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INTERACTIVE IMPACTS OF ENDOPHYTIC FUNGUS *TRICHODERMA HAMATUM* AND BIOCHAR ON SALINITY TOLERANCE OF ALFALFA (*MEDICAGO SATIVA* L.)

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Abstract

There are various reports available that confirm positive impact of biochar (BC) application with endophytic microbial strain to alleviate the adverse impact of abiotic stress on the plant development. Although, BC interactions with root-associated fungi are poorly understood. In the current study, we have used endophytic *Trichoderma hamatum* (TH) stain and BC to combat the salinity stress challenge in the alfalfa plant. As it is well known that, salinity stress adversely affected the morphological yields like length of root and shoot, dry and fresh weight of root and shoot, photosynthetic pigments, nodule dynamics and mineral contents in the alfalfa plants. In addition, salinity stress enhances the concentrations of stress markers such as malondialdehyde (MDA), H_2O_2, proline, and glycine betaine (GB) content while reduced the concentration of antioxidative enzymes such as superoxidedismutase activity (SOD) catalase (CAT), ascorbate peroxidase (APX) and glutathione reductase (GR). However the single of combined treatments of TH stain and BC showed modulatory impact of the morphological yields and enhanced the root fresh eight by 75%, 25% and 160%; shoot height by 68% 34% and 39% , photosynthetic pigments, Chl a increased by 34%, 24% and 48%; Chl b 21%,0%, 31%; Chl a+b 17%,15% and 18%; Carotenoids 55, 5% and 36% and total pigments 22%, 14% and 40% ; similarly nodule number increased by 78%, 10% and 128%, soluble proteins by 145%, 72% and 200%, leg haemoglobin content 50%,25% and 61%, and nodules nitrogenase activity 77%,31% and 109%; antioxidative enzymes SOD increased by 6%, 2% and 9%, catalase activity increased by 6%, 2% and 11%, APX activity increased by 19%, 7% and 29% and GR content increased by 10%, 1% and 24% after treatment of TH, BC and combined application of TH, BC respectively under saline conditions. However, reduced the concentration of osmolytes such as content of H_2O_2 was decreased by 39%, 27%,43%, MDA content decreased by 52%, 17% and 60%; the proline content decreased by 27%, 27% and 54% and the content of GB decreased by 19%, 10% and 65.30% after treatment with TH, BC and combination of TH and BC respectively. Although the extent of effect of treatments was lower in case of single treatments of BC, while maximum effect was observed in the combined application of TH and BC.

Key words: *Medicago sativa* L., Photosynthetic pigments, Mineral elements, Osmolytes, Antioxidative enzymes.

Introduction

In the current regime of changing climatic conditions and global warming ensuring food security for the rising global population is one of the most challenging tasks. In last two decades changing climatic conditions and advent of different stress factors poses negative impact on the agricultural productivity (Tchonkouang *et al*., 2024). Salinity is one of the challenging abiotic stress that affect approx., 1128 million hectares land worldwide and severally affect the agricultural productivity. Although previous studies stated use of brackish and coastal water for agricultural irrigations are the two major factors of salinity in the agricultural land (Du *et al*., 2023). The advent of salinity adversely affects the growth and survivality of plant irrespective of their growth stages (Mal & Panchal, 2024; Fatima *et al*., 2024). In general, the salinity stress affects the plant physiology via disturbing ionic balance, which also results in the disturbance of osmotic balance that results in the irregularity in cell physiology, water movement, lower relative water capacity, stomata closure etc. (Verma *et al*., 2021; Mal & Panchal, 2024). Additionally, stress factors especially salinity stress generate excessive amount of reactive oxygen species (ROS) which results in the degradation of cell macromolecules including the nucleic acid, cell wall plant cell death (EL-Bauome *et al*., 2024). Moreover, salinity stress also affects the biosynthetic pathways of the chlorophyll and other morphological yields such as stem and root growth (El-Taher *et al*., 2021). However, the degree or extent of adverse impact depend upon the concentration of salt or duration of the salinity exposure. The long-term exposure or elevated salt concentration adversely affect plant physiology and lead to the death of plants (Kumar *et al*., 2021). As the plants are sessile organisms, facing the risk of stresses, however the plants system developed or induced some defensive mechanisms to mitigate the challenges of stresses. Plant developed various types of modification in their physiological, metabolic, anatomical and morphological characteristics to resist and cope with stresses (Imran *et al*., 2021). The plant systems produce various types of antioxidative enzymes, that mediate crucial role in scavenging oxygen or nitrogen free radicals. In addition, led to modifications in photosynthetic organelles that help in managing water relations (El- Beltagi *et al*., 2023). To mitigate the challenges of salinity stress in last few years various approaches such as desalination, conservation of water and soil, mulching, salt resistant species, control of sea water intrusion and utilization of microbial species have been widely practiced (Ondrasek *et al*., 2022). However, among them, utilization of microbial species such as bacteria, fungi, cyanobacteria as plant or soil inoculants have been frequently used throughout the world (Kumawat *et al*., 2023). The utilization of microbial strains for the salinity stress management emerges as a sustainable and cost-effective approach. In addition, the symbiotic relation of the microbes with the plants also helps in modulating plant growth or protecting the plant from the pathogen invasion via synthesising phytohormones, nutrients acquisition, section of antimicrobial compounds etc. However, during the salinity stress conditions the microbial strains synthesizes the compounds or nutrients which help in resistance against the salinity stresses. In addition, the microbial strains synthesize various types of antioxidant enzymes such as superoxide dismutases (SOD), catalases (CAT), ascorbate peroxidase (APX), glutathione reductase (GR) that play significant role in quenching free radical of ROS generated during the stress conditions (Verma *et al*., 2021). However nowadays endophytic strains have been preferred to use as bio inoculants to mitigate the challenges of salinity stress. The better colonization efficacy and effective acclimatization potential make the endophytic strains more potent than other rhizospheric microbial species (Verma *et al*., 2021). It has been reported that all the plants have at least some endophytic microorganisms and their number or concentrations varies among different plant organs (Kumar *et al*., 2016; Mushtaq *et al*., 2023). In the recent past fungal strains including the *Trichoderma* have been used as inoculants to improve the agricultural productivity. In addition, different *Trichoderma* sp. wildly used in the sustainable agriculture (Guzmán-Guzmán *et al*., 2023). In the previous studies different authors reported role of *Trichoderma* as biofertlizers, or biocontrol agents in stimulating plant growth promotion or protecting plants from pathogen invasions (Abd El-Rahman and Mohamed, 2014). The utilization of *Trichodrm*a species directly or indirectly induces phytohormone modulation, nutrients acquisition, synthesis of antimicrobial compounds and antioxidative enzymes (Ahmad *et al*., 2015; Wahab *et al*., 2023). BC, generally referred as black carbon a rich in the nutrients or mineral compositions composed of different biomass source like kitchen, agro or vegetable wastes or other biological byproducts, currently used for the amendments of soil (Danesh *et al*., 2023). However, in the agricultural fields BC used to improves the soil productivity via supplementation of mineral nutrients, cations such as K^+, $Ca^{2+,}$ $Mg^{2+,}$ and P, making availability of nitrogen or phosphorous in the soil (Khedulkar *et al*., 2023). Alfalfa (*Medicago sativa* L.) is one of the legume forages, cultivated throughout the world, due to high protein content (Scasta *et al*., 2012). The elevated salt concentration negatively impacts the growth and productivity of alfaalfa. Although, studies showed that alfalfa species can tolerate moderate level of salinity (Ferreira *et al*., 2015). But studies also showed that enhanced concentration of salinity reduced the morphological yields such plant height, ratio of stem-leaf and biomass yields (Cornacchione *et al*., 2017). Therefore, the present study has been designed to evaluate the impact of salinity stress on the morphological yields, photosynthetic pigments, antioxidative markers, absorption of mineral nutrients of the alfalfa plant and how the single or combined treatment of TH and BC affect the growth and productivity of alfalfa plant under salinity stress.

