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Central Role of Salicylic Acid in Resistance of Wheat Against *Fusarium graminearum*

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Abstract Head blight caused by *Fusarium graminearum* (*F. graminearum*) is one of the major threats to wheat and barley around the world. The importance of this disease is due to a reduction in both grain yield and quality in infected plants. Currently, there is limited knowledge about the physiological mechanisms involved in plant resistance against this pathogen. To reveal the physiological mechanisms underlying the resistance to *F. graminearum*, spikes of resistant (Sumai3) and susceptible (Falat) wheat cultivars were analyzed 4 days after inoculation, as the first symptoms of pathogen infection appeared. *F. graminearum* inoculation resulted in a greater induction level and activity of salicylic acid (SA), callose, phenolic compounds, peroxidase, phenylalanine ammonia lyase (PAL), and polyphenol oxidase in resistant versus susceptible cultivars. Soil drench application to spikes of SA, 24 h before inoculation with *F. graminearum* alleviated *Fusarium* head blight symptoms in both resistant and susceptible cultivars. SA treated plants showed a significant increment in hydrogen peroxide (H_2O_2) production, lipid peroxidation, SA, and callose content. SA-induced H_2O_2 level seems to be related to increased superoxide dismutase and decreased catalase activities. In addition, real-time quantitative PCR analysis showed that SA pretreatment induced expression of PAL genes in both infected and non-infected head tissues of the susceptible and resistant cultivars. Our data showed that soil drench application of SA activates antioxidant defense responses and may subsequently induce systemic acquired resistance, which may contribute to the resistance against *F. graminearum*. These results provide novel insights about the physiological and molecular role of SA in plant resistance against hemi-biotrophic pathogen infection.

Keywords *Fusarium graminearum* · *Fusarium* head blight · Resistance · Salicylic acid · Systemic acquired resistance · Wheat

Introduction

Fusarium head blight (FHB) is one of the most destructive wheat diseases caused by *Fusarium* species such as *Fusarium graminearum* (*F. graminearum*). *F. graminearum* is a hemi-biotrophic phytopathogen with a biotrophic phase at the early stages of infection (Shin and others 2011). The fungus infects cereal inflorescence for a short period of time at the beginning of anthesis. Following pathogen establishment, the fungal hyphae can spread from spikelet to spikelet through the vascular bundles of the rachis (Parry and others 1995). When *Fusarium* species reach the rachis node, they produce several toxins such as deoxynivalenol (DON) that accumulate in the kernels of the infected spikelets and consequently, limit grain yield (Proctor and others 1995). Grains contaminated with *Fusarium* mycotoxins and their byproducts are unhealthy

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for human and animal consumption (Bai and Shaner 2004). FHB symptoms can be characterized by dark brown discoloration of the peduncle and shriveled kernels with a chalky appearance. As there is an increasing prevalence of FHB in global cereal growing regions, many researchers have been focused on studying it.

Molecular and physiological factors determine the susceptibility or resistance of wheat cultivars to *F. graminearum*. FHB-resistant wheat genotypes, like Sumai3, employ mechanisms to reduce pathogen growth on or within the plants. In spite of considerable published data, the basis for resistance against FHB is principally unknown and up to now, no major factor responsible for resistance has been identified. Generally, plants protect themselves against pathogen spread by complex strategies such as alterations in cell wall structure (Castro and Fontes 2005), production of reactive oxygen species (ROS), accumulation of secondary metabolites (Benner 1993; Bennett and Wallsgrove 1994; Heath 2000; Agrios 2005), and synthesis of defense proteins (Ebrahim and others 2011). The infected plants can protect themselves from adverse outcomes resulting from further penetration and spread of pathogens through changes in cell wall structure. Deposition of callose (β-(1,3)-glucan biopolymer) at the site of pathogen attack forms a structure that functions as an effective barrier against pathogen penetration (Luna and others 2011).

Moreover, production of ROS such as the superoxide anion ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2), as one of the earliest responses to pathogen attack, can trigger hypersensitive cell death. Abnormally high production of ROS causes damage to biomolecules, whereas ROS at low/moderate concentrations acts as a second messenger in signaling cascades that mediates several responses in plant cells including programmed cell death (Sharma and others 2012). The hypersensitive response (HR), as an early defense response, restricts pathogen infection to the site of attempted ingress by necrosis and cell death. Plants utilize efficient antioxidative enzymatic and non-enzymatic protective mechanisms to scavenge excess ROS. Several antioxidative enzymes including superoxide dismutase (SOD), catalase (CAT), and peroxidase (POX) are involved in detoxification of ROS (Zhang and others 1995; Lee and Lee 2000). In addition, phenolics and some other organic compounds serve as potent non-enzymatic antioxidants in cells. Polyphenol oxidase (PPO), which catalyzes the oxidation of phenols to quinines, contributes in plant defense against pathogens (Li and Steffens 2002). To confront pathogen attack, plants also induce production of secondary metabolites to control pathogen penetration and spread. The phenylpropanoid pathway sits across the border of primary and secondary metabolism. Phenylalanine ammonia lyase (PAL) functions as a critical enzyme in the phenylpropanoid pathway and it is the key enzyme involved in synthesis of several secondary compounds such as phenolics and lignins (Hemm and others 2004).

It is clear nowadays that SA plays a critical role in wheat defense against pathogen attack (Makandar and others 2012). SA acts as a signaling messenger and an intermediate molecule of the plant–pathogen perception and response process. SA is activated in systemic acquired resistance (SAR) and protects plants from further infection after an initial pathogen attack (Mauch-Mani and Métraux 1998). Plants with deficiency in SA signaling are incapable of developing SAR and do not show pathogen-related (PR) gene activation upon pathogen infection (Pieterse and others 2009). With such background, Makandar and others (2010, 2012) demonstrated that exogenous application of SA can be efficient in conferring resistance to wheat against *F. graminearum*.

Although vigorous efforts have been made to identify mechanisms of resistance against FHB, much remains to be elucidated about the physiological capability of the wheat plant against *F. graminearum* attack. Here, we performed a comparative study between resistant and susceptible wheat genotypes to distinguish the effects of some antioxidant and biosynthetic enzymes in response to *F. graminearum*. Moreover, we investigated the effect of exogenous application of SA, as a key factor in SAR, on wheat resistance to the pathogen infection. Finally, to identify possible SA-dependent/independent responses we assessed the influence of pretreatment with SA on the studied physiological parameters in inoculated susceptible and resistant wheat cultivars.

Materials and Methods

Plant and Fungal Growth Condition

Two wheat (*Triticum aestivum*) genotypes with contrasting levels of resistance and susceptibility to FHB were used in this study: an Iranian spring wheat cultivar, Falat, as a highly susceptible cultivar to FHB and a Chinese originated FHB-resistant cultivar, Sumai3. Seeds were germinated in autoclaved compost-peat-based planting mixture as five seeds per 2000 cm^3 plastic pot. Wheat plants were grown in a greenhouse under a temperature regime of 21 and 18 °C during the day and night, respectively. Macroconidia of the aggressive Iranian *F. graminearum* isolate 'F42' was cultured for 7 days in the dark at 25 °C on potato dextrose agar (PDA). To prepare the suspension, the fungus was grown in mung bean broth (that is, 4 g of mung beans boiled in 100 cm^3 of water) and was incubated for 2 days at a temperature of 22 °C. The mixture of macroconidia with fungal mycelium was filtered through four

layers of autoclaved cheesecloth. Spore concentration was determined using a hemocytometer and the suspension was adjusted to 10^5 spore cm^{-3} (Makandar and others 2006).

