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Supplementation of nano-biochar improved growth and physiological attributes in wheat seedlings exposed to salt stress through enhanced activity of hydrolysing and nitrogen metabolic enzymes and regulation of crucial metabolites

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ABSTRACT

Nano-biochar (NBC) amendment could be an effective solution for sustainable agricultural production in salt affected areas. Nano-biochar application have shown positive effects on plant germination. But the under veiling mechanism of germinating seeds under NBC application still needs to explore. In this regard, two wheat varieties were used to assess the effects of NBC application on growth, germination indices, hydrolyzing and nitrogen metabolic enzymes as well as on plant metabolites at early germination stage under salt stress. Petri-plate experiments were performed at The University of Lahore, and 4 levels of NBC supplementation (0 %, 1 %, 3 %, and 5 %) were used both under salt stress (80 mM) and non-stress (0 mM). The results clearly showed that salt stress reduced the growth parameters such as radicle length, plumule length, seedling weight and germination indices such as final germination percentage (FGP), germination index (GI), and germination rate index (GRI) but increased the germination mean time (GMT). NBC in all concentrations specifically 5 % NBC elevated growth and germination indices of wheat seeds as well as reduced the mean germination time of seed sprouting both under stress and non-stress conditions. Further, temporal variations were observed in hydrolyzing and nitrogen metabolic enzymes and results showed that salt stress exerted negative effect on activities of α-amylase, Protease and Nitrate reductase even though activities of these enzymes increased in a timely manner. But 5 % NBC application significantly improved the enzyme activities both under stress and non-stress conditions. To investigate the effect of NBC on plant metabolites, Total free amino acids, total protein contents and total soluble sugars were estimated using spectrophotometry and results clearly showed that salt stress decreased the total free amino acids and total soluble sugars content but an incline was observed in total proteins which were reduced in a timely manner. However, 5 % application of NBC increased the free amino acids and soluble sugar contents but decreased the values of total proteins under stress or non-stress conditions. These results suggests an active involvement of NBC to improve early growth and germination indices of seeds and enhance hydrolyzing enzymes and plant metabolites under stress conditions.

1. Introduction

Environmental stress is a significant component that limits plant development and crop yield, and has an impact on the sustainability of agriculture (Balasubramaniam et al., 2023). Another important limiting factor for sustainable agriculture is salinity, salinity levels influence around 33 % of irrigated agriculture to varying degrees, and this figure may surpass 50 % by 2050 (Ali et al., 2023; Ahangar et al., 2020). Over 6 % of the world's total land area is impacted by salt stress, and over 70 nations have been identified as having significant salinity-affected areas (Amini et al., 2016). By limiting vegetative growth and delaying fertilization and subsequent seed formation, salinity is the most important ecological stressor impacting agricultural productivity and sustainable development in semi-arid and arid environments (Ayangbenro and Babalola, 2021). According to Masi et

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al. (2021), the majority of plants are salt-sensitive glycophytes. Through excessive salinity, harmful ionic effects, or peroxidation, salt stress can damage seedling development and survival (Horton et al., 2021). By reducing the concentrations of germination rate promoters like gibberellin (Gas), increasing the concentrations of abscisic acid (ABA), and affecting the permeability and water transportation within the seed (Petrik et al. 2022, Masi et al. (2021)). For glycophyte species to adapt to saline environments during seed germination and continued fertilization, seed priming is a crucial physiological technique (Sing et al., 2022). In contrast, roughly 29 % of the world's land surface is degrading due to factors such desertification, erosion, pollution, acidification, sodification, and salinization (Jia et al., 2019), which will permanently reduce the capacity of soil to store carbon (Ferreira et al., 2022). A promising strategy to increase soil quality and consequently crop yield is the application of biochar, a stable, C-rich by product produced from biomass (kamali et al., 2022; Usevičiūtė and Baltrėnaitė-Gedienė, E. Baltrėnaitė-Gedienė, 2020). According to Zhang et al. (2020), using biochar is a good way to regulate crop performance. In general, applying biochar could boost crop productivity (Yadav et al., 2023), however there were few research on alkaline soil under drought stress. Most agricultural fields are now unproductive as a result of the introduction of contemporary agricultural techniques, such as the excessive use of chemicals to increase agricultural production which in turn increased the greenhouse gases (Salehi et al., 2016). In this regard, the use of biochar is an efficient technique for reducing atmospheric CO^2 and greenhouse gas emissions to promote plant growth (Lehmann et al., 2021; Guo et al., 2022). Biochar is a carbon-rich result of the oxygen-starved, low-temperature burning of carbon-containing biomass such as agricultural leftovers, stall bedding, cull lumber, and sawmill wastes (Agegnehu et al., 2017). In places with low soil fertility and frequent stress outbreaks, using nanobiochar can therefore be an ideal solution for enhancing crop development and yield (Mahmud et al., 2020). The manufacture of nanomaterials in a variety of sizes and shapes has been made possible by recent improvements in manufacturing techniques. Due to their beneficial chemical and physical characteristics, nanomaterials in biochar have attracted a lot of recent attention among modified biochars (Lamaming et al., 2022). Nanomaterials can have good or detrimental impact on a plant and can enter through either the apoplast or the synplast, although, carbon nanoparticles are more beneficial to plants (Ramanayaka et al., 2020). Because they have a larger surface area and higher microporosity than their counterpart macroparticles. The function of manufactured carbon nanoparticles in boosting the growth and metabolism of tomato has been clarified (Mubashir et al., 2023). The main objectives of treating salt-affected soils are fostering plant growth and regaining primary productivity (Mukhopadhyay et al., 2021), as, biochar would increase plant photosynthesis and soil primary production, removing additional CO_2 from the environment (Horton et al., 2021; Liu et al., 2023). Seed germination and seedling establishment are hastened by hydrolytic enzymes such as α-amylase, protease, and acid phosphatases (Anwar et al., 2021). However, despite significant progress in understanding biochar's beneficial impacts on plant growth, there are still a lot of unknowns, such its effects in soils influenced by salinity. In this work, the effects of biochar addition on two varieties of wheat (Akbar and Zincol) and their physiological parameters were investigated under the stress of salinity. The primary goal of this study was to test the hypothesis that administration of Nanobiochar (NBC) could enhance wheat seedlings' salinity tolerance at germination-stage by increasing their germination qualities and enhancing biochemical activities.

2. Material and methods

By increasing the soil's ability to store water, biotic interactions, and provision of macro- and micronutrients, nano-biochar may boost crop output through a number of processes. In order to assess the impact of nano-biochar on germination indices, hydrolyzing enzyme activities, and some biochemical characteristics, a *petri*-plate experiment was carried out in the Botany Laboratory, Institute of Molecular Biology and Biotechnology, University of Lahore, Pakistan. Nano-biochar was added to petri plates in varying amounts (0 %, 1 %, 3 %, and 5 %) in three replications of each treatment and were set up using a completely randomized design (CRD). Twenty seeds from each of the two wheat varieties—Akbar and Zincol—were placed in each petri plate. After every 24 h, four seeds were weighed from each petri dish, and it was noted how many seeds germinated after 120 h. Plumule length, Radicle length, Seed Weight, and Number of germinated Seeds were calculated as growth metrics. There were several germination indices that were determined, including final germination percentage (G), germination index (GI), germination rate index (GRI), mean germination time (MGT), and imbibition percentage. α-amylase, protease, nitrate reductase, total proteins, total free amino acids, and total sugar contents were measured using the four seeds that were removed from each petri dish after 24 h.

