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The Effect of *Trichoderma citrinoviride* Treatment under Salinity Combined to *Rhizoctonia solani* Infection in Strawberry (Fragaria x ananassa Duch.)

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Abstract: *Trihoderma citrinoviride* protects plants from diseases by functioning as antagonists of many pathogenic fungi or by triggering the antioxidant defense system in plants. In the present study, to uncover the possible alleviative role of *Trichoderma* against salinity and *Rhizoctonia solani* infection, strawberry plants were pretreated *Trichoderma citrinoviride* and then subjected to salinity, *R. solani* and combined salinity and *R. solani*. The effect of *T. citrinoviride* on the alleviation of the effects of salt stress and *Rhizoctonia solani* infection was investigated by analysing leaf dry weight, PSII efficiency, and the activity of some antioxidant enzymes in the leaves of strawberry plants. *T. citrinoviride* improved competitive capability against salinity and *R. solani* infection. It showed 79% inhibition of the growth of pathogen *R. solani*. *T. citrinoviride* reduced 63% of the severity of disease in the leaves. *Trichoderma* pretreatment maximized plant dry weight. The *T. citrinoviride*-pretreated plants showed higher levels of PSII efficiency (Fv/Fm). Decreased lipid peroxidation and H_2O_2 accumulation compared to untreated seedlings under salt stress and *R. solani* infection was observed. *Trichoderma*-pretreated and –untreated plants respond differently to salt stress and *R. solani* infection by means of antioxidant defense. As compared to untreated seedlings, treated seedlings showed significantly lower activities of antioxidant enzymes, superoxide dismutase (SOD), peroxidase (POX), cell wall peroxidase (CWPOX) under salt stress and *R. solani* infection, indicating that treated seedlings might sense lower stress as compared to untreated seedlings. The study reports the effective adaptive strategy and potential of *T. citrinoviride* in alleviating the negative impact of salt stress and *R. solani* infection in strawberry.

Keywords: antioxidant enzymes; biotic stress; *Rhizoctonia solani*; salinity; strawberry; *Trichoderma*

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1. Introduction

Plants live in a complex and ever-changing environment, where they constantly interact with biotic factors (herbivores and microbial pathogens) and abiotic factors (salinity, drought, high and low temperature, etc.) [1]. These factors negatively affect plant growth and development, which cause oxidative stress, leading to crop loss. Soil-borne pathogens are the major sources of biotic stress, and they increase the severity of this negative impact along with other stressors. When plants are exposed to a pathogen attack, parallel to biochemical and molecular responses, they mechanically strengthen their tissues in order to restrict the pathogen propagation [2,3]. In addition, cell wall reinforcement is stimulated by lignin and callose deposition in plants as a plant defense response and resistance against fungal pathogens such as *Rhizoctonia* [4]. Moreover, peroxidase, chitinase, and lignin formation are some of the defense mechanisms in the protection of tomatoes and rice against *Rhizoctonia* [5]. *Rhizoctonia* spp. is one of the most destructive soil-borne pathogens

and causes significant losses in agricultural crops such as strawberry [6], tomato [7], corn [8], and potato [9]. The pathogen is difficult to control due to soil origin and ecological behavior, high survival rate of sclerotin in soil under harsh environmental conditions, and extremely wide host range [10]. Therefore, currently, it is a worldwide demand to find sustainable solution to the black root rot diseases caused by *Rhizoctonia* caused decrease in crop yield [11].

Oxidative stress is caused not only by biotic stress but also by abiotic stress. Therefore, combination of both stresses may act synergistically and affect plant growth and crop yield to a higher extent. Their combined effects can reduce the average yield to even less than 50% in an agricultural production [12]. Moreover, Nath et al. [13] showed that the presence of abiotic stresses such as salinity, temperature, cold, pH, and drought significantly alter the susceptibility of the plant to biotic stress. Salinity is one of the most harmful abiotic stresses which causes excessive intake of sodium (Na^+) and chloride (Cl^-) ions, leading to perturbation in plant metabolism. Additionally, oxidative stress occurs due to the generation of Reactive Oxygen Species (ROS) [14]. The formation of ROS; superoxide anion ($O_2^{\cdot -}$), singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2), and hydroxyl (HO.) cause severe damage to the plant cells [15]. ROS molecules, which are highly toxic and reactive, cause cell death by causing damaging proteins, carbohydrates, lipids, and DNA [16]. Plants have evolved various mechanisms to protect themselves from these toxic molecules. One of these mechanisms is the antioxidant defense system; ROS-scavenging enzymatic antioxidants such as superoxide dismutase (SOD), catalase (CAT), peroxidase (POX), cell wall peroxidase (CWPOX), ascorbate peroxidase (APX), and glutathione reductase (GR) are scavenged by non-enzymatic low molecular metabolites such as ascorbate (ASH), reduced glutathione (GSH), α-tocopherol, carotenoids, and flavonoids [16]. A vast amount of studies indicated that these molecules are produced during metabolic processes in different compartments of the cell and when they are produced at excess levels during stress, the metabolic balance is disturbed. If they are not scavenged efficiently by antioxidant enzymes, impaired metabolic balance occurs within plant cells.

Recent studies increase our limited knowledge of the molecular principles and mechanisms underlying plant-microbe-environment interactions [17]. For instance, the positive effects of the soil-borne fungus *Trichoderma* [18], which colonize in the roots of many plants as opportunistic, harmless plant symbiotes and do not show pathogenic effects, have been identified recently. The use of *Trichoderma*, a bio-control agent, is increasing day by day and is the subject of many studies in order to prevent the intensive use of chemical drugs (pollutes the atmosphere, is harmful to the environment, has remaining residues, and is not economical [19] in the fight against plant diseases. *Trichoderma* spp. is among the most effective components of the rhizosphere in terms of both plant growth and health [20]. *Trichoderma* species are also very effective microorganisms in suppressing plant diseases with biological warfare mechanisms such as hyperparasitism, antibiosis, and competition. The most common types of *Trichoderma* include T. *harzianum*, *T. coningii*, *T. atroviride*, *T. reesei*, *T. viride* and *T. ghanense* [21]. Many of these species have been reported to be effective biocontrol agents against soil-borne pathogens, including *Rhizoctonia,* by exhibiting various forms of biological action to control plant pathogens [22].

So far, little is known about how biocontrol agents act to protect host cells and suppress excessive ROS production in plants under biotic stress [23]. Chowdappa et al. [24] and Kumar et al. [25] showed that oxidative stress was regulated in infected plants through the increase of antioxidant enzymes such as POX, CAT, and SOD in their studies with different Tricoderma species (*T. harzianum* and *T. virens*). Similar results have also been reported in *T. harzianum* by Youssef et al. [11] and in *T. atroviride* by Nawrocka et al. [26]. However, there is limited information about the effect of *T. citrinoviride* under stress conditions. The study by Yesilyurt et al. [27] is the only study examining the role of *T. citrinoviride* on antioxidant enzymes under salt stress in maize.

Strawberry is among the most popular fruits all over the world due to its delicious taste and high content of sugars, vitamins, minerals and carotenoids, as well as ascorbic

acid (Asc), phenolic compounds and other antioxidants that are beneficial for health [28]. The susceptibility of commercial strawberry cultivar to salt stress has been demonstrated by many studies [29,30]. Strawberry, which has a large cultivation area, naturally has many diseases and pests. *Rhizoctonia*, which causes black root rot disease, also affects strawberry plants. Although there are reports regarding the sole effects of both stresses, they are to date rare and limited. Additionally, to the best of our knowledge, there is limited information about how the interactions between plant and *T. citrinoviride* affect host response to a combination of abiotic and biotic stress or how aspects of the abiotic and biotic environment affect this plant-*T. citrinoviride* interactions. In order to address these, in the present study, the possible effect of *Trichoderma citrinoviride* on disease suppression, plant growth and antioxidant status of strawberry (Fragaria × ananassa Duch.) under combination of salinity and *Rhizoctonia solani* infection were investigated. With this aim, we determined plant growth, severity of disease, Fv/Fm ratio, H_2O_2 content, lipid peroxidation, NADPH oxidase enzyme activity and some of antioxidant enzyme activities (superoxide dismutase (SOD), peroxidase (POX), cell wall depend peroxidase (CWPOX)).

