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Trichoderma harzianum-fertilizer enhances the tolerance of date palm seedlings against soil salinity

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Abstract

This experiment has studied the role of *Trichoderma harzianum* (TH-fertilizer) at 30 and 60 g kg^{-1} soil to improve the tolerance of date palm seedlings (Barhee cv.) against different salinity levels at 0, 75, and 150 mM NaCl in sandy soils. Salt stress reduced plant height, root length, and leaf and root numbers. On the other hand, TH-fertilizer improved these parameters and the chlorophyll and dry matter. Application of 60 g kg^{-1} soi^{-1} had a better effect on seedling growth than 30 g kg $soil^{-1}$. Salt treatments increased the malondialdehyde, and hydrogen peroxide content, which was decreased with TH-fertilizer treatments. TH-fertilizer increased proline, soluble proteins, catalase, ascorbate peroxidase, peroxidase enzyme, K, and Na/K ratio, whereas abscisic acid decreased under salt stress. Therefore, TH-fertilizer has effectively adjusted plant growth under such conditions. following review seeks to shed some light on certain specifics about the biology, pathogenicity, physical traits, and significant secreted metabolites of the hosts of this fungus.

Keywords: Electrolyte leakage, Hydrogen peroxide, *Phoenix dactylifera* L., Sandy soil, Salt stress, *Trichoderma harzianum*.

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Introduction

Date palm *(Phoenix dactylifera* L.*)* grows well under severe arid conditions since it tolerates high temperatures and drought; thereby, it helps mitigate desertification (Aseeri et al., 2021). The lack of microorganisms in sandy soils has resulted in a lack of fertility (Gatiboni 2018). The addition of environmentally friendly fungi contributes to the availability of organic matter (AL-Mansour and Kalaivanan 2021). It improves soil fertility (Hoffland et al. 2020), which could create a suitable condition for improved date palm growth. However, other abiotic conditions could also affect young palm growth, such as drought and salinity (Shareef et al., 2021). Indeed, these were the reasons for the disappearance of many date palm cultivars in southern Iraq. Salinity as abiotic stress can affect plant growth by inducing osmotic and ionic imbalances within plant cells. Salt stress limits plant growth by causing toxicity, decreasing water availability, stabilizing plant reserves, and modulating the structural organization of the proteins (Shareef and Al-khayri 2021). Plant tolerance can be developed through conventional breeding or advanced molecular techniques; traditional methods are time-consuming, while new techniques are expensive. In the present global scenario of rapid population growth, the demand for food is increasing. It requires more reliable methods to enhance plant growth, such as fungi as a bio-fertilizer (Begum et al. 2019). The new trends of more green agriculture to reduce soil and food pollutants, increase food quality, and improve socio-economic and ecological sustainability, requires abandoning and using chemical fertilizers, pesticides, and industrial additives and switching to natural materials such as organic and bio-fertilizers (Aseeri et al., 2021). *Trichoderma sp.* (TH) are endophytic plant symbionts broadly used as bio fertilizers that boost plant growth and as biocontrol agents against plant diseases (Saba 2012). They are more effective when applied to environmentally-stressed plants (i.e., salinity, drought) (Evelin et al. 2019). *Trichoderma sp.* plays a vital role in improving soil characteristics, increasing the readiness and absorption of P, K, and micronutrients, and thus improving the nutritional status of the plant by increasing the activity of soil organisms or through added fungal inoculants that improve plant growth and productivity (Przybyłko, Kowalczyk, and Wrona 2021). Ikram et al. (2019) reported that some *T. harzianum* isolates stimulate plant growth through the secretion of plant growth regulators that induce other biochemical and physiological mechanisms, such as the absorption of plant nutrients. Applying *T. harzianum* in the root zone of citrus and date palm seedlings showed a prominent effect on root growth, resulting in improved vegetative growth characteristics (Al-Haiani et al. 2014; Ahmed et al. 2019). The role of *T. harzianum* was actually via stimulating the induction of auxin-

like substances and gibberellins and nutrient absorption (Selvaraj et al. 2008). The *Trichoderma* genus helps alleviate the adverse salinity stress conditions, induce metabolic machinery, and improve plant growth (Rubio et al., 2017; Yusnawan et al., 2021). For environmental stress-tolerant palms, applying mycorrhizal fungi appear to be a novel solution for agroecosystems in sandy and saline soils by improving the physiological aspect of plant growth. It is a technique that can generate agroecosystems and improve plant growth under environmental stresses. This study aimed to study the role of *T. harzianum* live fungus on growth characteristics of salt-stressed 'Barhee' date palm seedlings grown under Iraqi arid conditions.

