

## Zinc nanoparticles mediated biofortification and nutrient interactions in cowpea (*Vigna unguiculata* L.)

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Zinc deficiency remains a major constraint to crop nutritional quality and human health, particularly in grain legumes consumed as protein-rich staples. The present study evaluated the effectiveness of zinc oxide nanoparticles (ZnO-NPs) as a foliar biofortification strategy in cowpea (*Vigna unguiculata* L.), with emphasis on nutrient interactions, nutrient quality indices, and micronutrient partitioning. A pot experiment was conducted at Chaudhary Charan Singh Haryana Agricultural University, Hisar during the Kharif season of 2023-24 using the variety Kashi Kanchan under Completely Randomized Design with thirteen treatments and three replications. Zinc was applied as foliar spray at three zinc concentrations (400, 500 and 600 ppm) in both ZnO-NPs and bulk form, along with an untreated control. Foliar sprays were imposed at 25 and 50 DAS. Seed nutritional parameters, including protein, nitrogen, total soluble sugars, total phenolics, potassium, zinc, iron, copper, manganese, and zinc nutrient harvest index (Zn-NHI), were determined. Zinc application significantly improved seed nutritional quality, with ZnO-NPs treatments consistently outperforming bulk zinc. The highest protein (27.81%), nitrogen (4.45%), soluble sugars (39.57 mg g<sup>-1</sup>), phenolics (931.10 mg GAE 100 g<sup>-1</sup>), micronutrient concentrations, and Zn-NHI (61.72%) were recorded under 600 ppm ZnO-NPs applied twice which remained statistically at par with 500 ppm ZnO-NPs applied twice. Nutrient responses were further integrated using Z-score transformation and a Z-based Nutrient Quality Index (Z-NQI), supported by regression analysis where Z-score and NQI analyses clearly differentiated treatments, with 500 & 600 ppm ZnO-NPs (two sprays) achieving the significantly highest NQI, indicating superior nutrient balance and biofortification efficiency. Regression analysis revealed strong associations between NQI and protein, zinc, and Zn-NHI (R<sup>2</sup> up to 0.90), confirming zinc as a key driver of overall nutritional enhancement. The study demonstrates that ZnO-NPs, particularly at 500 ppm with two foliar applications, offer an efficient and integrative strategy for zinc biofortification and nutritional quality improvement in cowpea.

**Keywords:** Zinc Nutrient Harvest Index, Z-score Analysis, Regression analysis, Nutritional Quality Index, Foliar Application, ZnO-NPs and nutrient interaction

### Introduction

Micronutrient malnutrition, particularly zinc (Zn) deficiency, remains a major global health challenge affecting both human populations and agricultural productivity. Zinc plays a pivotal role in numerous physiological and biochemical processes in plants, including enzyme activation, protein synthesis, photosynthesis, and nitrogen metabolism<sup>1</sup>. In humans, inadequate dietary zinc intake leads to impaired immune function, growth retardation, and increased susceptibility to diseases. Grain legumes, such as cowpea, serve as an important source of dietary protein and micronutrients for millions of people, especially in developing regions. However, the inherent low zinc concentration in edible plant parts limits their contribution to alleviating zinc deficiency,

thereby necessitating effective biofortification strategies<sup>2</sup>. Biofortification through agronomic interventions has emerged as a practical and scalable approach to enhance micronutrient content in crops<sup>3</sup>. Conventional zinc fertilizers, though widely used, often exhibit low use efficiency due to soil fixation, leaching losses, and limited root uptake, particularly in alkaline and calcareous soils. These constraints not only reduce zinc availability to plants but also restrict the effectiveness of zinc biofortification programs. In recent years, nanotechnology has gained attention as a promising tool in agriculture, offering novel avenues to improve nutrient delivery, uptake efficiency, and physiological utilization within plants<sup>4</sup>.

Zinc oxide nanoparticles (Zn-NPs), owing to their ultra-small size, high surface area, and enhanced reactivity, have demonstrated superior performance over bulk zinc sources in terms of controlled release, improved absorption, and reduced losses<sup>5</sup>. The nano-

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enabled delivery of zinc has been reported to stimulate plant growth, enhance nutrient assimilation, and improve stress tolerance. More importantly, Zn-NPs offer significant potential for crop biofortification by facilitating greater zinc accumulation and balanced nutrient uptake, thereby improving overall nutritional quality<sup>6</sup>. Despite these advantages, comprehensive assessments of zinc nanoparticle-mediated biofortification, particularly from a multinutrient perspective, remain limited.

Evaluation of biofortification efficiency requires more than the assessment of individual nutrient concentrations. Nutrient Quality Indices (NQIs), derived from standardized nutrient values, provide an integrated and quantitative framework to assess nutrient balance, synergistic and antagonistic interactions, and overall nutritional quality of crops. When coupled with multivariate statistical tools, such as correlation analysis, regression modelling, and principal component analysis, NQIs enable a robust interpretation of complex nutrient dynamics under nano-fertilization regimes<sup>7</sup>. These approaches offer deeper insights into how zinc nanoparticles influence not only zinc accumulation but also the assimilation and interrelationships of essential macro- and micronutrients. In this context, the present study aims to evaluate zinc nanoparticle-mediated biofortification of cowpea using nutrient quality indices and multivariate analysis. The study focuses on elucidating the influence of Zn-NPs on nutrient quality, nutrient interactions, and biofortification efficiency, thereby providing a quantitative and integrative understanding of nano-enabled zinc nutrition in cowpea<sup>8</sup>. The findings are expected to contribute to the development of sustainable, nano-based biofortification strategies for improving crop nutritional quality and addressing micronutrient malnutrition.