Material and Methods

Plant, *Trichoderma* and biochar: The Alfalfa (*Medicago sativa* L., cv Nubaria-1) seeds obtained from Agricultural Research Center, Ministry of Agriculture, Giza, Egypt. The endophytic strain *Trichoderma hamatum* (Bonord.), Bainier, was previously isolated and identified by professor Abeer Hashem (Hashem *et al*., 2014). TH was subculture in the potato dextrose agar (PDA) medium for 2-3 times at the room temperature and further used for the experiment. Shells of the Indian white shrimp (*Fenneropenaeus indicus*) were collected from local seafood markets (Al Qatif, Saudi Arabia) and cleaned carefully then dried (80°C for 12 h). The dried samples were crushed and sieved (sieve in 0.15 mm, 100-mesh). The dried samples were subjected to slow pyrolysis at 500°C with a 40 min holding period to generate biochar according to the method described by Liu *et al*., (2021).

Experimental design: This research was conducted in a growth chamber at King Saud University, Riyadh, Saudi Arabia, College of Food and Agricultural Sciences. The seeds were then washed or surface sterilized (0.01% Mercury chloride or $HgCl_2$) for the 2 minutes then washed with distilled water three successive times. The seeds were then sown in the experimental pot for growing, then after treated with BC 1% and approximately 800 spores of endophytic TH using protocol of Hashem *et al*., (2016) and stored in the controlled condition. The pots were watered during time to time for proper growth of alfalfa seeds. After passing of 20 days the pots were divided in two groups, first group treated with normal tap water, while second group were treated with saline water having concentration of 125 mM NaCl. Further each group was further categorized into four subgroups: 1) no treatment (control); 2) treated with TH; 3) treated with BC and 4) treated with TH and BC combinedly.

Measurement of growth parameters: For the measurement of morphological yields in of alfalfa various parameters such as shoot height, Root Depth, shoot fresh weight, root fresh weight, shoot dry weight root dry weight, root/ stem ratio or the branch number were considered. After completion of one month of growth total five plants (randomly selected) were brought to the laboratory and manually measured the root length, shoot height. However, to measure the dry weight samples were oven-dried at 70°C for 24 h and then measured.

Estimation of photosynthetic pigments: The protocol of Vernon & Seely (1966) described in details by Lichtenthaler (1987) used for extraction and estimation of photosynthetic pigments (chlorophyll a, b and carotenoids using acetone (80%, v/v), the colour intensity was measured at 470, 652, and 665 nm spectrophotometrically.

Nodulation dynamics, nodule activity and total proteins content: The nodule dynamics of the alfalfa plants were evaluated using the standard protocol of Abd_Allah *et al*., (2015). Although the concentration of leghaemoglobin in the alfalfa root nodules were determined by Keilin & Wang (1945) protocol and the nitrogen contents in the alfalfa were determined by following the standard protocol of (Allen, 1953).

Estimation of mineral ion contents: The mineral ions contents (Na^+, K^+, P and Ca^{2+}) accumulated in the leaves of alfalfa were carried out using micro-Kjeldahl apparatus following the slandered protocol. To determine the nitrogen contents stranded protocol of Bremner (1960) were followed. However, P (Sen Tran *et al*., 1988), Na^+ Wolf (1982), K^+ (Page *et al*., 1982) and Ca^{2+} ions were estimated using protocol of (Hunter and Hall, 1953).

Determination of MDA, H_2O_2, proline, and Glycine beraine (GB) content: The methods described by Heath and Packer (1968), Mukherjee & Choudhuri (1983), Bates *et al*., (1973) and Habib *et al*., (2012) used for estimation of malondialdehyde (MDA), H_2O_2, proline and GB, respectively.

Determination of antioxidant enzymes: The assay of antioxidative enzymes activity estimated following standard protocols of Beauchamp & Fridovich (1971) for SOD, Aebi (1987) for CAT; Nakano & Asada, (1987) for APX and Aravind & Prasad, (2005) for GR, respectively.

Statistical analysis: The statistical analysis of experimental data was carried out using the software SPSS 26.00 (Gomez & Gomez, 1984) and the quantitative analyses were obtained after one–way ANOVA and the data's have been considered significance having value of $p<0.05$ with *Tukey's* post hoc test analysis.

Results

Morphological growth parameters: During the experiment, treatment of salt results in the decreased morphological yields in compared to the salt non-treated plant. The combined application of TH and BC showed maximum enhancement in the morphological yields in both salts treated and control plants in compared to alone use of TH or the BC. Although the impact of singly applied BC was lower in compared to the TH and combined application of TH and BC.

