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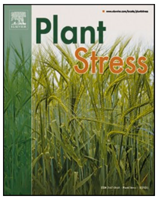

The role of *Trichoderma koningii* and *Trichoderma harzianum* in mitigating the combined stresses motivated by *Sclerotiniasclerotiorum* and salinity in common bean (*Phaseolusvulgaris*)

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ABSTRACT

Under natural conditions, crops typically suffer from severe challenges due to the increasing of abiotic and biotic stresses which severely affect plant growth and reduc crop yield. The present study investigated the single and combined impacts of *Sclerotinia sclerotiorum* and salinity stress on common bean (*Phaseolus vulgaris* L.) seedling which is scarcely studied. The study evaluated the in vitro and in vivo influence of two salinity tolerant *Trichoderma* isolates, *T. koningii* and *T. harzianum* against *S. sclerotiorum* under salinity stress. The results showed the ability of *T. koningii* and *T. harzianum* to grow and sporulate at high levels of salinity, 80 mM NaCl, without significantly impacting their ability to produce cell wall degrading enzymes, cellulase and chitinase. Amylase and proteinase (Prb1) genes were detected in *T. harzianum*. The in vitro assay revealed that both isolates could inhibit the growth of *S. sclerotiorum* under high salinity concentrations. In a greenhouse experiment, both *Trichoderma* isolates ameliorated the damaging impacts of *S. sclerotiorum* under salinity stress on common bean seedlings' germination and growth characteristics compared to their untreated control. Both bioagents significantly attenuated the damping-off and collar/stem rot percentages of infected common bean under salinity stress. Salinity stress intensified the effect of *S. sclerotiorum* on photosynthetic pigments, induced oxidative and nitrative stress, hampered ionic homeostasis, and deactivated antioxidants and defense-related molecules. On the other hand, *Trichoderma* isolates restrained the reduction of chlorophylls and carotenoids, ascorbate, reduced glutathione, flavonoids, phenolics, and various antioxidant enzymes, especially for single stresses and *T. harzianum*. All these upregulations reflected in keeping the cell membranes of common beans seedling more stable where the levels of lipid peroxidation and methylglyoxal due to the reduction of reactive oxygen species and upregulation of nitric oxide, which expressed better growth under pathogen attack or/and saline. The tested isolates, *T. koningii* and *T. harzianum* could be used as effective biological control against *S. sclerotiorum* on common beans in saline soils or areas irrigated with saline water.

Introduction

Common bean *(Phaseolus vulgaris* L.) is one of the most significant leguminous crops widely grown in all geographical areas, including South America, Europe, and Africa (Bevilacqua et al., 2020). It is the world's most important food legume, far more than chickpeas, lentils, faba bean, and cowpea (Schmutz et al., 2014). It serves as a critical plant protein source of vitamins, zinc, iron, and fiber for urban and rural areas, and it is a staple food crop in many developing nations due to its high nutritional value (Sofy et al., 2022).

Although common bean productivity enhanced worldwide, a considerable shortage still between the yield potential of the developed varieties and the yield obtained in the field, ultimately owing to abiotic and biotic stresses (Antwi-Boasiako et al., 2022) system; however, its

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production is affected by many biotic and abiotic stresses. *Sclerotinia sclerotiorum* (Lib.) de Bary causes damping off root and stem and fruit rot could infect more than 500 plant species (Antwi-Boasiako et al., 2022; Robison et al., 2018). The disease symptoms appeared as cottony-looking white mycelium on different parts of the plant, causing 100% yield loss and reducing the quality of sensitive common bean cultivars under suitable weather circumstances (Singh and Schwartz, 2010). Once *S. sclerotiorum* infects legume crops, it induces upregulation/downregulation of the defense-related genes linked to metabolic pathways affecting its susceptibility to infection (Westrick et al., 2019). So, eco-friendly management tactics are seriously needed to prevent *S. sclerotiorum* impacts on common bean development, physiological attributes, and yield potential.

In addition to *S. sclerotiorum,* common beans are sensitive to abiotic stressors such as soil salinization. Wekesa et al. (2022) stated that about 20% of cultivable and 33% of irrigation land is saline worldwide and it is estimated that by 2050 around 50% of the arable land will be saline. Assimakopoulou et al. (2015) reported that common beans are a salt-sensitive crop and suffer from yield reductions in soil containing salinity levels of less than 2 d Sm^{-1}. Several studies stated the negative impacts of salinity on seed germination, nodulation, decreasing nitrogen fixation, ionic homeostasis, and antioxidants via stimulating oxidative damage (Wekesa et al., 2022; Dawood et al., 2022a; Cokkizgin 2012). Due to the increasing potential of climate change, crops typically encounter a combination of abiotic and biotic stresses. In common bean, the combination of salinity and S. *sclerotium* infection are of scarce studied and the nature of this co-occurrence should be well-understood. The effect of combined stress factors on crops is not always additive because the nature of interactions between the stresses applied typically dictates the outcome. Plants tailor their responses to combined stress factors and exhibit several unique responses, along with other common responses (Ramu et al., 2016; Pandey et al., 2017). Therefore, to fully recognize the impact of combined abiotic and biotic stresses on common beans, it is important to understand the nature of such interactions.

These global challenges call for urgent but sustainable solutions, which are achieved by using biocontrol microorganisms. In the era of the green revolution, reliance on pesticides is not an appropriate strategy that negatively influences the environment, generates pesticide resistance pathogens, and targets beneficial organisms (Jacquet et al., 2022). However, biological control has dual beneficial effects on biotic and abiotic stresses besides being a more reliable and ecosystem-friendly management technique (Adeleke et al., 2022). The efficacy of biological control against biotic and abiotic stress has been reported in many studies (Carvalho et al., 2015; Abd El-Baki et al., 2022; Silva et al., 2023).

Trichoderma includes many species adapted to different habitats with high specific growth rates and resistance to multiple phytopathogenic fungi such as *Phytophthora, Rhizoctonia, Sclerotinia, Pythium, and Fusarium*, among others (Abdelrhim et al., 2023; Nysanth et al., 2022; Silva et al., 2022; Zin and Badaluddin, 2020; Elshahawy and El-Mohamedy, 2019; El Komy et al. 2015). *Trichoderma* inhibits the growth of pathogens by wrapping hyphae around hosts with appressorium-like structures and triggering the massive secretion of lytic enzymes such as glucanases, chitinases, proteases, and lipases that causes cell wall degradation of host fungi (Wu et al., 2017; Abbas et al., 2022).

Among many *Trichoderma* species *T. harzianum* is the most dominant and has a worldwide distribution. (Druzhinina et al., 2010; Blaszczyk et al., 2011). *T. harzianum* causes systemic resistance to infections and releases substances that stimulate plant growth and root development. It is frequently employed as a biocontrol and biofertilizer agent due to its advantageous interactions with plants (Cai et al., 2015). In addition to the biocontrol effect, *T. harzianum* increases photosynthetic rate, weight, shoot and root length, and the number of leaves and leaf area of treated plants (Zhang et al., 2013; Hashem et al., 2014; Domínguez et al., 2016). *T. harzianum* alleviated chilling and salinity stresses on tomatoes through enhancing photosynthetic rate, leaf water content, proline and growth rates as well as reducing the lipid peroxidation rate and electrolyte leakage (Rubio et al., 2017; Ghorbanpour et al., 2018). *T. koningii* is another important species of Trichoderma, it reduced the severity of diseases caused by the pathogens *Phaeomoniella chlamydospora* and *P. minimum* on grapevines (Pilar Martínez-Diz et al., 2021). *T. koningii* synthesizes Koninginins which has antimicrobial and antifungal activity and secrets viridins that suppressed spore germination of *Aspergillus niger, Botrytis allii, Colletotrichum lini, Fusarium caeruleum, Penicillium expansum*, and *Stachybotrys atra* (Singh et al., 2005; Souza et al., 2008). *T. koningii* has an important role in enhancing tomatoes resistance to abiotic stresses which reduced thermal stress via increasing starch, protein, and total phenol content, while reducing hydrogen peroxide via modulation of antioxidants (Tripathi et al., 2021). *Trichoderma spp*. has been reported to induce plant resistance and tolerance to various abiotic stresses such as temperature, drought, and salinity via stimulating root growth, increasing photosynthetic efficiency, enhancing nutrient uptake, and more effectively protecting plants from oxidative damage stimulating the antioxidant system (Sofy et al., 2022; Zhang et al., 2022). However, using *Trichoderma* under combined stress, i.e., abiotic and biotic stresses, are slightly studied.

This work aimed to; i) test the performance of *Trichoderma koningii* and *Trichoderma harzianum* to grow and sporulate on saline medium (up to 100 mM NaCl), ii) study the activity of fungal cell wall degrading enzymes, i.e., cellulase and chitinase produced by *T. koningii* and *T. harzianum* under salinity stress, iii) detecting amylase and proteinase (Prb1) genes in tested *Trichoderma* isolates. iv) testing the ability of *T. koningii* and *T. harzianum* to antagonize *S. sclerotium* in the presence of the saline medium, and v) illustrate the efficacy of the two *Trichoderma* to tolerate the damping off and collar/stem rot incidence of common bean (*Phaseolus vulgaris*) caused by *S. sclerotiorum* in presence or salinity using various morpho-physiological traits.

Material and methods

Source of Sclerotinia sclerotiorum and Trichoderma isolates

The isolate of *Sclerotinia sclerotiorum* (AUMC No. 11690) was obtained from the Mycological Center, Faculty of Science, Assiut University 71,516, Assiut, Egypt. A plug of obtained culture was plated on Potato Dextrose agar (PDA) in sterile Petri plates and kept in an incubator at 19 °C for five days. PDA was used for sub-culturing and maintaining *S. sclerotiorum* culture.

The two isolates, *T. koningii* (FUE3) (accession number KC200070) and *T. harzianum* (FUE15) (accession number KC200071), were isolated from the rhizosphere of cucumber plants. They were provided by Dr. Nada F. Hemeda, Genetics Department, Faculty of Agriculture, Fayoum University, and more details about isolates identification and characteristics available in the previous study (Hassan et al. (2015a). Once received a plug of each isolate was grown on PDA media and incubated for six days at 25 °C. During this study, PDA has been used for sub-culturing and maintaining the cultures of *Trichoderma* isolates.

Effect of salinity on the linear growth and sclerotia formation of S. sclerotiorum

The effect of salinity on the growth of S. sclerotiorum was displayed according to Sekmen Cetinel et al. (2021) with minor modifications. After preparing PDA medium, NaCl was added to the media where the concentrations were adjusted to five different concentrations 20, 40, 60, 80, and 100 mM NaCl; no NaCl was added to the control. The media was autoclaved and poured into 9 cm Petri plates. After solidification, a 5 mm diameter disc from five-day-old S. sclerotiorum, was placed separately in the center of the Petri plate for each concentration. The plates were placed in a completely randomized design in an incubator for 5 days at 20 °C. The diameter of the growing cultures (mm) was measured for each salt concentration and the control. The number of sclerotia was counted per plate for 14-day old culture. Five plates were used for each concentration; each considered one replicate. The percentage of growth inhibition (%) was calculated using the following equation:

$$Inhibitionofmyceliumgrowth(\%) = C - T/C \times 100.$$

C = growth diameter of the control plate and T = growth diameter of the treated plate.

Effect of salinity on the linear growth and sporulation of T. koningii and T. harzianum

The effect of salinity on the growth of *T. koningii* and *T. harzianum* was displayed according to Zhang et al. (2022) with minor modifications. The media and the process were implemented as described previously. The plates were incubated at 25 °C for 5 days. Five plates were used to present five replicates. The experiment was repeated twice. The percentage of growth inhibition (%) was calculated as described previously. The plates were incubated at 25 ± 2 °C for 7 days for sporulation assessment. 20 mL of sterilized water was added to each plate and the spores were harvested and the conidial suspension was filtered through three layers of cheesecloth. Spore concentration was measured using a hemacytometer (Norton and Harman, 1985).

Effect of salinity on the mycelium fresh and dry weight of S. sclerotiorum, T. koningii, and T. harzianum

Mycelium fresh and dry weight were assessed according to the method described by Satyadev et al. (2022) with minor modifications. 50 mL of potato dextrose broth (PDB) medium was poured into a 250 mL Erlenmeyer flask. NaCl was added to the media, and the concentration was adjusted to 20, 40, 60, 80, and 100 mM NaCl. NaCl free medium was used as a control; 0.5 cm diameter disc of 5 days old *S. sclerotiorum, T. koningii* and *T. harzianum* culture was placed gently on the surface of PDB media separately. The inoculated *S. sclerotiorum* and *Trichoderma* isolates flasks were incubated for five days at 20± 2 °C and 25 ± 2 °C, respectively. Five replicates were used for each treatment, each replicate was represented by one flask. For fresh weight assessment, the mycelium from liquid media was harvested using a filtration-based method described by Newell and Statzell-Tallman (1982) with slight modifications. Briefly, the fungal mycelium was filtrated using a 40-μm-mesh nylon screen, washed with 25 mL distilled water, recollected on Whatman filter paper no. 1, then vacuumed for 30 s to abandon free water from the samples. Subsequently, mycelia were kept in small Petri dishes to avoid rapid dehydration during fresh weighting. Fresh weight was assessed immediately after filtration. For dry weight assessment, the mycelia of tested fungal cultures were left to dry at 50 °C for 24 h and then reweighted. During the dry weight assay, fresh desiccant was maintained in the weighting chamber of the balance. The percentage of mycelium fresh and dry growth reduction was calculated as follows:

$$\text{Inhibition of mycelium fresh or dry weight } (\%) = \frac{\text{Mycelium fresh/dry weight of control} - \text{Mycelium fresh/dry weight in the treatment}}{\text{Mycelium fresh/dry weight of control}} \times 100$$

Antagonistic activity of T. koningii and T. harzianum against S. sclerotiorum under salinity stress

A dual culture assay was implemented according to Poosapati et al. (2014) to study the antagonistic between *T. koningii, and T. harzianum* and *S. sclerotiorum* under different levels of salinity. PDA media amended with five salinity concentrations (20, 40, 60, 80, 100 mM NaCl) was prepared as described previously. A 5 mm diameter disc from the five-day-old *T. koningii, and T. harzianum* culture was inoculated on PDA for each concentration separately on one side of the Petri plates (2 cm away from the edge). A 5 mm disc obtained from a seven-day-old PDA culture of pure single pathogenic isolate *S. sclerotiorum* was placed on the opposite side of the plate perpendicular to the *Trichoderma* isolates. The plates were incubated at 20 ± 2 °C for five days. NaCl free medium was used as a control. Five replicates were used for each treatment. Observations on the width of the inhibition zone and mycelia growth of the tested pathogens were recorded, and the percentage of the pathogen growth inhibition was calculated by using the formula proposed by Singh et al. (2002).

$$Growthinhibitionpercent(GI, \%) = C - T/C \times 100.$$

C = growth radius of the control plate and T = growth radius of the treated plate.

