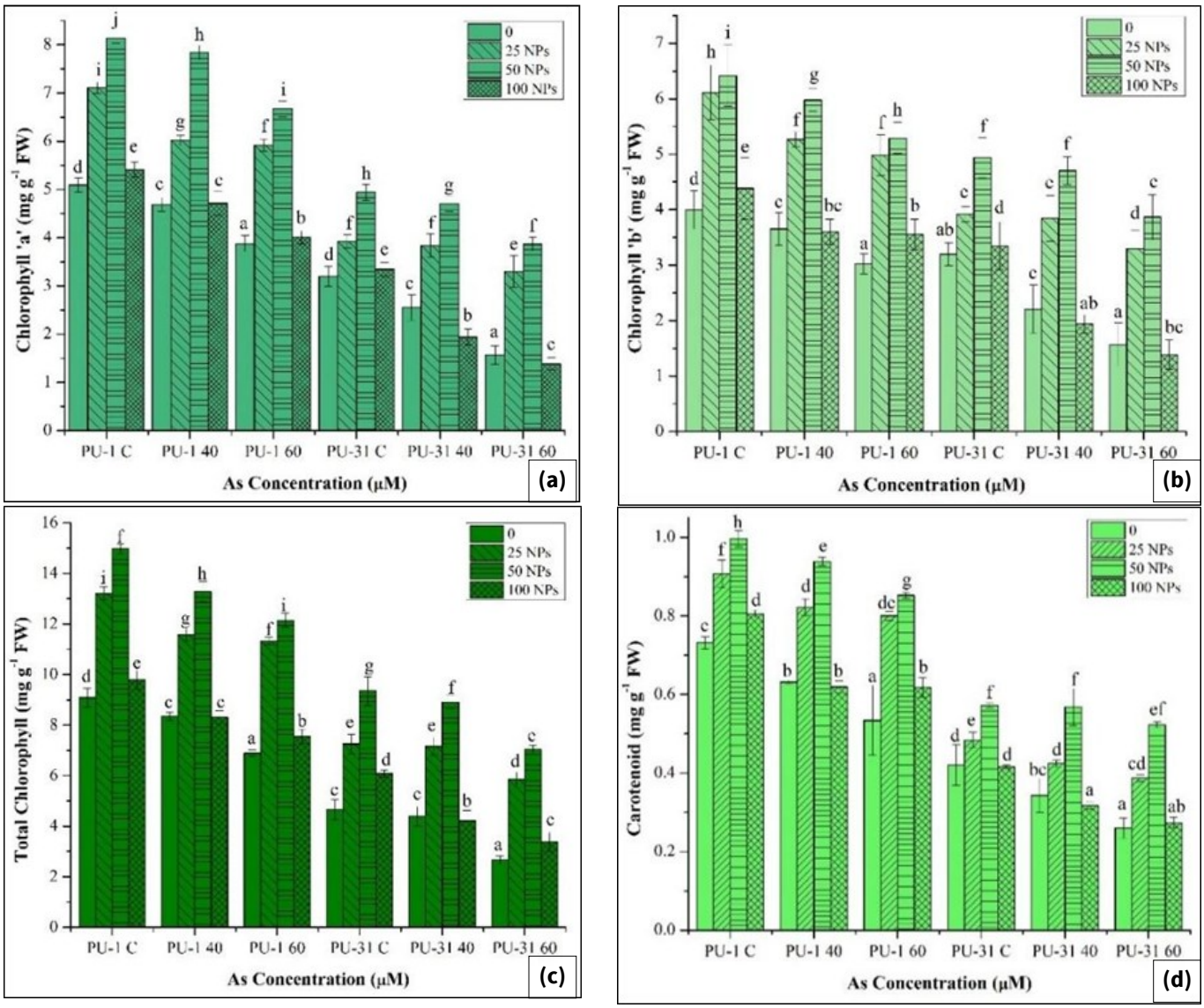


Fig. 1. Effect of arsenic (0, 40, 60 µM) and MNPs treatments (25, 50, 100 mg L^{-1}) on on photosynthetic pigments in PU-1 and PU-31 varieties. a: Chlorophyll-a; b: Chlorophyll-b; c: Total chlorophyll; d: Carotenoids in PU-1 and PU-31 varieties. Each column represents the mean ± standard deviation (n = 3). Distinct letters above the bars denote significant differences according to $p < 0.05$.

