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Salt stress amelioration and nutrient strengthening in spinach (*Spinacia oleracea* L.) via biochar amendment and zinc fortification: seed priming versus foliar application

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Soil salinity is a major nutritional challenge with poor agriculture production characterized by high sodium (Na^+) ions in the soil. Zinc oxide nanoparticles (ZnO NPs) and biochar have received attention as a sustainable strategy to reduce biotic and abiotic stress. However, there is a lack of information regarding the incorporation of ZnO NPs with biochar to ameliorate the salinity stress (0, 50,100 mM). Therefore, the current study aimed to investigate the potentials of ZnO NPs application (priming and foliar) alone and with a combination of biochar on the growth and nutrient availability of spinach plants under salinity stress. Results demonstrated that salinity stress at a higher rate (100 mM) showed maximum growth retardation by inducing oxidative stress, resulted in reduced photosynthetic rate and nutrient availability. ZnO NPs (priming and foliar) alone enhanced growth, chlorophyll contents and gas exchange parameters by improving the antioxidant enzymes activity of spinach under salinity stress. While, a significant and more pronounced effect was observed at combined treatments of ZnO NPs with biochar amendment. More importantly, ZnO NPs foliar application with biochar significantly reduced the Na^+ contents in root 57.69%, and leaves 61.27% of spinach as compared to the respective control. Furthermore, higher nutrient contents were also found at the combined treatment of ZnO NPs foliar application with biochar. Overall, ZnO NPs combined application with biochar proved to be an efficient and sustainable strategy to alleviate salinity stress and improve crop nutritional quality under salinity stress. We inferred that ZnO NPs foliar application with a combination of biochar is more effectual in improving crop nutritional status and salinity mitigation than priming treatments with a combination of biochar.

Keywords Salinity, Biochar, Chlorophyll pigments, Antioxidant enzymes activates, Nutrient contents

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Climate change is one of the pervasive challenges for agriculture that threatens global food security and environmental sustainability[1]. Approximately 40 million people worldwide are vulnerable to malnutrition as a result of the current climate change situation[2]. Salinity is the potential consequence of climate change which in turn affects agricultural crop production, necessary to sustain the growing global population[3]. Salinity inhibits plant growth by reducing the soil osmotic potential, which lowers the availability of water and raises the concentration of certain ions. The state of ionic imbalance has inhibitory effects on plant metabolism, stomatal closure, and chlorosis and disturbs photosynthesis, membrane permeability, and enzyme activity[4,5] and these effects altogether lead to the generation of reactive oxygen species (ROS) such as O^{-2} and H_2O_2[6,7]. In addition, plants grown under salinity stress showed deficient micronutrients such as zinc (Zn), copper (Cu), iron (Fe), and manganese (Mn) due to the fixation of these nutrients on clay part of soil[8], and higher soil pH[9,10]. The high concentration of salt in soil has deleterious effects on all stages of plant development, which eventually results in crop yield reduction. To fulfill the food requirement of the growing population there is a need to manage the salt-affected land. Multiple approaches have been established to boost the quality of salt-affected soils and optimize plant productivity to ensure sustainable agriculture.

Nanotechnology is an emerging field that involves the synthesis of nanoparticles (1–100 nm). The nanoparticles have achieved considerable attention in agriculture due to their potential for increasing crop growth under harsh environmental conditions including salt stress[11]. Seed priming and foliar application of nanoparticles (nano-fertilizers) are two strategies to alleviate stress responses during seed development and growth under salt affected soil[12]. Many studies reported zinc oxide nanoparticles (ZnO NPs) imperative roles in alleviating salinity stress. ZnO NPs foliar application positively affects morpho-physiological attributes of coffee and tomato plants grown under saline soil[13,14]. According to a study by Farouk and Al-Amri[15], ZnO NPs application on canola plants reduced the negative effects of salinity by improving the antioxidant enzyme activity, osmolytes synthesis, and ionic balance. Moreover, seed priming of NPs boost seed performance, and helps to overcome seed dormancy leading to improved crop growth under salt stressed condition[16]. The study conducted by Abou-Zeid et al.[12] demonstrated that seed priming with ZnO NPs improved wheat seedlings with a better photosynthetic rate. The potential toxicity of NPs particularly at elevated concentrations, could provide a significant barrier to the utilization of these materials as nano-fertilizers[17]. Therefore, the addition of low concentrations of NPs together with other amendments like biochar may be an effective strategy to improve growth and nutrient status in plants under salt stress. In addition of NPs, biochar amendment has been recognized as an effective strategy for restoring saline lands and improving plant tolerance to salt stress[18].

Biochar has excellent physicochemical, and biological characteristics and its amendment in soil facilitates nutrient uptake[19]. Biochar holds prominent properties including pH, cations exchange capacity (CEC), and bulk density along with many biological activities i.e. enzyme activity and microbial population[20,21]. Additionally, biochar contains a significant amount of trace elements (Fe, Zn, B, Cu, Mn), which increase nutrient absorption in plants[22]. Previous studies showed that biochar application increased the level of soil available potassium, phosphorus, and organic matter while decrease the concentrations of SO_4^{2-}, Na^+, Ca^{2+}, Mg^{2+}, and Cl^- in saline-alkaline soil[23]. The addition of biochar improves soil fertility and crop yield by remediating the salt affected soil[24]. Zhao et al.[25] reported that biochar amendment to saline-alkaline soil improved plant growth and salt tolerance in maize by boosting the absorption of essential nutrients (N, P, K, Ca, Mg). However, in plants, biochar promotes seed germination, and influences plant growth in response to abiotic stresses[26].

Spinach (*Spinacia oleracea* L.) is a globally cultivated leafy vegetable with 26.2 Mt of production annually[27]. Spinach being a glycophytic (salt sensitive) plant has relatively low salt tolerance with high nutritive values and important vitamins[28,29]. Salinity induced alterations in the nutritional composition of spinach, as well as physiological modifications[21,30]. Previous studies only focus on the use of a single amendment i.e. either biochar or a single module of nanoparticles, for plant growth and nutrient content under salinity. We hypothesized that ZnO NPs in combination with biochar may efficiently reduce the salt stress and improve the growth performance and nutrient content of spinach as compared to alone treatments. So, according to the best of our knowledge, there is not a single study on the combined use of biochar and double-module of ZnO NPs (foliar and priming) for alleviation of plant growth as well as nutrient availability under salinity stress. Consequently, the current study critically investigates the mechanisms underlying the combined application of ZnO NPs and biochar, administered through both foliar and seed priming methods, on the growth and physiology of spinach plants under salinity stress. Furthermore, we also assessed the oxidative stress and antioxidant enzymes to highlight the salinity tolerance in spinach by these treatments.

Materials and methods

Synthesis and characterization of ZnO nanoparticles

ZnO NPs were synthesized by sol–gel method by using zinc nitrate $Zn(NO_3)_2{\cdot}6H_2O$. In brief, 0.1 M of $Zn(NO_3)_2{\cdot}6H_2O$ (100 mL) solution was made and subjected to heating (60 °C) for 1 h with constant stirring. After this, the transparent solution was obtained (pH of 5.0). NH_4OH was added dropwise to adjust the pH to 8.5. The solution became cloudy due to the presence of dispersed particles and was again stirred for 30 min (60 °C), centrifuged (20 min) and the supernatant was discarded. Following this, the precipitates were filtered and dried (70 °C)[31]. The details of the nanoparticles characterized were analyzed by using scanning electron microscopy SEM (Model: Japan Hitachi Regulus 8100) utilized at 20 kV. The imaging condition nanoparticles were stick it directly to the conductive adhesive, and use Quorum SC7620 sputtering coater to spray gold for 45 s and the gold spraying was 10 mA.

Preparation of biochar and its characterization

The farmyard manure (FYM) used for biochar production was collected from the farm, located in Nankana, Punjab, Pakistan. FYM biochar was prepared at the Biochar Research Unit, Environmental Bio-geochemistry Laboratory, GCU, Faisalabad, Pakistan, using a muffle furnace (500 °C) by following the method described by James et al.[32]. The pyrolyzed biochar was cooled at room temperature, grounded, sieved (0.154 mm), and stored in a sealed bag. The biochar physicochemical properties of electrical conductivity (EC)[33], pH[34], organic matter (OM)[35], organic carbon (OC) from the OM (organic matter), total nitrogen (TN)[36], cation exchange capacity (CEC)[37] was determined shown in (Table 1). Furthermore, the detailed biochar characterization was done by using SEM–EDX with energy-dispersive X-ray spectroscopy (Japan Hitachi Regulus 8100) to determine porosity, pore size, pore shape, elemental mapping, and spot analysis. FTIR-Thermo Scientific Nicolet iS20) was done to identify various functional groups in biochar.

Soil collection and analysis

The soil used in the study was collected from an agriculture field with an approximate depth of 20 cm with the help of a stainless-steel blade. The soil was dried in open air over a week, and sieved by a 0.7 mm sieve to remove debris and roots that are present in the soil. The soil texture[38] EC and pH were determined. Whereas, soil water holding capacity was measured using a volumetric method adapted from Yargicoglu et al.[39]. Organic matter[40], $CaCO_3$[41] sodium adsorption ratio (SAR), and extractable K[42] CEC[43] concentration in soil were also analyzed. Mehlich 3 Extraction method[44] was followed to determine plant available nutrients in the soil. Metal concentration in soil was measured by the method by Park et al.[45] shown in (Table 2).

Soil treated with FYM biochar

Before starting the experiment, 1.5 kg of soil was added to plastic cylindrical pots (weight 54 g, diameter 1 cm, height 13 cm). To induce salinity stress different NaCl concentrations (0, 50 and 100 mM) were added in pots

Farmyard manure biochar		
Parameters	**Unit**	**Values**
pH	–	8.21 ± 084
Electrical conductivity (EC)	ds m^{-1}	2.15 ± 0.14
Cation exchange capacity (CEC)	mol kg^{-1}	34.81 ± 1.04
Total organic carbon (TOC)	g cm^{-3}	61.54 ± 5.84
Organic matter (OM)	g cm^{-3}	2.041 ± 1.24
Total nitrogen (TN)	(%)	2.51 ± 1.45

Table 1. The physicochemical analysis of biochar used in this study.

Bare soil			2% Biochar amend soil
Parameters	**Unit**	**Values**	**Values**
Basic properties			
pH	–	8.21 ± 1.04	8.39 ± 1.21
Electrical conductivity (EC)	µS cm^{-1}	1432 ± 0.54	1434 ± 2.0
Cation exchange capacity (CEC)	mol kg^{-1}	8.64 ± 0.54	8.26 ± 0.24
Total organic carbon (TOC)	g cm^{-3}	2.041 ± 1.24	2.51.84 ± 3.64
Organic matter (OM)	(%)	1.02 ± 2.45	3.54 ± 8.45
Soluble ions			
Sodium (Na^+)	$mmol_c$ L^{-1}	8.21 ± 2.8	–
Sodium adsorption ratio (SAR)	$mmol_c$ L^{-1}	6.01 ± 0.06	–
Calcium carbonate ($CaCO_3$)	%	1.41 ± 0.089	–
Nutrients concentrations		Available	
Potassium (K)	mg kg^{-1}	416.2 ± 12.5	465.7 ± 56.2
Nitrogen (N)	mg kg^{-1}	526.4 ± 51.54	575.8 ± 8.45
Phosphorus (P)	mg kg^{-1}	1509.4 ± 91.5	1610 ± 213.3
Zinc (Zn)	mg kg^{-1}	3.4 ± 1.40	4.1 ± 0.097
Metal concentrations		Available	
Cadmium (Cd)	mg kg^{-1}	0.032 ± 0.06	0.21 ± 0.14
Chromium (Cr)	mg kg^{-1}	0.0146 ± 0.021	0.131 ± 0.067

Table 2. The physicochemical analysis of soil used in this study.

as shown in several studies[46]. Then, the soil was initially treated with FYM biochar 2.0% (by weight) with 40% water holding capacity, and subsequently incubated for one month. The selection of these rates was also based on earlier experiments using similar application rates[47]. Following incubation, the soil was allowed to dry, and pH, EC, and CEC were again determined by[43]. Plant available nutrients (K, P, Mg, Ca, Cu, and Zn) were measured by the method proposed by Mehlich[44] and later analyzed by ICP-OES as shown in (Table 2).