2. Materials and Methods

2.1. Trichoderma Citrinoviride Isolation and Identification

T. citrinoviride used in this study was provided from The Culture Collection Unit of the Phytopathology subdivision at Ege University, Turkey. *Trichoderma* spp. was grown on potato dextrose agar (PDA). Plates were incubated at 23 °C for 1 week. Phenotypic characterizations of isolate were performed using various culture techniques such as green conidia formation, pigments, and colony appearance. Slide examination of fungal growth and conidiophores formation were performed under light microscope at the Fruit and Vine Fungal Disease Lab. in Bornova Plant Protection Research Institute. Phenotypic identification was determined according to Gams and Bissett [31]. After phenotypic identification, fungal culture was further verified by molecular methods using the primers described by White et al. [32]. ITS1 (5′-TCC GTA GGT GAA CCT GCG G-3′), ITS4 (5′-TCC TCC GCT TAT TGA TAT GC-3′) (White et al., 1990) and EF1 forw. (5′-ATGGGTAAGGAGGACAAGAC-3′), TEF1a rev. (3′-GCCATCCTTGGAGATACCAGC-5′). The amplification program carried out an initial denaturation at 94 °C for 3 min followed by 30 cycles of denaturation for 1 min at 95 °C, annealing for 1 min at 60 °C and extension for 1 min at 72 °C and a final elongation step of 10 min at 72 °C. PCR product was separated in 3% agarose gels in 1X TAE buffer at 75 V for 40 min, stained with ethidium bromide and visualized under UV light.

Sequencing of PCR: To determine the complete sequences of ITS1 and ITS4, they were sequenced with ABI prism automated DNA sequences. These sequences were used to identify the fungi with help of the BLAST program (www.ncbi.nih.gov/BLAST, accessed on 25 June 2021), and multiple sequence alignments were determined with the Clustal W program. Amplification of DNA samples with primer pairs ITS and tef1 was compared with the Ky764860.1 and Hg93, showing 98.1% and 99.7% similarity, respectively.

2.2. Rhizoctonia Solani Isolation and Identification

The isolate of *R. solani* were isolated from the roots of diseased plants of strawberry. The infected strawberry roots were surface sterilized in 1% sodium hypochlorite solution for 3 min, washed for 1 min two times with sterilized distilled water, and dried between two sterilized filter papers. The sterilized root fragments were transferred to PDA medium and were incubated at 25 °C for seven days. The mycelia growth was taken and transferred onto new PDA medium. Phenotypic characterizations of developed isolate were performed.

2.3. Bioassay Analysis

2.3.1. Bioassay of Trichoderma Isolate against *R. solani*

T. citrinoviride and *R. solani* species were enlarged on PDA medium for antagonistic activity. *T. citrinoviride* and *R. solani* strains were tested in vitro using 85 mm petri dishes containing 20 mL of PDA medium with pH 5.5 for antagonistic activity [33]. Mycelial discs

(5 mm in diameter) of *T. citrinoviride*, was placed on one edge of a petri dish containing PDA, while same size of *R. solani* was also placed at the periphery but on the opposing end of the same Petri dish. *R. solani* was enlarged at the edge of the plate for the control group. Antagonistic activity was tested 6 days after incubation. Four petri dishes per treatment were used, and they incubated at 28 °C and the percent inhibition of radial growth (PIRG) was recorded. PIRG was defined using the equation indicated below for all cultures that were measured [34].

$$\text{Percentage Inhibition of Radial Growth} = [(R1 - R2)/R1] \times 100 \text{ (R1 = Control colony of radius,}$$

$$\text{R2 = } \textit{Trichoderma}\text{-treated colony radius)}$$

2.3.2. Bioassay of Trichoderma Isolate against Salinity

Forty mM NaCl were added into petri dishes that each contained sterilized PDA. Petri dishes were sealed with parafilm and incubated in the dark at 25 °C for 4–7 days until the growth in the control plates reached the edge of the plates. The plates were then assessed by measuring the distances fungal cultures.

2.4. Plant Material and Treatments

Strawberry (Fragaria x ananassa Duch. cv. 'Rubigen') plants were obtained from Ege University, Faculty of Agriculture, Bornova, İzmir, Turkey. Three week old seedlings were planted into pots (16 cm × 14 cm) filled with torf + perlite + vermiculite mixture (7:2:1) and grown under natural day/night light conditions of the greenhouse. Pots were arranged in a completely randomized block design with three replicates for each treatment and four pots per replicate. Seedlings were watered regularly with the half-strength Hoagland solution and stress treatments were started when plants were 3 weeks old. Plants were divided into eight different treatment groups as follows; (1) control, (2) 40 mM NaCl, (3) *R. solani* infected, (4) 40 mM NaCl + *R. solani* infected, (5) *T. citrinoviride* pretreated, (6) *T. citrinoviride* pretreated + 40 mM NaCl, (7) *T. citrinoviride* + *R. solani*, (8) *T. citrinoviride* + NaCl+ *R. solani*. Strawberry plants were dip into solution of 2×10^{-6} cfu/mL spots suspension of *T. citrinoviride*. After *T. citrinoviride* inoculation, these seedlings were grown for two weeks. After two weeks, while salt treatment started by adding 40 mM NaCl into Hoagland solution, *R.solani* pathogen was inoculated with PDA pieces. For *R. solani* inoculation, 80 plugs (5 mm diameter) from 8 day old PDA cultured of *R. solani* containing abundant mycelium and sclerotia were mixed well in 1 cm of the soil surface and watered. Plant growth and disease incidence were taken on the 14th day of stress treatments. Disease incidence was estimated by both visual assessments of the plants and re-isolation of *R. solani* from the diseased root of the plants. Fully expanded leaves were sampled after 14 days for biochemical analyses, frozen in liquid nitrogen and stored at −80 °C until further analyses. For each treatment, at least 12 replicate plants were inoculated in a completely randomized experimental design and all experiments were repeated.

$$\text{Disesase Incidence (\%)} = [(\text{Number of infected plants/Total number of plants}) \times 100]$$

2.5. Plant Growth

Leaf samples were taken from each and was used for dry weight determination, and their dry weights were measured after samples were dried 70 °C for 72 h.

2.6. Fv/Fm (Maximal Efficiency of PSII Photochemistry)

The measurements were conducted after day 14 of salt stress and *R. solani* infection exposure. Leaves were dark-adapted for 30 min before the measurements. The maximum quantum efficiency of PSII readings (Fv/Fm (maximal efficiency of PSII photochemistry)) measured in second developed apical leaf and they were recorded by Plant Efficiency Analyser, P-Sensor type (Hansatech Fluorometer, Hansatech Instrument Ltd., Norfolk, UK)

2.7. Lipid Peroxidation (TBARS) Content

The level of lipid peroxidation in samples was done according to the method of Madhava Rao and Sresty [35], which determined the content of thiobarbituric acid reactive substances (TBARS). TBARS content was calculated from the absorbance at 532 nm and measurements were corrected for

non-specific turbidity by subtracting the absorbance at 600 nm. The concentration of TBARS was calculated using an extinction coefficient of 155 $mM^{-1}cm^{-1}$.

