

Research Article

Evaluation efficiency of salinity tolerant *Trichoderma harzianum* for alleviation of salinity stress in date palm (*Phoenix dactylifera* L.)

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Abstract

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This study aimed to determine the benefit of a bioagent (*Trichoderma harzianum*) to lessen the adverse effects of NaCl stress on date palm offshoots (*Phoenix dactylifera* L.) Varying concentrations of sodium chloride (NaCl) (0, 5, 10, 15, 20, and 25 dS m⁻¹) were used to observe the effect on photosynthetic pigments, antioxidant enzymes, total proline, total phenolics, and hydrogen peroxide in date palm offshoots in the presence and absent of the bioagent *T. harzianum*. Results of the study showed that enhancing the NaCl concentration from 10-20 dS m⁻¹ did not affect the mycelial growth colony of the *T. harzianum* in the potato dextrose agar (PDA) medium. The results indicated a significant increase in photosynthetic pigments (chlorophyll and carotenoid), levels of peroxidase and catalase enzymes, total proline, and total phenolic content in the date palm offshoots with the application of *T. harzianum*. A higher concentration of NaCl leads to a higher level of hydrogen peroxidation. Additionally, salt stimulated the production of antioxidant enzymes such as catalase and peroxidase. The study has indicated a significant reduction in salt stress's negative physiological and biochemical effects on date palm offshoots after applying the bioagent *T. harzianum*. This study showed that *Trichoderma*, with its ability to promote plant growth, may be employed to increase the growth of date palm offshoots developing under NaCl stress conditions.

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Introduction

Agricultural plants might lose up to 50% of their production due to repeated exposure to abiotic stresses. These stressors can alter a plant's biochemistry, appearance, and function and potentially cause the plant to die (Evelin et al., 2019; Gupta et al., 2021). Salt stress prevents plants from absorbing water from the soil, it can cause ionically and osmotically stressful conditions in them. Paraida and Das (2005) and Gupta and Huang (2014) proposed that nutritional disorders result from physiological processes associated with ion buildup disruptions. According to Liu et al. (2023), excessive salt can harm transpiring leaf cells and the plant's vascular system, which prevents the plant from

growing. It also reduces water absorption and impedes development due to osmotic effects. It is projected that by 2050, agricultural land's global sensitivity to salt will rise from 20% to 30% (OtLewska et al., 2020). The adverse impacts of soil salinity are exacerbated by elements, including intense and prolonged exposure, high temperatures, and limited rainfall (Etesami and Maheshwari, 2018; Verma et al., 2021). According to Kumar et al. (2020), salinity impacts several physiological and biochemical functions, such as the absorption of nutrients, the disruption of cell membranes, and tissue damage brought on by reactive oxygen species (ROS) and deficits in antioxidant enzymes. Salinity has an impact on several physiological functions that may hasten senescence,

including respiration, soluble proteins, pigments, the glycolysis pathway, the photosynthetic system, nitrogen fixation, and other functions (Parida and Das, 2005; Kumar et al., 2020).

According to Cetinel et al. (2021), fungus injection is a safe and ecologically friendly way to treat issues caused by salt. Alfano et al. (2007) and Mastouri et al. (2010) discuss the application of fungi as enhancers of nutrient absorption, plant development, and induction of systemic resistance, as well as biological controls of a variety of plant fungal diseases and pests. Based on the findings by Poosapati et al. (2014) and Singh et al. (2019), it is evident that soil salinity significantly hinders plant growth and development while also impacting soil microbe growth. Notably, specific salt-resistant *Trichoderma* species, as highlighted by Kumar et al. (2017) and Singh et al. (2019) offer potential solutions to this challenge. Studies by Rawat et al. (2016), Ikram et al. (2019), and Zhang et al. (2019), strongly supports the use of salt-tolerant *Trichoderma* to mitigate the adverse effects of salinity on crucial crops like rice, maize, wheat, tomato, and cucumber. Through their positive plant interactions, *Trichoderma* spp. effectively enhance salt tolerance, thereby reducing the dependence on chemical pesticides and fertilizers, ultimately advancing sustainable agriculture (Othman et al., 2022). This enhances the plants' absorption of water and nutrients. *Trichoderma* inhibits reactive oxygen species (ROS) in host plants, which lessens abiotic stress by encouraging the development of systemic and local defense mechanisms as well as antioxidant enzyme production (Verma et al., 2021).