The activity of cell wall degrading enzymes produced by T. koningii and T. harzianum under salinity stress

Chitinase activity

The production of chitinase *T. koningii* and *T. harzianum* under six levels of salinity starting from 0 mM to 100 mM NaCl with 20-unit intervals was evaluated according to Hassan et al. (2015b) with minor modification. A 5 mm disc from 5 days old culture of the two isolates was transferred into 200 mL of Czapeck-Dox medium supplemented with 10% glucose in 500 mL flask. The salt concentrations were adjusted by adding NaCl according to the required salinity level. Three flasks were used for each salt concentration, each representing one replicate. The flasks were placed in the incubator for 96 h at 25 °C. The obtained mycelium was harvested using filter paper filtration and washed three times with 2% of $MgCl_2$ and distilled water. The mycelium was transferred into a 100 mL flask containing 50 ml of Czapeck-Dox medium supplemented with 1.5% colloidal chitin. NaCl concentrations were adjusted according to the tested level. The flasks were incubated at 100 rpm on a rotary shaker for 96 h at 25 °C. Spectrophotometric assay of chitinase described by Ulhoa and Peberdy (1991) was used to production and the activity of chitinase in the obtained culture filtrates by using N-acetyl-d-glucosamine (GlcNAc) as standard. A volume (1 mL) of culture filtrates was mixed with 1 ml of 0.5% colloidal chitin and incubated with shaking for 24 h at 40 °C. The negative control was prepared as described by Mathivanan (1995), 1 mL of culture filtrate, boiled at 100 °C for 3 min, was mixed with 1 mL of 0.5% colloidal chitin. After incubation, the mixture was centrifuged for 5 min at 4000 rpm. A volume (1.5 mL) of modified reagent (dinitrosalicylic acid 1%, phenol 0.2%, sodium sulfite 0.05% and sodium hydroxide 1%) was added to 1.5 of obtained supernatant. The mixture was placed in a water bath at 80 °C for 5 min, A 0.5 mL of 40% Rochelle salt (potassium sodium tartrate solution) was added for color stabilization. The color intensity was measured using a spectrophotometer at 575 nm, according to Mathivanan et al. (1998). Each unit of enzyme activity was defined as the amount of enzyme required for forming one μmole of N-acetyl-glucosamine in one mL of the reaction under the standard assay conditions.

Cellulase activity

The cellulase activity produced by *T. koningii* and *T. harzianum* was measured under the salinity levels according to Hassan et al. (2015b) with minor modification. 200 mL of Czapeck-Dox medium supplemented with 10% glucose was poured into a 500 mL flask. The salt concentration was adjusted in each flask according to the tested salinity level. Three flasks were used for each concentration, each representing one replicate. A 5 mm disc of five days old of *T. koningii* and *T. harzianum* culture was added to each flask and incubated for 96 h at 25 °C. The culture was filtered using filter papers, and the obtained mycelium was washed three times with 2% of $MgCl_2$ and distilled water. The mycelium was transferred to 100 mL flasks containing 50 mL Czapeck-Dox medium supplemented with 1% carboxymethyl cellulose. The salt was adjusted by adding NaCl to each flask according to the salt treatments. The flasks were incubated for six days at 25 °C with shaking (100 rpm). The obtained mixture was centrifuged for 5 min at 4000 rpm. A 0.5 mL of culture filtrate was added to 1 mL 0.5 mM sodium citrate buffer pH 4.8 and 1 mL of 1% (w/v) carboxymethyl cellulose and incubated for 10 min at 50 °C (Melo et al., 1997). Alkaline dinitrosalicylic acid was added to the mixture to stop the reaction, and the absorbance was measured at 540 nm (Miller et al., 1960).

Effect of T. koningii and T. harzianum on germination, root, and shoot length, and vigor indexes of S. sclerotiorum-infected common bean in the presence and absence of salinity stress

The impact of *T. koningii* and *T. harzianum* on common beans challenged water salinity (80 mM NaCl) as abiotic stress, and *S. sclerotiorum* as biotic stress has been investigated. The experiment was implemented under greenhouse conditions Department of plant pathology, Minia University, Egypt. Spore suspension of *T. koningii* and *T. harzianum* was prepared according to Brandão et al. (2019) with minor modifications. Each *Trichoderma* isolate was grown on 25 g of barley grains moistened with 50 mL distilled water after autoclaving for 30 min at 121 °C. The inoculated flasks were incubated for one week at 25 °C and 12-hour photoperiod. An additional 50 mL of distilled water was added to each flask. The obtained suspension was filtered with cheesecloth to adjust spore suspensions at 1×10^7 conidia mL^{-1}, using a hemocytometer.

The inoculum of *S. sclerotiorum* was prepared according to El-Ashmony et al. (2022) with minor modifications. Twenty-five grams of barley and 50 mL of water were added into 250 ml flasks and autoclaved for 20 min at 121 °C. After cooling, three plugs of 5 days old *S. sclerotiorum* culture were added to each flask and incubated at 20 °C for three weeks. After incubation, the obtained inoculum was added to loamy clay soil at the rate (1% w/w). The loamy/clay soil has a pH ranging from 7.7 to 8.0 and 1.5–2.7 organic matter and total soluble nitrogen (15–40 ppm) according to (FAO, 2005). After mixing the inoculum into the soil, the pots were placed in the greenhouse and arranged in split plots with biocontrol agents as main plots. The experiment was divided into three main plots: No biocontrol agent, *T. koningii* and *T. harzianum*. In the plots treated with *Trichoderma* isolates, all the pots treated directly before planted with 1×10^7 mL^{-1} spore suspension of *T. koningii or T. harzianum* (50 mL/pot), the treatment repeated weekly.

Four treatments were applied to each plot; Control (The plants received only water, no biotic or abiotic stresses); *S. sclerotiorum* (The plants infected with pathogen); NaCl (80 mM) (Plants challenged with saline water only); *S. sclerotiorum* + NaCl (80 mM) (The plants challenged with the pathogen and salin water). Five replicates represented each treatment, one pot displayed as one replicate. Two weeks after sowing dates, the number of germinated seeds was counted, and the germination percentages, as well as damping-off characteristics, were calculated using the following equations:

$$\text{Germination}(\%) = \text{numberofgerminatedseeds} / \text{totalnumberofplantedseeds} \times 100$$

$$\text{Dampingoff}(\%) = \text{Totalnumberofplantedseeds} - \text{thenumberofgerminatedseeds} / \text{totalnumberofplantedseeds} \times 100$$

Four weeks after the sowing date, the whole plant was harvested, including aerial parts and the roots. The roots were washed thoroughly using tap water for soil removal. Fresh plants were used for physiological traits and enzymatic analysis after incubation in liquid nitrogen and then kept at –70 °C. The lengths and weights of the seedlings' roots and the shoot were measured. The vigor index of the seedlings was calculated using two measurements; First, the seedling vigor index based on the root and shoot length (SVIL) was evaluated according to Abdul-Baki and Anderson (1973) as follows:

SVIL= (shoot length + root length) × germination (%)

Second, the seedling vigor index based on the weight was calculated using the following equation:

SVIW=seedling dry mass at the end of the test × germination (%)

The collar/stem rot incidence was calculated as the percentage of plants with collar/stem rot symptoms. The severity of seedlings was assessed according to Hall and Phillips (1996). A scale of 0 to 4 was used where 0 = 1–25% of white mold on the infected stem, 2 = 26–50%, 3 = 51–75%, and 4 = 76–100%.

Effect of T. koningii and T. harzianum on common bean physiological and biochemical traits under S. sclerotiorum and salinity stresses

Pigmentation

The chlorophyll a, b and carotenoid content were estimated based on Lichtenthaler and Wellburn (1983).

Reactive oxygen species and stress biomarker traits

Reactive oxygen species (ROS) of leaves viz., hydrogen peroxide (H_2O_2), superoxide anion ($O_2^{\bullet -}$), and hydroxyl radical ($^{\bullet}OH$) were screened based on (Mukherjee and Choudhuri (1983); Elstner and Heupel (1976), and Halliwell et al. (1987), respectively. Lipid peroxidation was estimated in liquid nitrogen-frozen leaves at -80 ºC of artichoke plants using the thiobarbituric acid reaction by monitoring malondialdehyde (MDA) formation according to Madhava Rao and Sresty (2000). Methylglyoxal (MG) was estimated based on the method of Gilbert and Brandt (1975) in liquid nitrogen-frozen leaves at -80 ºC.

Defense-related secondary metabolites

Phenolic compounds in common bean leaves were determined based on the method of Kofalvi and Nassuth (1995) using a standard curve of gallic acid and expressed as mg/g FW. The flavonoid content was quantified based on the protocols of Zou et al. (2004), and the anthocyanins were detected based on the method of Krizek et al. (1993).

Signaling molecules nitric oxide and hydrogen sulfide, as well as salicylic acid

Nitric oxide (NO) content was quantified through the method explained by (Hu et al., 2003). The hydrogen sulfide (H_2S) content of common bean leaves (liquid nitrogen and frozen leaves at −80 °C) were estimated based on the method of Nashef et al. (1977). Salicylic acid (SA) content in common bean leaves was measured according to the method described by Warrier et al. (2013).

Defense related enzymes

Phenylalanine ammonia-lyase (PAL/ EC 4.3.1.5) activity was examined using the protocol of Havir and Hanson (1973). Polyphenol oxidase (PPO/EC 1.10.3.1) was determined using the protocol of Kumar and Khan (1982).

Antioxidant enzymes/molecules

Non-enzymatic antioxidants ascorbic acid (ASA) and reduced glutathione (GSH) were estimated on leaves, as cited by Jagota and Dani (1982) and Ellman (1959), respectively. Superoxide dismutase (SOD/EC.1.15.1.1) activity was quantified following the autoxidation of epinephrine, as mentioned by Misra and Fridovich (1972). Catalase (CAT/EC.1.11.1.6) activity was calculated following the breakdown of H_2O_2 for 1 min, where the decrease in absorbance was monitored at 240 nm using the protocol of Noctor et al. (2016). Ascorbate peroxidase (APX/EC.1.11.1.11) was detected in the previously prepared enzyme extract by monitoring the oxidation of ascorbate as a substrate in the presence of EDTA and H_2O_2 at 290 nm using the protocol of Silva et al. (2019). Guaiacol peroxidase (POD/ EC 1.11.1.7) activity was determined based on the protocol of Tatiana et al. (1999).

Molecular characterization of the proteinase-encoding (prb1) and amylase genes

The RNA from *T. koningii* and *T. harzianum* was extracted by using ABT total RNA mini extraction kit (Applied Biotechnology Co., Ismailia, Egypt). The isolated RNA was treated with DNaseI and purified by the RNeasy Mini Elute Cleanup kit 1741985 (Roche).

Primers were designed for coding two genes of Trichoderma, the specific primers of amylase gene are F 5′-CCACACAAGCGATAAGCACG-3′ and R 5′- GCCATTTGGTGACAACCGTC -3′, Product size is 635 bp, designing of primers according to the isolate with accession number XM_024922814.1. The specific primers of proteinase (Prb1) are F 5′-GCAGGTGAGCTACCCTTTGT-3′ and R 5′-AGGGGCAAAAACATCAACGC-3′, Product size is 440 bp, designing of primer according to the isolate with accession number M87518.1. The primers produced by AUGCT Company, China according to GenBank database of NCBI site.

The reverse transcription-polymerase chain reaction (RT-PCR) was conducted utilizing the One Step RT-PCR Kit, which was manufactured by the esteemed Biotech Corporation of China. The PCR product was examined through electrophoresis (Viterbo et al., 2002). DNA elution was performed using the QIA quick Gel Extraction Kit from Qiagen. Subsequently, sequencing products were resolved on HiSeq 2000/2500, which is sequencing platform provided by Macrogen, a reputable sequencing company. The nucleotide sequences were translated using Xpasy program (https://web.expasy.org/translate/.

Subsequently, a multiple sequence alignment was generated through Clustal W (http://www.ebi-ac.uk/ Clustal W), followed by computer analysis of the sequences. The deduced amino acid sequence from each gene was obtained by BLASTP Network Service (NCBI) and aligned with reference sequences from the NCBI database through MEGA X. The phylogenetic dendrogram was constructed with the aid of the MEGA X program, and the unweighted pair group method with arithmetic mean (UPGMA) was employed to generate the phylogenetic tree, in accordance with Kumar et al. (2018). Ultimately, the sequences of the two genes were registered in GenBank. http://www.pir.uniprot.org/.