2.8. Determination of H_2O_2 Content

The ferrous-xylenol orange (eFOX) assay was used to measure H_2O_2 [36] using eFOX reagent. In this assay, 1% ethanol is added to the reagent, which increases its sensitivity to H_2O_2 by 50% (eFOX). Samples were homogenized in ice-cold acetone, containing 25 mM H_2SO_4. Then, homogenates were centrifuged for 5 min at 3000× *g* at 4 °C. For 50 µL of supernatant, 950 µL eFOX reagent (250 µM ferrous ammonium sulphate, 100 µM xylenol orange, 100 µM sorbitol, 1% ethanol, *v*/*v*) was used. Reaction mixtures were incubated at room temperature for 30 min. The absorbance differences of the mixture at 550 and 800 nm were measured. The H_2O_2 concentration was calculated by using a standard curve with known H_2O_2 concentrations.

2.9. Enzyme Extractions and Assays

All assays were performed at 4 °C. Then, 0.1 g of the samples was grounded to fine powder by liquid nitrogen and homogenized in 500 µL of 50 mM Tris-HCl, pH 7.8, containing 0.1 mM ethylenediaminetetraacetic acid (EDTA), 0.1% (*w*/*v*) Triton-X100, 1 mM phenylmethanesulfonyl fluoride (PMSF), and polyvinylpyrrolidone (PVP; 1%, *w*/*v*). The homogenate was centrifuged at 14,000× *g* for 10 min at 4 °C. Supernatants were used for the determination of protein content and enzyme activities. Total soluble protein contents of the enzyme extracts were determined according to Bradford [37], using bovine serum albumin (BSA) as a standard. All spectrophotometric analyses were conducted on a Shimadzu UV 1700 spectrophotometer, Shimadzu Ltd., Tokyo, Japan.

2.9.1. NADPH Oxidase (NOX) Activity

NOX (EC 1.6.3. 1) activity was determined according to Jiang and Zhang [38]. The reaction mixture contained 50 mM Tris–HCl buffer, pH 7.5, 0.5 mM XTT, 100 µM NADPH•Na_4 and 20 µg of protein. XTT reduction was followed at 470 nm. The background production was determined by the presence of 50 U SOD. One unit of NOX was defined as 1 nmol ml^{-1} XTT oxidized min^{-1} ($E = 2.16 \times 10^4\ M^{-1}\ cm^{-1}$)

2.9.2. Superoxide Dismutase (SOD) Activity

SOD (EC 1.15.1.1) activity was measured according to Beuchamp and Fridovich [39], measuring its ability to inhibit photochemical reduction of NBT at 560 nm. The reaction mixture (3 mL) contained 0.033 mM NBT, 10 mM l-methionine, 0.66 mM EDTA Na_2 and 0.0033 mM riboflavin in 0.05 mM sodium phosphate buffer (pH 7.8). One unit of enzyme activity was defined as the quantity of SOD enzyme that inhibits 50% NBT photoreduction.

2.9.3. Peroxidase (POX) and Cell Wall Bound POX (CWPOX) Activity

POX (EC1.11.1.7) and Cell wall POX (CWPOX) activity was based on the method of Herzog and Fahimi [40]. For determination of both activities, the same homogenates were used with different pretreatments. After the enzyme extraction, centrifugation was performed at 14,000× *g* for 10 min at 4 °C and supernatants were taken for POX assay while, pellets were washed in 50 mM sodium phosphate pH 5.8 and centrifuged at 1000× *g* for 10 min at +4 °C. After centrifugation, pellets were resuspended with 1 mL dH_2O and 1 M NaCl was pipetted into the tubes and stirred for 2 h. After that, samples were centrifuged at 1000 rpm for 10 min and supernatants were used for CWPOX assay. The reaction mixture contained 3,3′-diaminobenzidine-tetra hydrochloride dihydrate solution containing 0.1% (*w*/*v*) gelatine and 150 mM Na-phosphate-citrate buffer (pH 4.4) and 0.6% H_2O_2. Absorbance was followed for 3 min at 465 nm. One unit of POX activity was calculated as the mmol H_2O_2 decomposed $mL^{-1}min^{-1}$.

2.9.4. Statistical Analysis

The experiments were repeated twice, and three biological replicates were used from each experiment for all analyses ($n = 6$). Results are expressed as the mean ± standard error of the mean. Groups were compared using Student's *t*-tests. In the figures, different letters above the bars indicate significant differences between the control and treatment groups at the $p \leq 0.05$ level according to the least significant difference (LSD) test. Multivariate analyses were done using the SPSS statistical analyses program (IBM SPSS Statistics 25.0, 2017, NY, U.S.). General Linear Model was performed

using LSD test for comparing *Trichoderma*-pretreated, salt stress-treated and *Rhizoctonia*-infected and control groups.

3. Results

3.1. Effects of Trichoderma Citrinoviride on the Growth of R. solani

T. citrinoviride isolate was screened against *R. solani* (Figure 1) and it showed antagonistic activity against *R. solani* in the dual culture. While *T. citrinoviride* exhibited rapid growth, it caused a high inhibition (79%) in *R. solani* growth (Figure 1A,B). On the other hand, *T. citrinoviride* were not affected under salt stress (Figure 1C).

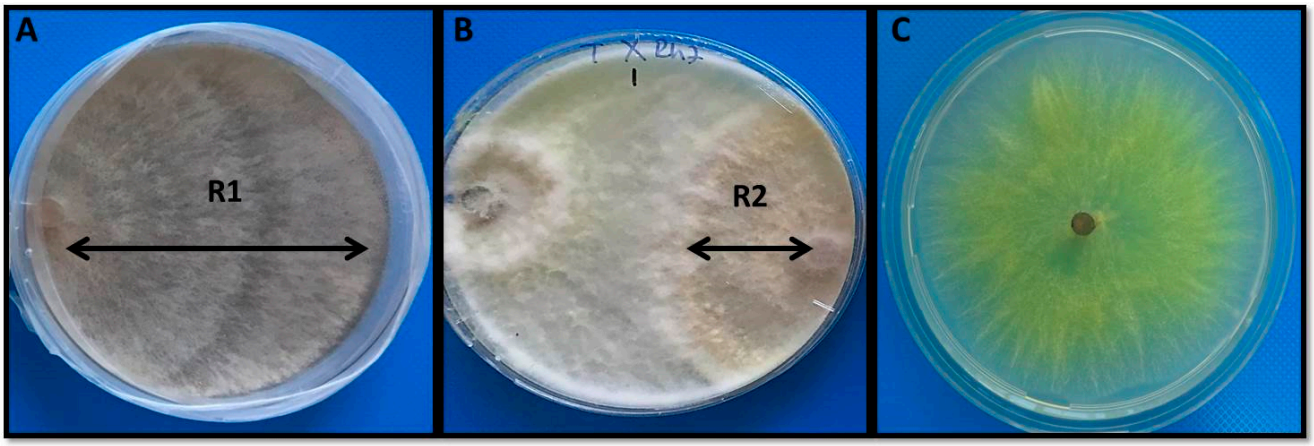

Figure 1. (**A**) Control plate with *R. solani* (**B**) Effect of *T. citrinoviride* suppressing *R. solani* growth (**C**) Growth of *T. citrinoviride* under 40 mM NaCl stress.

3.2. Disease Incidence

Plants treated with *Trichoderma citrinoviride* showed reduction in the development of the disease symptoms (T + R and T + S + R groups) as compared to untreated plants grown on infested soil (R and S + R groups) (Figure 2).While disease incidence in solely *R. solani*-infected plants was 88%, disease incidence in the *R. solani*-infected plants under salt stress reached 93%. Disease incidence was only 33% and 41% in T + R and T + S + R groups, respectively, which indicated the protective effect of *Trichoderma* pretreatment.