According to specialists in water resources cited in an Al-Hurra Iraq Channel program titled "The Water Crisis in Iraq," the country's yearly rainfall has significantly decreased due to rising temperatures. It is now at 35% and is predicted to reach 65% by 2050. The Gulf waters' salinity has grown from 35 to 50 parts per million due to climate change's effects on sea levels and water scarcity. The agricultural regions in southern Iraq, where date palm trees are widely planted, are at risk due to this (Al-Hurra Iraq/Washington, August 9, 2023).

The principal goals of the study were to 1. examine the effects of various NaCl stress levels on the growth of *Trichoderma* isolate, and 2. estimate the effects of inoculating *Trichoderma harzianum* on the development, biochemical alterations and physiological changes of date palm offshoots in saline environments.

Materials and Methods

A tolerance test using various concentrations of NaCl with T. harzianum

T. harzianum isolated and purified from the BioHealth WSG Administration and Distribution: Humintech GmbH, Germany. e-mail: info@humintech.com).

T. harzianum was cultivated in potato dextrose agar (PDA) for five days at 25 ± 2 °C. Mycelium discs (5 mm) were taken from the PDA Petri dish colony edge and positioned in the center of the plates (9 cm diameter) that contained PDA modified with different NaCl concentrations (0, 5, 10, 15, 20, and 25 dS m⁻¹). Pure sodium chloride was used to produce these concentrations. At 25 ± 2 °C, the plates were incubated. Five duplicates of each concentration (treatment) are present. The colonies' diameters were measured every day. According to Poosapati et al. (2014), the ability of *T. harzianum* isolate to salt tolerance was estimated as the percentage reduction of mycelial growth (IMG) colony calculated when the mycelium growth in control (0 dS m⁻¹) arrives at the edge of the plate, using the formula: %IMG = $[(C-N)/C]\times 100$, where N = diameter of mycelial growth colony on PDA with NaCl and C = diameter of mycelial growth colony on NaCl-free medium (0.0 dS m⁻¹).



Figure 1. The locations of the orchards of date palms that have been covered by the survey of the study.

T. harzianum effects on offshoots of date palm under greenhouse conditions

Experiments were conducted at the Date Palm Research Center at the University of Basra in greenhouses and laboratories during 2021 and 2022 (Figure 1). Different sodium chloride (NaCl) concentrations of 0, 10, 16, and 20 dS m⁻¹ (equivalent to 0, 125, 200, and 250 mM of NaCl concentrations) were evaluated both alone and in the presence of the fungus *Trichoderma harzianum*, along with control treated with distilled water. The date palm plantlets used in the experiment were ten months old. These variations were applied to the plants for six months, with the specific concentration added according to the soil water content of the experiment area. Each treatment consisted of three replicates, with two plantlets per pot. The conidia from *T. harzianum* were

harvested and suspended in 1 mL of solution after 7 days of culture.

According to Abdullah and Yusnawan (2020), the conidial suspensions were applied to the date palm offshoots using a randomized block design with four replicates for each *T. harzianum* treatment. As a control, untreated offshoots lacking the *Trichoderma* isolate were included in the experimental setup to provide a basis for comparison. Following this, we cultivated the date palm offshoots in soil with a high saline content and previously treated with 69 kilograms of nitrogen per hectare. The soil was characterized by a slightly high pH of 8.0. All other conditions regarding cultivation, fertilizer applications, and irrigation were maintained. Measurements of the following physiological traits were made after the experiment was finished.

Photosynthetic pigments content in offshoots of date palm leaves

Total chlorophyll (Chl) and carotenoids (Car) were extracted using the procedure outlined by Lichtenthaler and Buschmann (2001). After being crushed in liquid nitrogen, fresh leaves were extracted using a 90% (v/v) methanol and demineralized water solution. After five minutes of vortexing and eight seconds of sonication, the samples were centrifuged for ten seconds at 3000 rpm. The Hitachi Double Beam spectrophotometer (Tokyo, Japan) was used to determine the absorbance (A) of the resultant supernatants. Carotenoids and total chlorophyll concentrations were calculated and reported in μg per g of sample.