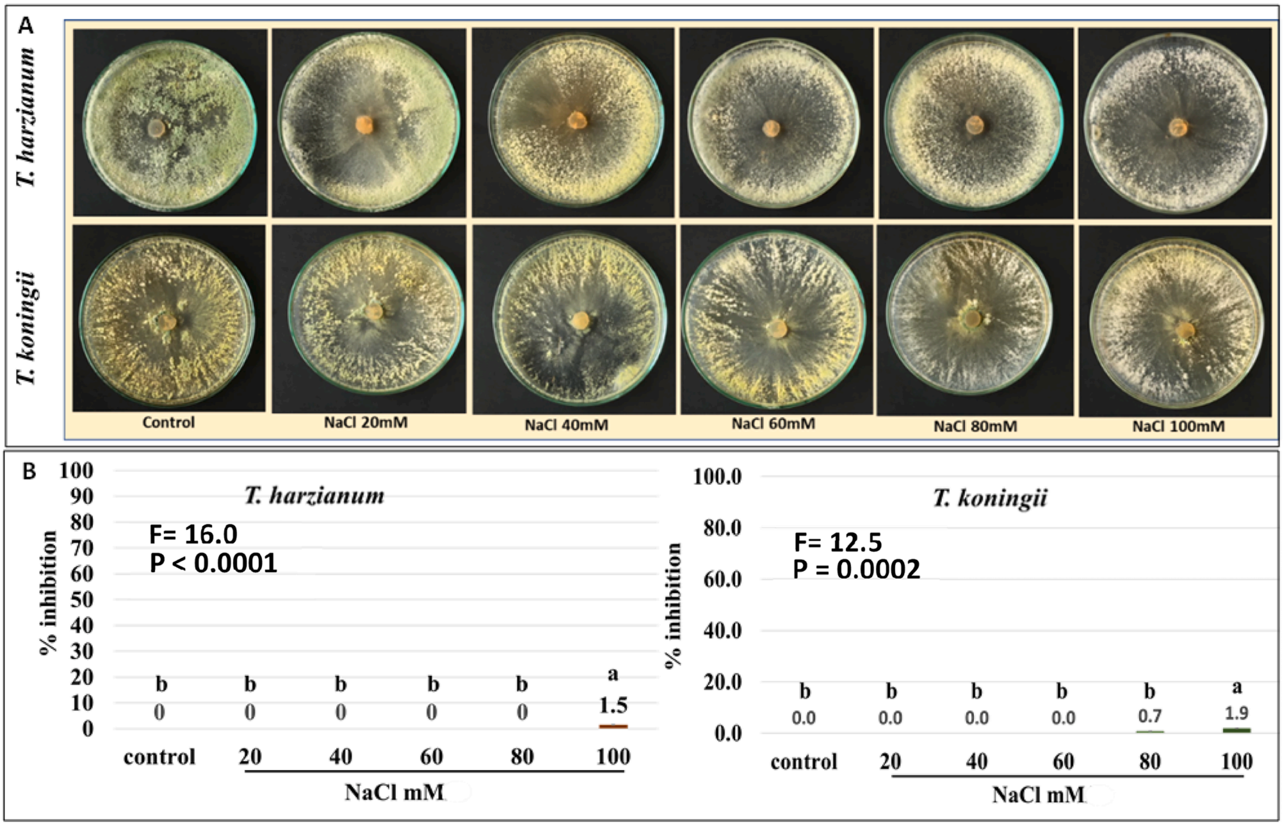

Fig. 1. The effect of NaCl at five levels (20, 40, 60, 80, and 100 mM) on; A and B) the inhibition percentage of *S. sclerotiorum* mycelium at different levels of NaCl, C, and D) mycelium fresh and dry weight of *S. sclerotiorum*, and E and F) sclerotia formation on nutrient agar (NA) media. The experiments were repeated twice independently. The data presented as means ± standard deviation of five biological replicates. Different letters indicate statistically significant differences among treatments, while the same letters signify no significant differences between them according to Tukey's honestly significant difference test ($p < 0.05$).

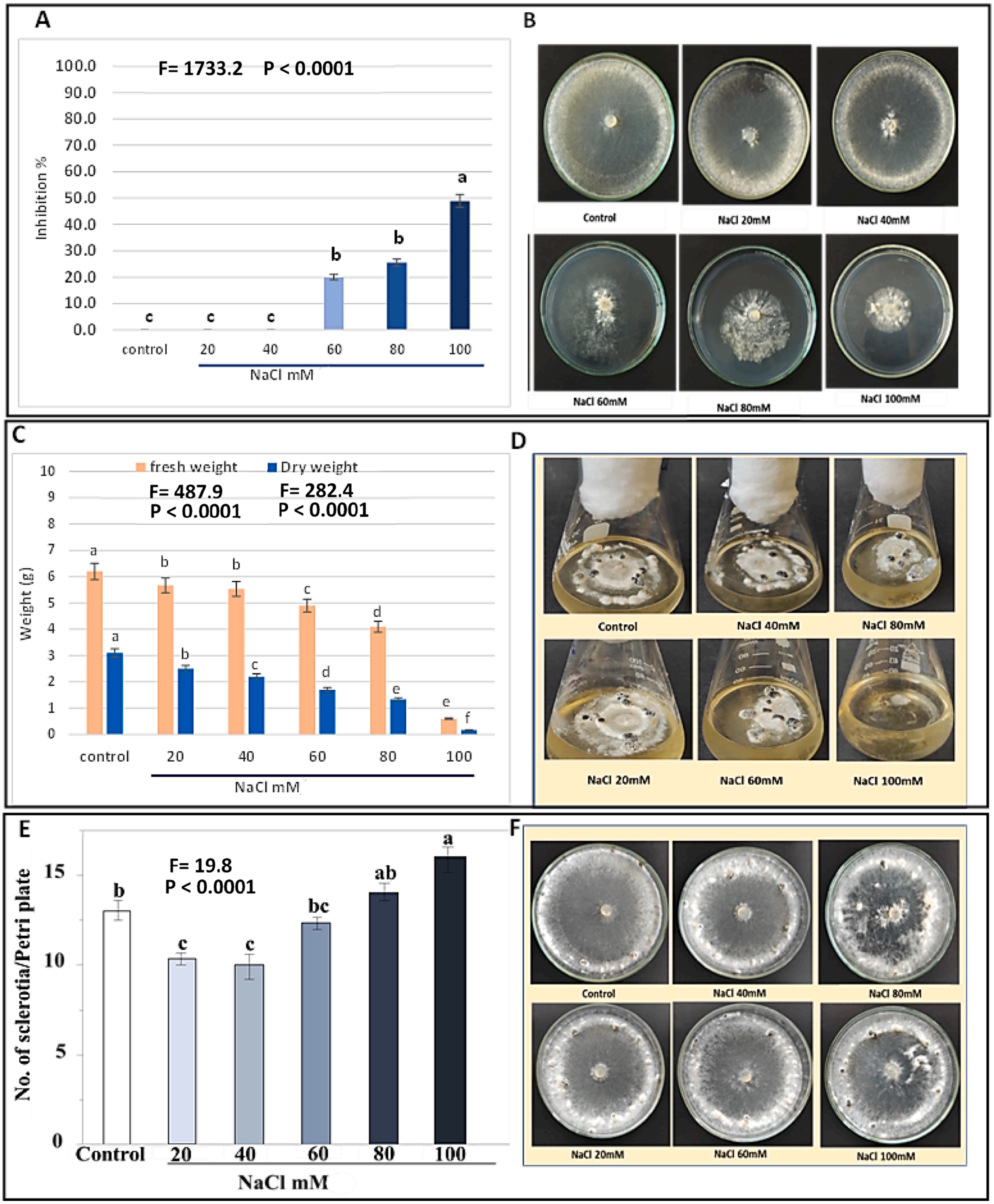

Fig. 2. A and B) The impact of five levels of NaCl, 20, 40, 60, 80, and 100 mM, on the growth of *T. koningii* and *T. harzianum*. The mycelium of *T. koningii* and *T. harzianum* cultures was measured after five days of incubation on PDA media amended with the five levels of NaCl; the control was grown in PDA containing distilled water. The data presented as means ± standard deviation (mean ± SD) of five biological replicates. Different letters indicate statistically significant differences among treatments, while the same letters signify no significant differences between them according to Tukey's honestly significant difference test ($p < 0.05$).

Statistical analysis

The in vitro and greenhouse experiments were conducted using a completely randomized design (CRD). All experiments were repeated at least twice, with five replicates for each treatment. Only three replicates were used for the enzyme's activity assay. One experiment's data were presented, as the values of each pair of repeated experiments were highly similar. The analysis of variance (ANOVA-one way) was used to test the significance different in laboratory experiments. However, ANOVA-two way was used to test the significant differences among different infection and treatment levels and the interaction in greenhouse experiment and with physiological traits. A Tukey's honestly significant difference (HSD) test was used for post-hoc analysis. The least significant difference was used to compare the means at $p \leq 0.05$. The data were analyzed using JMP data analysis software version 14.

Experimental results

Effect of salinity on the linear growth, mycelium fresh and dry weight and sclerotia formation of S. sclerotiorum

The influence of different levels of salinity, 20, 40, 60, 80, and 100 mM, on the growth of *S. sclerotiorum* culture was investigated. The results revealed that there was no significant effect of low levels of NaCl 20 and 40 mM on the linear growth of *S. sclerotiorum*. Then the effect intensified until maximal inhibition (48.9%) at 100 mM NaCl compared with other levels (Fig. 1a and b). No significant effect of low levels of NaCl 20 and 40 mM was observed on the fresh weight of *S. sclerotiorum* mycelium growing on nutrient broth (NB). NaCl at 60, 80 and 100 mM significantly reduced the weight of *S. sclerotiorum* fresh mycelium. The highest reduction of fresh mycelium weight was observed on NaCl at

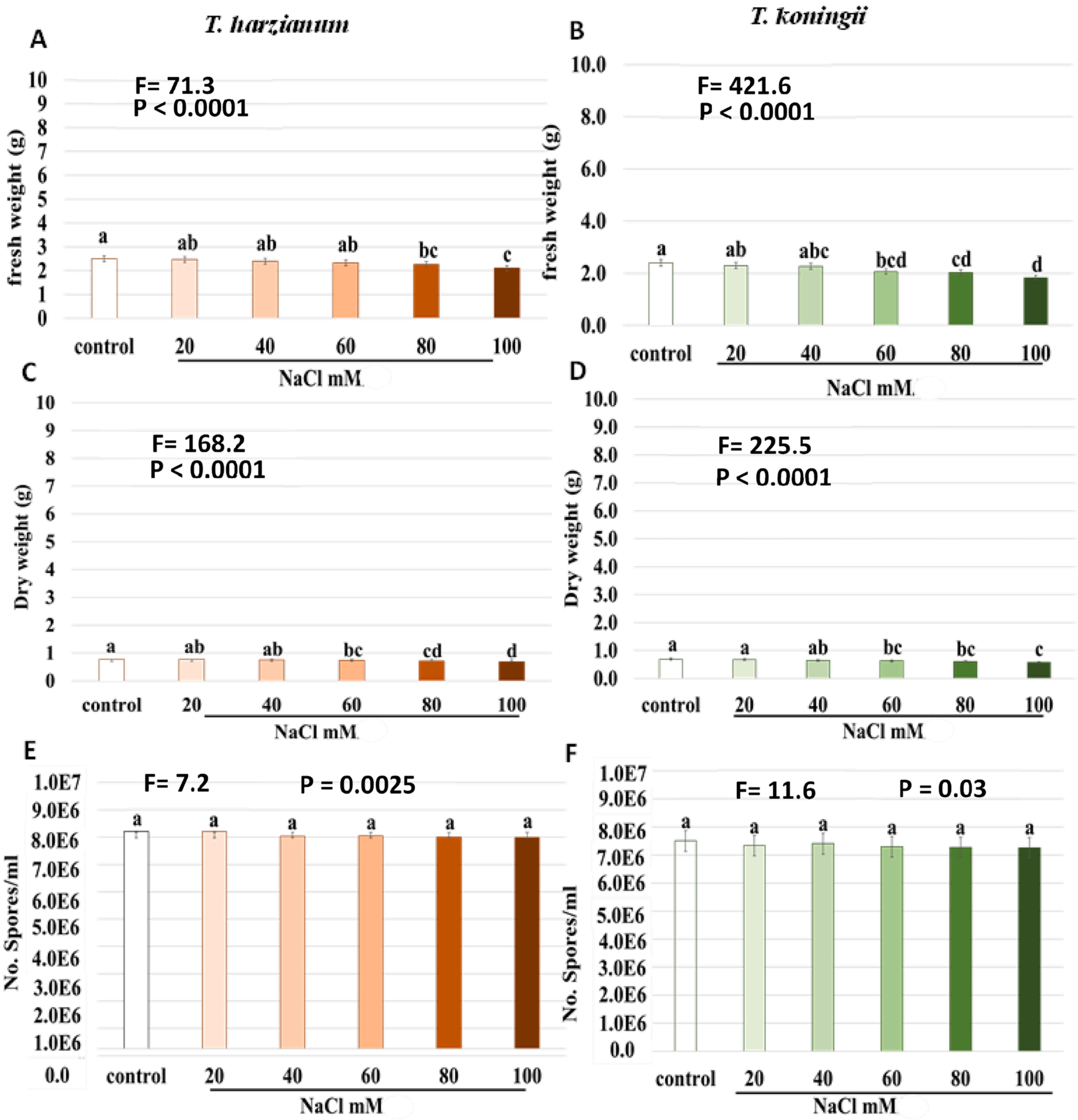

Fig. 3. The effect of NaCl at five levels (20, 40, 60, 80, and 100 mM) on mycelium fresh and dry weight, as well as sporulation of *T. harzianum* (A, C, & E) and *T. koningii* (B, D, & F), respectively. The data presented as means ± standard deviation of five biological replicates. Different letters indicate statistically significant differences among treatments, while the same letters mean no significant differences between them according to Tukey's honestly significant difference test ($p < 0.05$).

100 mM (0.6 gm). All tested levels of NaCl significantly reduced the dry weight of *S. sclerotiorum* mycelium with a high reduction observed at 100 mM NaCl) (0.17 gm) compared with other levels of NaCl (Fig. 1 c and d). The data presented in Fig. 1 d and f showed the number of formed sclerotia significantly increased under high salinity pressure (100 mM NaCl) (16/plate) compared with the control and other levels of salt. There was no significant impact of 60 and 80 mM NaCl on the number of formed sclerotia compared to the control.

Effect of salinity on the linear growth of T. koningii and T. harzianum

Salinity stress showed a slight effect on the growth of *T. koningii* and *T. harzianum* (Fig. 2). No inhibition was observed when *T. koningii* and *T. harzianum* were grown on 20,40, 60, and 80 mM NaCl. Only minor inhibitions, 1.9% and 1.5%, were observed when *T. koningii* and *T. harzianum* were grown on 100 mM NaCl compared to the control, respectively.

