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ORIGINAL ARTICLE

Evaluation of resistance and the role of some defense responses in wheat cultivars to Fusarium head blight

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Abstract

Fusarium graminearum and *F. culmorum* are the causal agents of Fusarium head blight (FHB) in cereal crops worldwide. Application of resistant cultivars is the most effective and economic method for management of FHB and reducing mycotoxin production in wheat. Understanding the physiological and biochemical mechanisms involved in basal resistance of wheat to FHB disease is limited. In this research, after screening resistance levels of eighteen wheat cultivars planted in Iran, Gaskozhen and Falat were identified as partially resistant and susceptible wheat cultivars against *Fusarium* spp., respectively. Also, we investigated the role of hydroxyl radical (OH^-), nitric oxide (NO), callose deposition, lipid peroxidation and protein content in basal resistance of wheat to the hemi-biotrophic and necrotrophic *Fusarium* species causing FHB. Nitric oxide as a signaling molecule may be involved in physiological and defensive processes in plants. Our results showed that NO generation increased in seedlings and spikes of wheat cultivars after inoculation with *Fusarium* species. We observed earlier and stronger callose deposition at early time points after infection by *Fusarium* spp. isolates than in non-infected plants, which was positively related to the resistance levels in wheat cultivars. Higher levels of OH^- and malondialdehyde (MDA) accumulation (as a marker of lipid peroxidation) were observed in the Falat than in the Gaskozhen cultivar, under non-infected and infected conditions. So, estimation of lipid peroxidation could be useful to evaluate cultivars' susceptibility. These findings can provide novel insights for better recognition of physiological and biochemical markers of FHB resistance, which could be used for rapid screening of resistance levels in wheat cultivars against this destructive fungal disease.

Key words: *Fusarium culmorum*, *Fusarium graminearum*, Fusarium head blight, resistance, wheat

Introduction

Wheat (*Triticum aestivum* L.) is one of the most important field crops and is consumed as a major dietary source worldwide. Wheat production and yield are limited by biotic and abiotic stresses (Bahieldin *et al.* 2005). Fusarium head blight (FHB) is an important and destructive disease of small grain cereals including wheat. It is caused by different species of *Fusarium* especially, *F. graminearum* and *F. culmorum* (Nielsen *et al.* 2011). The disease not only reduces the yield and quality, but also contaminates the product with various mycotoxins. Mycotoxins have various acute and chronic effects on human and animal health (Shin *et al.* 2014).

Several strategies have been used to manage FHB disease and reduce the risk of mycotoxin contamination, including crop rotation, genetic resistance, application of natural compounds, as well as chemical and biological control (Mesterházy 2014; Tian *et al.* 2016). Application of resistant cultivars, plant extracts and essential oils, such as thymol oil and Galla chinensis extract, are the most effective, economic, and environmentally safe ways to control plant diseases (Forrer *et al.* 2014; Khaledi *et al.* 2015; Lenc *et al.* 2015; Gill *et al.* 2016). To date, two main types of resistance to FHB are widely accepted: type I – resistance to initial infection, type II – resistance to fungal spread within the spike. Additionally, three other types of resistance were reported by Mesterházy *et al.* (1999): type III – resistance to deoxynivalenol (DON) accumulation, type IV – resistance to kernel infection, type V – tolerance. Different wheat genotypes express various levels of resistance against *Fusarium* spp. causing FHB (Mesterházy *et al.* 2005). Resistance to FHB is a complex trait, with polygenic inheritance and its expression is influenced by the environment (Liu *et al.* 2009; Ruan *et al.* 2012; Buerstmayr and Buerstmayr 2015).

After recognition of the pathogen, basal defense responses lead to activation of several resistance mechanisms such as production of reactive oxygen species (ROS) (Shetty *et al.* 2008; Khaledi *et al.* 2016), reactive nitrogen species (RNS) (Hong *et al.* 2008; Duan *et al.* 2015), deposition of callose (Ellinger *et al.* 2014), enzymatic and non-enzymatic antioxidants (Zhou *et al.* 2007; Khaledi *et al.* 2016). In plant-pathogen interactions, one of the earliest plant defense responses is production of ROS (Shetty *et al.* 2008). The most important ROS are superoxide anion (O_2^-), hydroxyl radical (OH^-), hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$) and the closely related RNS, nitric oxide (NO) (Shetty *et al.* 2008; Das and Roychoudhury 2014).