Determination of total soluble proline contents

Proline content was evaluated following the method of Bates et al. (1973). A 0.5 g of fresh-leaf sample was homogenized with 5 mL of 3% sulfosalicylic acid. Then, 3 mL of the sample was mixed with 3 mL of ninhydrin reagent and 3 mL of glacial acetic acid. This mixture was heated in a water bath with bubbles for an hour until it reached 90 °C and then rapidly cooled to 25 °C. A chromophore was produced by adding 4 mL of toluene to the cooled solution. The absorbance was determined at 520 nm using a UV-VIS spectrophotometer. Proline content was calculated and reported in μg per g of sample.

Determination of antioxidant enzymes (peroxidase and catalase)

To assess the activity of peroxidase (POD) and catalase (CAT) activities, one gram (1.0 g) of fresh leaves was crushed in 25 mL of sodium phosphate buffer (50 mM pH 7.8) and centrifuged for 20 minutes at 10,000 rpm, following the methods described by Kar and Mishra (1976) and Samantary (2002). The supernatant was collected, and the activity of POD and CAT was measured at 430 nm and 290 nm, respectively. Their activities were expressed as unit g^{-1} FW.

Determination of total phenolic contents

The total phenolic content of date palm offshoots was determined, with some modifications following the methods of Yusnawan and Inayati (2018). The phenolic compounds in date palm seedlings were measured as micrograms (μg) of gallic acid equivalent per gram of fresh weight using gallic acid as the standard.

Hydrogen peroxide (H_2O_2)

The method described by Tabatabai (1998) was used to extract hydrogen peroxide using cold acetone. The extract was quantitatively mixed with titanium tetrachloride and ammonia to generate a peroxide-Ti complex. The compound was dissolved in 2 M sulfuric acid after its separation via centrifugation. After determining the solution's absorbance at 420 nm, the H_2O_2 concentration was measured using the standard curve and expressed as $\mu\text{g g}^{-1}$ of FW.

Results

Growth Inhibition of *Trichoderma harzianum* in PDA containing NaCl concentration

T. harzianum treated with 0, 5, 10, 15, 20, and 25 dS m^{-1} of NaCl did not exhibit any significant changes in growth after 4 days of incubation (Figure 2). The rate at which *T. harzianum* underwent mycelial formation in response to 10, 15, 20, and 25 dS m^{-1} of NaCl did not exhibit any consistent difference. Without any hindrance, the *T. harzianum* in the control condition (which lacked extra NaCl) grew until the Petri plate was filled. The Petri plates were filled with all concentrations of mycelial growth from the isolate treated with NaCl when the incubation period was prolonged to five days.

The results of the photosynthetic pigments (Figures 3 and 4) revealed a significant inhibition in total chlorophyll and carotenoid contents under NaCl stress at 10, 16, and 20 dS m^{-1} , which were 9.24, 7.38, and 5.48 $\mu\text{g g}^{-1}$ FW for chlorophyll respectively, and 0.024, 0.018, and 0.014 $\mu\text{g g}^{-1}$ FW for carotenoid respectively. The inhibition of photosynthetic pigments was reversed by the application of *T. harzianum* to the offshoots, increasing in total chlorophyll and carotenoid contents, which were 10.98, 10.18, and 9.94 $\mu\text{g g}^{-1}$ FW for chlorophyll, and 0.024, 0.021, and 0.020 $\mu\text{g g}^{-1}$ FW for carotenoid, respectively. The harmful effects of NaCl stress on pigment production, which result in their breakdown and/or harm to the chloroplast thylakoid, may be connected to this suppression of the photosynthetic pigment concentrations.

The free proline content of date palm offshoots was evaluated in the study with and without the *T. harzianum* inoculum at various NaCl concentrations (10, 16, and 20 dS m^{-1} NaCl stress). The presence of *T. harzianum* in a saline condition significantly impacted the proline content. Seedlings treated with

T. harzianum showed the highest accumulation of proline content in all NaCl treatments (10, 16, and 20 dS m⁻¹ of NaCl stress), with 20 dS m⁻¹ NaCl producing the highest proline level (Figure 5).