On studying the impact of TH and BC on the alfalfa different parameters of morphological yields decreased after salt treatment in compared to the non-salt treated control plants. However, in case of control the shoot height increased by 16%, 3% and 15% after treatments with TH, BC and combined application of TH and BC respectively (Table 1). The salt treated plant showed enhancement in shoot height by 68%, 34% and 39% after treatment with TH, BC and combined application of TH and BC respectively. Although no much variation in root depth were observed in the non-salt treated alfalfa during all the three treatments. However, Root fresh weight decreased after the salt treatment in compared to control. The treatment of alfalfa with TH, BC and combined application of TH and BC enhanced the root fresh weight by 75%, 25% and 131% respectively, while after salt treatment enhancements were recorded as 83%, 50% and 160% respectively. Similarly, root dry weight was recorded enhanced by 31%, 25% and 80% in the control plant, however, 33%, 100% and 300% were recorded in the salinity stress plant after treatment with single application of TH, BC and combined application of TH and BC respectively. Although the R/S was found decreased in both control and salt stress plant. The lowest value was recorded in plant treated with BC (31%) followed by TH 19% and the combined application of TH and BC had approximately similar percentage like the control plant. However, during the salinity stress condition the R/S ratio decreased by 17%, 19% and 4% after treatment with Trichoderma, BC or combinedly TH + BC respectively. The branch number in the alfalfa plants also decreased in the salinity stress condition. However, the treatment of TH and BC singly or combinedly enhanced the branched number in the 84%, 38%, 84% under control condition and 48%, 4%,44% under salinity stress condition after treatment with of TH, BC and combined application of TH and BC respectively.

Impacts of endophytic TH and BC on the photosynthetic pigments: Salinity stress affected the chlorophyll contents, however the treatment of alfalfa plants with the endophytic fungal strain TH and BC singly or combination mitigate the negative effect of salinity stress. Although the impact of BC was minimum and combined application of TH and BC was maximum in recovering or improving the chlorophyll, carotenoids, or total pigment contents (Table 2). In case of control alfalfa plant the contents of Chl a was found enhanced by 24 %, 4% and 51%, however Chl b was found enhanced by 24%, 8% and 56% after treatment with TH, BC and TH + BC respectively. Similar trend was observed for Chl a+b 24%, 5%, 52%, carotenoids 60%, 30%, 80 %, total pigments 28 %, 8% and 55% after treatment with TH, BC and TH + BC respectively. However, after salt treatment overall the chlorophyll pigments were decreased in compared to the non–salt treated alfalfa, however the treatment of endophytic TH strains, BC and combined application of endophytic TH strain and BC enhanced the chlorophyll pigments, carotenoids and total pigments in the similar trends. Chl a increased by 34%, 24% and 48%; Chl b 21%,0%, 31%; Chl a+b 17%,15% and 18%; Carotenoids 55, 5% and 36% and total pigments 22%, 14% and 40% after treatments of TH, BC and TH + BC, respectively.

Nodule dynamics: Similar like morphological yields, salinity stress adversely affected the nodules number, soluble proteins, leghaemoglobin and nodule nitrogenise activities. The treatment of endophytic TH strain and BC singly or in combination positively impacted all the parameters. Although the impalement of salinity negatively impacted the nodules number, soluble proteins, leghaemoglobin and nodule nitrogenase activities in compared to the control or non-salt treated alfalfa plant. During treatment's combined application of TH + BC showed maximum impact, however single inoculation of BC showed minimum (Table 3). The control non-salt treated alfalfa the single treatment of TH, BC and combined application of TH and BC significantly enhanced the nodule number by 102%, 36% and 184%, soluble proteins by 50%,10% and 64%, leghaemoglobin content 83%, 29% and 111%, and nodules nitrogenase activity 94%,40% and 136% respectively. Similar types of trends were observed in the salt treated alfalfa plants although the concentration was decreased in compared to the non-salt treated plants. Single treatment of TH, BC and combined application of TH and BC significantly enhanced the nodule number by 78%, 10% and 128%, soluble proteins by 145%, 72% and 200%, leghaemoglobin content 50%,25% and 61%, and nodules nitrogenase activity 77%, 31% and 109% respectively.

Table 1. Impacts of endophytic *Trichoderma hamatum* and biochar on the morphological yields of alfalfa under saline conditions.

Treatment	**Without salt (control)**							
Parameters	**Shoot height (cm)**	**Root depth (cm)**	**Shoot fresh weight (g)**	**Root fresh weight (g)**	**Shoot dry weight (g)**	**Root dry weight (g)**	**R/S ratio**	**Branch number**
Plant	19.5 ± 0.96^{a}	15.5 ±0.84^{b}	0.42 ± 0.06^{a}	0.16 ± 0.003^{c}	0.23 ± 0.01^{c}	0.16 ± 0.01^{c}	0.81 ± 0.07^{a}	13.0 ± 0.5^{c}
P + *T. hamatum*	22.6 ± 1.58^{a}	15.5 ± 2.42^{b}	0.70 ± 0.142^{a}	0.27.89 ± 0.023^{b}	0.29.8 ± 0.01^{b}	0.21 ± 0.001^{b}	0.56 ± 0.09^{a}	24.00 ± 1.46^{a}
Plant + Biochar	20.2 ± 1.9^{a}	15.4 ± 2.7^{b}	0.59 ± 0.12^{a}	0.20 ± 0.02^{c}	0.24 ± 0.01^{c}	0.20 ±0.005^{bc}	0.66±0.15^{a}	18.33 ± 0.96^{b}
P + *T. hamatum* + Biochar	22.4 ± 3.21^{a}	20.2 ± 3.6^{a}	0.76 ± 0.01^{a}	0.37 ± 0.01^{a}	0.39 ± 0.02^{a}	0.30±0.01^{a}	0.81±0.06^{a}	24.67 ± 0.8^{a}
P	0.67	0.085	0.263	0.0003	0.006	0.001	0.33	0.0007
Treatment	**With salt**							
Parameters	**Shoot height (cm)**	**Root Depth (cm)**	**Shoot fresh weight (g)**	**Root fresh weight (g)**	**Shoot dry weight (g)**	**Root dry weight (g)**	**R/S ratio**	**Branch number**
Plant	16.33 ± 1.59^{b}	13.93 ± 1.37^{a}	0.35 ± 0.02^{b}	0.06 ± 0.01^{b}	0.06 ± 0.005^{c}	0.03 ± 0.004^{c}	0.92 ± 0.12^{a}	8.33 ± 0.86^{ab}
P + *T. hamatum*	27.40 ± 1.93^{a}	20.30 ± 1.49^{a}	0.56 ± 0.03^{a}	0.11 ± 0.003^{ab}	0.11 ± 0.006^{ab}	0.10 ± 0.005^{a}	0.77 ± 0.009^{a}	12.33 ± 1.06^{a}
Plant + Biochar	21.97 ± 1.10^{ab}	16.20 ± 1.74^{a}	0.47 ± 0.03^{ab}	0.09 ± 0.006^{b}	0.08 ± 0.004^{bc}	0.06 ± 0.002^{b}	0.75 ± 0.10^{a}	8.00 ± 0.8^{b}
P + *T. hamatum* + Biochar	22.83 ± 2.27^{ab}	19.70 ± 1.71^{a}	0.42 ± 0.12^{ab}	0.16 ± 0.01^{a}	0.14 ± 0.01^{a}	0.12 ± 0.009^{a}	0.89 ± 0.133^{a}	12.00 ± 1.15^{ab}
p	0.04	0.26	0.06	0.03	0.004	0.004	0.88	0.079