Experiment design

The pot experiment was conducted in a natural environment at district Sheikhupura, Punjab-Pakistan (31.8630° N and 73.6639° E) by following a completely randomized design (CRD) with three replicates per treatment. Seeds of spinach (*cv.* Spinach Prickly) were obtained from Ayub Agricultural Research Institute (AARI), Faisalabad, Pakistan and this experiment on plants was complied with national, international guidelines and legislation. The spinach seeds were disinfected with hydrogen peroxide (H_2O_2) solution 2.5%, (v/v by volume) for 5 min, and subsequently washed with d-H_2O. After washing the seeds were shifted to a priming media, controls were treated with DI water, and for NPs treatment (100 mg/L of distilled water) ZnO NPs were continuously aerated with aeration pump for 24 h. Following the priming procedure, the seeds were dried using a paper towel. Seven seeds were sown in each pot carefully. After germination, only well grown three seedlings per pot were retained. For foliar spray, the ZnO NPs solution was prepared with distilled water (DW) and ultra-sonicated for 30 min in water until a stable dispersion of NPs was obtained. ZnO NPs 0 and 100 mg/L of distilled water applied after two weeks of germination with the help of a hand-held spray bottle. The control treatments for foliar NPs were treated with DW. No plants died during planting, and no additional agronomic measures such as pesticides were taken. The analysis of all measured parameters was conducted four weeks following the application of ZnO NPs treatment, while the environmental stress persisted until the harvesting of the plants.

Growth parameters

After the completion of the pot experiment (7 weeks), plants of spinach were carefully up rooted and then washed gently with distilled water to remove the dust and deposits. Shoot and root length were measured via a meter scale. Meanwhile, several leaves per plant were recorded and dried at 80 °C in an oven for 48 h. After drying, shoot and root dry weight was recorded using an electrical balance (OHAUS-PR224).

Chlorophyll content and photosynthetic pigments

The chlorophyll content was measured followed by the method proposed by Arnon[48]. 0.5 g of fresh leaves were grounded with 80% (v/v by volume) acetone and centrifuged at 15,000 rpm under 4 °C to extract chlorophyll content. The absorbance was recorded at specific wavelengths of 645 nm, 663 nm and 470 nm (Labman LMSPUV1900 Double Beam UV–VIS Spectrophotometer). SPAD values were determined by SPAD meter (atLEAF CHL STD chlorophyll meter 502: FT Green LLC, Wilmington USA). Furthermore, gas exchange parameters (photosynthesis rate, transpiration rate, substomatal CO2 concentration, and water use efficiency) were recorded in day daytime (10:00 am to 12:00 pm) (Infrared gas analyzer (3051c Plant Photosynthesis meter).

Determination of antioxidant enzyme activities and oxidants

Sampling was done after the four weeks of germination to determination of the antioxidant enzymatic activities. Fresh leaves and roots (0.3 g) were grounded using a pre-chilled pestle and mortar on ice and homogenized with a 50 mM phosphate buffer solution (PBS) with pH 7.8. The resulting mixture was centrifuged (12,000 rpm) at 4 °C for 15 min and the supernatant was obtained[49]. The collected supernatant was further used for the determination of superoxide dismutase (SOD), catalase (CAT), and ascorbate peroxidase (APX). The SOD activity was determined by using a reaction solution containing NBT (75 µM), riboflavin (20 µM), EDTA (100 µM), and L. methionine (130 mM), with the enzyme extract[50]. The absorbance at 560 nm wavelength via spectrophotometer was measured. For CAT activity determination containing reaction solution with 50 mM PBS at pH 7.8, H_2O_2 (300 mM) and enzyme extract. The absorbance at 240 nm wavelength was to measure the activity (Labman LMSPUV1900 Double Beam UV–VIS Spectrophotometer) according to the method of Aebi[51]. The APX activity was measured by preparing a reaction solution containing PBS (H_2O_2) (300 mM), ascorbic acid (7.5 mM), and enzyme extract[52]. The measurement was recorded spectrophotometrically at 290 nm for 30 and 60 s (via Labman LMSPUV1900 Double Beam UV–VIS Spectrophotometer. The malondialdehyde (MDA) contents were determined by following the method proposed by Zhang and Kirkham[53]. In brief, the reaction solution containing was ground trichloroacetic acid (TCA) (0.5%) with thiobarbituric acid (TBA) (5%) mixed with enzyme extract, subjected to heating (95 °C) following centrifugation (4800 rpm) to collect the supernatant. The absorbance at 532 and 632 were taken to measure MDA contents. Electrolyte leakage (EL) in spinach leaves and roots was determined by following the method of Dionisio-Sese and Tobita[54]. Hydrogen peroxide (H_2O_2) was determined in spinach roots and leaves by following the method proposed by Jana and Choudhuri[55].

Measurement of total phenolic contents

0.5 g leaf samples were homogenized in 10 mL (80%) MeOH and incubated (12 h) in a shaking water bath After centrifuging at 4000 rpm, supernatant was collected for analysis. The Folin-Ciocalteu reagent method was followed to estimate the total phenolic content. Sodium carbonate solution (7.5%) was added after 5 min to the reaction mixture and incubated (90 min). Gallic acid was used as a standard and absorbance at 765 nm was used to assess phenolic content[56].

Na^+ and Nutrient contents

The above and below ground plant dried samples (0.1 g) were digested in H_2O_2 (8 mL) and of plasma pure HNO_3 (2 mL) mixture 4:1, v/v (by volume) in Teflon vessels using a hotplate until the solution became transparent and ICPOES was used to measure Na^+, micro and micronutrients[57].

Statistical analysis

The statistical analysis was conducted using the SPSS statistics software (IBM: Version 2020). The statistical differences between various treatments were evaluated using one-way analysis of variance (ANOVA) in the statistical software SPSS (Version 2020) and post-hoc Tukey's honestly significant difference (HSD) was employed for pairwise comparison. Furthermore, OriginPro, (Version 2023b, Origin lab corporation, Northampton MA. USA) for principal component analysis and Pearson correlation analysis were carried out to visualize data patterns.

Results

ZnO nanoparticles and FYM biochar characterizations

The SEM analysis was employed to investigate the surface morphology of ZnO NPs. The SEM images show the particle size 60 nm as shown in (Fig. 1a–c). The surface morphology structure of biochar derived from farmyard manure (Fig. 2a–d) shows a slightly rough and porous structure due to that numerous pores size provide additional space for microorganisms to improve the soil environment. Biochar effectively binds ions and molecules in its surface pores, facilitating their strong interaction with nutrients and their subsequent transfer into plants. SEM–EDX was used to identify the elemental composition of produced biochar. Figure S1b–g depicted results of

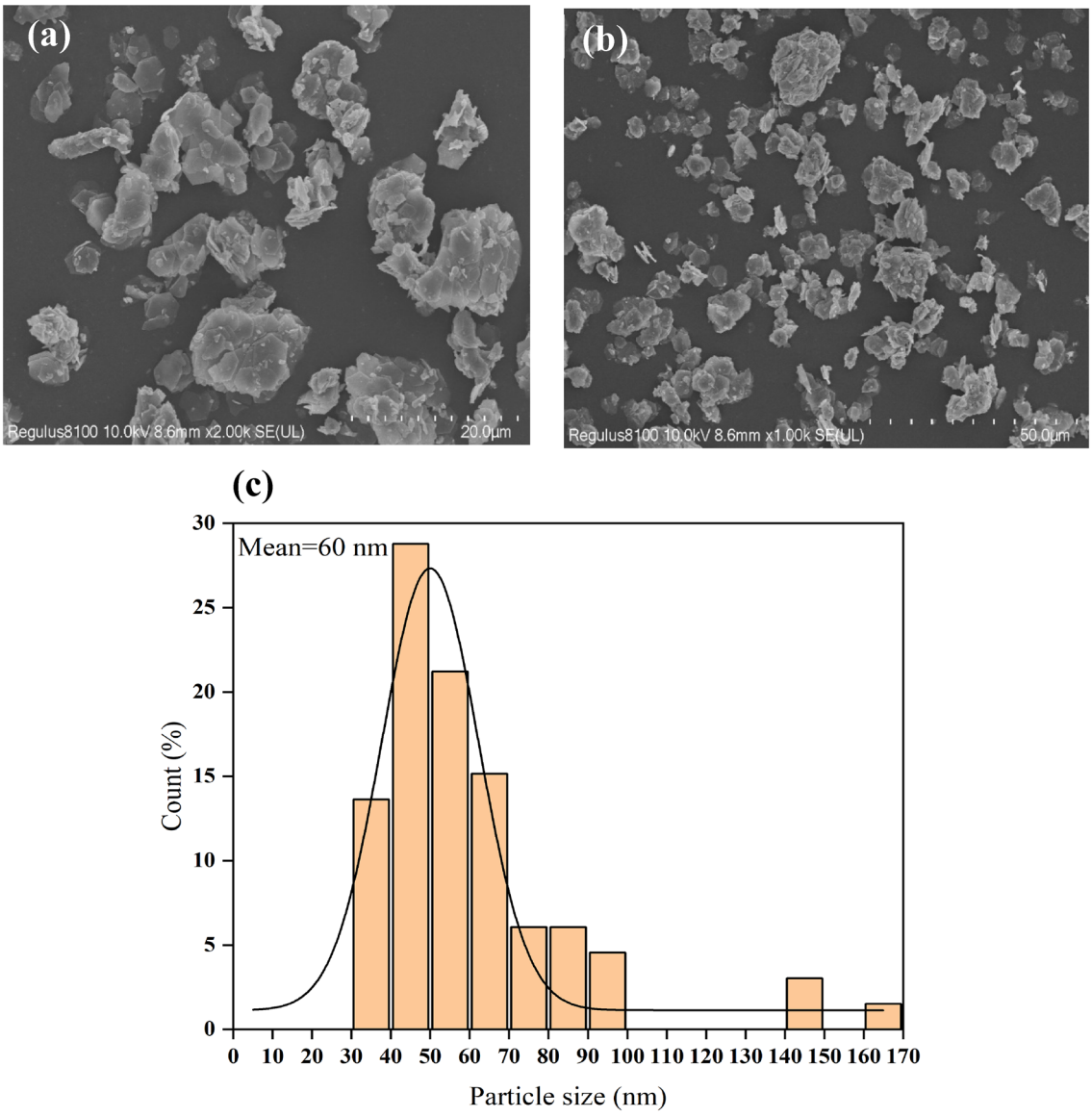

Figure 1. Scanning electron microscopy (SEM) images of ZnO NPs **(a, b)**, Particle size distribution **(c)**.