2.1. $\alpha-$amylase activity of seedlings

One gram of the frozen, liquid nitrogen-treated seedling material was homogenized in ten millilitres of phosphate buffer (pH 7.2), extracted with cold 1 % NaCl, and centrifuged at 4000 rpm for thirty minutes. The supernatant was taken to measure the enzyme activity using the technique described by Chrispeels and Varner (1967). The enzyme activity was determined as mg of hydrolysed starch per gram of fresh weight h^{-1}.

2.2. Protease enzyme activity

The measurement of protease activity was conducted following the guidelines suggested by Ainouz's (1970). According to which, 1 g of seedlings were homogenized, then, 1 % NaCl was added to a 0.2 M phosphate buffer (pH 7.5), and the mixture was centrifuged at 12,000 rpm for 30 min. After that, 0.2 M sodium phosphate buffer (pH 6.0) solution and 5 mL of 1 % casein solution were incubated at 50 °C. After 60 min, 1 mL of 40 % TCA was added to stop the reaction (Ainouz, 1970). After treating with the Folin Phenol reagent, the proteolytic activity of the sample was determined at 570 nm in the TCA soluble fraction (Lowry et al., 1951).

2.3. Nitrate reductase activity

Nitrate reductase activity was measured following the protocol of Sym (1984). Chopped leaf samples (0.5 g) were added in 4.5 ml of 0.2 *M* phosphate buffer (pH 7.0) and 0.5 mL of 0.02 M KNO_3. These samples were incubated in dark at 32 °C for 1 h following the mixing with 0.5 mL of sulphanilamide. Then immediately 0.5 mL of N (1-naphthyl)-ethylene diamine dihydrochloride was added after shaking. Pink diazo colored complex with NO_2 was produced which was diluted after 20 min with 5 mL distilled water leading to centrifugation for 5 min at 2000 rpm to remove turbidity. Absorbance was taken at 542 nm against a set of standards developed with $NaNO_2$.

2.4. Analysis of proteins and free amino acids

The method of Lowry et al. (1951) was used to calculate the total soluble proteins. After being pulverized seedlings (1.0 g) in 10 mL of 0.2 M phosphate buffer (pH 7.0) and filtered through nylon cloth were used. The plant filtrate was centrifuged at 10,000 rpm for five minutes after being precipitated with 10 % TCA in an equivalent amount. Then after resuspending the pellet with 0.1 mol NaOH dm^{-3}, the protein was evaluated. Using the Bovine Serum Albumin (BSA) solution standard curve, total proteins (mg/g) were determined (Bradford, 1976). Following Hamilton et al. (1943) description of the ninhydrin procedure, the total free amino acids were calculated. In order to do this, 1 mL of plant extract was combined with 1 mL each of 10 and 2 % ninhydrin solutions. The amino acid levels (mg/g fresh weight) were estimated using the standard curve of leucine solution after the OD was read at 570 nm using a spectrophotometer.

2.5. Estimation of total soluble sugars

Freshly prepared seedlings (1.0 g) were ground in 10 mL of 80 % ethanol (v/v) following centrifugation. The Supernatant (0.1 ml) with 3 ml freshly produced anthrone was heated at 97 °C for 15 min. Ice cold water was used to cool it down then on a spectrophotometer, the OD was measured at 625 nm. (Hitachi, 220, Japan).

2.6. Statistical analysis

The analysis of variance (ANOVA) was used to statistically assess the data using a computer based software Statistix 10.1. The differences among significant means were evaluated using the Tukey's HSD test to find the significant difference test at a 5 % probability level. Using the Microsoft Excel Bar-Graphs were made using the relationships between the variables.

3. Results

3.1. Nano-biochar enhanced the germination indices of wheat seedlings under salt stress

To investigate the effect of nano-biochar application on germinating wheat seeds, a *petri*-plate experiment was conducted and various growth and germination indices were measured at different time points (Fig. 2a-c and Table 2). It was observed that, salt stress decreased the weight of soaked seeds in Akbar variety gradually at 24 h and 48 h but a sharp decline was noted at 72 h (28.3 %), 96 h (37.7 %), and 120 h (36 %). However, application of 5 % NBC under salt stress (AS_8NBC_5) improved the seed weight by 17, 10, 21, 39.6 % at 24, 48, 72 and 96 h, respectively while 3 % NBC (AS_8NB_3) treatment improved the weight by 47 % as compared to only salt treated plants. In Zincol variety, salt stress significantly reduced the seed weight by 33, 24, 32.6 and 43 % at 48, 72, 96 and 120 h, respectively compared to only alkali treated plants. But, the addition of 1 % NBC (AS_8NBC_1) under salt stress increased the seed weight by 6 and 46 % at 24, 96 and 120 h, respectively. The application of 3 % NBC increased the seed weight by 8 % and 37 % at 48 h and 96 h, respectively compared to only alkali treated plants.

Salt stress significantly decreased the germination indices such as final germination percentage (FGP) and mean germination time (Fig. 2b, c). Salt stress drastically reduced the final germination percentage of wheat seeds by 62 % in Akbar and 58 % in Zincol variety at 48 h time point. 5 % NBC application increased the FGP by 70 %, while in Zincol variety, 5 % NBC application showed consistent improvement by 58 and 22 % at 48 and 72 h time points compared to only salt treated plants. Mean germination time of wheat seeds also influenced by salt stress. Intriguingly, salt stress decreased the MGT by 57.8 % in AKBAR and 18 % in ZINCOL as compared to control at 48 h, while increased MGT was recorded at other time points. 9, 10.4 and 9. 5 % in AKBAR at 72, 96 and 120 h, respectively and 9.12 % at 72 h increase in MGT was noted compared to control. The application of NBC at all concentrations, on the other hand decreased the MGT percentage compared to only salt treated seeds which clearly depicts the alleviation of salt stress effects on germinating wheat seeds.

3.2. Nano-biochar effect on seedling length and germination index under salt stress

To further investigate the role of NBC application at early growth stages of plant, plumule length and radicle length was measured (Fig. 1& 3a,b). The results displayed that, salt stress reduced the length of plumule by 54.4 % in Akbar and 58.27 % in Zincol but 5 % application of NBC increased the plumule length by 13–14 % without salt stress in both varieties. NBC treatment worked more efficiently under salt stress conditions as a sharp increase in plumule length was observed. Under salt stress, 3 % NBC application increased the plumule length by 58.27 and 61.91 % in Akbar and Zincol varieties respectively. Similar trend was observed for radicle length, as salt stress significantly reduced the length in range of 47–49 % in both varieties, and 5 % NBC application improved the radicle length by 27 % under control conditions in both varieties. Under salt stress, highest increase (58 and 59 %) in radicle length was observed in 3 % NBC treated seeds in both varieties, respectively.

Further, germination index (GI) and germination rate index (GRI) were calculated (Fig. 3c, d). In consistent with our previous results, salt stress decreased the germination index in both varieties but 5 % application of NBC improved the GI rates by 11 and 24 % without salt stress in both varieties, respectively. Under salt stress, 5 % NBC also increased the germination index in range of 24 to 26 % in both verities, respectively. Similarly, GRI rates were declined by 10 % under salt treatment . Interestingly, 5 % application of NBC increased the germination rate index in both salt treated and non-treated plants. Under control conditions, 5 % NBC increased GRI by 5.46 and 13.14 % and in salt stress GRI increased by 15.46 and 16.34 % in both varieties, respectively. Overall, in germination indices, 3 and 5 % application of NBC displayed better results and NBC exhibited good performance under stress conditions compared to control conditions.