Fungal Inoculation and FHB Disease Rating

Point inoculation of wheat spikelets was done with 10 µl of spore suspension at 6–7 weeks after germination at anthesis according to Zadoks stages of 65–69 (Zadoks and others 1974). 10 µl of either *F. graminearum* suspension or mung bean broth (mock treatment) was injected between the palea and lemma. To assess disease symptoms, the central spikelets, one on either side of the rachis, were inoculated. For physiological analysis, three alternate pairs of spikelets (six spikelets per spike) around the middle of the spike were inoculated (Gunnaiah and others 2012). For each replication of each treatment, 30 spikes were inoculated. Moisture zip-lock bags were used to cover the inoculated spikes and were removed 3 days after the inoculation. Following inoculation with *F. graminearum*, plants were evaluated for FHB disease as percent spikelets infected with FHB for 28 days at periodic intervals (Makandar and others 2012). Experiments were repeated three times, each with three replications. Statistical analysis was performed in SPSS software using *t* test to reveal significant differences ($P \leq 0.05$) in visual symptoms among different samples.

Chemical Treatment of Plants

Soil drench applications of sodium salicylate were carried out as described by Makandar and others (2012). Briefly, stock solution of 10 mM sodium salicylate (Sigma-Aldrich) was prepared in water and diluted to a final concentration of 200 µM. The soil was left at room temperature to get semi-dry to the state that the plant was still turgid and then 500 cm^3 of 200 µM sodium salicylate was added to the soil in the 2000 cm^3 pots. In all treatments, irrigation was done 24 h prior to inoculation experiments. Plants (about 50 days old, at anthesis) of similar size were divided into 4 sets for the study. Each set consisted of 30 plant replicates. Experimental sets were as follows: Set No. 1 (Control): plants irrigated with water and inoculated with mock, Set No. 2 (+*F. graminearum*): plants irrigated with water and inoculated with *F. graminearum*, Set No. 3 (+SA): plants irrigated with 200 µM SA and inoculated with mock, Set No. 4 (+SA + *F. graminearum*), plants irrigated with 200 µM of SA and subsequently inoculated by *F. graminearum*. Sampling for molecular and physiological analysis was done 4 days after inoculation when the first symptoms of infection appeared.

Determination of H_2O_2 Content

H_2O_2 content was determined according to the methods of Velikova and others (2000). Treated and control spikelet tissues (0.4 g) were homogenized in 1 cm^3 of 0.1 % (w/v) trichloroacetic acid (TCA) on ice and centrifuged at 12,000×*g* for 15 min. 1 cm^3 of potassium phosphate buffer and 1 cm^3 of potassium iodide (KI) were added to 0.5 cm^3 aliquot of the supernatant. The absorbance of the supernatant was recorded at 390 nm and H_2O_2 content was calculated using a standard curve.

Determination of Antioxidant Enzymes Activity

Plant materials (that is, spikelets) were homogenized at 4 °C with a pestle and mortar in 0.1 M of Tris–HCl buffer (pH 8.9) to estimate different enzyme activities. The Tris–HCl buffer contained 0.5 mM NaCl, 5 mM 1,4 dithiotheritol (DTT), and 5 mM ethylenediaminetetraacetic acid (EDTA). The homogenate was then centrifuged at 13,000×*g* (J2-21 M, Beckman, Palo Alto, USA) for 30 min at 4 °C and the obtained supernatant was kept at −70 °C and later used for enzyme assays. A UV–Vis recording spectrophotometer (UV-160, Shimadzu, and Tokyo, Japan) was used to detect enzymes activity.

Estimation of SOD (EC 1.15.1.1) activity was performed by monitoring the inhibition of photochemical reduction of nitroblue tetrazolium (NBT) in a reaction mixture, as described by Giannopolitis and Ries (1977). The reaction mixture contained 50 mM sodium phosphate buffer (pH 7.5), 13 mM methionine, 75 µM NBT, 75 µM riboflavin, 0.1 mM EDTA, and 0.1 cm^3 of enzyme extract. For 14 min, the reaction mixture was irradiated and at 560 nm the absorbance was read comparing to the non-irradiated blank. One unit of SOD was defined as the amount of enzyme which caused 50 % inhibition of NBT reduction under the assay condition, and the results were reported in the [unit mg^{-1} (protein)]. Proteins were determined according to Bradford (1976), using serum albumin as a standard.

According to Aebi (Aebi and Catalase 1974) protocol, CAT (EC 1.11.1.6) activity was assayed from the rate of H_2O_2 decomposition as measured by the decrease in absorbance at 240 nm. The reaction mixture comprised 50 mM potassium phosphate buffer (pH 7.0), 15 mM H_2O_2, and 20 µl of protein extract. Activity was expressed as units (µmol of H_2O_2 decomposed per min per mg protein [unit mg^{-1} (protein)].

POX (EC 1.11.1.7) activity was measured according to the method of Abeles and Biles's (1991). The reaction mixture comprised 4 cm^3 of 0.2 M acetate buffer (pH 4.8), 0.4 cm^3 of H_2O_2 (3 %), 0.2 cm^3 of 20 mM benzidine, and 0.05 cm^3 of enzyme extract. The increase in absorbance

was recorded at 530 nm. The POX activity was defined as 1 μM of benzidine oxidized per min per mg protein [Unit mg^{-1} (protein)].

Determination of PPO and PAL Activity

PPO (EC 1.14.18.1) activity was estimated according to the method of Raymond and others (1993) at 40 °C. The reaction mixture contained 2.5 cm^3 of 0.2 M sodium phosphate buffer (pH 6.8), 0.2 cm^3 of 20 mM pyrogallol and 0.1 cm^3 of enzymes extract. The increase in absorbance was recorded at 430 nm. The PPO activity was defined as 1 μM of pyrogallol oxidized per min per mg protein [unit mg^{-1} (protein)].

PAL activity was measured based on the rate of cinnamic acid production, as described by Ochoa-Alejo and Gómez-Peralta (1993). 1 cm^3 of the extraction buffer, 0.5 cm^3 of 10 mM L-phenylalanine, 0.4 cm^3 of double distilled water and 0.1 cm^3 of enzyme extract were incubated at 37 °C for 1 h. By addition of 0.5 cm^3 of 6 M HCl, the reaction was terminated and the product was extracted using 5 cm^3 of ethyl acetate. This was then followed by evaporation to remove the extracting solvent. The solid residue was suspended in 3 cm^3 of 0.05 M NaOH and the cinnamic acid concentration in it was determined with a spectrophotometer with absorbance measured at 290 nm. Each unit of PAL activity is equal to 1 μmol of cinnamic acid produced per min.

Determination of Lipoxygenase (LOX) Activity

LOX activity was assayed based on Grossman and Zakut's (1979) method. Pure linoleic acid (10 μl) was suspended in 25 cm^3 of 0.1 M sodium tetraborate comprising 0.1 % Tween 20 by sonication (Sekhar and Reddy 1982). In a spectrophotometer cuvette, the substrate (0.1 cm^3) was vigorously shaken with 2.9 cm^3 of 0.1 M phosphate buffer at pH 4–5. By adding 0.1 cm^3 of enzyme extract the reaction was started and the increase in absorbance was recorded at 234 nm. Activity was expressed as unit (ΔA_{234} min^{-1}) mg^{-1} protein. Protein content was determined according to Bradford (1976), using serum albumin as standard.