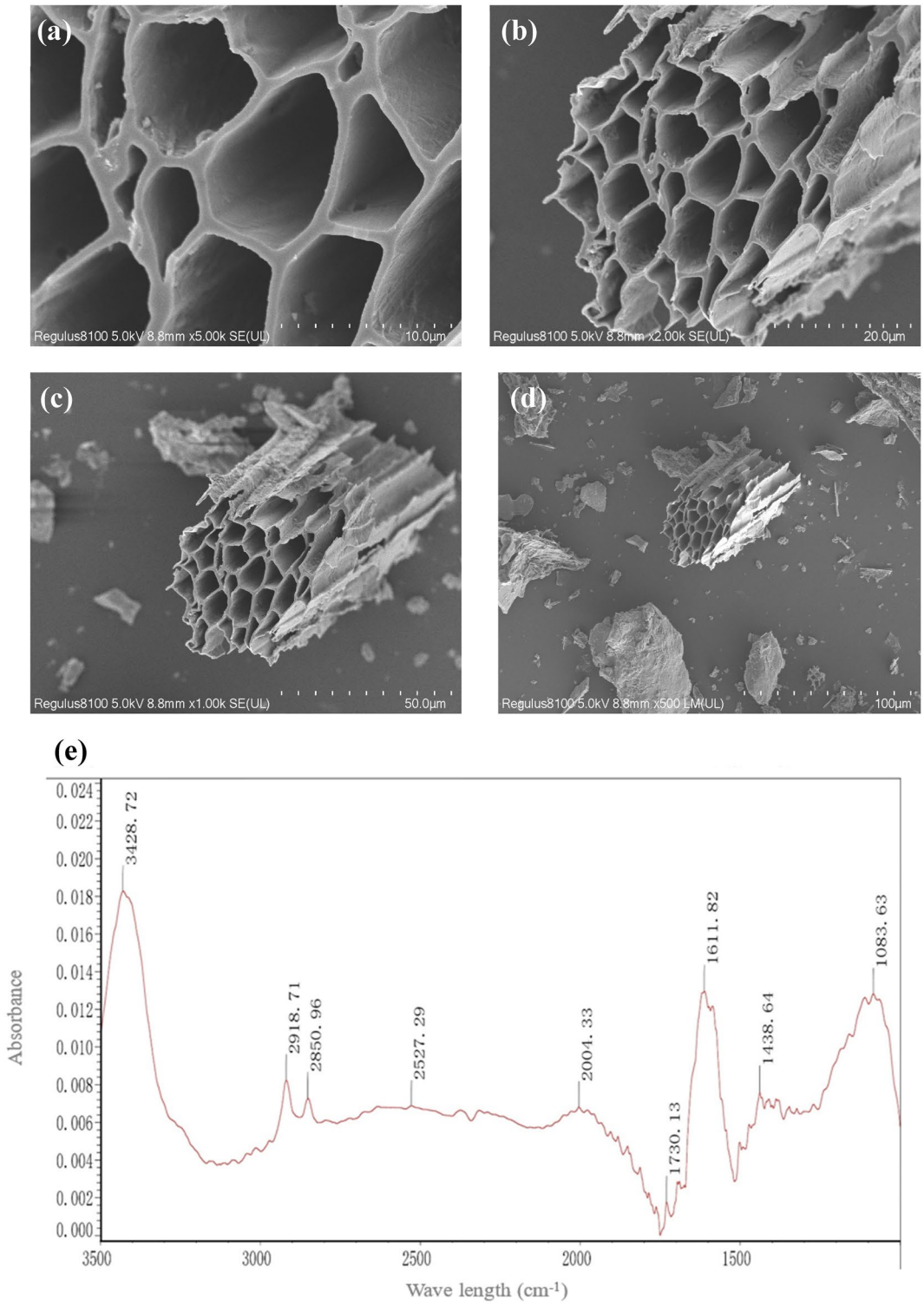

Figure 2. Scanning electron microscopy (SEM) analysis of farmyard manure biochar produced at temperature 500 °C (**a**) Image of pores size and shape at 10 µm (**b**) Image of surface at 20 µm (**c**) Pores ranging at 50 µm and 1000X (**d**) Top view of a surface image of biochar at 100 µm and FTIR spectra of farmyard manure biochar (**e**).

SEM–EDX mapping confirmed the presence of carbon (C), nitrogen (N), Sulphur (S), oxygen (O), potassium (K) thereby verifying that manure-based biochar improve the soil nutrient and SEM–EDX spectra analysis shown the peaks for carbon (C-22.87%), potassium (K-77.17%) and Sulphur (S-0.04%) elements in the spectra (Fig. S2a–c). By employing SEM–EDX, it was observed that FYM biochar may supply a considerable amount of nutrients like

carbon (C-22.87%), potassium (K-77.17%), and Sulphur (S-0.04%) to plants, in addition to other properties of adsorption. The results of our analysis revealed various elements such as carbon (C) and nitrogen (N) Sulphur (S), oxygen (O), and potassium (K). FTIR was a convenient method to identify functional groups of biochar shown in (Fig. 2e). The strong peaks observed at 3428.72 cm^{-1} represent –OH. The peak at 2918.71 cm^{-1} is attributed to C–H. The peak 2527.29 cm^{-1} is associated with O–H stretching. The peak 2004.33 is C–H stretching. The peak at 1730.13 cm^{-1} is C = O stretching. The peak 16,611.82 cm^{-1} is C = C stretching. The peak at 1438.64 cm^{-1} is O–H. The peak at 1038.63 is C–O. Our results also depict the presence of functional groups on the surface of FYM biochar. A possible explanation is that biochar reduced the amount of Na^+ through its negative surface charges thus reducing the Na^+ uptake and transport to the aerial part of spinach.

Effect of ZnO NPs (foliar/priming) alone and with biochar on plant growth attributes in the spinach

The Results revealed that salt stress significantly ($p \leq 0.05$) reduced plant growth attributes. However, the priming and foliar ZnO NPs with and without biochar greatly improved the growth attributes of spinach plants under salt stress (0, 50 and 100 mM). Our results highlighted that biochar interaction with both priming and foliar ZnO NPs significantly enhanced all growth parameters of spinach as compared to the alone application of NPs under salt stress shown in (Fig. 3). Salt stress at 100 mM concentration followed by 50 mM concentration significantly reduced all measured growth attributes compared to control without NPs and biochar however, the maximum decrease was observed in at 100 mM salt concentration. Specifically, the shoot length decreased by, 40.38%, and root length by 66.93%, the shoot fresh weight by 70.81%, the shoot dry weight by 49.51%, root fresh weight by 57.40%, root dry weight by 68.92% and number of leaves by 36.63% respectively, at 100 mM salt concentration compared to control without NPs and biochar. In the case of 100 mM salt stress, the priming ZnO NPs exhibited improved shoot length 21.99%, root length 22.17%, root fresh weight 30.20%, root dry weight 28.15%, shoot fresh weight 28.50%, shoot dry weight by 30.59% as well as number of leaves 22.33% as compared to their respective control treatments. It was observed that combined application of ZnO NPs with biochar treatment significantly ($p \leq 0.05$) and efficiently enhanced all growth parameters shoot length 35.50%, root length 40.65%, root fresh weight 54.78%, root dry weight 60.78%, shoot fresh weight 68.89%, shoot dry weight 51.82% and number of leaves 48.98% under 100 mM salt stress when compared to respective control. The foliar application of ZnO NPs alone increased the shoot length by 24.90%, root length 36.70%, root fresh weight 48.37%, root dry weigh 50 28%, shoot fresh weight 47.42%, shoot dry weight 58.04% and number of leaves 41.11% 100 mM salt stress as compared to their respective control. Our results revealed that combine treatment of foliar ZnO NPs with biochar showed a remarkable and significant ($p \leq 0.05$) increase in shoot length by 48.90%, root length 58.70%, root fresh weight 75.59%, root dry weigh 69.78%, shoot fresh weight 59.42%, shoot dry weight 61.34% and number of leaves 59.21% under 100 mM salt stress as compared to respective control as shown in (Fig. 3).

Effect of ZnO NPs (foliar/priming) alone and with biochar on photosynthetic contents and gas exchange parameters in the spinach

Our results showed that salt stress significantly reduced the SPAD values, chlorophyll a (chl a), chlorophyll b (chl b) and carotenoids (Car) content in spinach plant. A prominent decrease was observed in the SPAD value, chl a, chl b and Car content by 56.48, 65.80%, 74.60, 69.88% and 74.28% respectively, under salt stress 100 mM with respect to control without NPs (priming and foliar) and biochar treatment shown in (Fig. 4). Similarly, a significant decrease in photosynthetic rate (Pn) 61.12%, stomatal conductance (gs) 72.34%, transpiration rate (Tr) 62.34% and as well as water use efficiency (WUE) 74.62% under salt stress 100 mM with respective control (Table 3). In current study, we observed that chlorophyll and photosynthetic efficiency greatly enhanced by the application of priming and foliar ZnO NPs and effect was more pronounced with combined application of nanoparticles (priming and foliar) and biochar treatments. The priming ZnO NPs enhanced SPAD values by 29.52%, chl a 32.20%, chl b 34.41%, Car 36.13%, Pn 28.79%, gs 21.91%, Tr 33.57% and WUE 43.72%, under salt stress (100 mM) as compared to their respective control. Similarly, the foliar application of ZnO NPs alone enhanced SPAD values by 48.96% chl a 56.43%, chl b 62.43%, Car 59.34%, Pn 44.81%, gs 39.84%, Tr 71.63% and WUE 62.8%, under salt stress (100 mM) as compared to their respective control. At priming ZnO NPs with biochar treatment a significant improvement was observed in SPAD values by 40.80% chl a 69.12%, chl b 61.05%, Car 48.34%, Pn 57.67%, gs 69.76%, Tr 61.93% and WUE 67.53% respectively, under 100 mM salt stress as compared to respective control. However, our results revealed further improvement in these parameters was observed at foliar ZnO NPs with biochar treatment with an increase of 62.57% in SPAD values, 78.56 chl a, 74.93% chl b, 59.76% Car, 81.34% Pn, 82.84% gs, 75.50% Tr and 89.56% in WUE as compared to the respective control.

Effect of ZnO NPs (foliar/priming) alone and with biochar on antioxidant enzyme activities in the spinach

The antioxidant activities were analyzed in the present study depicted in (Figs. 5 and 6). The results indicated that salt stress significantly reduced the activities of antioxidant enzymes and ZnO NPs priming and foliar application with and without biochar application increased antioxidant enzyme activities as shown in (Figs. 5 and 6). Specifically, at 100 mM salt stress significantly reduced the roots and leaves SOD activity by 63.78, 59.10%, POD activity 71.07, 69.06%, CAT activity 65.60, 74.43%, APX activity 73.87, 65.15% respectively, as compared to control. The priming ZnO NPs treatment alone increased roots and leaves SOD activity by 33.15, 29.17%, POD activity 23.71, 37.31%, CAT activity 24.81, 41.77%, APX activity 33.15, 51.87%, respectively, under salt stress (100 mM) as compared to respective control. However, this increase in spinach root and leaves SOD activity 49.89, 61.76%, POD activity 51.78, 61.15%, CAT activity 60. 17, 69.28% and APX activity 58.34, 73.07% was improved significantly ($p \leq 0.05$) at foliar ZnO NPs alone treatment under 100 mM salt stress as compared to

