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Disease Severity, Resistance Analysis, and Expression Profiling of Pathogenesis-Related Protein Genes after the Inoculation of *Fusarium equiseti* in Wheat

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Abstract: Wheat (*Triticum aestivum*) is an important cereal crop, grown throughout the temperate and in some tropical and sub-tropical zones, at higher elevations. Several biotic and abiotic factors influence the production of wheat. In the present study, two wheat varieties have been subjected to disease severity and resistance analysis against *Fusarium equiseti*. Disease severity analysis revealed Shafaq-2006 to be more resistant than Sahar-2006. Both varieties were further subjected to the expression analysis of six important defense-related genes by RT-PCR and quantitative real-time PCR. This analysis revealed that PR-1, TLP, Chitinase, and β-1,3-glucanase genes were highly expressed in Shafaq-2006 and possibly play a significant role in its defense mechanism. In addition, biochemical and physiochemical parameters were also studied to further explore the difference between resistant and susceptible varieties. With total proline and protein contents, sugar and chlorophyll contents also increased significantly in resistant variety. Likewise, higher relative water content, total plant length, and the high root–shoot ratio was observed in resistant plants, compared to susceptible wheat plants. These increases in chemical and physiological parameters might be related to the activation of the defense mechanism due to the higher expression of PR genes in the resistant wheat variety. These genes can further be employed for cloning into wheat and other transgenic crops to develop resistance against *F. equiseti*.

Keywords: plant defense mechanism; plant disease; pathogenicity; *Triticum aesitivum*; Shafaq-2006; Sahar-2006

1. Introduction

Wheat (*Triticum aestivum*) is regarded one of the major cereal crops that is being challenged by various biotic and abiotic stresses which influence and disturb metabolic processes in plants. Plants can be affected by many abiotic factors, including excessive temperature, nutrient deficiency, ultraviolet radiations, drought, pollution, and lack of oxygen as well as biotic agents, such as bacteria, fungi, viruses, and insects [1,2]. Various

fungal species have been known to cause severe diseases in plants. As a predominant soil saprophyte, *F. equiseti* is associated with fruit rots as well as dead and dying plant tissues. It also acts as an important pathogenic agent on various agricultural plants [3]. It is a typically soil-borne pathogen that frequently exists in subtropical and warm temperate regions [4]. *F. equiseti* is known to cause different diseases in many plants, such as root rot in winter, wheat and stem rot in maize [5], and Fusarium head blight in barley and wheat [6,7]. In a previous report, *F. equiseti* isolates obtained from saline sea-bed soil samples exhibited pathogenicity during seedling pre-emergence [8]. To overcome the influences of these unusual pathogens or environmental stresses, plants activate different defense systems [9].

Plants have established several constitutive and inducible defense mechanisms, including the expression of different genes in response to pest/pathogen infection. Hypersensitive reaction (HR) is the most effective response, which induces cell necrosis quickly around the infection site. This response is related to an integrated and coordinated set of metabolic changes involved in the inhibition of further pathogen entrance [10]. Biotic stresses affect the growth and development of plants and in response, they change the expression of different genes in their defense [11]. Plants trigger various signaling pathways soon after recognizing external changes and convert physical stress into a biochemical response. As a result, they stimulate the expression of stress-response genes [12].

Under stress, several proteins are accumulated in plants that are known as pathogenesis-related (PR) proteins. PR proteins are studied to be coded by the host plant specifically under biotic stresses [13]. These proteins are not only accumulated in the infected leaves, but are also produced systemically, associated with the development of systemic acquired resistance (SAR). It has been found that PR-proteins induce in many species of various plant families [14], which suggests their general role in the adaptation to different biotic stresses. Many studies show that the PR-proteins are accumulated more in resistant plants as compared to susceptible plants [15]. Artificial constitutive over-expression of PR-proteins has been proved to be very useful in inducing stress resistance [9].

Several PR-1 proteins are identified in Arabidopsis (*Arabidopsis thaliana*), rice (*Oryza sativa*), barley (*Hordeum vulgare*), wheat, maize (*Zea mays*), tomato (*Solanum lycopersicum*), and pepper (*Capsicum annuum*) [16]. Among PR-proteins, PR-1 is the most abundant, which is accumulated in about 1–2% of the total protein contents in a leaf [17]. PR-1 gene expression serves as a molecular marker that indicates a defense response against pathogens [17]. Thionins are usually small (5 kDa) cysteine-rich PR proteins initially isolated from cereals. They have widespread in vitro antibacterial and antifungal activity [18]. Thionin accumulation in the cell wall of resistant wheat spikes indicates its role in plant defense [19]. Thaumatin-like proteins (TLPs) in plants are associated with developmental processes and defense against phytopathogens and elicitors [20]. Due to the antifungal property of TLPs, they have been efficiently used in genetic engineering for producing disease-resistant plants [21]. Chitinases are accumulated in plants in response to fungal infection as well as other abiotic and biotic stressors [22]. Studies have reported that the expression of Chitinase increases against phyto-pathogens and its induction is stronger in resistant varieties of wheat [23], sugar beet (*Beta vulgaris*) [24] and tomato [25]. The Chitinase gene has been reported to be induced in response to fungal infection in maize and wheat [26]. Plant β-1,3-glucanases are members of the PR-2 family of pathogenesis-related proteins, which play an important function in plant defensive responses against pathogen infection [22]. Plant β-1,3-glucanases have been recognized as one of the important components of defense mechanism against phytopathogens [27]. They are strongly induced when plants respond to infection or wounding by fungal, viral, or bacterial pathogens [28]. Another group of PR proteins is the plant defensins, which have been identified in different plant families, including the *Solanaceae, Brassicaceae,* and *Fabaceae*. These proteins are either constitutively expressed in reproductive or storage organs or induced in the result of injury or pathogen attack, as part of a systemic defense response [29]. The antifungal activity of defensins has been studied extensively, while only a few plant defensins have been known to prevent the growth of bacteria [30].