Determination of Malondialdehyde (MDA)

According to Heath and Packer (1968) method, the MDA content was measured in relation to thiobarbituric acid reactive substances. The spikelets (0.5 g) were homogenized in 5 cm^3 of 0.1 % (w/v) trichloroacetic acid (TCA) and centrifuged at 10,000×g for 20 min. To 1 cm^3 aliquot of the supernatant, 4 cm^3 of 0.5 % thiobarbituric acid (TBA) in 20 % TCA was added. The resulting mixture was heated at 95 °C for 30 min and then was quickly cooled in an ice bath. The absorbance was determined at 532 and 600 nm, after centrifugation at 10,000×g for 15 min. The value for non-specific absorption at 600 nm was then subtracted from that of 532 nm. The concentration of MDA was calculated using absorption coefficient of 155 mM^{-1} cm^{-1}.

SA Quantification by HPLC

Sample preparation for quantification and extraction of SA was carried out based on Wen and others (2005). Briefly, 1 g of treated and control spikelet tissues were extracted with methanol/water/trifluoroacetic acid (TFA) (50:50:0.1) mixed solvent, and the volume of the turbid fluid was adjusted to 10 cm^3 accurately. The mixture was centrifuged at 3000 rpm for 5 min, and filtered through a 0.45-μm nylon filter. Three replications per each treatment were used for the estimation of total SA content. Chromatographic separations were performed on an Agilent 1200 series high-performance liquid chromatography (HPLC) system including a quaternary pump and a degasser equipped with a G1315D diode array detector and a G1321A fluorescence detector. Separations were carried out on a C_{18} column (250 × 4.6 mm, with 5.0 m particle size) from Waters Company (Massachusetts, USA). The flow rate of the mobile phase was kept at 0.5 cm^3/min. Mobile phase A was water containing 0.02 % TFA, and phase B was methanol containing 0.02 % TFA. The temperature of column was controlled at 25 °C. Injection volume was 10 μl and samples detection was performed at 305 nm (Wen and others 2005).

Determination of Callose Content

Control and treated spikelet tissues (1 g) were extracted using ethanol and callose content was determined based on the method of Kohle and others (1985). The mixture was centrifuged at 12000 g for 20 min. The pellet was then dissolved in 300–350 μl dimethyl sulfoxide (DMSO). The samples were centrifuged for 5 min at 12,000×g after cooling at room temperature. One hundred cm^3 of supernatant was supplemented with 200 cm^3 of 1 M NaOH and 1.2 cm^3 of loading mixture [400 cm^3 0.1 % (w/v) aniline blue in water; 590 cm^3 1 M glycine/NaOH (pH 9.5); and 210 cm^3 1 M HCl] to quantitatively determine the extracted callose. Using a fluorescence spectrophotometer (Shimadzu spectrofluorophotometer RF-5000) at 479 nm emission wavelength and 393 nm excitation, the total fluorescence of samples was determined.

Estimation of Total Antioxidant Activity Using DPPH Scavenging Assay

Free radical scavenging activity of spikelets was measured according to a modified method of Shimada and others (1992). Spikelet tissue (0.1 g) was extracted in 1 cm^3 methanol 80 %. Then 0.1 cm^3 of plant extract was added to 3.9 cm^3 of 80 ppm of 1,1-diphenyl-2-picrylhydrazyl (DPPH) solution. After being shaken vigorously, the mixture was allowed to sit in the dark for 30 min. The absorbance was recorded at 517 nm. Using the following equation the free radical scavenging activity was calculated:

$$\% \text{ inhibition of DPPH radical} = \left[\left(\text{Absorbance of control} - \text{Absorbance of sample}\right) / \left(\text{Absorbance of control}\right)\right] \times 100.$$

PAL Gene Expression Analyses

Total RNA was extracted from control and treated wheat spikelets using RNX-plus kit (RN7713C, CinnaGen, Iran) according to the manufacturer's instruction with slight optimization. The quality and concentration of RNA samples were examined by EB-stained agarose gel electrophoresis and spectrophotometer analysis. Total RNA was treated with DNaseI (Fermentase, Germany) to remove DNA contamination before cDNA synthesis according to the manufacturer's instructions. Three micrograms of DNase-treated RNA was reverse transcribed into complementary DNA (cDNA) using Revert Aid^{TM} Reverse Transcriptase (Fermentas, Germany), oligo dT18 and random hexamer primers (MWG, Germany) in a total volume of 20 µl reaction mixture, according to the manufacturer's instructions. The mRNA expression level of PAL (Accession number: AY005474.1) was measured by quantitative real-time PCR using the primers (Forward: CATGAACG AGCGCAAGCACATCG & Reverse: AACGTAGTAT ACCAGGAACCTCC), which were designed using PRIMER EXPRESS software (Applied Biosystems). The relative expression level was quantified in comparison with the house keeping gene β-actin (Accession number: JQ269668.1) as an internal control (Forward: TGTT CCAGCCATCTCATGTTGG & Reverse: TCATG CGAT CAGCAATTCCAGG). Quantitative real-time PCR was performed using Applied Biosystems 7500 Real-Time PCR System (Applied Biosystem/MDS SCIEX, Foster City, CA, USA), with 10 ng cDNA, 10 µl of SYBR Green I master mix (Takara, Shiga, Japan), and 200 nM of forward and reverse primers up to final reaction volumes of 20 µl, according to the manufacturer's instructions. The PCR was performed through the following instruction: an initial denaturation at 95 °C for 5 min, followed by 40 cycles of denaturation at 95 °C for 10 s, annealing at 60 °C for 30 s, and extension at 72 °C for 30 s. The specificity of the PCR products was examined by melting curve analysis, restriction endonuclease digestion followed by 12 % polyacrylamide gel electrophoresis. The mean of ΔCT for two cultivars after treatment and the control condition was calculated and finally the relative expression of the PAL gene was estimated by the ratio formula (ratio $= 2^{-\Delta\Delta Ct}$) as described by Livak and Schmittgen (2001). The PAL gene expression level was expressed relative to the appropriate control. All experiments were repeated at least twice ($n \geq 2$) and a serial dilutions of cDNA was examined to obtain a standard curve for each primer pair.

Statistical Analysis

Experiments were repeated three times, with three replications each. Statistical calculations were performed with SPSS-20 (SPSS, Chicago, IL, USA). Tests for significant differences among physiological parameters under different treatments were conducted using analysis of variance (ANOVA) with Duncan's multiple range tests at the 0.05 level of confidence.

Results

In the present study, we examined several physiological and biochemical responses of susceptible and resistant wheat genotypes to *F. graminearum* infection. Inoculated spikes were monitored and disease symptoms were recorded for 28 days after inoculation (Fig. 1). The first symptoms were observable as brownish spots at the base of the glumes and ultimately the glumes became bleached. The effect of SA on plant responses to pathogenesis in susceptible and resistant cultivars under *F. graminearum* inoculation was investigated.