Effect of salinity on the mycelium fresh and dry weight and sporulation of T. koningii and T. harzianum

The low salt concentrations of 20 and 40 mM showed no significant effect on the fresh and dry weight of *T. koningii* and *T. harzianum.* Salt concentrations below 100 mM almost showed a similar effect on the fresh and dry mycelium weight of tested *Trichoderma* isolates (Fig. 3). A significantly lower fresh (1.8 and 2.1 g) and dry (0.6 and 0.7 g) weight of *T. koningii* and *T. harzianum* was observed when grown on the highest salt concentration 100 mM compared to the control (2.4 and 2.5 g) (0.7 and 0.8 g), respectively. *Trichoderma* isolates showed similar sporulation performance on the different levels of salinity. In general, salt levels had no significant impact on the sporulation of *T. koningii* and *T. harzianum* compared to each other and the control. The average spore number of *T. harzianum* and *T. koningii* in high saline media 100 mM was 8.0×10^6 and 7.3×10^6 compared to 8.2×10^6 and *T. koningii* 7.5×10^6 mL^{-1} in control, respectively.

Chitinase and cellulase activity of T. koningii and T. harzianum under salinity stress

The activity of chitinase and cellulase of *T. koningii* and *T. harzianum* was monitored under different levels of salinity (Fig. 4). No significant effect between 0, 20, 40, 60, and 80 mM NaCl was observed in chitinase activity produced by *T. koningii* and *T. harzianum*. In comparison, the significant effect was recorded only at 100 mM NaCl. *T. koningii* showed the highest chitinase (0.0173 mg/mL) and cellulase (0.038 mg/mL) activities at 20 mM NaCl. However, *T. harzianum* expressed the highest chitinase activity (0.0168 mg/mL) under control and the highest cellulase activity (0.033 mg/mL) under 20 mM NaCl.

The antagonistic effect of T. koningii and T. harzianum against S. sclerotiorum under salinity stress

Salinity stress generally did not affect the parasitism ability of *T. koningii* and *T. harzianum* against *S. sclerotiorum* (Fig. 5). In addition, the results of the in vitro antagonistic experiment revealed that the high growth inhibition of *S. sclerotiorum* 64.1 and 63.7% achieved by *T. koningii* and *T. harzianum* grown on 80 mM NaCl compared to 32.6 and 50% in the control plates containing 0 NaCl, respectively. *T. harzianum* showed similar antagonistic performance on 20, 40, 60, 80, and 100 mM NaCl against *S. sclerotiorum*, with no significant effect observed between all salinity levels and the control (0.0 NaCl). However, *T. koningii* showed different antagonistic performance against *S. sclerotiorum,* the highest growth inhibition (75%) of *S. sclerotiorum* was observed at 20 mM NaCl.

Effect of T. koningii and T. harzianum on germination percentage, root and shoot length, and vigor indexes of S. sclerotiorum-infected common bean under salinity stress

The role of *T. koningii* and *T. harzianum* against biotic stress expressed by *S. sclerotiorum* and abiotic stress expressed by salinity was investigated on two common bean varieties, Bronco and Nebraska. In general, treating *S. sclerotiorum*-infected and salt-treated Bronco and Nebraska seedlings with *T. harzianum* and *T. koningii* significantly enhanced the germination of the seeds and increased the vigor index based on plant length (VI) and the vigor index based on seedling weight (VII) compared with untreated-infected seedlings (Table 1).

The highest germination percentage (G%) was observed in both varieties, Bronco and Nebraska, when seedlings were treated with *T. harzianum* (99.7 and 99.9 %, respectively), followed by *T. koningii* (99.5 and 99.7 %, respectively) compared with all other treatments. In contrast, the lowest G% (47.5 and 50.0%) was observed on Bronco and Nebraska seedlings challenged with both *S. sclerotiorum* and salt (80 mM), respectively. Adding *T. harzianum* to soils challenged with the coexistence of *S. sclerotiorum*-infected and salt-treated seedlings significantly increased the G% in Bronco to (96.0 and 95.3 %) and in Nebraska

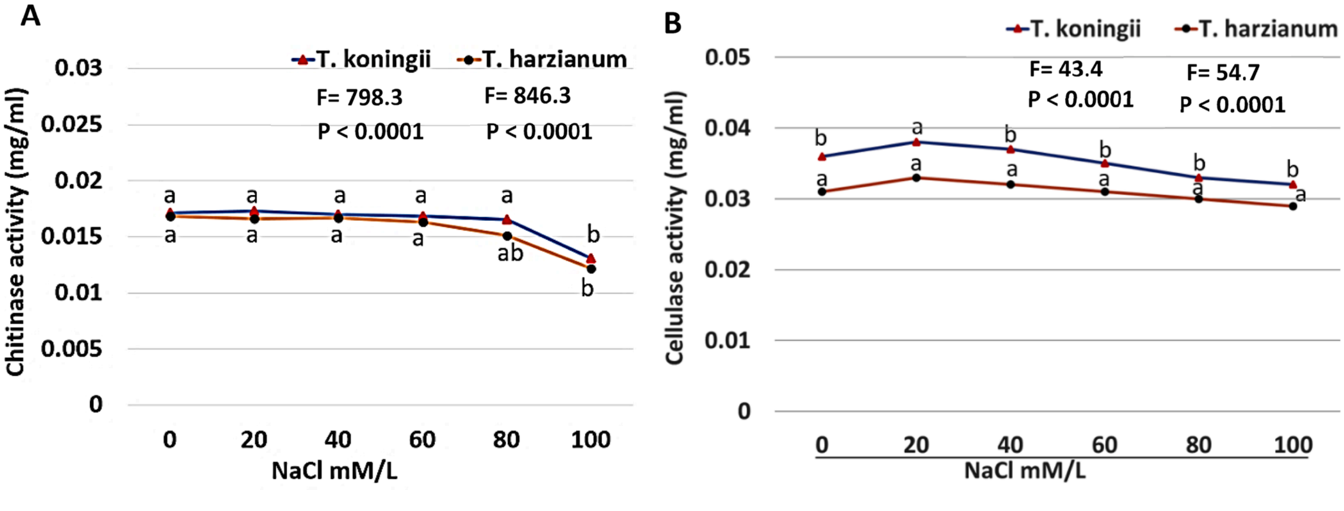

Fig. 4. The effect of NaCl at five levels (20, 40, 60, 80, and 100 mM) on A) chitinase activity (A) and cellulase (B) activities produced by *T. koningii* and *T. harzianum.* The data presented as means ± standard deviation (mean ± SD) of three biological replicates. Different letters indicate statistically significant differences among treatments, while the same letters mean no significant differences between them according to Tukey's honestly significant difference test ($p < 0.05$).

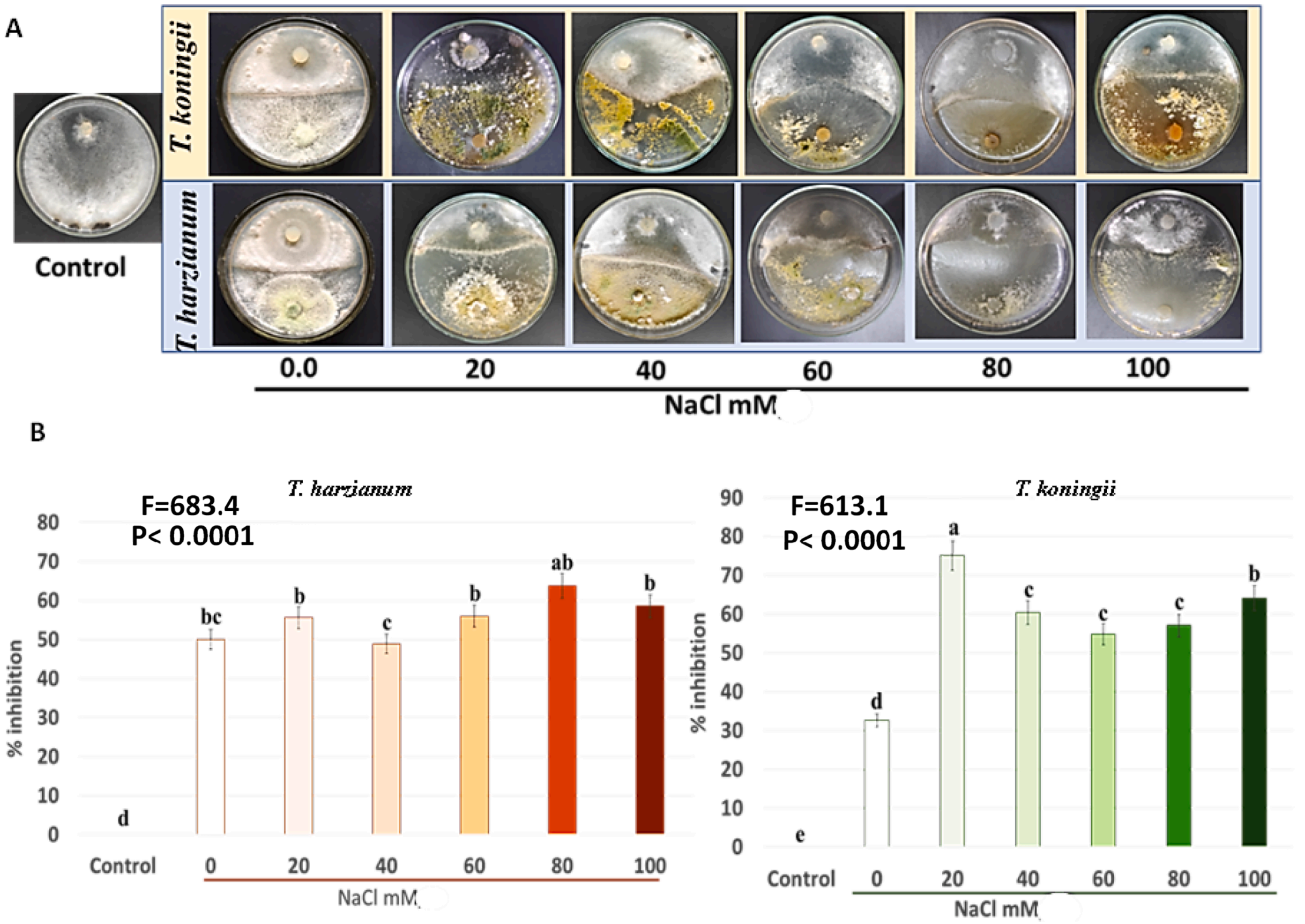

Fig. 5. A and B. The antagonistic effect of T. koningii and T. harzianum against S. sclerotiorum under salinity stress was assessed as an inhibition percentage. Plates with only S. sclerotiorum culture presented the control, and 20.0 to 100 mM NaCl with 20 mM intervals represented the studied salinity levels. The data presented as means ± standard deviation (mean ± SD) of five biological replicates. Different letters indicate statistically significant differences among treatments, while the same letters mean no significant differences between them according to Tukey's honestly significant difference test ($p < 0.05$).

to (97%) compared to similar treatment without application of Trichoderma, Bronco seedlings (65.0% and 80.0%) and Nebraska seedling (75.0 and 83.8%), respectively. In addition, both *T. harzianum* and *T. koningii* significantly increased G% and seedling vigor indexes (VI and VII) of Bronco and Nebraska seedlings challenged with both *S. sclerotiorum* and salt compared with Trichoderma-untreated seedlings under the same stresses. The vigor index (VI) of Bronco and Nebraska seedlings challenged with a combined pathogen and salt stresses was (359.4 and 471.6) compared with (1212.0 and 1314.2) and (1433.9, and 1626.0) when the diseased seedlings treated with *T. harzianum* and *T. koningii*, respectively. Similar effects of *T. harzianum* and *T. koningii* were observed on the seedling vigor index (VII) values, which significantly increased in *Trichoderma* treated seedlings compared with Trichoderma-untreated diseased seedlings.

The impact of *Trichoderma* isolates on the damping off and the collar/stem rot of the two common beans varieties Bronco and Nebraska has been studied (Table 1 and Fig. 6). No damping off was recorded in non-infected control and non-infected *Trichoderma* treated pots. A significantly high damping-off percentage was observed in Bronco and Nebraska seedlings challenged with *S. sclerotiorum* and salt simultaneously or separately compared with control and Trichoderma-treated seedlings. Bronco and Nebraska seeds challenged with *S. sclerotiorum* and salt showed 52.5 and 50.0 % damping off, respectively. Treating the seeds with *T. harzianum* and *T. koningii* decreased the damping off to 14.5 and 10.8% and 11.8 and 8.0 in Bronco and Nebraska, respectively. Compared with non-infected control, a high percentage of collar/stem rot was observed in *S. sclerotiorum*-infected seedlings. In addition, salinity intensified the incidence percentages of collar/stem rot in *S. sclerotiorum*-infected seedlings to 86.3 and 80.0 compared to 75.0 and 65.0 in non-salinity-infected Bronco and Nebraska seedlings, respectively. Seedlings treated with *T. koningii* and *T. harzianum* showed significantly lower collar/stem rot (%) than Trichoderma-untreated seedlings. The percentage of collar/stem rot in Bronco and Nebraska seedlings challenged with *S. sclerotiorum* and salinity significantly decreased to 18.0 and 12.5 % in *T. harzianum*-treated seedling and to 22.5 and 16.5% in *T. koningii*-treated seedling, respectively.

Biochemical and physiological responses of common bean plants to trichoderma species under salinity and sclerotia infection

As provided in Fig. 7, the Chl a content was significantly reduced in a similar manner under salinity (Sal) and/or *S. sclerotiorum*-infected plants (Inf) infection compared to control plants. In contrast, the most adverse effect on Chl b and carotenoids was observed under combined stress of salinity and *S. sclerotiorum* infection (Sal+Inf). *T. koningii* and *T. harzianum* significantly increased the photosynthetic pigments in control (unstressed plants) compared with Trichoderma-untreated seedlings. Although, *T. koningii* and *T. harzianum* restrained the reduction in pigments composition under biotic and abiotic stresses, the increment of Chl a and Chl b elicited by the application of *Trichoderma*

Table 1

Effect of *T. harzianum* and *T. koningii* on the germination, vigor index, damping off and collar/stem rot of *S. sclerotiorum*-infected common bean under salinity stress*.