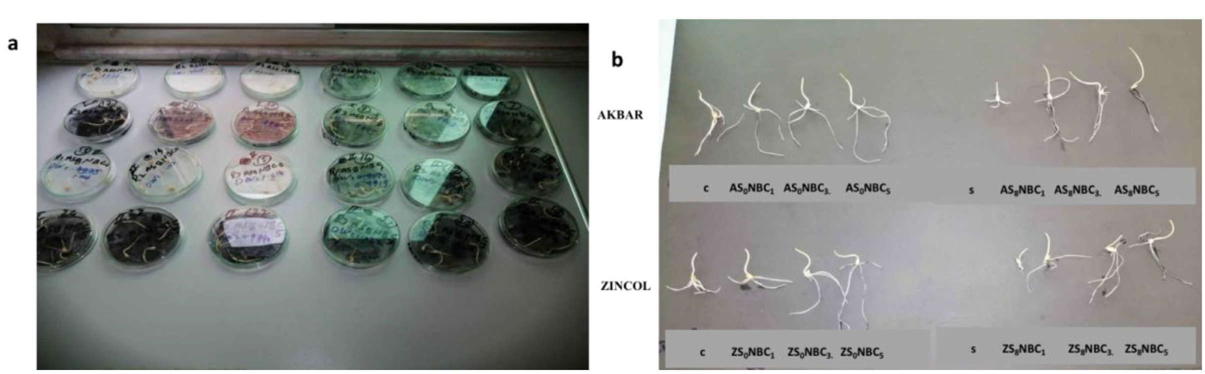

Fig. 1. A *petri*-plate experiment (a) showing Effect of nano-biochar on wheat seedlings plumule and radicle lengths with and without salt stress.

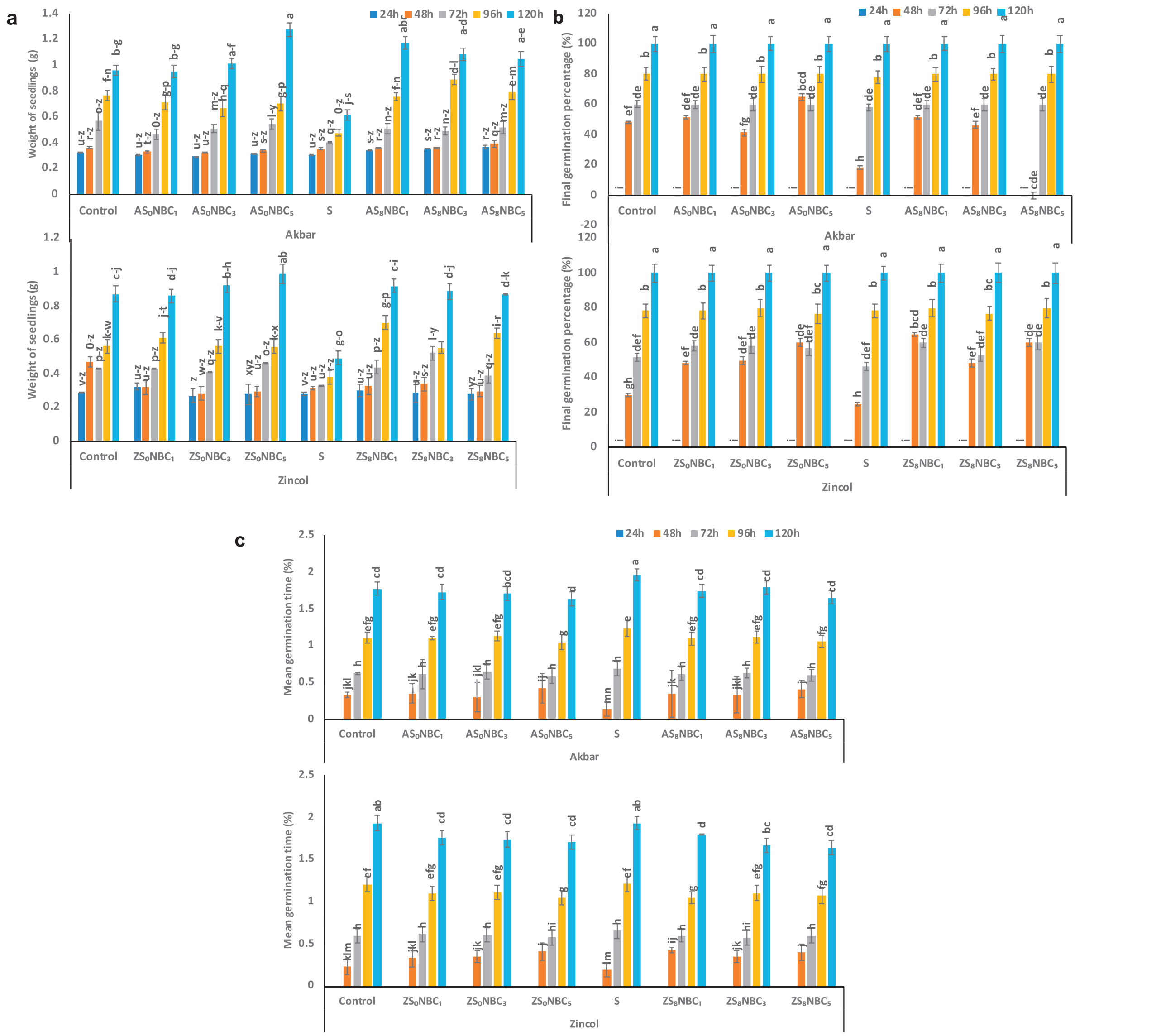

Fig. 2. Effect of nano-biochar on biomass and germination attributes of wheat seedlings. Determination of (a) weight of seeds (b) Final germination percentage (c) Mean germination time under applied nano-biochar at different time points with and without salt stress. AS_0, AS_8, ZS_0 and ZS_8 represents 0 mM and 80 mM (8 dS/m) salt stress in Akbar and Zincol varieties, respectively. NBC_1, NBC_3 and NBC_5 represents 1 %, 3 % and 5 % irrigation of nano-biochar solution. Each value is a mean of 4 replicates. Graph bars represent mean values ± error bars showing standard errors. Different letters a, b, c and so on represent significant differences in the mean values at $p < 0.05$.

3.3. *Nano-biochar effect on hydrolysing and nitrogen metabolic enzymes activities under salt stress*

To understand the underneath mechanism of germinating wheat seeds under application of NBC, hydrolysing enzymes activities were determined at different time points (Fig. 4 and Table 1). Our results clearly showed that salt stress exerts negative effect on enzyme activities as a sharp reduction in α-amylase activity at 24 h time point was noted in both control and salt treated plants but after that gradual reduction was observed. Salt stress reduced the α-amylase activity by 24.72, 22.51, 19, 16.91 and 16.52 % in Akbar and 25.81, 24.42, 19.12, 17, and 16.28 % in Zincol at 24, 48, 72, 96 and 120 h, respectively. In line with our previous results trend, 5 % NBC application without salt stress enhanced the α-amylase activity by 31.81, 29.6, 26.25, 23.79 and 21 % in Akbar and 31, 29.9, 27.8, 24.9 and 22.4 % in Zincol at 24, 48, 72, 96 and 120 h, respectively. Whereas, 5 % NBC application under salt stress improved the α-amylase activity by 47.7, 45.3, 41.61, 37.8 and 35.2 % in Akbar and 47.5, 45.7, 40,2, 36.3 and 33.74 % in Zincol at 24, 48, 72, 96 and 120 h, respectively.