Materials and Methods

Experiment

The 'Barhee' date palm seeds used in this research were received from Basrah University, Iraq, Date Palm Research Center. Seed germination was conducted in 2020 in sandy soil, was prepared, washed well with water and sterilized with formalin, and then filled with sterilized growth medium by autoclaving; at a rate of one plant per pot and in the nursery room under 28±2 °C for three months. Afterward, every seedling was transferred into pots (4 L). *T. harzianum* fungus (TH) (imported from Al-Baraka Company for Organic Agricultural Supplies, Jordan) was added to the soil in a commercially prepared Biocont-T(Granular) at a rate of 30 and 60 g kg^{-1} soil, taking into account that one gram of the inoculant contains 19 × 107 spores. Seedlings were left to grow in the nursery at 29±2 °C, with 23% relative humidity (RH), and photoperiod maintained at 12 $h.d^{-1}$ with 1350 µmol m^{-2} s^{-1} light intensity. Each week, the pots irrigated with 0, 75, and 150 mM NaCl solution. After six months, the number of leaves and roots, plant height, leaf, and root length were determined. To calculate the dry weight, the leaves and roots were separated, weighed, and then oven-dried at 72 °C for 48 h.

Chlorophyll content: According to Lichtenthaler & Wellburn (1983), 100 mg of fresh leaves were squashed in 10 ml $(CH_3)_2CO$ acetone (80%) and centrifuged at 2000 rpm for five minutes. Chlorophyll content was colorimetry estimated at 663 nm.

Malondialdehyde (MDA) content: According to Davey et al. (2005), leaves (0.2 g) were homogenized with ten volumes of 80% ethanol on ice and then centrifuged at 14000 xg for 15 min. The supernatant was mixed with an equal volume of 0.70% (w/v) thiobarbituric acid (TBA) solution containing 200% (w/v) trichloroacetic acid and 0.01% oxytoluenes and then incubated at

97°C for 20 min. After cooling and subsequent centrifugation, 5 µl of the supernatant was used for HPLC analysis using an ODS column (4.6 mm) equilibrated with 35% methanol in 60 mM potassium phosphate buffer (pH=6.8). MDA was eluted at 1.5 ml.min^{-1} and quantified at 540 nm. Chemically prepared MDA from acid-hydrolysis of tetra ethoxy propane was used for calibration.

Electrolyte leakage (EL): One gram of leaves was mixed with 12 ml water and saved with shaker overnight at 27 ºC. By an electrical conductivity meter; the first conductivity (C1) was determined at 27 ºC. The solution was then autoclaved for 15 minutes and cooled to 26 ºC to determine the second conductivity (C2). Once assessed, the membrane stability was assessed to determine the C1: C2 ratio (Lutts et al., 1995).

Hydrogen peroxide (H_2O_2): Hydrogen peroxide was extracted with cold acetone according to the procedure described by Tabatabai (1998). The extract was quantitatively mixed with titanium tetrachloride and ammonia to produce a peroxide-Ti complex. The complex was collected through centrifugation and dissolved in 2 M sulfuric acid. The absorbance of the solution was measured at 420 nm, and H_2O_2 content was calculated according to the standard curve.

Soluble proteins: Fresh leaves (0.8 g) were ground with liquid nitrogen before the thawed homogenate was cool-centrifuged (4 °C) at 14 000 × g for 12 min. Amounts of proteins were assayed in the supernatant, according to Bradford et al. (1976). The absorbance was measured at 600 nm on a UV spectrophotometer. Standards were prepared from bovine serum albumin, and total amounts of soluble proteins were calculated as mg g^{-1}.

Proline concentration: Proline content was assessed according to Bates et al. (1973). The leaf sample (0.5 g) was homogenized with 5 mL of 3% sulfosalicylic acid. The sample was separated, and 3 mL was mixed with ninhydrin reagent (3 ml) and glacial acetic acid (3 ml). This mix was warmed in a bubbling water bath for an hour until it reached 90 °C and quickly cooled to 25 °C. A chromophore was shaped by adding 4 mL toluene to the cold solution. The absorbance was measured at 520 nm using the UV-VIS spectrophotometer. Proline solution (0-10 µg mL^{-1}) was used as a standard.

