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Comparative analysis of chemical fertilizers, nanoparticles and biofertilizers on the growth and yield responses of mustard cultivars

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ABSTRACT

Background: The present research is based on the hypothesis that foliar spray of zinc oxide nanoparticles (ZnO NPs) or application of biofertilizers such as vesicular arbuscular mycorrhiza (VAM) or arbuscular mycorrhizal fungi (AMF) and phosphate solubilizing bacteria (PSBs) are more efficient than generally used chemical fertilizers supplementation such as nitrogen (N), phosphorus (P) and supplementation of zinc (Zn).

Methods: In this study, six mustard varieties, *Brassica juncea* var. Alankar, Pusa Jai Kisan, Varuna, Sakha, Rohini, and Pusa Bold were tested for their comparative growth responses. Out of the six tested varieties, only two screened varieties (Alankar and Rohini) were further tested for their comparative growth responses among the foliar spray of zinc oxide nanoparticles (ZnO NPs), soil-applied chemical fertilizers to supplement nitrogen (N) phosphorus (P) and zinc (Zn) as zinc sulfate and soil-applied biofertilizers as phosphate solubilizing bacteria (PSBs) and vesicular arbuscular mycorrhiza (VAM) or arbuscular mycorrhizal fungi (AMF).

Results: The results revealed that out of these three treatments, ZnO NPs significantly ($p \leq 0.05$) increased the growth morphology of the two mustard varieties, followed by VAM/AMF and PSBs, which were followed by chemical supplementation of N, P, and Zn. The effects were more pronounced in Alankar than in the Rohini variety of mustard.

Conclusions: From the present study, it is concluded that foliar spray of ZnO NPs and the application of biofertilizers can be a potent alternative to costly chemical fertilizers in the cultivation of mustard crops.

KEYWORDS

Zinc-oxide Nanoparticles; Phosphate Solubilizing Bacteria; Vesicular Arbuscular Mycorrhiza; Arbuscular Mycorrhizal Fungi; Chlorophyll; Proline; Mustard-Varieties

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Introduction

Mustard (*Brassica juncea* L.) is one of the important oilseed crops grown in the Rabi (winter) season and contributes to 25% of the oilseed economy of India [1,2]. The use of chemical fertilizers in arable soil is a routine practice in modern agriculture to supplement the depleting nutrients from natural soil fertility, among them nitrogen, phosphorus, potassium (NPK) and zinc (Zn) are more common [3,4]. Mustard varieties also respond to chemical fertilizers, particularly N and P. The recommended dose of chemical fertilizers is crop-specific; excess or less application may lead to suboptimal production of lethal effects. Increased population growth and demand for food supply required higher use of chemical fertilizers, a costly input [5]. Additionally, excess use of these fertilizers polluted all three spheres of the environment [6]. Potential substitutes, biofertilizers, including arbuscular mycorrhizal fungi (AMF) and phosphate-solubilizing bacteria (PSBs), are cost-effective, pollution-free, renewable, and safe for crops [7]. Arbuscular mycorrhizae supplement nitrate and phosphate ions along with other metal ions within the rhizosphere [8]. Phosphate solubilizing bacteria unlock the phosphate from the complex soil composites and solubilize them with the help of phytase enzyme, while AM fungi besides facilitating critical minerals modify host root architecture. These biofertilizers also secrete phytohormones in the host rhizosphere and protect them from soil borne pathogens [7,8]. Zinc plays a functional role in many physiological processes, in biochemical reactions, such as metalloenzymes [3,9], in the biosynthesis of proteins and chlorophyll, and in immune responses in animal systems [10]. Several metalloenzymes and biochemical reactions require Zn as a cofactor in cell metabolism [11]. Among the critical elements, nitrogen is an important part of the functional and structural part, i.e., proteins, secondary metabolites, coenzymes, and other molecules; phosphorus has a major role in nucleotide biosynthesis and energy transactions and signaling, while potassium regulates cell osmolarity and ion exchange [12]. Zinc deficiency in crops is common and often represented as zinc hunger [13].

In part, Zn hunger is prevalent due to the plant's inefficacy of absorbing and translocating it [14] or soil deficiency [13]; thus, Zn fertilization improves the production and quality of produce in several crop plants. Therefore, to mitigate issues such as the limited availability of soil nutrients, high rates of loss of soil-applied fertilizers, and constraints on nutrient delivery to plant organs due to environmental conditions

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during critical growth stages. Foliar fertilization for sustainable crop management has recently been well-addressed [15]. Fertilization through foliar spray has been proven to alleviate micronutrient deficiencies, reduce toxicity, and avoid fertilizer-related pollution [16-19]. Zn-induced phytotoxicity can directly reduce photosynthesis [20] or can create nutritional imbalance by interacting with other nutrients [21].