In protease activity, similar trends were observed as salt stress decreased the protease enzyme activity at all time points compared to control plants while 5 % NBC application significantly enhanced the activity with and without salt stress. Salt stress reduced the protease enzyme activity by 42.2, 55.8, 53.9, 49.3 and 54.9 % in Akbar and 50.8, 53.6, 45.8, 36.9 and 41 % in Zincol at 24, 48, 72, 96 and 120 h, respectively. 5 % NBC application without salt stress regulated the protease enzyme activity by 68, 56.6, 52.8, 45.8 and 44.2 % in Akbar and 66.6, 67.5, 62.8, 56.6 and 59 % in Zincol at 24, 48, 72, 96 and 120 h, respectively. Similarly, 5 % NBC application under salt stress improved the protease enzyme activity by 80, 77.5, 74.9, 71.3 and 74.34 % in Akbar and 82.5, 83.9, 77.05, 86.2 and 71 % in Zincol at 24, 48, 72, 96 and 120 h, respectively.

Further, NBC application positively regulated Nitrate reductase enzyme activity (NR), as salt stress declined enzyme activity, but 5 % NBC application showed highest values. It was calculated that, salt stress reduced the NR activity by 12.5, 18.6, 15.3, 12.8 and 14.7 % in Akbar and 54.7, 16.4, 13.47, 11.2 and 12.8 % in Zincol at 24, 48, 72, 96 and 120 h, respectively. Following previous trend, 5 % NBC

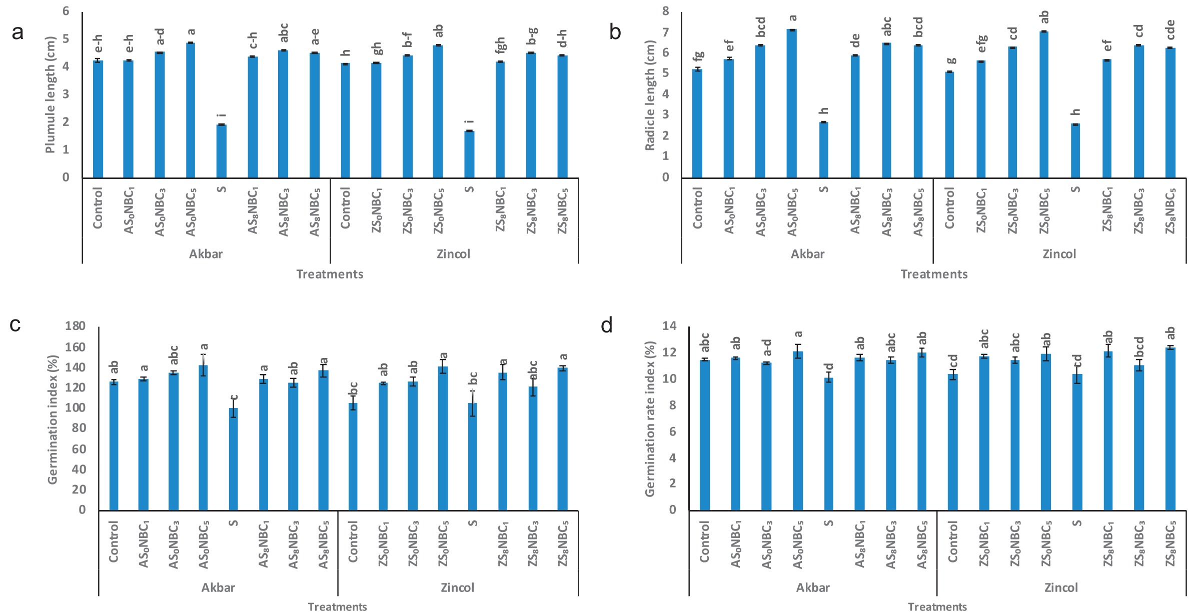

Fig. 3. The impact of nano-biochar irrigation on seedling length and germination index (a) Plumule length (b) Radicle length (c) Germination index (d) Germination rate index under applied nano-biochar with and without salt stress. AS_0, AS_8, ZS_0 and ZS_8 represents 0 mM and 80 mM (8 dS/m) salt stress in Akbar and Zincol varieties, respectively. NBC_1, NBC_3 and NBC_5 represents 1 %, 3 % and 5 % irrigation of nano-biochar solution. Each value is a mean of 4 replicates. Graph bars represent mean values ± error bars showing standard errors. Different letters a, b, c and so on represent significant differences in the mean values at $p < 0.05$.

application without salt stress regulated the NR enzyme activity by 54.5, 44.7, 40, 35.8 and 39 % in Akbar and 42, 46, 41.2, 36.8 and 40.2 % in Zincol at 24, 48, 72, 96 and 120 h, respectively. Whereas, 5 % NBC application under salt stress improved the NR enzyme activity by 58.6, 54, 48.3, 43 and 47 % in Akbar and 72.6, 53.6, 47.8, 42.5 and 46.5 % in Zincol at 24, 48, 72, 96 and 120 h, respectively.

These results suggested that NBC application at seedling stage could positively enhance the hydrolysing enzyme activities as well exert positive impacts on nitrogen metabolism.

3.4. Nano-biochar application enhanced the plant metabolites under salt stress in germinating wheat seedlings

To investigate the effects of NBC supplementation on metabolism of germinating wheat seeds, the total free amino acids, total soluble sugars and total soluble proteins were analysed (Fig. 5). Our results clearly showed that salt stress exerts negative effect on plant metabolites but NBC application significantly improved the metabolites contents under stress and non-stress conditions. Salt stress reduced the total free amino acids by 45.9, 30.2, 25.8, 22.5 and 24 % in Akbar and 56, 33.9, 28.3, 24.3 and 26 % in Zincol at 24, 48, 72, 96 and 120 h, respectively. In line with our previous results trend, 5 % NBC application without salt stress enhanced the total free amino acids by 44.7, 34.7, 31., 28.4 % and 29.7 % in Akbar and 27.7, 18.8, 16.26, 14.28 and 15.2 % in Zincol at 24, 48, 72, 96 and 120 h, respectively. Whereas, here 3 % NBC application under salt stress improved the total free amino acids by 65.2, 48.8, 43.4, 39 and 41.4 % in Akbar and 5 % NBC improved this parameter by 67.8, 45.8, 39.4, 34.5 and 36.85 % in Zincol at 24, 48, 72, 96 and 120 h, respectively.

In contrast with protease activity trend, salt stress increased the total proteins by 12.3, 20.6, 29.8, 39.8 and 49 % in Akbar and 12.8, 21, 29.7, 38.4 and 49 % in Zincol at 24, 48, 72, 96 and 120 h, respectively. While, NBC application overall reduced the total protein contents at all time points such as 5 % NBC application without salt stress decreased the total protein contents by 25.8, 29.4, 34.2, 40.9 and 50.8 % in Akbar and 25, 28.6, 33.24, 39.5 and 48.9 % in Zincol at 24, 48, 72, 96 and 120 h, respectively. Similar in trend, 5 % NBC application decreased the total protein contents by 30, 42, 49, 58.7 and 68 % in Akbar and by 28.8, 41.7, 47.3, 53.8 and 61.6 % reduction in Zincol was observed under salt stress.

Total soluble sugars displayed same trend with α-amylase activity as expected. Salt stress reduced the total soluble sugars by 28.7, 25.9, 21.3, 18.7 and 12.6 % in Akbar and 30, 28.2, 21.6, 19 and 17.9 % in Zincol at 24, 48, 72, 96 and 120 h, respectively. 5 % NBC application without salt stress enhanced the total soluble sugars by 35, 32.6, 28.5, 25.7 and 22.7 % in Akbar and 34.5, 33, 30.3, 26.9 and 24 % in Zincol at 24, 48, 72, 96 and 120 h, respectively. Whereas, 5 % NBC application under salt stress enhanced the content of total soluble sugars by 52.2, 49.3, 44, 39.8 and 32.7 % in Akbar and 52.9, 50.6, 43.9, 39.41 and 36.3 % in Zincol at 24, 48, 72, 96 and 120 h, respectively.