The first symptoms of FHB appeared 4 days after inoculation (dai) in both genotypes. Infection in Sumai3 reached a peak of 20 % in 24 dai and did not progress any further. Results indicated that all of the spikelets of Falat were infected at 28 dai. Soil drench application of SA one day before inoculation curtailed FHB infection in Falat and Sumai3 by about 70 and 10 %, respectively (Fig. 1). Thus, SA showed a significant effect in inducing resistance in the susceptible and resistant wheat cultivars.

Once the first symptom of the disease appeared, inoculated spikes were collected at 4 dai. This is believed to be the time for the biotrophic phase of the pathogen. The appearance of symptoms provided a confirmation that the pathogen had penetrated the host tissues and infection was successful. HPLC analysis showed that SA content in

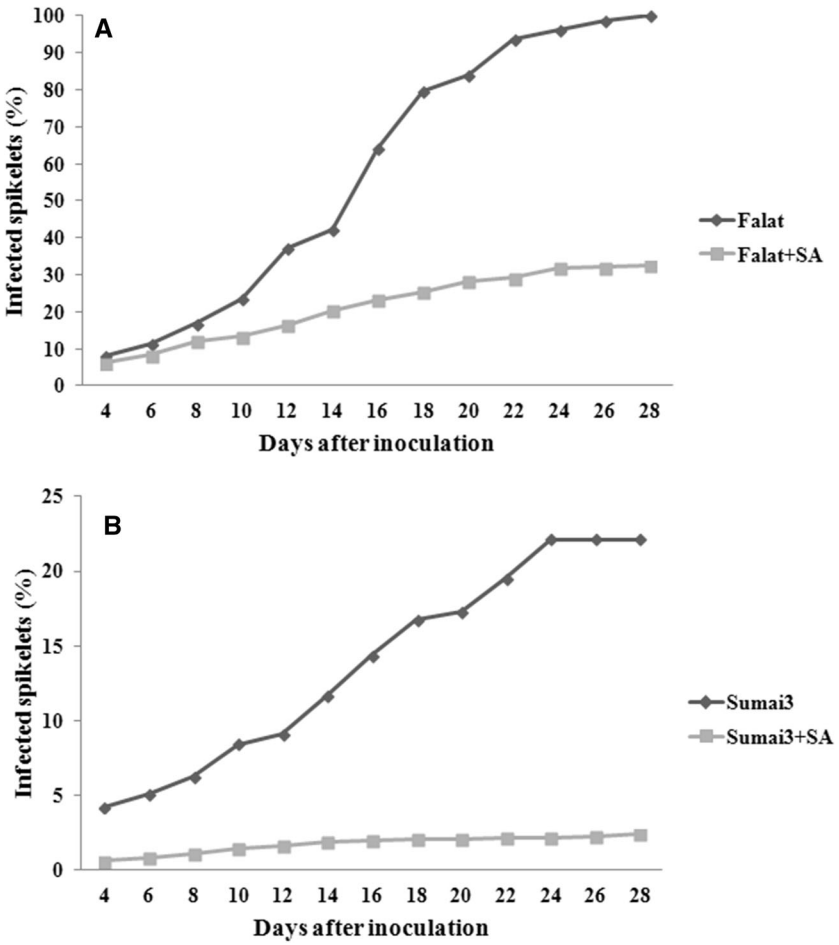

Fig. 1 FHB disease rating in (**a**), Falat (susceptible) and (**b**), Sumai3 (resistant) wheat cultivars irrigated with salicylic acid (SA) and with water as a control 1 day before inoculation. Exogenous application of SA enhances resistance to *Fusarium* head blight. Diseased rating was recorded as the percentage of infected spikelets in each spike. At minimum, 30 plants per cultivars were tested for each treatment. *Columns* indicate mean ± SE of thirty replicates. The groups are +Fig (plants irrigated with water and inoculated with *F. graminearum*) and +SA + Fg (plants irrigated with 200 μM sodium salicylate solution 1 day before inoculation with *F. graminearum*)

spikelets of Sumai3 was significantly (about threefold) higher in comparison with Falat at the beginning and at 4 days after mock inoculation (no significant changes were seen during the period after inoculation in both cultivars). In addition, infected spikelets of Sumai3 showed increased SA content, whereas the infected spikelets of Falat showed no increase in SA content (Fig. 2). Following SA treatment of Falat, its SA content was significantly induced, by about

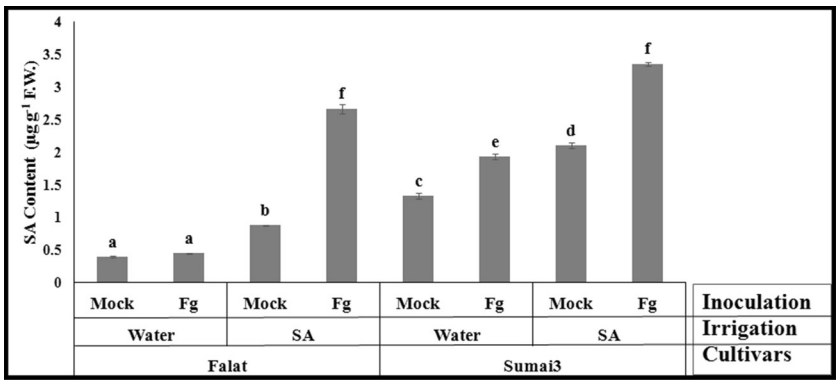

Fig. 2 Effect of *F. graminearum* inoculation and soil drench application of SA on content of SA in spikelets of Falat (Susceptible) and Sumai3 (Resistant) wheat cultivars at 4 days after inoculation. Irrigation by SA solution was done 1 day before inoculation. *Columns* indicate mean ± SE based on three replicates. Means with *different letters* for each cultivar indicate a significant difference at $P \leq 0.05$ using Duncan multiple range test. The groups are Mock (plants inoculated with mung bean broth), Fg (plants inoculated with *F. graminearum*), SA (plants irrigated with 200 μM sodium salicylate solution 1 day before inoculation), and Water (plants irrigated with water 1 day before inoculation)

2- and 5-folds in mock-inoculated and infected plants, respectively, as compared to the control. SA treatment also caused induction of SA content in mock and *F. graminearum* inoculated Sumai3.

Basal H_2O_2 content in Sumai3 (resistant) was about twofold higher compared to Falat. Both *F. graminearum*-infected Falat and *F. graminearum*-infected Sumai3 showed an increase in H_2O_2 at 4 dai. Irrigating Falat and Sumai3 with SA solution enhanced H_2O_2 production in comparison with their controls (Fig. 3a).

Antioxidant enzyme activity exhibited completely different patterns in response to *F. graminearum* inoculation in resistant and susceptible cultivars (Table 1). SOD activity decreased about sixfold in *F. graminearum*-infected Falat, whereas it increased about threefold in *F. graminearum*-infected Sumai3 as compared to their respective controls. Moreover, Falat inoculation following soil drench application of SA caused induction of SOD activity up to fourfold after *F. graminearum* inoculation when compared to inoculated plants irrigated with water. Results also indicated that following SA treatment in Sumai3, SOD activity was significantly increased, by about threefold in *F. graminearum*-inoculated plants, when compared with control plants.

POX activity in Sumai3 spikelets was significantly higher (about fivefold) in comparison with Falat. *F. graminearum* inoculation also caused enhancement of POX activity in both cultivars; however, this increase was much higher in Sumai3 than Falat (Table 1). Furthermore, POX activity was enhanced by SA treatment in Falat and Sumai3, in both the mock and *F. graminearum* inoculations.

