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Research Article

Methionine-coated nano zinc oxide: A novel nanopriming agent to enhance antioxidant defence, and agronomic traits in arsenic-stressed rice

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Abstract

Arsenic contamination significantly affects rice yield and production. Recent studies have highlighted the potential of nanoparticles to mitigate heavy metal stress in cereals, although concerns about their phytotoxicity in agricultural systems have emerged. Coating nanoparticles may enhance their biocompatibility and reduce toxicity. In this study, we synthesized nano zinc oxide (ZnONPs) and methionine-coated nano zinc oxide (Met-ZnONPs), characterizing their properties using UV-Vis spectroscopy and transmission electron microscopy (TEM). Met-ZnONPs exhibited a blue shift and quantum confinement when characterized through UV-Vis and TEM. We aimed to compare the effects of seed priming with ZnONPs and Met-ZnONPs, hypothesizing that methionine coatings would enhance efficacy. Rice seeds were primed for 24 hours with either ZnONPs or Met-ZnONPs before sowing under both arsenic stress and non-stress conditions. We monitored intrinsic arsenic levels in soil and irrigation water and assessed arsenic content in rice grains post-

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harvest. Priming with 25 ppm Met-ZnONPs increased plant height, fresh weight, and activities of key antioxidant enzymes (ascorbate peroxidase, glutathione reductase, monodehydroascorbate reductase, and dehydroascorbate reductase) by 13.73%, 19.36%, 19.57%, 25.19%, 17.17%, and 14.4% respectively, compared to increases of 10.24%, 3.82%, 3.63%, 11.26%, 16.35%, 9.94%, and 7.06% for 50 ppm ZnONPs. Furthermore, 50 ppm Met-ZnONPs resulted in a 47.89% reduction in grain arsenic and a 36% decrease in hydrogen peroxide levels, while ZnONPs alone showed reductions of 22.28% in grain arsenic and 32% in hydrogen peroxide. These findings suggest that coating nanoparticles can enhance crop production by improving their biocompatibility and mitigating phytotoxic effects.

Keywords: abiotic stress; amino acid coated nanoparticles; ascorbate glutathione cycle; seed priming; yield of rice

Introduction

Arsenic contamination is a major environmental and health concern, particularly in rice-growing systems where flooded conditions promote arsenic uptake and accumulation in the grain (Moulick *et al*., 2021; Singh *et al*., 2023). Heavy metals such as arsenic impair seed germination, enzymatic activity, and nutrient acquisition, resulting in reduced crop productivity.

In plants, antioxidants play a critical role in defending against oxidative stress caused by environmental factors. The ascorbate-glutathione (AsA-GSH) cycle is central to this defence system. Ascorbate reduces ROS directly, while glutathione, through its reduced (GSH) and oxidized (GSSG) states, regenerates ascorbate, maintaining a balance that supports sustained antioxidant activity (Ishtiaq *et al*., 2023; Rajput *et al*., 2021). The glutathione pool, comprising both GSH and GSSG, is essential for cellular redox homeostasis. Methionine, an amino acid with antioxidant properties, contributes indirectly by promoting the synthesis of compounds like glutathione. When used as a coating for zinc oxide nanoparticles, methionine may enhance the delivery and efficacy of zinc in plants, potentially bolstering antioxidant defences and stress tolerance (Nuzaiba *et al*., 2023).

Under heavy metal stress, the seed priming technique facilitates the seed germination process, as reported by several researchers (Weber *et al*., 2021; Waqas Mazhar *et al*., 2024 not present in references). Priming offers several benefits, such as better germination, improved stress tolerance, enhanced nutrient acquisition, and a better antioxidant defence system. By using biocompatible and bio-rational materials for seed priming, along with proper management practices, farmers can mitigate the impacts of heavy metal stress (Mazhar *et al*., 2023; Waqas Mazhar *et al*., 2024).

The use of nanotechnology has emerged as a fascinating field for agricultural biologists, as it has led to the exploration of several cost-effective, bio-rational, and biocompatible nanomaterials. Zinc nanomaterials have been applied in various areas of agriculture, including biotic and abiotic stress, showing encouraging results (Mazhar *et al*., 2022; Waqas Mazhar *et al*., 2024). Although the application of nanomaterials in agriculture is proving highly encouraging, several studies have documented the phytotoxicity and environmental concerns associated with these nanomaterials. The application of nanomaterials in agriculture raises concerns regarding their accumulation in soil and water, as well as concerns about their biocompatibility (Ma *et al*., 2023; Bordin *et al*., 2024).

One possible solution to improve the biocompatibility of nanomaterials is the use of coatings. Studies have reported several significant benefits of coated nanomaterials (Warerkar *et al*., 2024). Coating nanomaterials can improve their stability and biocompatibility, preventing their agglomeration and maintaining their bio-rationality while performing a protective role (Saleem and Zaidi, 2020).

While numerous studies have explored the effects of zinc nanoparticles in mitigating environmental stress in plants (Mazhar *et al*., 2022), the use of methionine-coated zinc oxide nanoparticles remains unexplored. In the present study, we have used methionine amino acid coatings on zinc nanomaterials and studied the comparative effects of seed priming with coated and uncoated nanomaterials under arsenic stress in rice plants. Methionine is an essential amino acid and can serve as a functional coating material for nanomaterials. Methionine coatings can improve the biocompatibility of nanomaterials, reducing potential cytotoxicity and enhancing their compatibility with biological systems. Furthermore, methionine coatings can improve dispersion and enhance rationality (Nuzaiba *et al*., 2023).

The aim of this comparative study is to investigate the effects of nano zinc oxide (ZnONPs) and methionine-coated nano zinc oxide (Met-ZnONPs) on rice plants under arsenic stress through seed priming. The objectives of the study are to evaluate the impact of seed priming with these nanoparticles on the antioxidant defence system of rice plants and to determine whether the methionine coating enhances the beneficial effects of ZnONPs in conferring arsenic stress tolerance. The hypothesis for this study is that seed priming with methionine-coated nano zinc oxide will be more beneficial compared to uncoated ZnONPs in improving the antioxidant defence of rice plants under arsenic stress, leading to increased arsenic stress tolerance, reduced phytotoxicity, and enhanced biocompatibility culminating into better crop yield.

Material and methods

Experimental layout

The pot trials were conducted in the Department of Botany at Mirpur University of Science and Technology (MUST) in District Bhimber (33°9′0.72″N, 73°44′41.53″E), Azad Jammu and Kashmir (AJK), Pakistan, replicating Mazhar *et al*., (2022). Experimental conditions included an average day temperature of 34±1 °C and a night temperature of 27±1 °C, with a 12-hour photoperiod. Seeds of the IRRI-6 rice variety, sourced from the National Agricultural Research Centre (NARC) in Islamabad, were washed in distilled water three times to eliminate excess solution. For soil preparation, approximately 10 kg of the top 10-20 cm of soil was collected from Bhimber farmland, air-dried, sieved through a 2 mm mesh, and studied using protocol as discussed (Sparks *et al*., 2020). The studied parameters regarding soil quality are listed in table 1. The intrinsic arsenic contents of irrigation water and soil were monitored before giving arsenic treatment (Table 1).

Table 1. Studied parameters for the experimental soil used to grow rice plants

Parameter	Value	Unit
Ph	7.35	-
Electrical Conductivity (EC)	2.09	dS m^{-1}
Organic Matter	1.99	%
Sand	34	%
Silt	43	%
Clay	23	%
Soil Intrinsic Arsenic	3	mg kg^{-1}
Nitrogen (N)	25	mg kg^{-1}
Phosphorus (P)	12	mg kg^{-1}
Potassium (K)	110	mg kg^{-1}
Calcium (Ca)	1260	mg kg^{-1}
Magnesium (Mg)	120	mg kg^{-1}
Iron (Fe)	15	mg kg^{-1}
Zinc (Zn)	1.09	mg kg^{-1}

The pot experiment was arranged in a completely randomized design (CRD) with a 2 × 5 factorial arrangements. Factor A was arsenic level (0 and 24 mg As kg^{-1} dry soil), and Factor B was priming treatment (unprimed control, ZnONPs 25 ppm, ZnONPs 50 ppm, Met-ZnONPs 25 ppm, and Met-ZnONPs 50 ppm). Each treatment combination was applied to three pots, with five seedlings per pot. Three representative seedlings per pot were sampled for measurements, and their values were averaged to produce a single value per pot. The pot was considered the experimental unit for all statistical analyses. In total, the experiment included 30 pots and 150 seedlings (Mazhar *et al*., 2022).