Thirty different wheat varieties have been cultivated in Pakistan; of these, 22 have been considered as high-yielding and eight as low-yielding [31]. Shafaq-2006 is currently the major cultivated wheat variety that exhibits resistance against aphids [32]. It has also been reported as a high-yielding variety with a durable resistance mechanism against yellow rusts [32]. Sahar-2006 is susceptible in its reactions against different inoculum [33]. Under natural field conditions, this variety behaves as moderately resistant to abiotic stresses [34]. This experiment has been designed to study the behavior of important pathogenesis-related genes in two different wheat varieties (Shafaq-2006 and Sahar-2006). These genes can further be used for cloning into wheat and other transgenic crops to create resistance to *F. equiseti*.

2. Materials and Methods

2.1. Fungus Culture and Inoculum Preparation

The identified fungus strain *Fusarium equiseti* was obtained from the National Agriculture Research Center (NARC), Islamabad, Pakistan. *F. equiseti* was grown on Sabouraud Dextrose Agar (SDA) medium for seven days at 25 °C in an incubator (Figure 1). Pure culture of this fungus was obtained by pouring 5 mL of sterilized distilled water and scraping the agar surface with the help of a spatula to isolate fungal spores in petri dishes. Subsequently, the spore suspension was filtered using a muslin cloth, and the spore number was counted and adjusted to 10^7 mL^{-1} by hemocytometer. This culture was further used for the inoculation.

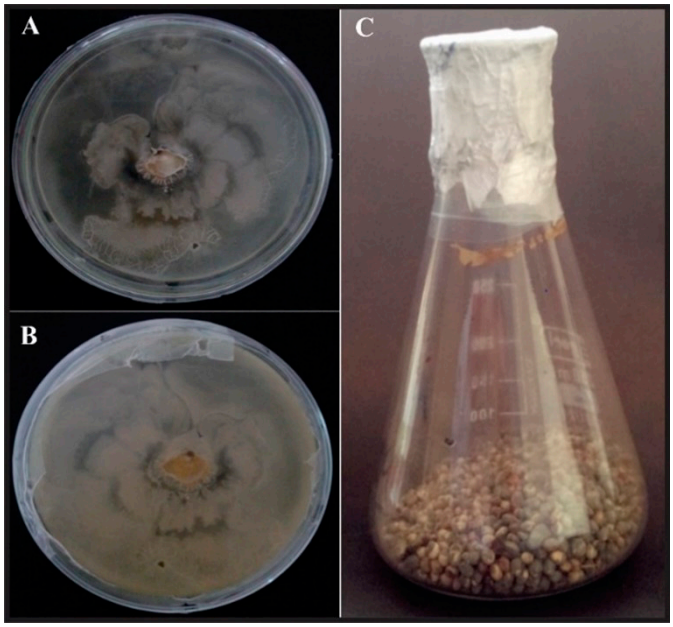

Figure 1. Growth of *Fusarium equiseti* on SDA at 25 °C and inoculum preparation. (**A**) Plate from front side, (**B**) Plate from reverse side, and (**C**) Inoculation preparation of fungus.

2.2. Seed Sterilization and Soil Inoculation

The seeds of two wheat varieties viz. Shafaq-2006 and Sahar-2006 were collected from the National Agriculture Research Center (NARC), Islamabad. Seeds of both wheat varieties were soaked in 75% ethanol for 3 min for surface sterilization and washed thoroughly with distilled water, three times. The sterilized seeds (eight seeds per pot) were sown in plastic pots for 21 days in a growth chamber at 25 °C, 80% relative humidity and 14 h photoperiod. *F. equiseti* was used for the standard systemic inoculation method [35]. Briefly, sorghum seeds were sterilized in 70% ethanol, rinsed thrice with distilled water, and soaked overnight in distilled water. The next day, sorghum seeds were dried on filter papers and autoclaved at 121 °C for 21 min. Autoclaved sorghum seeds were inoculated by mixing 1.15 g of *F. equiseti* spore suspension (filtered using a muslin cloth) in a flask

and kept in an incubator at 25 °C for 15 days. The flask was shaken daily to mix the fungus evenly with the sorghum seeds. Two grams of inoculated sorghum seeds were mixed in one kg of autoclaved soil. Each one kg fungal inoculated soil was used for four pots and seeds of each wheat variety were sown in respective pots. In addition, the seeds of both wheat varieties were also grown in non-inoculated soil (autoclaved soil without fungal inoculation), which served as control. The plants were irrigated on a daily bases to maintain soil humidity. The experiment involved three replicates for each treatment, including control. Harvesting was done 3 weeks after sowing.

2.3. Disease Severity Analysis

Lesions were counted at the time of harvesting by using a standard protocol [36]. All the plants were observed carefully to calculate disease severity in percentage.

2.4. Biochemical Parameters

Different biochemical parameters were measured to see biochemical changes in resistant and susceptible wheat varieties. Leaves of both varieties were used to measure sugar contents, following the protocol of Dubois et al. [37], with slight modifications [38]. The methods of Bates et al. [39] and Lowry et al. [40] were followed to determine proline and protein contents, respectively. The method of Arnon [41] was used to determine chlorophyll contents.