Callose deposition frequently occurs as a consequence of ROS burst (Zhang *et al.* 2009). Accumulation of ROS contributes to the induction of defense genes, and cell wall reinforcement by callose deposition (Yi *et al.* 2014). Generation of ROS leads to callose deposition at sites of penetration, which is recognized as an early defense response of a host to microbial pathogens (Altinok and Dikilitas 2014).

Active reinforcement of the cell wall through deposition of cell wall appositions, known as papillae, at sites of interaction with pathogens appears to be a common component of the pathogen-associated molecular patterns (PAMP) triggered immunity response (Nicaise *et al.* 2009; Underwood 2012; Voigt 2014). Compounds commonly associated with papillae include: callose, phenolics including lignin and phenolic conjugates such as phenolic-polyamines, ROS, peroxidases, cell wall structural proteins such as arabinogalactan proteins and hydroxyproline-rich glycoproteins, and cell wall polymers including pectin and xyloglucans (Paris *et al.* 2007; Hematy *et al.* 2009).

Plants exhibit physiological or biochemical and structural changes in cell walls in response to biotic and abiotic stress. Mechanical wounding, physiological stress and phytopathogen infection can induce callose synthesis (Tortora *et al.* 2012). Callose, a linear β-1,3-glucan with some β-1,6-branches, plays important roles during a variety of processes in plant development and in response to multiple biotic and abiotic stresses (Chen and Kim 2009). Callose deposition is typically triggered by conserved PAMPs (Gomez-Gomez *et al.* 1999). Wheat cultivars, which were partially resistant to *F. graminearum*, showed increased callose deposition in the transition zone of the spikelet's rachilla and rachis (Ribichich *et al.* 2000). Callose accumulation at sites of pathogen penetration is known as a physical barrier to slow pathogen invasion (Jones and Dangl 2006).

Hydrogen peroxide can react with metal ions via the Fenton pathway to generate the extremely toxic and highly reactive OH^-, which can react indiscriminately with all macromolecules such as DNA, lipids, proteins and carbohydrates (Imlay 2003). Hydroxyl radical could be involved in initiating the oxidation of polyunsaturated phospholipids, thus leading to impairment of membrane function (Schneider *et al.* 2008; Ayala *et al.* 2014). Malondialdehyde (MDA) is one of the final products of lipid peroxidation, which is an indicator of oxidative damage in plant cell membranes induced by stress (Singh *et al.* 2012; Wang *et al.* 2014). It is known that besides drought, pathogenic fungi can also change the MDA content in plants (Chen *et al.* 2008; Noorbakhsh and Taheri 2016).

The NO, similar to ROS, is a small redox signal and ubiquitous bioactive molecule, which is known as a relatively stable radical but rapidly reacts with other radicals including ROS (Hill *et al.* 2010). The NO and NO-derived RNS are produced in the chloroplasts and mitochondria (Galatro *et al.* 2013). Nitric oxide functions as a signaling molecule and plays an important role during interaction of plant and pathogen (Guo *et al.* 2004; Qiao *et al.* 2015). This gaseous molecule is a signaling messenger involved in plant responses to different stresses (Gaupels *et al.* 2011). Like ROS, NO is an important messenger in many physiological processes and defense reactions in cooperation with ROS (Hong *et al.* 2008). Nitric oxide interacts with ROS and is involved in stomatal closure and pathogen defense (Mur *et al.* 2013). Reactive nitrogen species are important signal transduction molecules in wheat defense against biotic and abiotic stress (Guo *et al.* 2004; Mur *et al.* 2013; Duan *et al.* 2015; Qiao *et al.* 2015).