Nano zinc oxide preparation and coatings with methionine

ZnONPs were prepared by heating and stirring a solution of zinc chloride precursor while slowly adding 1 M aqueous sodium hydroxide drop by drop. After stirring for 30 minutes, a white precipitate formed, which was collected under vacuum and washed thrice with ice-cold deionized water before being dried overnight in an oven. Characterization of the prepared ZnONPs was conducted using UV-visible spectroscopy and TEM. The ZnONPs were further processed by grinding them with a pestle and mortar, then stored in an air-tight container. Coating of the ZnONPs with L-methionine involved grinding the dry ZnONPs (100.2 g, 25 nm) with L-methionine (200.0 g) in a colloidal mixer. This process produced a diffused melt that solidified into a white solid in approximately 15 minutes. The resulting mixture of Met-ZnONPs, was further ground to a fine powder and stored under a nitrogen atmosphere for seed priming use. UV-visible spectroscopy and TEM were employed for characterization of M-Nano-ZnO (Alkhtib *et al*., 2020).

Factors studied: Seed priming and arsenic stress

Two factors were included in the pot trials: two levels of arsenic stress and five levels of seed priming treatments. Different seed treatment concentrations of ZnONPs and Met-ZnONPs were prepared, including 0 ppm as a control treatment, while other trials consisted of 25 and 50 ppm each for ZnONPs and Met-ZnONPs. Initially, a 25 mg and 50 mg of ZnONPs and Met-ZnONPs was placed in a litre of deionized water and sonicated for 30 minutes to achieve uniform dispersions. After sonication, the particle size and dispersion stability were confirmed using UV-visible spectroscopy and TEM, ensuring that no significant aggregation occurred. The pH of the priming suspensions was measured and found to be neutral (7.0); no further pH adjustment was necessary, as the neutral pH does not adversely affect seed germination or seedling vigour. The desired concentrations of ZnONPs and Met-ZnONPs were obtained, and seeds were soaked in their respective solutions for 24 hours under dark conditions maintaining continuous aeration (Waqas Mazhar *et al*., 2024; Mazhar *et al*., 2022).

The concentrations of the priming agent (25 and 50 ppm) and the priming duration of 24 hours were selected based on both preliminary optimization trials and literature reports. In preliminary trials, a range of concentrations (10, 25, 50, 75, 100 ppm) and durations (12, 24, and 48 hours) were tested, and 25 and 50 ppm with a 24-hour duration resulted in the highest germination rates and seedling vigour without causing toxicity. These values are also supported by previous studies in bitter gourd (Waqas Mazhar *et al*., 2024) and mung bean (Mazhar *et al*., 2022; Ishtiaq *et al*., 2023), which reported that high concentrations are less effective, whereas low-to-moderate concentrations of nanopriming agents enhance seed germination and early growth. To determine an arsenic level capable of inducing a measurable stress response in rice, a preliminary experiment tested four target soil arsenic concentrations i.e., 18, 20, 22, and 24 mg As kg^{-1} dry soil, corresponding to 240.3, 267.1, 293.8, and 320.5 μmol As kg^{-1}, respectively (Table 2). These concentrations were prepared by adding 31.22, 34.70, 38.17, and 41.64 mg $NaAsO_2$ kg^{-1} dry soil. Among these treatments, 24 mg As kg^{-1} produced the most pronounced reduction in seedling vigour (reflected by weaker early growth and reduced root-shoot development) and was therefore selected for the main experiment. Fourteen-day-old seedlings were subsequently transplanted into the treated soil and maintained under the described growth conditions (Zhao *et al*., 2023).

Table 2. Arsenic toxicity levels in the soil for preliminary experiment

Target As level (mg As kg^{-1} soil)	Actual As added (mg As kg^{-1} soil)	Moles of As required (mol As kg^{-1} soil)	Mass sodium arsenite ($NaAsO_2$) required (mg kg^{-1} soil)
18	18	0.0002403	31.22
20	20	0.0002671	34.70
22	22	0.0002938	38.17
24	24	0.0003205	41.64

Calculations used: As atomic mass = 74.9216 g·mol^{-1}; $NaAsO_2$ molar mass = 129.9094 g·mol^{-1}

Total chlorophyll assay

The total chlorophyll content of each experimental trial's plants was assayed following the protocol devised by Arnon (1949). Fresh leaves (approximately 0.25 g) were collected from each treatment and placed overnight for chlorophyll extraction with 80% acetone at 0.4 °C. These extractions were then centrifuged at 10,000 rpm for 5 minutes. The supernatant obtained was used to measure absorption patterns at wavelengths of 663 nm, 645 nm, and 480 nm using a spectrophotometer (Hitachi-U2001, Tokyo, Japan).

Extraction and study of the antioxidants

Antioxidant enzymes were extracted from the harvested leaf samples. Approximately 0.5 g of leaf tissue was macerated in a mortar and pestle with 5 mL of 50 mM cold phosphate buffer. The homogenate was then filtered and subjected to centrifugation at 15,000 rpm for 20 minutes at 4 °C. This extract was used to assess the activities of antioxidant enzymes (Panda, 2012).

Superoxide dismutase (SOD) activity was determined based on the inhibition of nitroblue tetrazolium (NBT) photoreduction at 560 nm. The reaction mixture comprised 50 µL enzyme extract, 50 µM NBT, 1.3 µM riboflavin (Vitamin B2), 13 mM methionine, 75 nM EDTA, and 50 mM phosphate buffer (pH 7.8). To initiate the reaction, the solution was exposed to a 30 W fluorescent light source within a chamber for 15 minutes. The formation of blue formazan was measured at 560 nm with a UV-visible spectrophotometer (Giannopolitis and Ries, 1977). Observation on peroxidase (POD) functions relies on the oxidation of guaiacol. The reaction mixture contained 50 mM phosphate buffer, 20 mM guaiacol, 40 mM H_2O_2, and 100 µL of enzyme extract, with absorbance recorded at 470 nm after 20 seconds (Chance and Maehly, 1955). For determination of catalase (CAT) functions, reaction mixture included 50 mM phosphate buffer (pH 7.8), 59 mM H_2O_2, and 0.1 mL enzyme extract. The reduction in absorbance, indicative of H_2O_2 decomposition, was used to estimate CAT activity (Chance and Maehly, 1955).

Hydrogen peroxide contents

Fresh leaf samples (0.1g) were homogenized with 5 mL of 0.1% trichloroacetic acid on an ice bath. The extract was then centrifuged at 12,000 rpm for 5 minutes. Next, 0.5 mL of the test extract and 0.5 mL of Potassium Phosphate Buffer were mixed in a test tube. To this mixture, 1 mL of 1 M Potassium Iodide was added and shaken well before taking readings at 390 nm using a spectrophotometer (Velikova *et al*., 2000). Hydrogen peroxide content was quantified using a standard calibration curve prepared with known concentrations of H_2O_2 (0-100 µM). The absorbance at 390 nm was converted into H_2O_2 content using the slope of the standard curve, and results are expressed as nmol H_2O_2 per gram fresh weight (nmol g^{-1} FW).

Estimation of proline values

Proline standard solutions with known concentrations ranging from 0 to 100 µg/mL were prepared using toluene as a solvent. Tissue samples or plant extracts were homogenized in a suitable buffer. The homogenate was then mixed with sulfosalicylic acid and centrifuged to precipitate proteins. The supernatant was collected and mixed with glacial acetic acid and ninhydrin reagent. After heating and cooling, toluene was

added to extract the chromophore. The absorbance of the pink chromophore was measured at 520 nm using a spectrophotometer. The proline concentration in the samples was determined based on the calibration curve generated using standard proline solutions (Bates *et al*., 1973).

Estimation of seed starch contents, seed protein, and seed arsenic contents

Starch content was determined using an iodine test as described by Bates *et al*. (1943), with absorbance measured at 660 nm. Protein content in powdered rice caryopsis was analysed using the Biuret method with bovine serum albumin as the standard, and absorbance recorded at 540 nm (Lin *et al*., 2005). Arsenic content was quantified in samples dried at 80 °C for 48 hours, digested with HNO3/HClO4 (3:1, v/v), and analysed using an Atomic Absorption Spectrophotometer (GBC 92 plus) (Costa *et al*., 2016).