## Material and Methods

The proposed study was conducted at Research Farm, Department of Vegetable Science, CCS Haryana Agricultural University, Hisar during Kharif season, 2023-24. During the crop season, the maximum temperature reported was 45°C, while the minimum temperature varied from 19.4°C. The morning relative humidity remained consistently high, ranging from 82 to 102 per cent, whereas the evening relative humidity increased from 64 to 86 per cent. Considerable variation in rainfall was observed, with heavy rainfall peaks during SMW 28 and 31, indicating the occurrence of intense monsoon showers. The cowpea variety 'Kashi Kanchan' was selected due to its wide adaptability, stable performance in north India and responsiveness to nutrient management, making it suitable for evaluating the effects of nano- and bulk zinc fertilization under controlled conditions. Five earthen pots per treatment per replication of 30 cm diameter filled with 17 kg of air-dried soil were used for raising the crop. The soil used for filling the pots was sandy loam in texture with pH 7.8, organic carbon 0.25 %, available nitrogen 132 kg/ha, phosphorus 13.80 kg/ha, potassium 238 kg/ha, iron 1.05 ppm and zinc 0.36 ppm. The soil was collected from the surface layer (0–15 cm) of the research farm, airdried, gently crushed and passed through a 2-mm sieve to ensure uniformity. Three doses of zinc oxide (400, 500 and 600 ppm) were applied in both nanoparticles and bulk foam along with a recommended dose of fertilizer taking control (no zinc oxide application) as a check as mentioned in Table-1. Zinc oxide nanoparticles (ZnO-NPs; white powder, hexagonal structure, 97% purity, particle size <50 nm, -30mv zeta potential and highly stable in solution) were procured from Sigma-Aldrich, USA, while bulk zinc

Table 1 — Different treatment combinations followed during experiment

No. of treatment	Treatment detail	Time of application
T <sub>1</sub>	Recommended dose of fertilizer (RDF)	As Basal dose
T <sub>2</sub>	RDF + 400 ppm ZnO NPs	1 spray: - At 25 DAS
T <sub>3</sub>	RDF + 400 ppm Bulk Zn	1 spray: - At 25 DAS
T <sub>4</sub>	RDF + 400 ppm ZnO NPs	2 sprays: - At 25 DAS & 50 DAS
T <sub>5</sub>	RDF + 400 ppm Bulk Zn	2 sprays: - At 25 DAS & 50 DAS
T <sub>6</sub>	RDF + 500 ppm ZnO NPs	1 spray: - At 25 DAS
T <sub>7</sub>	RDF + 500 ppm Bulk Zn	1 spray: - At 25 DAS
T <sub>8</sub>	RDF + 500 ppm ZnO NPs	2 sprays: - At 25 DAS & 50 DAS
T <sub>9</sub>	RDF + 500 ppm Bulk Zn	2 sprays: - At 25 DAS & 50 DAS
T <sub>10</sub>	RDF + 600 ppm ZnO NPs	1 spray: - At 25 DAS
T <sub>11</sub>	RDF + 600 ppm Bulk Zn	1 spray: - At 25 DAS
T <sub>12</sub>	RDF + 600 ppm ZnO NPs	2 sprays: - At 25 DAS & 50 DAS
T <sub>13</sub>	RDF + 600 ppm Bulk Zn	2 sprays: - At 25 DAS & 50 DAS

was supplied as zinc sulphate heptahydrate ( $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ ) of analytical grade. Fresh spray solutions were prepared prior to each foliar application. For nano zinc treatments, the required quantity of ZnO-NP powder was dispersed in distilled water and subjected to ultrasonication for 25–30 minutes at 20KHz using a probe sonicator to ensure uniform dispersion and minimize agglomeration. For bulk zinc treatments, the calculated amount of  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$  was dissolved in distilled water and the solution was also ultrasonicated for the same duration to maintain uniformity in spray preparation. After sonication, the volume was adjusted to obtain the desired zinc concentrations as per treatment details. A non-ionic surfactant (Tween-20 @ 0.05%) was added to both nano ZnO suspension and bulk  $\text{ZnSO}_4$  solution after sonication, followed by gentle mixing without further sonication. These fertilizers were applied as foliar spray at two crop growth stages *i.e.*, 25 days after sowing (pre-flowering) and 50 days after sowing (flowering) using a fine mist hand sprayer during early morning hours. All recommended agronomic practices were followed timely for successfully raising the crop. Recommended dose of fertilizers *i.e.*, 20 kg/ha of nitrogen and 40 kg/ha of phosphorus was applied as basal dose. At physiological maturity, plants were harvested and seeds from each replication were collected, cleaned, oven-dried at 65–70 °C until constant weight was achieved and finely ground. Observations were recorded on a replication-wise basis, and mean values were computed for statistical analysis. The experimental data were analyzed following the procedure appropriate for a Completely Randomized Design to test the significance of treatment effects and analysis of variance. Zinc (Zn), iron (Fe), manganese (Mn), copper (Cu), and potassium (K) contents of the cowpea seeds were determined after appropriate acid digestion using standard laboratory analytical procedures. Nitrogen (N) content was estimated by the Kjeldahl method, and crude protein content (%) was calculated by multiplying the nitrogen content by a conversion factor of 6.25. All nutritional parameters were estimated on a dry weight basis and expressed in appropriate units. The detailed procedure for the estimation of the above-mentioned nutrients is given below.

#### Nitrogen and Protein content (%)

Nitrogen content in seed samples of each treatment was calculated using Kjeldahl method. A known quantity (0.5 g) of Digestion of finely ground seed

sample was done with concentrated sulphuric acid in the presence of a digestion mixture until digest became clear and colorless. The digested sample was distilled with excess sodium hydroxide and the liberated ammonia was absorbed in boric acid solution. The absorbed ammonia was titrated against standard sulphuric acid. Protein content (%) was calculated by multiplying the nitrogen content by a conversion factor of 6.25.

#### Total Soluble Sugar Content ( $\text{mg g}^{-1}$ )

Approximately 100 mg finely ground seed powder was used per treatment per replication for estimation of total soluble sugar content. The seed powder was transferred into a centrifuge tube and extracted with 10 ml of 80 per cent ethanol. The mixture was centrifuged at 3000 rpm for 10 minutes, and the supernatant was collected. The extraction was repeated twice, and the supernatants were pooled. An aliquot of 1 ml extract was taken in a test tube, to which 4 ml of freshly prepared anthrone reagent was added. The contents were mixed thoroughly and heated in a boiling water bath for 10 minutes. After heating, the tubes were cooled rapidly to room temperature. The absorbance of the developed green colour was measured at 620 nm using a spectrophotometer against a reagent blank. A standard curve was prepared using known concentrations of glucose. Absorbance values of the standards were plotted against glucose concentration to obtain the standard curve, which was used for estimating total soluble sugar content in seed samples. Total soluble sugar content was expressed as milligrams of sugar per gram of seed dry weight ( $\text{mg g}^{-1}$ ).