3.3. Plant Growth Analysis

Leaf dry weight used as a parameter for not only complaining the ameliorative effect of *Trichoderma* treatment but also for analyzing the difference between infected and uninfected plants (Figure 3). Salinity decreased plant growth significantly. Nontreated *Trichoderma* and uninfected strawberry plants showed 13.3% reduction in leaf dry weight at 40 mM NaCl. Furthermore, *R. solani*-infected plants had a significant decline in the total leaf dry weight. Leaf dry weight decreased by 49% in solely *R. solani*-infected plants, as compared to that of the control, but the rate of decline did not change when *R. solani* infection was combined with salinity. On the other hand, *Trichoderma* pretreatment significantly ameliorated (10%) the inhibitory effect of 40 mM NaCl on the leaf dry weight as compared to the control (Figure 3). The ameliorative effect of *Trichoderma* on leaf growth of *R solani* infected plants was higher by 26% as compared to that of NaCl-treated plants In *Trichoderma*-treated plants, leaf growth inhibition was reduced by only 34% under combined effects of salt and pathogen stress as compared to control. Therefore, remarkably, *Trichoderma* neutralized the inhibitory effect of *R. solani* in 40 mM NaCl. Furthermore the infection severity in 40 mM NaCl-treated plants infected with *R. solani* was also reduced in the groups pretreated with *Trichoderma*.

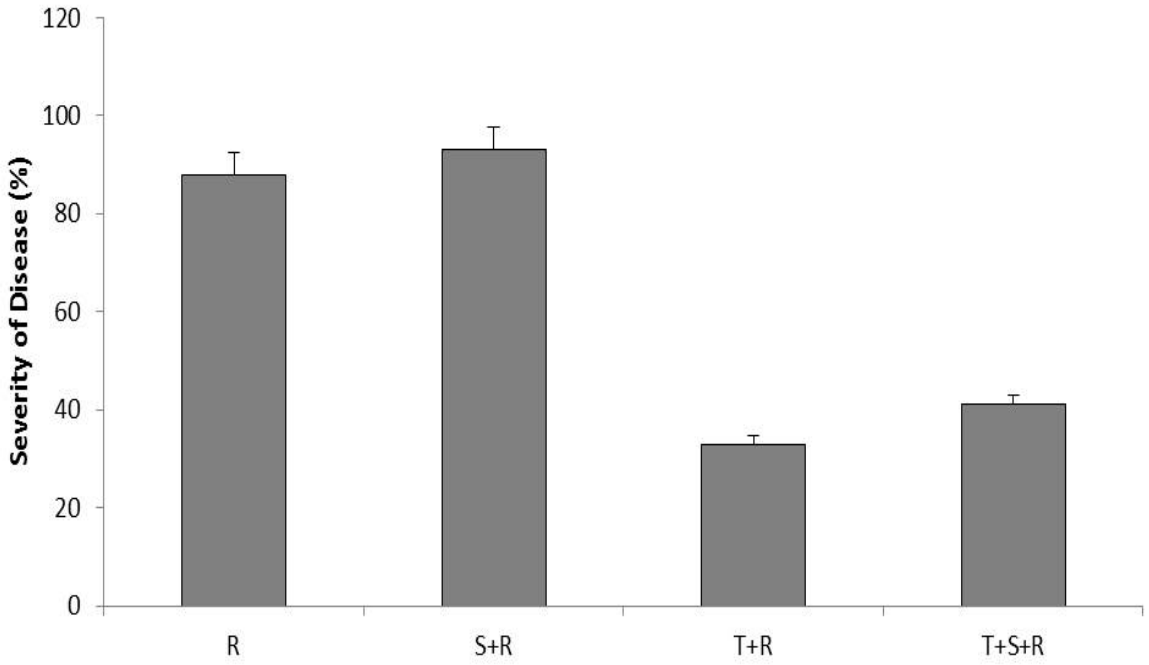

Figure 2. The effect of *T. citrinoviride* on severity of disease in strawberry plants which were subjected to abiotic stress (40 mM NaCl) and biotic stress (*R. solani*) either separately or in combination. Different letters indicates significant differences between treatments ($p < 0.05$). R, *R. solani* infected; S + R, NaCl + *R. solani*; T + R, *T. citrinoviride* + *R. solani*; T + S + R, *T. citrinoviride* +NaCl + *R. solani*.

3.4. Maximum Quantum Yield of PSII (Fv/Fm)

The *Trichoderma* treatment slightly enhanced Fv/Fm under normal conditions as compared to the control. On the other hand, *Trichoderma* pretreatment caused a significant increase in Fv/Fm in the leaves of plants which were subjected to salt stress and *R.solani* infection either separately or in combination as compared to *Trichoderma*-untreated plants under abiotic and biotic stress (Table 1). Fv/Fm values were decreased by 9.5% and 9% in *R. solani* infected groups and when *R. solani* infection is combined with salinity, respectively. Solely salt stress treatment did not change Fv/Fm values (Figure 4). Remarkably, *Trichoderma* treatment under abiotic and biotic stress restored the Fv/Fm values close to control levels when compared to all *Trichoderma*-untreated plants under stress.

3.5. H_2O_2 Content

Only salt stress enhanced the H_2O_2 content by 31%, while only *R. solani* infection increased it by 87% as compared to the control. Moreover, a combination of salt stress and *R. solani* infection increased the H_2O_2 by 74% in the leaves of strawberry plants as compared to the control (Figure 5). *Trichoderma* treatment decreased H_2O_2 content to control levels under salt stress. Furthermore, *Trichoderma* treatment decreased H_2O_2 content by 46.7% in *R. solani*-infected plants, as compared to in that of solely *R. solani*-infected plants (Table 1). Similarly, in 40 mM NaCl *R. solani*-infected plants, *Trichoderma* decreased the H_2O_2 content by 26.3%, as compared to *R. solani* infected plants under salt stress (Table 1).