Abscisic acid (ABA) content:

One gram of leaf tissues was homogenized in 70 % methanol at 4 °C. Under a vacuum, the concentrate was separated and dissipated with Whatman channel paper (No.1). The pH of the aqueous stage was adjusted to 8 using phosphate buffer (0.2 mol). The aqueous stage was divided twice with methanol. A rotary evaporator was used to eliminate the methanol stage, and then pH was adjusted to 2.5 using HCl (1 N). The concentrate injection specified ABA into a turnaround stage of HPLC equipped with a C12 column in an isocratic elution mode using a convenient stage including $(CH_3)_2CO$: H_2O (26:74) with 30 mmol phosphoric acids, according to Tang et al. (2011). Sodium hydroxide (1 N) was used to maintain pH at 4. The transition rate was 0.6 $ml.min^{-1}$, and the elution of ABA was determined at 270 nm under 25 ºC.

Antioxidant enzymes:

Activities of peroxidase (POD), ascorbate peroxidase (APX), and catalases (CAT) were determined using the protocol of Radić et al. (2009) using a spectrophotometer.

Mineral concentrations:

The method of Cresser and Parsons (1979) determines the mineral concentration. The solution was transparent and utilized for conclusions of K and Na concentrations with an emanation flame photometer.

Statistical analysis

The experiment was designed in a completely randomized design of Trichoderma-fertilizer and salts treatments; five replicates each. The results were subjected to the analysis of variance (ANOVA), and means were compared using the Duncan's range multiple tests (DMRT) at $P \leq 0.01$ by SPSS-23 statistical analysis software (Chicago, USA).

Results

The response of plant growth to TH- fertilizer under salinity conditions

TH-fertilizer treatments increased plant height, root length, and the number of leaves and roots of stressed and non-stressed plants; however, stressed plants have shown lower growth parameter values than non-stressed ones. Applying TH-fertilizer (60 g) generally increased plant height,

root length, number of leaves, and roots of the non-stressed plants compared to the other concentrations. The same effect was noticed under salinity conditions (Table 1). TH-fertilizer treatments increased the chlorophyll and dry matter under stress and natural conditions. The chlorophyll content and dry weight gradually decreased as salt levels increased. (Fig. 1). Also, TH-fertilizer (60 g) combined with NaCl (75 mM) significantly increased chlorophyll and dry matter contents compared to other salt stress treatments.

Table 1 Role of TH- fertilizer under salt stress to enhance No. of leaves, Length of leaves, No. of roots, and Length of roots of date palm seedlings.

Treatments	Leaf number	Leaf length (cm)	Roots number	Root length (cm)
0 NaCl (control)	5.12±0.12 c	27.68±0.59 cd	5.15±0.17 cd	26.35±0.59 c
0 NaCl + TH-30	6.12±0.12 b	32.02±1.02 b	6.17±0.17 b	41.02±5.02 b
0 NaCl + TH-60	7.12±0.12 a	35.35±0.59 a	7.12±0.12 a	67.68±7.65 a
75 NaCl	4.12±0.12 d	26.68±0.59 d	4.35±0.59 de	25.68±0.59 c
75 NaCl + TH-30	6.08±0.13 b	28.35±0.59 c	5.89±0.33 bc	31.02±1.02 c
75 NaCl + TH-60	6.35±0.59 b	31.02±1.02 b	6.12±0.12 b	61.02±10.2 a
150 NaCl	4.08±0.12 d	25.35±0.59 e	4.19±0.18 d	26.02±1.02 c
150 NaCl + TH-30	5.05±0.07 c	28.02±1.02 cd	4.75±0.47 de	32.02±1.02 c
150 NaCl + TH-60	6.05±0.07 b	28.35±0.59 c	5.86±0.29 bc	46.02±5.02 b

Means of 5 replicates ±SD after one year of application. After a Duncan test, a column with various letters is unique at $p \leq 0.01$.