Treatments		G%		VI/Plant length		VII/Dry weight		Damping off		Collar/stem rot%	
		Bronco	Nebraska	Bronco	Nebraska	Bronco	Nebraska	Bronco	Nebraska	Bronco	Nebraska
No *Trichoderma*	Control	99.0^{a}	99.5^{a}	1955.0^{c}	2213.8^{c}	324.0^{fg}	377.9^{f}	0.0 g	0.0 g	0.0^{d}	0.0^{e}
	S. sclerotiorum	65.0^{f}	75.0^{f}	585.6^{h}	789.6^{j}	107.0^{j}	142.8^{h}	35.0^{b}	25.0^{b}	75.0^{b}	65.0^{b}
	NaCl (80 mM)	80.0^{e}	83.8^{e}	849.4 g	1143.3^{i}	154.4^{i}	201.0 g	20.0^{d}	16.3^{c}	0.0^{d}	0.0^{e}
	S. sclerotiorum + NaCl (80 mM)	47.5 g	50.0 g	359.4^{i}	471.6^{k}	34.9^{k}	50.4^{i}	52.5^{a}	50.0^{a}	86.3^{a}	80.0^{a}
T. koningii	Control	99.5^{a}	99.7^{a}	2220.2^{b}	2521.8^{b}	502.2^{b}	590.5^{bc}	0.0 g	0.0 g	0.0^{d}	0.0^{e}
	S. sclerotiorum	92.3^{bc}	93.3^{bcd}	1206.4^{f}	1454.8 g	350.5^{ef}	454.5^{e}	7.8^{ef}	6.8^{def}	17.5^{c}	13.0^{cd}
	NaCl (80 mM)	92.0^{bcd}	95.3^{abc}	1504.1^{e}	1685.9^{e}	375.0^{de}	535.8^{cd}	8.0^{def}	4.8^{efg}	0.0^{d}	0.0^{e}
	S. sclerotiorum + NaCl (80 mM)	85.5^{de}	89.3^{d}	1212.0^{f}	1314.2^{h}	247.8^{h}	415.4^{ef}	14.5^{cd}	10.8^{d}	22.5^{c}	16.5^{c}
T. harzianum	Control	99.7^{a}	99.9^{a}	2484.4^{a}	2654.8^{a}	553.2^{a}	679.3^{a}	0.0 g	0.0 g	0.0^{d}	0.0^{e}
	S. sclerotiorum	96.0^{ab}	97.0^{ab}	1384.7^{e}	1598.1^{f}	403.2^{d}	601.3^{b}	4.0^{fg}	3.0^{fg}	15.0^{c}	11.0^{d}
	NaCl (80 mM)	95.3^{ab}	97.0^{ab}	1645.6^{d}	1794.6^{d}	447.8^{c}	553.0^{bcd}	4.8^{fg}	3.0^{fg}	0.0^{d}	0.0^{e}
	S. sclerotiorum+NaCl (80 mM)	88.3^{cd}	92.0^{cd}	1433.9^{e}	1626.0^{ef}	306.6 g	513.0^{d}	11.8^{de}	8.0^{de}	18.0^{c}	12.5^{cd}
F & P-Value Trichoderma		$F = 317.8$	$F = 465.8$	$F = 911.9$	$F = 2402.1$	$F = 1958.9$	$F = 1227.4$	$F = 321.9$	$F = 469.0$	$F = 484.0$	$F = 1241.4$
		$P < 0.0001$	$P<0.0001$	$P < 0.0001$	$P < 0.0001$	$P < 0.0001$	$P < 0.0001$	$P < 0.0001$	$P < 0.0001$	$P < 0.0001$	$P < 0.0001$
Stress		$F = 186.7$	$F = 271.7$	$F = 1253.6$	$F = 3991.2$	$F = 854.3$	$F = 185.8$	$F = 190.9$	$F = 274.5$	$F = 573.7$	$F = 1173.1$
		$P < 0.0001$	$P<0.0001$	$P < 0.0001$	$P < 0.0001$	$P < 0.0001$	$P < 0.0001$	$P < 0.0001$	$P < 0.0001$	$P < 0.0001$	$P < 0.0001$
Trichoderma × Stress		$F = 53.8$	$F = 98.5$	$F = 24.6$	$F = 93.4$	$F = 7.1$	$F = 16.3$	$F = 53.3$	$F = 98.6$	$F = 162.5$	$F = 423.2$
		$P < 0.0001$	$P<0.0001$	$P < 0.0001$	$P < 0.0001$	$P < 0.0001$	$P < 0.0001$	$P < 0.0001$	$P < 0.0001$	$P < 0.0001$	$P < 0.0001$

[*] Values presented as the means of five replicates. Different letters indicate statistically significant differences among treatments, while the same letters signify no significant differences between them according to a Tukey's honestly significant difference test ($p < 0.05$).

isolates was insignificant under biotic and abiotic compared to Trichoderma-untreated seedlings.

There is no significant change in sodium content was observed in *S. sclerotiorum*-infected plants while it is significantly accumulated under salinity stress. Furthermore, when salinity was co-applied with *S. sclerotiorum*, Na content significantly increased. The percent accumulation of sodium was 9, 97, and 116% relative to non-stressed plants. On the other hand, a reduction of sodium accumulation was observed when *Trichoderma* isolates applied to the plants treated with salinity and *salinity + S. sclerotiorum,* the reduction was significant, in case of *T. harzianum* but not *T. koningii,* compared to the control (Fig. 7).

The application of *T. koningii* and *T. harzianum* alleviated the reduction of potassium content under stress conditions (Inf, Sal, and Inf+Sal) from (7.6, 5.4, 4.1 mg/g DW) to be (9.5, 7.9, and 5.8 mg/g DW) for *T. koningii* and (10.3, 9.1, and 6.9 mg/g DW) for *T. harzianum* (T8), respectively. The effect of both bio elicitors on increasing potassium under normal conditions was non-significant.

For the reactive oxygen species and membrane biomarkers, the application of single stresses enhanced their production. The effect was severe under combined stress (Fig. 8). In this regard, the accumulation percentages were (36.8, 65.4, and 80.3%) for hydrogen peroxide, (27.7, 62.2, and 91.7 %) for superoxide anion, (153.7, 81, and 147 %) for hydroxyl radical, (18, 50.2, and 73.8%) for MG, and (16.1, 31.1, and 50.2%) for lipid peroxidation under Inf, Sal, and Inf+Sal, respectively compared to control plants. Both *Trichoderma* species significantly attenuated the production of damaging indexes (H_2O_2, $O_2^{\cdot -}$, OH, MG, and lipid peroxidation), and the effect was more prominent for *T. harzianum* than *T. koningii.*

The non-enzymatic antioxidants, GSH and AsA, declined under various stresses but significantly for the combined stress. When *T. harzianum* and *T. koningii* were applied, the contents of GSH and AsA were increased compared to their corresponding level (Fig. 9).

As demonstrated in Fig. 9, the NO content is differentially stimulated by biotic or/and abiotic stresses. In this regard, NO increased significantly with the applied stressors but maximally under combined stress compared to unstressed plants. When *Trichoderma* species were applied, NO was obviously reduced to be (2.8, 3.5, and 3.7) for *T. koningii* or (2.2, 2.5, and 2.3) for *T. harzianum* compared to their corresponding stress (4.2, 3.7, and 4.9) under INF, SAL, and INF+SAL, respectively.

The signaling molecule, H_2S, presented in Fig. 9 salinity or/and *Sclerotia* infection induced non-significant stimulation of H_2S content. While the applied bio elicitors tended to increase the endogenous H_2S content further, and a highly significant effect was recorded under Sal and Inf+Sal.

The phytohormone, SA, was dramatically retarded with a significant effect under Inf+Sal, followed by SAL, and the lowest effect was recorded under *Sclerotia* infection. The utilization of the two selected *Trichoderma* species curtailed the reduction in SA induced by both stresses, where *T. harzianum has* the highest alleviation effect on SA, especially on combined stress (Fig. 9).

Salinity or/and fungal infection differentially affected the secondary metabolic products, as presented in (Fig. 9). Although salinity stress increased the phenolic compounds and Inf+Sal reduced their content, such changes were non-significant compared to non-stressed plants. Bio-elicitor applications enhanced the bio-contents of phenolic compounds relative to their corresponding treatment (Fig. 9). The potential impact of the applied stressors was observed on flavonoids and anthocyanins, where a highly significant reduction was reported compared to the unstressed plants. Compared to the control, the flavonoid contents (85.7, 76, and 70.1%) and anthocyanins were (75.6, 60.8, and 55%) for Inf, Sal, and Inf+Sal, respectively, relative to the control. On the other hand, the protectant effect of both microbes induced by the accumulation of anthocyanins and flavonoids where their values promoted higher their corresponding treatment as well as the control plants (Fig. 9).

The antioxidant enzymes of common bean plants were impacted drastically by the applied stressors, especially the combined stresses

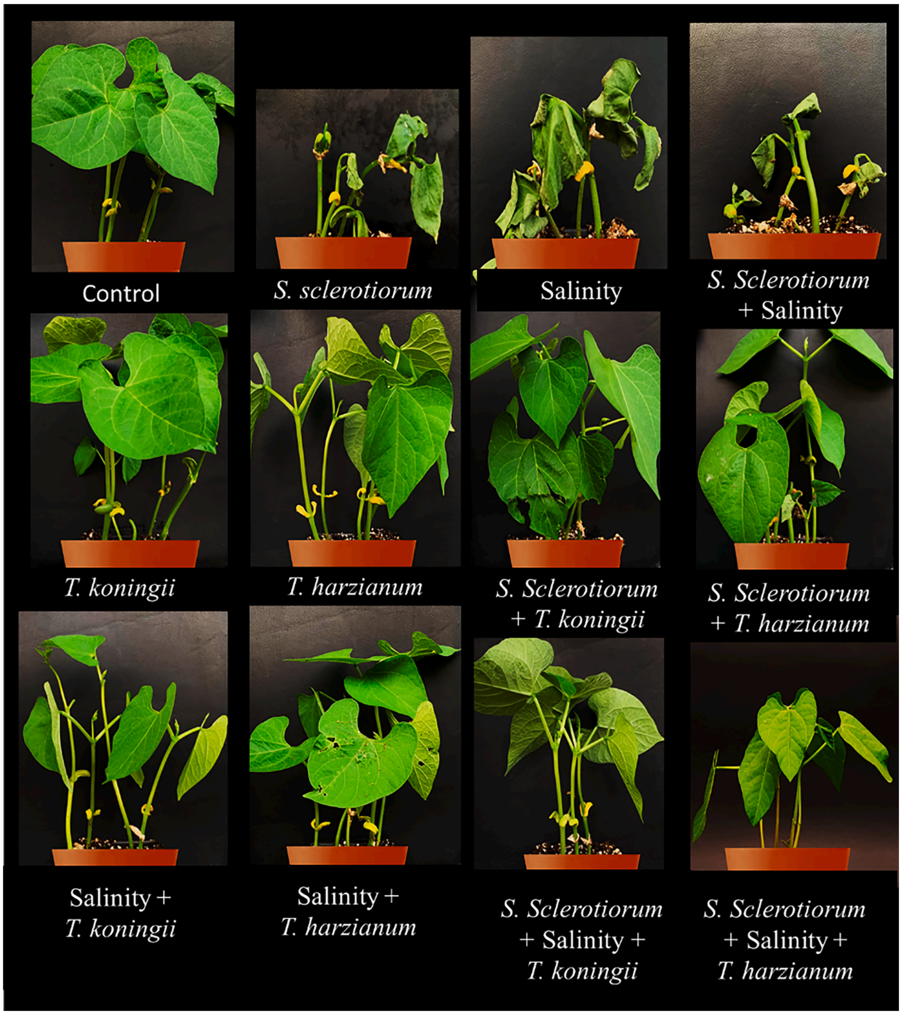

Fig. 6. The effect of *T. harzianum* and *T. koningii* on two weeks old common bean seedling challenged with *S. sclerotiorum* or/and salinity separately o simultaneously.

(Fig. 10). The applied stresses significantly reduced SOD (15, 19, and 29%), CAT (29, 35, and 43%), POD (20, 31, and 49%), APX (20, 33, and 53%) for Inf, Sal, and Inf+Sal, respectively. However, the bio-controlling agents restrained the reduction of their activities and enhanced them to be higher than the control.

The effect of single stresses on PAL and PPO activities was non-significantly changed, but the co-stresses retarded their activities highly significantly. On the other hand, using both microbes enhanced their activities, where the highest effect was recorded for single stress, and all activities were mostly higher than the control. In general, *T. harzianum* and *T. koningii* induced the resistance of common bean seedling against combined stresses introduced by *S. sclerotiorum* and salinity by different ways (Fig. 11).

RT-PCR detection of amylase and proteinase (Prb1) genes

The detection of the amylase and proteinase (Prb1) genes in *T. harzianum* and *T. koningii* involved the amplification of their cDNA using primers designed based on the amylase sequence (accession number XM_024922814.1) and Prb1 (accession number M87518.1). For amylase, one fragment with an approximately 635 bp length was successfully amplified from *T. harzianum* DNA (Fig. 12a), and its specific band was observed in the *T. harzianum* (KC200071). Upon conducting DNA sequencing analysis, the partial amylase gene isolated from *T. harzianum* strain was found to have a length of 635 bp and encodes 206 amino acids. The amplification process of proteinase (Prb1) yielded a single fragment of approximately 440 bp from *T. harzianum* DNA (Fig. 12a). Notably, the specific band appeared only in *T. harzianum* (KC200071). Further analysis through DNA sequencing revealed that the length of the partial proteinase (Prb1) gene isolated from *T. harzianum* strain was 440 bp, which encodes 154 amino acids residues.

Sequence alignment and phylogenetic analysis of the amylase and proteinase (Prb1) genes

Utilizing the computational program Blast p for homology search showed that there exists a significant homology between the amylase from *T. harzianum* isolate and numerous amylase genes in other *T. harzianum* isolates, i.e., *T. harzianum* (XP-024,777,254), *T. harzianum* (PKK54011), *T. harzianum* (PNP53146), and *T. harzianum* (KKO99378). The similarity is higher than 94% (Fig. 12b). The amylase sequence of the *T. harzianum* (KC200071) strain has a GenBank accession number of OQ935355.1. In contrast, the amylase protein sequence of *T. harzianum* (KC200071) strain has a GenBank accession number of WGV33815.1.