Effect on bioactive compounds

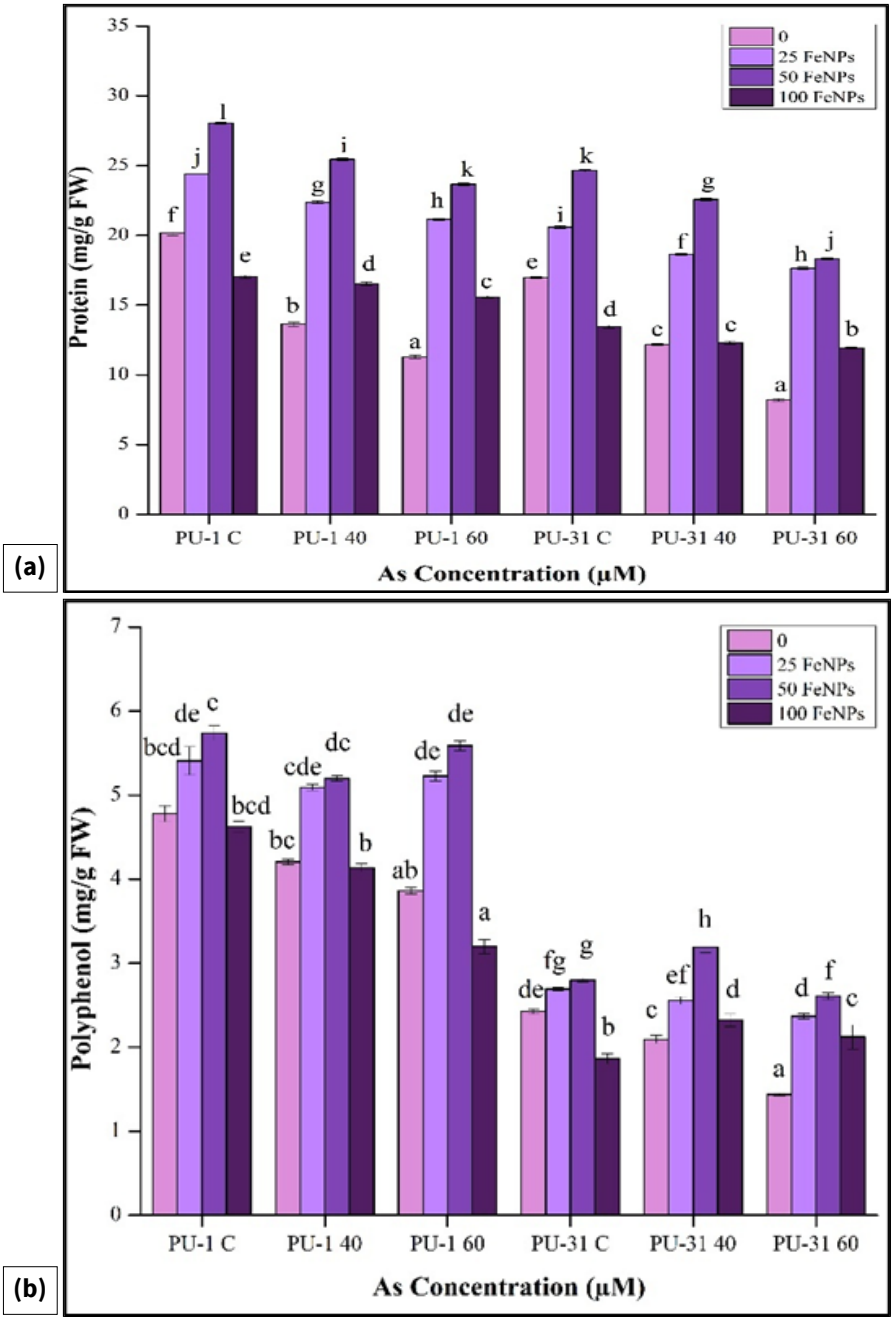
Under As stress, protein levels decreased to 11.29 mg g^{-1} FW in PU-1 and 8.21 mg g^{-1} FW in PU-31 at 60 µM As compared to the control. The MNPs assisted in increasing protein levels with the greatest effect at 50 mg L^{-1} MNPs, where protein levels increased by 23.66 µM mg^{-1} FW in PU-1 and 17.64 µM mg^{-1}FW in PU-31 when under 60 µM As (Fig. 2a). Similarly, polyphenol content decreased under As stress, with a polyphenol content of 3.86 mg g^{-1} FW in PU-1 and 1.43 mg g^{-1} FW in PU-31 under 60 µM As stress as compared to control. The addition of MNPs was known to significantly raise the polyphenol accumulation, with again, the highest improvement at 50 mg L^{-1} MNPs, resulting in increases of 5.58 mg g^{-1} FW in PU-1 and 2.61 mg g^{-1} FW in PU-31 under 60 µM As (Fig. 2b). As like other measures, flavonoids showed decreases under As stress, with total flavonoid content of 16.32 mg g^{-1} FW in PU-1 and 10.89 mg g^{-1} FW in PU-31 at 60 µM As stress compared to the control. However, the addition of MNPs significantly elevated flavonoid content, with the greatest rise observed at 50 mg L^{-1} MNPs, reaching 29.48 mg g^{-1} FW in PU-1 and 23.12 mg g^{-1} FW in PU-31 under 60 µM As stress (Fig. 2c). As shown in Table 2, all physio-biochemical parameters significantly influenced under As stress in both varieties.