#### Total Phenolic Content ( $\text{mg GAE g}^{-1} \text{DW}$ )

Approximately 100 mg finely ground seed powder was used per treatment per replication for estimation. The powdered sample was extracted with 10 ml of 80 per cent methanol by thorough mixing. The mixture was centrifuged at 3000 rpm for 10 minutes, and the supernatant was collected for analysis. An aliquot of 0.5 ml of the extract was taken in a test tube, to which 2.5 ml of ten-fold diluted Folin–Ciocalteu reagent was added. After allowing the mixture to stand for 5 minutes, 2.0 ml of 7.5 per cent sodium carbonate solution was added. The contents were mixed thoroughly and incubated at room temperature for 30 minutes for colour development. The absorbance of the resulting blue colour was measured at 765 nm using a spectrophotometer against a reagent blank.

A standard calibration curve was prepared using different concentrations of gallic acid. Absorbance values were plotted against Gallic acid concentrations, and the standard curve was used to calculate the total phenolic content of the samples. Total phenolic content was expressed as milligrams of gallic acid equivalents per gram of sample dry weight (mg GAE g<sup>-1</sup> DW).

#### Potassium content (%)

Potassium content was determined by flame photometric method after wet digestion. For this estimation, 0.5 g of ground seed sample was digested with di-acid mixture (nitric acid and perchloric acid in the ratio of 9:4) on a hot plate until the clear solution was obtained. After cooling, the digest was diluted with distilled water and the volume was made up to 50 ml. The solution was filtered through Whatman No. 42 filter paper. Potassium content in the filtrate was calculated using a flame photometer after calibration with standard potassium solutions, and results were expressed as percentage on dry weight basis.

#### Zinc, Iron, Copper and Manganese content (ppm)

The same di-acid digested extract used for potassium estimation was used for the estimation of zinc, iron, copper and manganese. The concentrations of these micronutrients were determined using an Atomic Absorption Spectrophotometer (AAS). Standard solutions of zinc, iron, copper and manganese were prepared for calibration of the instrument. The digested seed extracts were aspirated into the AAS and absorbance was observed at their respective wavelengths. The amount of each micronutrient was determined by comparing sample readings with the standard curve and expressed as mg kg<sup>-1</sup> (ppm) on dry weight basis.

#### Zn-Nutrient Harvest Index (NHI)

Zinc content of straw and seeds collected from each treatment across three replications was determined after di acid digestion using nitric acid and perchloric acid mixture using AAS and expressed in ppm as described above. The Zn-NHI was calculated using formula

$$\text{Zn-NHI} = \frac{\text{Zinc uptake in seed} \times 100}{\text{Total zinc uptake by entire plant (seed + straw)}}$$

#### Z-score transformation and Z-based Nutrient Quality Index

Measured nutrient concentration data were subjected to Z-score transformation to standardize

variables measured in different units prior to index construction. Z-scores were calculated using treatment-wise mean values of each nutrient across all treatments according to the following equation:

$$Z_{ij} = \frac{X_{ij} - \mu_j}{\sigma_j}$$

Where,  $X_{ij}$  denotes the observed mean value of the  $j^{\text{th}}$  nutrient under the  $i^{\text{th}}$  treatment,  $\mu_j$  represents the overall mean,  $\sigma_j$  represents the standard deviation of the  $j^{\text{th}}$  nutrient across treatments

The Z-based Nutrient Quality Index (Z-NQI) was calculated for each treatment as the arithmetic mean of the Z-scores of all nutrients included in the analysis:

$$\text{Z-NQI}_i = 1/n \sum_{j=1}^n Z_{ij}$$

Where  $n$  is the total number of nutrients considered.

Treatments were ranked based on Z-NQI values in descending order, with the highest Z-NQI assigned Rank 1.

## Results and Discussion

Zinc plays a crucial role in improving seed nutritional quality through its involvement in enzymatic activity, protein synthesis, and micronutrient metabolism. In the present investigation, different doses of bulk zinc and zinc oxide nanoparticles were evaluated to assess their influence on seed nutritional parameters. The treatments exhibited significant variation for protein, soluble sugars, phenolics, macronutrients, micronutrients, and zinc nutrient harvest index, indicating the effectiveness of zinc application in improving seed quality. The mean performance of the treatments assessed using one-way analysis of variance is given in Table 2 and discussed below in brief.

Protein content ranged from 23.74% to 27.81% among the treatments. The lowest protein content was recorded in the control (23.74%). Foliar application of zinc significantly enhanced protein content, with zinc oxide nanoparticles treatments performing better than bulk zinc. The maximum protein content (27.81%) was recorded in 600 ppm ZnO-NPs with two sprays, which was statistically at par with 500 ppm ZnO-NPs with two sprays and superior to control and bulk zinc treatments. Nitrogen also follows the same trend ranging from 3.80% in control to 4.45% with two sprays of 500 & 600 ppm ZnO-NPs. The increase in

Table 2 — Effect of foliar application of zinc oxide nanoparticles and bulk zinc on seed biochemical and nutritional parameters