To unleash the full potential of plant performance, nanoparticle fertilizer represents a new and effective technique of nutrient delivery. This is crucial for creating more sustainable crop systems globally [22,23]. Nanoparticles are defined as particles with a size of less than 100 nm in at least one dimension [24]. Improvements in seed germination, seedling growth, biomass, total nitrogen content, protein and sugar contents, photosynthetic efficiency, and nutrient uptake are all documented as positive impacts of nanoparticles (NPs) on plant growth in crops such as cucumber, mung, spinach, wheat, and tomato [25-29]. Research shows that NPs can enter plant tissues and then move inside a plant's body systemically [30-32]. Among the different NPs used, ZnO NPs are currently the fourth most widely used in the world [33]. Due to their unique characteristics compared to conventional Zn fertilizers, ZnO NPs can also serve as cutting-edge Zn fertilizers. Uncertainty exists regarding the process through which ZnO NPs enter plants. Studies have demonstrated that foliar ZnO NP and $ZnSO_4$ spraying on wheat increased the Zn content in grains while leaving no traces of ZnO NP in them [29]. With slower delivery of micronutrients and a reduced risk of soil pollution and other environmental hazards compared to applying chemical fertilizers directly to the soil, nanoscale fertilization may be able to prevent the symptoms of phytotoxicity in plants [34]. In addition, nanoscale fertilizer application requires a lesser amount of fertilizer than conventional ones used through soil [35]. Even in stressed regimes, the use of nanofertilizers has been proven to have positive impacts on plant growth compared to normal conditions [32,35-40]. However, whether it is a nano application, these effects depend on concentration.

The present research work is based on the hypothesis that foliar spraying of ZnO NPs or application of biofertilizers such as VAM or AMF and PSBs are more efficient and promote plant growth better than generally used chemical fertilizer supplementation like nitrogen (N), phosphorus (P) and supplementation with zinc (Zn).

Therefore, the present study aimed to compare the impacts of various soil-applied chemical fertilizers, such as N, P, Zn, foliar spray of ZnO NPs, and soil-applied bio-fertilizers such as phosphate solubilizing bacteria (PSBs) and arbuscular mycorrhizal fungi (AMF), on the growth and biochemical responses of mustard cultivars.

Materials and Methods

Experimental site and design

The present experiment was performed in the Botany Department of Tilakdhari College, Jaunpur, state Uttar Pradesh (25° 73' N latitude, 82°68' E longitude at an elevation of 96 m above mean sea level). The 25 × 25 cm earthen pots were filled with 3 kg of field soil with the properties given in Table 1. The recommended dose of fertilizers was mixed with the soil present in the pot. The experiment was conducted under ambient environmental conditions in September-February 2020.

Pots were placed in a randomized completely block design (RCBD) where the experiment consisted of two factors and five replicates (2×7×5). first factor is two varieties of *Brassica juncea* (L.). The second factor included seven levels of fertilizer treatments (control, ZnO NPs, Zn, N, P, PSB, and AMF) and five replicates for each treatment randomly distributed in block (RBD). The total experimental units were 70(2×7×5=70).

Table 1. Chemical characteristics of the soil before sowing.

Texture	Sandy loam
pH	7.8
CEC (meq/l)	3.56
EC (dSm^{-1})	1
Organic carbon (%)	0.32
Available N (kg/ha)	106
Available P (kg/ha)	15
Available K (kg/ha)	230
Available Zn (kg/ha)	0.34

Materials and experimental treatment plan

The authentic seeds of *Brassica juncea* (L.) Czern and Coss cv. Alankar and Rohini were selected based on previous experiments and were procured from the National Seed Corporation Ltd., New Delhi, India. The cultural strains of biofertilizers (*Glomus intraradices*) inoculum and PSB *Pseudomonas aeruginosa*) were procured from the Agriculture Department Seed Distribution Unit, District Agriculture Office, Quarsi Road, Aligarh. The nanoparticles (ZnO-NPs) were purchased from Sigma-Aldrich Chemicals Pvt. Ltd. India. 100 mM stock solution of ZnO-NPs was prepared by dissolving its required amount in 10 ml DDW in a 100 ml volumetric flask, and making up total volume 100 ml by adding DDW. The working concentrations of NPs were prepared by diluting this stock solution of ZnO-NPs as per requirement.