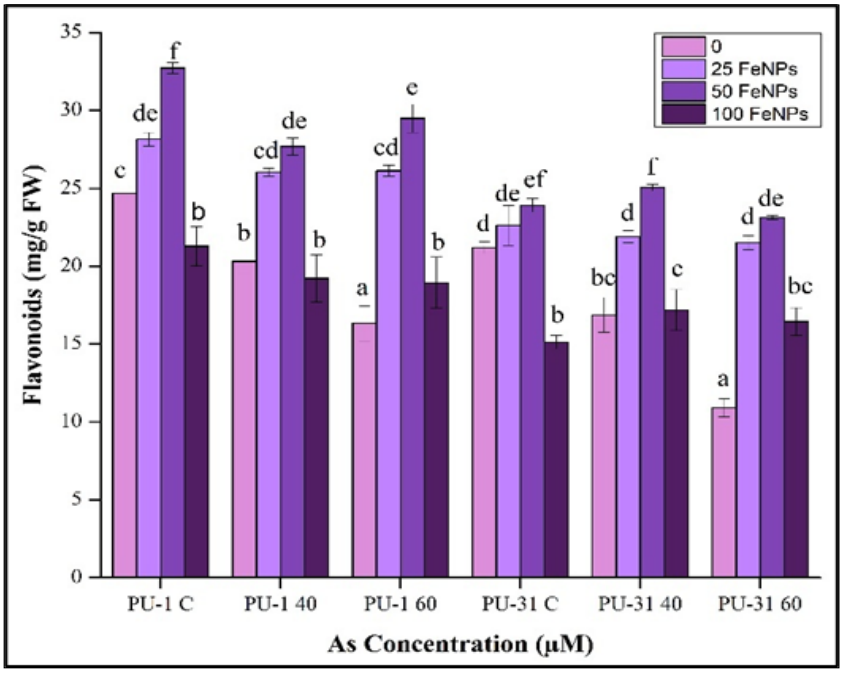
Photosynthetic efficiency analysis

To analyse photosynthetic efficiency, 25-day-old plants were treated with MNPs under As stress (40, 60 µM). Regression analysis illustrating the relationship between ΔF/Fm′ and ETR across PPFD levels is presented in Fig. 3. MNPs significantly enhanced photosynthetic efficiency in PU-1 (Fig. 3a) and PU-31 (Fig. 3b) under both control and arsenic-stressed conditions. As MNPs concentrations increased from 25 to 50 mg L^{-1}, photosynthetic rates increased in PU-1 (Fig. 3a) ETR_{max} (26–44 %), $\Delta F/Fm'_{sat}$ (4-8 %) and PU-31 (Fig. 3b) ETR_{max} (13–26 %), $\Delta F/Fm'_{sat}$ (2–4 %), under 40 µM and 60 µM conditions compared to the control.

Reverse transcription quantitative polymerase chain reaction assay for quantification of the *psbA* gene

The *psbA* gene's expression was measured in relation to the *Actin* gene. The reverse transcription quantitative polymerase chain reaction (RT-qPCR) was utilized to evaluate relative alteration of gene expression using the ΔΔCt methodology, also referred to as the





(c)