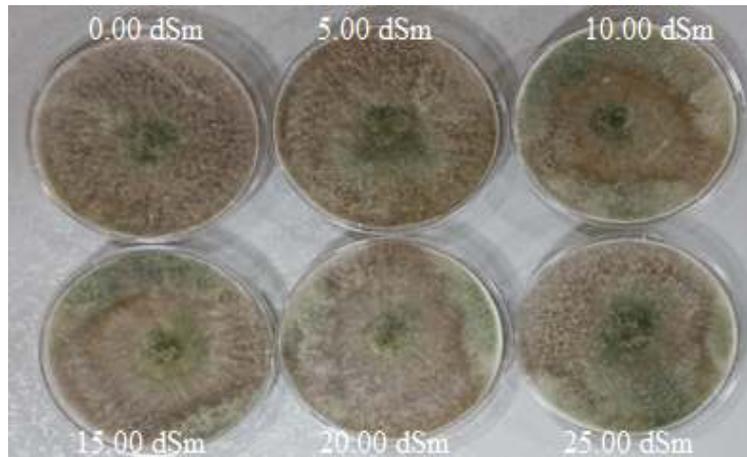


Figure 2. The effect of varying doses of NaCl stress on the *T. harzianum* radial mycelial growing colony on PDA medium at 25±2 °C in the laboratory. Each treatment is the average of four replicates.

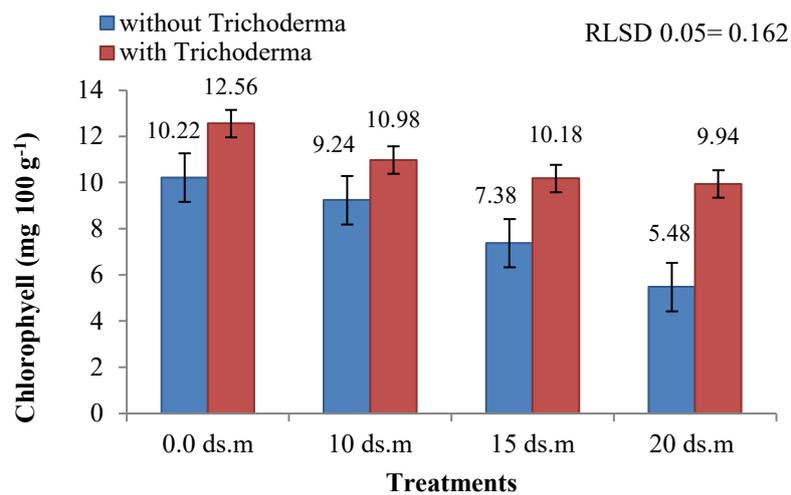


Figure 3. Effect of *Trichoderma harzianum* treatments on chlorophyll content of date palm seedling (*Phoenix dictylofera* L.) under saline conditions.

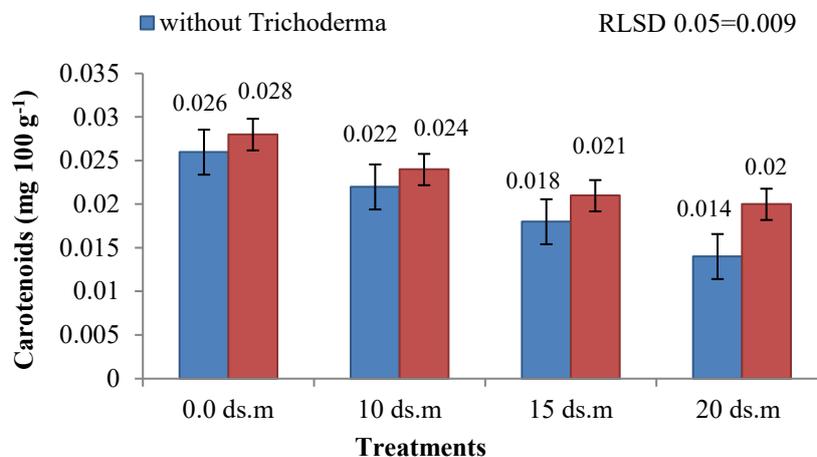


Figure 4. Effect of *Trichoderma harzianum* treatments on carotenoid content of date palm seedling (*Phoenix dictylofera* L.) under saline conditions.