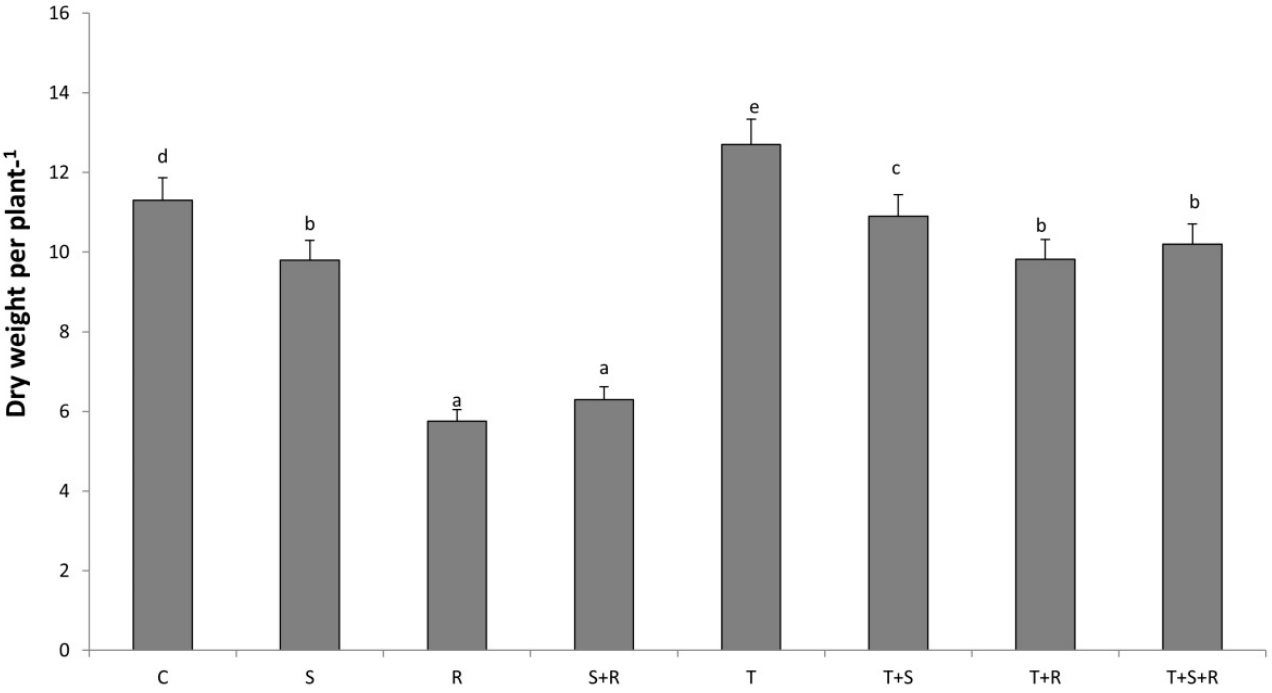

Figure 3. The effect of *T. citrinoviride* on leaf dry weight of strawberry plants which were subjected to abiotic stress (40 mM NaCl) and biotic stress (*R. solani*) either separately or in combination. Different letters indicate significant differences between the treatments (($p < 0.05$). C, control; S, NaCl-treated; R, *R. solani*-infected; S + R, Salt stress + *R. solani;* T, *T. citrinoviride*-treated; T + S, *T. citrinoviride* + NaCl; T + R, *T. citrinoviride* + *R. solani*; T + S + R, *T. citrinoviride*+ NaCl + *R. solani*.

Table 1. Results of multiple comparisons by ANOVA for Trichoderma (T), Salt (S), Rhizoctonia (R), control (C) and their interactions for dry weight, Fv/Fm, H_2O_2, TBARS, NOX, SOD, POX, CWPOX values. Multivariate analysis through general linear model (GLM) was performed using the LDS test considering F values at 95% confidence level (***: $p < 0.001$, **: $p < 0.01$, *: $p < 0.05$, ns: non-significant).

Dependent Variables	Independent Variables				
	T		T + S	T + R	T + S + R
	C	T + S + R	S	R	S + R
Dry weight	7.807 ***	0.677 *	5.303 *	10.361 **	10.361 **
Fv/Fm	1.193 *	1.958 *	5.423 *	17.999 **	17.999 **
H_2O_2	0.912 *	96.136 **	8.432 **	1.518 *	1.518 *
TBARS	0.129 ns	5.666 *	2.153 *	1.008 *	1.008 *
NOX	0.319 ns	1.469 *	1.224 *	1.304 *	1.304 *
SOD	0.033 ns	1.130 *	0.927 *	0.075 ns	0.075 ns
POX	25.056 **	0.547 *	0.587 *	2.134 **	2.134 **
CWPOX	2.618 *	21.837 **	1.227 *	3.515 *	3.515 *

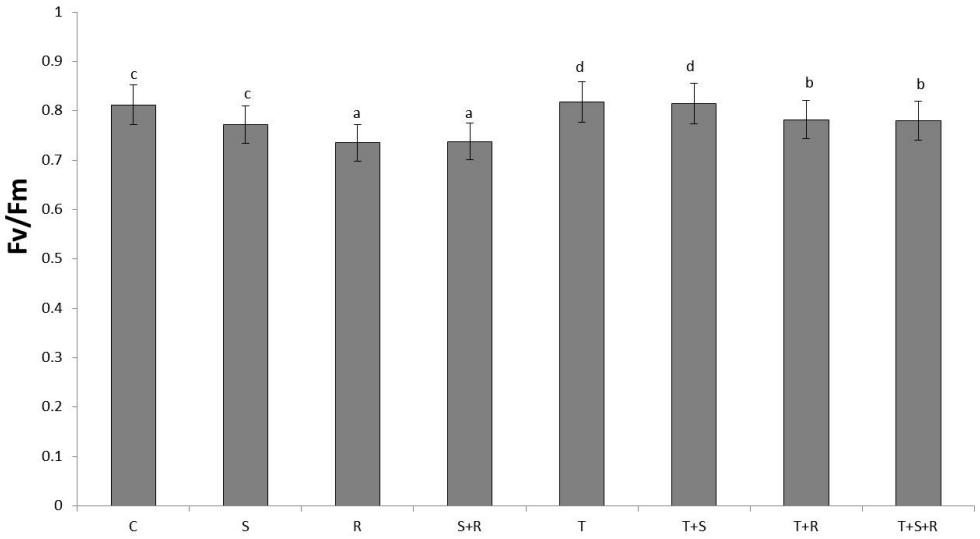

Figure 4. The effect of *T. citrinoviride* on Fv/Fm (PSII efficiency) in the leaves of strawberry which were subjected to abiotic stress (40 mM NaCl) and biotic stress *(R. solani)* either separately or in combination. Different letters indicates significant differences between treatments ($p < 0.05$). C, control; S, NaCl-treated; R, *R. solani*-infected; S + R, Salt stress + *R. solani;* T, *T. citrinoviride*-treated; T + S, *T. citrinoviride* + NaCl; T + R, *T. citrinoviride* + *R. solani*; T + S + R, *T. citrinoviride*+ NaCl + *R. solani*.

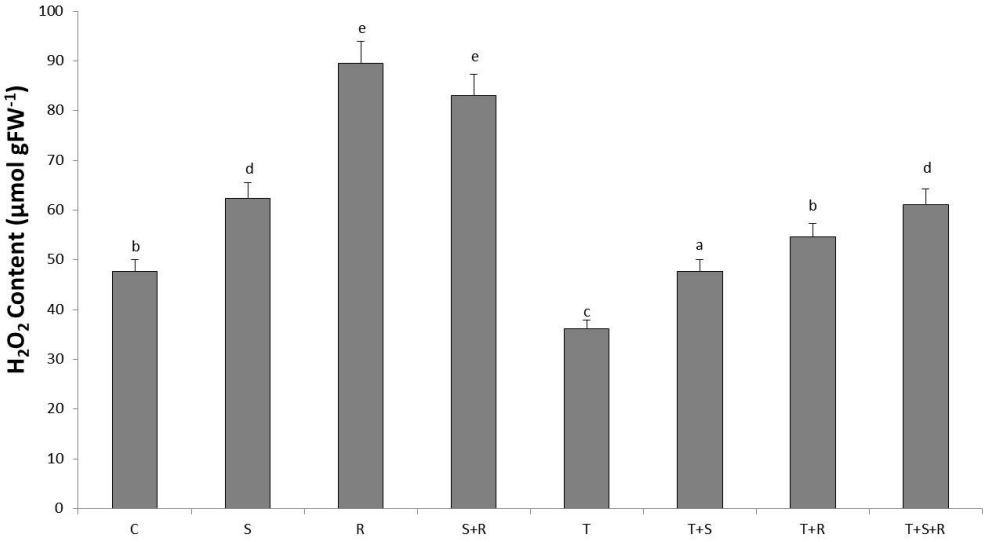

Figure 5. The effect of *T. citrinoviride* on H_2O_2 content in the leaves of strawberry plants which were subjected to abiotic stress (40 mM NaCl) and biotic stress *(R. solani)* either separately or in combination. Different letters indicates significant differences between treatments ($p < 0.05$). C, control; S, NaCl-treated; R, *R. solani*-infected; S + R, Salt stress + *R. solani;* T, *T. citrinoviride*-treated; T + S, *T. citrinoviride* + NaCl; T + R, *T. citrinoviride* + *R. solani*; T + S + R, *T. citrinoviride*+ NaCl + *R. solani*.

3.6. Lipid Peroxidation

The TBARS content was increased by 18.3% in salt-stressed plants as compared to the control (Figure 6).However; it remained at control levels in *Trichoderma* treated plants. *R. solani* infection caused a significant increase (48.4%) in TBARS content as compared to control. Furthermore, the highest lipid peroxidation level was found in 40 mM NaCl-treated plants infected with *R. solani*. However, the rate of increment in TBARS content of this group was not different from that of *R. solani*-treated plants under normal conditions (Figure 6). On the other hand, *Trichoderma* pretreatment decreased the TBARS content

by 9% and 10% in solely NaCl-treated and solely *R. solani*-infected plants respectively, as compared to untreated-*Trichoderma* plants under stress (Table 1). Furthermore, *Trichoderma* prevented excessive increase in TBARS content in 40 mM NaCl-treated plants infected with *R. solani*. In these plants, TBARS content was decreased by 26.7% in *Trichoderma* pretreated groups, as compared to those of 40 mM NaCl-treated plants infected with *R. solani* (Table 1).