Healthy, uniform-sized seeds were surface sterilized with a 0.01% solution of mercuric chloride for 5 min to disinfect from surface pathogens and then washed repeatedly with double distilled water (DDW). To check the percent germination of seeds, a germination test was also conducted. Seeds of two mustard varieties, Alankar and Rohini, were sown in pot soils. The soil analysis was conducted before the experiment presented in Table 1. Eight seeds per pot were sown and then thinned to three plants per pot one week after germination, selecting robust growing similar plants.

Among the six treatments (excluding control) of plants, three sets were maintained for the two mustard varieties. Five pots for each treatment were maintained as replicates (n=5). Mustard plants were irrigated with tap water as needed (Figure 1).

1. The first set of plants was foliar sprayed with ZnO NPs (4 millimoles aqueous solution).
2. For the next two different sets, AM fungus and PSB were applied. Fifty grams of Rhode grass cultured AM fungus; *Glomus intraradices* inoculum, was added to the soil around the seed to provide 500 IP (infective propagules) per pot. As a PSB, a suspension culture of *Pseudomonas aeruginosa* was used for the treatment of seeds. One milliliter of nutrient broth (Mannitol 10g, Yeast extract 1.0g, K_2HPO_4 0.5g,

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$MgSO_4.7H_2O$ 0.2g, NaCl 0.1g per liter of DDW) suspension contained approximately 1.5×10^9 cfu per ml of media. Seeds were coated with this suspension culture and dried in a cool shady place before sowing.

3. For three different sets, N, P, and Zn were amended in the pot soil as per recommended doses of 120, 60, and 25 kg/ha taking urea, single superphosphate, and $ZnSO_4$ as fertilizers. The fertilizer requirement per kg pot soil was calculated* as 72, 104, and 19 mg, respectively.

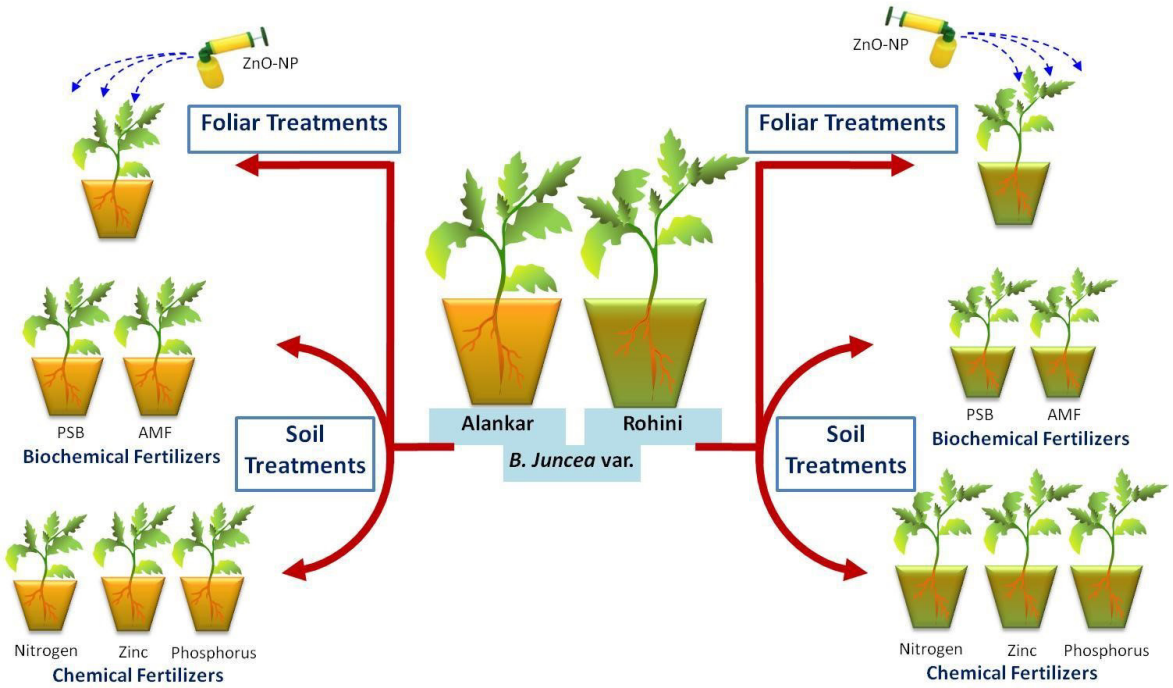

Figure 1. Treatment plan of the present work on two mustard varieties.