2.5. Physiological Parameters

Different physiological parameters were also studied to analyze the effects of *F. equiseti* on resistant and susceptible wheat varieties. A measuring tape was used to measure the length of roots and shoots of both varieties during harvesting, and their fresh weight was recorded. Fresh samples were kept in an oven at 70 °C for 24 h to calculate their dry weight.

Leaf relative water contents (RWCs) of plants were measured by using the standard method of Whetherley [42]. For this purpose, the fresh leaf weight of the samples was measured and placed in distilled water. After 24 h, the weight of fully turgid leaves was recorded, and leaves were kept in an oven at 70 °C. The dry weight of these samples was determined after 72 h to calculate leaf RWCs.

2.6. RNA Extraction, Quantification, and cDNA Synthesis

Total RNA was extracted from leaves of three-week-old plants using a Thermo scientific® Gene JET plant RNA purification kit. By using Nanodrop, the quantification of RNA samples was performed and stored at –80 °C for further use. Total RNA was subjected to cDNA synthesis by using a Thermo scientific® cDNA synthesis kit and stored at –80 °C.

2.7. Primer Designing, RT-PCR, and qRT-PCR Analysis

Primers were designed to amplify six PR-family genes (Table 1) using Primer3 Input (http://bioinfo.ut.ee/primer3-0.4.0/, accessed on 20 December 2019).

Table 1. Primer sequences used for RT-PCR and wheat genes they are targeting.

Gene	Accession Number	Forward Primer (5′ → 3′)	Reverse Primer (5′ → 3′)	Product Size (bp)
PR-1	HM489878.1	GCCAGCTACTACTCTCTCCG	AGGTATCCCATGCACGACTC	175
Thionins	AY253444.1	AAGCACTTCTGGATTTCGCC	CATCCTGTTCATCGCTGCAG	168
TLP	KJ764822.1	TTCCTCCTCCTGGCTGTTTT	ATATCCTCCCGGCTTTGGTG	175
Chitinase 2	AB029935.1	ACGGCGATATGGTTCTGGAT	TAGCGCTTGTAGAACCCGAT	209
β-1,3-glucanase	DQ090946.1	CTACAGGTCCAAGGGCATCA	GCGGCGATGTACTTGATGTT	210
Defensin	KJ551546.1	TGTCCAATAAGAACTGCGCG	TGGTTCCATGGGCTAGCTAG	161
Actin	GQ339780.1	GAGAAGCTCGCATATGTGGC	TCCAGCAGCTTCCATACCA	180

RT-PCR was performed to check primer specificity for the amplification of six selected genes, such as PR-1 (HM489878.1), Thionins (AY253444.1), Chitinase 2 (AB029935.1), β-1,3-glucanase (DQ090946.1), Thaumatin like protein (TLP) (KJ764822.1), and Defensin

(KJ551546.1) from both varieties. The actin gene (GQ339780.1) was used as a reference control. Total cDNA was used as a template for the RT-PCR.

RT-PCR was performed in a 25-µL reaction mixture comprising of 16 µL water, 2.50 µL 10× PCR buffer, 1.50 µL dNTPs, 1.50 µL $MgCl_2$, 1 µL template, 0.50 µL Taq polymerase, and 1 µL from each forward and reverse primers (Thermo scientific®). The following thermal profile was set up for the reactions: 94 °C for 5 min, followed by 25 cycles of 94 °C for 40 s, 49 °C for 1 min, and 72 °C for 1 min, and the final extension at 72 °C for 5 min. 1.50% agarose gel was used to run the PCR product.

The qRT-PCR was carried out by using Applied Biosystems 7300 Real-Time PCR System. A volume of 3 µL first strand cDNAs and SYBR Green PCR Master Mix (Thermo scientific®) was used to perform thermal cycling with following conditions: initial denaturation at 95 °C for 1 min, 40 cycles of denaturation at 95 °C for 15 s, annealing at 49 °C for 15 s, and extension at 72 °C for 45 s. Relative quantitation was calculated and normalized to the housekeeping Actin gene.

2.8. Statistical Data Analysis

The analysis of collected data was carried out by using the Statistics 8.1 software. Statistical analyses were performed using two-way ANOVA followed by the Tukey test. The results are presented as mean ± SDa. The differences among groups were considered statistically significant at a p-value < 0.05.

3. Results

3.1. Disease Severity Profiling

Both varieties showed symptoms on leaves after 15–20 days of germination in *F. equiseti* inoculated soil. No symptoms appeared on control plants. These results confirm the successful systemic inoculation of fungus. Shafaq-2006 showed less disease symptoms than Sahar-2006. In addition, comparing to control, the less number of plants were observed under fungal treatments, where most of the plants of susceptible variety (Sahar-2006) died as compared to resistant variety (Shafaq-2006) (Figure 2). For Shafaq-2006, 2% diseased plants were observed, whereas 18.80% diseased plants were found for Sahar-2006, as shown in Table 2.