Proteins play important roles in recognition and defense against pathogens (Zhang *et al.* 2013b; Zhu *et al.* 2010). Production of the proteins which are involved in primary metabolism, oxidative stress, detoxification, and signal transduction as well as some proteins with other functions, increased in response to fungal infection (Yang *et al.* 2011). Zhang *et al.* (2013b) reported that differentially expressed proteins may be involved in complicated processes to defend against fungal infection in FHB-resistant genotypes by degrading fungal cell walls and strengthening plant cell walls. Proteomic analysis of wheat spikes in resistant cultivar – *F. graminearum* interaction revealed accumulation of plant proteins involved in oxidative stress, pathogenesis-related (PR) responses and nitrogen metabolisms (Zhou *et al.* 2005).

Despite the economic importance of FHB and mycotoxins in wheat, the current understanding of resistance cultivars and defense mechanisms in wheat defense against different *Fusarium* species causing FHB is limited. Therefore, the objectives of this study were to: i) screen and identify the sources of resistance in Iranian wheat cultivars against FHB; ii) examine the effect of *Fusarium* species causing FHB on defense responses of wheat by inoculating wheat spikes and leaf segments; iii) investigate changes of OH^- and NO at two growth stages of wheat; and iv) to compare the MDA, callose and protein contents in partially resistant and susceptible wheat cultivars as a part of defense mechanisms involved in this pathosystem.

Materials and Methods

Wheat cultivars and plant growth conditions

Fifteen spring wheat (*Triticum aestivum* L.) cultivars including Falat, Roshan, Sivand, Kouhdasht, Morvarid, Gonbad, Shiroodi, Tajan, Atrak, Arta, N87-20, Ofogh, Pishtaz, Sirvan, Sumai3 and three winter wheat cultivars including Gaskozhen, Zare and Mihan with different levels of FHB resistance, obtained from the Agricultural Research Center of Khorasan Razavi, Tehran, Golestan and Yazd provinces in Iran were used. These cultivars are commonly planted in Iran because of their good quality and high yield under the climatic conditions of this country. The seeds were surface sterilized with 1% sodium hypochlorite for 1 min, rinsed three times with sterile distilled water and incubated for 5 days on wet, sterile filter paper in Petri dishes at 25°C. Each germinated seed was sown in a 15-cm-diameter plastic pot filled with potting soil, which had been autoclaved at 121°C for a minimum of 30 min at 100 kPa (15 psi) on 2 successive days and grown under greenhouse conditions (30±4°C; 16 : 8 h L : D photoperiod). The winter wheat cultivars require vernalization to initiate flowering. These cultivars were vernalized in a germination tray for 6 weeks at 4°C in a growth chamber (Bernardo *et al.* 2007). After vernalization, the seedlings were transplanted. The soil used in this experiment, was a combination of clay, sand and farmyard manure at a ratio of 2 : 1 : 1 (v/v/v).

Fungal isolates and inoculum preparation

Isolates FH1 of *F. graminearum* and FH9 of *F. culmorum* belonging to the nivalenol (NIV) chemotype obtained from symptomatic wheat plants in the Golestan province of Iran and deposited in the fungal culture collection of Ferdowsi University of Mashhad, were used to determine sources of resistance to FHB (Khaledi *et al.* 2017). The isolates were grown at 25°C with alternate cycles of 12 : 12 h (L : D) on potato dextrose agar (PDA). Fungal inocula were produced on mung bean broth (MBB) and synthetic nutrient agar (SNA) media as described by Zhang *et al.* (2013a) and Koch *et al.* (2013), respectively. Conidial suspensions were diluted with autoclaved water to a final concentration of 1×10^5 conidia · ml^{-1} containing 0.05% (v/v) Tween 20.