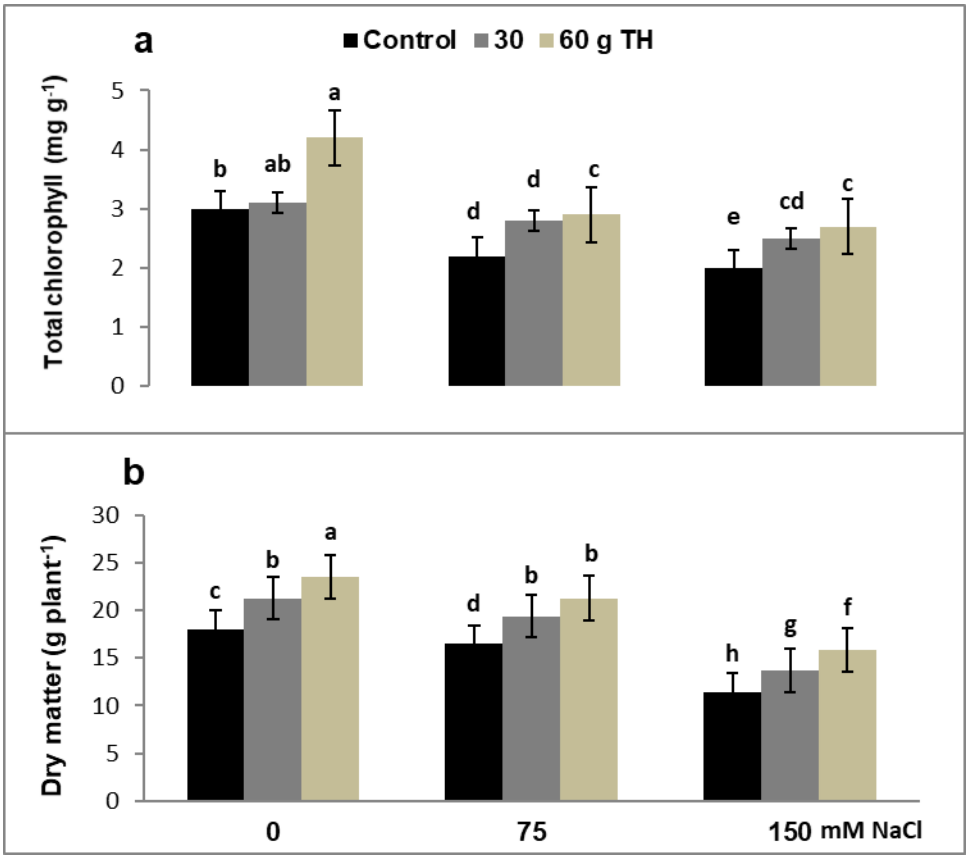

Fig. 1 Role of TH- fertilizer under salt stress to enhance total chlorophyll (a) and dry weight (b) to 5 replicates ±SD of date palm seedlings, after one year of application. A column with various letters is unique at $p \leq 0.01$ after a Duncan test.

TH-fertilizers enhance antioxidant defense and reduce Na-induced oxidative damages

Figure 2 showed that salinity treatments expanded MDA, EL, and H_2O_2 contents. Their contents decreased with TH-fertilizer compared to salt stress treatments. TH- fertilizer (60 g) generally decreased MDA, EL, and H_2O_2 contents under NaCl (150 mM) compared to the other treatments. TH-fertilizer insignificantly affected H_2O_2 content at NaCl (75 mM) (Fig. 2).