*Taking field soil per hectare (~36,00,000 kg), bulk density of soil (1.2 t/m³), and layer depth of 0.3 m, the following formula was used.

Nutrient requirement = 100/Nutrient content of fertilizer (%) × Recommended dose

Methodology

At 60 days after sowing (DAS), the plants were sampled to study the following growth features.

Growth analysis

The root and shoot lengths of the two varieties were measured using a meter scale. The ratio of the shoot by root length was calculated by dividing the lengths of the two. The fresh and dry mass of roots and shoots was measured with an electronic balance. To analyze the dry mass, the uprooted plants (roots and shoots) were placed in an oven at 80°C for 72 h and wrapped in butter paper. The dried plants were then weighed to record plant dry mass. The leaf area of randomly selected leaves from each variety was determined by the graph paper method of Pandey and Singh [41].

Total chlorophyll and proline content in leaves

The leaf's total chlorophyll content was estimated in finely cut fresh leaves following the method of Mackinney [42]. The leaf proline content in fresh tissue was determined by following the method of Bates et al. [43].

Activity of Carbonic anhydrase (CA) and Nitrate reductase (NR) enzyme

Carbonic anhydrase activity (CA, E.C. 4.2.1.1) and nitrate reductase activity (NR, E.C. 1.6.6.1) were determined by following Dwivedi and Randhawa [13] and Jaworski [44] in fresh leaf samples.

Statistical analysis

The experiment was conducted according to a simple randomized block design (SRBD). Each treatment was replicated five times (n=5), and three plants per pot were maintained where each pot was considered a replicate. Treatment means were compared by analysis of variance using R ver. 3.1.0 for Windows. The least significant difference (LSD) between treatment means was calculated at a 5% probability level ($p< 0.05$).

Results

Growth parameters

Most of the (bio)fertilizer treatments (ZnO NPs, Zn, N, P, PSBs or AMF) promoted the growth (length, fresh mass, dry mass of root and shoot and leaf area) parameters in both varieties in a treatment-dependent manner at 60 DAS (Figure 2). However, the maximum stimulation of most of the growth parameters is achieved by ZnO NPs followed by either PSB or AMF. However, the Alankar variety outperformed the treatment here. The root dry mass of Alankar for ZnO NPs, PSB, and AMF was 88%, 62%, and 86%, respectively, while for shoot dry mass it was 83%, 72%, and 80%, compared to control plants. For the same treatments leaf area improvement of Alankar was 35%, 28%, and 34%, and for Rohini, it was 33%, 23%, and 28%, respectively. The ratio of the shoot by root length showed different responses for the treatments.