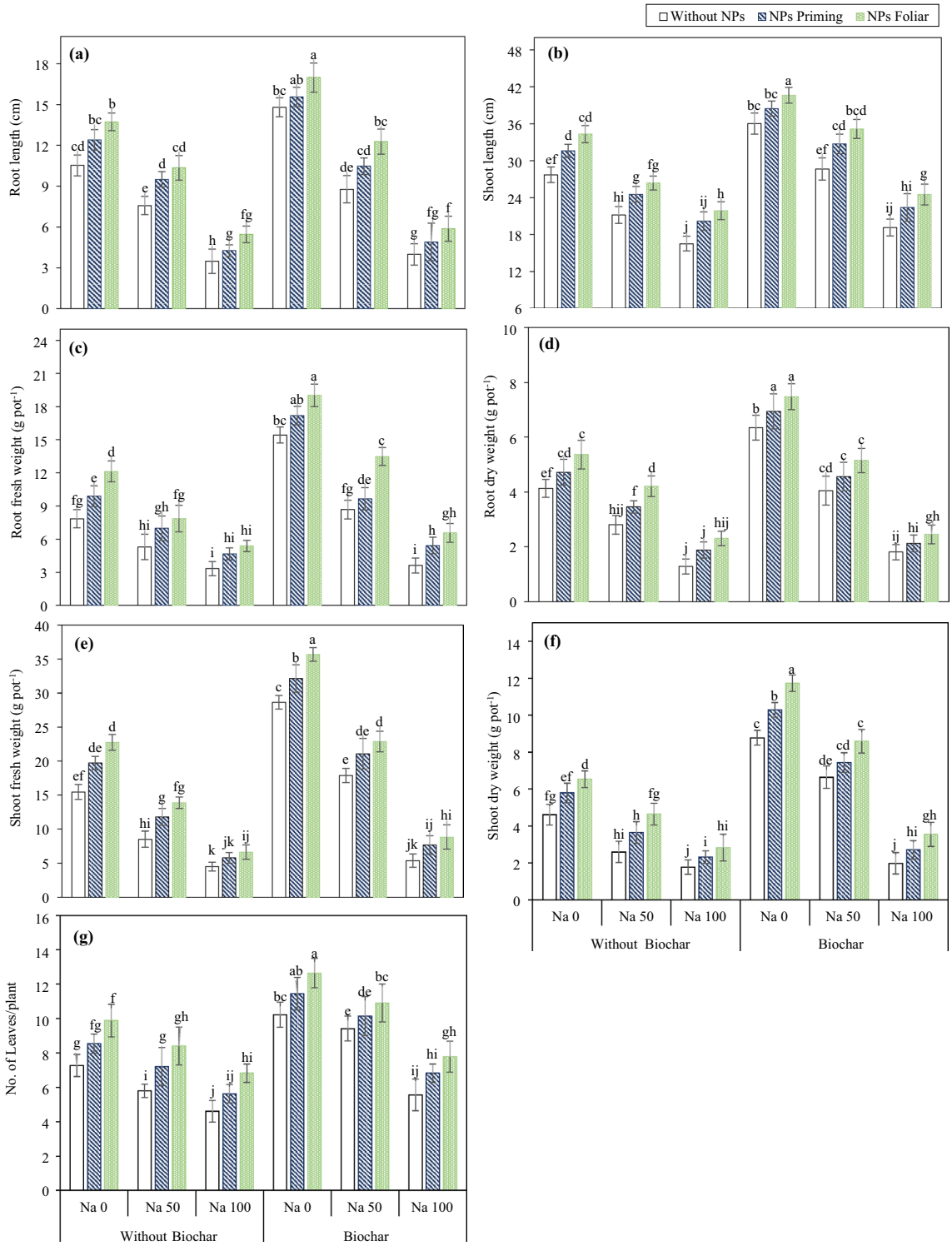

Figure 3. Effect of ZnO NPs (0, 100 mg/L priming, and foliar 0, 100 mg/L) alone and combined with biochar on (**a**) root length, (**b**) shoot length, (**c**) root fresh weight, (**d**) root dry weight, (**e**) shoot fresh weight, (**f**) shoot dry weight and (**g**) number of leaves of spinach grown under salinity stress (0, 50 and 100 mM). Error bars denoted the standard deviations of the measured data having three replications ($n = 3$). Small letters on the error bars denoted the statistical significance among the treatments determined by Tukey's test ($p \leq 0.05$).

their respective control. The combined treatment of priming ZnO NPs with biochar enhanced root and leaves SOD activity 55.95, 44.98%, POD activity 59.81, 61.87%, CAT activity 43.87, 52.87%, APX activity 53.76, 67.87%, respectively, under salt stress (100 mM) as compared to control. Whereas, combined treatment of foliar ZnO

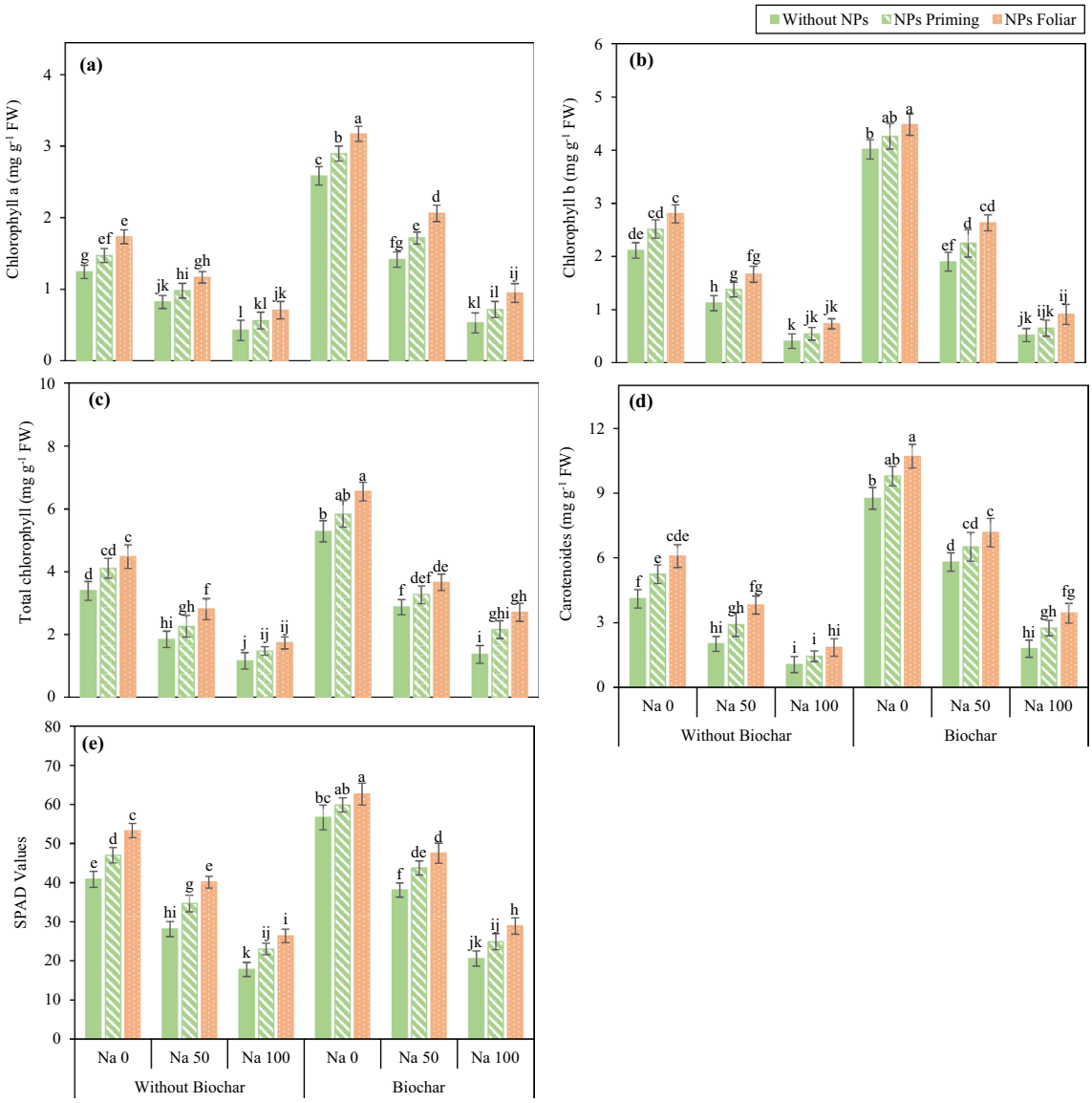

Figure 4. Effect of ZnO NPs (0, 100 mg/L priming, and foliar 0, 100 mg/L) alone and combined with biochar on (**a**) chlorophyll a, (**b**) chlorophyll b, (**c**) total chlorophyll, (**d**) carotenoids and (**e**) SPAD values of spinach grown under salinity stress (0, 50 and 100 mM). Error bars denoted the standard deviations of the measured data having three replications ($n = 3$). Small letters on the error bars denoted the statistical significance among the treatments determined by Tukey's test ($p \leq 0.05$).

NPs with biochar showed significant($p \leq 0.05$) and remarkable enhancement by increasing roots and leaves SOD activity 71.24, 79.41%, POD activity 83.4, 77.87%, CAT activity 73.89, 86.67% and APX activity 81.14, 87.04% respectively, under salt stress (100 mM) as compared to respective control.

Effect of ZnO NPs (foliar/priming) alone and with biochar on oxidative stress in the spinach

The oxidative stress was analyzed in spinach roots and leaves to measure the efficiency of ZnO NPs either alone or in combination with biochar to reduce the toxicity of salt stress. The salt stress induced oxidative stress in the spinach plant which was evident from the increasing level of hydrogen peroxide (H_2O_2), malondialdehyde (MDA), and electrolyte leakage (EL) in both roots and leaves of the spinach plant shown in (Figs. 7 and 8). The salt stress caused a significant increase in root and leaf H_2O_2 by 73.45, 68.98%, MDA 78.02, 67.89%, EL 54.34, 48.52% respectively, under 100 mM salt stress as compared to control. On the other hand, ZnO NPs (priming and foliar) decreased the H_2O_2, MDA, and EL content in spinach either alone or with biochar treatment. At priming ZnO NPs alone, the H_2O_2 contents in roots and leaves were reduced by 8.45, 11.79%, MDA 9.55, 12.89%, and EL 9.89, 7.89% respectively, while foliar ZnO NPs alone decreased the roots H_2O_2 19.33, MDA 14.89 and EL 20.03% and leaves H_2O_2 22.08, MDA 21.99 and EL 18.62% respectively, under salt stress (100 mM) as compared to respective control. The combined treatment of priming ZnO NPs with biochar showed a significant ($p \leq 0.05$) reduction in roots and leaves of H_2O_2 43.19, 23.83%, MDA 51.19, 54.61% andEL 58.15, 46.86% respectively,

Treatments	Stomatal conductance (mmol m^{-2} s^{-1})	Photosynthesis rate (µmol m^{-2} s^{-1})	Transpiration rate (mmol m^{-2} s^{-1})	Water use efficiency (%)
Without biochar				
Control	1.021 ± 0.153fg	14.62 ± 0.99g	0.852 ± 0.09hi	5.77 ± 0.85fg
Na 0, NPs Priming (100 mg/L)	1.511 ± 0.150de	16.71 ± 1.31fg	1.216 ± 0.18ef	8.71 ± 1.01de
Na 0, NPs Foliar (100 mg/L)	1.996 ± 0.135bc	21.51 ± 1.05e	1.571 ± 0.10cd	11.78 ± 1.20c
Na 50, NPs 0	0.583 ± 0.199hi	10.44 ± 1.35i	0.544 ± 0.07jk	3.66 ± 0.87hi
Na 50, NPs Priming (100 mg/L)	0.843 ± 0.144g	13.74 ± 1.15gh	0.880 ± 0.09h	4.96 ± 0.65gh
Na 50, NPs Foliar (100 mg/L)	1.132 ± 0.097f	16.64 ± 1.06fg	1.077 ± 0.10fg	7.21 ± 1.23efg
Na 100, NPs 0	0.167 ± 0.057jkl	5.68 ± 0.80k	0.327 ± 0.12l	1.46 ± 0.61k
Na 100, NPs Priming (100 mg/L)	0.204 ± 0.010jk	7.32 ± 1.02jk	0.436 ± 0.06k	2.11 ± 0.93j
Na 100, NPs Foliar (100 mg/L)	0.234 ± 0.030jk	8.23 ± 0.70jk	0.561 ± 0.11ij	2.52 ± 0.63ij
Biochar				
Sole	2.152 ± 0.156b	30.34 ± 1.31c	1.846 ± 0.10bc	12.64 ± 1.15bc
Na 0, NPs Priming (100 mg/L)	2.264 ± 0.123ab	33.70 ± 1.09b	2.081 ± 0.07ab	14.72 ± 0.86b
Na 0, NPs Foliar (100 mg/L)	2.440 ± 0.081a	37.77 ± 1.20a	2.270 ± 0.12a	17.58 ± 0.94a
Na 50, NPs 0	1.148 ± 0.096f	17.44 ± 1.44f	0.965 ± 0.09gh	5.84 ± 0.99f
Na 50, NPs Priming (100 mg/L)	1.428 ± 0.090e	22.95 ± 1.71e	1.228 ± 0.11ef	8.26 ± 1.59ef
Na 50, NPs Foliar (100 mg/L)	1.741 ± 0.111cd	26.07 ± 2.04d	1.411 ± 0.21de	10.58 ± 1.51cd
Na 100, NPs 0	0.184 ± 0.030l	6.64 ± 1.06j	0.396 ± 0.13kl	1.81 ± 0.86jk
Na 100, NPs Priming (100 mg/L)	0.291 ± 0.060j	10.05 ± 1.23ij	0.529 ± 0.10jk	2.45 ± 0.49ij
Na 100, NPs Foliar (100 mg/L)	0.349 ± 0.055i	11.25 ± 2.03hi	0.667 ± 0.17hij	2.86 ± 0.66ij