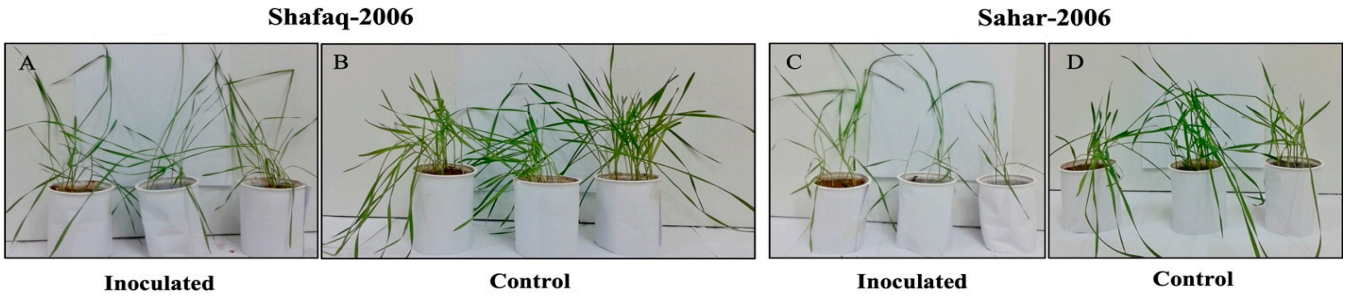

Figure 2. Disease severity analysis of the resistant (Shafaq-2006), and susceptible (Sahar-2006) wheat varieties. (**A**) Shafaq-2006 (Inoculated), (**B**) Shafaq-2006 (Control), (**C**) Sahar-2006 (Inoculated), and (**D**) Sahar-2006 (Control).

Table 2. Mean number of leaf spots and diseased plants' percentage.

Variety	Treatment	Mean Number of Spots per Leaf	Diseased Plants (%)
Shafaq-2006	Control	0	0
	Inoculated	4	2
Sahar-2006	Control	0	0
	Inoculated	16.60	18.80

3.2. Biochemical Parameters

Different biochemical parameters were measured in order to analyze the effects of *F. equiseti* on susceptible and resistant wheat varieties. Total proline and protein contents significantly increased by 44.66% and 68.07%, respectively, in the resistant variety (Shafaq-2006) as compared to the susceptible variety (m) under *F. equiseti* inoculation. The amount of total proline contents was the same in control plants of both varieties (Figure 3A), but the total proline contents of inoculated Sahar-2006 decreased 29.28%, compared to control. While a significant difference was observed in protein contents of both varieties in control plants. High protein contents (32.58%) were observed in control plants of Shafaq-2006 than Sahar-2006 (Figure 3B). The analysis of sugar contents showed a significant difference between the inoculated and control wheat varieties. Where, a significant increase in the sugar contents of Shafaq-2006 was recorded, compared to control. Similarly, a significant difference was observed in the sugar contents of both inoculated varieties, where Shafaq-2006 exhibited 51.10% higher sugar contents, compared to Sahar-2006. Additionally, the sugar content of Shafaq-2006 increased by 35.50% compared to that of Sahar-2006 under control conditions (Figure 3C). However, the sugar content of Sahar-2006 significantly decreased by 18.58% under fungal inoculation, compared to control.