Data are the representation of mean of three replicates ± Std errors; different letters in the column showed significant difference $p<0.05$ observed during *Tukey post hoc* analysis

Table 2. Impacts of endophytic *Trichoderma hamatum* and biochar on the photosynthetic pigments (mg/g fresh weight) of alfalfa under saline condition.

Treatment	**Without salt**					
Parameters	**Ch a**	**Ch b**	**a + b**	**a / b**	**Carotenoids**	**Total pigs**
Plant	0.45 ± 0.003^{c}	0.25 ± 0.003^{d}	0.70 ± 0.006^{d}	1.77 ± 0.02^{a}	0.10 ± 0.005^{c}	0.80 ± 0.01^{d}
P + *T. hamatum*	0.56 ± 0.002^{b}	0.31 ± 0.002^{b}	0.87 ± 0.003^{b}	1.81 ± 0.01^{a}	0.16 ± 0.003^{ab}	1.03 ± 0.006^{b}
Plant + Biochar	0.47 ± 0.001^{c}	0.27 ± 0.001^{c}	0.74 ± 0.002^{c}	1.78 ± 0.008^{a}	0.13 ± 0.001^{bc}	0.87 ± 0.003^{c}
P + *T. hamatum* + Biochar	0.68^{a}	0.39^{a}	1.07 ± 0.00 1^{a}	1.75 ± 0.005^{a}	0.18^{a}	1.24 ± 0.002^{a}
p	0.003	0.014	0.0183	0.73	0.0128	0.008
Treatment	**With salt**					
Parameters	**Ch a**	**Ch b**	**a + b**	**a / b**	**Carotenoids**	**Total pigs**
Plant	0.29 ± 0.009^{c}	0.19 ± 0.004^{d}	0.48 ± 0.01^{d}	1.48 ± 0.01^{b}	0.19 ± 0.008^{b}	0.67 ± 0.022^{c}
P + *T. hamatum*	0.39 ± 0.005^{b}	0.23 ± 0.002^{b}	0.62 ± 0.008^{b}	1.74 ± 0.01^{a}	0.20 ± 0.004^{b}	0.82 ± 0.01^{b}
Plant + Biochar	0.36 ± 0.003^{b}	0.21 ± 0.001^{c}	0.57 ± 0.004^{c}	1.71 ± 0.006^{a}	0.20 ± 0.002^{b}	0.77 ± 0.007^{b}
P + *T. hamatum* + Biochar	0.43 ± 0.001^{a}	0.25^{a}	0.68 ± 0.002^{a}	1.76 ± 0.003^{a}	0.26 ± 0.001^{a}	0.94 ± 0.004^{a}
p	0.0037	0.0001	0.0025	0.0021	0.0073	0.0076

Data are the representation of mean ± Std errors; However different letters in the column denoted significance differences ($p<0.05$) among them observed during *Tukey post hoc* analysis

Table 3. Impacts of endophytic *Trichoderma hamatum* and biochar on the nodulation dynamics and nodule activity of alfalfa under saline conditions.

Treatment	**Without salt (control)**			
Parameters	**Nodules (number/plant root)**	**Soluble protein (mg/ g fresh wt)**	**leghaemoglobin concentration (mg/g fresh wt)**	**Nodule nitrogenase activities (C_2H_4/ g fresh wt/ hout)**
Plant	23.0 ± 2.3^{d}	0.28 ± 0.009^{b}	10.02 ± 0.069^{d}	0.50 ± 0.009^{d}
P + *T. hamatum*	48.0 ± 1.73^{b}	0.42 ± 0.01^{a}	18.40 ± 0.2^{b}	0.97 ± 0.02^{b}
Plant + Biochar	31.33 ± 2.22^{c}	0.31 ± 0.005^{b}	12.93 ± 0.04^{c}	0.70 ± 0.01^{c}
P + *T. hamatum* + Biochar	65.67 ± 1.20^{a}	0.46 ± 0.02^{a}	21.22 ± 0.17^{a}	1.18 ± 0.02^{a}
p	0.0099	0.000108	0.006	0.0019
Treatment	**With salt**			
Parameters	**Nodules (number/plant root)**	**Soluble protein (mg/ g fresh wt)**	**leghaemoglobin concentration (mg/g fresh wt)**	**Nodule nitrogenase activities (C_2H_4/ g fresh wt/ hout)**
Plant	9.33 ± 1.33^{c}	0.12 ± 0.011^{d}	5.59 ± 0.103^{d}	0.219 ± 0.07^{d}
P + *T. hamatum*	16.67 ± 0.88^{b}	0.269 ± 0.007^{b}	8.46 ± 0.2^{b}	0.389 ± 0.008^{b}
Plant + Biochar	10.33 ± 0.88^{c}	0.19 ± 0.002^{c}	7.03 ± 0.04^{c}	0.29 ± 0.05^{c}
P + *T. hamatum* + Biochar	21.33 ± 0.88^{a}	0.33 ± 0.01^{a}	9.05 ± 0.05^{a}	0.46 ± 0.009^{a}
p	0.0094	0.0059	0.0046	0.0012

Table 4. Impacts of endophytic *T. hamatum* and biochar on the elements accumulation in leaf (mg/g dry weight) of alfalfa under saline conditions.