Trichoderma strain prb1 exhibited a high degree of homology with several prb1 genes of other *T. harzianum* isolates, *T. harzianum* (PNP44437), *T. harzianum* (XP_024778051), *T. harzianum* (PKK44130), and *T. harzianum* (KKO96730), exceeding 95% (Fig. 12c). The proteinase (Prb1) gene, comprising 440 bp, encodes 154 amino acids. The GenBank accession number assigned to the proteinase (Prb1) sequence of *T. harzianum* (KC200071) strain is OR027044.

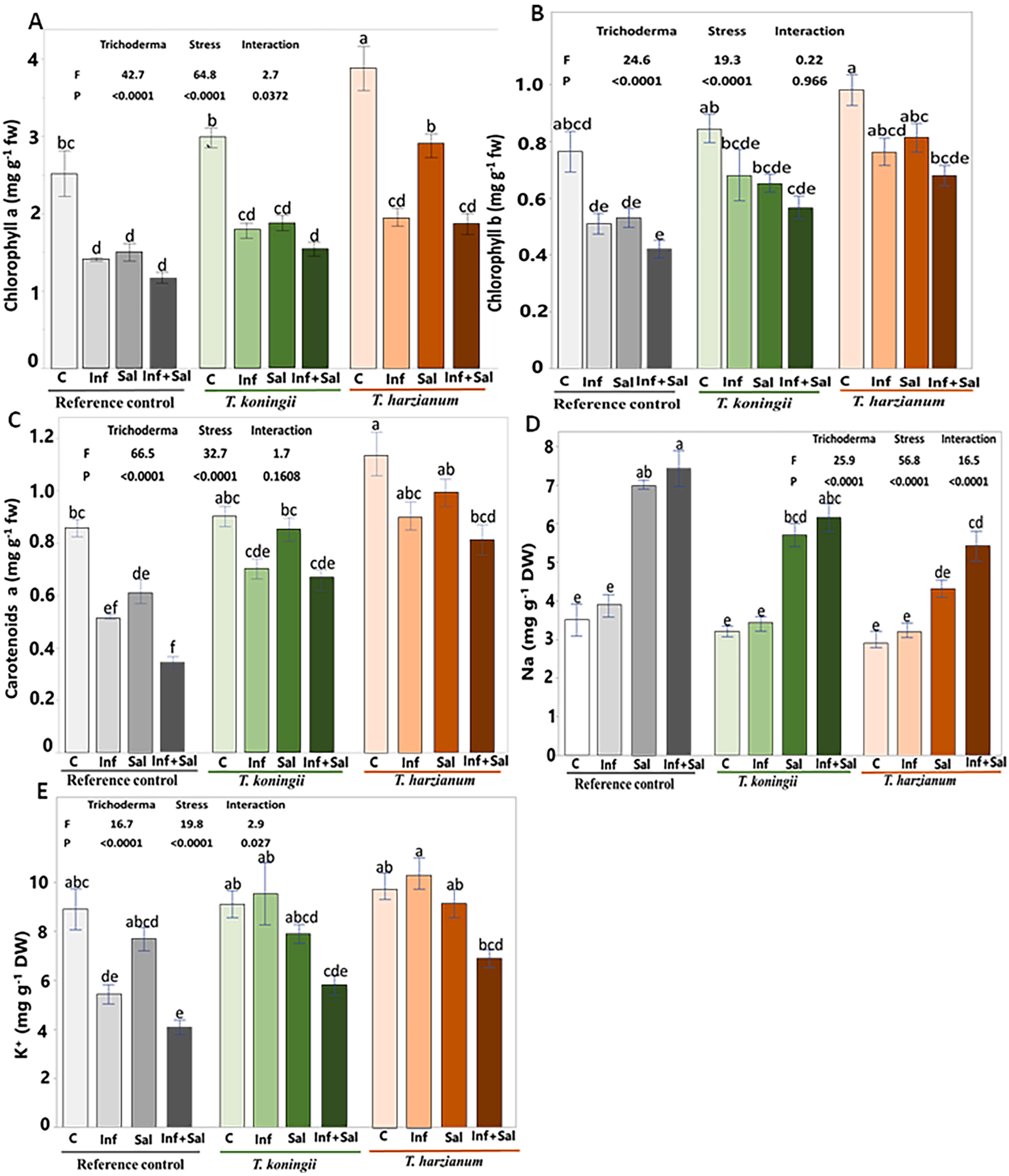

Fig. 7. Effect of *T. harzianum* and *T. koningii* application on common bean content of A) Chlorophyll a, B) Chlorophyll b and C) Carotenoids, D) Sodium (Na) content, E) Potassium (K) content, under infection with *S. sclerotiorum* and salinity stresses. The data presented are the means ± the standard deviation (mean ± SD) of five biological replicates. Different letters indicate statistically significant differences among treatments, while the same letters mean no significant differences between them according to Tukey's honestly significant difference test ($p < 0.05$).

Discussion

Trichoderma, a soil-borne fungus, are active and non-virulent components and plays a key role in anti-phytopathogen employing mechanisms such as antibiosis, induced systematic resistance, mycoparasitism, and competition (Rahman et al., 2023). *Trichoderma* colonizes roots and modulate plant-soil-environment interactions directly or indirectly (Tyśkiewicz et al., 2022). This study investigates the role of *T. harzianum* and *T. koningii* in alleviating the dual stresses of *S. sclerotiorum* as biotic and salinity as abiotic stress on common bean seedlings. High salinity affected the growth and mycelium fresh and dry weight of *S. sclerotiorum* but encouraged sclerotia formation. Previous studies reported that

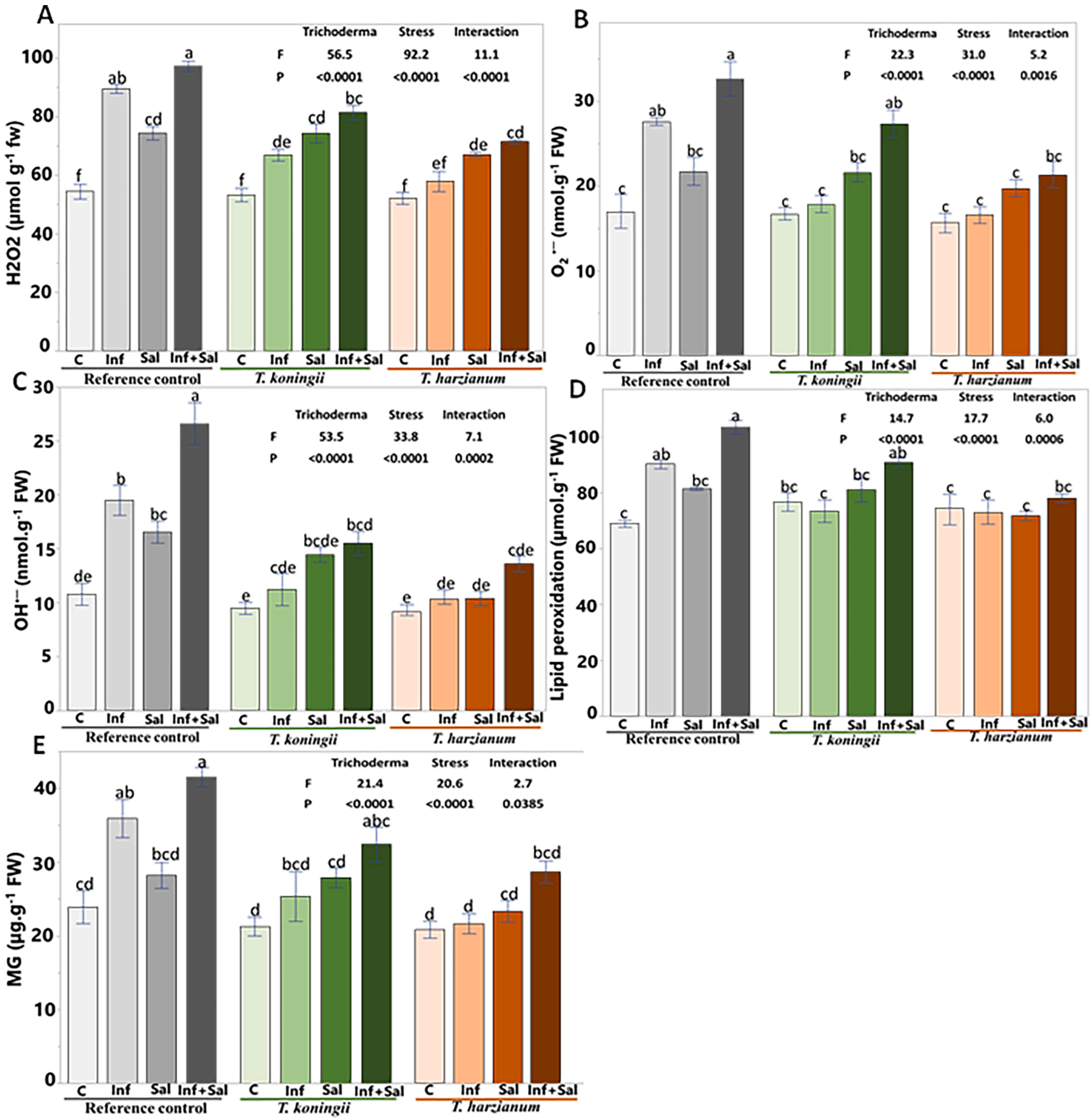

Fig. 8. Effect of *T. harzianum* and *T. koningii* application under salinity and/or *S. sclerotiorum* on common bean contents of hydrogen peroxide (A), superoxide anion (B), hydroxyl radical (C), lipid peroxidation (D), and methylglyoxal (E). The data presented are the means ± the standard deviation (mean ± SD) of five biological replicates. Different letters indicate statistically significant differences among treatments, while the same letters means no significant differences between them according to Tukey's honestly significant difference test ($p < 0.05$).

environmental factors, i.e., salinity, can change the activity and performance of bioagents or pathogens in soil medium (Kamesh Krishnamoorthy et al. 2017; Guo et al., 2018).

Interestingly, neither *T. harzianum* nor *T. koningii* was affected by salinity levels up to 80 mM NaCl, where both isolates showed regular growth and sporulation performance. Furthermore, both *T. harzianum* and *T. koningii* are sufficiently antagonized by *S. sclerotiorum* under the tested levels of NaCl salinity. Such utilization of salinity-tolerant *Trichoderma* species as bio inputs microbial products in triggering plant tolerance could be effective management to various plant biotic and abiotic stresses (Singh et al., 2019; Adusumilli and Kolli, 2022; Cheng et al., 2023). The tolerance of *T. harzianum* and *T. koningii* to salinity stress and antagonizing *S. sclerotiorum* could be accounted for the ability of both *Trichoderma* to secret cell wall degrading enzymes (i.e., chitinase and cellulase), even under high salt concentration, which strengthens their ability to suppress the growth and development of *S. sclerotiorum* in vitro and in vivo. As with other fungi, the cell wall of *S. sclerotiorum* is composed mostly of cellulose (L-1,4-glucan), l-1,3-glucan, l-1,6-glucan, and chitin. Therefore, secretion of hydrolytic exoenzymes, such as cellulase and chitinase, by the tested *Trichoderma* isolates enhances the parasitism and the degradation of the mycelia of phytopathogenic fungi such as *S. sclerotiorum* (Aline et al., 2000; Sood et al., 2020).

The detected genes, proteinase (Prb1) and amylase were added to the armament of *T. harzianum*, which enhanced its antagonistic activity. The gene (prb1) coding for proteinase is a basic hydrolytic enzyme produced by *T. harzianum* (Xiao et al., 2023), hence inducing mycoparasitism and

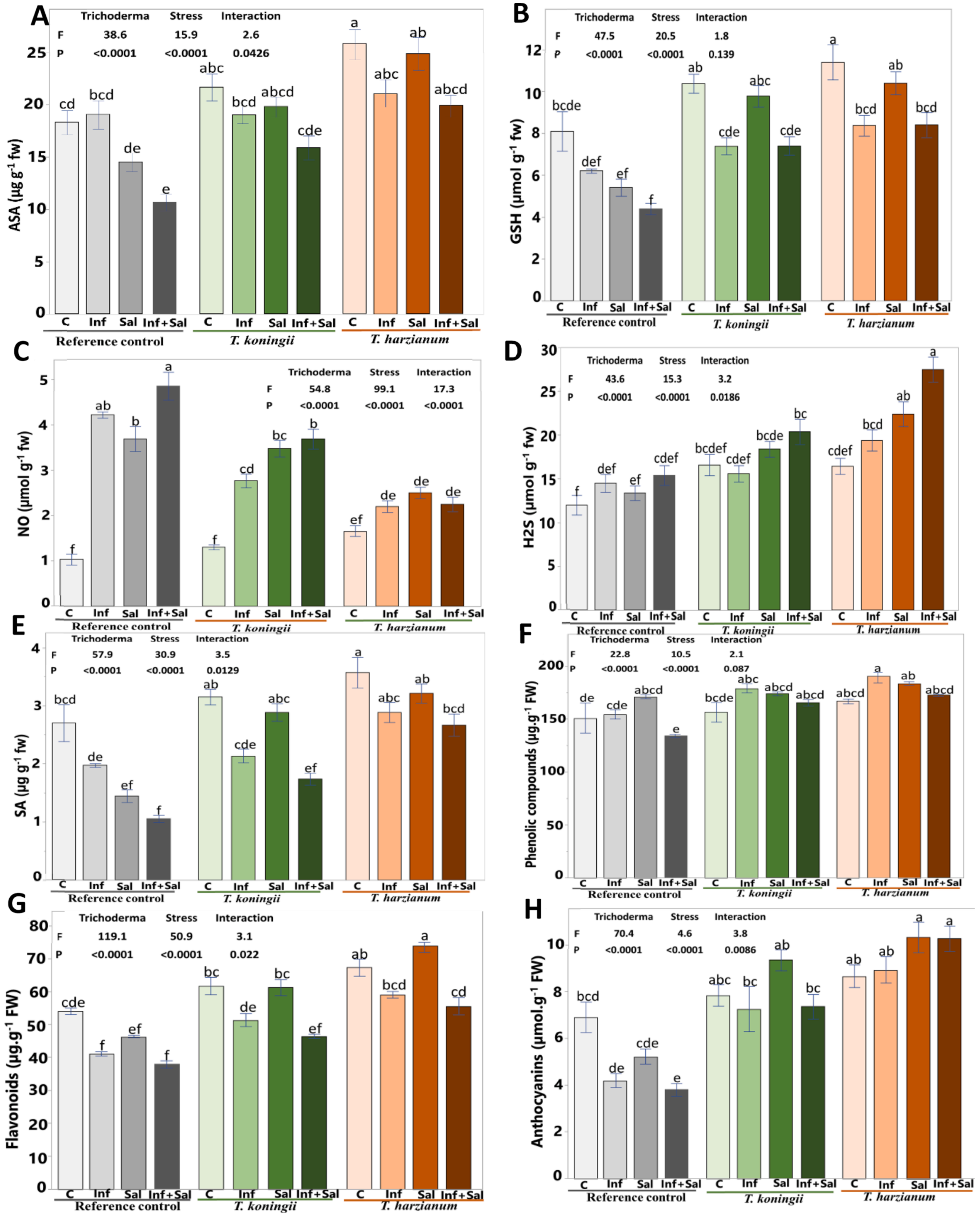

Fig. 9. Effect of *T. harzianum* and *T. koningii* application under salinity and/or *S. sclerotiorum* on common bean contents of ascorbate (A), reduced glutathione (B), nitric oxide (C), hydrogen sulfide (D) salicylic acid (E), phenolic compounds (F), flavonoids (G), and anthocyanins (H). The data presented are the means ± the standard deviation (mean ± SD) of five biological replicates. Different letters indicate statistically significant differences among treatments, while the same letters means no significant differences between them according to Tukey's honestly significant difference test ($p < 0.05$).