Results for CAT activity were different from the other antioxidant enzymes. *F. graminearum* infection induced CAT activity in the susceptible cultivar and reduced it in the resistant one (Table 1). Furthermore, SA pretreatment reduced CAT activity by about threefold in infected Falat and twofold in infected Sumai3.

For PPO, the changes were more modest than with other enzymatic activities. *F. graminearum* infection reduced PPO activity in Falat, whereas increased it in Sumai3. Treatment of Falat and Sumai3 with SA also caused an increase in PPO activity (Table 1).

It was found that *F. graminearum* infection causes a significant increase of MDA production in both cultivars (Fig. 3b). Additionally, SA pretreated susceptible and resistant cultivars showed an increment of MDA content in *F. graminearum*- and mock-inoculated spikeletes.

In this step, to have a more conclusive study, some non-enzymatic antioxidant activity was also investigated. Our data showed that significant changes in activity occurred in response to *F. graminearum* in both cultivars. DPPH radical scavenging activity increased in both cultivars after *F. graminearum* inoculation, but this induction was higher in the resistant cultivar when compared to the susceptible one (Fig. 4a). Besides, SA treatment increased DPPH radical

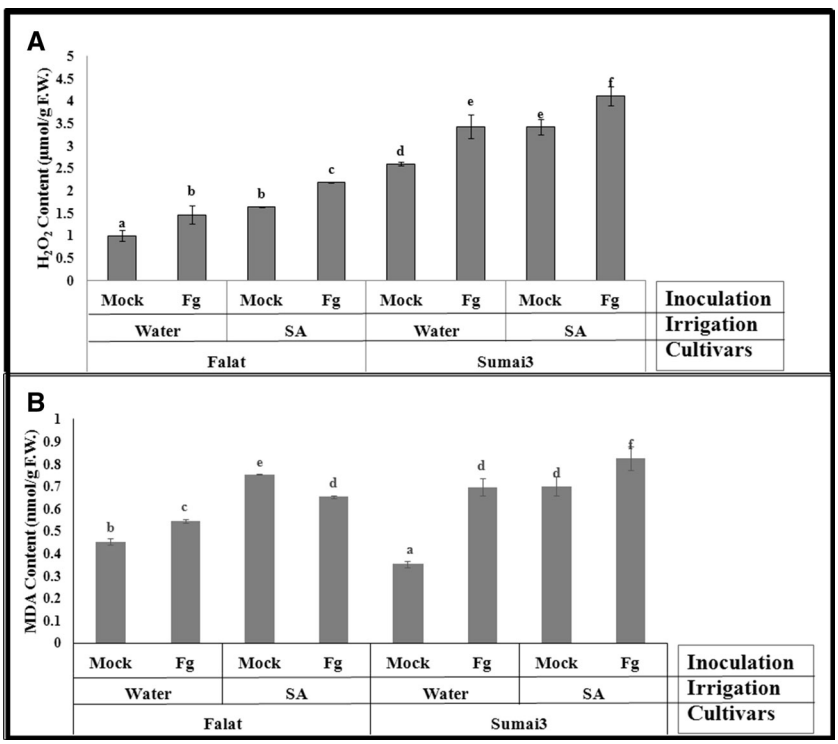

Fig. 3 Effect of *F. graminearum* inoculation and soil drench application of SA on content of **a** hydrogen peroxide (H_2O_2) and **b** malondialdehyde (MDA) in spikelets of susceptible (Falat) and resistant (Sumai3) wheat cultivars at 4 days after inoculation. Irrigation by SA solution was done 1 day before inoculation. *Columns* indicate mean ± SE based on three replicates. Means with *different letters* for each cultivar indicate a significant difference at $P \leq 0.05$ using Duncan multiple range test. The groups are Mock (plants inoculated with mung bean broth), Fg (plants inoculated with *F. graminearum*), SA (plants irrigated with 200 µM sodium salicylate solution 1 day before inoculation), and Water (plants irrigated with water 1 day before inoculation)

Table 1 Specific activity of four antioxidative enzyme in Falat (susceptible) and Sumai3 (resistant) wheat cultivars treated with *F. graminearum* and salicylic acid

Treatment	Genotypes	Antioxidant enzymes activity [U mg^{-1}(protein)]			
		SOD	POX	CAT	PPO
Control	Falat	0.061 ± 0.000d	0.037 ± 0.003a	0.089 ± 0.001d	0.027 ± 0.000b
	Sumai3	0.053 ± 0.005c	0.190 ± 0.000d	0.158 ± 0.000g	0.026 ± 0.000b
+*F. graminearum*	Falat	0.011 ± 0.001a	0.039 ± 0.000ab	0.115 ± 0.000f	0.021 ± 0.000a
	Sumai3	0.167 ± 0.005g	0.730 ± 0.001f	0.078 ± 0.000c	0.052 ± 0.000d
+SA	Falat	0.075 ± 0.000e	0.048 ± 0.002bc	0.052 ± 0.000b	0.038 ± 0.000d
	Sumai3	0.081 ± 0.000f	0.690 ± 0.001e	0.096 ± 0.000e	0.064 ± 0.000e
+SA + *F. graminearum*	Falat	0.043 ± 0.000b	0.055 ± 0.001c	0.033 ± 0.000a	0.036 ± 0.000c
	Sumai3	0.178 ± 0.00h	0.660 ± 0.002g	0.054 ± 0.000b	0.106 ± 0.000f

Irrigation by SA solution was done 1 day before inoculation. Data are the means (±SE) of three replicates. Means with different letters for each cultivar indicate a significant difference at $P \leq 0.05$ using Duncan multiple range test. The groups are Control (plants irrigated with water and inoculated with mock), +Fg (plants irrigated with water and inoculated with *F. graminearum*), +SA (plants irrigated with 200 μM sodium salicylate solution and inoculated with mock), and +SA + Fg (plants irrigated with 200 μM sodium salicylate solution and inoculated with *F. graminearum*)

SOD superoxide dismutase, *POX* peroxidase, *CAT* catalase, *PPO* polyphenol oxidase