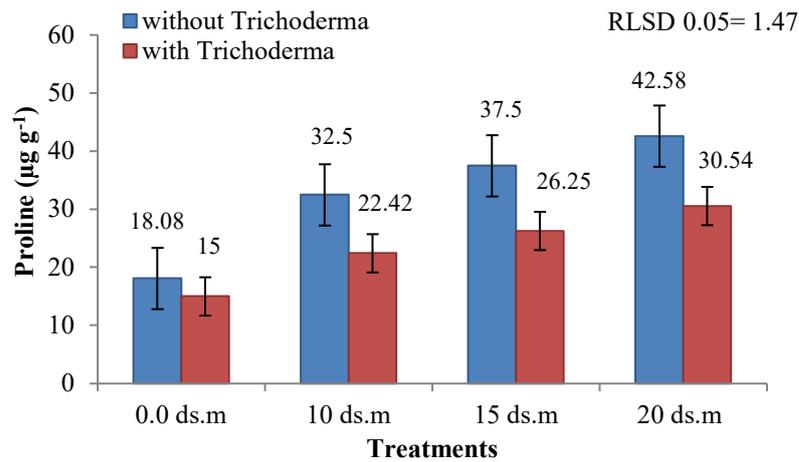


Figure 5. Effect of *Trichoderma harzianum* treatments on proline content of date palm offshoots (*Phoenix dactylifera* L.) under saline conditions.

The activity of POD and CAT antioxidant enzymes augmented significantly when exposed to NaCl stresses of 10, 16, and 20 dS m in comparison to the control (without NaCl). Under NaCl stress, the levels of POD and CAT recorded 14.88, 15.56, and 18.78 unit g⁻¹, respectively, and 11.82, 12.13, and 14.93 unit g⁻¹ respectively for CAT. These values increased more significantly when *T. harzianum* was applied with NaCl stress, recording 24.94, 28.75, and 30.46 unit g⁻¹ for POD, respectively, and 16.30, 20.88, and 21.63 unit g⁻¹ respectively for CAT (Figures 6 and 7). This suggests that POD and CAT may help date palm offshoots to sustain NaCl stress. Additionally, the index values differed significantly from the control index when *T. harzianum* was treated with 10, 16, and 20 dS.m of NaCl stress. Furthermore, the results in (Figure 8) showed that treating date palm offshoots with *T. harzianum* increased the total phenolic content compared to the control. Under salinity stress, date palm seedlings coupled with *T. harzianum* exhibited higher levels of phenolic compounds than those under 10, 16, and 20 dS.m of NaCl stress. When

T. harzianum was applied to date palm offshoots exposed to 10, 16, and 20 dS.m of NaCl stress, respectively, the phenolic content increased by 18.4%, 24.5%, and 24.2%, respectively, compared to the offshoots under NaCl stress only.

As a consequence of superoxide radicals scavenging, hydrogen peroxide (H₂O₂), a toxic compound injurious to plants, is produced due to salt exposure. Figure 9 shows that under 10, 16, and 20 dS m of NaCl stress, offshoots of date palms treated or not with *T. harzianum* had an essential increase in H₂O₂ content. Significantly higher levels were observed in offshoots of date palms under NaCl levels (5.16, 5.28, and 5.53 µg.g FW). However, *T. harzianum* treatment reduced H₂O₂ accumulation, where under NaCl stress, the H₂O₂ content of control offshoots of date palms was significantly higher than that of *T. harzianum*-treated offshoots of date palms. The minimum level of H₂O₂ was observed in offshoots of date palm treated with *T. harzianum* (3.30, 3.38, and 3.42 µg g FW) grown under saline soil (10, 16, and 20 dS m⁻¹ NaCl stress) or control conditions (nonsaline soil).