Treatment	**Without salt (control)**			
Parameters	**Na^{+}**	**K^{+}**	**K/Na**	**Ca^{2+}**
Plant	71.11 ± 0.07^{a}	35.73 ± 0.3^{c}	5.03 ± 0.02^{d}	7.84 ± 0.17^{d}
P + *T. hamatum*	46.3 ± 0.22^{c}	41.19 ± 0.24^{b}	8.94 ± 0.41^{b}	8.93 ± 0.099^{b}
Plant + Biochar	59.9 ± 0.07^{b}	41.28 ± 0.49^{b}	6.90 ± 0.009^{c}	8.37 ± 0.10^{c}
P + *T. hamatum* + Biochar	32.9 ± 0.00^{d}	44.62 ± 0.43^{a}	13.59 ± 0.2^{a}	9.32 ± 0.02^{a}
p	0.0019	0.0023	0.0026	0.0077
Treatment	**With salt**			
Parameters	**Na+**	**K^{+}**	**K/ Na**	**Ca^{2+}**
Plant	53.68 ± 0.68^{a}	21.11 ± 0.11^{d}	0.39 ± 0.006^{d}	4.08 ± 0.06^{c}
P + *T. hamatum*	20.81 ± 1.07^{c}	27.95 ± 0.40^{b}	1.35 ± 0.005^{b}	6.02 ± 0.006^{b}
Plant + Biochar	24.91 ± 0.15^{b}	26.48 ± 0.31^{c}	1.06 ± 0.006^{c}	5.94 ± 0.13^{b}
P + *T. hamatum* + Biochar	17.43 ± 0.37^{d}	30.68 ± 0.22^{a}	1.76 ± 0.02^{a}	6.77 ± 0.02^{a}
p	0.0083	0.0073	0.0087	0.0091

Data are the representation of mean ± std errors; However different letters in the column denoted significance differences ($p<0.05$) among them observed during *Tukey post hoc* analysis

Table 5. Impacts of endophytic *T. hamatum* and biochar on the oxidative stress marker of alfalfa under saline conditions.

Treatment	**Without salt**			
Parameters	**H_2O_2 (µg/ g fresh weight)**	**MDA (nmol/ g fresh weight)**	**Proline µmol / g fresh weight**	**GB (µMol/g fresh weight)**
Plant	240.07 ± 2.83^{a}	19.67 ± 0.07^{a}	13.47 ± 0.44^{b}	26.90 ± 0.55^{b}
P + *T. hamatum*	204.5 ± 4.5^{c}	16.23 ± 0.43^{b}	15.77 ± 0.20^{a}	29.40 ± 0.15^{a}
Plant + Biochar	228.13 ± 1.72^{b}	19.27 ± 0.44^{a}	13.37 ± 0.24^{b}	27.07 ± 0.14^{b}
P + *T. hamatum* + Biochar	187.80 ± 3.08^{d}	15.47 ± 0.17^{b}	16.13 ± 0.14^{a}	30.27 ± 0.24^{a}
p	0.0012	0.0005	0.00015	0.00014
Treatment	**With salt**			
Parameters	**H_2O_2 (µg/ g fresh weight)**	**MDA (nmol/ g fresh weight)**	**Proline (µmol / g fresh weight)**	**GB (µMol/g fresh weight)**
Plant	438.33 ± 7.2^{a}	78.80 ± 3.1^{a}	50.23 ± 0.82^{a}	50.17 ± 0.75^{a}
P + *T. hamatum*	269.50 ± 1.10^{c}	38.13 ± 1.29^{c}	26.73 ± 2.91^{c}	40.93 ± 0.43^{b}
Plant + Biochar	321.23 ± 2.42^{b}	66.13 ± 0.50^{b}	36.50 ± 0.55^{b}	48.57 ± 0.86^{a}
P + *T. hamatum* + Biochar	251.17 ± 1.47^{d}	31.63 ± 0.66^{d}	23.07 ± 0.20^{c}	34.70 ± 0.34^{c}
p	0.0028	0.0014	0.0070	0.004

Table 6. Impacts of endophytic *T. hamatum* and biochar on the antioxidant enzymes of alfalfa under saline conditions.

Treatment	**Without salt (control plant)**			
Parameters	**SOD (EU/ mg protein)**	**CAT (EU/ mg protein)**	**APX (EU/ mg protein)**	**GR (EU/ mg protein enzymes)**
Plant	63.99 ± 1.07^{d}	53.29 ± 0.51^{a}	6.20 ± 0.04^{d}	11.85 ± 0.11^{c}
P + *T. hamatum*	69.17 ± 0.55^{b}	58.19 ± 0.11^{a}	7.96 ± 0.03^{b}	12.75 ± 0.14^{b}
Plant + Biochar	66.57 ± 0.31^{c}	55.66 ± 0.06^{a}	6.99 ± 0.02^{c}	12.05 ± 0.08^{c}
P + *T. hamatum* + Biochar	71.90 ± 0.17^{a}	202.12 ± 0.20^{a}	8.38 ± 0.05^{a}	13.50 ± 0.13^{a}
p	0.0002	0.440	0.0036	0.0034
Treatment	**With salt**			
Parameters	**SOD (EU/ mg protein)**	**CAT (EU/ mg protein)**	**APX (EU/ mg protein)**	**GR (EU/ mg protein enzymes)**
Plant	88.96 ± 0.68^{d}	71.1 ± 0.11^{d}	10.93 ± 0.06^{d}	13.86 ± 0.07^{c}
P + *T. hamatum*	94.78 ± 0.28^{b}	75.80 ± 0.24^{b}	13.02 ± 0.11^{b}	15.26 ± 0.188^{b}
Plant + Biochar	91.13 ± 0.16^{c}	72.83 ± 0.14^{c}	11.74 ± 0.06^{c}	14.03 ± 0.10^{c}
P + *T. hamatum* + Biochar	97.57 ± 0.25^{a}	79.15 ± 0.08^{a}	14.16 ± 0.05^{a}	17.20 ± 0.04^{a}
p	0.0020	0.0015	0.0077	0.0059