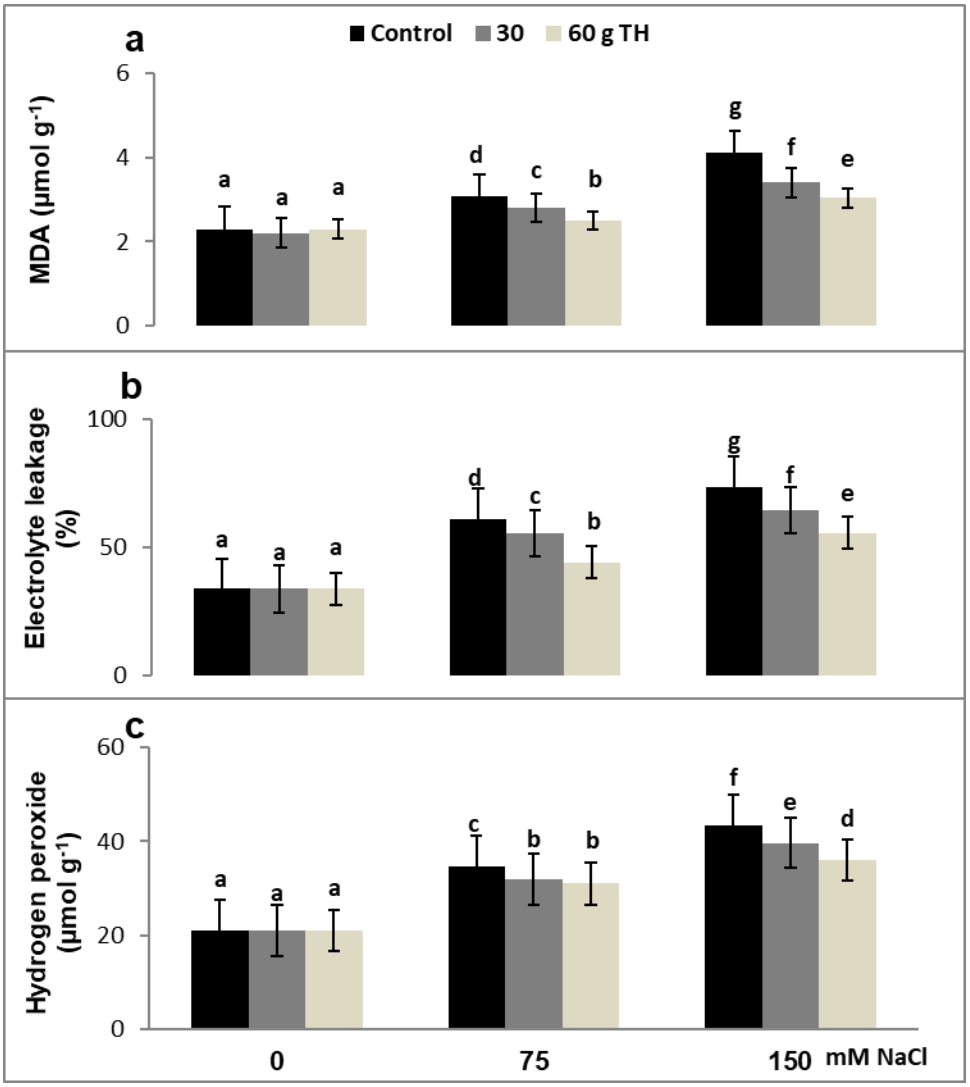

Fig. 2 Role of TH- fertilizer under salt stress to enhance MDA (a), electrolyte leakage (b), and hydrogen peroxide (c) to 5 replicates ±SD of date palm seedlings, after one year of application. A column with various letters is unique at $p \leq 0.01$ after a Duncan test.

Soluble protein levels have increased with increased NaCl concentrations. TH-fertilizer significantly increased the soluble proteins of the stressed plants. Also, Proline had been continuously increased with increased concentrations of NaCl. TH-fertilizer (60 g) decreased proline content at NaCl (150 mM) (Fig. 3). In addition, TH-fertilizer decreased ABA levels under salinity conditions (Fig. 3).