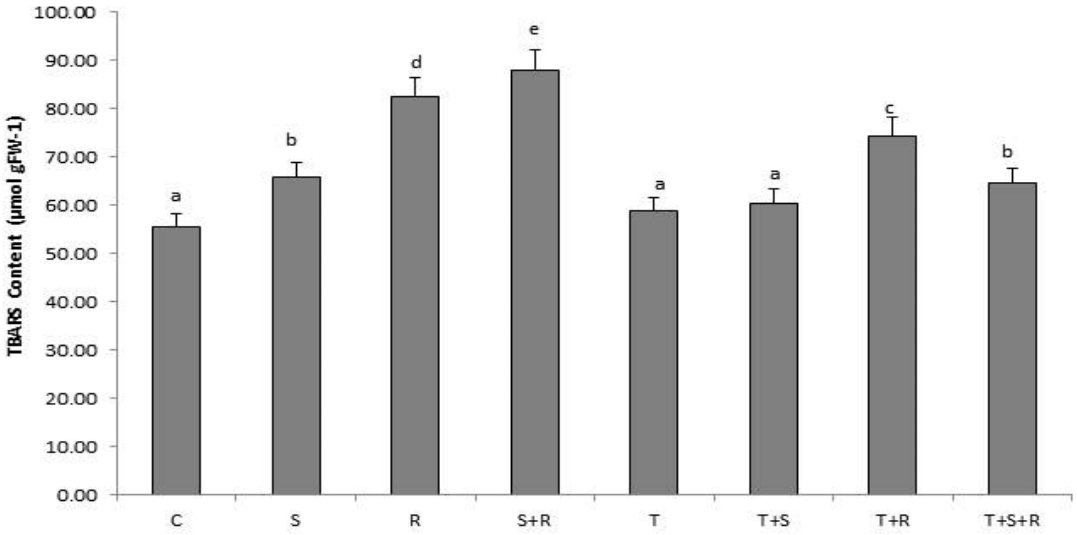

Figure 6. The effect of *T. citrinoviride* on TBARS content in the leaves of strawberry plants which were subjected to abiotic stress (40 mM NaCl) and biotic stress *(R. solani)* either separately or in combination. Different letters indicates significant differences between treatments ($p < 0.05$). C, control; S, NaCl-treated; R, *R. solani*-infected; S + R, Salt stress + *R. solani;* T, *T. citrinoviride*-treated; T + S, *T. citrinoviride* + NaCl; T + R, *T. citrinoviride* + *R. solani*; T + S + R, *T. citrinoviride*+ NaCl + *R. solani*.

3.7. NADPH Oxidase (NOX) Activity

NOX activity was enhanced in the leaves of strawberry plants which were subjected to 40 mM NaCl and *R. solani* infection either separately or in combination (Figure 7). The NOX activity was increased by 1.8-fold in NaCl-stressed plants as compared to control groups. However, *R. solani* infection resulted in higher levels of NOX activity. NOX activity was increased by 2.6 fold in solely *R. solani*-infected plants while it was increased by 4.6-fold in *R. solani*-infected plants under salt stress as compared to that of control. Accordingly, the highest NOX activity was measured in 40 mM NaCl + *R. solani*-infected plants. On the other hand, *Trichoderma* pretreatment caused a significant decrease (2.8-fold) in the leaves of strawberry plants under salt stress as compared to solely NaCl-treated plants (Table 1). Thus, NOX activity remained at control levels in *Trichoderma*-treated plants under salt stress. Furthermore, *Trichoderma* pretreatment decreased NOX activity by 2-fold in *R. solani*-infected plants, as compared to that of solely *R. solani*-infected plants (Table 1). Similarly, in 40 mM NaCl *R. solani*-infected plants, *Trichoderma* pretreatment significantly decreased NOX activity by 3-fold, as compared to *R. solani* infection under salt stress (Table 1).

3.8. Antioxidant Enzyme Activities

3.8.1. SOD Activity

A remarkable increase was observed in SOD activity in the leaves of strawberry plants subjected to salt stress and *R. solani* infection either separately or in combination as compared to the control. Among the *Trichoderma*-untreated plants, the highest SOD activity was observed in solely *R. solani*-infected plants (Figure 8A). SOD activity was increased by 28.7% in the NaCl-treated plants in *Trichoderma* pretreated group as compared to solely NaCl-treated plants. However *Trichoderma* did not significantly change SOD activity in the

leaves of *Rhizoctonia*-infected plants in *Trichoderma* pretreated group, as compared to solely *Rhizoctonia*-infected plants (Table 1). When *R. solani* infection is combined with salinity, SOD activity in the *Trichoderma* pretreated groups was increased by 28.5% as compared to in that of solely 40 mM NaCl + *R. solani*-treated plants.

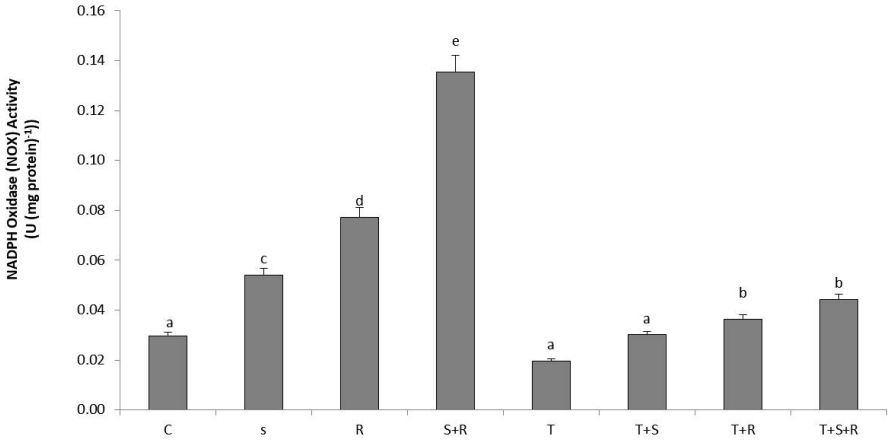

Figure 7. The effect of *T. citrinoviride* on NOX activity in the leaves of strawberry plants which were subjected to abiotic stress (40 mM NaCl) and biotic stress *(R. solani)* either separately or in combination. Different letters indicates significant differences between treatments ($p < 0.05$). C, control; S, NaCl-treated; R, *R. solani*-infected; S + R, Salt stress + *R. solani;* T, *T. citrinoviride*-treated; T + S, *T. citrinoviride* + NaCl; T + R, *T. citrinoviride* + *R. solani*; T + S + R, *T. citrinoviride*+ NaCl + *R. solani*.