Table 3. Effect of ZnO NPs (0, 100 mg/L priming and foliar 0, 100 mg/L) alone and combined with biochar on gas exchange parameters (stomatal conductance, photosynthetic rate, transpiration rate and water use efficency) of spinach grown under salinity stress (0, 50 and 100 mM). The values depict the average of three replicates ($n = 3$) with standard deviation. Small letters in the each column indicate that values are significant different form each other according to Tukey's test at probability level $p \leq 0.05$.

under salt stress (100 mM). At foliar ZnO NPs with biochar combined treatment further reduction ($p \leq 0.05$) in roots and leaves H_2O_2 55.16, 58.35% MDA 68.12, 49.56% and EL 58.21, 61.31% respectively, under salt stress (100 mM) as compared to respective control.

Effect of ZnO NPs (foliar/priming) alone and with biochar on total phenolics in the spinach

Our results depicted that salt concentration significantly reduced the total phenolics in spinach roots and shoots with increasing concentration compared to the control. The lowest concentration was found at 100 mM salt stress as compared to the control. Whereas, the ZnO NPs (priming, foliar) with and without biochar significantly increased total phenolics under salt stress (100 mM) as shown in (Fig. 9). The combined treatment of ZnO NPs priming and foliar with biochar yielded remarkable and significant ($p \leq 0.05$) results as compared to alone application treatments of nanoparticles. Specifically, ZnO NPs priming and foliar alone treatments increased the total phenolics in the roots by 70.37, 93.77% and in shoot by 32.65,65.27% respectively, under salt stress (100 mM) as compared to their respective control. The priming ZnO NPs with biochar increased root total phenolics by 70.73% and shoot phenolics by 120.14% respective to their control. Furthermore, the maximum increased total phenolics was observed at foliar ZnO NPs with biochar which was 92.76% in root and 148.13% in leaves under salt stress (100 mM) as compared to the respective control.

Effect of ZnO NPs (foliar/priming) alone and with biochar on nutrient contents in the spinach

Our results showed that the concentration of macro and micronutrients in the roots and leaves of spinach was decreased by increasing salt stress concentrations. However, significantly ($p \leq 0.05$) lower concentrations were observed under higher salt stress (100 mM) as compared to control. The application of ZnO NPs (priming, foliar) alone increased nutrients in spinach root and leaves and this increase was further enhanced significantly ($p \leq 0.05$) with the combined application of NPs with biochar treatments, as shown in (Table 4). The priming ZnO NPs alone increased spinach root and leaves Zn 48.69, 41.60%, Fe 39.40, 36.83%, Mn 23.10, 42.96%, Mg 47.99, 36.38%, K 24.78, 34.05% respectively. While, foliar ZnO NPs alone significantly ($p \leq 0.05$) increased spinach root and leaves Zn 59.46, 60.54%, Fe 48.30, 56.93%, Mn 43.66, 62.48%, Mg 59.60, 48.38%, K 41.95, 53.18% respectively under salt stress (100 mM) as compared to respective control. The combined application of priming ZnO NPs with biochar further increased the root and leaves Zn 67.40, 58.03%, Fe 52.08, 58.13%, Mn 43.37, 64.58%, Mg 61.51, 58.48%, K 49.59, 61.32% respectively as compared to respective control. The highest increment was observed at foliar ZnO NPs with biochar treatment which was 71.82%, 64.89%, 78.06%, 69.43%, and 80.89%, in the root Zn, Fe, Mn, Mg, and K respectively, as compared to respective control. Similarly, the prominent increased contents of leaves Zn 81.78%, Fe 78.50%, Mn 79.8%, Mg 73.14%, and K 84.60% recorded at combined treatment of foliar ZnO NPs with biochar under 100 mM salt stress as compared to respective control. Overall, results

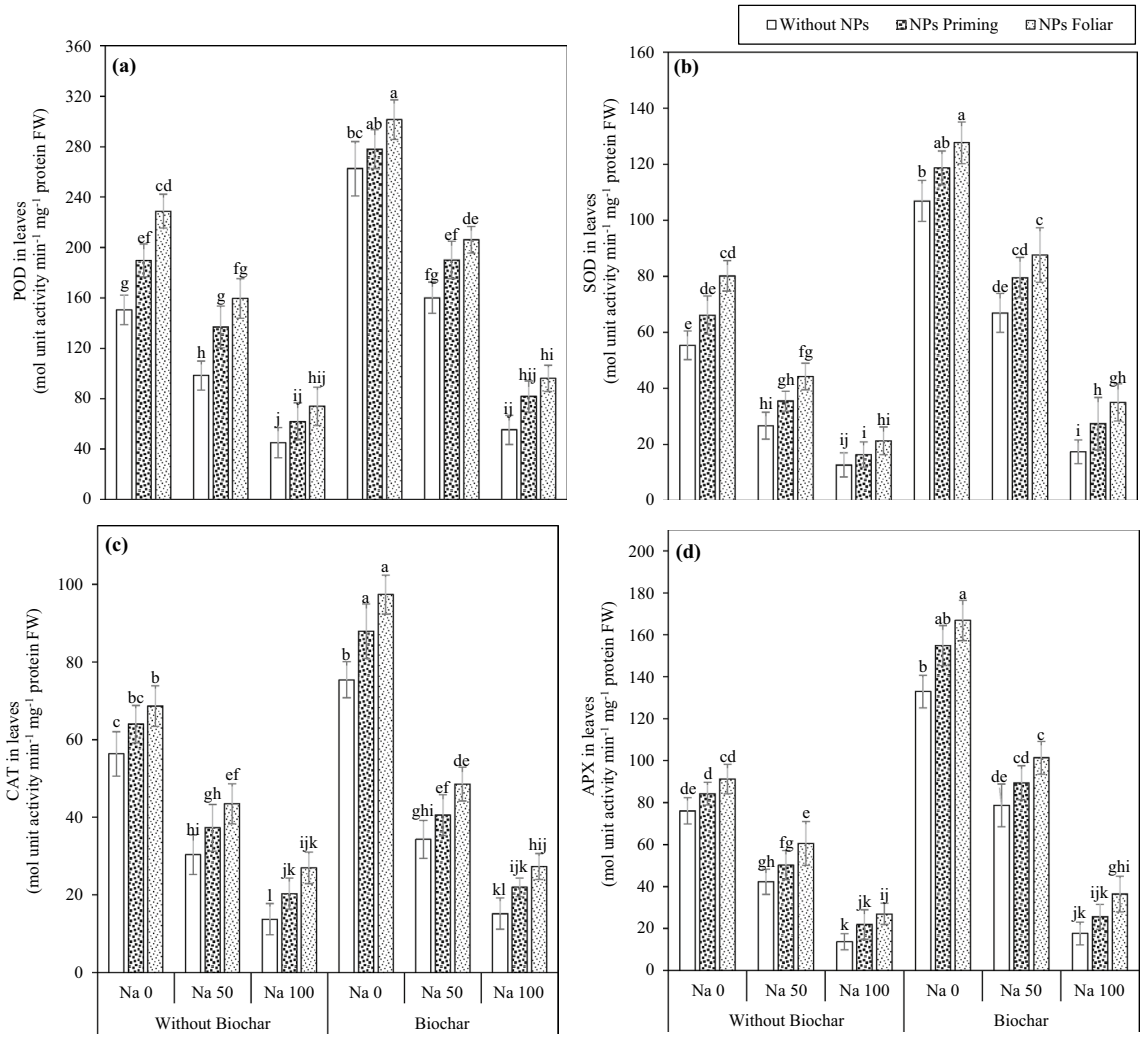

Figure 5. Effect of ZnO NPs (0, 100 mg/L priming, and foliar 0, 100 mg/L) alone and combined with biochar on (**a**) peroxidase (POD) in leaves, (**b**) superoxide dismutase (SOD) in leaves, (**c**) catalase (CAT) in leaves, and (**d**) ascorbate peroxidase (APX) in leaves of spinach grown under salinity stress (0, 50 and 100 mM). Error bars denoted the standard deviations of the measured data having three replications ($n = 3$). Small letters on the error bars denoted the statistical significance among the treatments determined by Tukey's test ($p \leq 0.05$).

demonstrated that the combine application of foliar ZnO NPs significantly ($p \leq 0.05$) showed more remarkable results with the combined treatment of biochar compared to all other treatments.

Effect of ZnO NPs (foliar/priming) alone and with biochar on Na^+ content in spinach

The Na^+ concentration of the root and leaves of spinach was increased with increasing concentrations of salt stress. Notably, the highest concentration of Na^+ in plant roots was found under 100 mM salt stress. A similar trend in Na^+ content was observed in spinach leaves ZnO NPs in both priming and foliar treatment either alone or in combination with biochar reduced the uptake of Na^+ from roots to leaves under salt stress (100 mM) (Fig. 10). ZnO NPs priming and foliar alone treatments reduced Na^+ content in the roots by 9.32, 21.71% and Na^+ in shoot by 12.19, 27.40% respectively, under salt stress (100 mM) as compared to their respective control. The combined treatment of ZnO NPs priming and foliar with biochar yielded more positive and significant ($p \leq 0.05$) results as compared to alone application treatments of nanoparticles. The priming ZnO NPs with biochar reduced Na^+ content in root by 49.70%, shoot 45.44%, respective to their control. Furthermore, the maximum reduction in Na^+ content was observed at foliar ZnO NPs with biochar which was 57.69% root and 61.27% decrease in leaves under salt stress (100 mM) as compared to the respective control.

Pearson correlation analysis

The Pearson correlation analysis identified multiple significant correlations among the investigated variables. Furthermore, the correlation between Na^+ contents and key plant parameters was carried out using Pearson correlation (Fig. 11). Results demonstrated that the strong negative ($p \leq 0.05$) association between Na^+ levels and key plant parameters including shoot length, chlorophyll contents, and nutrients can be attributed to a complex interplay of oxidative stress and plant physiological responses.