Treatments	Total Protein (%)	Total soluble sugar (mg/g)	Total Phenolics (mg GAE/100g dry weight)	K (%)	N (%)	Zinc (ppm)	Zn- NHI (%)	Iron (ppm)	Copper (ppm)	Manganese (ppm)
T1: Control	23.74	34.10	769.12	1.89	3.80	42.80	58.28	62.15	10.92	22.44
T2: 400 ppm ZnO nanoparticles -1 spray	26.68	38.22	882.34	1.79	4.27	52.40	61.00	68.53	11.82	24.47
T3: 400 ppm Bulk Zn-1 spray	24.45	35.08	794.21	1.87	3.91	43.60	58.70	63.70	11.14	22.94
T4: 400 ppm ZnO nanoparticles -2 sprays	27.58	39.21	917.82	1.75	4.41	55.20	61.51	70.13	12.10	25.09
T5: 400 ppm Bulk Zn-2 sprays	25.20	36.00	825.31	1.80	4.03	46.50	60.11	65.05	11.39	23.74
T6: 500 ppm ZnO nanoparticles -1 spray	27.08	38.69	898.65	1.77	4.33	53.74	61.24	69.24	11.95	24.75
T7: 500 ppm Bulk Zn-1 spray	24.71	35.38	804.21	1.86	3.95	44.54	58.83	64.17	11.22	23.26
T8: 500 ppm ZnO nanoparticles -2 sprays	27.80	39.54	930.21	1.74	4.45	56.48	61.70	70.75	12.19	25.28
T9: 500 ppm Bulk Zn -2 sprays	25.40	36.28	835.21	1.83	4.06	47.41	60.23	65.50	11.45	23.95
T10: 600 ppm ZnO nanoparticles -1 spray	27.10	38.70	900.15	1.77	4.34	53.75	61.25	69.23	11.96	24.74
T11: 600 ppm Bulk Zn-1 spray	24.93	35.65	813.11	1.85	3.99	45.44	58.95	64.50	11.29	23.46
T12: 600 ppm ZnO nanoparticles- 2 sprays	27.81	39.57	931.10	1.74	4.45	56.52	61.72	70.78	12.20	25.30
T13: 600 ppm Bulk Zn-2 sprays	25.57	36.52	843.41	1.82	4.09	48.26	60.35	65.90	11.50	24.13
C. D. at 5%	0.16	0.22	8.06	0.01	0.03	0.81	0.11	0.48	0.06	0.20

seed protein and nitrogen content under ZnO-NP treatments is attributed to central role of zinc in nitrogen metabolism and protein synthesis. Zinc acts as a cofactor for enzymes involved in amino acid biosynthesis and gene expression, thereby enhancing nitrogen assimilation and protein accumulation<sup>9</sup>.

Total soluble sugar content varied from 34.10 mg g<sup>-1</sup> in control to 39.57 mg g<sup>-1</sup> at 600 ppm ZnO-NPs with two sprays, which was statistically superior to control and bulk zinc treatments but at par with 500 ppm ZnO- NPs with two sprays as confirmed by the CD value (0.22). Zinc oxide nanoparticle treatments consistently recorded higher soluble sugar content, while bulk zinc treatments showed moderate improvement. Improved soluble sugar content further indicate enhanced photosynthetic efficiency and assimilates partitioning, as zinc regulates carbonic anhydrase activity and chlorophyll synthesis<sup>10</sup>. Total phenolic content showed significant variation among treatments and ranged from 769 to 931.10 mg GAE 100 g<sup>-1</sup>. The

significantly highest phenolic content (931 mg GAE 100 g<sup>-1</sup>) was recorded in 500 and 600 ppm ZnO-NPs with two sprays, which were statistically superior to control and bulk zinc treatments. Higher phenolic content under ZnO-NP treatments suggests stimulation of secondary metabolism and antioxidant pathways, likely through zinc-mediated activation of phenylalanine ammonia-lyase<sup>11</sup>.

Potassium content of seed showed a consistent decreasing trend with zinc fertilization, particularly under zinc oxide nanoparticle treatments. The potassium content ranged from 1.89 % in control to 1.74 % under ZnO-NPs applied at 500 and 600 ppm with two sprays. The highest potassium content was recorded in the control (1.89%), while progressive application of zinc resulted in a gradual reduction in seed potassium concentration. Among the bulk zinc treatments, potassium content decreased from 1.87% at 400 ppm (one spray) to 1.82% at 600 ppm (two sprays). A more pronounced decline was observed under zinc oxide nanoparticle treatments,

where potassium content decreased from 1.79% at 400 ppm (one spray) to 1.74% at 500 and 600 ppm with two sprays. The reduction in potassium concentration under zinc fertilization may be attributed to a dilution effect, wherein enhanced seed growth, protein synthesis, and micronutrient accumulation resulted in a relative decrease in potassium concentration<sup>12</sup>. Zinc application, particularly in nanoparticle form, improves metabolic activity and seed biomass, leading to a dilution of potassium on a concentration basis. In addition, potassium being a highly mobile nutrient is preferentially retained in vegetative tissues for physiological processes, resulting in limited remobilization towards the seed under enhanced zinc nutrition<sup>13</sup>.

An analysis of micronutrient dynamics revealed significant variation among treatments, indicating a clear influence of zinc application on micronutrient accumulation illustrated in Fig. 1. Seed zinc, iron, copper and manganese concentration ranged from 42.80 to 56.52, 62.15 to 70.78 ppm, 10.92 to 12.20 ppm and 22.44 to 25.30 ppm respectively. The significantly highest zinc (56.52 ppm), iron (70.78 ppm), copper (12.20 ppm) and manganese (25.30 ppm) content was recorded in 600 ppm ZnO-NPs with two sprays, closely followed by 500 ppm ZnO-NPs with two sprays, treatments while the lowest value for each micronutrient was observed in the control. Zinc nutrient harvest index ranged from 58.28% to 61.72%. The maximum value (61.72%) was recorded under 600 ppm ZnO-NPs with two sprays, which was statistically comparable to 500 ppm ZnO-NPs with two sprays but superior to control and bulk zinc treatments. Similar trends were observed by<sup>14</sup>. This indicates improved zinc partitioning towards seed

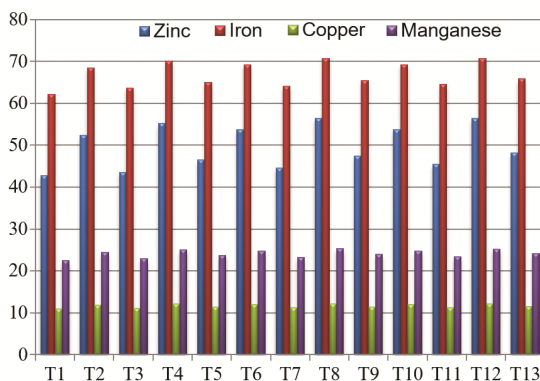


Fig. 1 — Micronutrient accumulation in cowpea seeds as influenced by zinc fertilization.

under nanoparticle application. Enhanced accumulation of zinc, iron, copper, and manganese, along with higher zinc nutrient harvest index under ZnO-NPs treatments, indicates improved micronutrient uptake and partitioning towards seeds, supporting the effectiveness of ZnO-NPs for agronomic biofortification<sup>15</sup>.