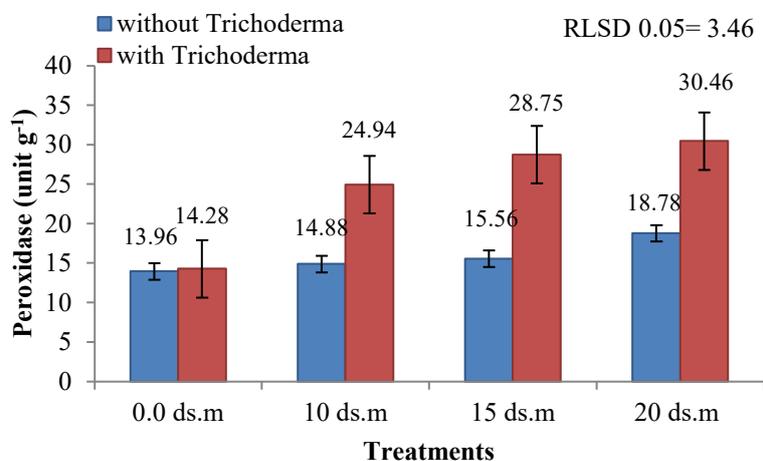


Figure 6. Effect of *Trichoderma harzianum* treatments on peroxidase (POD), enzyme levels of date palm offshoots (*Phoenix dactylifera* L.) under saline conditions.

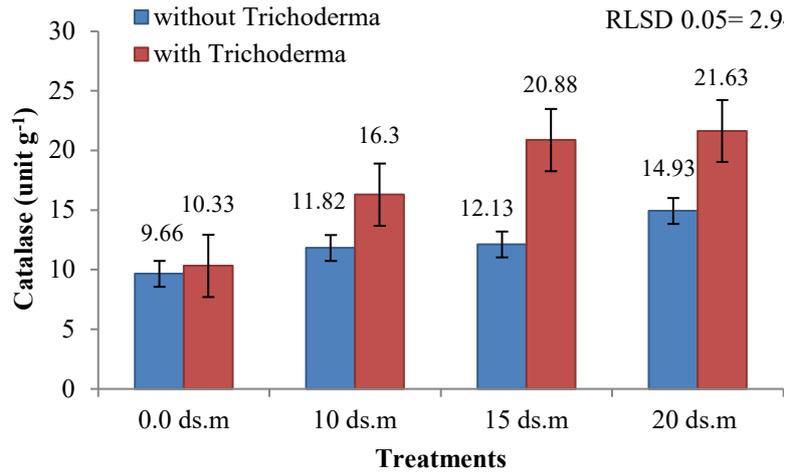


Figure 7. Effect of *Trichoderma harzianum* treatments on catalase (CAT) enzyme levels of date palm offshoots (*Phoenix dictylofera* L.) under saline conditions.

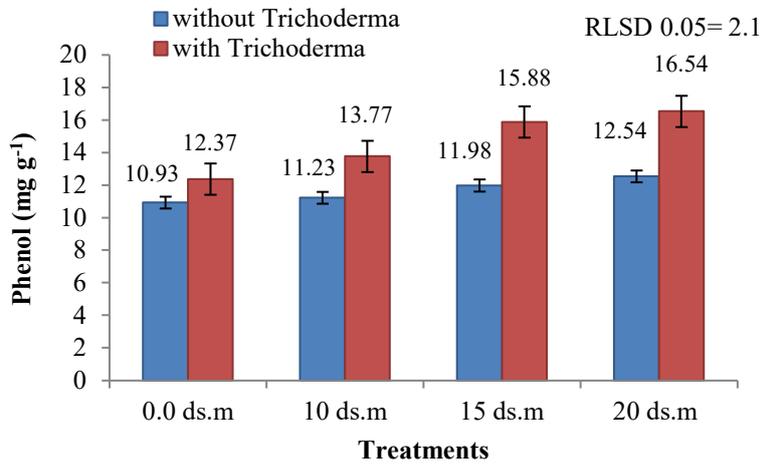


Figure 8. Effect of *Trichoderma harzianum* treatments on phenolic content of date palm offshoots (*Phoenix dictylofera* L.) under saline conditions.