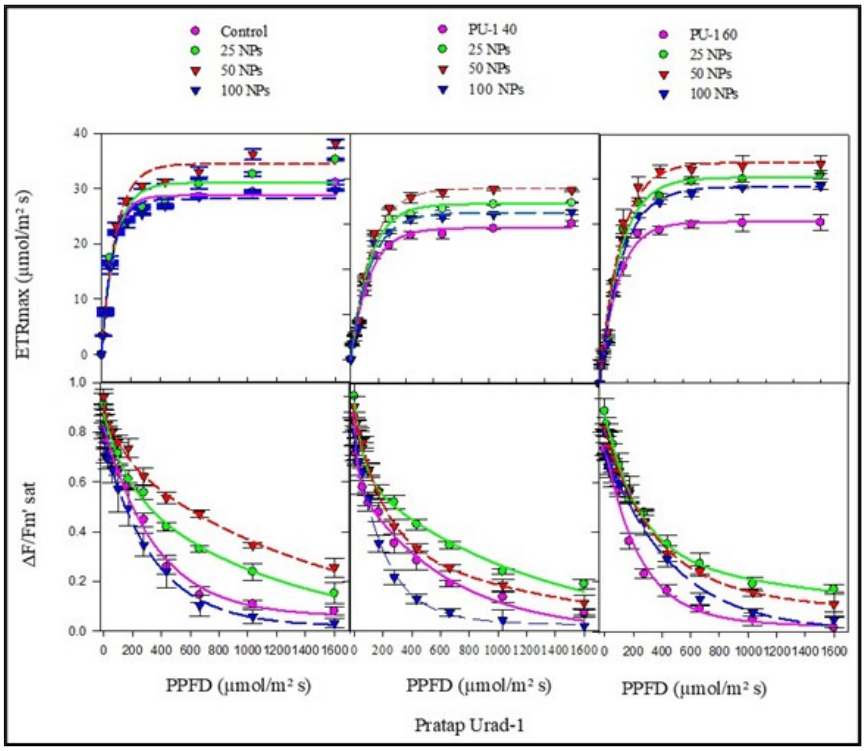
Fig. 2. Effect of arsenic (0,40,60 µM) and MNPs treatment (25, 50 and 100 mg L^{-1}). a: Protein; b: Polyphenol; c: Flavonoids, on PU-1 and PU-31 varieties respectively. Each column represents the mean ± standard deviation (n = 3). Distinct letters above the bars denote significant differences according to $p < 0.05$.

Table 2. Percent variability (two-way ANOVA) in measured parameters caused by varieties (PU-1 and PU-31), concentrations (As, MNPs and As+MNPs) and their interaction (variety × concentrations) in black gram

Source of variation			
Parameters	**Variety**	**Concentrations**	**Variety × concentrations**
Chlorophyll-a	330.039*	90.115*	11.699*
Chlorophyll-b	145.466*	89.688*	3.124**
Total Chlorophyll	366.890*	20.904*	1.196NS
Carotenoids	102.041*	63.892*	1.002NS
Protein	154.267*	450.321*	.545NS
Polyphenol	146.331*	68.318*	110.582*
Flavonoids	260.096*	195.262*	137.556*
ETR_{max}	21.641*	208.751*	112.909*
$\Delta F/Fm'_{sat}$	289.191*	3.667*	1.040NS

*Represents significance at $^{*}p < 0.05$, $^{**}p < 0.01$.

NS represents non-significant.



(a)