4. Discussion

The main purpose of the study was to evaluate the effect of salt stress in relation to the germination attributes and biochemical parameters of two varieties of wheat, i-e, Akbar and Zincol under the influence of Nanobiochar (NBC). Results showed that seed weight, final germination percentage, length of plumule and radical, germination index and germination rate index decreased in both varieties under salt stress while under the influence of NBC application specifically at the concentration of 5 %, these parameters increased despite of stress. So, plant germination rate may have been reduced because of the negative effect of increased levels of salt stress. The current results are consistent with the research conducted by Dehnavi et al. (2020) who worked on seed germination and seedling development on Sorghum under salinity. The osmotic effect of increased salt stress prevents plants from retaining the proper nutritional requirements necessary for improved plant growth, which might diminish seed germination and root emergence. Similar to this, biochar nanoparticles made rice seedling roots and shoots grow longer (Zheng et al., 2020). Marzouk (2017) provided evidence that root development in date palm seedlings was enhanced by the use of olive pomace waste

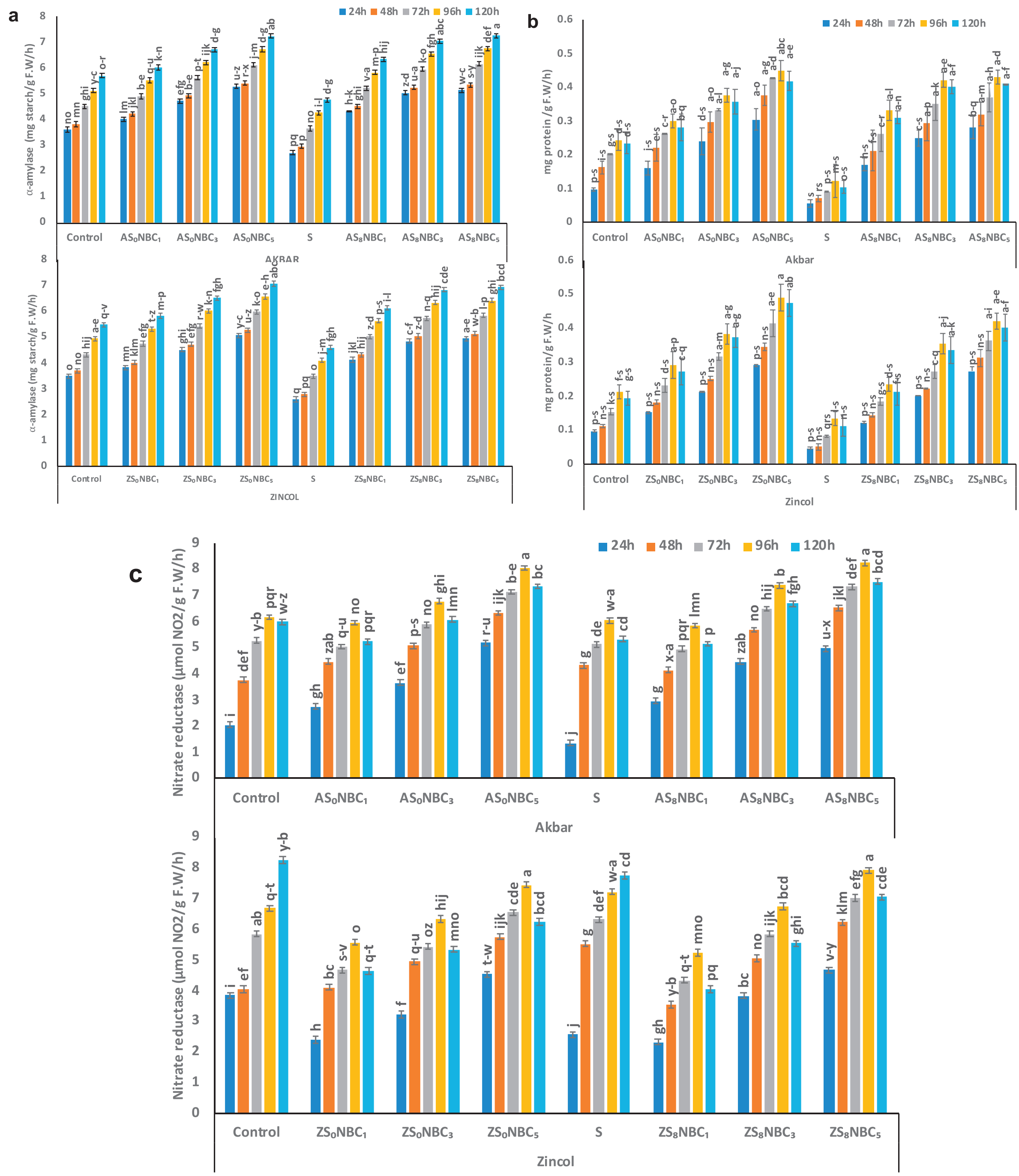

Fig. 4. The impact of nano-biochar irrigation on hydrolyzing and nitrogen metabolic enzyme activities (a) *α*-amylase enzyme activity (b) Protease enzyme activity (c) Nitrate reductae enzyme activity in wheat seedlings at different time points with and without salt stress. AS_0, AS_8, ZS_0 and ZS_8 represents 0 mM and 80 mM (8 dS/m) salt stress in Akbar and Zincol varieties, respectively. NBC_1, NBC_3 and NBC_5 represents 1 %, 3 % and 5 % irrigation of nano-biochar solution. Each value is a mean of 4 replicates. Graph bars represent mean values ± error bars showing standard errors. Different letters a, b, c and so on represent significant differences in the mean values at $p < 0.05$.

biochar. This is because plants responded favourably to the application of biochar, the increase in plant growth may also be attributable to the fertilizer-biochar synergy effect, increased nutrient availability, balanced microorganisms, and substrate with superior characteristics (Ali et al., 2021). Talha et al. (2023) also reported that nanobiochar boosted the development of *Brassica chinensis* L. in stressed soil and enhanced the germination percentage of seeds in solution. The current research also revealed that under slat stress, the mean germination time of both types rose; however, at a concentration of 5 % NBC, both varieties under study showed a considerable reduction in these parameters.

Sugar content and *α*-amylase activity decreased in the seedlings of both studied varieties under salt stress, however, a considerable decrease was seen at 24 h, and a progressive decline was also seen in both varieties at other time points till 120 h. Although, an increase was evident when the seedlings were treated with NBC, exhibiting maximum increase at 5 % NBC despite of stresses or non-stressed environment. These results are corroborated by Vitale et al. (2021), who claimed that the enhanced enzymatic activity in germination was due to the fact that seeds need more amount of ATP or other energy-rich molecules for appropriate growth and development. Developing embryos are the primary source of energy for seeds to

Table 1
Effect of nano-biochar applications (0, 1, 3 and 5 %) on different attributes of wheat seedlings at different time points with non-stress and stress conditions. Various abbreviations used are as follows; FAA = free amino acids, TP = total phenolics, TS = total sugars, NR = Nitrate reductase, MGT = mean germination time, G% = germination percentage.