Data are the representation of mean ± Std errors; However different letters in the column denoted significance differences ($p<0.05$) among them observed during *Tukey post hoc* analysis

Mineral ion contents: The data presented in the Table 4 showed that treatment of endophytic TH strain and BC singly or in combination positively impacted mineral contents of Na^+, K^+, K/ Na, and Ca^{2+} in the leaves of control or salt treated alfalfa plants. Although the treatment of salt reduces the overall contents of the mineral elements in compared to non-salt treated alfaalfa plants except Na^+. The treatment of salt significantly decreased the contents of K+, K/ Na, and Ca $^{2+}$. However, the treatment of TH strain and BC singly or in combination positively modulates the contents of mineral elements except Na^+. In case of Na^+ the content was decreased by 35%, 16% and 56%, K^+ concentration increased by 15%, 15% and 24% ratio of K/Na increased by 77%, 37% and 170%, and the concentration of Ca^{2+} increased by 13%, 6% and 18% after treatment with TH, BC and combination of TH + BC respectively. Similar types of trends were observed in the salt treated alfalfa leaves the concentration of Na^+ was reduced by 62%, 54% and 68%, concentration of K^+ was increased by 32 %, 25% and 45%; ration of K/Na was increased by 246%, 171% and 351% and Ca^{2+} was increased by 47%, 45% and 65% after treatment with TH, BC and combination of TH + BC respectively.

Oxidative stress markers: The onset of salinity stress enhanced the oxidative stress enzymes or markers. In this study the salt treatment enhanced the oxidative stress markers such H_2O_2, MDA, Proline or Glycine betaine contents in compared to the control. However, the treatment of endophytic TH strain and BC singly or in combination reduced the contents (Table 5). During the experiment in control (non-salt treated alfalfa) the content H_2O_2 decreased by 15%, 5% and 22%, the content of MDA decreased by 18%, 3% and 22%. The concentration of proline was increased by 17%, decreased by 1% and increased by 19%, and the content of GD was increased by 9%, 3% and 12% after treatment with TH, BC and combination of TH + BC respectively. Increase of salt treated plant similar types of trends was observed, the content of H_2O_2 was decreased by 39%, 27%,43%, MDA content decreased by 52%, 17% and 60%; the proline content decreased by 27%, 27% and 54% and the content of GB decreased by 19%, 10% and 65.30% after treatment with TH, BC and combination of TH + BC, respectively.

Data are the representation of mean ± Std errors; However different letters in the column denoted significance differences ($p<0.05$) among them observed during *Tukey post hoc* analysis.

Antioxidant enzymes activity: During experiment the activity of antioxidative enzymes increased with single TH, BC or combined treatment of TH + BC in both non-salt treated or salt treated alfalfa plant (Table 6). In case of non-salt treated, SOD activity significantly increased by 8%, 4% and 12%, catalase activity increased by 9%,4% and 6%, APX activity increased by 28%, 12%, 35% and GR content increased by 7%, 1% and 13% after treatment with TH, BC and combination of TH + BC respectively. Similar types of trend were observed in salt treated plant the activity of SOD increased by 6%, 2% and 9%, catalase activity increased by 6 %, 2% and 11%, APX activity increased by 19%, 7% and 29% and GR activity increased by 10%, 1% and 24% after treatment with TH, BC and combination of TH + BC respectively.

Discussion

Plants are a sessile organism, highly susceptible against the biotic and abiotic stress, that showed adverse detrimental on the plants irrespective of their plant growth stages (Solanki *et al.*, 2023). The saline conditions generally imbalance the mineral ions concentration which results in the imbalance of ions or causes specific ion toxicity or changes in osmotic potential. In addition, results in the decrement of osmotic potential that makes difficult for plants to absorb water, which can disrupt normal physiological processes and impede growth (Acosta-Motos *et al.*, 2017). Although the level of toxicity and their effect on plants depends upon the concentration and exposure time (Taiz *et al.*, 2015). The excessive concentration of salt in the soil causes lower solute potential, which ultimately lowered water potential and the plant continue to absorb water and maintain cellular turgor pressure under these saline conditions, because for the survival it is mandatory that their internal water potential should be lower than the surrounding soil (Zhao *et al.*, 2021).

Morphological growth parameters: In this study, salt treatments result in the lowering of morphological yields in compared to control, however, control or the salt treated plant showed enhancement in the morphological yields after single or combined treatment of TH and BC. In the previous experiments authors also reported stimulatory impact of BC on the plants. The treatment of BC modulates the bioavailability of mineral ions to the plants (Hossain Sani *et al.*, 2020). In addition, modulates the phytohormones levels especially indole acetic acid or gibberellin that directly play crucial role in root growth initiation, cell division, enhancement in shoot and root height or other morphological parameters (Langeroodi *et al.*, 2019). The strain of *Trichoderma* also reported for the significant contribution in enhancing the morphological yields via modulating the phytohormones level, enhancing nutrients acquisition to the plants (Khadka & Uphoff, 2019). However previously published report also stated that utilization of TH enhanced the morphological yields especially the root growth and branching via synthesis of indole acetic acid (Hashem *et al.*, 2016). Further, Hashem *et al.*, (2019) reported the role of *Trichoderma* in diminishing the ABA accumulation during the stress condition and promoting cytokinin that helps in modulating the plant shoot heights. The application of TH and BC combinedly show stimulatory effect in plant growth or morphological yields under saline conditions. The utilization of *Trichoderma* helps in absorbing water or nutrients from the deep soil even under the saline conditions with the help of mycelium, however utilization of BC full fill the requirements of essential nutrients (Sun *et al.*, 2020). Additionally, BC helps in maintaining soil moisture or relative water content (Langeroodi *et al.*, 2019).