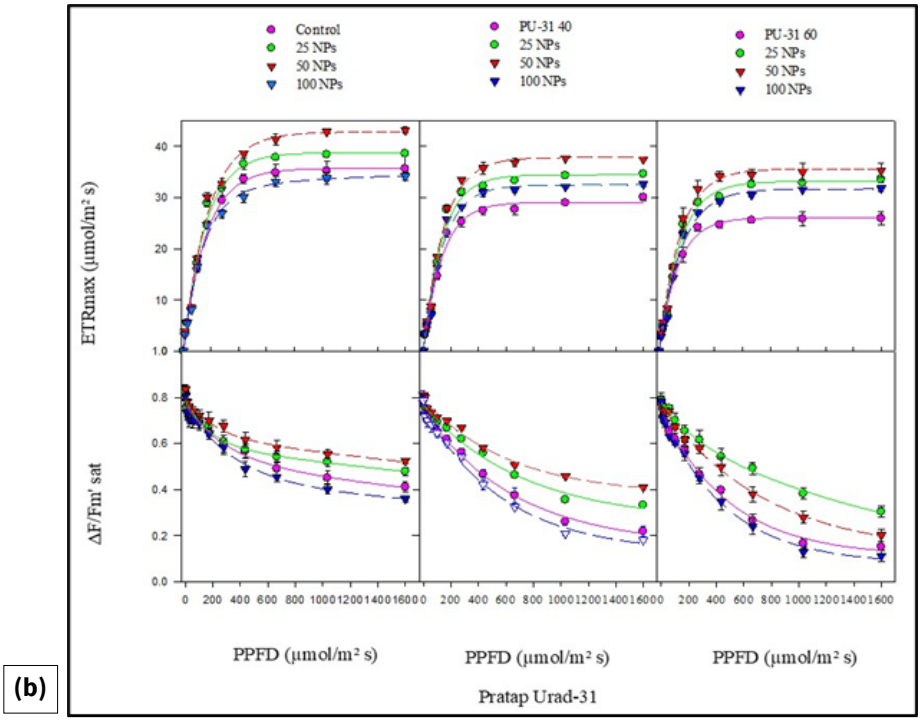


Fig. 3. The effects of arsenic (0, 40 and 60 µM) and MNPs treatment (25, 50 and 100 mg L^{-1}) are depicted by regression lines of PPFD dependence of apparent ETR_{max} and $\Delta F/Fm'_{sat}$ in (a) PU-1 and (b) PU-31 varieties, respectively. Graphs show averages with standard error (SE), indicating significant differences $p \leq 0.05$, $p \leq 0.005$ between treatments for each variable (n = 3). Separately for each variable ($p < 0.05$; n = 3).

$2^{\wedge-\Delta\Delta Ct}$ method. The Ct value obtained following the PCR cycle reveals the sample's threshold level. The expression of the *psbA* gene in both varieties of black gram was downregulated, with a greater degree of downregulation observed in the PU-31 variety compared to the PU-1 variety under As stress at concentrations of 40 and 60 µM. The use of 25 and 50 mg L^{-1} of MNPs led to a 2.93- and 2.58-fold, 3.64- and 2.94-fold upregulation of *psbA* gene expression in PU-1 and 1.40 and 1.35-fold, 1.91 and 1.71-fold upregulation in PU-31, respectively, under 40 and 60 µM stress compared to the controls (Fig. 4a and b). Increasing the concentration of NPs leads to a higher log-fold change across all As concentrations, suggesting a potential mitigating effect of NPs against As-induced changes. The highest log-fold change is observed with 50 mg L^{-1} NPs at 60 µM As. Arsenic toxicity negatively impacts plant growth, while the application of biosynthesised MNPs enhances chlorophyll pigment concentration, improves photosynthetic efficiency and upregulates the expression of the *psbA* gene. Foliar application of MNPs to black gram protected this legume crop and its productivity from As stress through upregulation of *psbA*, the protein-coding gene for the D1 protein in PSII, a decrease in reactive oxygen species (ROS) and maintained photosynthesis performance (Fig. 5).

Discussion

In an attempt to lessen the harmful effects of heavy metals and promote plant growth, several studies have examined the interactions between NPs and plants. However, it is yet unknown how MNPs reduce the toxicity of As in black gram varieties. In this