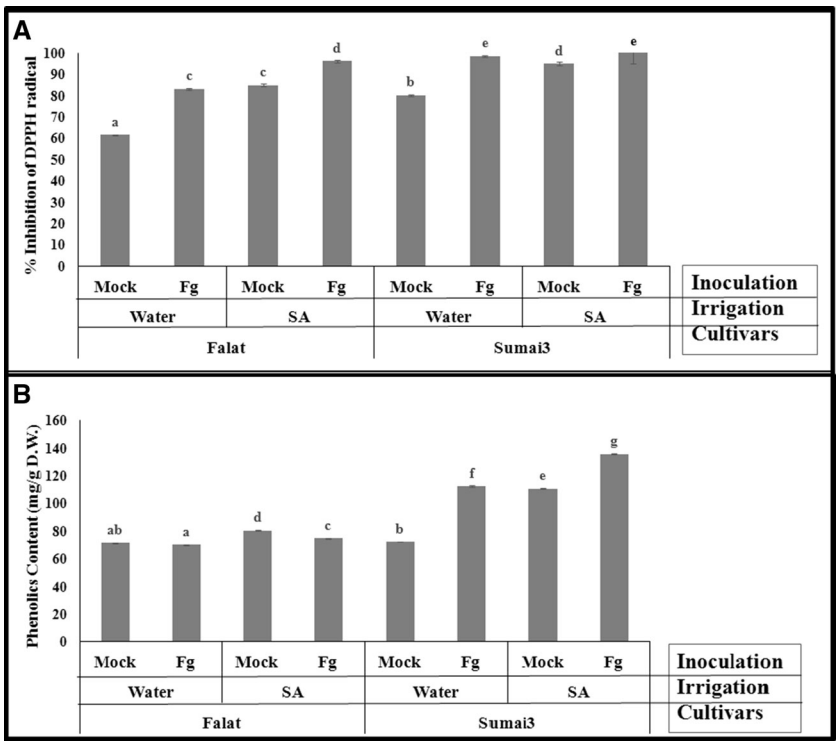

Fig. 4 Impact of *F. graminearum* inoculation and soil drench application of SA on **a** DPPH scavenging activity and **b** total phenolics content in susceptible (Falat) and resistant (Sumai3) wheat cultivars at 4 days after inoculation. Irrigation by SA solution was done 1 day before inoculation. *Columns* indicate mean ± SE based on three replicates. Means with *different letters* for each cultivar indicate a significant difference at $P \leq 0.05$ using Duncan multiple range test. The groups are Mock (plants inoculated with mung bean broth), Fg (plants inoculated with *F. graminearum*), SA (plants irrigated with 200 μM sodium salicylate solution 1 day before inoculation), and Water (plants irrigated with water 1 day before inoculation)

scavenging activity in both inoculated and non-inoculated tissues of both cultivars. Our observations revealed that phenolic content was enhanced significantly following *F. graminearum* inoculation in Sumai3 (Fig. 4b). Whereas, no significant changes between inoculated and non-inoculated Falat was observed. Additionally, SA treatment also enhanced phenolic content significantly in Falat and Sumai3.

To better understand the underlying mechanisms of resistance against *F. graminearum*, the activities of PAL and LOX were investigated as key enzymes in production of SA and jasmonic acid (JA). Infection with *F. graminearum* significantly increased PAL activity and gene expression in both cultivars (Fig. 5). This increase was more pronounced in Sumai3 in comparison with Falat. In addition, pretreatment with SA increased PAL activity and

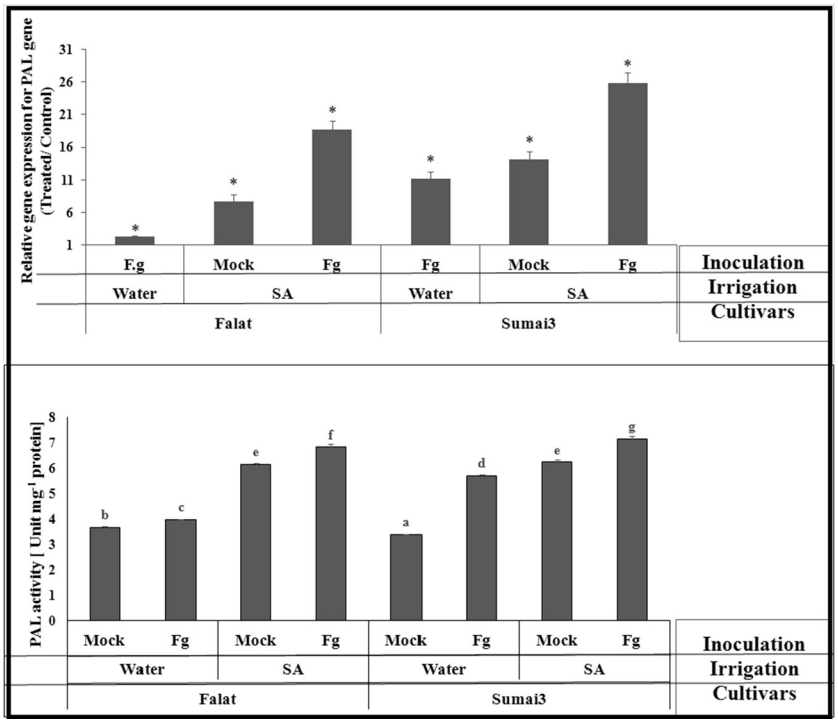

Fig. 5 **a** RT-qPCR analyses of phenylalanine ammonia lyase (PAL) gene transcript in Falat (susceptible) and Sumai3 (resistant) wheat cultivars treated with *F. graminearum* and SA. Irrigation by SA solution was done 1 day before inoculation. The relative fold change of target gene transcript was calculated using the comparative cycle threshold method. Treated samples were quantified relative to the control (mock inoculated) at the same time point. *β-Actin* was used as an endogenous control to normalize the data for input RNA difference between the various samples. Mean values and standard error (±SE) for at least twice ($n \geq 2$) independent assays are shown. *Asterisk* indicates significant difference between control and treated samples: * <0.05. **b** Activity of PAL in Falat and Sumai3 infected by *F. graminearum* and effect of salicylic acid treatment on PAL activity. *Columns* indicate mean ± SE based on three replicates. The groups are Mock (plants inoculated with mung bean broth), Fg (plants inoculated with *F. graminearum*), SA (plants irrigated with 200 µM sodium salicylate solution 1 day before inoculation), and Water (plants irrigated with water 1 day before inoculation)

gene expression by about 3- and 7-fold in the susceptible cultivar, respectively. Results also indicated that following SA treatment in Sumai3, PAL activity and gene expression were significantly induced in *F. graminearum*-inoculated plants when compared with control plants.

Basal LOX activity in spikeletes of Falat was about fourfold higher than in Sumai3 (Fig. 6). The results also showed a decrease in LOX activity in both cultivars after inoculation with *F. graminearum* and SA treatment (Fig. 6).

We found that *F. graminearum* treatment increased callose content in both cultivars as compared to control plants irrigated with water (Fig. 7). In addition, SA treated infected plants of both cultivars showed about 1.5-fold more callose content than infected plants irrigated with water.

Taken together, these results indicated that *F. graminearum* inoculation resulted in alteration in antioxidant systems in both cultivars and when the results obtained for Sumai3 were compared with those for Falat, a greater induction level and activity was observed in Sumai3 for the majority of the parameters, including SA and phenolic content, DPPH radical scavenging, PAL, and POX activities. The protective effect of SA in Falat and Sumai3 suggested that SA pretreatment increased activation of defense signaling in infected Falat and Sumai3. SA pretreatment appears to have induced a certain level of resistance to FHB in Falat by triggering defense signaling. Moreover, it was observed that activities of SOD and PPO decreased in infected Falat, whereas SA pretreatment could induce the activity of these enzymes in infected Falat with *F. graminearum*.