The overall mean performance indicated that zinc oxide nanoparticles were significantly superior to bulk zinc in improving seed nutritional quality. Among the treatments, 500 ppm ZnO-NPs with two sprays proved to be the most effective, recording significantly higher values for protein, soluble sugars, phenolics, zinc content, and zinc nutrient harvest index.

#### Z-Score Analysis of Individual Nutritional Parameters

While mean values provide absolute differences among treatments, Z-score transformation enabled standardized comparison across nutrients with different units and magnitudes. Z-score analysis clearly amplified treatment effects and revealed distinct nutrient enrichment patterns under ZnO-NPs treatments<sup>16</sup>. Z-score analysis of biochemical and nutritional parameters of cowpea seed is mentioned in Table-3.

Z-nitrogen and protein values ranged from  $\approx -1.61$  in control to  $\approx +1.30$  under ZnO-NPs treatments. Bulk Zn treatments showed mostly negative values ( $\approx -1.11$  to  $-0.31$ ), indicating inefficient nitrogen assimilation. The highest positive Z-N values were observed under 600 ppm (1.30) and 500 ppm (1.29) ZnO-NPs with two sprays, confirming zinc-induced activation of nitrogen metabolism and improved nitrate reductase activity. ZnO-NPs with two sprays, confirming enhanced nitrogen metabolism and amino-acid synthesis due to improved zinc bioavailability<sup>17</sup>.

Z-total phenolic content values ranged from  $\approx -1.59$  in control to  $\approx +1.34$  under ZnO-NPs treatments, indicating a pronounced influence of zinc form and spray frequency on phenolic metabolism. Bulk Zn treatments exhibited predominantly negative Z-TPC values ( $\approx -1.14$  to  $-0.25$ ), reflecting limited stimulation of secondary metabolite synthesis under conventional zinc sources. The highest positive Z-TPC values were recorded under 600 ppm (1.34) and 500 ppm (1.32) ZnO-NPs with two sprays, indicating maximum accumulation of phenolic compounds. This enhancement may be attributed to zinc-induced activation of key enzymes of the phenylpropanoid pathway, improved antioxidant defense mechanisms, and enhanced metabolic efficiency under nanoparticle

Table 3 — Z-score analysis of biochemical and nutritional parameters of cowpea seed under various foliar zinc application treatments

Treatments	Z-Protein	Z-TSS	Z-Phenolics	Z-N	Z-Zn	Z-Zn (NHI)	Z-Fe	Z-Cu	Z-Mn	Z-K	NQI	Rank
T1: Control	-1.61	-1.60	-1.59	-1.61	-1.36	-1.62	-1.59	-1.63	-1.83	1.55	-12.94	13
T2: 400 ppm ZnO nanoparticles -1 spray	0.49	0.56	0.45	0.49	0.52	0.56	0.54	0.45	0.38	-0.37	4.10	6
T3: 400 ppm Bulk Zn-1 spray	-1.11	-1.09	-1.14	-1.11	-1.20	-1.29	-1.07	-1.12	-1.28	1.16	-9.27	12
T4: 400 ppm ZnO nanoparticles -2 sprays	1.12	1.08	1.09	1.12	1.07	0.97	1.08	1.09	1.05	-1.13	8.58	3
T5: 400 ppm Bulk Zn-2 sprays	-0.58	-0.60	-0.57	-0.58	-0.63	-0.15	-0.61	-0.54	-0.41	0.59	-4.11	9
T6: 500 ppm ZnO nanoparticles -1 spray	0.76	0.81	0.74	0.76	0.78	0.76	0.78	0.75	0.68	-0.75	6.11	5
T7: 500 ppm Bulk Zn-1 spray	-0.93	-0.93	-0.95	-0.93	-1.02	-1.18	-0.91	-0.93	-0.93	0.97	-7.77	11
T8: 500 ppm ZnO nanoparticles -2 sprays	1.29	1.26	1.32	1.29	1.32	1.13	1.29	1.30	1.26	-1.33	10.25	2
T9: 500 ppm Bulk Zn -2 sprays	-0.44	-0.46	-0.39	-0.44	-0.45	-0.05	-0.46	-0.40	-0.18	0.40	-2.90	8
T10: 600 ppm ZnO nanoparticles -1 spray	0.81	0.81	0.78	0.81	0.78	0.76	0.78	0.77	0.67	-0.75	6.23	4
T11: 600 ppm Bulk Zn-1 spray	-0.75	-0.79	-0.79	-0.75	-0.84	-1.08	-0.99	-0.77	-0.71	0.78	-6.56	10
T12: 600 ppm ZnO nanoparticles- 2 sprays	1.30	1.27	1.34	1.30	1.33	1.14	1.30	1.32	1.28	-1.33	10.28	1
T13: 600 ppm Bulk Zn-2 sprays	-0.31	-0.33	-0.25	-0.31	-0.29	0.04	-0.80	-0.29	-0.011	0.20	-2.52	7

zinc applications. Z-TSS values ranged from  $\approx -1.60$  in control to  $\approx +1.27$  under ZnO-NPs treatments, indicating strong enhancement of carbohydrate metabolism. Bulk Zn treatments recorded negative Z-TSS values ( $\approx -1.09$  to  $-0.33$ ), reflecting reduced photosynthate production. The highest Z-TSS values were observed under 600 ppm (1.27) and 500 ppm (1.26) ZnO-NPs with two sprays, confirming improved photosynthetic efficiency and assimilate translocation<sup>18</sup>.