Photosynthetic pigments: In the previous published report, it has been mentioned that abiotic stress conditions can lead to the degradation or reduction of photosynthetic pigments especially the chlorophyll or carotenoids. The reason for this reduction in chlorophyll yields are the inhibitions of enzymes such as δ- aminolevulinic acid dehydratase and protochlorophyllide reductase. Additionally, studies also showed that salinity also adversely affect or degrade the enzymes such as RuBP (ribulose-1,5-bisphosphate) carboxylase and ATP synthase, which play crucial role in electron transport chain (Amanullah & Khan, 2023). The degradation of these enzymes resulted in damage of thylakoids membrane or chloroplasts and the imbalance stomata conductance. In addition, the salinity stress affects the conductance or concentration of some essential ions such as Mg^{2+}, $Fe^{2+,}$ Zn^{2+} etc, which are required for the synthesis of chlorophyll (Ahmad *et al*., 2015). Similarly, the synthesis of carotenoids also decreased during the salinity stress conditions due to adverse impact of salinity on the carotenoid's precursors β-carotene and zeaxanthin (Akladious & Mohamed, 2018). In our experiment, the salinity stress condition decreased the photosynthetic pigments such as Chl, carotenoids or the total pigment in compared to the control. However, application TH and BC singly or combination enhanced the photosynthetic pigments. Although the extent of enhancement was lower in single application of BC and maximum observed in the co-inoculation of TH or BC. The application of BC improves the soil physiochemical characteristics properties, which are required for the optimum growth of plants (Batool *et al*., 2022). These improvements help in restoring the chlorophyll synthesis. Although co-inoculation of TH and BC showed promising results in compared to single application as the BC indirectly improves the soil physiochemical properties supports the growth of natural microflora including the fungal species, which improves the water holding capacity of the plants, absorption of mineral nutrients required for the synthesis of chlorophyll molecules during the stress conditions (Batool *et al*., 2022; Amanullah & Khan, 2023). Similar types of results improved photosynthetic pigments were reported by Ahmad *et al*., (2015) after application of TH under saline conditions in *Brassica juncea*. Morever, Kumar *et al*., (2020) reported the significant effect of single or the combined application of TH, BC, in improving photosynthetic pigments in spinach under salinity stress.

Nodules, leghaemoglobins and nodule nitrogenase activity: In our experiment, salinity stress condition decreased the number of nodules, leghaemoglobin, and nodule nitrogenase activity. However alone or co-inoculation of TH and BC enhanced the nodule numbers, leghaemoglobin and nodule nitrogenase activity. It has been generally observed that salinity stress condition generally impairs the signaling pathways between plant roots and the *Trichoderma* species, which is required for the nodule formation. However, during the salinity stress the elevated level of salt concentration increased the osmotic pressure and also causes ion toxicity, which results in the damaging of nodule cells (Mushtaq *et al*., 2021). Leghaemoglobin is crucial for transporting oxygen to the nitrogen-fixing bacteria in the nodules while maintaining a low oxygen concentration to protect the nitrogenase enzyme. However, the onset of salinity stress lowers the leghaemoglobin synthesis via impaired oxygen transport (Verma *et al*., 2023). In addition, the decreased leghaemoglobin levels under salinity stress lead to higher oxygen concentrations in the nodules, which inactivate the nitrogenase enzyme, and ultimately reduced the nitrogen fixation efficiency. As the nitrogenase enzymes is highly prone to the oxygen presence and enhancement in the oxygen level can lead to the inactivation of enzyme activity (Quilliam *et al*., 2012; Ma *et al*., 2019). Salinity stress generally decreased the leghaemoglobin's synthesis and damage the structures and functions of nodules. Additionally, as for the normal functioning of nitrogenase optimum energy is required but the salinity stress condition reduced overall the energy availability by impairing photosynthesis pigments. In addition, enhanced concentration of salt ions generally imbalances the cell homeostasis and disrupt the normal enzymatic activity of nitrogenase (Tejera *et al*., 2004).

In this study alone or co-inoculation of TH and BC positively modulates the number of nodules, leghaemoglobin and nodule nitrogenase activity nr salinity stress conditions. It has been generally observed that BC mediate role in availability of mineral elements and also play crucial role in absorbing excess salt concentration (Ma *et al*., 2019). In addition, provide favourable conditions for the rhizobium and other nitrogen fixing microbe for the colonization and nodule formation (Ma *et al*., 2019). However, *Trichoderma* also play pivotal role in improving root architecture, making it easier for rhizobia to establish effective nodulation (Farhangi-Abriz & Torabian, 2018). BC also play pivotal role in the efficient synthesis and functioning of leghemoglobins, which are crucial for oxygen transport in nodules (Farhangi-Abriz & Torabian, 2018). However, *Trichoderma* enhance the antioxidant defence system in plants, reducing oxidative damage caused by salinity stress. This can help maintain the integrity and functionality of leghaemoglobin in the nodules, ensuring proper oxygen regulation for nitrogen fixation.

Nodule nitrogenase activity: Application of BC also help in providing a stable and favourable microenvironment within the nodules, protecting the nitrogenase enzyme from inactivation by high oxygen levels (Quilliam *et al*., 2012). Whereas, it has been assumed that *Trichoderma* enhances enhancing energy system and nutrient uptake under the salinity stress condition, which favour the nodule nitrogenase activity and ultimately nitrogen fixation processes (Ma *et al*., 2019).

Mineral elements: In the experiment salinity stress condition decreased the K^+, K/ Na, and Ca^{2+} in compared to the control except Na^+, However, alone or co-inoculation of TH and BC enhanced the mineral concentration in the alfalfa leaves. In the previous studies it has been reported that application of BC significantly enhanced the physicochemical properties of soil. As the BC are mineral rich and contains different ions such as Ca^{2+}, Mg^+, which play pivotal role in ion exchange with the salt ions. In addition, also act as a catalyst for various physiochemical or biochemical phenomenon (Abd El-Naby *et al*., 2019). Current finding of our results showed combined application of TH and BC enhanced the concentration Na^+ ions and decreased the concentration of other mineral ions after single or combined application of TH and BC. Similar types of observation were also reported by Wang *et al*., (2013) after single or combined application of *Trichoderma* and BC in the spinach plants. Also, it has been reported enhancement in N, P, and K the essential nutrients after treatments of *Trichoderma* and organic amendments in the Canola plants. Hossain Sani *et al*., (2020) similar observation in the tomato plant. Also reported similar types of observation in the spinach plant and also found elevated concentration of Na^+ and decreased concentration of other minerals ions. Although the enhanced level of Na^+ ions has been found in the plant may be due to the rooting Na^+ ions or improved soil (Wang *et al*., 2013). However, the concentration of K^+ ions was found maximum in case of both the control or the salt treated plants after treatment of singly or combinedly with *Trichoderma* and BC. In the previous study Wang *et al*., (2014) reported that the combined application improves the ion exchange capacity of K+ and limit the Na^+ entry, which results in the enhanced uptake of K^+ in the plants (Wang *et al*., 2014). The application of BC help in lowering the concentration of Na^+ due to high absorption potential but mediate significant role in ion exchange with Ca^{2+} and Mg^{2+} (Hashem *et al*., 2016).