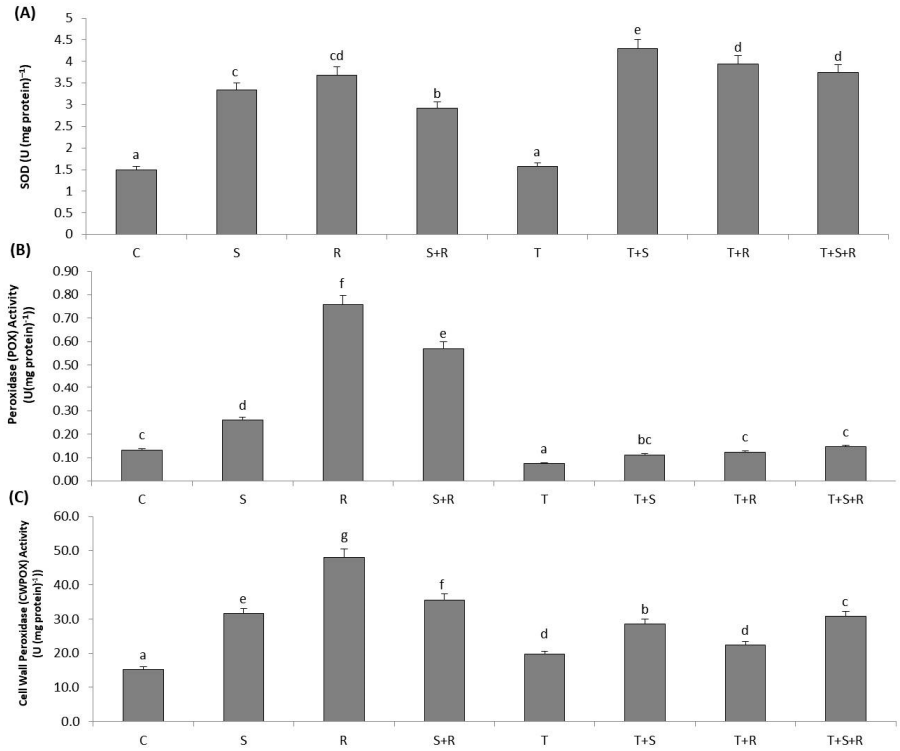

Figure 8. The effect of *T. citrinoviride* on SOD (**A**), POX (**B**) and CWPOX (**C**) activities in the leaves of strawberry plants which were subjected to abiotic stress (40 mM NaCl) and biotic stress *(R. solani)* either separately or in combination. Different letters indicates significant differences between treatments ($p < 0.05$). C, control; S, NaCl-treated; R, *R. solani*-infected; S + R, Salt stress + *R. solani;* T, *T. citrinoviride*-treated; T + S, *T. citrinoviride* + NaCl; T + R, *T. citrinoviride* + *R. solani*; T + S + R, *T. citrinoviride*+ NaCl + *R. solani*.

3.8.2. POX Activity

POX activity increased in solely NaCl-treated plants and the rate of increment was 2-fold as compared to that of the control (Figure 8B). Furthermore, all *R. solani*-infected plants either solely or combined with salinity (R and S + R) have exhibited higher levels of POX activity, as compared to that of solely NaCl-treated plants. The highest rate of increment in POX activity was 5.7-fold in solely *R. solani*-infected plants as compared to the control. On the other hand, *Trichoderma* treatment decreased in POX activity by 58%, 84% and 74% in the plants which were subjected to salt stress and *R. solani* infection either separately or in combination (Table 1). Thus, *Trichoderma* treatment under stress restored the POX activity values close to control levels.

3.8.3. CWPOX Activity

CWPOX activity was enhanced by 2-fold in the plants under salt stress (Figure 8C). However, *R. solani* infection resulted in the higher levels of POX whether it was alone or in combination with 40 mM NaCl. On the other hand, *Trichoderma* pretreatment prevented excess increase in the CWPOX activity in the all stress-treated plants (Table 1). Even, after *Trichoderma* treatment, CWPOX activity content in all stress groups was decreased almost to control levels.

4. Discussion

Salinity and pathogen attacks are two important constraints of plant growth and development [41]. Inoculation with beneficial fungal endophytes such as *Trichoderma* species has been found effective under both stresses to increase productivity. Therefore, understanding the interactions between plants and these fungal endophytes having influence on plant growth and stress tolerance is required. In the current study, we established correlations between the *Trichoderma citrinoviride* and the plant antioxidant enzyme profile in the strawberry plants which were subjected salt stress and *R. solani* infection either separately, or in combination. To the best of our knowledge, this study is the first to analyze the effect of abiotic and biotic stresses on induced resistance mechanisms by *Trichoderma* pretreatment in strawberry.

Recently, Krause et al. [42] demonstrated that *T. hamamatum* was able to inhibit *R. solani* infecting radish in the greenhouse. *T. harzianum*, another strain of *Trichoderma*, have effective antimicrobial activity against *R. solani* causing black root rot in bean [43]. According to our results, *T. citrinoviride* pretreatment suppressed *R. solani* infection therefore, it might be considered as a potential biocontrol agent due to the antifungal activity. On the other hand, it is known that high salinity can increase the disease incidence of plants or susceptibility of the plants to pathogen [44,45]. In the present study, we investigated the effects of *T. citrinoviride* pretreatment and salt stress (40 mM NaCl) on the plant's predisposition to disease. Exposure to another stress factor such as salinity under *R. solani* infection did not increase the severity of the disease. Salt stress did not affect the final disease incidence in strawberry infected with *R. solani* as reported in cyclamen infected with *F. oxyporum* f. sp. cyclaminis by Elmer [46]. However, disease incidence in Chili pepper cv. Tequilla Sunrise infected with *Phytophthora capsici* was increased with salinity [47].

In our study, strawberry plants which were subjected to 40 mM NaCl and *R. solani* infection either separately or in combination declined in leaf dry weight. Growth inhibition under different NaCl salinity was reported in different plants as well, including strawberry [48,49] cucumber [41], and cowpea [50]. Similar to salt stress, *R. solani* infection was also reported to cause growth inhibition in different plant species such as cucumber [51], bean [43], and tomato [11]. The main reason of this inhibition in the growth under the effect of salt stress and *R. solani* infection might be attributed to the increased osmotic stress, plant water retention disturbance, deficiency of nutrients, increased respiration rate, decreased photosynthetic activity and decrease in activation of the natural plant-defense mechanisms leading to reduced total biomass production, as was previously reported by Nostar et al. [41], Hashem, and Abd Allah. [52] and Saidi Moradi et al. [53]. Promotion

of plant growth by different *Trichoderma* species such as *T. harzianum*, *T. longibrachiatum*, *T. asperellum* under salt stress and *R. solani* infection, separately, in cucumber [54], rice [55], wheat [56], bean [43], and cotton [25] has been reported. We also found that the negative effects caused by salt stress and *R. solani* infection were mitigated by *Trichoderma* treatment. In the present study, *T. citrinoviride* increased the leaf dry weight of strawberry plants. Our results corroborated with the findings of Yesilyurt et al. [27] who reported that *T. citrinoviride* alleviates the adverse effects of salt stress in maize growth. In previous study, ameliorative effect of T. *citrinoviride* on maize growth under salt stress was attributed to the efficient role of *Trichoderma* in photosynthesis mechanism and osmolyte accumulation which is known to promote plant growth [27]. Another possible reason for improved plant growth by *Trichoderma* might be due to decreased ROS induced-oxidative damage in stressed plants that was also evident by lower lipid peroxidation levels.

It is known that Fv/Fm is a favorable parameter which allows detection of any damage on PSII causing photo-inhibition [57]. In our study, contrary to plants infected with *R. solani* (R and R + S), salt stress-treated seedlings did not show a remarkable decrease of the Fv/Fm ratio. Low Fv/Fm values in *R. solani* infected plants under salt stress generally might be indicative of some detrimental effects to the PSII reaction center [27,58,59]. *Trichoderma* treatment increased Fv/Fm ratio in NaCl-treated and *R. solani*-infected plants, suggesting that *Trichoderma* might be effective in improving photosynthetic apparatus under both abiotic and biotic stress.