Plant inoculation and disease evaluation

In the greenhouse experiments, a pathogenicity test on wheat spikes was carried out using the method described by Yoshida *et al.* (2007). At the flowering stage (ZGS 64 to 65), 10 ml of a spore suspension (1×10^5 conidia · ml^{-1}) amended with 0.05% Tween 20 was sprayed on the spikes of each plant. The inoculated plants were incubated overnight in a greenhouse at 18–25°C, with 90–100% humidity. Then, the plants were placed in a plastic bag for 3 days to maintain high relative humidity. Control plants were treated with sterile distilled water containing 0.05% (v/v) Tween 20. Inoculated wheat heads were evaluated after 10 days and the FHB disease severity was estimated. In all cases, when lesions developed, the pathogen was reisolated from infected plants. Disease severity was measured as the percentage of infected spikelet(s) within the spike using a 0 to 5 scale (0 – no disease, 1 – to 20%, 2 – to 40%, 3 – to 60%, 4 – to 80% and 5 – more than 80% disease severity). Disease incidence was expressed as the percentage of spikes infected in the plot, from 0% (no infection) to 100% (indicating all spikes examined were infected). FHB index was calculated as the combination of disease severity and disease incidence (Amarasinghe *et al.* 2013). Each test had four replicates arranged in a randomized complete block design, and the experiment was repeated three times.

In the detached leaf bioassay, 4-cm length segments from the mid-section were prepared from the apical leaf of 4-week-old wheat plants. Each leaf segment was placed adaxial surface uppermost on the surface of 0.5% water agar as described by Browne and Cooke (2004). Leaf segments were inoculated at the center of the adaxial surface with 5 µl inoculum suspension of 1×10^5 conidia · m^{-1} containing 0.05% (v/v) Tween 20. Control leaf segments were inoculated using a drop of sterile distilled water containing 0.05% Tween 20 without the fungus. Petri dishes were incubated at 25°C with a 12 : 12 h (L : D) cycle. After 5 days, the length of necrotic lesions was measured. The test included four replicates for each isolate and the experiment was repeated three times.

Callose deposition assay

Quantification of callose deposition was performed as described by Yi *et al.* (2014). Leaves of 4-week-old wheat plants at various time points after the pathogen inoculation were sampled and investigated for callose deposition. Briefly, leaf segments were incubated for at least 24 h in 95% ethanol until all tissues were transparent, then washed in 0.07 M phosphate buffer (pH 9) for 15 min, and incubated for 1–2 h in 0.07 M phosphate buffer containing 0.01% aniline blue (Sigma) prior to microscopic analysis. Observations were performed with a fluorescence microscope (Olympus BX51) using UV filter.

In addition, the callose content was quantitatively measured by the method of Hirano *et al.* (2004). Twenty mg plant tissues were washed once with 96% ethanol and three times with 20% ethanol which contained 5% polyvinylpolypyrrolidone (PVP, w/v). To solubilize the callose, 1 ml of 1 M NaOH was added to the washed tissues and the tubes were heated at 80°C for 15 min. The extract was then centrifuged at 10,000 × *g* for 15 min and the supernatant was assayed for callose. The callose content was quantified spectrofluorometrically at excitation and emission wavelengths of 393 and 484 nm, respectively. Curdlan (β-1,3-glucan) was used to prepare a calibration curve. Callose content was expressed as curdlan equivalents (CE) per mg fresh leaf weight (FW) ($\mu g\ CE \cdot mg^{-1}\ FW$).

Quantitative measurement of OH^-

The OH^- content was assayed using the method of Halliwell *et al.* (1987). Fresh plant tissue (50 mg) was homogenized on ice with 1 ml of 10 mM phosphate buffer (pH 7.4) containing 15 mM 2-deoxyderibose, at 37°C for 2 h. Following incubation, an aliquot of 0.7 ml from the above mixture was added to the reaction mixture containing 3 ml of 0.5% (w/v) thiobarbituric acid (TBA, 1% stock solution made in 5 mM NaOH) and 1 ml of glacial acetic acid. The contents of the reaction mixture were heated in a water bath for 30 min at 100°C, and then cooled down to 4°C for 10 min. Absorbance of the reaction mixture was measured at 532 nm. The OH^- content was calculated using the molar extinction coefficient ($155\ mM^{-1} \cdot cm^{-1}$), and expressed as $nmol \cdot g^{-1}$ FW.