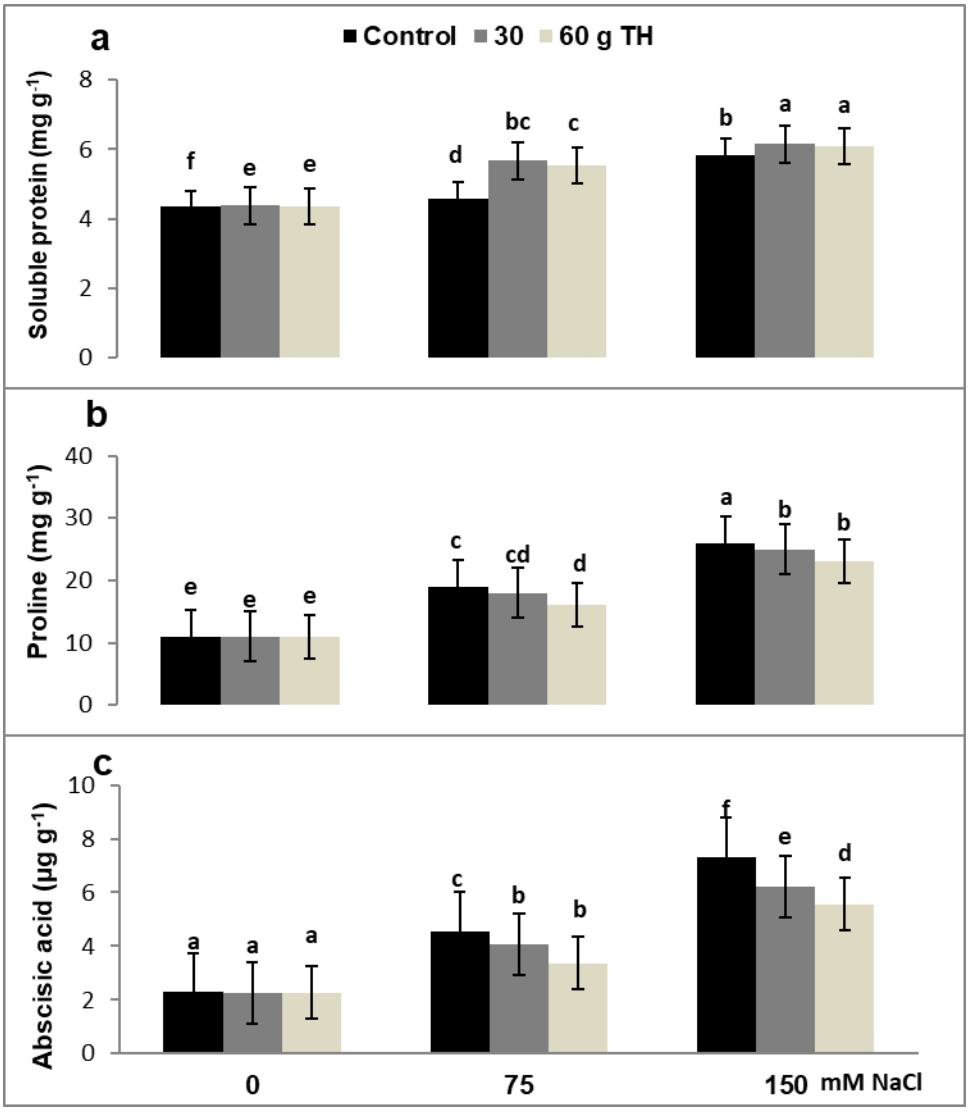

Fig. 3 Role of TH- fertilizer under salt stress to enhance soluble protein (a), proline (b), and abscisic acid (c) to 5 replicates ±SD of date palm seedlings, after one year of application. A column with various letters is unique at $p \leq 0.01$ after a Duncan test.

Both TH-fertilizers and the high level of NaCl led to high enzyme activities (Fig. 4). The most pronounced effect on CAT and APX was noticed at NaCl (150 mM), but POD activity was the most at TH-fertilizer (60 g) (Fig. 4).

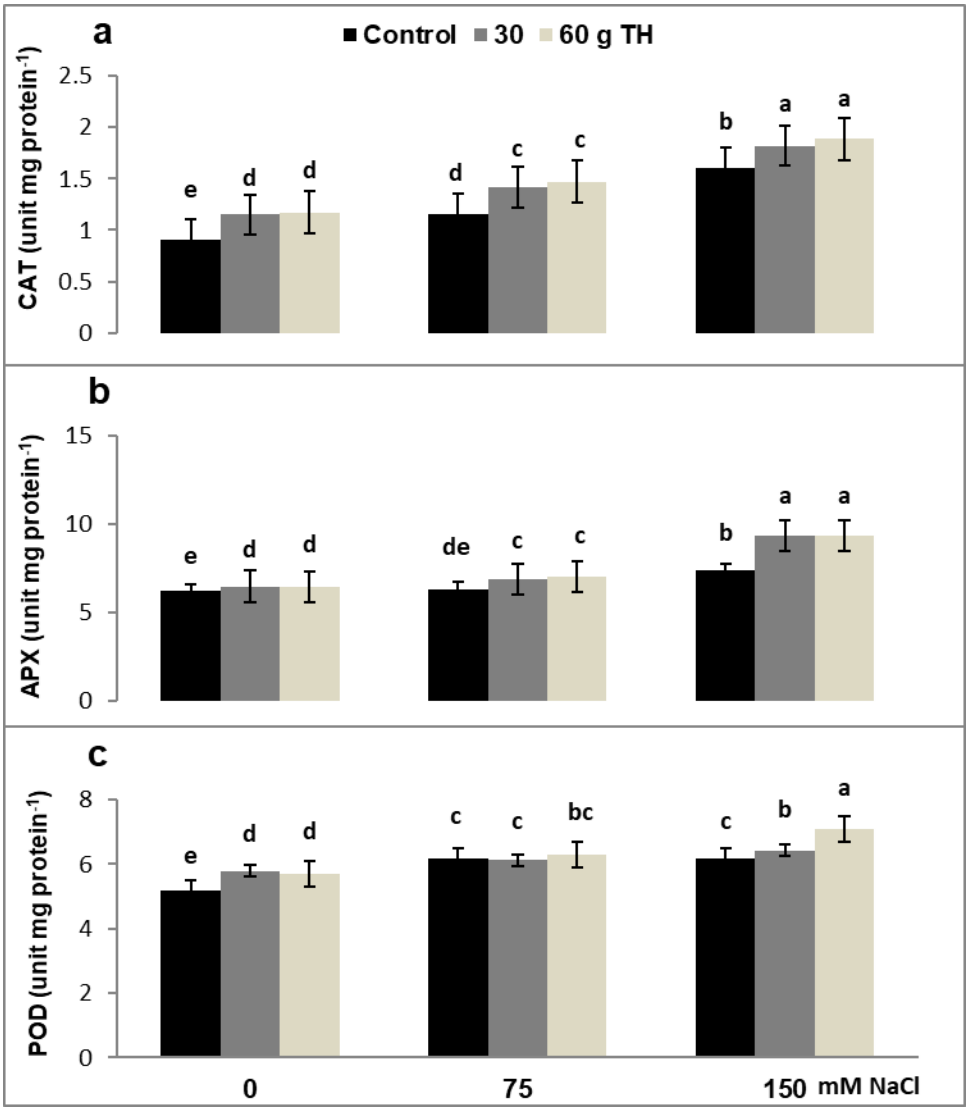

Fig. 4 Role of TH- fertilizer under salt stress to enhance the activity of catalase (a), superoxide dismutase (b), and peroxidase (c) to 5 replicates ±SD of date palm seedlings, after one year of application. A column with various letters is unique at $p \leq 0.01$ after a Duncan test.