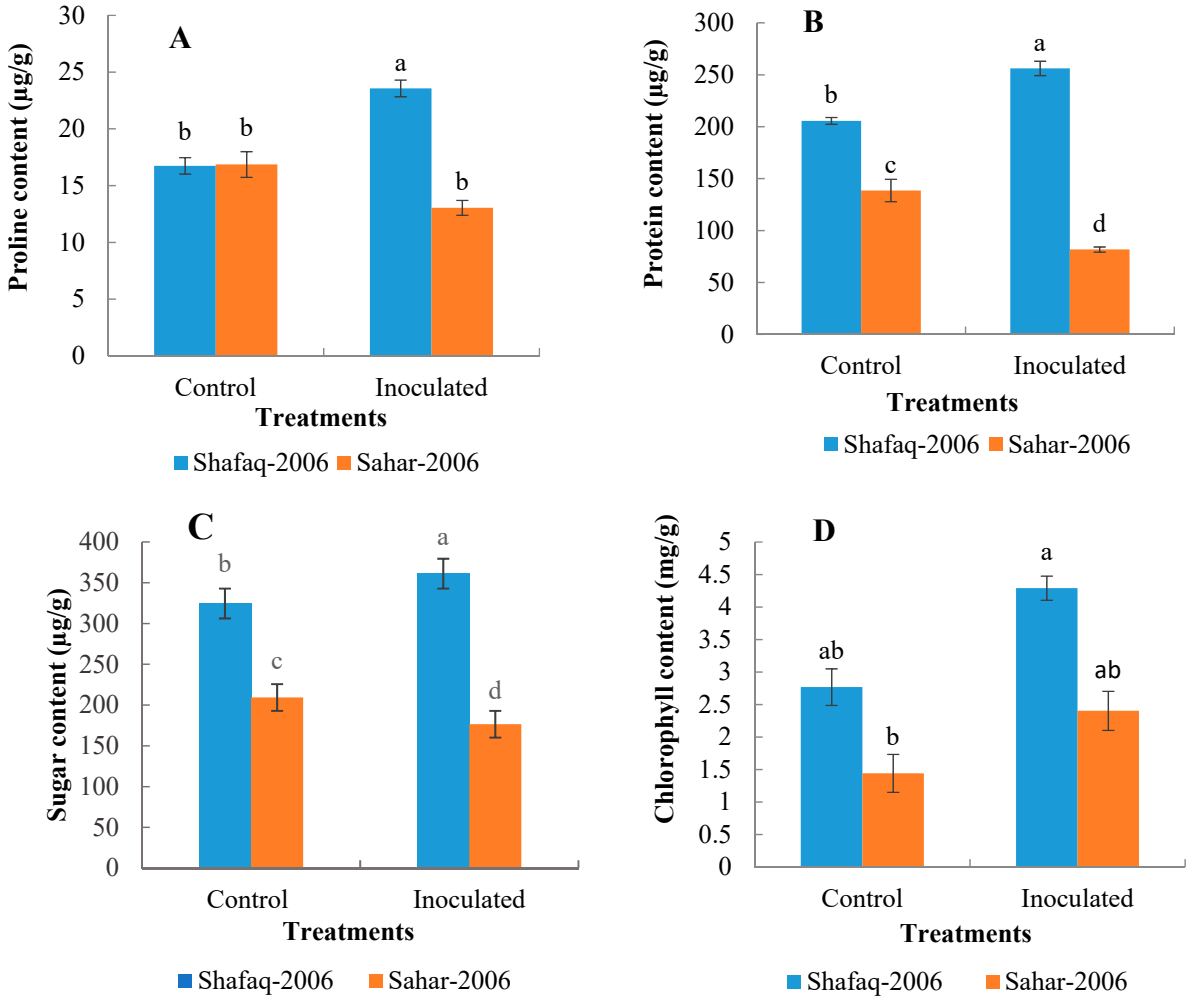

Figure 3. Measurement of the biochemical parameters, such as (**A**) Proline contents, (**B**) Protein content, (**C**) Sugar content, and (**D**) Chlorophyll content after the systemic inoculation of *Fusarium equiseti* in the Shafaq-2006 and Sahar-2006. All the means sharing common letter(s) are not significantly different at $p < 0.05$ and vertical bars represent the standard error of means (n = 3), while all the means with different letter(s) are significantly different at $p < 0.05$.

Although there was not a significant difference in total chlorophyll contents of both varieties, the chlorophyll contents of Shafaq-2006 increased by 43.99% to that of Sahar-2006,

which exhibited a considerable reduction in chlorophyll contents under fungal inoculation. Higher chlorophyll contents were observed in the control plants of resistant variety than susceptible one (Figure 3D). However, the inoculated Sahar-2006 exhibited 40.02% higher total chlorophyll contents as compared to control.

3.3. Physiological Parameters

Different physiological parameters were also measured to assess the effect of *F. equiseti*. The RWC of Shafaq-2006 was observed to be significantly higher under inoculation as well as control conditions, compared to Sahar-2006. In control, the higher RWCs (36.15%) were observed in Shafaq-2006, compared to Sahar-2006. However, an increase in RWC was noted under inoculated treatment, where Shafaq-2006 exhibited 55.40% increase in RWC, compared to Sahar-2006 (Figure 4A). However, there was no significant difference in RWC of Sahar-2006 under inoculation and control conditions. In the case of root–shoot ratio, both varieties did not show a significant difference between their inoculated and control plants. The root–shoot ratio of inoculated Sahar-2006 rather decreased than control. However, a little increase in the root–shoot ratio of inoculated Shafaq-2006 was recorded, compared to control. However, the results exhibited a significant difference in inoculated treatment, where in, the root–shoot ratio of Shafaq-2006 increased by 58.11% in comparison with Sahar-2006 (Figure 4B). In addition, plant length was also analyzed, which clearly indicated the susceptibility and resistance of Sahar-2006 and Shafaq-2006 to *F. equiseti*, respectively. Because the plant length of inoculated Shafaq-2006 was significantly increased by 20.68%, compared to control, it increased by 55.21% to that of Sahar-2006 under *F. equiseti* inoculation treatment. However, Shafaq-2006 exhibited a higher plant length (15.94%), compared to Sahar-2006 in control (Figure 4C). Moreover, the plant length of inoculated Sahar-2006 also decreased by 48.86% compared to that of control.

3.4. Expression Analysis by RT-PCR and qRT-PCR

RT-PCR was used in order to check the bands' intensity of six genes, including PR-1, Thionins, Thaumatin-like protein (TLP), Chitinase 2, β-1,3-glucanase, and Defensin in inoculated Shafaq-2006 wheat variety. PR-1 and TLP genes were observed to be more expressed in inoculated Shafaq-2006 by observing higher bands' intensity. Moreover, the bands' intensity of β-1,3 glucanase and Chitinase 2 was observed to be higher (Figure 5). These results indicate a possible role of these genes in resistance against *F. equiseti* stress in Shafaq-2006. Low bands' intensity of Thionins and Defensins genes were observed, which shows that these genes do not play any considerable role against *F. equiseti* stress in Shafaq-2006.

The expression analysis of six genes, including PR-1, Thionins, Thaumatin-like protein (TLP), Chitinase 2, β-1,3-glucanase, and Defensin was observed by qRT-PCR. The qRT-PCR analysis showed the higher expression of PR-1 in inoculated Shafaq-2006. The expression levels of TLP, β-1,3 glucanase, and Chitinase were also observed to be higher in the inoculated Shafaq-2006 (Figure 6). It indicates that these genes are involved in resistance against *F. equiseti* in Shafaq-2006. The expression level of Defensins and Thionins was observed to be lower in Shafaq-2006 as compared to other genes, which shows that these genes do not play any considerable role in resistance against *F. equiseti* stress in inoculated Shafaq-2006. In the case of Sahar-2006, the results of the qRT-PCR analysis showed minute expression of these genes under stress, thus suggesting that these genes do not play a role in conferring resistance against *F. equiseti* stress.