Discussion

Investigation to find wheat traits that provide resistance against *F. graminearum* has attracted a great deal of attention. Although transcriptomic and proteomic studies of resistance are at the center of such investigations, they suffer from some limitations. As an example, in transcriptomic studies, differentially expressed genes are not

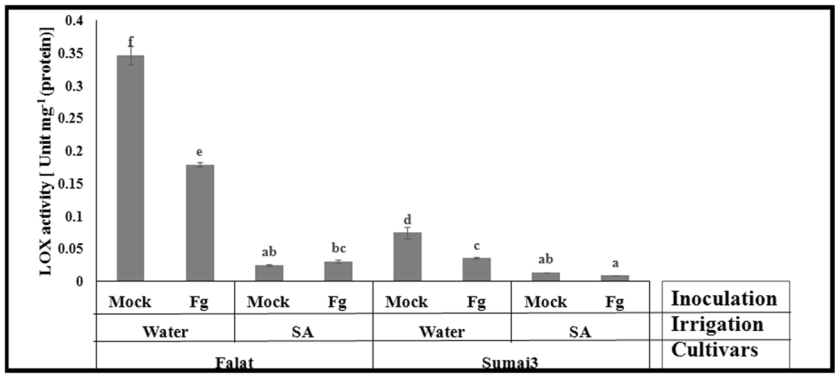

Fig. 6 Impact of *F. graminearum* inoculation and soil drench application of SA on LOX activity in Falat (susceptible) and Sumai3 (resistant) wheat cultivars at 4 days after inoculation. Irrigation by SA solution was done 1 day before inoculation. *Columns* indicate mean ± SE based on three replicates. Means with different letters for each cultivar indicate a significant difference at $P \leq 0.05$ using Duncan multiple range test. The groups are Mock (plants inoculated with mung bean broth), Fg (plants inoculated with *F. graminearum*), SA (plants irrigated with 200 µM sodium salicylate solution 1 day before inoculation), and Water (plants irrigated with water 1 day before inoculation)

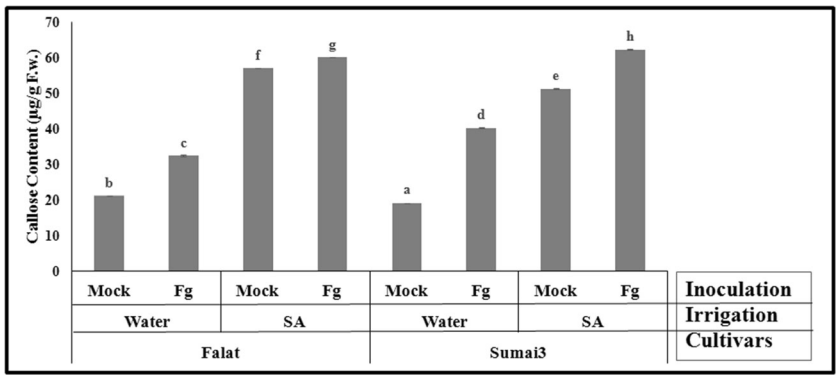

Fig. 7 Effect of *F. graminearum* and SA treatment on callose content in susceptible (Falat) and resistant (Sumai3) wheat cultivars at 4 days after inoculation. Irrigation by SA solution was done one day before inoculation. *Columns* indicate mean ± SE based on three replicates. Means with *different letters* for each cultivar indicate a significant difference at $P \leq 0.05$ using Duncan multiple range test. The groups are Mock (plants inoculated with mung bean broth), Fg (plants inoculated with *F. graminearum*), SA (plants irrigated with 200 µM sodium salicylate solution 1 day before inoculation), and Water (plants irrigated with water 1 day before inoculation)

necessarily causal to a particular trait or response. Moreover, not all the genes that are involved in a response or trait are detected due to expression changes (Wertheim 2012). Study of physiological responses of plants to stresses can partly unravel obscure issues regarding complicated pathways involved in the modulation of molecular signaling. In the current study, several physiological parameters have been investigated to better understand the differences between susceptible and resistant genotypes treated by *F. graminearum*. This study, for the first time, reports the H_2O_2-related mechanisms involved in basal resistance of wheat against *F. graminearum*.

There is compelling evidence showing that SOD along with other oxidative stress defense-related enzymes can eliminate detrimental effects of ROS, as an early response against plant–pathogen interactions (Helepciuc and others 2014). SOD protects cells from oxidative stress by converting the destructive superoxide radical into a less dangerous form, hydrogen peroxide and molecular oxygen. Hence, it constitutes the first line of defense against ROS-induced oxidative burst. Subsequently, the peroxide molecules are degraded by CAT and POX (Xu and others 2013). Decreased SOD activity can lead to uncontrolled production of oxygen-free radicals (ROS) and lipid peroxidation of membranes which in turn would result in harmful ion leakage.

According to our results, activity of some antioxidant enzymes, such as SOD and PPO is greatly enhanced after *F. graminearum* inoculation in the resistant genotype, whereas it decreased in the susceptible genotype. Similarly, increased activity of SOD in the resistant genotype in response to *Fusarium* infection was reported by García-Limones and others (2002). This is in agreement with our observations in the resistant wheat inoculated by *F.*

graminearum. Hence, it is plausible that the increased activity of SOD can be interpreted as an attempt to overcome the oxidative stress.

Defensive roles of PPO in plants were reviewed by Constabel and Barbehenn (2008). The beneficial role of PPO during plant pathogenesis can be seen in light of the fact that primarily toxicity of PPO-produced quinones for host cell and pathogen may accelerate cell death (hypersensitive response). Direct toxicity of quinones against pathogens has also been proposed by Mayer and Harel (1979). Secondly, PPO forms a physical barrier by consumption of ROS as a substrate within the cell wall (Li and Steffens 2002; Mohammadi and Kazemi 2002). The barrier is formed through cross-linking proteins or other phenolics. Raju and others (2008) reported higher activities of PPO in the resistant cultivar of chickpea treated by *F. oxysporum*. This is assumed to be involved in restricting plant disease development by formation of lignin (Mohammadi and Kazemi 2002). Similar results were obtained by Silva and others (2008) in a similar study on *Coffea arabica*.

Further, our results showed that *F. graminearum* infection may lead to increased SA content as well as PAL and POX activities in both resistant and susceptible cultivars. POX as a ROS scavenger and cell wall modifier showed higher activity in Sumai3 as compared to Falat. POXs have been reported to be mainly present in the cell wall and to catalyze oxidation of various organic compounds like phenolics, lignin, or suberin. These are effective in construction, rigidification, and eventual lignification of the cell wall against pathogen penetration. Pathogen-induced POX activity has been reported in cucumbers (Chen and others 2000), rice (Reimers and others 1992), tomatos (Mohan and others 1993), and tobacco (Goy and others 1992).

According to our results, basal resistance to *F. graminearum* in the resistant cultivar is associated with induction of H_2O_2 production, enhanced POX activity, and phenolic accumulation.

PAL is a key plant enzyme at the entry point of the phenylpropanoid pathway. It should be noted that an increase in PAL activity may be related to the plant defense system through biosynthesis of active metabolites, such as phytoalexins, phenols, lignins, and salicylic acid in plant defense pathways (Mandal and others 2009). This study demonstrated that activity of PAL was induced by the pathogen in both cultivars, while, this increase was stronger in Sumai3 than Falat inoculated with *F. graminearum*. According to the results, a direct correlation was observed between the increase of PAL activity and induction of its gene expression in both cultivars. These observations suggested that PAL gene expression correlates with PAL activity. Current findings also proposed PAL activity to play an important role in resistance to head blight. Similarly, Chen and others (2000) reported that high levels of PAL were induced in cucumber roots inoculated with *Pythium aphanidermatum*. Furthermore, SA -induced PAL gene expression and activity in susceptible and resistant cultivars suggests a positive feedback in SA production.