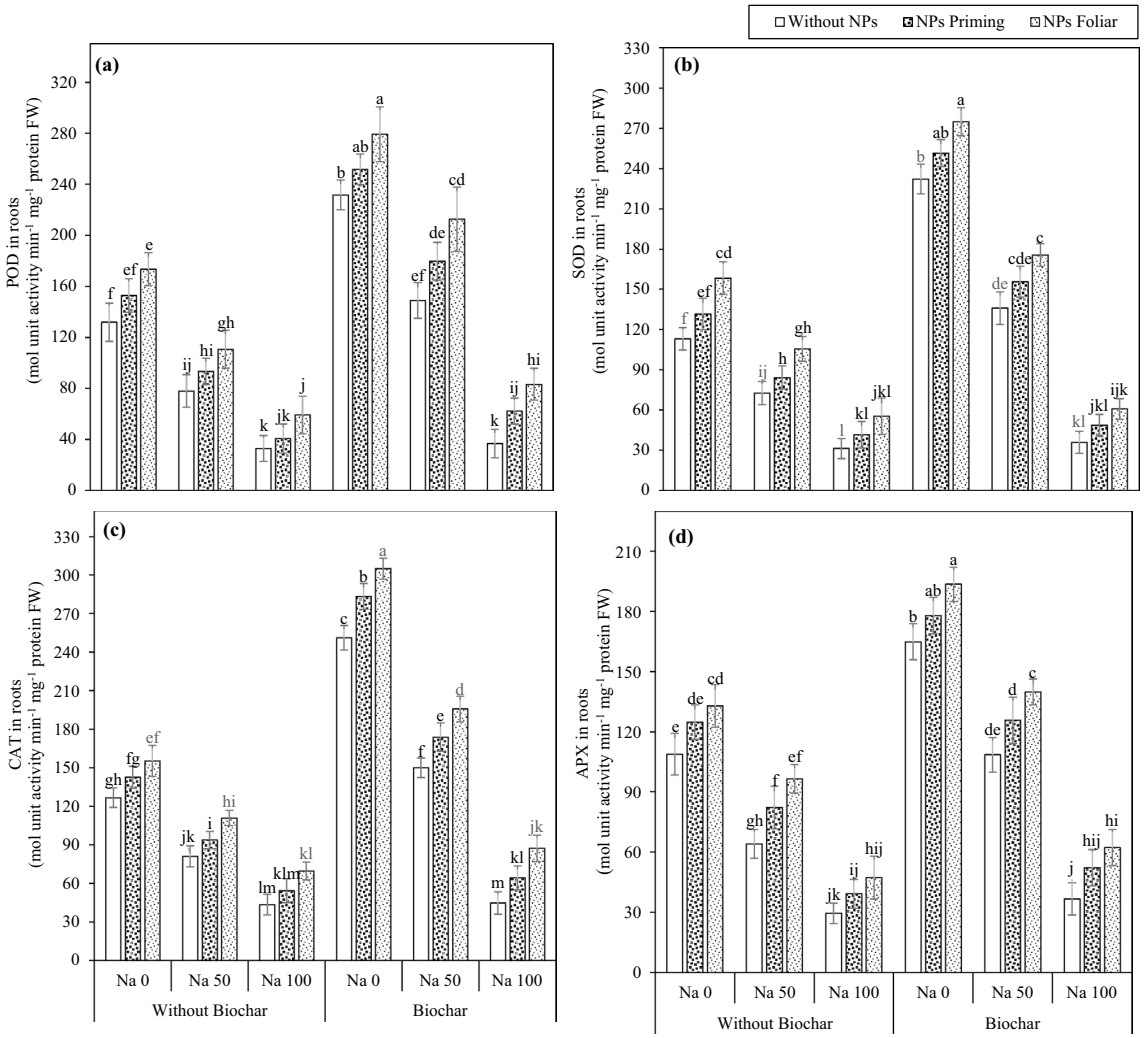

Figure 6. Effect of ZnO NPs (0, 100 mg/L priming, and foliar 0, 100 mg/L) alone and combined with biochar on (**a**) peroxidase (POD) in roots, (**b**) superoxide dismutase (SOD) in roots, (**c**) catalase (CAT) in roots, and (**d**) ascorbate peroxidase (APX) in roots of spinach grown under salinity stress (0, 50 and 100 mM). Error bars denoted the standard deviations of the measured data having three replications ($n=3$). Small letters on the error bars denoted the statistical significance among the treatments determined by Tukey's test ($p \leq 0.05$).

Principal component analysis

Principal component analysis (PCA) was performed on the dataset, identifying two principal components (PC1 and PC2) that explained a significant portion of the total variance. PC1 explained 93.3% of the variability showing that it carries most of the information within the dataset. PC2 explained an additional 3.0% of the variance (Fig. 12).

Discussion

The present study aimed to investigate the impact of ZnO NPs, applied through both priming and foliar methods, individually and in combination with biochar, in alleviating salt stress effects on spinach. Results revealed that salt stress significantly reduced plant morphological parameters (Fig. 3). The priming and foliar ZnO NPs with and without biochar greatly improved the growth attributes of spinach plants under salt stress. Previous studies by Silva et al.[58] and Liu et al.[59] have demonstrated the efficacy of biochar in improving soil structure and nutrient retention, leading to notable advancements in plant height, leaf area, and overall biomass. The presence of elements like C, N, S, Na, and Cl in biochar indicates a positive role in improving crop nutritional quality and soil fertility, ultimately improving crop growth[60,61]. The role of ZnO NPs in influencing key plant growth parameters has been highlighted by previous Studies[62,63]. Our results are in line with Ali et al.[46] who depicted that 100 mg/L ZnO NPs efficiently ameliorated the salt stress (100 mM) and improved biomass of barley.

Current results revealed that salt stress significantly reduced the SPAD values, chlorophyll, carotenoid contents, and photosynthetic efficiency in spinach plants. However, priming and foliar application of ZnO NPs significantly boost chlorophyll levels and photosynthetic efficiency and the effect was more pronounced with the combined application of nanoparticles and biochar treatments (Fig. 4, Table 2). Previous studies such as Arruda et al.[64] and Yang et al.[65], have reported the vulnerability of chlorophyll and photosynthesis to salinity stress, with

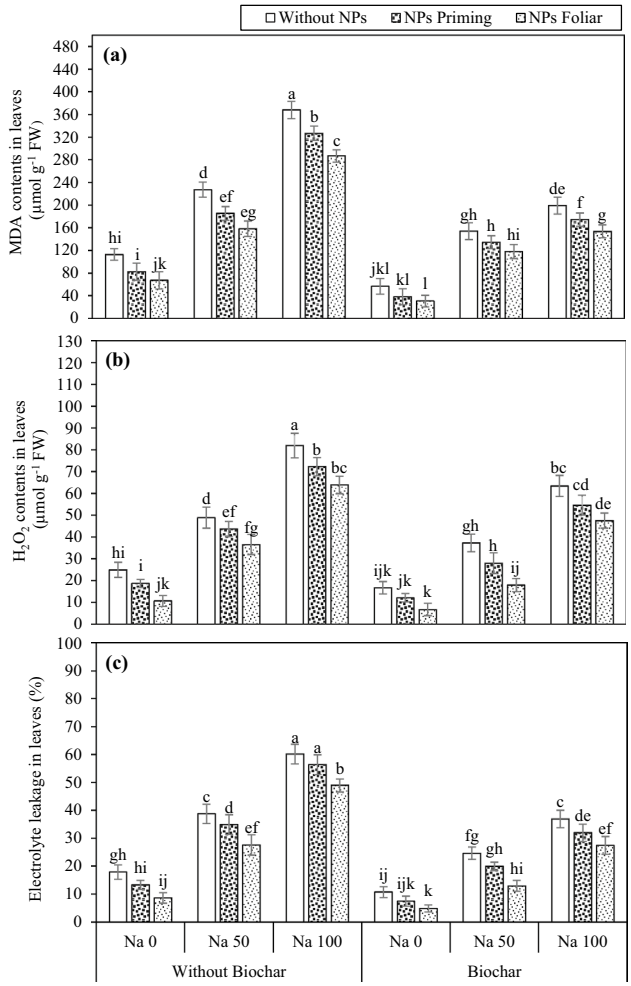

Figure 7. Effect of ZnO NPs (0, 100 mg/L priming, and foliar 0, 100 mg/L) alone and combined with biochar on (**a**) malondialdehyde contents (MDA) in leaves, (**b**) hydrogen peroxide contents (H_2O_2) in leaves and (**c**) electrolyte leakage (EL) in leaves of spinach grown under salinity stress (0, 50 and 100 mM). Error bars denoted the standard deviations of the measured data having three replications ($n = 3$). Small letters on the error bars denoted the statistical significance among the treatments determined by Tukey's test ($p \leq 0.05$).

decreases in chlorophyll content and photosynthetic rates being common manifestations. Our findings align with those of Chen et al.[66] and Helaoui et al.[67], who demonstrated that biochar incorporation contributed to the maintenance of chlorophyll content and the preservation of efficient photosynthetic activity under saline conditions. Additionally, NPs as elucidated by Chen et al.[68] and Ali et al.[46], have shown potential in enhancing photosynthetic processes by mitigating oxidative stress. ZnO NPs enhanced the uptake of essential nutrients, leading to the restoration of photosynthesis in plants under salinity stress[69].

Electrolyte leakage, H_2O_2, and MDA contents are indicators of oxidative stress and negatively affect plant growth[46,70]. The salt stress induces oxidative stress in spinach plants which was evident from increasing levels of reactive oxygen species (ROS) including H_2O_2, MDA, and EL) in both leaves and roots of spinach plants shown in (Figs. 7 and 8). Previous investigations by Yasemin et al.[71] and Lamsaadi et al.[72] have highlighted the role of EL, H_2O_2 and MDA content as reliable indicators of oxidative damage in plants exposed to salinity stress. On the other hand, ZnO NPs decreased the ROS in spinach either alone or with biochar treatment. This aligns with the observations of Din et al.[73], who reported a reduction in EL, H_2O_2, and MDA content in plants treated with biochar under saline conditions. Furthermore, nanoparticles, as explored by[74] and Junedi et al.[75], exhibited antioxidant properties, mitigating oxidative stress in plant cells. The ZnO NPs facilitate Fe^{+2} ions absorption in different plant tissues via the xylem and phloem. This uptake and distribution of Fe^{+2}, facilitated by ZnO NPs plays a vital role in the alleviation of oxidative stress caused by salinity[69]. These results underscore the potential of biochar and nanoparticles in ameliorating oxidative stress, providing a protective shield against salinity-induced cellular damage. Antioxidants enzymes activities (SOD, POD, CAT, and APX) are the indicator of plants natural defensive system against any type of stress. Our findings indicated that salt stress significantly reduced the activities of antioxidant enzymes activities, but ZnO NPs priming and foliar application with and without biochar application increased antioxidant enzyme activities shown in (Figs. 5 and 6). Many studies have

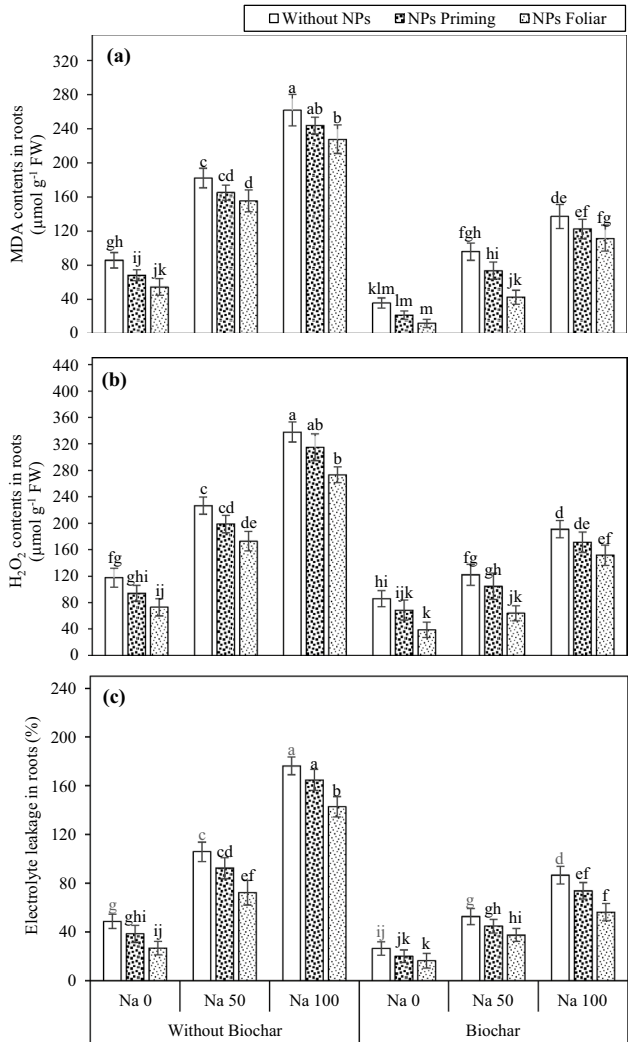

Figure 8. Effect of ZnO NPs (0, 100 mg/L priming, and foliar 0, 100 mg/L) alone and combined with biochar on (**a**) malondialdehyde MDA contents in roots, (**b**) hydrogen peroxide contents (H_2O_2) in roots and (**c**) electrolyte leakage in roots of spinach grown under salinity stress (0, 50 and 100 mM). Error bars denoted the standard deviations of the measured data having three replications ($n = 3$). Small letters on the error bars denoted the statistical significance among the treatments determined by Tukey's test ($p \leq 0.05$).