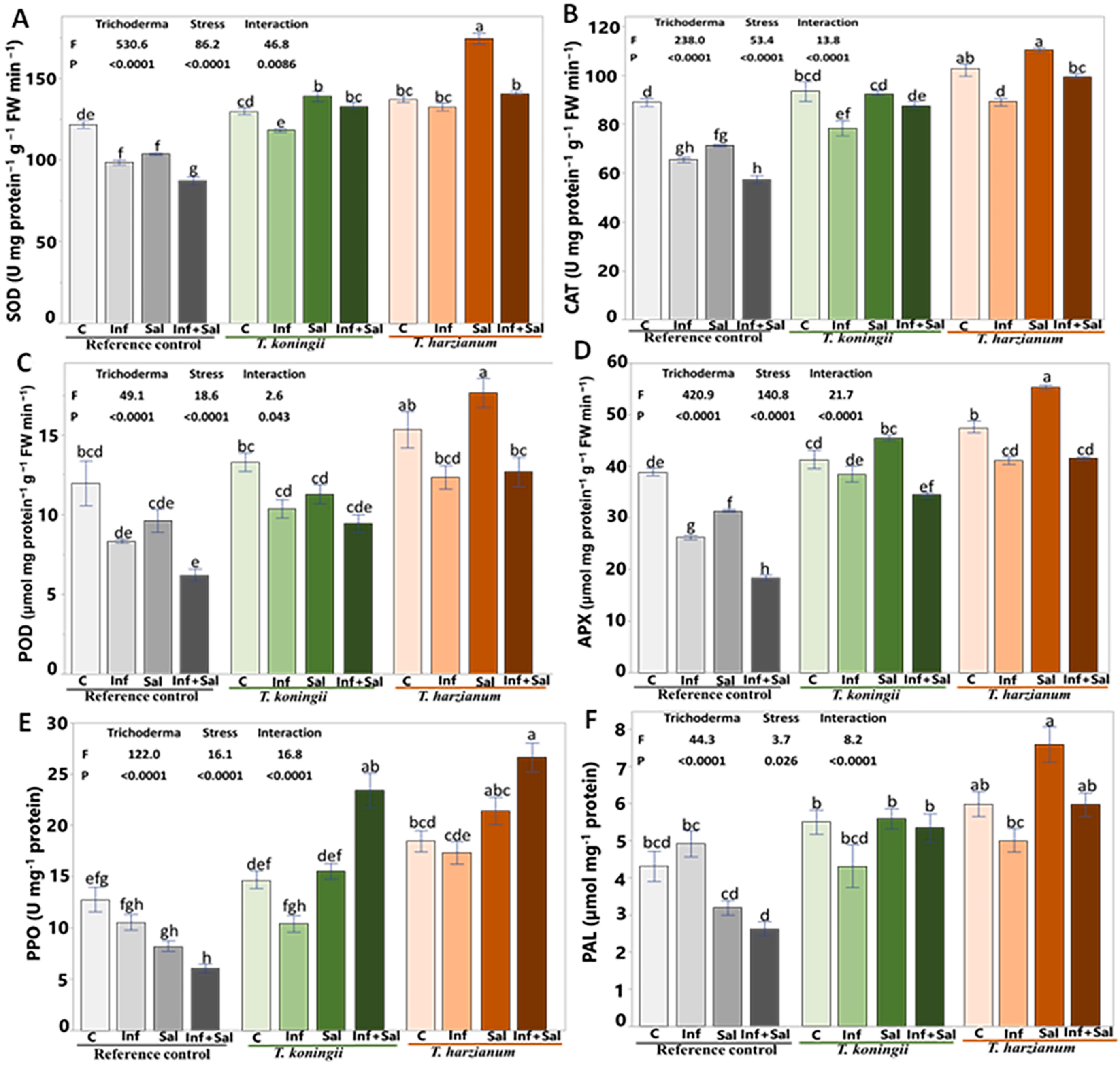

Fig. 10. Effect of *T. harzianum* and *T. koningii* application under salinity and/or *S. sclerotiorum* on common bean antioxidative activity of superoxide dismutase (A), catalase (B), guaiacol peroxide (C), ascorbate peroxidase (D), phenylalanine ammonialyase (E), and polyphenol oxidase (F). The data presented are the means ± the standard deviation (mean ± SD) of five biological replicates. Different letters indicate statistically significant differences among treatments, while the same letters signify no significant differences between them according to Tukey's honestly significant difference test ($p < 0.05$).

antagonistic activity (Flores et al., 1997). Proteinase is one of the important hydrolytic exoenzymes that has a remarkable role in the interaction between *Trichoderma* and fungal phytopathogens and plays a critical role in mycoparasitism (Fan et al., 2014; Dou et al., 2014). Despite its limited mycoparasitism role, amylase mediates hydrolysis of starch or any other readily available related K-glucan to keep *Trichoderma* surviving in the soil (Aline et al., 2000). In addition, Stirling (2018) stated that the release of compounds like polysaccharide-degrading enzymes is a prominent mechanism of antagonism that instigates systemic acquired resistance (SAR) and induced systematic resistance (ISR) in plants. Thus, *T. harzianum* was more powerful due to the presence of amylase and proteinase genes, and these could give higher affinity to highly affect the pathogen in the presence or absence of salinity besides inducing the resistance against salinity stress.

The impact of *T. harzianum* and *T. koningii* in suppressing damping-off and collar/stem rot under high salinity stress has been investigated. The in vivo experiment revealed that damping off and collar/stem rot% of common bean was drastically increased when the seedling was challenged with *S. sclerotiorum* and NaCl (80 mM) simultaneously compared to their single effect. A similar trend was observed on the vigor index of common bean seedlings.

In the present study, the role of both *Trichoderma* isolates in inducing host plant defense mechanisms and resistance was investigated, besides figuring out the damaging impacts of single or combined stresses. In the present study, compared to single salinity or pathogen infection stresses,

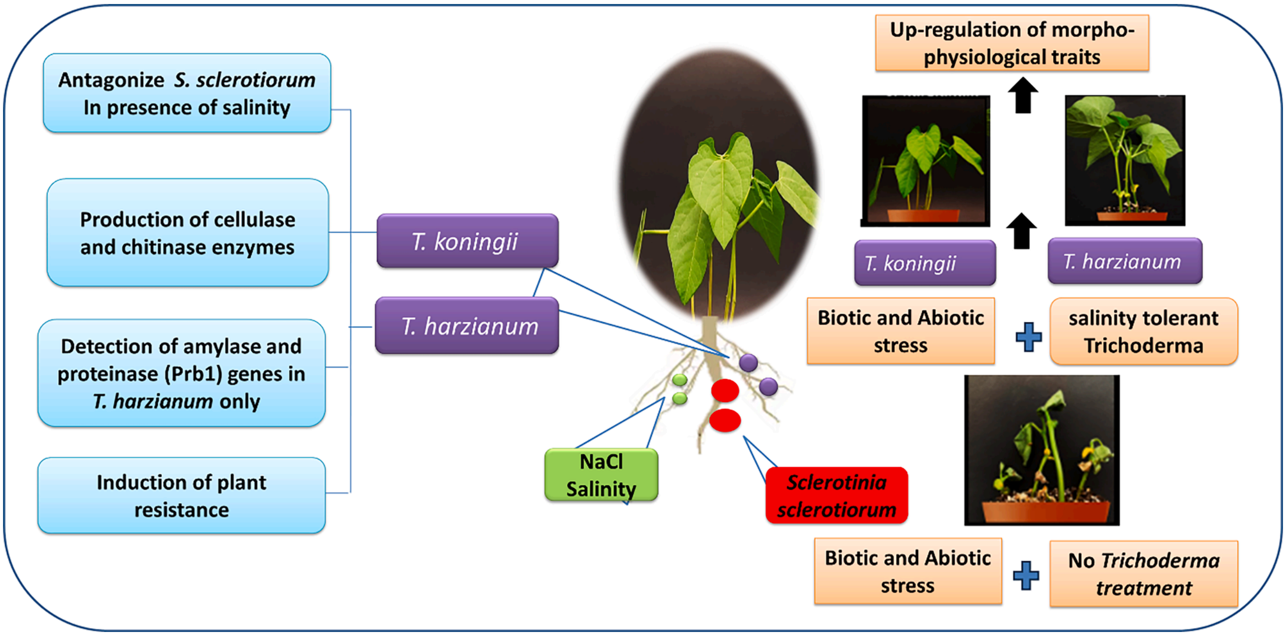

Fig. 11. Different mechanisms of *T. harzianum* and *T. koningii* to enhance plant resistance against biotic (*S. sclerotiorum*) and abiotic (salinity) stresses. .

soil salinization resulted in enhanced susceptibility of common bean to pathogen infection when present in combination. This effect was detected in various physiological processes, such as high retardation of the photosynthetic pigments under combined stress. *lerotiorum*, it results in the reduction of its chlorophyll content. Perveen et al. (2010) recorded a similar reduction of chlorophyll under *S. sclerotiorum* and attributed this impact to the degeneration of the chloroplast due to secretion of the oxalic acid by the Sclerotinia which causes the rupturing of the chloroplast membrane. At the same time, salinization of soil reduced the chlorophyll *b*iosynthesis and chlorophyll-producing enzymes due to increasing Na^+ and Cl^- ions (Ragaey et al., 2022) as well as enhanced chlorophyllase activity due to the accumulation of ROS (Dawood et al., 2021), which deteriorated the pigment–protein complexes (Sofy et al., 2022). Thus, single or combined stresses limited photosynthetic efficiency and restricted the C supply needed by plant cells for normal growth, reducing the fresh and dry weight of the plant's shoot and root, especially under combined stress and, to a lesser extent, single stresses. However, *Trichoderma* restricted the reduction in photosynthesis and induced an increase in the germination% and the vigor index as well as decreasing damping off and collar/stem rot%.

The positive effect of *T. harzianum* or *T. koningii* to combined stresses could be ascribed to high salinity tolerance and stability of cellulase and chitinase activity under high salinity, and the production of proteinase and amylase that has been confirmed during the study, explains the significant impact of both isolates against *S. sclerotiorum*. Similarly, various studies have reported that most *Trichoderma* spp. can produce bioactive substances which antagonistic effects on plant-pathogenic fungi, including secondary metabolites and cell wall-degrading enzymes, can effectively improve crop resistance, reduce plant diseases, and promote plant growth (Druzhinina et al., 2018; Kubicek et al., 2019; Xiao et al., 2023).

In addition to mycoparasitism, *Trichoderma spp*. induced common bean resistance to the single or combined stresses of *S. sclerotiorum* and salinity. *T. harzianum* had the efficacy to enhance K^+ uptake and limited sodium accumulation under salinity or combined stress, which reflected in better membrane status; however, this effect was non-significant for *T. koningii.* In support of our study, Sofy et al. (2022) stated that *T. harzianum* enabled the soybean plants to control Na^+ accumulation and mitigate the negative impacts of salinity on K^+ absorption. On the other hand, high accumulation of Na^+ and reduced K^+ ions, hence ionic imbalance which is generated for salinity-stressed plants and more potentially for combined stress, may create membrane depolarization and plasma membrane disintegration due to shortage of essential minerals (such as K^+, Ca^{2+}, Mg^{2+}, etc.) needed for membrane fluidity.

In soils that experienced *Trichoderma*, herein, they effectively lessened the oxidative damage under the three stresses applied where H_2O_2, $O_2^{\bullet -}$, and $^{\bullet}OH$ and their membrane impairments indexes (MDA and MG) were declined, and the greatest effect was recorded for *Trichoderma* rich in amylase and protein-coding genes, *T. harzianum.* Our results supported the findings of Zhang et al. (2022) that the application of *T. atroviride* significantly increased the content of chlorophyll and soluble protein and reduced the MDA content in cucumber under salt stress or root rot caused by *Fusarium oxysporum* of cucumber. *S. sclerotiorum* or/and salinity stress increase the accumulation of H_2O_2, $O_2^{\bullet -}$, and $^{\bullet}OH$ contents and the magnitude were the highest when both stress co-occurred.

The pathogenesis of *S. sclerotiorum* was associated with the accumulation of ROS (Chaves et al., 2021). However, salinity stress disrupts the electron transport chain leading to oxidative damage and overproduction of ROS in plants (Sheteiwy et al., 2022, 2023). his triggering of ROS enhanced the oxidative reaction to lipids, proteins, and macromolecules. Therefore, high cellular damage from salinity and/or *S. sclerotiorum* infection was depicted on common beans, as indicated by great levels of MG, reactive ketoaldehydes, and MDA, a lipid peroxidation marker. Similar reports on the damaging effect of salinity or *S. sclerotiorum* as single stresses on ROS and the related oxidative damage were reported (Sofy et al., 2022; Dawood et al., 2022b; Zhang et al., 2022; Chaves et al., 2021).