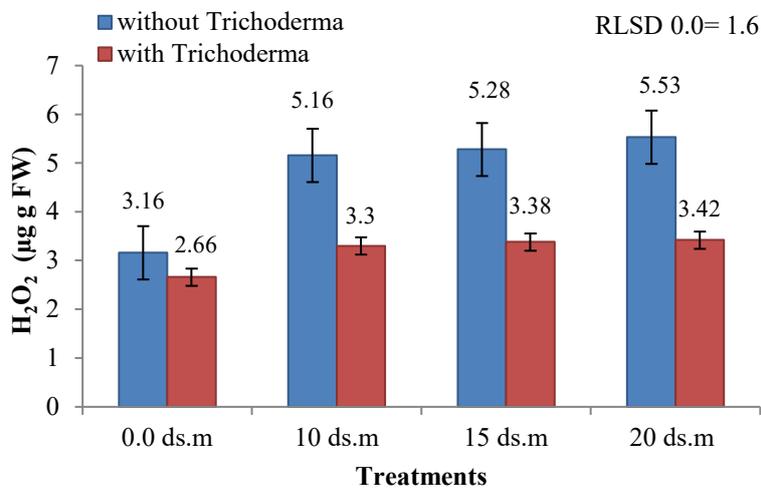


Figure 9. Effect of *Trichoderma harzianum* treatments on hydrogen peroxide levels (H₂O₂) of date palm offshoots (*Phoenix dictylofera* L.) under saline conditions.

Discussion

According to the experimental findings, the *T. harzianum* isolate was not sensitive to exposure to NaCl. This research demonstrated that the growing mycelium of *T. harzianum* was unaffected by an increase in NaCl content. After four days of incubation, the *Trichoderma* isolate achieved a maximum linear growth of 8.8 cm at 20 dS m⁻¹ of NaCl, the highest salt concentration in the research by Contreras-Cornejo et al. (2016). This corresponds with the findings of this study, which showed 9.0 cm of linear growth in the *T. harzianum* isolate grown on plates treated with all concentrations of NaCl stress.

Variation in the salt tolerance of *Trichoderma* across native and commercial isolates has been observed. Zahran (1997) states that filamentous fungi may alter their physiology and appearance due to their high resistance to hydric stress and increasing osmotic pressure. According to Killham (1994), microorganisms can respond to osmotic stress in two ways. Both of these tactics cause ions required for metabolism to accumulate by either assembling solutes in the cell to counterbalance the increase in osmotic pressure or selectively blocking aggregated solutes (NaCl). The results of this study align with those of Kumar et al. (2017), who investigated the salt tolerance of 70 *Trichoderma* spp isolates to different concentrations of NaCl. The data showed that 92.8% (65) of the isolates were resistant to a 5% NaCl concentration, with 45.7% (32) tolerating a 10% NaCl concentration. Poosapati et al. (2014) reported that all tested *Trichoderma* isolates, except at 1M NaCl concentration, showed significant resistance and maximum mean colony growth at all salt concentration levels.

Plants experiencing salinity stress exhibit high concentrations of reactive oxygen species, damaging their photosystems and cellular processes (Harman et al., 2019). Numerous studies have explored photosynthetic traits in salt-stressed environments. Hashem et al. (2014) reported that salt stress reduces photosynthesis, impacts photosynthetic components, and alters chloroplast ultrastructure. NaCl also interferes with crucial photosynthesis enzymes like phosphoenolpyruvate carboxylase and rubisco (Santos et al., 2004; Al Hassan et al., 2015). Improving photosynthetic efficiency in crop plants is essential. Long et al. (2018) found that early attempts at implementing changes were effective. Additionally, photosynthetic pigment concentrations increased when *Trichoderma* spp. colonized the roots.

In a study by Alexandru et al. (2013), it was found that various *Trichoderma* species (*T. harzianum*, *T. koningii*, *T. longibrachiatum*, and *T. tomentosum*) enhanced photosynthesis and chlorophyll in tomato plantlets. The increased chlorophyll content in *Trichoderma*-treated roots and leaves of date palm offshoots is consistent with previous findings. The results of this study indicated that the application of *T. harzianum* treatment increased photosynthesis

pigments, aligning with previous research showing that *Trichoderma* inoculation promoted plant growth and increased photosynthetic pigment (Kumar et al., 2017). This improvement came about as a result of the stimulation of chlorophyll production and the activation of the chlorophyllase enzyme, which caused the breakdown of chlorophyll (Santos et al., 2004). Common reactions to abiotic stress include decreased photosynthetic pigments, slowed root development, and disturbed mineral absorption (Hashem et al., 2014; Al Hassan et al., 2015). During the vegetative growth phase, the treated date palm offshoots increased carotenoid content, which was more noticeable than during the regenerative growth phase. According to Das and Roychoudhury (2014), this rise emphasizes the function of carotenoids as reactive oxygen species (ROS) scavengers in oxidative stress tolerance mechanisms and in shielding membranes from stress.