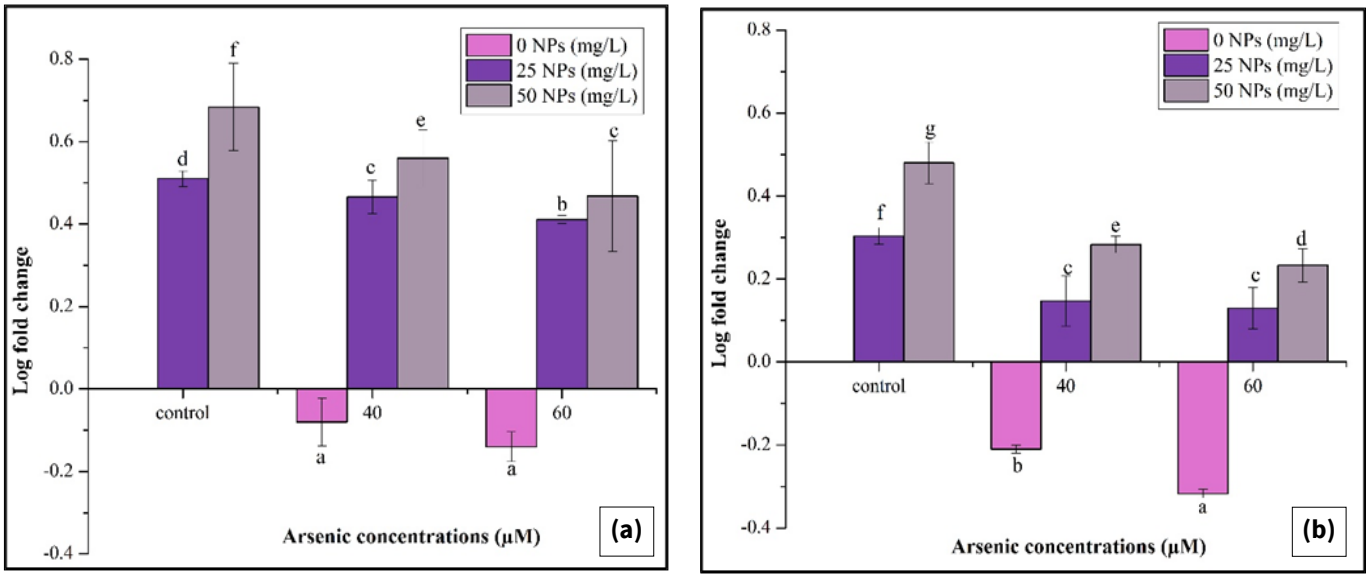


Fig. 4. Treatment with arsenic (0, 40, 60 µM) and MNPs (25, 50, 100 mg L^{-1}) affected *psbA* gene expression in black gram. a: PU-1; b: PU-31. Values are expressed as mean ± standard deviation (n = 3). Different letters above the bars indicate significant differences at $p < 0.05$.

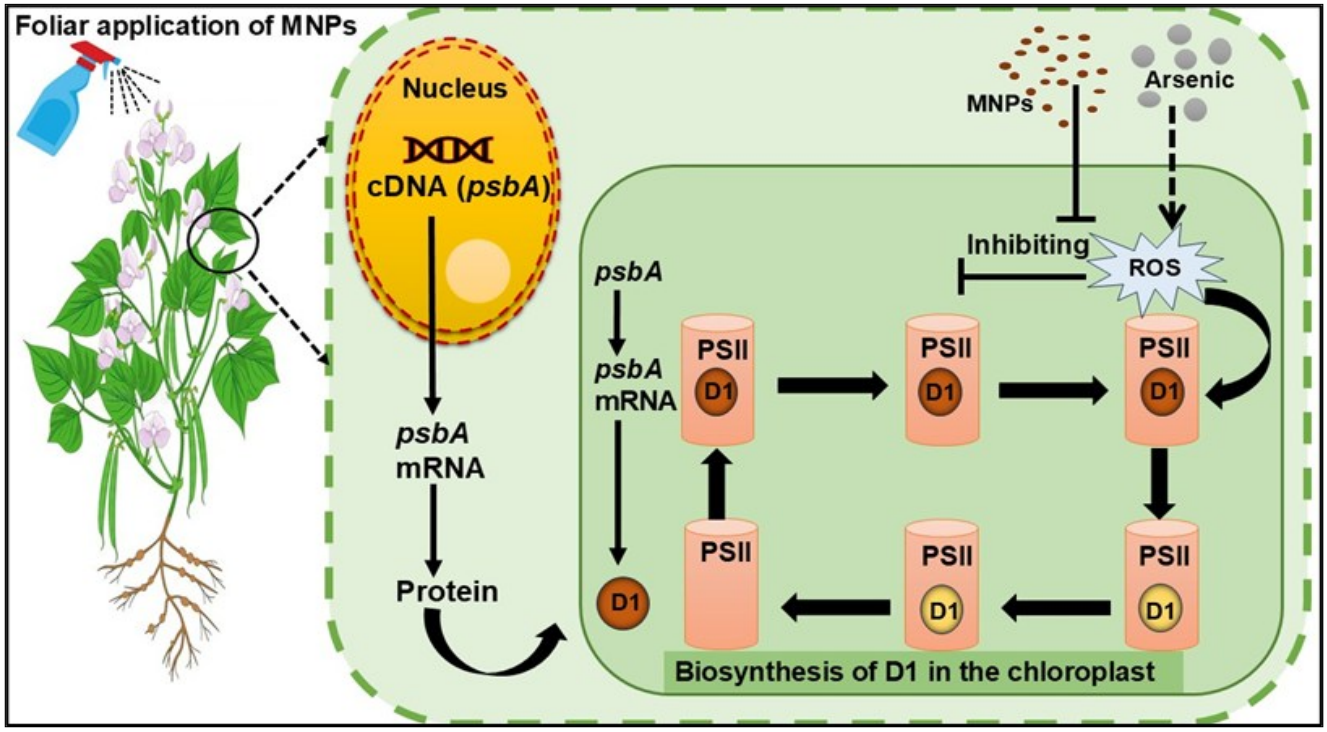


Fig. 5. Protective role of magnetite nanoparticles (MNPs) in black gram under arsenic stress. Foliar-applied MNPs enhance *psbA* expression and D1 protein biosynthesis in Photosystem II (PSII), while inhibiting arsenic-induced reactive oxygen species (ROS) generation and maintaining photosynthetic efficiency.

study, we examined the content of photosynthetic pigments, polyphenol, flavonoids, protein, photosynthetic efficiency and *psbA* gene expression in black gram varieties at different As and MNPs concentrations.