Source	df	Protease	FAA	TP	α-amylase	TS	NR	MGT	G%
Genotype (Gen)	1	0.2219***	6.43626***	0.0803**	2.056***	2.056***	0.8286***	0.0001ns	37.60ns
Time	4	0.2999***	7.84742***	1.0237***	34.961***	34.962***	82.2584***	22.7853***	68,015.52***
Gen*Time	4	0.0322***	0.00003ns	0.0039ns	0.002ns	0.002ns	0.0001ns	0.0038ns	20.94ns
Condition (Cond)	1	0.0513***	1.72936***	0.0054ns	0.420***	0.420***	0.1383***	0.0008ns	8.44ns
Gen*Cond	1	0.0018ns	0.21616***	0.0327ns	0.009ns	0.009ns	0.0032ns	0.0027ns	87.60*
Time*Cond	4	0.0032ns	0.00003ns	0.0983***	0.002ns	0.002ns	0.0001ns	0.0022ns	14.17ns
Gen*Time*Cond	4	0.0040ns	0.00003ns	0.0039ns	0.001ns	0.001ns	0.0001ns	0.0069*	49.58*
Application (Appl)	3	0.4726***	5.21714***	2.5646***	47.160***	47.157***	126.1186***	0.0205***	528.99***
Gen*Appl	3	0.0198***	0.32263***	0.0077ns	0.007ns	0.007ns	0.0094ns	0.0009ns	25.94ns
Time*Appl	12	0.0161***	0.00003ns	0.008ns	0.001ns	0.001ns	0.0001ns	0.0524***	408.85***
Gen*Time*Appl	12	0.0111***	0.00003ns	0.0024ns	0.001ns	0.001ns	0.0001ns	0.0027ns	20.38ns
Cond*Appl	3	0.0155**	0.85170***	0.9421***	4.729***	4.730***	5.7857***	0.0032ns	74.55*
Gen*Cond*Appl	3	0.0079ns	0.23815***	0.0068ns	0.012ns	0.012ns	0.0152ns	0.0017ns	62.60*
Time*Cond*Appl	12	0.0008ns	0.00003ns	0.008ns	0.001ns	0.002ns	0.0001ns	0.0091***	76.11***
Gen*Time*Cond*Appl	12	0.0003ns	0.00003ns	0.0024ns	0.002ns	0.002ns	0.0001ns	0.0050**	27.36ns
Error	160	0.003<-	0.012<-	0.0087<-	0.007<-	0.007<-	0.0096<-	0.002<-	19.48<-

germinate (Zaynab et al., 2021). The primary enzymes involved in the beginning of starch hydrolysis in the endosperm of a seed include α-amylase, α -glucosidase, and starch phosphorylase (Collins et al., 2021). Additionally, α -amylase is an important enzyme because of its ability to play a role in hydrolyzing starch into amylopectin and amylose (Galanakis, 2022).

In the current study, it has been observed that under stressed conditions, protease enzymes demonstrated higher values than in the seedlings grown under normal condition. The total free amino acid followed the same trend, while the total soluble protein decreased under stressed condition. Protease enzymes also promote the generation of reactive oxygen species (ROS), which have been discovered during plant exposure and response to abiotic challenges, particularly water shortage stress (Liu et al., 2020; Ali and Baek, 2020). It primarily controls a variety of physiological and metabolic processes that result in protein turnover in plants. Furthermore, the creation and expression of recombinant protease inhibitors (RPIs) has resulted from the art of recognizing the role of proteolytic enzymes and their inhibitors in plant metabolic systems. Recombinant protease inhibitors are increased protein molecules produced by transgenic plants that are utilized to reduce the negative effects of proteolysis during plant stress exposure (Mangena 2022). As a result, it controls a variety of stress-related reactions, including senescence and the ability to tolerate biotic and abiotic stress (Sharma and Gayen, 2021).

In non-stressed condition, the breakdown of stored proteins in germination seeds leads to the creation of amino acids. To meet the demand for the creation of new proteins and various other

Table 2
Effect of nano-biochar applications (0, 1, 3 and 5 %) on growth and germination indexes of wheat seedlings at different time points with non-stress and stress conditions. Various abbreviations used are as follows; PL = plumule length, RL = radical length, GI = germination index, GRI = germination rate index.

Source	df	PL	RL	GI	GRI
Genotype (Gen)	1	0.291***	0.235***	52.08ns	0.30ns
Condition (Cond)	4	5.313***	7.642***	30.08ns	0.07ns
Application (Appl)	4	6.698***	18.600***	1594.39***	4.23***
Gen*Cond	1	0.037***	0.016***	243.00*	0.70*
Gen*Appl	4	0.000ns	0.002ns	76.14ns	0.21ns
Cond*Appl	4	4.005***	4.420***	224.81*	0.60*
Gen*Cond*Appl	3	0.000ns	0.002ns	159.72ns	0.50*
Error	32	0.0007<-	0.0007<-	56.64<-	0.16<-

biomolecules, such as the enzymes required to regulate apices growth, the hydrolysis of proteins and their conversion to amino acids are regulated. According to the findings of the current study, the proteins content increased in stress and gradually decreased with increasing time lines. But, as the result of stress, amino acid conversion was delayed, according to the current study's findings. The current findings are in line with some of the earlier research on *Acalypha indica* and *Oryza sativa* when Protein content decrease over time under stress (Venkatachalam et al., 2017; Jayasri and Suthindhiran, 2017).

The effects of salt stress reduced nitrate reductase activity, whereas the application of NBC increased this activity in both studied varieties of wheat. However, when NBC concentrations increased, nitrate reductase activity also increased which is a positive sign to enhance the seed germination as NR enzyme provides specific signals to promote seed germination. In a recent review, an extensive cross-talk has been presented among NO and Ca_{2+} signalling pathways which clearly showed involvement of nitric oxide in Ca^{2+} mobilization across membranes and helps in signal transduction (Mariyam et al., 2023).

In addition to lowering the cost of planting crops, increasing the nitrogen use efficiency (NUE) of crops can also lower the energy required to produce chemical fertilizers, which has a significant positive impact on reducing the effects of climate change (Al Tawaha et al., 2020: Yoon et al., 2020). As opposed to applying chemical fertilizer as the only source of nitrogen, the combined application of biochar and chemical nitrogen fertilizer (such as urea) can effectively increase crop yield and NUE by slowing down nitrogen release, regulating microbial diversity, and stimulating nitrification while inhibiting denitrification (Verma and Reddy, 2020; Liao et al., 2020; Yu et al., 2020).

There are many research focusing on the improved soil nitrogen availability brought about by the application of biochar, but there are few data on molecular signals or root phenotypes connected to the increase in nitrogen availability in the rhizosphere (Cui et al., 2018). According to a recent study, soil biophysical and chemical characteristics such mineral nitrogen, moisture, and temperature have an impact on the architecture and phenotypic of roots. As a result, there are significant functional differences within root orders due to the remarkable phenotypic flexibility of plant roots in their cellular structure, architecture, cell types, morphologies, metabolisms, and biochemical profiles. Application of biochar may increase these discrepancies under both stressed and non-stressed conditions (Feng et al., 2021).