Study of AsA-GSH cycle and glutathione substrate pool

The activity of enzymes in the AsA-GSH cycle was studied following Nakano and Asada (1981). APX activity was measured at 290 nm for two minutes using a spectrophotometer. GR activity was determined by measuring absorbance colour intensity at 340 nm for three minutes. MDHAR and DHAR activity was measured following recommended procedure (Ishtiaq *et al*., 2023). For analysis on glutathione, fresh leaf material was ground in 0.1 M HCl (2 mL) containing EDTA. The extract was collected by centrifugation, and a reaction mixture was prepared using phosphate buffer, DTNB, the extract, and NADPH. The absorbance of the reaction mixes was measured at 412 nm (Griffith, 1980).

Observations on growth and yield outcomes

Plant length was measured using a scale or meter rod, and the measurements were recorded in centimetres or millimetres as needed. Samples were carefully labelled according to each treatment applied, and their lengths were recorded in a notebook. Plant fresh weights were recorded using both a manual electronic and digital balance. For observations on yield, tiller count, panicle count, spikelet count per panicle, panicle length, and 1000 grain weights were recorded. Furthermore, straw and paddy yield of the plants were also monitored (Mazhar *et al*., 2022).

Statistical analysis

The data analysis included: (i) the calculation of least significant difference (LSD) at the 5% level, with mean separation; (ii) a two-way ANOVA to examine mean square and p-values, assessing the effects of individual factors - stress and treatment - as well as their interaction; (iii) construction of a Spearman correlation matrix; (iv) principal component analysis (PCA) of the dataset; and (v) computation of mean and standard error and making bar charts using Microsoft Excel Professional plus 2016. The PCA and correlation matrix were generated using XLSTAT software version 2014 (Addinsoft, Paris, France), while ANOVA and LSD analysis were conducted with Co-Stat version 6.3 (Berkley, CA, USA) (Ishtiaq *et al*., 2022; Maqbool *et al*., 2023; Mushtaq *et al*., 2023; Sardar *et al*., 2023).

Results

Blue shift and quantum confinement effect

The TEM examination revealed that the uncoated ZnONPs are larger (measured with a 0.5 µm scale bar) and methionine-coated ZnONPs are smaller (measured with a 0.2 µm scale bar) (Figure 1A; Figure 1B). Zn nanostructures were more porous with methionine coating (Figure 1B). Furthermore, from the results of TEM examination, one can infer that methionine coating has reduced the effective size of the nanostructures under observation. The results show that quantum confinement effect where the electronic properties change

with the particle size, has operated. Larger nanoparticles typically absorb light at longer wavelengths (red shift), as seen with the uncoated Zn absorbing at 376 nm. Smaller nanomaterials absorb at shorter wavelengths (blue shift), as seen with the Met-ZnONPs absorbing at 340 nm (Figure 1).

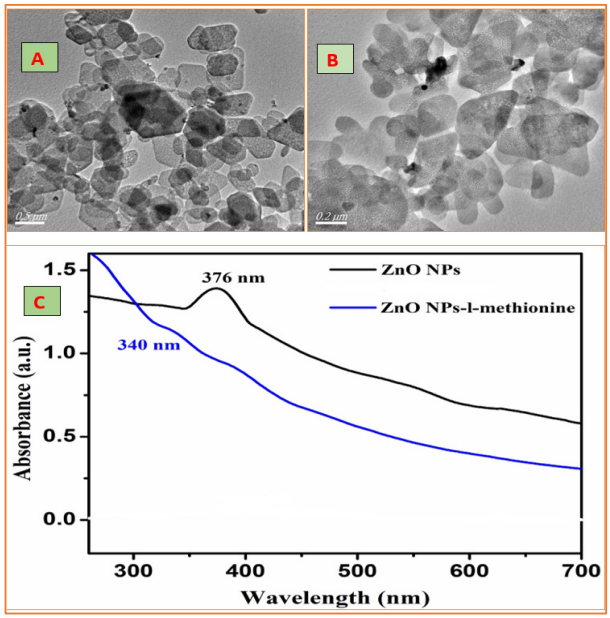


Figure 1. Characterization details of the Zn nanomaterials used in the study TEM micrograph of the Zn nanostructures (A), TEM micrograph of the uncoated ZnO (B), TEM micrograph of the Methionine coated ZnO, (C) UV-Vis spectroscopic observations related to coated and uncoated Zn nanostructures

The optical properties of the synthesized nano zinc oxide either coated or uncoated were studied. The UV-Vis result showed that methionine coatings resulted in the change of optical properties of the nano zinc oxide (Figure 1C). Zinc nanomaterials typically absorb UV light due to their wide band gap (about 3.37 eV), which corresponds to the absorbance in the UV region as shown in our results. The methionine coating however, results to a blue shift (a shift toward shorter wavelengths, from 376 nm to 340 nm).

Total chlorophyll contents and hydrogen peroxide levels

Under stress conditions, the photosynthetic machinery of plants undergoes damage, leading to a significant increase in the production of reactive oxygen species (ROS). Upon examination of these parameters, it was observed that arsenic stress caused a substantial decrease in the total chlorophyll content of rice plants by 36.64% (Figure 2A) and an increase in hydrogen peroxide content by 21.01% (Figure 2B). However, all seed priming treatments resulted in significant increases in total chlorophyll content and decreases in hydrogen peroxide levels (a form of ROS). The effect of Met-ZnONPs was superior to that of ZnONPs alone. The higher concentration of both treatments, i.e., 50 ppm, proved to be the most effective. Specifically, priming with 50 ppm ZnONPs led to a 37% increase in total chlorophyll content and a 32% decrease in hydrogen peroxide levels. In comparison, the same concentration of Met-ZnONPs resulted in a 45% increase in total chlorophyll content and a 36% decrease in hydrogen peroxide levels. The results are according to our hypothesis suggesting methionine loaded nano-zinc as a superior option compared to ZnONPs alone.

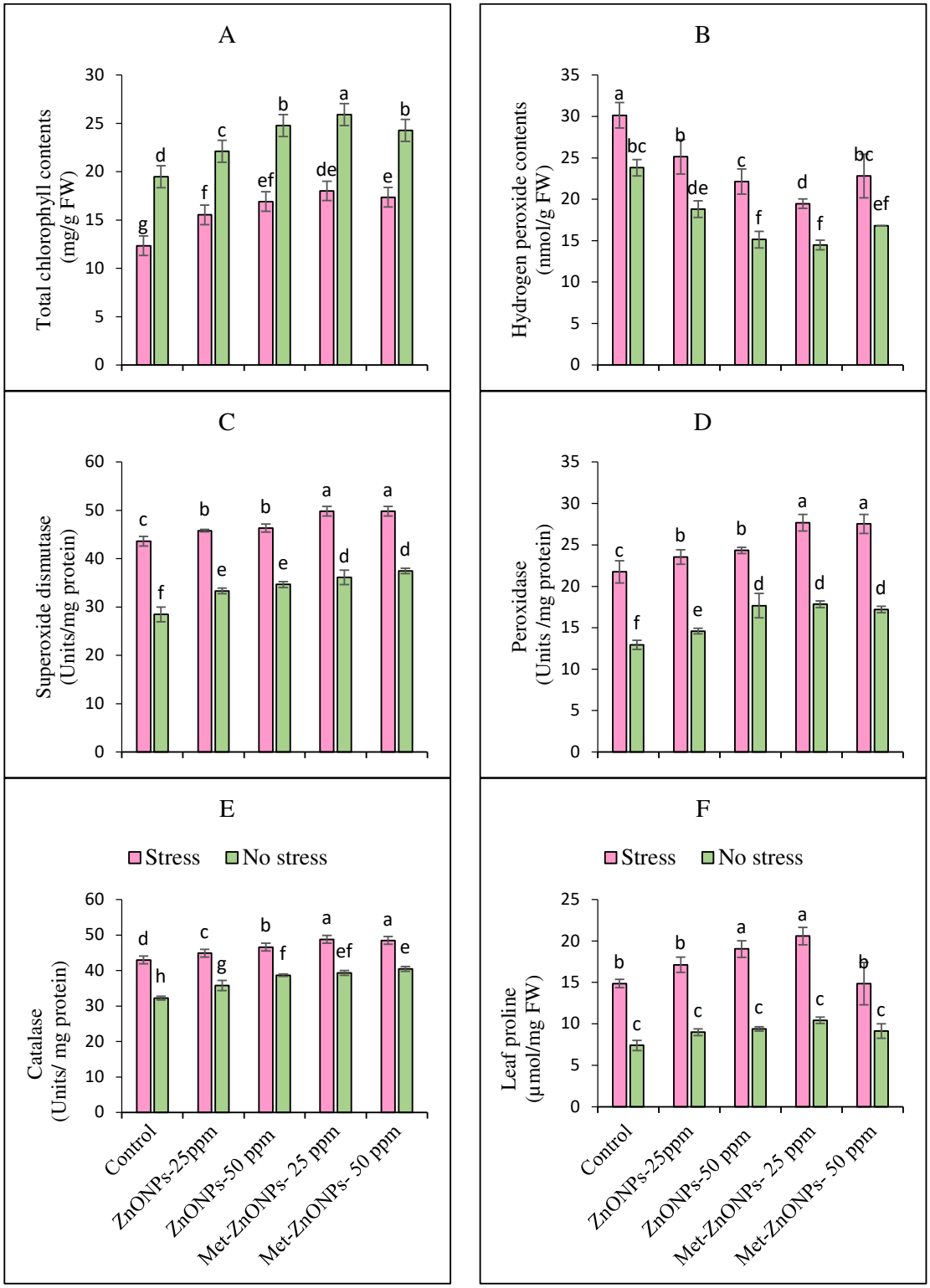


Figure 2. Physio-biochemical attributes of rice plants as affected through seed priming with nano zinc oxide and methionine coated nano zinc oxide (A) total chlorophyll contents (B) hydrogen peroxide (C) Superoxide dismutase functions (D) peroxidase functions (E) Catalase functions (F) Leaf proline contents