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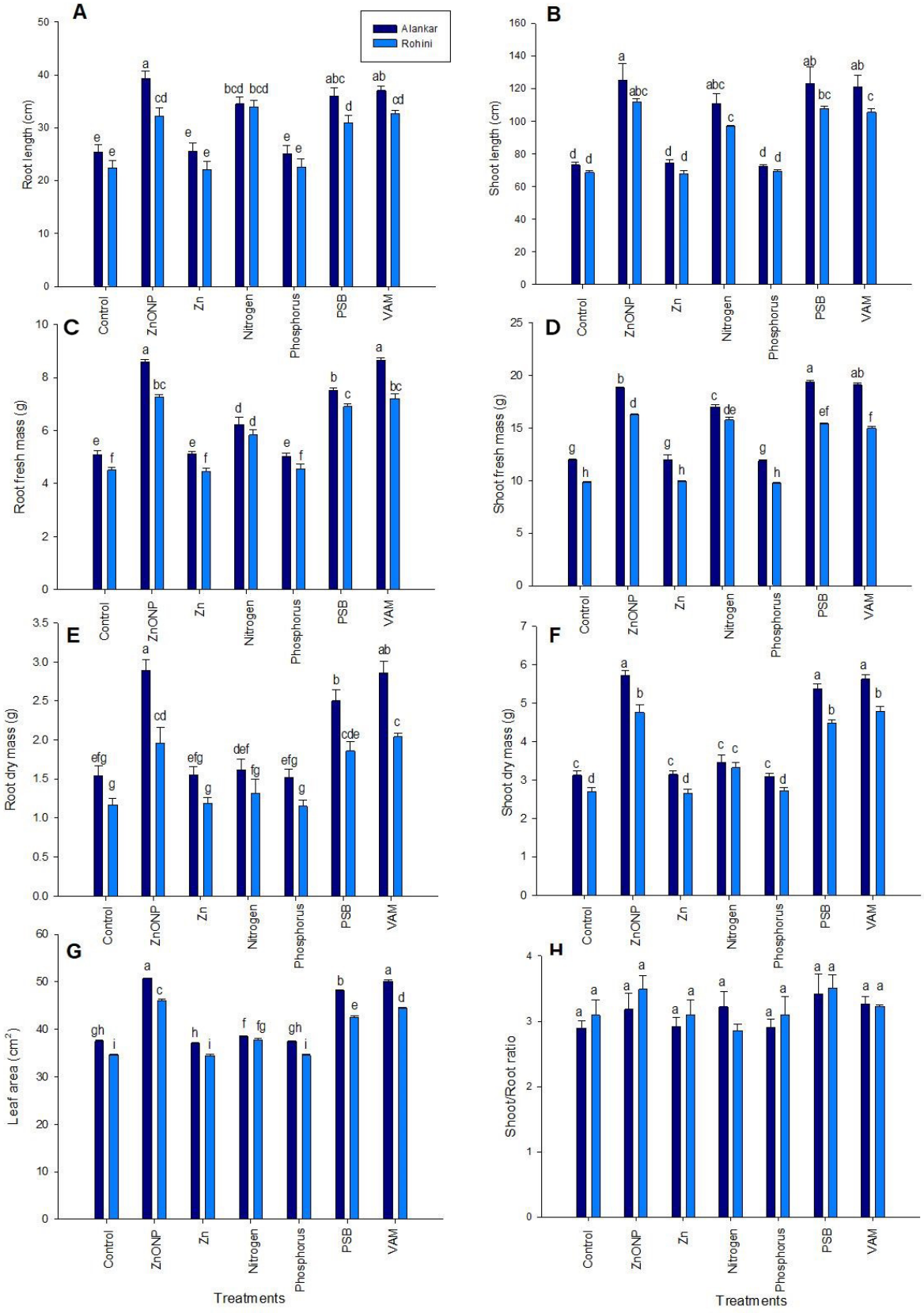

Figure 2. Effect of foliar and soil mediated chemical and biofertilizers (Zn, Nitrogen, PSB, and AM) on two different mustard varieties *Brassica juncea* var. Alankar and Rohini on (A, B) lengths (cm), (C, D) fresh masses (g), (E, F) dry masses of roots and shoots, (G) leaf area, and (H) shoot/root dry mass ratio at 60 days after sowing (DAS). Data are presented as the treatment mean ± standard error (n = 5). The different letters above the bars show that data are significantly different at p ≤0.05 by Duncan's multiple range test (DMRT).

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Total chlorophyll content in leaves

The total chlorophyll content (Figure 3A) in leaves increased significantly (p≤0.05) when the two varieties were foliar sprayed with ZnO NPs. Alankar registered a 28% increase, while Rohini reflected a 19% increase. The PSB and AMF treatments also significantly (p≤0.05) increased the leaf chlorophyll contents by 28% and 34% in Alankar and 10% and 16% in Rohini, respectively, compared to the control plants. No significant (p≤ 0.05) increase in leaf chlorophyll level was noticed against soil-mediated N, P, and Zn treatments in the two varieties of mustard.