emphasized the vital function of these enzymes in reducing oxidative stress by eliminating reactive oxygen species (ROS)[76,77]. The use of biochar has shown a significant improvement in the activities of antioxidant enzymes. The results are consistent with the research conducted by Nawaz et al.[78] and Kumari and Malaviya et al.[79] which showed that biochar can enhance the activities of antioxidant enzymes, hence strengthening the plant's ability to defend against oxidative stress. Moreover, nanoparticles, as explored by Adrees et al.[80] and Hussain et al.[81], have been shown to enhance the activities of antioxidant enzymes in wheat plants. This enhancement in antioxidant enzyme activities underscores the role of biochar and nanoparticles and provides an effective countermeasure against salinity-induced oxidative stress.

Salinity stress often disrupts nutrient uptake mechanisms, impacting the availability of essential nutrients. The results of the current study indicated that the concentration of macro and micronutrients in the roots and leaves of spinach was decreased by increasing salt stress concentrations and a significant decrease in nutrients was observed under higher salt stress (100 mM) as compared to control.Our results also demonstrated strong negative correlation Na^+ levels and nutrient content (Fig. 11). In this scenario, higher Na^+ concentrations suggest a reduction in the uptake of essential nutrients. The observed negative association between shoot length and certain variables may be attributed to impaired cellular integrity, which might impede the uptake of nutrients and water. Previous studies by Ntanasi[82] and Huang et al.[83] have emphasized the sensitivity of nutrient uptake to salinity stress in plants. However, the incorporation of biochar into the soil exhibited a notable improvement in nutrient uptake efficiency. Biochar enhances nutrient uptake by plants by improving soil structure, regulating the pH, improving water retention, and through reduction of nutrient immobilization. Biochar formed under higher temperatures settings exhibits a multitude of high-quality surface pores, allowing for efficient transmission and accommodation of several nutrients[84] thereby, preventing the leaching of nutrients[85–87]. The utilization of

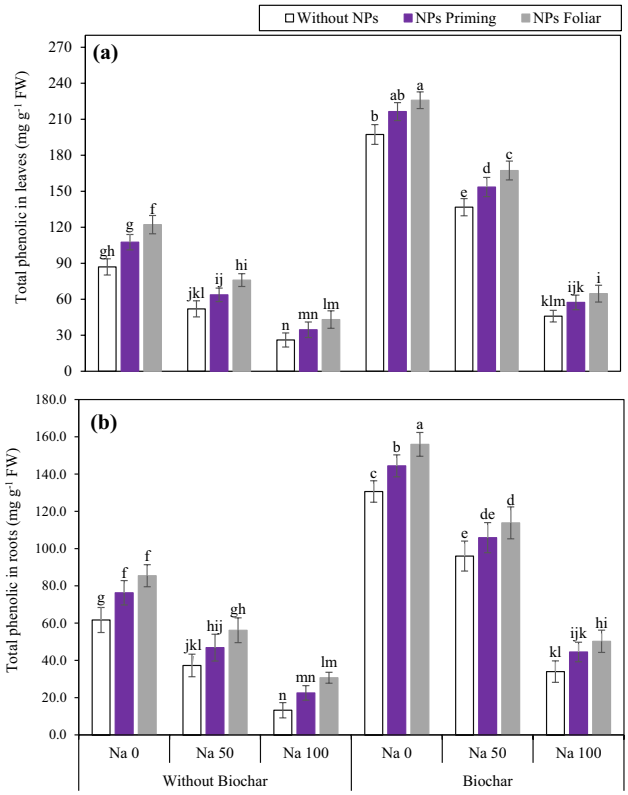

Figure 9. Effect of ZnO NPs (0, 100 mg/L priming, and foliar 0, 100 mg/L) alone and combined with biochar on (**a**) total phenolic in leaves and (**b**) total phenolic in roots of spinach grown under salinity stress (0, 50 and 100 mM). Error bars denoted the standard deviations of the measured data having three replications ($n = 3$). Small letters on the error bars denoted the statistical significance among the treatments determined by Tukey's test ($p \leq 0.05$).

ZnO NPs alone resulted in a higher level of nutrients in both the root and leaves of spinach and this increase was further enhanced ($p \leq 0.05$) with the combined application of NPs with biochar treatments, as shown in Table 4. Due to smaller size and greater adsorption capacity, NPs can facilitate in transportation of nutrients in cells. Moreover, NPs modulate genes that improve nutrient uptake and promote the nutrient assimilation-related process in plants in saline environments. Previous studies such as Ijaz et al.[88] and Ali et al.[46] have demonstrated the potential to enhance nutrient uptake through improved root nutrient assimilation processes.

Salinity leads to a higher accumulation of sodium ions (Na^+) in plant tissues, which disturbs the ionic balance inside cells and hinders important physiological functions. Our findings showed that the Na^+ concentration of root and leaves of spinach was increased with increasing concentrations of salt stress (Fig. 10). Our study confirmed a substantial increase in sodium uptake by plants subjected to salinity stress, indicative of the challenges posed by salt accumulation. However, the incorporation of biochar into the soil demonstrated a remarkable reduction in sodium uptake. Biochar has a higher cation exchange capacity due to which it has a greater potential to reduce the uptake of Na^{+2} ions by plants. In addition, biochar mitigates salt stress in plants through its sorption capacity[89] and it depends on the functional groups[90,91]. These findings are consistent with the research conducted by Din et al.[73] and Wu et al.[92], emphasizing the significance of biochar in reducing (Na^+) accumulation in plants exposed to salt stress. In addition, the use of nanoparticles has been studied by Junedi et al.[75] and Soliman et al.[93]. have shown promise in regulating ion transport processes, including sodium uptake. The reduction in Na^{+2} ions by plants may be because ZnO NPs enhance the uptake of essential nutrients, which resulted in the completion of the uptake of Na^{+2} ions and essential nutrients. So, in this way, there would be a reduction in the uptake of Na^{+2} ions by plants due to the application of ZnO NPs. Moreover, ZnO NPs might have enhanced the antioxidant enzymatic activities in plants, resulting in the hindrance of Na^{+2} ions uptake by plants. Notably, Na^+ uptake, a critical factor in salinity stress, was effectively mitigated by the combined use of biochar and nanoparticles. Overall the current study exhibited that the foliar application of ZnO NPs was more efficient than that of seed priming and previous studies such as Ali et al.[94], Parveen and Siddiqui[95] and Adrees et al.[96] also validate our findings. Foliar application of nanoparticles surpasses seed priming in enhancing plant resilience, primarily due to its efficient absorption mechanism and targeted actions within the plant[97] due to their small size and unique physicochemical properties enable rapid penetration through the leaf surfaces, utilizing cuticles and stomata for entry[98]. Furthermore, foliar spray facilitates a more uniform distribution of nanoparticles, ensuring consistent coverage across various plant tissues[99]. The outcome of the present study underscores the role of biochar and

Treatments	Shoots					Roots				
	Micronutrients (μ g g^{-1} DW)			Macronutrients (mg g^{-1} DW)		Micronutrients (μ g g^{-1} DW)			Macronutrients (mg g^{-1} DW)	
	Zn	Fe	Mn	Mg	K	Zn	Fe	Mn	Mg	K
Without biochar										
Control	131.0 ± 8.28fg	88.4 ± 5.3ef	156.2 ± 12.11ef	3956.6 ± 332.18gh	4210.4 ± 278.39efg	64.36 ± 4.32g	952.57 ± 88.8de	234.9 ± 17.61d	3532.6 ± 309.87f.	2646.1 ± 197.04fg
Na 0, NPs Priming (100 mg/L)	144.8 ± 8.90ef	113.3 ± 10.5d	181.9 ± 16.30de	4486.3 ± 342.30efg	4942.5 ± 319.57ef	73.01 ± 3.89fg	1094.6 ± 95.2cd	246.8 ± 15.70cd	3883.5 ± 240.97ef	2925.2 ± 288.74ef
Na 0, NP Foliar (100 mg/L)	166.8 ± 8.50de	132.1 ± 10.2c	207.0 ± 19.42c	5150.4 ± 264.02de	5568.1 ± 303.14de	81.67 ± 2.80f	1241.5 ± 111.01c	265.9 ± 13.93c	4461.4 ± 432.68e	3216.8 ± 233.46de
Na 50, NP 0	85.96 ± 8.23ij	46.8 ± 7.8hij	63.6 ± 10.01gh	2253.7 ± 198.30ij	2516.8 ± 302.78ij	41.33 ± 4.56hi	601.17 ± 86.2gh	119.2 ± 13.61fg	1877.8 ± 115.92 hij	1453.5 ± 109.53ij
Na 50, NP Priming (100 mg/L)	99.23 ± 5.04hi	55.0 ± 8.4gh	80.2 ± 13.69g	3082.8 ± 217.57hi	3225.9 ± 234.04hi	47.62 ± 3.58h	731.27 ± 64.2efg	138.0 ± 8.68f	2253.6 ± 98.30gh	1830.1 ± 166.89h
Na 50, NP Foliar (100 mg/L)	117.58 ± 8.04g	68.4 ± 5.9fg	119.9 ± 16.65f.	3679.4 ± 248.98h	3973.6 ± 183.89ghi	60.94 ± 4.20g	831.90 ± 160.2ef	152.6 ± 11.67ef	2640.1 ± 170.92g	2221.2 ± 152.75gh
Na 100, NP 0	32.67 ± 5.80m	20.7 ± 6.8k	28.6 ± 7.05i	1180.7 ± 313.69l	1474.1 ± 213.80k	21.21 ± 2.52j	390.3 ± 33.6i	52.5 ± 8.56j	1058.5 ± 219.84k	795.1 ± 145.78m
Na 100, NP Priming (100 mg/L)	46.30 ± 6.32klm	28.3 ± 7.6jk	43.2 ± 9.68hi	1610.3 ± 214.65jkl	2064.6 ± 205.18ijk	31.53 ± 2.94hij	544.0 ± 50.2hi	64.6 ± 6.85i	1640.5 ± 170.32ijk	992.01 ± 174.06kl
Na 100, NP Foliar (100 mg/L)	57.01 ± 5.37kl	35.0 ± 6.3ijk	50.9 ± 9.08ghi	1933.6 ± 451.31jk	2582.5 ± 305.85hij	37.41 ± 2.68ghi	619.3 ± 110.9fgh	85.4 ± 8.20hi	1874.6 ± 190.64hij	1208.2 ± 168.13jk
Biochar										
Sole	244.4 ± 11.37b	218.5 ± 8.0b	315.3 ± 14.79b	7412.9 ± 779.90c	9153.9 ± 613.59c	135.3 ± 6.54c	1540.3 ± 115.5b	379.2 ± 16.61b	7564.8 ± 242.08c	4903.8 ± 194.91b
Na 0, NP Priming (100 mg/L)	255.2 ± 13.55b	239.9 ± 11.5 ab	342.6 ± 16.70ab	8416.9 ± 631.49b	10,892.5 ± 1356.23b	152.9 ± 10.56b	1627.2 ± 112.3b	404.7 ± 17.37b	8850.5 ± 360.99b	5211.5 ± 172.52ab
Na 0, NP Foliar (100 mg/L)	276.2 ± 11.28a	256.4 ± 13.4a	368.5 ± 27.62a	9466.9 ± 536.27a	12,579.2 ± 642.97a	168.6 ± 3.61a	1861.3 ± 93.1a	443.3 ± 20.23a	10,568.8 ± 428.51a	5524.8 ± 268.44a
Na 50, NP 0	156.6 ± 9.48e	96.5 ± 9.2de	152.5 ± 11.14ef	4149.4 ± 268.23fgh	4703.5 ± 504.41efg	84.6 ± 7.85ef	965.0 ± 111.1de	175.9 ± 10.26e	5458.1 ± 315.49d	3118.2 ± 256.46def
Na 50, NP Priming (100 mg/L)	173.9 ± 11.01cd	114.5 ± 10.1d	171.5 ± 17.33de	4898.2 ± 510.31ef	5474.5 ± 640.84ef	96.0 ± 10.08e	1075.1 ± 108.1cd	227.0 ± 13.82d	6114.5 ± 145.21d	3617.01 ± 150.42d
Na 50, NP Foliar (100 mg/L)	185.9 ± 12.07c	129.2 ± 12.9cd	192.5 ± 17.10cd	5487.6 ± 273.52d	6603.1 ± 707.89d	109.3 ± 7.51d	1251.7 ± 104.3c	259.3 ± 16.60cd	7103.9 ± 689.68c	4152.3 ± 98.70c
Na 100, NP 0	34.70 ± 5.01mn	23.9 ± 6.8jk	33.5 ± 8.56ij	1416.1 ± 212.27kl	1630.5 ± 365.44jk	24.9 ± 1.73ij	484.9 ± 68.1hi	63.1 ± 11.05ij	1194.3 ± 237.61jk	861.5 ± 86.15lm
Na 100, NP Priming (100 mg/L)	54.3 ± 9.17kl	39.7 ± 9.43hij	51.4 ± 8.87ghi	1986.8 ± 474.28ijk	2510.8 ± 650.55ij	37.2 ± 5.12hi	632.6 ± 88.3fgh	84.2 ± 5.51hi	1741.3 ± 267.48ij	1292.8 ± 220.30 jk
Na 100, NP Foliar (100 mg/L)	67.6 ± 10.11j	52.8 ± 11.49ghi	64.2 ± 13.24gh	2377.9 ± 600.09ij	3369.8 ± 806.42ghi	43.01 ± 3.81hi	731.4 ± 92.3efg	98.2 ± 11.50gh	2040.2 ± 386.52ghi	1514.7 ± 304.55ij