TH-fertilizer enhances mineral uptake

Both K and Na/K ratios decreased, but Na increased significantly with increased salinity levels (Table 2). TH-fertilizer increased K and Na/K ratio significantly under NaCl stress. TH-fertilizer (60 g) with salt stress increased potassium concentration. TH-fertilizer (60 g) reduced sodium either conditions of salinity or natural. Na/K ratio increased significantly by TH-fertilizer (60 g) in natural or salinity conditions.

Table 2 Role of TH- fertilizer under salt stress to enhance sodium, potassium, and K/Na ratio of date palm seedlings.

Treatments	Na (mg g^{-1} DW)	K (mg g^{-1} DW)	K /Na Ratio (%)
0 NaCl (control)	2.92±0.01 f	13.02±0.01 a	5.14±0.01 c
0 NaCl + TH-30	2.87±0.01 g	13.13±0.05 a	5.27±0.02 c
0 NaCl + TH-60	2.18±0.01 h	14.25±0.01 a	7.27±0.04 a
75 NaCl	4.27±0.01 d	11.23±0.01 bc	3.41±0.02 g
75 NaCl + TH-30	4.17±0.01 e	13.24±0.01 b	3.95±0.08 b
75 NaCl + TH-60	4.25±0.02 d	13.46±0.01 b	3.95±0.01 b
150 NaCl	8.60±0.02 a	10.11±0.01 cd	2.06±0.04 h
150 NaCl + TH-30	4.69±0.01 b	12.43±0.01 cd	3.44±0.04 f
150 NaCl + TH-60	4.59±0.01 c	12.75±0.02 bc	3.58±0.05 e

Means of 5 replicates ±SD after one year of application. After a Duncan test, a column with various letters is unique at $p \leq 0.01$.

Discussion

Salinity is a significant abiotic factor that affects plant growth and productivity. One of the Salinity stress signs is osmotic stress, which directly impacts nutrient deficiency and affects different physiological and biochemical processes that eventually impact plant development (Shareef & Al-Khayri, 2021). The utilization of *Trichoderma* fungus as a root colonizing agent was reported to help improve root development, particularly under unfavorable ecological conditions (Shoresh, Harman, and Mastouri 2010), such as drought, salinity, and heat stress (Mastouri, Björkman, and Harman 2012). It enhances plant absorption of water and nutrients, particularly N and P (Cai et al. 2015). *Trichoderma* fungus has been effectively utilized as a biostimulant for edible plants, and it is typically non-harmful to humans (López-Bucio et al., 2015). Generally, salt stress decreases the development of date palm seedlings since the high levels of NaCl disturb the ionic equilibrium of plant cells. *Trichoderma* fungus has been reported

to mitigate the deleterious effects of salinity (Shareef 2020); *Trichoderma* releases various compounds that stimulate plant's resistance to biotic and abiotic stresses (Mastouri et al. 2012). The reduction in leaf and root growth in salt-stressed plants could be referred to as the reduced rate of water supply and photosynthesis, accompanied by the limited carbohydrate supply required for growth. In general, inhibited root growth under salinity conditions has inhibited vegetative growth (Zhao et al. 2020). The *Trichoderma*-cultured seedlings have shown the highest root length compared to all other culture media. *Trichoderma* produces phytohormones-like materials such as zeatin and related gibberellins, reflected in enhanced root growth and water absorption (Illescas et al., 2021). Moreover, *Trichoderma* helps reduce the toxic elements of the soil, improving soil characteristics (Mastouri et al., 2012). Plant growth activity (e.g., height or biomass) mitigates Na accumulation in the leaves. *Trichoderma* can dissolve many nutrients such as P, Fe, Cu, Mn, and Zn and make them available for plants in some soils (Altomare et al. 1999). Saeed et al. (2011) found that *T. harzianum* caused a significant increase in the availability of Fe, Zn, Mn, and C, increasing the plant's vegetative growth. *Trichoderma* decreases oxidized mineral ions to raise their solubility and availability to the plants (Lombardi et al. 2020). High NaCl reduced the chlorophyll content of the plant by increasing the chlorophyllase degrading activity (Zhao et al., 2020), and the present study showed that TH-fertilizer improved seedlings' chlorophyll content and dry matter under salinity conditions. Electrolyte leakage (EL) has increased with increased NaCl levels that damage the root function due to increased membrane damage (Silva et al. 2007). *Trichoderma*-treated plants showed a reduction in EL, associated with reduced H_2O_2 levels and lipids peroxidation under salinity conditions (Shareef & Al-Khayri, 2021). Yasmeen and Siddiqui (2017) found that root colonization by *T. harzianum* has improved the activity of the antioxidant enzymes (e.g., CAT, SOD, and POX), which work as scavengers for scavenger's ROS and lead to membrane stability. Accumulating proline under salinity conditions protects the cell by adjusting the osmotic level with the vacuole and the extreme climate. Proline also interfaces with cellular molecules like enzymes to stabilize their structure and functions (Rawat et al., 2011). MDA generally indicates plant's oxidative stress and increases with increased NaCl levels in plant tissue. Lipid peroxidation is the primary indicator of the high inactive free radicals, and MDA is the main component of the peroxidation process (Ayala, Muñoz, and Argüelles 2014). The results demonstrated that MDA accumulation increased in the untreated plants than in the treated ones under salinity. TH-fertilizer reduced MDA content, which may be due to the increased expression of stress-related enzymes such as