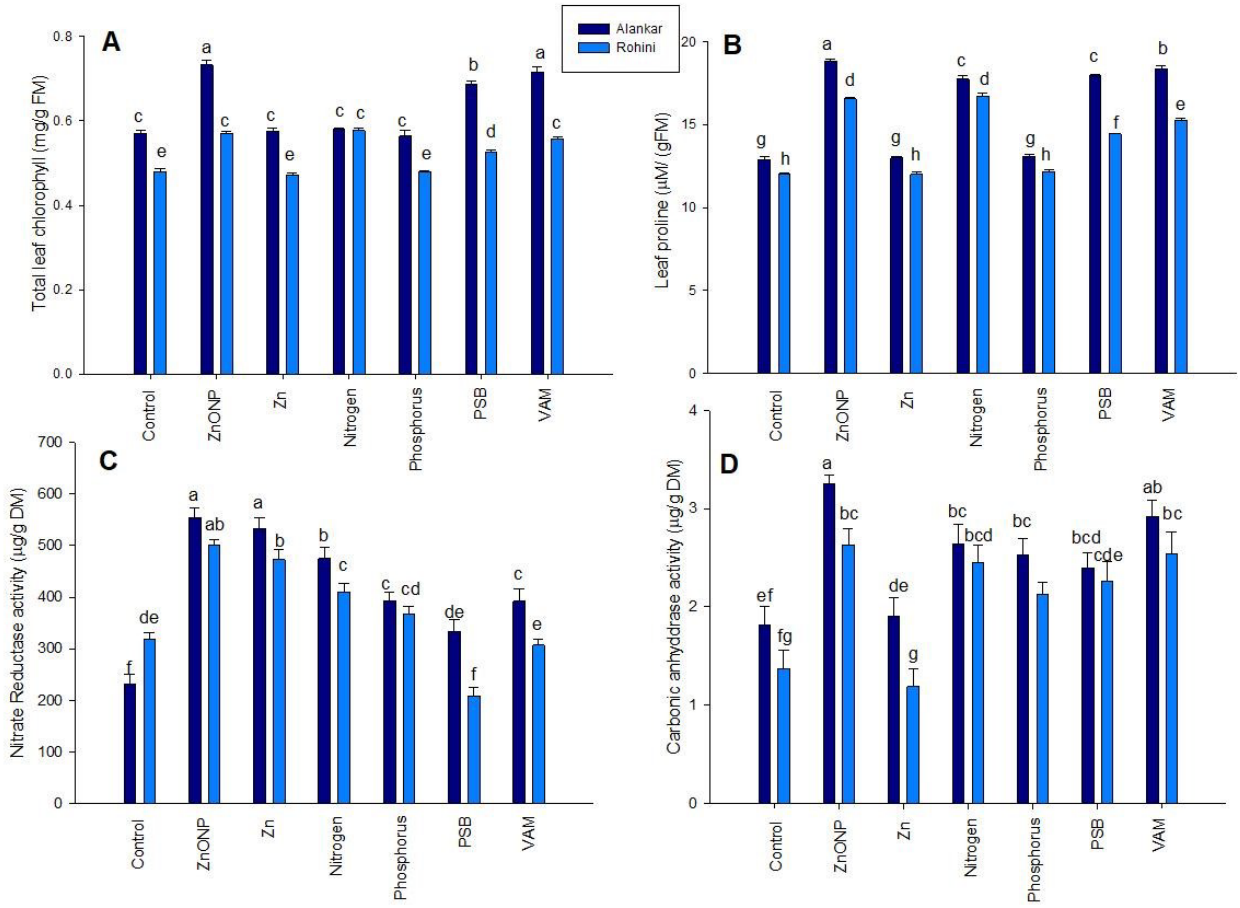

Figure 3. Effect of foliar and soil mediated chemical and biofertilizers (Zn, Nitrogen, PSB, and AM fungi) on two different mustard varieties *Brassica juncea* var. Alankar and Rohini on (A) total chlorophyll, (B) proline level, (C) nitrate reductase activity, and (D) carbonic anhydrase activity at 60 days after sowing (DAS). Data are presented as the treatment mean ± standard error (n = 5). The different letters above the bars show that data are significantly different at p≤ 0.05 by Duncan's multiple range test (DMRT).

Proline content in leaves

A significant (p≤0.05) increase in leaf proline content (Figure 3B) was recorded against the leaf-sprayed ZnO NPs and the soil-mediated two biofertilizers (PSB and AMF/VAM) and nitrogen treatment in the Alankar and Rohini varieties of mustard plants. The data indicated that proline accumulation was higher in Alankar than in Rohini against the given treatments. For the above treatments, the increase was 46%, 39%, 42%, and 37%, respectively, for Alankar. For Rohini, the increase was lesser, and in the order of ZnO NPs>AMF>PSB>N, an insignificant increase of proline was registered for the treatments of soil-mediated Zn and P recorded compared to control plants in the two varieties.

Nitrate reductase (NR) and carbonic anhydrase (CA) activity

The two mustard varieties; Alankar and Rohini, showed a significant (p≤0.05) increase in NR and CA activity (Figure 3C and 3D) compared to most of the treatments. The increase in the activity of these enzymes against all the treatments of chemicals and biofertilizers was higher in Rohini than in Alankar. For NR activity, ZnO NP was followed by Zn and N treatments in the two varieties, and for CA, it was followed by AMF/VAM and PSBs, respectively. For NR activity, the increase against ZnO NP treatment was 140% and 111% in Alankar. For the Zn and N treatments, however, the increase in NR activity was 99% and 72%, respectively, compared to the control plants. The CA activity was 79% and 58% for the two varieties, Alankar and Rohini, respectively.