Z-zinc values showed wide variation from  $\approx -1.36$  in control to  $\approx +1.33$  under ZnO-NPs treatments, clearly differentiating zinc sources. Bulk Zn treatments showed negative values ( $\approx -1.20$  to  $-0.29$ ). The maximum Z-Zn values were recorded under 600 ppm (1.33) and 500 ppm (1.32) ZnO-NPs with two sprays, demonstrating superior zinc uptake and internal utilization from nanoparticle formulations. Z-iron values ranged from  $\approx -1.59$  in control to  $\approx +1.30$  under ZnO-NPs treatments. Bulk Zn treatments exhibited mostly negative Z-Fe values ( $\approx -1.07$  to  $-0.80$ ), indicating sub-optimal iron uptake. The maximum Z-iron values were observed under 600 ppm (1.30) and 500 ppm (1.29) ZnO-NPs with two sprays, suggesting improved iron absorption and translocation facilitated by zinc-mediated enhancement of membrane permeability and nutrient synergism. Z-copper values ranged from  $\approx -1.63$  in control to  $\approx +1.32$  under ZnO-NPs treatments, indicating improved copper nutrition with nanoparticles zinc application. Bulk Zn treatments recorded negative Z-Cu values ( $\approx -1.12$  to  $-0.29$ ), suggesting poor copper availability. The highest Z-Cu values were observed under 600 ppm (1.32) and

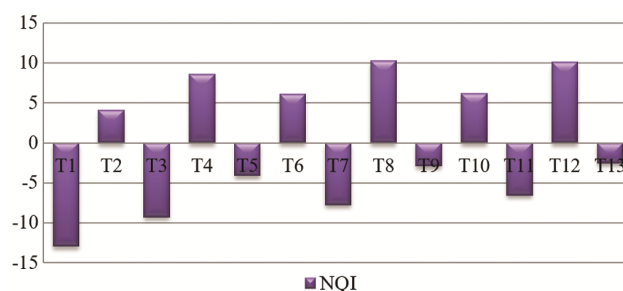


Fig. 2 — Nutrient Quality Index (NQI) across treatments.

500 ppm (1.30) ZnO-NPs with two sprays, indicating enhanced micronutrient uptake and improved enzymatic activity related to redox processes. Z-manganese values varied from  $\approx -1.83$  in control to  $\approx +1.28$  under ZnO-NPs treatments. Bulk Zn treatments exhibited negative Z-Mn values ( $\approx -1.28$  to  $0.01$ ), reflecting sub-optimal manganese absorption. The maximum Z-Mn values were recorded under 600 ppm (1.28) and 500 ppm (1.26) ZnO-NPs with two sprays, suggesting improved photosynthetic activity and enzyme activation due to better manganese availability<sup>19</sup>.

The Z-score approach was used to standardize individual nutrient responses and integrate them into a composite Nutrient Quality Index (NQI), enabling comparison among diverse zinc treatments irrespective of scale differences as depicted in Fig. 2. Positive NQI values indicate superior nutrient balance and improved nutritional quality, whereas negative values reflect nutrient imbalance or sub-optimal performance<sup>20</sup>. In the present study, a wide variation in NQI values was observed across treatments, ranging from  $-12.94$  in control (T1) to  $+10.28$  in 600

and 10.25 in 500 ppm ZnO nanoparticles with two sprays (T12 & T8). The control treatment recorded the most negative NQI, indicating poor nutrient balance in the absence of zinc supplementation. This highlights the essential role of zinc in maintaining nutrient homeostasis<sup>21</sup>. Among zinc treatments, ZnO nanoparticle (ZnO-NPs) applications consistently produced higher Z-score-based NQI values compared to bulk Zn, irrespective of concentration and spray frequency. At 400 ppm, ZnO-NPs applied as two sprays (T4) showed a positive NQI (+8.58), whereas the corresponding bulk Zn treatment (T5) remained negative (-4.11), suggesting greater nutrient use efficiency of nanoparticulate zinc. At 500 and 600 ppm concentration, ZnO-NPs treatments showed a pronounced improvement in NQI, with recording the highest NQI. In contrast, bulk Zn at the same concentration, even with two sprays (T9), failed to achieve positive NQI values (-2.90). This trend indicates enhanced foliar absorption, translocation, and physiological utilization of zinc when applied in nanoparticle form. A similar pattern was observed at 600 ppm, where ZnO-NPs treatments (T10 and T12) recorded positive NQI values (+6.23 and +10.28, respectively), while bulk Zn treatments (T11 and T13) continued to exhibit negative NQI values. The superiority of two sprays over one spray was evident across ZnO-NPs treatments, indicating that repeated foliar application improves zinc availability during critical growth stages. Overall, the Z-score-based NQI clearly differentiated treatments based on their nutrient performance. ZnO nanoparticles, particularly at 500 and 600 ppm with two foliar sprays,

significantly enhanced nutrient quality compared to bulk zinc sources. The negative NQI values observed in bulk Zn treatments suggest limited solubility and lower bioavailability, leading to inefficient nutrient assimilation.

To facilitate integrated interpretation of multinutrient responses across zinc treatments, Z-score-standardized nutrient values were visualized using heat map analysis as depicted in Fig 3. Heat map representation enabled clear differentiation of nutrient enrichment and deficiency patterns among treatments, particularly highlighting the superior performance of zinc oxide nanoparticle applications<sup>22</sup>. Treatments receiving ZnO-NPs, especially at 500 and 600 ppm with two foliar sprays, exhibited consistently positive Z-scores across protein, nitrogen, zinc, zinc nutrient harvest index, iron, copper and manganese, whereas control and bulk zinc treatments showed predominantly negative or near-zero Z-scores. Such visualization of standardized data is widely used in biological and agronomic studies to interpret complex multivariate datasets and to identify synergistic and antagonistic nutrient interaction patterns that may not be evident from individual mean comparisons alone<sup>23</sup>. The observed Z-score patterns further corroborate the Z-based Nutrient Quality Index (NQI) results, confirming the enhanced nutrient balance and biofortification efficiency under nanoparticle-mediated zinc application.