CAT, SOD, and POX. Under NaCl stress conditions, when ROS are delivered, the detoxification proteins produced by *Trichoderma* work as scavenging enzymes and act as anti-oxidative protectors to the cells. Thus, TH-fertilizer directly improves the plant's defense mechanism. The present study indicated that TH-fertilizer plays an essential role in plant response to salinity. *Trichoderma* colonizes the root tissues and induces morphological and biochemical changes in the root system, improving the plant's defense mechanism against salinity by enhancing cell homeostasis and restoring growth and development (Rawat et al., 2011). Knowing the instrument that underlies the plant salt reaction to Trichoderma inoculant can help design new reinforcement strategies using Trichoderma in saline soil. *T. harzianum* improved the growth parameters of date palm seedlings due to the effect of the fungus in increasing the availability of nutrients in the soil to the plant and an increase in the plant's growth rate in height and biomass, and root system. *T. harzianum* leads to improving the physical, compound and natural properties of soil (Rawat et al. 2011). Bargaz et al. (2018) reported that the optimal use of soil microorganisms is a safe environmental alternative to chemical fertilizers. Bal and Altintas (2008) found that *T. harzianum* fungus stimulates plant growth by building the plant's organic mass.

Conclusions

TH-fertilizer's application led to improved growth of date palm seedlings, which could be attributed that increased plant's nutrient absorption, which was positively reflected in root and vegetative growth under salinity conditions. The role of TH could be due to dissolving and isolating Na and Cl ions and improving N absorption in sandy soils

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سماد *Trichoderma harzianum* يعزز تحمل شتلات نخيل التمر ضد ملوحة التربة

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الخلاصة

درست هذه التجربة دور سماد *Trichoderma harzianum* بتركيز 30 و 60 غم كغم$^{-1}$ من التربة لتحسين تحمل شتلات نخيل التمر (صنف البرحي) ضد مستويات الملوحة المختلفة بتركيز 0 ، 75 ، 150 ملي مول كلوريد الصوديوم في تربة رملية. قلل الإجهاد الملحي من ارتفاع النبات وطول الجذر وعدد الأوراق والجذور. من ناحية أخرى، قام سماد TH– بتحسين هذه الصفات إضافة الى الكلوروفيل والمادة الجافة. كان لتطبيق سماد الترايكوديرم بتركيز 60 غم كغم$^{-1}$ من التربة تأثير أفضل على نمو الشتلات من 30 غم كغم$^{-1}$. زادت معاملات الملح من محتوى MDA ومحتوى بيروكسيد الهيدروجين والذي انخفض مع ارتفاع معاملات التسميد. زاد سماد الترايكوديرم من محتوى البرولين والبروتينات الذائبة والانزيمات الكاتلايز وبيروكسيديز أسكوربات والبيروكسيديز وتركيز عنصر البوتاسيوم ونسبة الصوديوم/البوتاسيوم، بينما انخفض حامض الأبسيسك تحت اجهاد الملوحة. لذلك، فإن سماد الترايكوديرم عدّل بشكل فعال نمو النبات في ظل هذه الظروف.

الكلمات المفتاحية: نفاذية الاغشية، بيروكسيد الهيدروجين، *Phoenix dactylifera* L.، التربة الرملية، إجهاد الملوحة، *Trichoderma harzianum*