The proline content in untreated date palm offshoots exposed to salt stress significantly decreased, whereas *T. harzianum*-treated plants exhibited an increase in proline content. It is suggested that in salt-tolerant plants, the accumulation of proline helps protect cells by maintaining osmolyte balance (Rawat et al., 2013; Ikarm et al., 2019). Elevated H₂O₂ generation damages lipid and protein molecules under abiotic stress conditions such as salinity (Siddiqui et al., 2014; Zhang and Gan, 2016). *Trichoderma* treatment is believed to reduce H₂O₂ generation in salt stress conditions due to proline accumulation. When various NaCl concentrations (0, 70, 150, and 240 mM) were applied, stress-induced metabolite levels increased in rice leaves. Pre-treating seedlings with *Trichoderma* reduced MDA and H₂O₂ contents while increasing proline and phenolic concentrations (Rawat et al., 2016).

Under salt stress, the activity of peroxidase (POD) and catalase (CAT) increased, with further enhancement observed after *Trichoderma* application. POD acts by converting H₂O₂ into water to prevent membrane damage, while CAT transforms H₂O₂ into water and molecular oxygen. The increased activity of these antioxidant enzymes facilitates the removal of ROS, thereby reducing oxidative damage to plants. Applying *Trichoderma* increases the activity of antioxidant enzymes, which helps scavenge ROS generated during salt stress and lessen damage caused by stress. This enhanced activity helps minimize oxidative damage to cell membranes caused by ROS (Hoyos-Carvajal et al., 2009). This suggests that *Trichoderma* therapy reduces cellular damage by lowering H₂O₂ levels. Studies have shown that *T. atroviride* and *T. harzianum* can reduce H₂O₂ generation in maize and rice roots under stress conditions (Guler et al., 2016; Shukla et al., 2012). According to Rawat et al. (2016) and Zhang et al. (2019), who used *T. harzianum* to cure cucumber and chickpea under salt stress, the results of this study are consistent with theirs. Lower H₂O₂ levels in *T. harzianum*-treated seedlings suggest a more

effective cellular mechanism for quenching free radicals, providing defense against oxidative stress. Investigating the potential presence of antioxidant enzymes in date palm seedlings that could reduce high levels of toxic ROS, such as catalase or peroxidase, warrants further exploration (Ikarm et al., 2019). The application of *Trichoderma* alleviated salinity stress, possibly by enhancing the uptake of plant nutrients through increased enzyme activities like peroxidase, catalase, and reduced glutathione (Ikarm et al., 2019; Zhang et al., 2016).

Plants subjected to salt stress conditions often synthesize and accumulate secondary metabolites like polyphenols, phenols, phenolic acids, flavonoids, anthocyanins, and lignin to shield themselves from further harm (Kumar et al., 2020). According to Shiade and Boelt (2020) and Kalleli et al. (2022), phenolic compounds are important for enhancing oxidative processes because they neutralize singlet oxygen, absorb and neutralize free radicals, lower peroxides, and lessen the effects of salt. Similarly, compared to salt-susceptible genotypes, salt-tolerant chickpeas have higher levels of antioxidant enzymes like glutathione reductase, ascorbate peroxidase, catalase, and superoxide dismutase, which helps to lessen the damage that salt stress causes from reactive oxygen species (ROS) (Lo and Lin, 2002). The exogenous application of *Trichoderma* reduced salt stress in cucumber, likely by reducing ROS production in treated plants. Elevated levels of H₂O₂, a toxic compound resulting from salt exposure, contribute to lipid peroxidation and membrane injury in plants (Rawat et al., 2016; Zhang et al., 2016).

Conclusions

The study revealed that all tested concentrations of NaCl had no adverse effects on the mycelial growth colony of *T. harzianum* on the PDA medium. The results of the experiment showed that the presence of *T. harzianum* mitigated the negative impacts and enhanced the overall performance of the date palm offshoots under saline conditions. These results underscore the promise of utilizing *Trichoderma* as a biocontrol agent to boost the tolerance of date palm offshoots to salinity stress in agricultural settings. *T. harzianum* emerged as the most effective isolate, showing significant potential in alleviating abiotic stress in date palm offshoots affected by salinity.

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