Besides the enzymatic antioxidant system, induction of non-enzymatic antioxidants in the resistance genotype was observed. With regard to DPPH-free radical scavenging activity and phenolic content in response to *F. graminearum*, Sumai3 is potent in controlling abnormal ROS production. By contrast, a corresponding increase in phenolics content was not observed in *F. graminearum* inoculated spikelet of Falat. Increased total phenolic content in the resistant cultivar can play an important role in resistance and defense against *F. graminearum*. These results are in accordance with Ramamoorthy and others (2002) who reported a significant increase of phenolic content of tomato roots after *F. oxysporum* infection.

The favorable role of SA as a signal biomolecule in wheat defense against FHB was confirmed based on a recent study (Makandar and others 2012). According to this study, SA-triggered responsive genes enable the basal resistance of wheat against *F. graminearum*. Thus, SA was applied to induce resistance in susceptible Falat and resistant Sumai3 against FHB. It was found that soil drench application of SA prior to pathogen inoculation activated systemic resistance against *F. graminearum* and significantly reduced disease severity.

ROS and H_2O_2 trigger many defense responses and are involved in SA accumulation and cell death. This is supported by studies indicating that H_2O_2 treatment induces SA accumulation in tobacco (Leon and others 1995) and cell death in soybean cells (Shirasu and others 1997). Moreover, SA treatment caused induction of H_2O_2 and cell death in suspension cells of soybean infected with pathogen (Shirasu and others 1997).

As reported in several other studies, similarly an increase in H_2O_2 level was observed in the present work, following SA pretreatment (Wang and Liu 2012; Harfouche and others 2008; Chao and others 2010). Promotion of H_2O_2 accumulation by SA has been suggested to be related to increased SOD activity or the inhibition of enzymes responsible for H_2O_2 scavenging (Kang and others 2003; Krantev and others 2008; Chao and others 2010; Hayat and others 2010). H_2O_2 has been shown to be involved in defense against pathogens by triggering a hypersensitive response (Heath 2000).

The observation that POX, SOD, PPO, and PAL activities were strongly affected by application of SA in both cultivars supported the idea that their responses can be SA dependent. A similar result was also reported by Mandal and others (2009). Accordingly, exogenous application of SA to tomato infected by *F. oxysporum* resulted in an

increase in PAL and POX activities (Mandal and others 2009). In other studies, Xue and others (2014) and He and Wolyn (2005) reported that SA treatment in *Phaseolus vulgaris* and *Asparagus* plants enhanced PAL and POX activities, respectively, upon *F. oxysporum* infection. They suggested that enhanced activities of the aforementioned enzymes can strengthen the cell wall and restrict subsequent fungal penetration and infection. In our study, the activities of PAL and POX strongly increased in the SA-treated plants compared to the control; hence, it could provide a mechanism for enhanced resistance of wheat against *F. graminearum*.

Accumulations of phenolic compounds in the infected plants treated with SA can play an important role in resistance and defense against *F. graminearum*. The rise in phenolic compound content after treatment with SA may be due to increased PAL activity, as PAL has been reported to be associated with synthesis of phenolic compounds via phenylpropanoid pathway (Hahlbrock and Scheel 1989). Our results are also in accordance with the study by Ali and others (2007) on *Panax ginseng*. They showed that higher levels of phenolics were associated with higher levels of antioxidant activity.

Sood and others (2013) observed an increase in the levels of phenolic content in rice in response to SA treatments against sheath blight disease. Considering the antioxidative role of phenolic compounds in plant physiology, the simultaneous increase of phenolics levels and DPPH free radical scavenging activity suggests that the increased free radical scavenging activity might be due to the increased phenolic compound levels induced by SA treatment.

Callose and lignin are effective physical barriers and known as HR markers that are induced at infection sites to restrict pathogen spread (Zimmerli and others 2000; Donofrio and Delaney 2001; Roetschi and others 2001; Ton and Mauch-Mani 2004). It was shown that callose deposition in response to pathogen inoculation was higher in Sumai3 and that SA pretreatment increased its callose content in both genotypes. In line with these findings, Nishimura and others (2003) also showed that callose is produced in *Arabidopsis* plants inoculated with *Blumeria graminis*.

LOX enzyme is responsible for membrane degradation by deoxygenation of polyunsaturated fatty acids. *F. graminearum* infection resulted in a decrease and an increase in LOX activity in Sumai3 and Falat cultivars, respectively. In addition, SA treatment led to decreased LOX activity in both cultivars. A previous study by Nalam (2012) showed that the loss of function of *LOX1* and *LOX5* mutants resulted in enhanced resistance to *F. graminearum* infection. These mutants exhibited reduced cell death and a delayed hypersensitive response when challenged with an avirulent strain of *Pseudomonas syringae* pv tomato. Similarly, it was reported that silencing of the wheat 9-LOX homolog, TaLpx1 resulted in an enhanced resistance to *F. graminearum*.

Other studies on *Arabidopsis* showed that 9-LOXs caused susceptibility to *F. graminearum* through suppression of SA-mediated defense responses that are critical for basal resistance to this fungus, as also suggested by Nalam (2012). Nevertheless, some reports have indicated increased activity and expression of LOX against pathogen attack in other resistant cultivars of wheat. Devi and others (2000) and Mhaske and others (2013) reported that LOX activity increased in castor (*Ricinus communis*)- and pigeon pea (*Cajanus cajan*)-resistant genotypes after pathogen inoculation, respectively. One possible explanation of this discrepancy might be that monocots and dicots possess different defense strategies against pathogens. Moreover, the increased LOX activity may generate signal molecules such as JA, methyl JA/or lipid peroxides, which coordinately trigger specific defense responses. This signal molecule is mainly produced in response to necrotrophic pathogens (Mhaske and others 2013).

Conclusion

Our study provides an overview and novel insights into the *F. graminearum*–wheat interaction by analysis of enzymatic and non-enzymatic antioxidative pathways and some enzymes such as PAL and LOX which are involved in production of signaling molecules, including SA and JA. Our results showed that there are different physiological and biochemical response patterns against *F. graminearum* infection in the susceptible and resistant wheat cultivars compared, Falat and Sumai3. FHB resistant cultivar showed a large increase in phenolics and SA contents and activity of SOD, PPO, POX, and PAL in response to *F. graminearum* inoculation. The current findings suggest that these differences are associated with resistance. SA treatments of both susceptible and resistant cultivars of wheat from distinct genetic backgrounds render them more resistant to *F. graminearum* infection by altering the levels of the mentioned parameters regardless of genotype. It is indicated that a central role (whether direct or indirect) for the SA signaling pathway in activating wheat defense response against FHB is possible. The current results can provide novel insights for better recognition of the responsible mechanisms to regulate FHB resistance in wheat. The direct effect of SA is suggested to be further examined on the above-mentioned enzyme activities and their gene expression in conferring resistance to pathogens. We propose that the ability of the resistant cultivar in channeling ROS from $O_2^{\cdot-}$ to H_2O_2 as a substrate for POX

could be a determining factor for cell wall solidification (a defensive barrier) to enhance resistance against pathogen penetration. This defensive barrier was observed to be strengthened by callose deposition in the cell wall. LOX as a key enzyme in JA biosynthesis showed no role in basal resistance of wheat against FHB. Finally, our study suggested that the resistant wheat can direct ROS from a threat (a destructive factor) to an opportunity (a constrictive factor) by using the key regulator, that is, SA. We propose that SA played this role through regulation of the plant antioxidant system.

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Compliance with ethical standards

Conflict of interest The authors declare that there is no conflict of interest in this study.

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