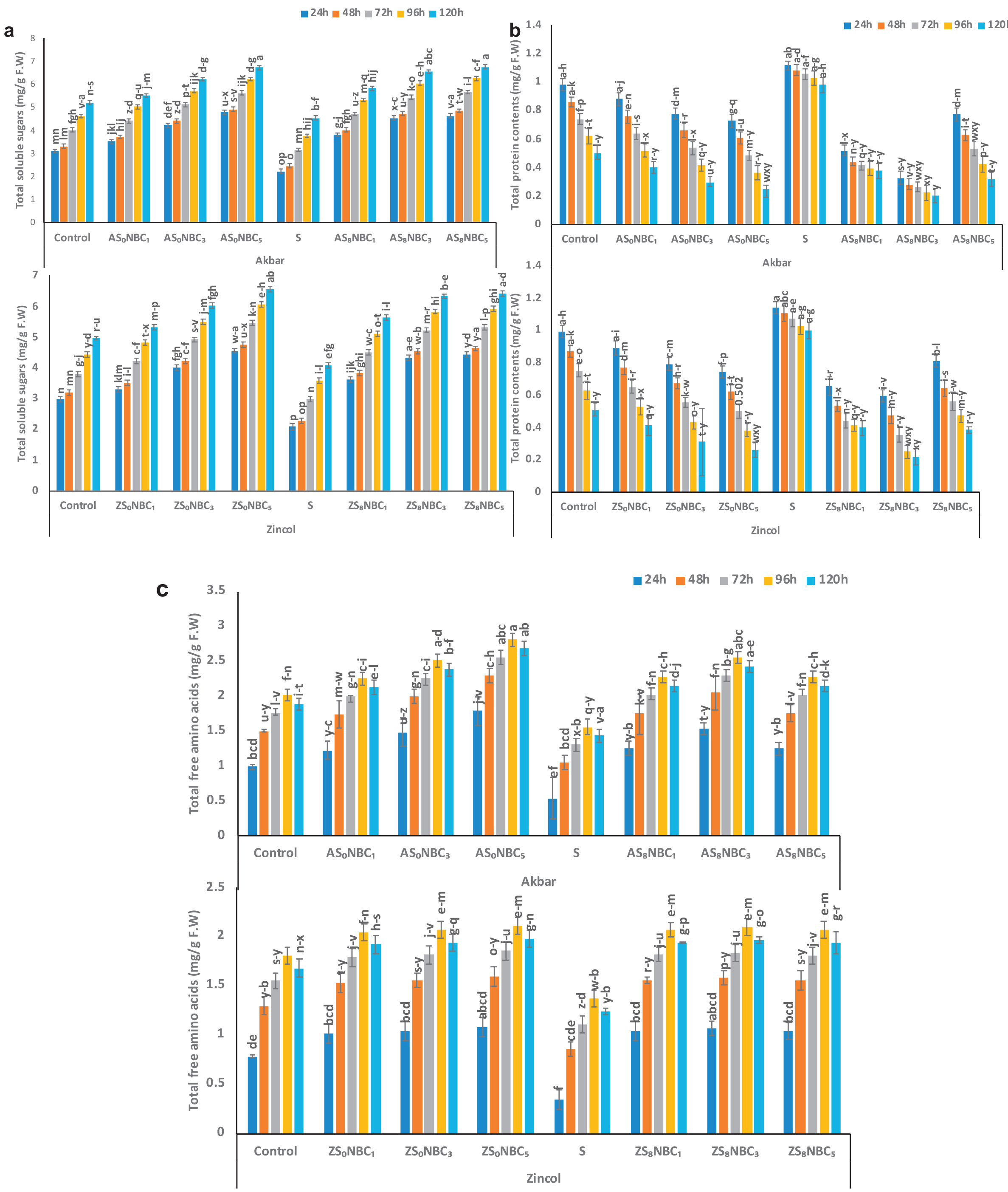

Fig. 5. The impact of nano-biochar irrigation on primary metabolites (a) Total soluble sugars (b) Total soluble proteins (c) Total free amino acids in wheat seedlings at different time points with and without salt stress. AS_0, AS_8, ZS_0 and ZS_8 represents 0 mM and 80 mM (8 dS/m) salt stress in Akbar and Zincol varieties, respectively. NBC_1, NBC_3 and NBC_5 represents 1 %, 3 % and 5 % irrigation of nano-biochar solution. Each value is a mean of 4 replicates. Graph bars represent mean values ± error bars showing standard errors. Different letters a, b, c and so on represent significant differences in the mean values at $p < 0.05$.

5. Conclusion

Our study tested different concentrations of NBC on seeds of two wheat varieties and assessed its subsequent effects on different germination indices, hydrolysing enzymes and plant metabolites. The results displayed that NBC application boosted wheat seedlings tolerance to stress by increased activities of α-amylase, Protease and Nitrate reductase enzymes related to starch and protein degradation which in turn upgrade contents of plant primary metabolites as well as showed positive contribution towards nitrogen metabolism. The positive changes at cellular level, results in improvement of seed germination indices, increase in plumule and radicle lengths as well as reduced the mean germination time of wheat seedlings under NBC application. However, a comprehensive studies on NBC adhesion and translocation, its crosstalk with phytohormones which contributes in production of secondary metabolites are still far behind.

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Declaration of competing interest

All the authors declare no conflict of interest.

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References

Ahanger, M.A., Aziz, U., Alsahli, A.A., Alyemeni, M.N., Ahmad, P., 2020. Influence of exogenous salicylic acid and nitric oxide on growth, photosynthesis, and ascorbate glutathione cycle in salt stressed Vigna angularis. Biomolecules 10, 42–46.

Agegnehu, G., Srivastava, A.K., Bird, M.I., 2017. The role of biochar and biochar-compost in improving soil quality and crop performance: a review. Appl. Soil Ecol. 119, 156–170.

Ainouz, I.I., 1970. Preliminary studies on proteins of Vigna sinesis. Anais da Acad. Bras. Ciências 42 (1), 97–101 v.n.p.1970.

Al Tawaha A.R., Singh S., Singh V., Kafeel U., Irfan M., Kumari A, Imran, Amanullah D.R., Al Tawaha A.R., Qaisi A., et al. (2020). Improving water use efficiency and nitrogen use efficiency in rice through breeding and genomics approaches. p. 307−37.

Ali, L., Xiukang, W., Naveed, M., Ashraf, S., Nadeem, S.M., Haider, F.U., Mustafa, A, 2021. Impact of biochar application on germination behavior and early growth of maize seedlings: insights from a growth room experiment. Appl. Sci. 11 (24), 11666.

Ali, M.S., Baek, K.H., 2020. Protective roles of cytosolic and plastidal proteosomes on abiotic stress and pathogen invasion. Plan. Theory 9, 832.

Amini, S., Ghadiri, H., Chen, C., Marschner, P., 2016. Salt-affected soils, reclamation, carbon dynamics, and biochar: a review. J. Soils Sedim. 16 (3), 939–953.

Anwar, S., Shafiq, F., Nisa, Z.U., Usman, U., Ashraf, M.Y., Ali, N., 2021. Effect of cadmium stress on seed germination, plant growth and hydrolyzing enzymes activities in mungbean seedlings. J. Seed Sci. 43, e202143042.

Ayangbenro, A.S., Babalola, O.O., 2021. Reclamation of arid and semi-arid soils: the role of plant growth-promoting archaea and bacteria. Curr. Plant Biol. 25, 100173.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72 (1−2), 248–254 v.n.p..

Balasubramaniam, T., Shen, G., Esmaeili, N., Zhang, H, 2023. Plants. response mechanisms to salinity stress. Plants 12 (12), 2253. https://doi.org/10.3390/plants12122253.

Chrispeels, M.J., Varner, J.E., 1967. Gibberellic acid-enhanced synthesis and release of α-amylase and ribonuclease by isolated barley and aleurone layers. Plant Physiol. 42 (3), 398–406.

Collins, H.M., Betts, N.S., Dockter, C., Berkowitz, O., Braumann, I., Cuesta-Seijo, J.A., Skadhauge, B., Whelan, J., Bulone, V., Fincher, G.B., 2021. Genes that mediate starch metabolism in developing and germinated barley grain. Front. Plant Sci. 12, 641325.

Cui, E.P., Gao, F., Liu, Y., Fan, X.Y., Li, Z.Y., Du, Z.J., Hu, C., Neal, A.L., 2018. Amendment soil with biochar to control antibiotic resistance genes under unconventional water resources irrigation: proceed with caution. Environ. Pollut. 240, 475–484.

Dehnavi, R.A., Zahedi, M., Ludwiczak, A., Cardenas Perez, S., Piernik, A., 2020. Effect of salinity on seed germination and seedling development of sorghum (Sorghum bicolor (L.) Moench) genotypes. Agronomy 10, 859.