In this study, As stress caused a decline in photosynthetic pigment levels, whereas application of MNPs at lower concentrations (25 and 50 mg L^{-1}) boosted pigment content in both black gram varieties (Fig. 1). A decrease in photosynthetic pigments indicates the level of metal toxicity since they are extremely susceptible to heavy metal stress. Exposure to 60 µM arsenic declined Chl-a, Chl-b, total Chl and carotenoid quantities by approximately 23 %, 24 %, 24 % and 26 % in the tolerant variety PU-1 and by 50 %, 28 %, 30 % and 22 % in the sensitive variety PU-31, respectively. This decrease highlights the inability of plants to maintain pigment synthesis under high As stress, likely due to enhanced enzymatic degradation of Chl (6). On the other hand, MNPs treatment at 60 µM As increased these pigments by 34 %, 32 %, 33 % and 17 % in PU-1 and by 20 %, 18 %, 51 % and 24 % in PU -31, respectively. These findings align with previous research that demonstrated elevated pigment levels in crops like fenugreek (27) and *Vigna radiata* L. (11) after foliar metal NPs treatment.

The level of protein is an important factor that reflects the physiological status of stressed plants. In this study, a sharp drop in the level of protein has been recorded in As-treated plants (Fig. 2a). A decline in protein content may result from increased protease activity during As stress, leading to distortion in protein architecture (28). Whereas MNPs supplementation enhanced the protein content progressively, both in control and stressed plants. Similar observations have also been recorded in fenugreek (27).

The most important non-enzymatic antioxidants that scavenge free radicals include flavonoids and polyphenols, which are chemicals derived from L-phenylalanine. Polyphenols are categorised as secondary metabolites that form in the cytosol through L-phenylalanine and malonyl-CoA, whereas flavonoids are made up of aromatic ring structures with -OCH_3 and -OH groups (29). Under abiotic stress, flavonoid biosynthetic genes are induced to activate a defensive response that increases the accumulation of flavonoids. Phenolics scavenge ROS by hydrogen transfer, disrupt radical chain reactions and chelate metal ions via carboxyl and hydroxyl groups, thereby reducing oxidative damage (30). Flavonoids also inhibit lipid peroxidation, providing additional protection. Elevated flavonoid and polyphenol levels under As stress safeguard cellular components, as observed in rice and *Vicia faba* L. (31, 32). In this study, foliar treatment of MNPs boosted flavonoid and polyphenol production in As-stressed black gram, improving stress tolerance. Comparable effects of FeNPs on antioxidant activity were also reported in wheat (33).

Iron is an important element for plants, especially for photosynthesis, because it helps to generate Chl and thereby improves the photosynthesis efficiency (18). Iron is part of cytochromes in PSII, which act as electron transporters and protect PSII from photoinhibition. Furthermore, Fe is part of several important metalloenzymes, including those involved in the electron transport chain, which are necessary for many metabolic processes. In addition to its role in Chl biosynthesis, Fe is equally important for maintaining the structure and function of chloroplasts. The increase in pigment levels and photosynthetic rate resulting from MNPs treatment is probably because of the reduction in oxidative damage (27).

The effects of As toxicity declined the capacity of roots to take up water and ions, limiting stomatal activity, photosynthesis and transpiration (1). Chlorophyll fluorescence is a widely used method of evaluating photosynthetic performance due to the ease of operating fluorometers. Photosynthesis-related traits are reliable substitutions for plant resilience to abiotic stress (34). In current research, exposure to As had a negative impact on Chl content in the leaf and the effective quantum yield of PSII, which decreased photosynthesis. The decline in photosynthetic pigments and

efficiency was observed to be stronger in the sensitive varieties PU-31. A similar decline in photosynthetic parameters has also been reported in rice seedlings under As stress (35).