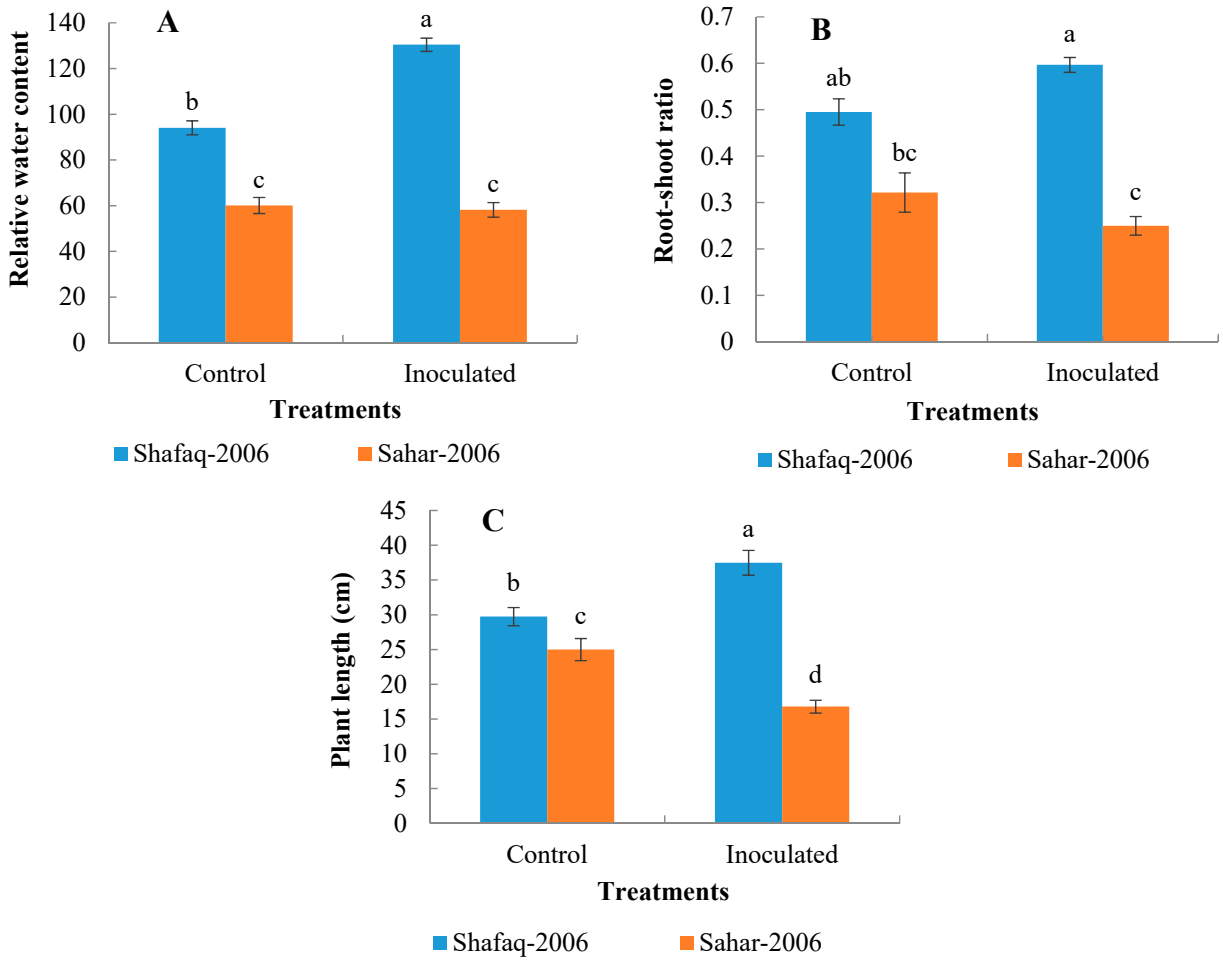

Figure 4. Measurement of the physiological parameters, such as (**A**) RWCs, (**B**) Root–shoot ratio, and (**C**) Plant length after the systemic inoculation of *Fusarium equiseti* in the Shafaq-2006 and Sahar-2006. All the means sharing common letter(s) are not significantly different at $p < 0.05$ and vertical bars represent the standard error of means (n = 3), while all the means with different letter(s) are significantly different at $p < 0.05$.

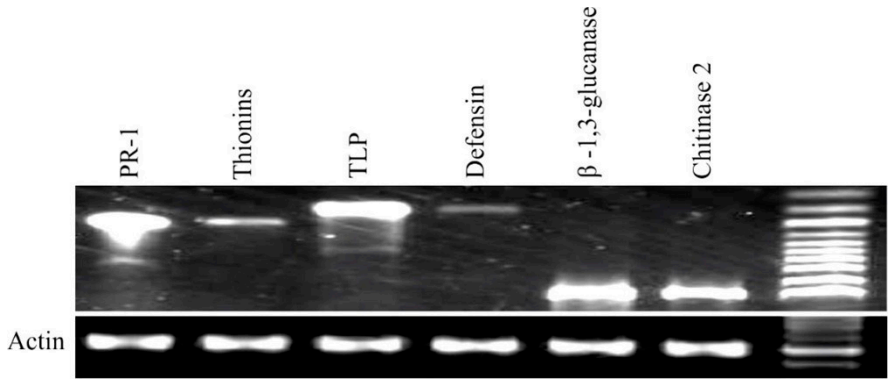

Figure 5. RT-PCR analysis of six PR-family genes in the Shafaq-2006 after the inoculation of *Fusarium equiseti*. Actin was used as a control.