Effect on antioxidant defence enzymes and proline levels

The investigation aimed to evaluate the functions of antioxidant enzymes in rice, namely Superoxide dismutase (SOD) (Figure 2C), Peroxidase (POD) (Figure 2D), and Catalase (CAT) (Figure 2E), in response to arsenic stress conditions. The results revealed that under arsenic stress, the activities of SOD, POD, and CAT enzymes were heightened by 34%, 40%, and 24.98%, respectively. Interestingly, priming with both ZnONPs and Met-ZnONPs further amplified the activities of SOD, indicating an enhanced defence mechanism against oxidative stress. Particularly, treatments with Met-ZnONPs exhibited superior effects compared to those with ZnONPs alone. The priming concentration of 50 ppm ZnONPs and 25 ppm Met-ZnONPs demonstrated more significant improvements in the functions of SOD, POD, and CAT. At a concentration of 50 ppm, ZnONPs increased the functions of SOD, POD, and CAT by 5.86%, 10.64%, and 7.79%, respectively. Conversely, priming with Met-ZnONPs resulted in even higher enhancements, with increases in SOD, POD, and CAT activities by 12.41%, 21.02%, and 11.34%, respectively. Furthermore, the treatments resulted in elevated proline content (Figure 2F). This elevation in proline levels suggests an enhanced ability of the plants to maintain cellular integrity and cope with osmotic stress induced by arsenic.

Effects on ascorbate-glutathione (AsA-GSH) cycle and glutathione substrate pool

The study investigated the effects of seed priming with ZnONPs and Met-ZnONPs on key enzymes involved in the AsA-GSH cycle in rice plants. Function of APX, GR, MDHAR, and DHAR in the cycle were examined under stress levels. The results demonstrated that all treatments significantly increased the activities of these enzymes, indicating an enhanced antioxidant defence system in response to arsenic stress. Under arsenic stress, the contents of APX, GR, MDHAR, and DHAR were increased by 48%, 48.61%, 25.5%, and 21.8% (Figure 3A; Figure 3B; Figure 3C; Figure 4D), respectively. Furthermore, priming with 50 ppm ZnONPs led to additional increases in the functions of these enzymes by 11.26%, 16.35%, 9.94%, and 7.06%, respectively. However, the effects of 25 ppm Met-ZnONPs were superior among all treatments, increasing the contents of APX, GR, MDHAR, and DHAR by 19.57%, 25.19%, 17.17%, and 14.4%, respectively. Both ZnONPs and Met- ZnONPs treatments increased APX activity, with the latter showing a higher percentage increase compared to ZnONPs alone, indicating its superior effectiveness in enhancing the antioxidant defence system. Similarly, the activities of GR, MDHAR, and DHAR enzymes were significantly elevated by both treatments, with Met- ZnONPs exhibiting a higher percentage increase compared to ZnONPs alone. Under arsenic stress, the levels of GSH and GSSG increased by 13.96% and 27.50%, respectively (Figure 3E; Figure 3F). Furthermore, treatment with 50 ppm ZnONPs enhanced these levels by an additional 9% and 11%, respectively. However, the most significant improvements in enhancing GSH and GSSG levels in rice plants were observed with the application of 25 ppm ZnONPs, leading to increases of 17.45% and 16.34%, respectively. The treatment with Met- ZnONPs resulted in a higher percentage increase in GSH content compared to ZnONPs alone, indicating the superior efficacy in enhancing the plant's antioxidant defence system. The elevated GSH levels reflect an improved antioxidant defence system, while the increase in GSSG suggests an enhanced recycling of GSH.

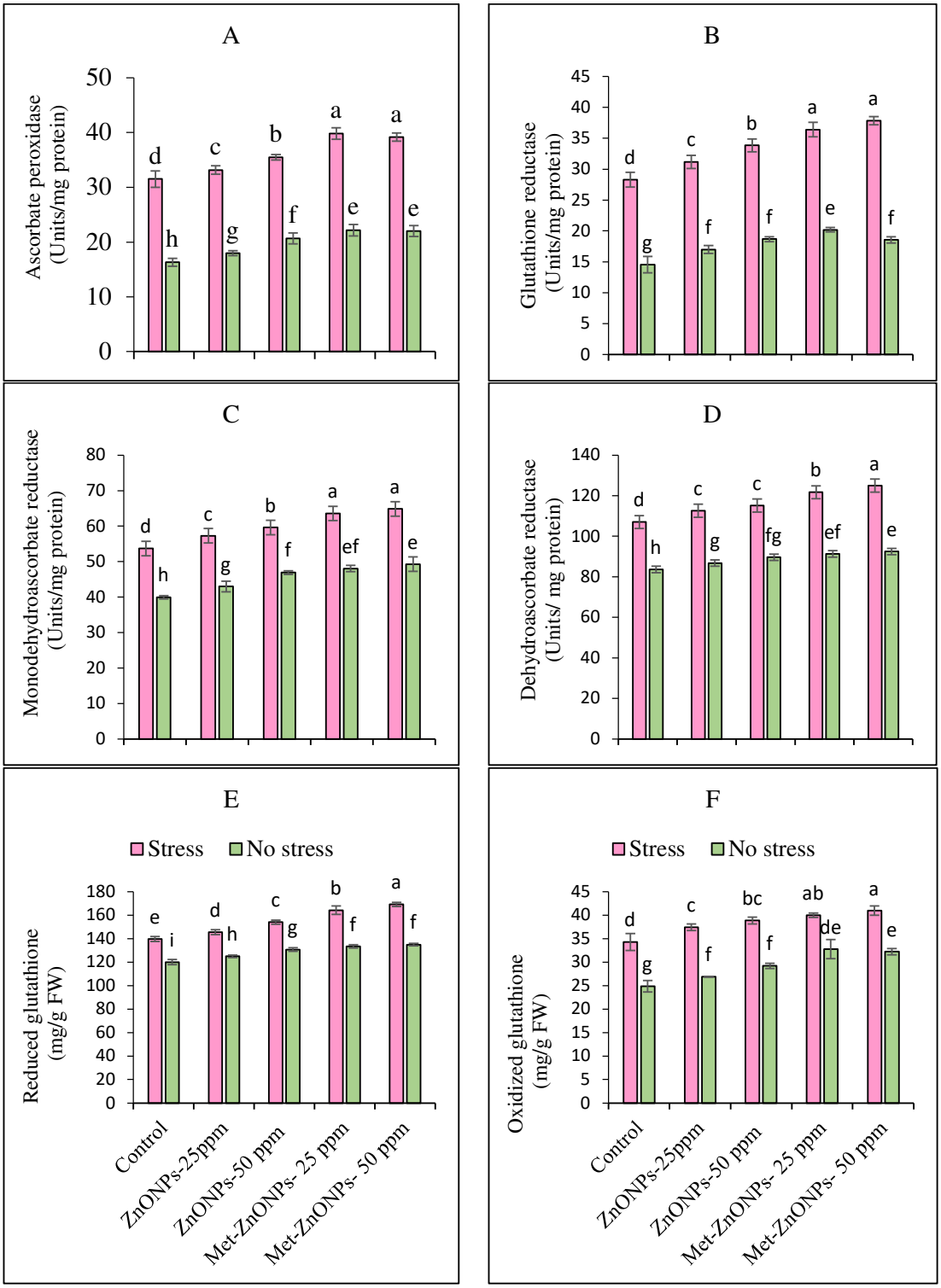
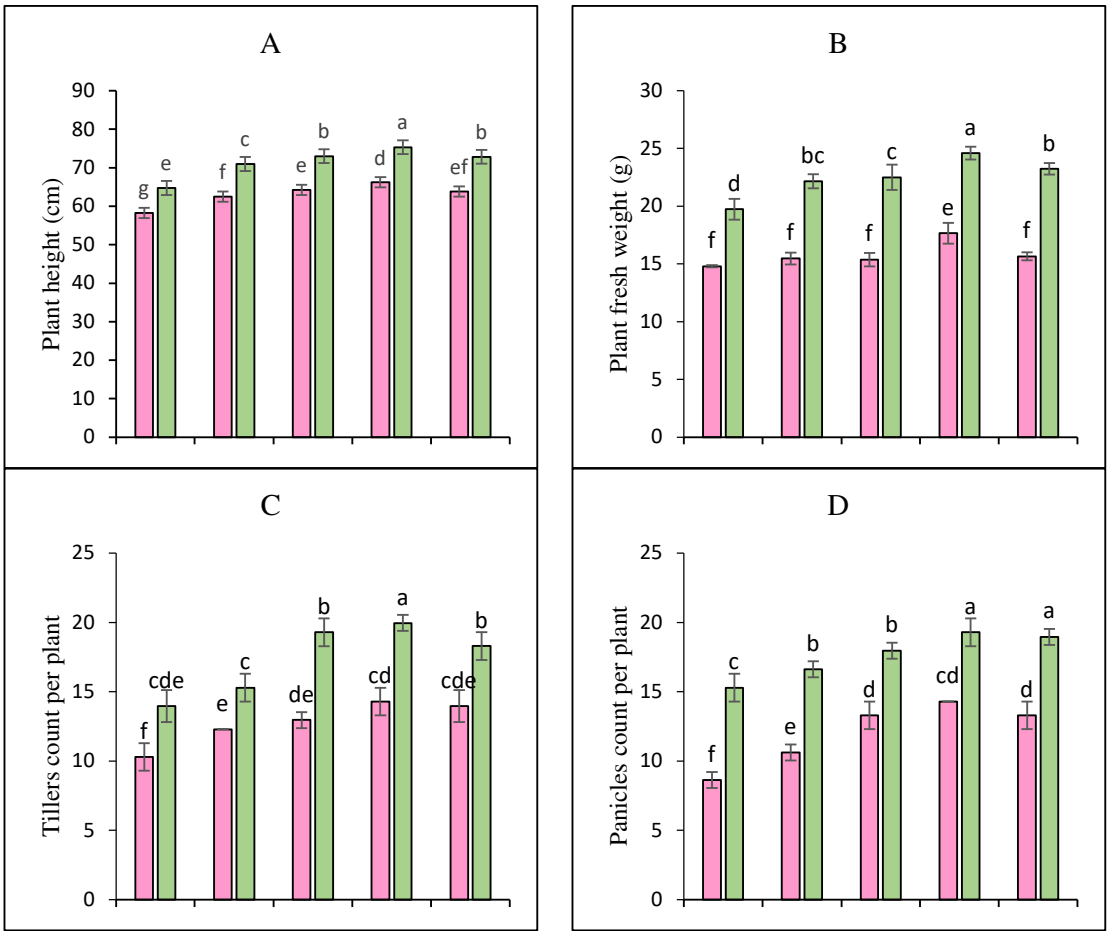