Discussion

Our agricultural system depends on the supplementation of primary nutrients (such as N, P, and K) to maximize crop output and support modern agriculture [45]. Mineral ion uptake properties show variation among plant species and cultivars [46]. In mustard plants, the growing seeds and leaves compete for nitrogen, and the size of the nitrogen pool in the vegetative sections largely determines seed set, seed growth, and

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final seed production [47,48]. Nitrogen supply influences several growth parameters, produces more robust growth and development, and increases plant height, number of flowering branches, total plant weight, and leaf area, all of which cumulatively enhance the yield output [49,50]. Brassica growth and yield improved with the application of 100–130 kg/ha nitrogen, while yield also increased at the same rate with the application of phosphorus [51-54]. However, this demand is typically higher in arid and semiarid environments [55,56]. As stated above, phosphorus has a greater impact on yield than nitrogen and potassium. Phosphorus is a component of nucleic acids, cell signaling, and membrane phospholipids. It also plays a role in energy metabolism, cell division, and the formation of several coenzymes, including ATP, NAD(P)H, and GTP [57]. P deficiency manifests as visible purplish pigmentation on leaves, young, stunted stems, early leaf shedding, and reduced seed output [58,59]. Single, double, and triple superphosphate (SSP, DSP, TSP), ammonium phosphate, dicalcium phosphate, basic slag, calcium meta-phosphate, rock phosphate, bone meal, etc., are the main sources of plant phosphorus [60]. The application of chemical fertilizers also poses a serious threat to nitrogen and other chemical pollution in soil and water bodies, leading to eutrophication [61]. To avoid nitrogen pollution and eutrophication of nutrients in water bodies, the application of nanosized nutrients, such as nanofertilizers, nanobiochar, and essential element nanoparticles, through foliar spraying has become a trend in recent studies because it minimizes the loss of nutrients and allows them to be efficiently absorbed by plant leaves due to their nano size, which saves the environment and expenses of farmers [18, 62].

The findings of the present study demonstrated that the use of ZnO NPs improved the growth of two varieties, including the root and shoot length, their ratio, fresh and dry weight, and leaf area (Figure 2). Significant differences were seen in the foliar delivery of ZnO NPs compared to soil amendment of Zn, which may be a cost-effective method for providing nutrients to the plants. After nitrogen, phosphorus, and potassium, Zn is regarded as the nutrient that limits yield the most both globally and in Indian soils [63]. According to estimates, 36.5% of Indian soils lack Zn [64]. While it is normal practice in modern agriculture to add fertilizers to complement natural soil fertility, temperate and tropical soils frequently continue to be low in micronutrients, particularly Zn [4,65]. Two forms of Zn influenced mustard growth differently. In general, foliar treatments with 4 mM ZnO NPs brought significant improvement in growth parameters compared to Zn, N, or P given through soil and control plants. The growth promotion was even higher than that with biofertilizers, PSBs, and AM fungi. Zinc from ZnO NPs can accumulate in the leaves through foliar feeding, making these NPs potentially useful sources of Zn for plants to employ in metabolic processes [66,67]. According to a recent study by [68], the predominant channel for wheat and sunflower (*Helianthus annuus L.*) to absorb ZnO NPs under experimental conditions was through the leaf cuticle. In addition, ZnO NPs are used as nanofertilizers, which may be a more effective and slow-releasing source of Zn than conventional fertilizers or other sources of Zn [66,69,70]. According to a study by [71], applying ZnO NPs to the soil at various concentrations increased the Zn content of wheat tissues under normal or water-stress conditions. Afterwards, Adrees et al. [72] demonstrated that foliar exposure to ZnO NPs enhanced wheat development through foliar application. The larger weights of the plants may be a factor in the enhanced availability of Zn as NPs compared to Zn applied to the soil. The mustard plants' growth and antioxidant enzyme activities were improved when ZnO NPs were sprayed [73]. The intensification of the metabolism aided by Zn is what causes the rise in dry mass. Enzymes, including dehydrogenases, aldolases, isomerases, transphosphorylases, and RNA and DNA polymerases, all require zinc to function [74]. Moreover, it contributes to tryptophan production, cell division, membrane structure maintenance, and photosynthesis and functions as a regulatory cofactor in protein synthesis [3,9]. Several species have been the subject of ZnO NPs experiments, and the overall beneficial interactions have been previously characterized [35,74-76]. An increase in the FW and DW of seedlings growing in the presence of ZnO NPs was observed in earlier studies [77]. Reduced growth and plant biomass, restriction of cell elongation and division, wilting, curling, and rolling of young leaves, chlorotic and necrotic leaf tips, and suppression of root growth are all signs of Zn toxicity [78,79]. According to the findings of Rossi et al. [80] on coffee plants treated with ZnO NPs, the photosynthetic apparatus was enhanced. In the present study, positive interactions were found between ZnO NPs and the net carbon assimilation rate and stomatal conductance.