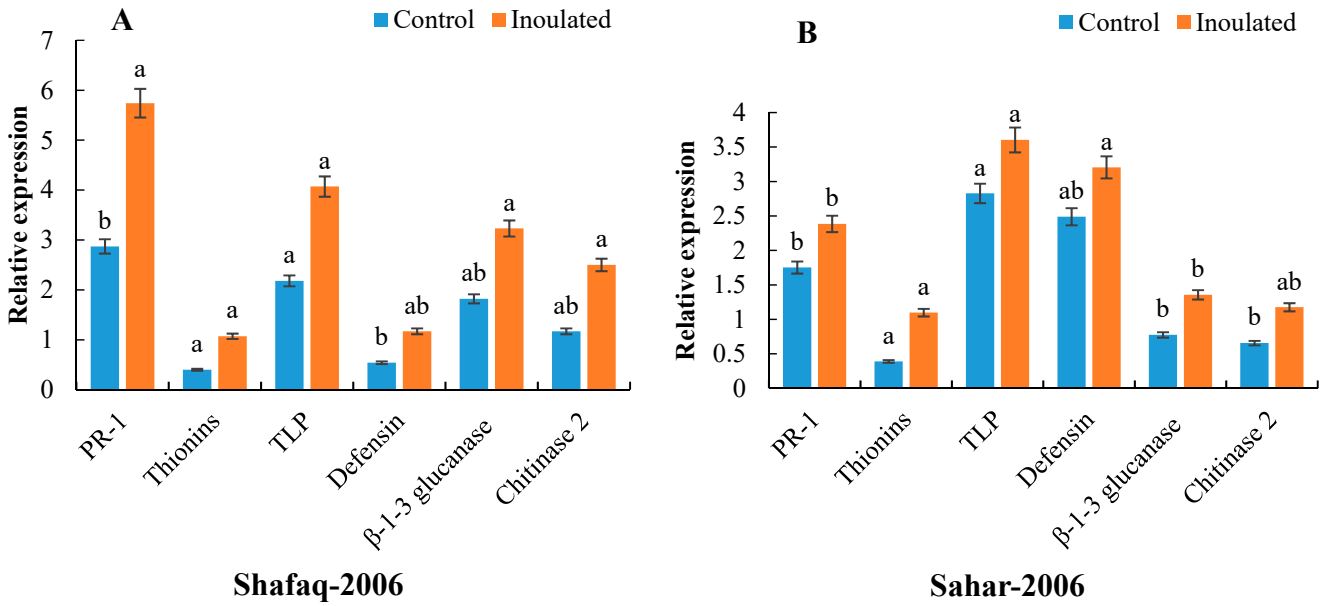

Figure 6. Relative expression of pathogenesis-related genes by qRT-PCR in inoculated and control wheat varieties. (**A**) Relative expression of pathogenesis-related genes in Shafaq-2006. (**B**) Relative expression of pathogenesis-related genes in Sahar-2006. All the means sharing common letter(s) are not significantly different at $p < 0.05$ and vertical bars represent standard error of means (n = 3), while all the means with different letter(s) are significantly different at $p < 0.05$.

4. Discussion

This experiment was conducted to examine the disease severity and expression of defense-related genes in wheat. Disease severity analysis proved Shafaq-2006 to be resistant, while Sahar-2006 was found susceptible to *F. equiseti*. Although Shafaq-2006 has not been reported to be fungus resistant before, previous studies have described it to be aphid resistant [32]. Sahar-2006 has been described to be susceptible in its reactions against the pressure of inoculum, especially with root inoculation methods [33].

In this experiment, we have observed higher proline contents in the inoculated resistant variety and a reduction in proline contents were observed in the inoculated susceptible variety. According to Claussen [43], a certain stress level is required for proline accumulation. Under various stress conditions, for instance, high salinity, drought, and biotic stress, proline accumulates in high concentration [44]. Studies have shown that proline contents in wheat were increased under water stress [45,46]. As a compatible solute, proline is involved in osmotic adjustment and mainly accumulates in most of the plants in stress condition [47]. Protein contents were observed to be increased in inoculated resistant variety, while a decrease in protein contents was observed in susceptible variety after infection. The analysis of the present results revealed that the protein contents in Shafaq-2006 were increased to that of Sahar-2006 in control as well as stress condition. Under *F. equiseti* stress (inoculation), the protein contents of Sahar-2006 were highly decreased; in contrast, protein contents of Shafaq-2006 significantly increased by 68.07% more than Sahar-2006. Thus, it has been reported that the increase in total protein contents plays an important role in plant defense [48]. Moreover, the higher sugar contents were recorded for the resistant wheat variety both in inoculated and control conditions. Similarly, Mohammadkhani and Heidari [49] reported the higher sugar and proline contents of wheat under stress. Sugar also plays a primary role in the plant defense mechanism against pathogens [50]. The sugar level is correlated with disease reaction in many plants and the high sugar level is considered responsible for the resistance to disease [50]. Generally, some pathogenic infections bring change in photosynthetic rate and respiratory pathway that cause fluctuation in sugar content [51]. Moreover, the increase in total chlorophyll contents was observed in inoculated Shafaq-2006, while Sahar-2006 exhibited a considerable reduction in total chlorophyll contents as compared to Shafaq-2006 after *F. equiseti* infection. The results

of the present study are in accordance with Manghwar et al. [52], who reported higher total chlorophyll contents in resistant wheat variety than the susceptible one after fungal infection. Interestingly, the total chlorophyll contents of Sahar-2006 also increased under fungal stress, compared to control plants. An increase in chlorophyll content might be due to the presence of a large number of bundle sheath chloroplast in the leaves of inoculated plants [52,53]. The reduction in chlorophyll contents in stress condition has been regarded as a typical symptom of oxidative stress that might be the result of chlorophyll degradation and pigment photo-oxidation [54]. In general, the resistant wheat variety (Shafaq-2006) exhibited higher biochemical parameters in the *F. equiseti* inoculated soil. These results are supported by many studies, which report that the increase in biochemical parameters under stress might be associated with a plant defense mechanism against pathogens [52,55,56].