Figure 3. Effects of seed priming with nano-zinc oxide either uncoated or coated with methionine on the enzymes of ascorbate glutathione cycle and glutathione substrate pool (A) Ascorbate peroxidase functions (B) Glutathione reductase functions (C) Monodehydroascorbate reductase functions (D) Dehydroascorbate reductase functions (E) Reduced glutathione contents and (F) Oxidized glutathione contents

Growth and yield attributes of rice plants

Arsenic stress caused a reduction in height and fresh weights of rice plants exposed to arsenic contamination. Quantitatively, arsenic stress led to a decrease in plant height (Figure 4A), and fresh weight (Figure 4B) by approximately 10%, and 25%, respectively. The application of Met-ZnONPs showed a more significant increase in plant height compared to ZnONPs alone. The most effective treatments for enhancing growth parameters were found to be 50 ppm ZnONPs and 25 ppm Met-ZnONPs. Specifically, 50 ppm ZnONPs resulted in an increase in plant height, and fresh weight by approximately 10.24%, and 3.82% respectively. In comparison, the 25 ppm Met-ZnONPs led to an increase in these parameters by approximately 13.73%, and 19.36% respectively. These results prove superiority of methionine loaded nano-zinc oxide compared to ZnONPs alone. In the study, Met-ZnONPs treatments at both 25 ppm and 50 ppm significantly enhanced yield attributes compared to ZnONPs alone, supporting the hypothesis. For tiller count per plant (Figure 4C), Met-ZnONPs-25 ppm led to a 38.83% increase over the control, whereas ZnONPs-50 ppm achieved only 25.87%. Panicle count per plant (Figure 4D), showed even greater differences, with Met-ZnONPs-25 ppm increasing the count by 65.64% over the control, compared to 54.01% for ZnONPs-50 ppm. Spikelet count per panicle (Figure 4E), and panicle length (Figure 4F), showed improvements as well, with spikelet count per panicle increasing by 18.33% under Met-ZnONPs-25 ppm compared to 13.33% with ZnONPs-50 ppm. A detailed comparison for the five treatments and their effects on yield attributes have been shown in table 3.



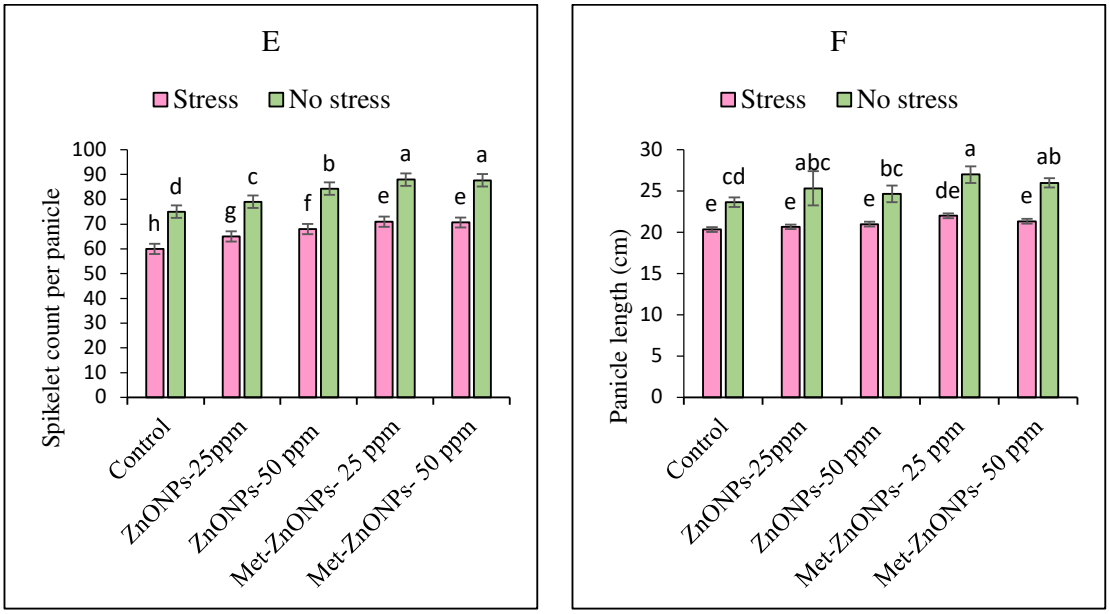


Figure 4. Yield attributes of rice plants followed in the current experiment (A) plant height (B) plant fresh weight (C)tiller count per plants (D) panicle count per plant (E) spikelet count per panicle (F) panicle length

Table 3. Percentage increase in the yield attributes of arsenic grown rice plants as affected through various seed priming treatments studied in the experiment

Attribute	ZnONPs-25ppm (%)	ZnONPs-50ppm (%)	Met-ZnONPs-25ppm (%)	Met-ZnONPs-50ppm (%)
Tillers count per plant	19.42	25.87	38.83	35.66
Panicle count per plant	23.14	54.01	65.64	54.01
Thousand caryopsis weight	5.18	8.64	18.99	14.67
Paddy yield of rice	6.39	13.69	18.72	18.72
Straw yield of rice	7.72	13.84	18.32	20.27
Caryopsis protein	1.02	2.73	6.14	4.43
Caryopsis starch contents	1.48	2.12	3.28	3
Length of the panicle	1.64	3.27	8.21	4.93
Spikelet count per panicle	8.33	13.33	18.33	17.78

Paddy yield (Figure 5A) was 18.72% higher in both Met-ZnONPs treatments, while ZnONPs-50 ppm resulted in a 13.69% increase. Other yield attributes also favoured Met-ZnONPs treatments. Straw yield (Figure 5B) rose by 20.27% under Met-ZnONPs-50 ppm, versus 13.84% under ZnONPs-50 ppm. Protein (Figure 5C) and starch contents (Figure5D) in the rice caryopsis saw modest but meaningful increases with Met-ZnONPs-25 ppm at 3.28% and 8.21% for protein and starch, respectively, surpassing ZnONPs treatments. The 1000-grain weight (Figure 5E) was also higher in Met-ZnONPs-25 ppm at an 18.99% increase, compared to an 8.64% increase in ZnONPs-50 ppm. These results indicate the superior efficacy of Met-ZnONPs as a seed priming treatment for enhancing yield components in arsenic-stressed rice.