In the present study, the efficacy of treatments followed the pattern of ZnO NPs>AMF>PSBs>N and increased the leaf chlorophyll level, proline content, NR, and CA activity (Figure 3 A-D). Nitrogen is a key nutrient component that gives crops their lush green color by increasing the amount of chlorophyll in the leaves and boosts biomass by increasing carbon fixation. However, depending on factors such as soil type, climate, management practices, when nitrogen is applied, cultivars, etc., nitrogen fertilizer needs can vary greatly [81]. Zn is a cofactor of carbonic anhydrase, which raises the amount of CO2 in the chloroplast and, as a result, also increases the ability of the Rubisco enzyme to carboxylate [82]. Different macro-and micronutrient uptake can be affected by zinc's effects on absorption [83,84]. Zn typically causes severe Fe deficiency chlorosis in dicots on acidic soils. Crops such as lettuce, mustard, and beet are particularly vulnerable to too much soil Zn [85]. Zn transport and uptake by leaves were also investigated. Typically, ZnO NPs enter the leaf system through wounds, hydathodes, cuticle penetration, and stomata [10]. This is evident from the data, which reveal that ZnO NPs markedly increased Zn levels in the leaf, while $ZnSO_4$ did not significantly accumulate when compared to the control. It results from the effects of adding P and other minerals, as well as phytohormones secreted by PSBs and AM fungi in the root zone. Positive effects of ZnO NPs were also studied on the seed germination and vegetative growth in different crops of *Arachis hypogea* [86], *Vigna radiata* [87,88], *Cicer arietinum* [89], *Glycine max* [90], *Helianthus annuus* [91], *Lycopersicon esculentum* [92], *Sesamum indicum* [93], *Brassica nigra* [94] and *Brassica juncea* [95]. PGPR, such as PSB, proves useful in enhancing crop productivity by making nutrients more bioavailable in the soil with chemical secretion in the rhizosphere [96]. Alone and combination of AM fungi with biocontrol fungi or nanoparticles also prove effective in increasing the crop productivity in plants by increasing the phosphorus and other nutrients available in the soil by releasing chemicals in the soil that change the pH and amount of available organic matter content in the soil [97-99]. Combinations of PGPR and AMF improved the crop

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productivity in various plants and also helped to manage growth under stress conditions [100-103]. Although the interaction of *Glomus* species with mustard plants is not common, recent studies clearly show that it helps the plants to increase their resistance against pathogens and increase crop productivity by regulating enzymatic activities in plants and increasing the amount of nutrients and organic matter in the soil [104-106].

Conclusions

Foliar exposure to ZnO NPs may be thought of as both an efficient and different method to increase productivity compared to other treatments. Nanoparticles have microscopic size and large surface area, which help maximize their uptake and translocation as nutrients in plants via foliar spray. As a result, ZnO NPs had more favorable effects on plant growth, morphology, development, physiology, and metabolism than traditional Zn salt because nanoparticles induce the genes involved in nutrient assimilation pathways. It may also be crucial to research how ZnO NPs affect other nutrients necessary for plant health as well as the general ecology of the rhizosphere. When compared to chemical fertilizer applications of P or even N, biofertilizers such as AMF/VAM and PSBs are also preferable because their chemical activity solubilizes and increases the bioavailability of nutrients in the soil, ultimately enhancing the growth of the treated plants. Further research is required to grow different crop species in the field under diverse agroclimatic circumstances to determine the cost-effectiveness and adaptability of foliar ZnO NP exposure.

However, a future aspect of this research is to determine the growth and yield responses of crops upon exposure to the combination of nanoparticles and biofertilizers.

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Authors' contributions

Avshesh Kumar: Conceptualization, methodology, writing, original data preparation; Rajkumar Yadav: Data Analysis and Statistical Analysis; Adnan Khan: Reviewing and correction in the manuscript; Mohd Irfan: Editing and drafting of the manuscript; Syed Aiman Hasan: Graphs and graphical abstract preparation.

Disclosure statement

No potential conflict of interest was reported by the authors.

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