In response to oxidative stress, the plants developed antioxidant systems (enzymes and molecules) to catabolize ROS. The single and compound stresses adversely affect the antioxidant molecules (ascorbic acid and reduced glutathione) and enzymes (SOD, CAT, POD, APX, PPO and PAL), revealing an imbalance in ROS detoxification, especially under the combined stress. Such reduction of antioxidant activity may show over production and accumulation of H_2O_2, and enhanced proteolysis caused by peroxisomal endopeptidases induced by oxidative stress (Shoaib et al., 2018). CAT activity decremented might be resulted from increasing H_2O_2 content and disturbance in redox balance with deactivation of catalase due to prevention of new enzyme synthesis at high salinity stress (Yamane et al., 2010). Disturbance in plant physiology

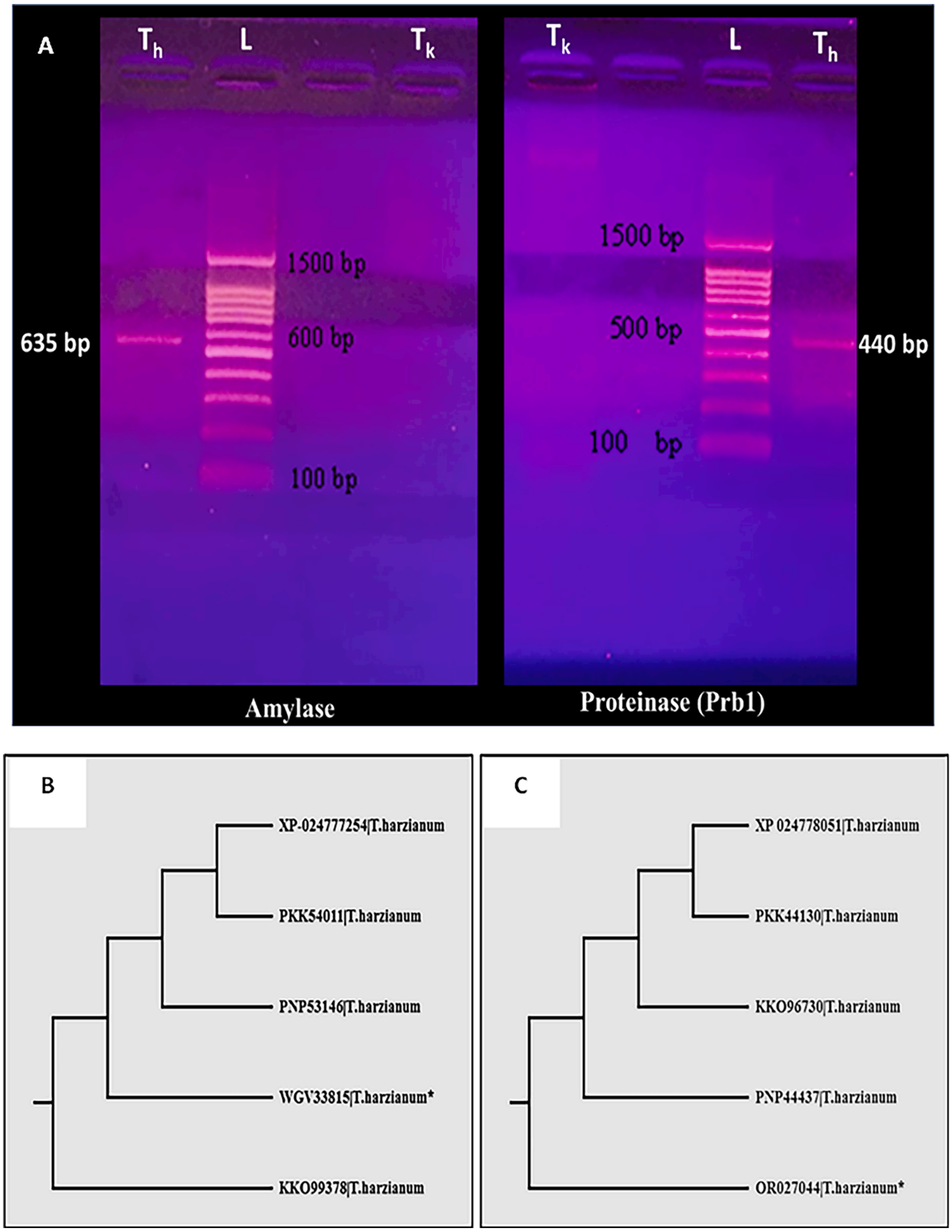

Fig. 12. A) Agarose gel analysis of specific-PCR products from amplification of amylase and proteinase (Prb1) genes of *T. harzianum* isolate (T_h), *T. koningii* (T_k), 100 bp DNA Ladder (L). *T. harzianum* tested for amylase showed a band size of 635pb. No band was detected for *T. koningii. T. harzianum* tested for proteinase (Prb1) showed a band size of 440pb. No band was detected for *T. koningii.* B). A rooted phylogenetic tree (UPGMA) was generated using utilizing MEGA X programs to demonstrate the relationship between A) the amylase gene originating from various *Trichoderma* strains and C) the proteinase (Prb1) gene sourced from various *Trichoderma* strains. The tree adheres to the UPGMA approach and is prominently displayed for further academic inquiry.

successfully establishes the consequences of the pathogens or salinity inside the host and the weakening of the plant defense system.

The induced systemic resistance triggered by *Trichoderma* can induce host plants to produce defense responses. In this sense, *Trichoderma*-treated plants restrained the reduction of antioxidant enzymes/molecules under salinity or/and *S. sclerotiorum* infection, where the effect of both bio-elicitors stimulated the activity of SOD, CAT, POD, APX, PAL, and PPO to be higher than non-stressed plants besides the accumulation of reduced glutathione and ascorbic acid. This could be the main factor in maintaining the ROS lower than their corresponding stressed plants, especially that of combined stress. The less cellular damage caused by salinity or/and *S. sclerotiorum* infection for Trichoderma-treated plants is possibly due to the lower fungal colonization and attenuation of the impacts of salinity on common bean tissues. In this regard, Singh et al. (2014) demonstrated that tomato plants treated with *Trichoderma* obtained tolerance toward root rot by up-regulating the activities of POD, polyphenol oxidase, and phenylalanine ammonialyase, by increasing the content of total phenols. These results suggest that metabolites released by *Trichoderma* isolates not only act directly on the pathogen but also trigger the plant to release defense-related compounds. Also, the inoculation of wheat plants with T. longibrachiatum under salinity stimulated the activities and gene expression of SOD, CAT, and POD (Zhang et al., 2016). Abdelrhim et al. (2023) denoted the role of *Trichoderma* in the stimulation of antioxidant enzymes which reduced ROS and lipid peroxidation of coriander against *Rhizoctonia solani.* Thus, *Trichoderma* modulated the antioxidant system as induction of common beans resistance against pathogen infection and growth promoting against salt stress.

Secondary metabolites were reported numerously to be involved in ROS-catabolism as antioxidants under abiotic/biotic stresses (Dawood et al., 2022a,b; Sheteiwey et al., 2022; Abdelrahim et al., 2021). Although total phenolics were increased under *S. sclerotiorum*, it seemed that its level was not enough to provide resistance against the invading pathogen. Salinity stress reduced the phenolic content, and the situation became worthy under combined stress which is the concomitant susceptibility of common bean to salinity and fungal infection. However, *Trichoderma* application reduced the negative impacts of pathogen infection by increasing phenolic compounds besides other regulations where phenolics are among the main compounds produced via the phenylpropanoid pathway during the cell wall modification process and are effective antioxidants in plants (Bagy et al., 2019). The increased levels of phenolics may provide an adequate substrate for oxidative reactions catalyzed by peroxidase, which results in lignification and makes the plant cells unfavorable for further pathogen progress (Abdelrhim et al., 2021). Phenolics may act as soluble antimicrobial compounds or cross-link with callose and proteins into cell walls, inhibiting pathogens' penetration and absorption of nutrients (Abdelrhim et al., 2023). It has been reported that the attenuation of PAL activity may result in high content of some phenolic components, which impedes flavonoid biosynthesis (Shoaib et al., 2018). In the same par, salinity or fungal infection reduced the flavonoids and anthocyanin content, while their content was alleviated in the presence of *Trichoderma* concomitant to PAL activation. Trichoderma's role in increasing phenolics' contents under salinity stress/pathogen infection was also observed (Sofy et al., 2022; Abdelrhim et al., 2023).

As a signaling molecule, NO has various regulatory roles in plants when present at certain levels. But the cytotoxic levels of salinity or *S. sclerotiorum* generated a burst of ROS as well as reactive nitrogen species, NO, that exceeded the plants' manageable threshold limit, causing nitrosative stress that damages the cellular structures and membranes (Dawood and Azooz, 2019, 2020). However, at higher concentrations, NO negatively affects chlorophyll and photosynthesis (Antoniou et al., 2013). Also, fungal infection is potentially associated with NO generation and metabolism during plant–fungal pathogen interactions (Cánovas et al., 2016). The combined stress of biotic and abiotic stress received the most damaging impacts of ROS, NO, and their consequences on common bean plants.

On the other hand, *Trichoderma*, up-regulated the level of NO under stress conditions and lowered extensively relative to the corresponding stressed plants. Such up-regulation of NO may be implicated in establishing the symbiotic association between plants and beneficial microbes to enhance plant growth and development (Khan et al., 2023a). This correlated with the ability of NO to induce SAR, which prepares the plant to respond more potentially to microbial infection (Khan et al., 2023b). SA augmented the defense against several pathogens and facilitates plants' tolerance to abiotic stress. However, the reduction of SA in the fungal-infected or salinity-stressed plants and highly significant when co-existed could be associated with the negative impacts on PAL activity, the enzyme involved in SA production as well as attenuation of defense-related molecules of common bean. This revealed the susceptibility of common plants to single or combined stress and maybe various changes to environmental conditions.

In the presence of the tested *Trichoderma* isolates, the immune system of common bean plants was mitigated, where up-regulation of SA was denoted. Jimenez-Perez et al. (2022) stated that SA up-regulates pathogen defense and stimulates the systemic acquired resistance in connection with the signaling molecule, NO. In this regard, the functions of both signaling molecules are interlinked to determine plant development. SA induces the scavenging of NO and its related compounds, while NO enhances the SA level, hence reducing NO-mediated oxidative stress (Mehmood et al., 2021). Furthermore, the SA-ROS plays a key role in transcriptional reprogramming during the plant defense responses (Abdelrhim et al., 2021, 2022).

The signaling molecule, H_2S, accumulates in plants under various stress. Also, the detection of higher endogenous H_2S levels in stressed plants treated with *T. koningii* and *T. harzianum* compared to their corresponding stress plants supported the role of this defense-related signaling molecule in resistance of common bean plants to salinity or/and *S. sclerotiorum*. Similar to NO, H_2S is efficient ROS scavengers and act either directly or indirectly by strengthening the antioxidant system by increasing the levels of antioxidant enzymes (SOD, APX, and GR), GSH content, and the metabolism of ROS ($^{\bullet}OH$, H_2O_2, and $O_2^{\bullet-}$), and reducing membrane lipid peroxidation by regulating intercellular responses (Corpas et al., 2019). Thus, Trichoderma's instigation of SA and hydrogen sulfide could incite tolerance against fungal infection and/or salinity via mediated NO functionality. The mechanism of *T. harzianum* and *T. koningii* on alleviation of the damaging impact of salinity and *S. sclerotiorum* of common bean was diagrammed in supplemented Fig. 1.

Conclusion

The role of two salinity-tolerant *Trichoderma* isolates, *T. harzianum* and *T. koningii*, have been investigated in alleviating the dual stress, *S. sclerotiorum* and salinity on common bean seedlings. The in vitro study showed high tolerance of *T. harzianum* and *T. koningii* to salinity which both isolates could grow and sporulate on saline media. Both isolates showed high antagonism against *S. sclerotiorum*, and produced highly active cell wall lytic enzymes, chitinase, and cellulose, under high salinity stress. However, *T. harzianum* was more powerful due to the presence of amylase and proteinase (Prb1) genes; these could give higher affinity to highly affect the pathogen in the presence or absence of salinity besides inducing the resistance against salinity stress. Thus, *T. harzianum* is more beneficial than *T. koningii* in the up-regulation of various physiological processes, especially for combined stress. The greenhouse experiment confirmed the successful role of *T. harzianum* and *T. koningii* in suppressing the stress of *S. sclerotiorum* and salinity and improving common bean seedlings' physiological traits compared to Trichoderma-untreated seedlings. Both *Trichoderma* isolates induced the resistance of stressed common bean seedlings by stimulating photosynthetic pigment, salicylic acid, hydrogen sulfide secondary metabolites, and antioxidants which aided in restricting oxidative and nitrosative stresses and membrane damage criteria.

Prospects

Although substantial studies investigated the impacts of *Trichoderma spp*. on single stresses of salt or fungal infection by *S. sclerotiorum* of common bean, many questions still need to be answered. We still have much to learn about how combined stresses and biological control agents interact.

CRediT authorship contribution statement

Abdelrazek S. Abdelrhim: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. **Nada F. Hemeda:** . **Mai Ali Mwaheb:** Data curation, Methodology, Validation, Writing – review & editing. **Maha O.A. Omar:** Data curation, Formal analysis, Investigation, Methodology, Project administration. **Mona F.A. Dawood:** Conceptualization, Investigation, Methodology, Project administration, Software, Writing – original draft, Writing – review & editing.

Declaration of competing interest

Abdelrazek S. Abdelrhim, Nada F. Hemeda , Mai Ali Mwaheb, Maha O. A. Omar, Mona F. A. Dawood declare that there was no conflict of interest from the authors in the research article entitled "**The role of**

***Trichoderma koningii* and *Trichoderma harzianum* in mitigating the combined stresses motivated by *Sclerotinia sclerotiorum* and salinity in common bean (*Phaseolus vulgaris*)”.**

Data availability

Data will be made available on request.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.stress.2024.100370.

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