An increase in RWCs was observed in Shafaq-2006, while low RWCs were observed in Sahar-2006 after *F. equiseti* inoculation. The increase in RWCs of the resistant wheat variety might be related to its higher protein production and total chlorophyll contents, which can promote the photosynthetic capacity by enhancing the gas exchange ability and water status under stress [57,58]. An increase in the total length of the plant was observed in the inoculated resistant variety. In the inoculated susceptible variety, the total length was significantly reduced. This decrease might be due to the fact that the water stress reduces the rate of plant height and leaf appearance [59]. Additionally, a higher root–shoot ratio (58.11%) of plants was observed in the inoculated resistant variety compared to the susceptible variety. Similarly, the root–shoot ratio of sorghum enhanced under water stress [60], whereas the root–shoot ratio of the inoculated susceptible variety was reduced. The reduction in the root length is an obvious plant response to fungal infection [61]. The studies have reported that the stress conditions at the seeding stage decrease the weight of endosperm and retard the growth of radicle, coleoptile, root, and shoot [62]. In brief, by analyzing the results of biochemical and physiological parameters, it could be concluded that Shafaq-2006 is resistant while Sahar-2006 is susceptible against *F. equiseti*. Furthermore, to assess the defense mechanism of resistant wheat variety and the expression of defense-related genes, both the inoculated and control wheat varieties were subjected to RT-PCR and qRT-PCR.

The high bands' intensity of PR-1, TLP, chitinase 2, and β-1,3-glucanase genes were observed by RT-PCR in the inoculated Shafaq-2006 wheat variety. These results indicate a possible role of these genes in the resistance against *F. equiseti* stress in Shafaq-2006. Low band intensities of Thionins and Defensins genes were observed. The high expression of PR-1, TLP, chitinase 2, and β-1,3-glucanase genes was shown during the qRT-PCR in Shafaq-2006 variety, while thionins and defensins did not show any expression. The expression of these genes was inconsiderable in the inoculated and control Sahar-2006 varieties. This might be the reason why Sahar-2006 could not overcome the infection caused by *F. equiseti* after the inoculation. As compared to Shafaq-2006, the effect of infection on Sahar-2006 was prominent, higher concentration of proline, reduced chlorophyll, sugar, protein, and water contents in addition to the overall reduction in growth rate showed acute effects of *F. equiseti.* RT-PCR and qRT-PCR results have shown that PR-1, TLP, Chitinase, and β-1,3-glucanase genes might be involved in playing major roles in inducing disease resistance in inoculated resistant plants. It has also been shown by the results that thionins and defensins do not play any considerable role in disease resistance of Shafaq-2006 against *F. equiseti*. Higher levels of these genes' expression may have reduced the infection against *F. equiseti*. Several PR proteins, including PR-1, 2, 3, 4, and 5 have been reported to inhibit the growth of fungi. PR-1 inhibits the growth of *Pseudomonas syringae* bacterium and *Cercospora nicotianae* fungus as well as oomycetes, including *Peronospora tabacina* and *Phytophthora parasitica* [17]. PR-1 proteins are involved in the thickening of the cell wall and may provide resistance against the pathogen spread in the apoplast [63]. In plants, the overexpression of TLP genes has been found to provide enhanced tolerance against fungal pathogens [64,65]. Thaumatin-like proteins (TLPs) are not normally expressed constitutively in healthy plants, but are induced exclusively in response to a

pathogenic attack or wounding [66]. The Chitinase gene is thought to play a dual role in fungal growth inhibition by digesting the cell wall and releasing pathogen-borne elicitors that are involved in inducing further defense reactions in the host. Overexpressions of β-1,3-glucanases and Chitinases are well-known examples of protection conferred by the transgenic expression of plant antifungal genes [65,67]. Chitinases expressed at high levels appear to be effective against plant-pathogenic fungi [68]. Chitinase genes are upregulated by *F. graminearum* during the early stages of barley and wheat spikes' infection [69]. Plant β-1,3-glucanases have been proposed as important components of plant defense mechanisms against pathogens [27]. Plant β-1,3-glucanases are thought to be involved in playing a key role in plant defense responses to pathogen infection [22].

5. Conclusions

It is concluded that Shafaq-2006 is a more resistant variety than Sahar-2006 against *F. equiseti*. All the PR-family genes do not play an equal role in the defense mechanism of Shafaq-2006. The higher expression of PR-1, TLP, Chitinase, and β-1,3-glucanase genes in Shafaq-2006 predicts their possible role in the defense mechanism of this variety against *F. equiseti*. These overexpressed genes might have created disease resistance by activating a defense mechanism and improving the production of different biochemicals such as protein, proline, chlorophyll, and sugar contents and also physiological parameters, such as RWC, total length of the plant, root and shoot length, and fresh and dry weight of root and shoot. Further research needs to be done to overexpress these genes by genetic engineering in Shafaq-2006 in order to increase its immunity against *F. equiseti* and other fungi.

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