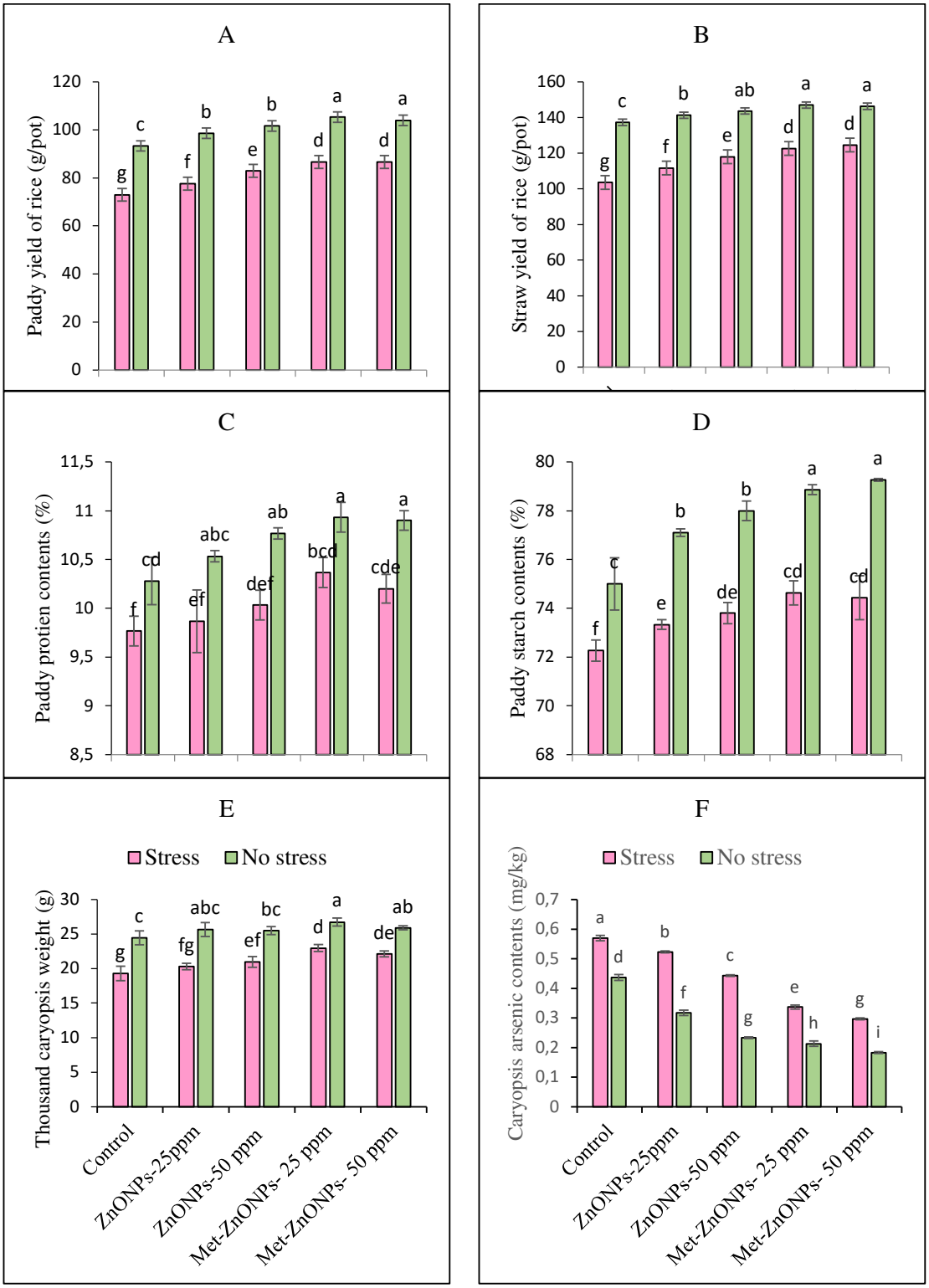


Figure 5. Yield attributes and arsenic contents of rice plants followed in the current experiment (A) paddy yield of rice (B) straw yield of rice (C) Caryopsis protein contents (D) caryopsis starch contents (E) thousand caryopsis weight (F) Caryopsis arsenic contents

Caryopsis arsenic values

Arsenic content in rice grains was evaluated, and the results (Figure 5F) indicate that arsenic levels were significantly elevated in the absence of a seed priming treatment with nano zinc oxide. Seed priming with ZnONPs significantly reduced arsenic content, while priming with Met-ZnONPs further decreased arsenic levels, demonstrating that Met-ZnONPs served as a more effective pre-conditioning treatment than ZnONPs alone. Under stress conditions, ZnONPs at 25 ppm led to an 8.25% decrease in arsenic content compared to the control, while ZnONPs at 50 ppm resulted in a more substantial 22.28% reduction. Methionine coating on ZnONPs further enhanced arsenic reduction, with Met-ZnONPs at 25 ppm showing a 40.88% decrease, and at 50 ppm achieving the highest reduction of 47.89%. In no-stress conditions, ZnONPs at 25 ppm and 50 ppm decreased arsenic content by 27.47% and 46.67%, respectively, relative to the control. Again, the addition of methionine coating markedly improved arsenic reduction. Met-ZnONPs at 25 ppm reduced arsenic levels by 51.26%, while Met-ZnONPs at 50 ppm achieved the highest arsenic reduction of 58.13%.

Statistical interpretation of the results

The Spearman correlation matrix (Table 4) shows a significant correlation among the variables examined. This positive correlation reinforces that the treatments were effective in mitigating arsenic stress's detrimental effects on plant growth and development. A two-way ANOVA revealed significant effects of both arsenic stress and zinc nano-priming on the measured parameters, along with a notable interaction between these two factors. This interaction indicates that the effectiveness of the treatments varied depending on the level of arsenic stress, highlighting the differential impact of the treatments under varying stress intensities (Table 5). For arsenic stress, which had two levels, the degrees of freedom (df) was 1 (df = n - 1). For treatments with five levels, the degrees of freedom were 4. The significance of the individual and interactive effects was denoted as follows: * ($p < 0.05$), ** ($p < 0.01$), and *** ($p < 0.001$).