Oxidative stress markers: The abiotic stress conditions like salinity or draught lead to the generation of ROS, which adversely affect the plant via disrupting membrane potential, lipid peroxidation and negatively impacting plant genetic materials (Hu *et al*., 2012. El-Beltagi *et al*., 2023). However, the osmoprotectants protect the plants during the oxidative stress conditions via protecting cell membrane, nucleic acids or other biomolecules (Farhangi-Abriz & Torabian, 2017). The application of BC or *Trichoderma* protect the plant from salinity stress conditions via improving the osmoprotectants contents in the alfalfa plant. Similar type of observation was also reported by Amanullah & Khan (2023) after application of *T. asperellum* and BC in the maize plant. Plants synthesizes extensive amount of reactive oxygen or reactive nitrogen species under stress conditions. During the salinity stress plants also releases various types of oxidative stress markers like H_2O_2, MDA, proline, GB etc (Ahanger & Agarwal, 2017; Mohamed *et al*., 2018). In the present study alfalfa plants releases these oxidative stress markers after utilization of single or co-inoculation of TH and BC reduces the contents H_2O_2, MDA. In the previous studies stated that BC helps in maintaining the integrity of plasma membrane and also play crucial role in water uptake or water use efficacy that helps in lowering or minimizing the oxidative stress (Hafez *et al*., 2020). Farhangi-Abriz & Torabian (2017) also reported the potential of BC in lowering H_2O_2, and MDA content during salinity stress. Authors also reported the role of *Trichoderma* in reducing the oxidative stress markers. For example, Amanullah and Khan (2023) evaluated the *Trichoderma* role in reducing MDA content in maize under salinity stress conditions and reported *Trichoderma* reduces the content of H_2O_2 in the spinach under saline condition. Plants accumulate proline in their cells, which acts as an osmoprotectant and help in maintaining the osmotic and turgor pressure in the cell. The elevated concentration of proline also provide shield to the plants against the oxidative stress. Glycine betaine is an organic compound that helps plants cope with salinity stress. When plants face high salt levels, glycine betaine accumulates in their cells, protecting cellular structures and enzymes from damage (Ashraf & Foolad, 2007).

Antioxidative enzymes markers: The concentration of antioxidative enzymes like SOD, CAT, APX, GR, had been decreased during the salinity stress. However, the single of combined application of Trichoderma and BC enhanced the concentration of antioxidative enzymes. SOD induces the dismutation of superoxide radicals (O_2^-) into H_2O_2 and O2, which results in the lowering of superoxide radicals and protect the cell from oxidative stress. In previous studies various authors reported enhancement in the concentration of SOD with rising concentration of saltlike tomato (Abdel Latef & Chaoxing 2011), chickpea (Rasool *et al*., 2013). However, catalase is required for quenching the free radicals generated during oxidative stress conditions. In addition, catalase play an essential role in catalysing the reaction especially breraking down of H_2O_2 into H_2O and O_2 and protect the plant cell from oxidative stress. (Van Breusegem *et al*., 2001). Although the APX also play similar role but have higher affinity for H_2O_2, making it potentially more significant in managing or detoxifying ROS during stress. APX scavenge the free radicals generating during the salinity stress conditions via detoxifying H_2O_2 concentration by converting H_20 and O_2. In addition, the regeneration of Ascorbate: APX is part of the ascorbate-glutathione cycle, where ascorbate is regenerated from its oxidized form, dehydroascorbate, ensuring a continuous supply of ascorbate for H_2O_2 detoxification (Brotman *et al*., 2013). Ascorbate is an essential antioxidant in the ascorbate-glutathione cycle. Studies have shown that plants treated with *Trichoderma* accumulate higher levels of reduced ascorbate. In addition, the treatment of *Trichoderma* results in the enhancement of expression pattern of stress genes such as monodehydroascorbate reductase, (MDAR), APX1, and GST that significantly enhances the antioxidant system to combat NaCl-induced oxidative stress (Brotman *et al*., 2013). Glutathione also play significant role in reducing H_2O_2 to water in the ascorbate-glutathione cycle (Noctor *et al*., 2002). Herouart *et al*., (1993) found that glutathione induces Cu/Zn superoxide dismutase expression pattern in tobacco plant. Studies have also shown that glutathione related enzymes get induced after treatment with *Trichoderma* (Shoresh & Harman, 2008). GR is crucial for maintaining the GSH/GSSG ratio, which is necessary for ascorbate regeneration and the activation of several important enzymes involved in CO_2 fixation (Bierbaumer *et al*., 2023). The protective function of glutathione transferase may stem from its role in eliminating 4-hydroxyalkenals (membrane lipid peroxides) and propanol by conjugating them with GSH (Berhane *et al*., 1994). Additionally, glutathione transferase has been reported to directly detoxify lipid peroxides, as some glutathione transferases possess glutathione peroxidase activity (Forman *et al*., 2009).

Conclusion

The present study showed that the combined application of TH and BC can be recommend for salinity stress management and growth promotion in the alfalfa plants. The studies showed that salinity stress adversely affect the morphological yields, photosynthetic pigments, nodule dynamics and mineral contents in the alfalfa plants. In addition, the salinity stress conditions enhanced the concentrations of osmolytes such as malondialdehyde

(MDA), H_2O_2, proline, and glycine betaine content while reduced the concentration of antioxidative enzymes such as SOD, CAT, APX and GR. However, the single of combined treatments of TH stain and BC showed modulatory impact of the morphological yields, photosynthetic pigments, mineral elements and antioxidative enzymes. The finding of the experiments showed potential of TH and BC in salinity stress management and growth promotion potential of plants under salinity stress condition.

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