**Simple Regression Analysis between Nutritional Traits and Nutrient Quality Index (NQI)**

Simple linear regression analysis was employed to evaluate the association between individual nutritional

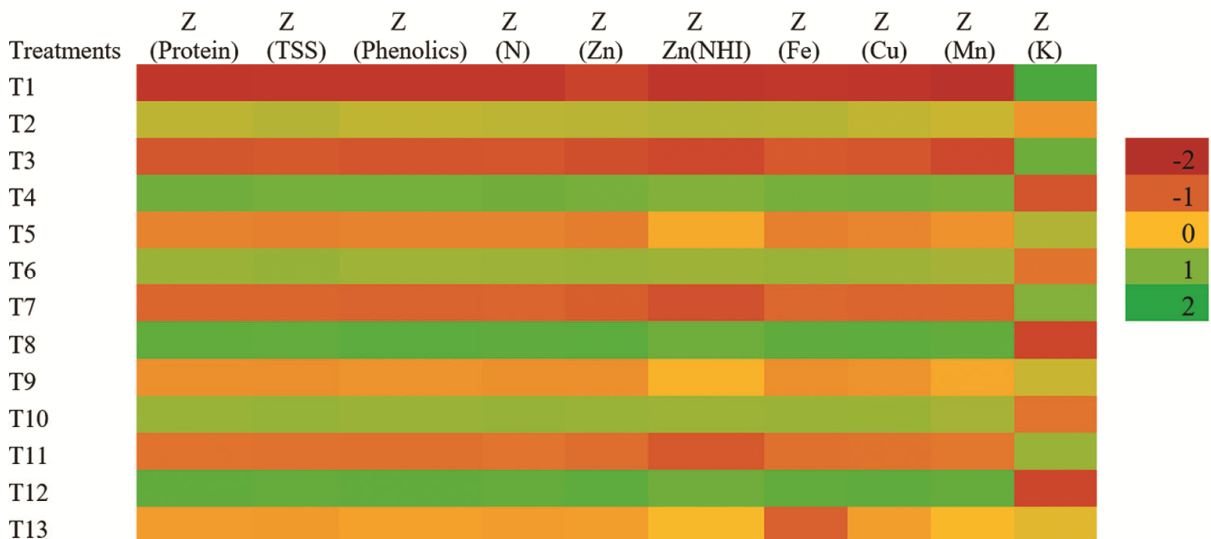


Fig. 3 — Z-score-based heatmap illustrating nutrient enrichment and depletion patterns across treatments.

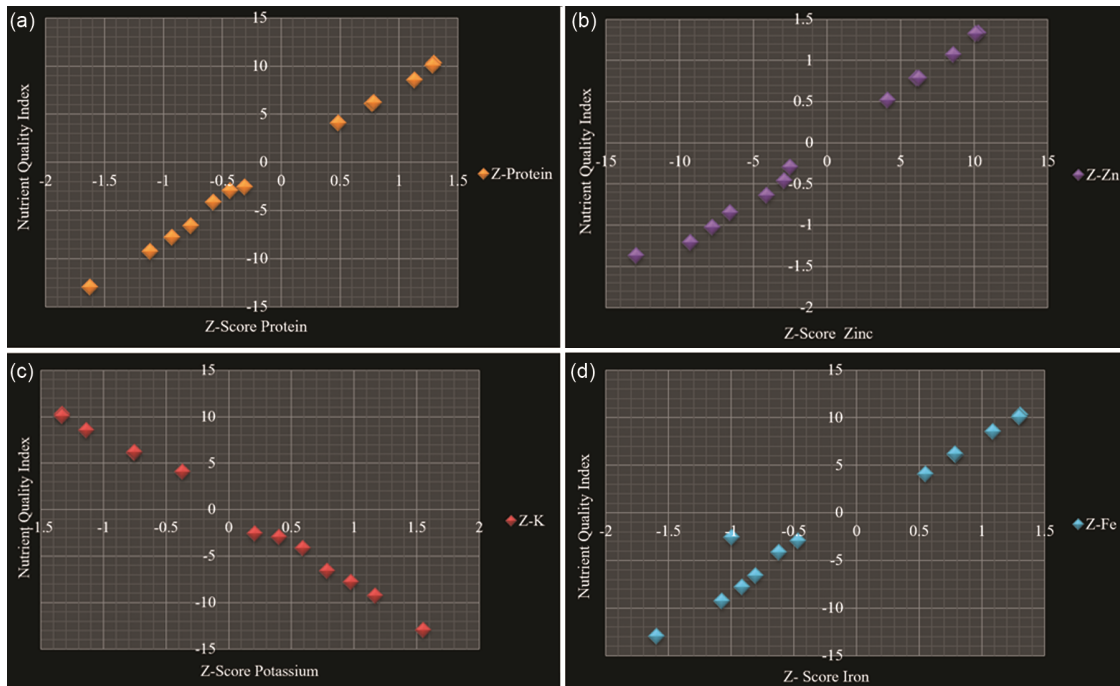


Fig. 4(a) — Linear association between standardized protein content and Nutrient Quality Index; (b) Linear association between standardized zinc content and Nutrient Quality Index; (c) Linear association between standardized potassium content and Nutrient Quality Index; (d) Linear association between standardized iron content and Nutrient Quality Index.

traits and Nutrient Quality Index (NQi) across zinc biofortification treatments presented in Fig 4 (a) – (d). The purpose of this analysis was to assess the direction and relative contribution of individual nutritional parameters to overall nutritional quality, rather than to establish predictive or causal relationships. This approach is justified because NQi represents an integrated index derived from standardized nutritional traits.

Simple regression analysis revealed moderate to very strong associations between individual nutritional traits and the Z-score-based Nutrient Quality Index (NQi), with coefficients of determination ( $R^2$ ) ranging from 0.56 to 0.90 as shown in Fig. 5. This range is consistent with values reported in agronomic and nutritional biofortification studies, where multiple quality attributes respond coherently to micronutrient interventions and are integrated through composite indices<sup>24</sup>.