Table 4. Effect of ZnO NPs (0, 100 mg/L priming and foliar 0, 100 mg/L) alone and combined with biochar on macronutrients and micronutrients in different parts of spinach grown under salinity stress (0, 50 and 100 mM). The values depict the average of three replicates ($n = 3$) with standard deviation. Small letters in the each column indicate that values are significant different form each other according to Tukey's test at probability level $p \leq 0.05$.

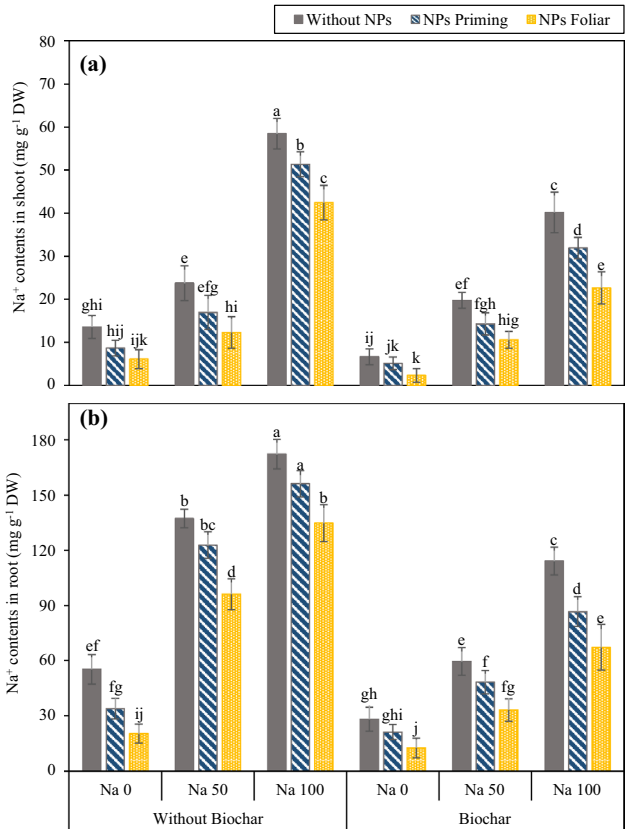

Figure 10. Effect of ZnO NPs (0, 100 mg/L priming, and foliar 0, 100 mg/L) alone and combined with biochar on (**a**) Na^+ contents in leaves and (**b**) Na^+ contents in roots of spinach grown under salinity stress (0, 50 and 100 mM). Error bars denoted the standard deviations of the measured data having three replications ($n = 3$). Small letters on the error bars denoted the statistical significance among the treatments determined by Tukey's test ($p \leq 0.05$).

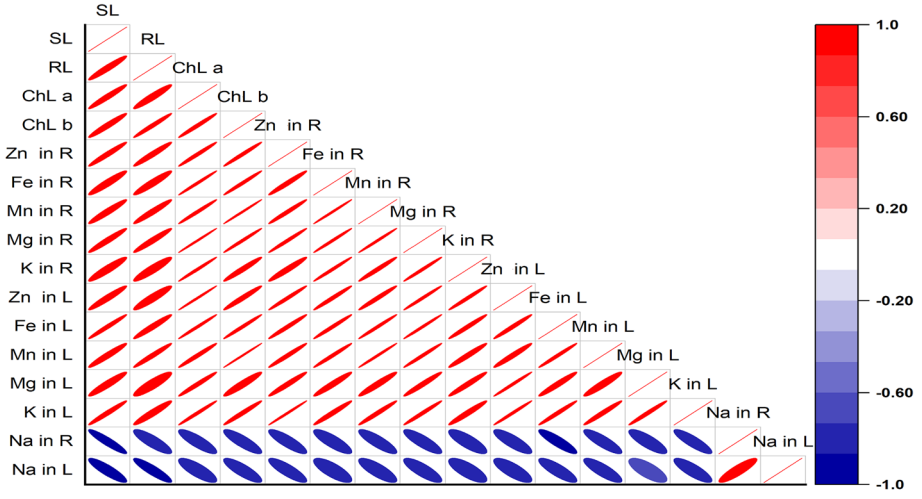

Figure 11. Pearson correlation for the studied spinach different parameters. The results are displayed in a correlation matrix, where negative correlations are indicated by blue while positive correlations are indicated by red.

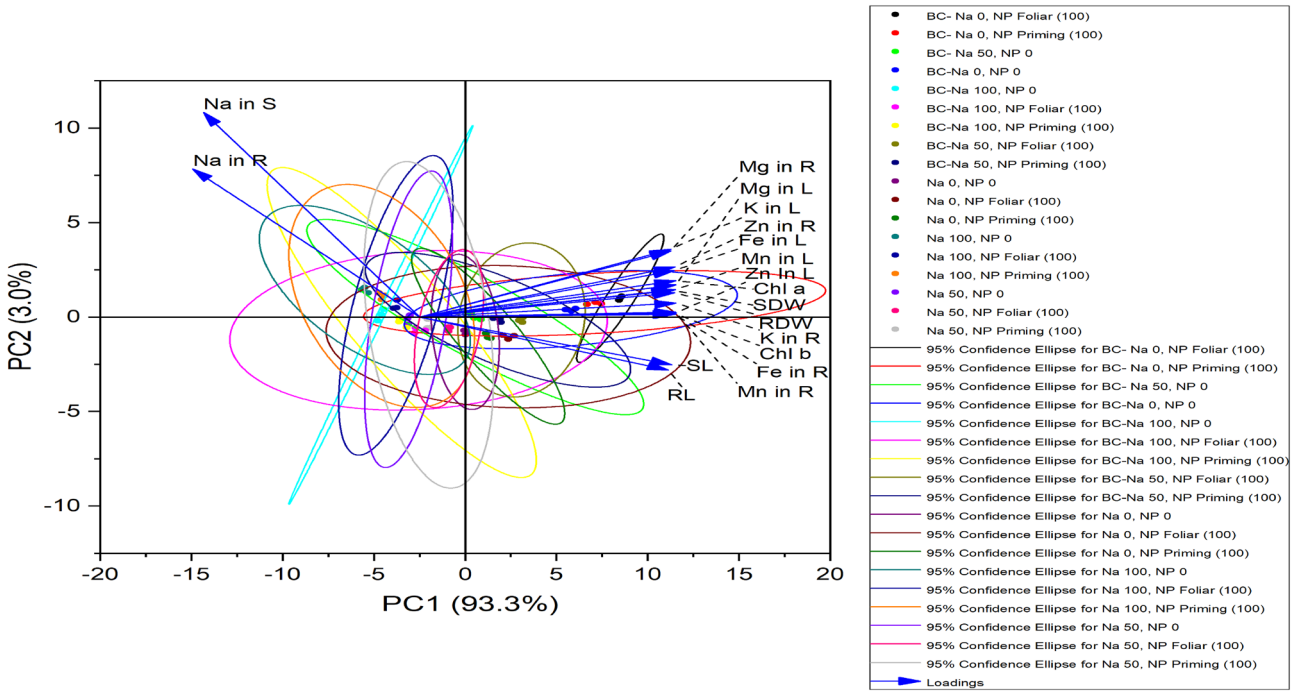

Figure 12. Principal component analysis for studied spinach different parameters.

nanoparticles not only in mitigating the negative impacts of salinity stress but also in promoting the sustained nutritional well-being of plants in challenging environments.

Conclusion

The current study highlighted that ZnO NPs (priming and foliar) could improve spinach growth performance under salinity stress. However, ZnO NPs with combination of biochar amendments remarkably improved plant growth and physiological attributes under salinity stress. Combination of ZnO NPs with biochar significantly improved growth performance by augmentation of chlorophyll content, gas exchange parameters and antioxidant enzymes activity such as SOD, CAT, APX in spinach under saline soil. The favorable response in reducing the soil Na^+ content was found higher when foliar ZnO NPs combined with biochar amendment. Similarly, the higher nutrient accumulation was also observed at combined treatments of ZnO NPs with biochar, notably at foliar ZnO NPs treatment with biochar. Conclusively, the incorporation ZnO NPs with biochar amendment is crucial for maintaining soil and crop nutrient balance for improving soil quality under salinity stress. Furthermore, studies at field level with diverse crops, biochar, and NPs are required to assess their ameliorative effect under salinity before any recommendations to farmers.

Data availability

All data generated or analyzed during the study are included in this article.

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Author contributions

S.A.: Performed the design of experiment, formal analysis, carried out the experiment and original drafted the manuscript. A.K.S.: Writing—review and editing. A.H.: Writing—review and editing. L.Z.: Data visualization. S.O.A.: Formal analysis. A.A.: Formal analysis, revising it critically for intellectual content. K.A.A.-G.: Writing—review and editing. M.A.A.: Investigation. S.A. and P.K.S.: Supervision, experiment design, resources, funding acquisition, investigation.

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Competing interests

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