Table 4. Spearman correlation matrix of the studied variables of rice plants grown under arsenic contaminated environment and treated with nano zinc oxide and methionine coated nano zinc oxide

Var.	As-C	H_2O_2	SOD	POD	CAT	PRO	T.C	NOT/P	NOP/P	1000 Wt	PYR	SYR	CP	CS	PL	NSPP	APX	GR	MDHAR	DHAR	GSH	GSSG
As-C	1																					
H_2O_2	0.81*	1																				
SOD	0.35	0.29	1																			
POD	0.35	0.24	0.96*	1																		
CAT	0.35	0.27	0.99*	0.97*	1																	
PRO	0.50*	0.15	0.77*	0.84*	0.79*	1																
T.C	-0.91*	-0.88*	-0.55*	-0.54*	-0.54*	-0.55*	1															
NOT/P	-0.88*	-0.91*	-0.38*	-0.36	-0.38*	-0.35	0.92*	1														
NOP/P	-0.88*	-0.91*	-0.52*	-0.51*	-0.50*	-0.45*	0.97*	0.93*	1													
1000 Wt	-0.85*	-0.85*	-0.55*	-0.55*	-0.55*	-0.49*	0.95*	0.91*	0.95*	1												
PYR	-0.92*	-0.88*	-0.52*	-0.53*	-0.51*	-0.52*	0.98*	0.94*	0.97*	0.96*	1											
SYR	-0.93*	-0.88*	-0.53*	-0.54*	-0.52*	-0.52*	0.96*	0.93*	0.98*	0.95*	0.98*	1										
CP	-0.81*	-0.91*	-0.42*	-0.40*	-0.41*	-0.32	0.88*	0.94*	0.94*	0.91*	0.93*	0.92*	1									
CS	-0.91*	-0.89*	-0.49*	-0.50*	-0.48*	-0.51*	0.97*	0.94*	0.97*	0.95*	0.99*	0.98*	0.93*	1								
PL	-0.75*	-0.79*	-0.59*	-0.62*	-0.59*	-0.49*	0.86*	0.84*	0.89*	0.90*	0.90*	0.89*	0.87*	0.89*	1							
NSPP	-0.90*	-0.89*	-0.53*	-0.52*	-0.52*	-0.48*	0.95*	0.94*	0.98*	0.95*	0.99*	0.98*	0.94*	0.98*	0.91*	1						
APX	0.36	0.25	0.97*	0.98*	0.98*	0.84*	-0.54*	-0.37*	-0.50*	-0.54*	-0.51*	-0.52*	-0.39*	-0.49*	-0.58*	-0.51*	1					
GR	0.33	0.27	0.97*	0.98*	0.97*	0.79*	-0.54*	-0.38*	-0.52*	-0.55*	-0.52*	-0.53*	-0.42*	-0.50*	-0.59*	-0.52*	0.98*	1				
MDHAR	0.35	0.27	0.98*	0.97*	0.98*	0.81*	-0.55*	-0.36	-0.51*	-0.55*	-0.51*	-0.52*	-0.39*	-0.49*	-0.59*	-0.52*	0.99*	0.97*	1			
DHAR	0.34	0.29	0.99*	0.96*	0.99*	0.76*	-0.55*	-0.38*	-0.52*	-0.56*	-0.52*	-0.53*	-0.42*	-0.48*	-0.59*	-0.52*	0.97*	0.98*	0.98*	1		
GSH	0.34	0.29	0.99*	0.96*	0.99*	0.77*	-0.55*	-0.37*	-0.52*	-0.56*	-0.51*	-0.52*	-0.42*	-0.49*	-0.60*	-0.52*	0.98*	0.98*	0.99*	0.99*	1	
GSSG	0.29	0.24	0.98*	0.95*	0.97*	0.77*	-0.50*	-0.33	-0.48*	-0.52*	-0.48*	-0.48*	-0.37*	-0.45*	-0.56*	-0.48*	0.97*	0.97*	0.98*	0.97*	0.98*	1

Asterisks (*) indicate statistically significant correlations at $p < 0.05$

Table 5. Two-way ANOVA table showing mean square and p values of the different variables of rice plants studied under arsenic stress and seed priming treatments as factors

Variation source	[a]*df*	PH	PFW	As-C	CP	CS	GSH	GSSG	GR
Arsenic stress (AS)	1	517.50[b]*** (0.000)	316.875*** (0.000)	0.185 *** (0.000)	2.970*** (0.000)	113.685*** (0.000)	5072.60*** (0.000)	656.136*** (0.000)	1884.48*** (0.000)
Nanopriming (NP)	4	72.799*** (0.000)	11.977*** (0.000)	0.069*** (0.000)	0.389*** (0.000)	10.371*** (0.000)	509.366*** (0.000)	52.712*** (0.000)	50.845*** (0.000)
Interaction AS X NP	4	1.659 ns (0.1066)	1.439* (0.0351)	0.003*** (0.000)	0.0107 ns (0.924)	0.885 * (0.0385)	60.996*** (0.000)	3.213 NS (0.0598)	7.129*** (0.000)
Error	20	0.756	0.450	0.00013	0.049	0.285	3.817	1.188	0.806
Variation source	**[a]*df***	**TC**	**SOD**	**POD**	**APX**	**CAT**	**H_2O_2**	**PRO**	**MDHAR**
Arsenic stress (AS)	1	381.547*** (0.000)	1304.84*** (0.000)	614.08*** (0.000)	1954.392*** (0.000)	621.985*** (0.000)	270*** (0.000)	517.497*** (0.000)	1565.29*** (0.000)
Nanopriming (NP)	4	33.050*** (0.000)	55.132*** (0.000)	30.908*** (0.000)	57.153*** (0.000)	48.794*** (0.000)	87.533*** (0.000)	17.609* (0.020)	104.294*** (0.000)
Interaction AS X NP	4	0.357 ns (0.772)	2.273 ns (0.079)	1.979 ns (0.076)	2.099 ns (0.0792)	1.808* (0.044)	0.5 ns (0.903)	5.153 ns (0.391)	1.763 ns (0.442)
Error	20	0.796	0.926	0.796	0.855	0.609	1.966	4.762	1.806
Variation Source	**[a]*df***	**NOT/P**	**NOP/P**	**1000 CW**	**PYR**	**PL**	**SYR**	**NSPP**	**DHAR**
Arsenic stress (AS)	1	145.2*** (0.000)	229.633*** (0.000)	145.2*** (0.000)	2688.53*** (0.000)	136.533*** (0.000)	5386.8*** (0.000)	1809.63*** (0.000)	5743.6 ***(0.000)
Nanopriming (NP)	4	23.833*** (0.022)	23.416*** (0.000)	7.728*** (0.000)	170.283*** (0.000)	5.283* (0.032)	232.7*** (0.000)	153.78*** (0.000)	171.574*** (0.000)
Interaction AS X NP	4	1.866 ns (0.101)	1.216 ns (0.112)	0.953 ns (0.156)	4.283 ns (0.161)	0.783 ns (0.743)	34.633*** (0.000)	2.883 ns (0.1031)	20.762*** (0.000)
Error	20	0.833	0.566	0.511	2.333	1.6	2.933	1.3	2.344

[a] df. Degree of freedom, ns non-significant; [b] *, **, and *** = significant at 0.05, 0.01, and 0.001 levels, respectively

The bi-plot analysis (Figure 6) provided additional insights, with clustering patterns that highlighted similarities and distinctions among variables and treatments. The PCA bi-plot (Figure 6) revealed clear groupings, where growth and yield parameters clustered together, while antioxidant defence enzymes formed a separate cluster. The aggregation of growth and yield attributes indicates that these parameters were negatively impacted by arsenic stress but showed improvement with seed priming treatments. Similarly, the clustering of antioxidant defence enzymes and the glutathione pool suggests that these variables increased in response to arsenic exposure and were further enhanced by seed priming. Additionally, hydrogen peroxide and arsenic content appeared closely aligned on the biplot, reflecting their concurrent increase under arsenic contamination. However, both variables demonstrated a tendency to decrease following seed priming treatment (Figure 6). Together, these findings illustrate the comprehensive impact of seed priming treatments on improving plant resilience to arsenic stress, with clear benefits across growth, yield, and antioxidant defence parameters.

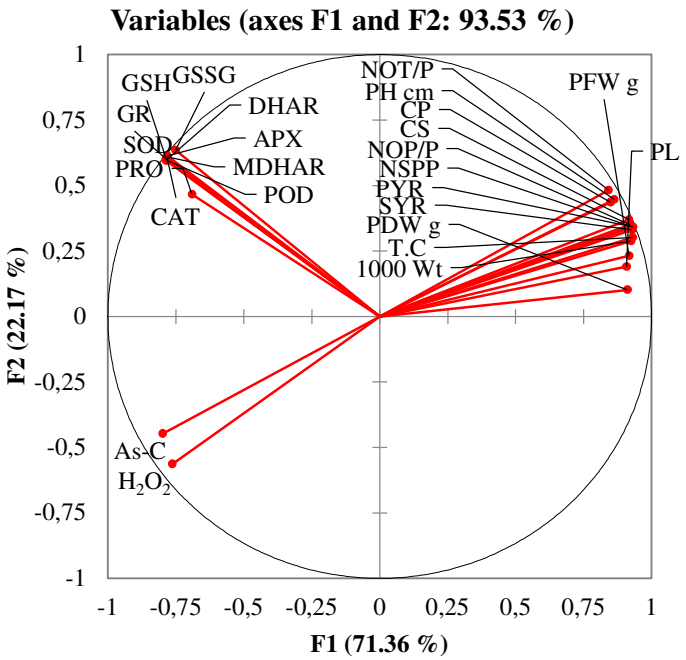


Figure 6. Principal components analysis plot of the variables of arsenic affected rice plants as affected by seed priming treatments with methionine coated zinc oxide nanoparticles and uncoated zinc oxide nanoparticles

Discussion

The methionine coating resulted to a shift toward shorter wavelengths, from 376 nm to 340 nm in the optical properties of nano zinc oxide. The difference in absorbance might be due to the methionine coating on nano-zinc oxide, which modifies the optical properties (Liu *et al.*, 2021). The presence of the methionine coating causes peak broadening, leading to the observed deviation in the spectra. Furthermore, TEM examination showed that Met-ZnONPs had a porous appearance. Methionine contains a polar side chain (Joshi*et al.*, 2020), which likely interacted with nano-zinc oxide, creating spaces on the surface and resulting in a porous appearance. Studies have shown that nanoparticle porosity can enhance the release of Zn^{2+} ions, which may have helped rice plants thrive better under heavy metal stress (Foroozesh and Kumar, 2020).