Previous studies have also shown that pigment composition and chloroplast ultrastructure are changed by heavy metal toxicity with consequences for photosynthetic rate, RuBisCO activity, ETR and PSII efficiency (36). In this study, treating MNPs at concentrations of 25–50 mg L^{-1} increased photosynthetic traits such as ETR_{max} and $\Delta F/Fm'_{sat}$ under As stress in both varieties of black gram (Fig. 5), which shows MNPs mitigated the effects of As-induced inactivation and damage to the photosynthetic machinery. Conversely, a higher dose of MNPs (100 mg L^{-1}) caused a reduction in pigment content and efficiency, indicating both toxicity and a potential disruption of the electron transport chain. Abiotic stress has shown similar patterns in wheat and pea plants (37, 17).

Magnetite nanoparticles enhance photosynthetic capacity and efficiency by stimulating key photosynthetic pathways and associated enzymes. They promote optimal thylakoid organisation and PSII activity by limiting excessive accumulation of ROS within chloroplasts and mitigating the harmful effects of ultraviolet-B radiation. Furthermore, MNPs improve ETR, enhance Chl biosynthesis, increase light absorption and promote photophosphorylation, thereby collectively improving CO_2 fixation and overall photosynthetic performance. In addition, MNPs upregulate the expression of genes involved in photosynthetic energy production, which contributes to enhanced photosynthesis under stress conditions. The beneficial effects of MNPs are largely attributed to the gradual release of iron ions (Fe^{2+}/Fe^{3+}), which serve as essential cofactors in photosynthetic processes. Iron plays a critical role in the activation of ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) and in maintaining electron transport through ferredoxin. Insufficient iron availability reduces ferredoxin activity, which in turn limits RuBisCO activation and overall photosynthetic efficiency (38, 39).

The present investigation confirmed a downregulation in the expression of the *psbA* gene in black gram cultivars exposed to As stress. The *psbA* gene is associated with the gene coding for the D1 protein of PSII, which plays an important role in protecting PSII from injury induced by oxidative stress during biotic and abiotic stress. The oxidative stress induced by As exposure led to a suppression in *psbA* expression, resulting in a decrease in photosynthetic rates and photosynthetic efficiency of PSII (Fig. 5). Similar downregulation has been observed under abiotic stress in studies of PSI and PSII-related genes, including treatments involving aluminium fluoride (40) and drought (41). The *psbA* gene, among other roles, also monitors the redox balance of the photosynthetic apparatus and the by-products, such as ROS, of photosynthesis and contributes to regulating the functioning of the antioxidant enzymes.

Iron oxide NPs are known to improve photosynthetic performance by enhancing the expression of photosystem related genes and restoring metabolic functions. This effect may be attributed to the gradual release of Fe^{2+}/Fe^{3+} ions, which serve as essential cofactors in electron transport and chloroplast enzymatic processes. Additionally, the MNPs reduce oxidative stress by activating antioxidant defence system, thereby minimising damage to the D1 protein and facilitating efficient PSII repair (42). This finding was consistent with the results of previous researchers, who noticed *psbA* upregulation in barley treated with MNPs (12). Similarly, previous reports states that the expression of photosynthetic genes increased in chickpea after titanium NPs application (43) and in wheat following MNPs treatment (44). The FeNPs mitigate salt stress by enhancing photosynthesis in peanut (45).

However, at 100 mg L^{-1}, the MNPs treatment caused a decline in Chl content, photosynthetic efficiency, protein, polyphenol and flavonoid content. Under certain conditions, NPs can enhance plants' antioxidant enzyme activity, improving their ability to withstand environmental stresses; excessive NPs may cause oxidative stress, affecting plant growth and development (46). While generally considered less toxic, iron oxide NPs can induce oxidative stress and alter root morphology and microbial community structure at higher concentrations (47). They tend to aggregate in soil, reducing mobility but posing risks through prolonged exposure and accumulation. Although iron-based NPs are generally considered less toxic compared to other metal NPs. Therefore, future research should focus on long-term field studies, dose optimisation and comprehensive risk assessment to ensure the safe and sustainable use of NPs in agriculture.

Conclusion

This study emphasises the potential of biosynthesised MNPs in mitigating the adverse effects of As stress on black gram varieties (PU-1 and PU-31). The nanoparticles not only improved photosynthetic pigments and efficiency but also restored the expression of the critical *psbA* gene involved in PSII function. These findings highlight a promising nanotechnology-based strategy for enhancing plant tolerance to environmental stress. Future research should focus on validating these results under field conditions and exploring the underlying molecular mechanisms through advanced approaches such as transcriptomic analysis.

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Authors' contributions

NK and VS conceptualised the research topic. GG conducted the experiments and prepared the first draft of the manuscript. NK and VS reviewed and edited the manuscript. All authors read and approved the final version of the manuscript.

Compliance with ethical standards

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