Protein and nitrogen exhibited the strong associations with NQi ( $R^2 = 0.86$ ), indicating that improvements in overall nutritional quality were closely linked with enhanced nitrogen assimilation and protein biosynthesis. Similar strong relationships between protein content and composite nutritional indices have been reported in zinc-biofortified cereals and legumes, where zinc fertilization improves

nitrogen metabolism through enhanced enzyme activation and ribosomal function, leading to higher protein accumulation<sup>25</sup>. Protein is therefore widely recognized as a central integrative component of nutritional quality in biofortification studies.

Zinc concentration and zinc nutrient harvest index (Zn-NHI) showed strongest associations with NQi ( $R^2 = 0.90$ ), highlighting zinc as a primary driver of nutritional enhancement. These findings are in agreement with previous reports demonstrating that both zinc accumulation and zinc partitioning efficiency are critical determinants of seed nutritional density<sup>26</sup>. Reported  $R^2$  values in the range of 0.80–0.90 are commonly considered optimal indicators of effective zinc biofortification, reflecting strong biological relevance without statistical redundancy<sup>27</sup>. Iron exhibited a moderate to strong association with NQi ( $R^2 = 0.78$ ), suggesting a synergistic response to zinc biofortification rather than a direct proportional relationship<sup>28</sup>. This magnitude of association is consistent with literature describing zinc-mediated improvements in iron uptake and utilization through enhanced root growth, transporter activity, and membrane stability. Iron responses are often secondary to zinc application, and moderate  $R^2$  values reflect complementary micronutrient interactions rather than identical

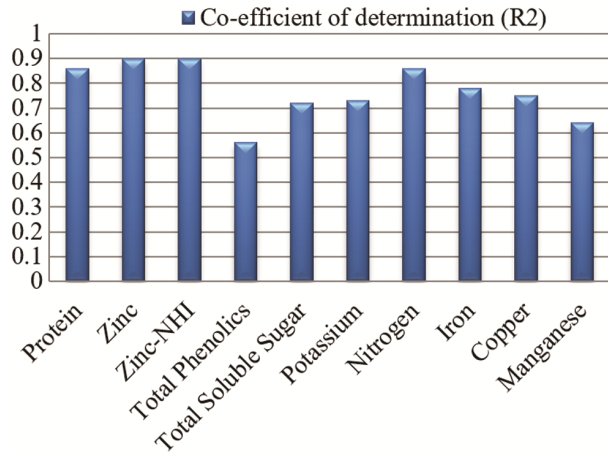


Fig. 5 — Relative contribution of nutritional traits to Nutrient Quality Index based on R<sup>2</sup> values.

accumulation patterns<sup>29</sup>. Copper and manganese showed moderate to strong positive associations (R<sup>2</sup> = 0.75 and 0.64, respectively) with NQI, indicating supportive roles in determining overall nutritional quality. Similar R<sup>2</sup> ranges have been reported for secondary micronutrients in biofortification studies, where these elements contribute through enzymatic activation, redox balance, and photosynthetic regulation rather than bulk accumulation<sup>30</sup>.

Total soluble sugars and Total phenolic content exhibited moderate positive associations with NQI (R<sup>2</sup> = 0.72 and 0.56), indicating that improvements in carbohydrate metabolism and secondary metabolite synthesis contributed to enhanced nutritional quality. Comparable R<sup>2</sup> values have been reported in studies linking micronutrient nutrition with photosynthetic efficiency, assimilate translocation, and antioxidant metabolism. As functional and secondary quality traits, sugars and phenolics are expected to show moderate rather than dominant associations with composite nutritional indices<sup>31</sup>.

In contrast, potassium showed a medium negative association with NQI indicating a consistent dilution or antagonistic effect under zinc biofortification. Negative associations between potassium and micronutrient-based quality indices have been widely documented, particularly under conditions of enhanced reproductive sink strength and increased nutrient density, where mobile macronutrients are redistributed or diluted on a concentration basis<sup>32</sup>. The observed R<sup>2</sup> value reflects a biologically meaningful inverse relationship rather than random variation.

Overall, the observed R<sup>2</sup> range (0.56–0.90) falls within the optimal and literature-supported range for

descriptive regression involving composite nutritional indices. These values indicate strong biological coherence among nutritional traits under zinc biofortification, while avoiding the unrealistically high coefficients often associated with circular or over-fitted models. The use of R<sup>2</sup> values, rather than regression slopes, aligns with recommended practices for composite indices allow robust, biologically meaningful interpretation of nutrient interactions<sup>33</sup>.

### Conclusion

The present investigation conclusively establishes zinc oxide nanoparticles as a superior zinc source over conventional bulk zinc for enhancing seed nutritional quality and micronutrient biofortification in cowpea. Foliar application of ZnO-NPs significantly improved protein, nitrogen, soluble sugars, phenolics, zinc and associated micronutrients, while also enhancing zinc partitioning towards seeds, as reflected by higher Zn-NHI values. Among all treatments, 500 ppm ZnO-NPs applied twice proved optimal, achieving the best balance between nutrient accumulation, metabolic efficiency, and micronutrient allocation. The integration of Z-score transformation and Z-based Nutrient Quality Index provided a robust, standardized framework to evaluate multinutrient responses and clearly discriminate treatment performance. Positive NQI values under ZnO-NP treatments, contrasted with negative values under bulk zinc, highlight the superior bioavailability and physiological efficiency of nanoparticle zinc. Regression analysis further confirmed that improvements in overall nutritional quality were primarily driven by enhanced protein synthesis, zinc accumulation, and zinc harvest efficiency. Overall, the findings support the potential of ZnO-NPs as an effective, scalable, and sustainable agronomic biofortification strategy for cowpea. Adoption of nano-enabled zinc fertilization, particularly at optimized concentrations and spray schedules, can substantially contribute to improving crop nutritional density and addressing micronutrient malnutrition in zinc-deficient regions. Future studies should validate ZnO nanoparticles across diverse agroclimatic conditions and cropping seasons, while assessing their long-term environmental safety. Further research on the physiological and molecular mechanisms of nutrient uptake, interactions with other micronutrients, field-scale performance, economic feasibility, and

bioavailability are needed to establish nano-enabled biofortification as a sustainable strategy for improving nutritional security.

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