Among ROS molecules, H_2O_2 is produced both under abiotic/biotic stresses and normal conditions. It can either be a toxic molecule causing irreversible damage to the plant cell or can be a secondary messenger regulating the antioxidative defense [60]. In the present study, we found a significant increase in H_2O_2 levels in response to salt stress and *R. solani* infection However, *Trichoderma* treatment reversed the accumulation of H_2O_2 in the *R solani*-infected plants under salt stress evident by a decrease in lipid peroxidation, which is an important oxidative stress marker. Therefore, we can propose that, *Trichoderma* treatment resulted in the improvement of cellular damage through decreased H_2O_2 levels. Guler et al. [59] and Shukla et al. [61] also found that *Trichoderma atroviride* and *Trichoderma harzianum* reduced H_2O_2 production in maize and rice roots under stress conditions.

Lipid peroxidation is an indicator of free radical-induced oxidative damage on cell membranes and expressed as TBARS content. Significant differences in lipid peroxidation levels were recorded in the leaves of stress-treated groups. Salt stress triggered lipid peroxidation, which might reflect the effect of salt stress on altering membrane lipid composition as indicated by previous studies in strawberry [48,49]. In the present study, plants infected with *R. solani* had the higher TBARS content, as compared to NaCl-treated plants. The highest lipid peroxidation level was determined in the *R. solani*- infected plants under salt stress. These results showed that strawberry plants have better performance in diminishing the effects of salt stress-induced oxidative stress, as compared to the harmful effects of *R. solani*-induced oxidative stress.

Trichoderma treatment resulted in a significant decrease in lipid peroxidation level in the leaves of all plants under salt stress and *R. solani* infection. These results suggest that *T. citrinoviride* can protect strawberry plants against salt and *R. solani*-dependent oxidative damage. This protective effect was very prominent, especially in *R. solani*–infected groups (R, S + R, T + S + R). These results are in a strong agreement with the results of Zhang et al. [62] who found decreased levels of lipid peroxidation in cucumber plants under salt stress treated with *T. harzianum*. Cell membrane stability is known to be correlated with abiotic and biotic stress tolerance. With this respect, salt-tolerance and pathogen resistance in *Trichoderma* treated plants might be resulted from lower TBARS accumulation, which decreased the symptoms of cellular damage.

Previous studies have shown that ther educed TBARS levels in the different *Trichoderma* species-pretreated seedlings might also be resulted from the elevated activities of antioxidant enzymes and the other protective molecules, the synthesis of compounds involved in eliminating the ROS molecules associated with lipid peroxidation [57,63].

Therefore, in the present study, we also determined the effect of *T. citrinoviride* on the activities of antioxidant enzymes in the leaves of strawberry plants under abiotic stress, biotic stress, and the combination of both stresses.

An increase in the activities of NOX, SOD, POX, and CWPOX during abiotic and biotic stress might be resulted from the induction of biosynthesis of these enzymes via the production of O_2^- and H_2O_2 [16]. Hence, these inductions can decrease the steady-state level of ROS levels in cells alleviating oxidative damage. This is reflected in a lower degree of lipid peroxidation in salt-treated plants. In contrast, the *R. solani*-infected groups all exhibited a greater extent of lipid peroxidation due to lack of efficient H_2O_2 detoxification mechanisms.

The NOX is among the main ROS sources in plant cells following the recognition of pathogens [64]. It catalyzes the formation of O_2^-, which is then converted to H_2O_2 [65]. Studies on different plants species have demonstrated that plasma membrane-associated NOXs are the main enzymatic sources of apoplast H_2O_2 accumulation [66,67]. Several lines of evidence suggest that the plasma membrane-associated NADPH oxidase might be essential for H_2O_2 accumulation. The combination of NaCl stress and *R. solani* infection increased NOX activity and induced H_2O_2 accumulation. The highest rate of increment in NOX was observed in *R. solani*-infected plants under salt stress, which have the highest H_2O_2 accumulation. We can speculate that at least some of the H_2O_2 production induced by salt stress and *R. solani* infection might be originated from enhanced NOX activity. Previous studies have indicated that NOX-dependent H_2O_2 accumulation plays a crucial role in both defense responses against pathogens such as restriction of the area of infection in wheat [67] and mediating NaCl-induced SOS pathway in *Arabidopsis* [68].

SOD, POX, and CWPOX are key components of the antioxidant defense of the plants [16]. SOD, catalyzed dismutation of O_2- to H_2O_2, is the most effective enzymatic antioxidant involved in stress tolerance. In order to reduce O_2^- and H_2O_2 damage, POX catalyzes the dismutation of H_2O_2 to water [16]. In this study, an increase was found in SOD activity in all strawberry plants under abiotic and biotic stress. Similarly, Keutgen and Pawelzik [69] found increased SOD activity in strawberry plants subjected to 40 and 80 mM NaCl. Similarly, SOD activity was increased also in tomato which was infected by *R. solani* [11]. We found decreased SOD activity in *Trichoderma* treated strawberry plants subjected to combined NaCl and *R. solani* stresses. These results are in strong conformation with the results of Yesilyurt et al. [27] who reported decreased SOD in *T. citrinoviride*-treated maize under salt stress. Contrary to these, in a previous study, it was found that biocontrol agents such as *Bacillus amyloliquefaciens* were able to induce SOD activity at a sufficient level to induce host protection [11,70].

Apart from their role in catalyzing the breakdown of H_2O_2, POXs have been reported to be involved in lignification and subarization processes [71]. Furthermore, POX activity has been reported to increase in different plants under salt stress and pathogen. Likewise, in this study, an increase was found in the POX activity of all *Trihoderma*-untreated plants under abiotic and biotic stress. The highest POX activity was observed in the plants infected with *R. solani* (R and S + R groups). In agreement with our results, Paranidharan et al. [72] found increased POX activities in rice in response to infection by *Rhizoctonia solani*. According to these results, we can speculate that increased lignin synthesis during both salt stress and pathogen infection in order to establish an apoplastic barrier and to make the cell wall less permeable to water loss might also be the case in our study [73]. On the other hand, POX activity of the all *Trichoderma*-treated plants under stress was lower than that of the control levels. Decreased activity of POX might reveal that *T. citrinoviride* might prevent or reduce lignification processes resulting from salt stress and *R. solani* infection. Therefore, it can reduce water loss as reported by Yesilyurt et al. [27] who reported higher RWC content in leaves of *T. citrinoviride*-treated maize plants under salt stress. There is an inverse relationship between growth rate and CWPOX. Several authors have shown that a reduction of growth is associated by increased CWPOX activity, which resulted in increased cell wall lignification [74,75]. Up to date, there is no information on how CWPOX activity is related to the growth responses in strawberry plants under combined salinity

and pathogen infection. Accordingly, the effect of *Trichoderma* pretreatment on its activity is unknown. In the present study, both salt stress and *R. solani* infection increased CWPOX activity. This increment in CWPOX in the leaves of *R. solani*-infected plants strawberry could reflect the modification of mechanical properties of the cell wall, as it was also previously reported in *Brassica juncea* under Cd stress by Verma et al. [76]. We suggest that reduction growth in *R. solani*-infected plants might be attributed to increased activity of CWPOX, which led to generation of hydroxyl radicals in cell walls mediating extension growth [77]. *Trichoderma* treatment significantly decreased the CWPOX activity in the salt-treated, *R. solani*-infected and salt + *R.solani*-infected plants compared to *Trichoderma*-untreated plants. These indicated the alleviative effect of *Trichoderma* treatment findings on plant growth under salt stress and *R. solani* infection.

5. Conclusions

We conclude that *Trichoderma treatment* enabled a reduced infection rate of black root rot, stimulated growth and resistance to salt stress and *R. solani* infection in strawberry. Furthermore, *Trichoderma* reduced H_2O_2 content, which might be responsible for the protection of membrane lipids from peroxidation. Although a significant interaction between salt stress, *R. solani* infection and antioxidant enzyme activity was found, Trichoderma treatment tends to reduce these effects and it is plausible to propose that different mechanisms might also be acting on its mode of action. *T. citrinoviride* treatment may be an alternative/additional way to improve yield and production under abiotic and biotic stresses.

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