Feng, L., Xu, W., Tang, G., et al., 2021. Biochar induced improvement in root system architecture enhances nutrient assimilation by cotton plant seedlings. BMC Plant Biol. 21, 269.

Ferreira, C.S.S., Seifollahi-Aghmiuni, S., Destouni, G., Ghajarnia, N., Kalantari, Z., 2022. Soil degradation in the European Mediterranean region: processes, status and consequences. Sci. Total Environ. 805, 150106.

Galanakis, C.M., 2022. Sustainable applications for the valorization of cereal processing by-products. Foods. 11, 241.

Guo, Z., Chen, X., Zhang, Y, 2022. Impact of environmental regulation perception on farmers' agricultural green production technology adoption: a new perspective of social capital. Technol. Soc. 71, 102085.

Hamilton, P.B., Van slyke, D.D., Lemish, S., 1943. The gasometric determination of free amino acids in blood filtrates by the ninhydrincarbondioxide method. J. Biol. Chem. 150, 231–250 v.p..

Horton, P., Long, S.P., Smith, P., Banwart, S.A., Beerling, D.J., 2021. Technologies todeliver food and climate security through agriculture. Nat. Plants. 7 (3), 250–255.

Jayasri, M.A., Suthindhiran, K., 2017. Effect of zinc and lead on the physiological and biochemical properties of aquatic plant *Lemna minor*: its potential role in phytoremediation. Appl. Water Sci. 7, 1247–1253.

Jia, G., Shevliakova, E., Artaxo, P., De-Docoudré, N., Houghton, R., House, J., Kitajima, K., Lennard, C., Popp, A., Sirin, A., Sukumar, R., Verchot, L., & Sporre, M. (2019). Land−climate interactions. In special report on climate change and land: an IPCC special report on climate change, desertification, land degradation, sustainable land management, food security, and greenhouse gas fluxes in terrestrial ecosystems (pp. 133−206). IPCC. https://www.ipcc.ch/srccl/chapter/chapter-2/

Kamali, A., Heidari, S., Golzary, A., Tavakoli, O., Wood, D.A., 2022. Optimized catalytic pyrolysis of refinery waste sludge to yield clean high quality oil products. Fuel 328, 125292.

Lamaming, J., Saalah, S., Rajin, M., Ismail, N.M., Yaser, A.Z., 2022. A review on bamboo as an adsorbent for removal of pollutants for wastewater treatment. Int. J. Chem. Eng. 2022.

Lehmann, J., Cowie, A., Masiello, C.A., Kammann, C., Woolf, D., Amonette, J.E., Cayuela, M.L., Camps-Arbestain, M., Whitman, T., 2021. Biochar in climate change mitigation. Nat. Geosci. 14 (12), 883–892.

Liao J., Liu X., Hu A., Song H., Chen X., Zhang Z. (2020). Effects of biochar-based controlled release nitrogen fertilizer on nitrogen-use efficiency of oilseed rape (*Brassica napus* L.). Scientific report, -Uk.; 10:1.

Liu, Q., Meki, K., Zheng, H., et al., 2023. Biochar application in remediating salt-affected soil to achieve carbon neutrality and abate climate change. Biochar. 5, 45.

Liu, W., Li, Y., Feng, Y., et al., 2020. The effectiveness of nanobiochar for reducing phytotoxicity and improving soil remediation in cadmium-contaminated soil. Sci. Rep. 10, 858.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265–275.

Mangena, P., 2022. Pleiotropic effects of recombinant protease inhibitors in plants. Front. Plant Sci. 13, 1–12.

Marzouk, E., 2017. Soil-less seed germination and root growth of date palm affected by biochar and metal nanoparticles. J. Soil Sci. Agric. Eng. 8, 77–84.

Mariyam, S., Bhardwaj, R., Khan, N.A., Sahi, S.V., Seth, C.S., 2023. Review on nitric oxide at the forefront of rapid systemic signaling in mitigation of salinity stress in plants: crosstalk with calcium and hydrogen peroxide. Plant Sci., 111835.

Mubashir, A., Nisa, Z.U., Shah, A.A., Kiran, M., Hussain, I., Ali, N., AbdElgawad, H., 2023. Effect of foliar application of nano-nutrients solution on growth and biochemical attributes of tomato (*Solanum lycopersicum*) under drought stress. Front. Plant Sci. 13, 1066790.

Mukhopadhyay, R., Sarkar, B., Jat, H.S., Sharma, P.C., Bolan, N.S., 2021. Soil salinity under climate change: challenges for sustainable agriculture and food security. J. Environ. Manage. 280, 111736.

Sharma, P., Gayen, D., 2021. Plant protease as regulator and signaling molecule for enhancing environmental stress-tolerance. Plant Cell Rep. 40, 2081–2095.

Singh, A., Sengar, R.S., Rajput, V.D., Minkina, T., Singh, R.K., 2022. Zinc oxide nanoparticles improve salt tolerance in rice seedlings by improving physiological and biochemical indices. Agriculture 12 (7), 1014.

Sym, G.J., 1984. Optimisation of the in-vivo assay conditions for nitrate reductase in barley (Hordeum vulgare L. cv. Igri). J. Sci. Food Agric. 35 (7), 725–730.

Talha, M., Shani, M.Y., Ashraf, M.Y., De Mastro, F., Brunetti, G., Khan, M.K.R., Gillani, S.W.U.H.S., Khan, A., Abbas, S., Cocozza, C., 2023. Lead toxicity-mediated growth and metabolic alterations at early seedling stages of Maize (Zea mays L.). Plants 12 (18), 3335.

Usevičiūtė, E.B.G, 2020. Dependence of pyrolysis temperature and lignocellulosic physical-chemical properties of biochar on its wettability. Biomass Convers. Biorefinery 1–19.

Venkatachalam, P., Jayalakshmi, N., Geetha, N., Sahi, S.V., Sharma, N.C., Rene, E.R., Favas, P.J., 2017. Accumulation efficiency, genotoxicity and antioxidant defense mechanisms in medicinal plant *Acalypha indica* L. under lead stress. Chemosphere 171, 544–553.

Verma, B., Reddy, M.S., 2020. Biochar augmentation improves ectomycorrhizal colonisation, plant growth and soil fertility. Soil Res. 58 (7), 673–682.

Vitale, E., Velikova, V., Tsonev, T., Ferrandino, I., Capriello, T., Arena, C., 2021. The Interplay between light quality and biostimulant application affects the antioxidant capacity and photosynthetic traits of soybean (Glycine max L. Merrill). Plants 10, 861.

Yoon, D.K., Ishiyama, K., Suganami, M., Tazoe, Y., Watanabe, M., Imaruoka, S., Ogura, M., Ishida, H., Suzuki, Y., Obara, M., et al., 2020. Transgenic rice overproducing Rubisco exhibits increased yields with improved nitrogen-use efficiency in an experimental paddy field. Nat. Food 1 (2), 134–139.

Yu, P., Li, Q., Huang, L., Qin, K., Niu, G., gu, M., 2020. The effects of mixed hardwood biochar, mycorrhizae, and fertigation on container tomato and pepper plant growth. Sustainability. 12 (17), 7072.

Zaynab, M., Pan, D., Fatima, M., Sharif, Y., Chen, S., Chen, W., 2021. Proteomics analysis of Cyclobalanopsis gilva provides new insights of low seed germination. Biochemistry 180, 68–78.

Zhang, L., Wang, Y., Mao, J., Chen, B., 2020. Effects of biochar nanoparticles on seed germination and seedling growth. Environ. Pollut. 256, 113409.