Seed preconditioning with both coated and uncoated nano-zinc oxide significantly improved the growth and biomass of rice under both stress and no-stress conditions. However, the LSD test results showed that Met-ZnONPs outperformed ZnONPs. Nano-zinc oxide, when used as a seed priming material, causes a slow release of zinc as a micronutrient. Literature reports that zinc is essential in promoting physiological processes, which may contribute to the improved physiological performance and growth of the rice plants (Wessels *et al.*, 2021; Mazhar *et al.*, 2022). Methionine coatings further enhanced the growth parameters observed, including plant height and weight. Studies indicate that methionine is a crucial amino acid involved in protein synthesis, nutrient uptake, and stress response (Elango, 2020). The methionine coating increases nano-zinc oxide stability and biocompatibility, promoting uptake by rice roots. Coatings on nanomaterials form a protective layer, reducing cytotoxicity and enhancing biocompatibility, which may have improved rice plant performance under arsenic contamination (Imani *et al.*, 2020).

In addition to better growth, improved agronomic features in rice plants, such as panicle count, tiller count, panicle length, spikelet count per panicle, and grain weight, were observed as a result of nano-zinc oxide

priming. These results are in good agreement with studies conducted by Mazhar *et al*., (2022). These agronomic traits were further enhanced by Met-ZnONPs, as shown by the LSD test results. Literature suggests that ZnONPs can increase nutrient uptake by promoting root growth, potentially supporting rice plant branching and tillering, which in turn increases panicle count per plant (Zhang *et al*., 2021). Seed priming with nano-zinc oxide also improved the nutritional quality of rice caryopsis. Carbohydrate and protein contents were significantly higher in the Met-ZnONPs treatment than with ZnONPs alone. Zinc is involved in starch synthesis and accumulation in grains, and its availability during critical growth stages positively influences starch levels. Adequate zinc also promotes protein synthesis, resulting in higher protein content in rice grains. Grain arsenic content was reduced with nano-zinc oxide priming, with a further reduction observed using methionine-coated particles. ZnONPs have been found to influence the expression of genes involved in metal transport and detoxification in plants. It is plausible that the rice plants upregulated genes related to arsenic detoxification (Chauhan *et al*., 2020), reducing arsenic uptake. Additionally, methionine's antioxidant properties may contribute to grain quality by mitigating oxidative stress and preserving grain integrity.

Oxidative damage occurs due to an imbalance between ROS production and its detoxification by antioxidant enzymes. Elevated levels of hydrogen peroxide and ROS can harm cellular components. In our study, seed priming elevated SOD, POD, and CAT functions, aiding in ROS scavenging by acting as electron acceptors and quenchers of hydrogen peroxide. Met-ZnONPs were especially effective in boosting the antioxidant defence of the plants. Studies have shown that methionine itself acts as an antioxidant with ROS-detoxifying properties, suggesting that methionine coatings provided an additional antioxidant source for ROS detoxification (Dabaibeh, 2024).

Total chlorophyll and proline levels increased with nano-zinc oxide seed priming. Numerous studies highlight zinc's positive impact on chlorophyll stability (Mazhar *et al*., 2022; Waqas Mazhar *et al*., 2024). Proline content increased under arsenic stress and further increased with ZnONPs priming, with Met-ZnONPs amplifying these effects. Proline is an osmoprotectant and biocompatible solute, assisting in the cross-talk between plant and environmental stressors. It acts as a free radical scavenger, stabilizes cell structure, and regulates cellular osmotic balance, enhancing tolerance to heavy metal stress (Ghosh *et al*., 2022).

Enzyme functions in the AsA-GSH cycle were also studied in this experiment. The results indicate increased activities of the AsA-GSH cycle enzymes, including APX, GR, MDHAR, and DHAR, which were further enhanced by priming treatments. The AsA-GSH cycle regulates antioxidant defence and ROS balance (Ishtiaq *et al*., 2023), detoxifying hydrogen peroxide and regenerating ascorbate and glutathione. The APX enzyme, the first in the AsA-GSH cycle, converts toxic ROS, such as hydrogen peroxide, into water using AsA as a reducing agent (Jiang *et al*., 2022). The GR enzyme regenerates the reduced form of GSH from oxidized GSSG using NADPH as a cofactor. MDHAR reduces monodehydroascorbate to AsA, while DHAR performs another reduction to convert dehydroascorbate back to AsA. The presence of Zn^{2+} contributes to activating the AsA-GSH cycle (Ishtiaq *et al*., 2023), suggesting that nano-zinc oxide seed priming may have provided essential Zn for cycle activation. Methionine coatings on the nanoparticles further facilitated the cycle's antioxidant role, modulating AsA-GSH cycle enzymes for arsenic tolerance in rice (Ahmad *et al*., 2020).

The glutathione pool of the rice plants was examined, focusing on GSH and GSSG levels. Enhanced GSH production was linked to increased AsA-GSH cycle enzyme activity. GSH plays a critical role in plant stress tolerance by maintaining redox homeostasis (Averill-Bates, 2023; Ishtiaq *et al*., 2023). GSH oxidation leads to GSSG formation, and a higher cellular glutathione pool supports a more reducing environment, promoting efficient ROS removal (Ishtiaq *et al*., 2023). Increased GSH activity boosts cells' ability to withstand oxidative stress, maintaining an optimal cellular redox state. GR activity helps regenerate the GSH pool from GSSG. Since methionine contains sulphur and is a precursor for synthesizing sulphur-containing compounds like GSH, methionine coatings on ZnONPs may further increase their efficacy in regulating GSH and GSSG activities. Methionine availability can impact GSH production, affecting APX, GR, MDHAR, and DHAR activities (Mandal *et al*., 2022).

Conclusion

Conclusively, the findings of this study suggest that nanomaterials can achieve greater biocompatibility when coated with bio-rational materials like methionine, as demonstrated here. Specifically, Met-ZnONPs proved more effective than ZnONPs alone in enhancing growth and agronomic traits. Seed priming with Met-ZnONPs increased total chlorophyll content and reduced hydrogen peroxide levels. The antioxidant defence system, particularly the AsA-GSH cycle enzymes, was significantly activated in rice plants raised with zinc nanomaterial seed priming. Supporting our hypothesis, arsenic content in rice was significantly lower in plants primed with Met-ZnONPs compared to those with ZnONPs alone. Additionally, seed priming led to the accumulation of the compatible solute proline, aiding rice in tolerating arsenic-contaminated environments. Future studies should conduct long-term field trials across different rice-growing regions to comprehend the Met-ZnONPs suitability in agricultural systems. This would validate the lab findings in real-world conditions and help understand the broader agronomic impacts of Met-ZnONPs on yield, soil health, and crop quality.

Authors' Contributions

Conceptualization: MWM, MI, MM, LAS, LAA, ST, AA, AA, RA, MA, TH, FIJ, MZA, SS; Data curation: MWM, MI, MM, LAS, LAA, ST, AA, AA, RA, MA; Formal analysis: AA, ST, RA, MA, TH; Funding acquisition: LAS, LAA, AA, SS; Investigation: MWM, MI, MM, ST, MA, TH; Methodology: MWM, MI, MM, ST, RA, TH; Resources: LAS, LAA, AA, SS; Software: RA, MA, TH, MZA, SS; Supervision: MI, LAS, SS; Validation: MWM, TH, MA, RA, MZA; Visualization: MWM, MM, LAA, SS; Writing - original draft: MWM, MI, MM, ST; Writing - review & editing: LAS, LAA, AA, RA, SS.

All authors have read and approved the final manuscript

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Data Availability Statement

The data that support the finding of this study are available from first author upon reasonable request.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article

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