Estimation of lipid peroxidation

A quantitative index of lipid peroxidation, MDA content, was estimated according to Hodges *et al.* (1999). Briefly, 1.0 g of leaf tissue was homogenized in 20 ml 96% ethanol : water (80 : 20; v/v), followed by centrifugation at 3,000 × *g* for 10 min. Two 0.5 ml aliquots of the alcoholic extract were taken; one was mixed with 0.5 ml (i) + TBA solution containing 20% trichloroacetic acid, 0.01% butylated hydroxytoluene (BHT) and 0.65% TBA, and the other was mixed with (ii) – TBA solution that had the same composition as solution (i) but without TBA. The mixture was heated at 95°C for 25 min, cooled and then centrifuged at 4000 × *g* for 10 min. Absorbance was measured at 440, 532 and 600 nm. The MDA equivalent was derived from the absorbance according to Hodges *et al.* (1999).

Determination of endogenous NO content

Nitric oxide content was determined according to Murphy and Noack (1994). Fresh plant tissue (3 g) was incubated with 100 units of catalase and 100 units of superoxide dismutase for 5 min to remove endogenous ROS before the addition of 10 ml of oxyhemoglobin (5 mM). After 2 min of incubation, NO was measured spectrophotometrically based on the conversion of oxyhemoglobin to methemoglobin. Absorbance was determined at 550 nm and NO content was expressed as $\mu mol \cdot ml^{-1} \cdot g^{-1}$ FW.

Estimation of total protein

The protein concentration was determined as described by Bradford (1976) using bovine serum albumin as a standard.

Statistical analysis

All experiments included three independent repetitions carried out with four replications in each repetition. The means were separated using Duncan's multiple range tests at $p < 0.05$, where the F-value was significant. Statistical analysis was performed with statistical package for the social sciences (SPSS; version 23) software.

Results

Greenhouse evaluation of FHB resistance in wheat cultivars and virulence of *Fusarium* isolates

The results of evaluating resistance of 18 wheat cultivars to the isolates FH1 of *F. graminearum* and FH9 of *F. culmorum* revealed significant differences in the resistance levels of various cultivars to the pathogens (Fig. 1, Table 1). *Triticum aestivum* L. cv. Gaskozhen showed the lowest disease progress with an average FHB index of 16.5±0.82 and mean lesion length of 13±1.18 on the leaf segments among all tested cultivars. A significant difference was not observed between the Sumai 3 and Gaskozhen cultivars. The Falat cultivar showed

Table 1. Average of FHB index and leaf lesion length caused by *Fusarium* isolates on each wheat cultivar and each isolate on all cultivars

Cultivars/Isolates	Average of FHB index	Average of leaf lesion length
Wheat cultivars		
Falat	55.5 ± 1.19 a	35.3 ± 2.13 a
Pishtaz	42.5 ± 1.37 e	27.5 ± 1.86 c
Gaskozhen	16.5 ± 0.82 h	13.0 ± 1.18 g
Zare	51.0 ± 1.00 b	29.0 ± 2.31 b
Mihan	29.5 ± 1.76 f	23.0 ± 2.04 f
Kouhdasht	48.0 ± 0.95 c	28.5 ± 1.06 c
Morvarid	20.5 ± 0.90 g	17.0 ± 2.80 e
Gonbad	46.0 ± 0.51 d	28.5 ± 4.31 c
N87-20	47.0 ± 0.60 c	29.0 ± 0.44 b
Ofogh	47.5 ± 1.44 c	26.0 ± 2.35 d
Sivand	46.0 ± 1.21 cd	29.0 ± 1.13 b
Roshan	44.5 ± 0.95 de	29.0 ± 2.59 bc
Sirvan	51.5 ± 0.57 b	28.0 ± 0.97 b
Arta	43.5 ± 0.90 e	29.0 ± 1.02 b
Atrak	41.0 ± 1.44 f	30.5 ± 1.51 b
Shiroodi	45.0 ± 0.68 d	28.0 ± 1.03 c
Tajan	43.0 ± 0.71 e	29.5 ± 1.03 b
Sumai3	17.0 ± 0.88 h	13.0 ± 1.30 g
Isolates		
FH1	52.8 ± 1.22 a	32.3 ± 0.89 a
FH9	33.7 ± 0.88 b	23.2 ± 0.77 b

Averages ± standard error (SE) are given in each column. Different letters indicate significant differences according to Duncan analysis using SPSS software ($p < 0.05$).
FH1 – isolate of *F. graminearum*, FH9 – isolate of *F. culmorum*

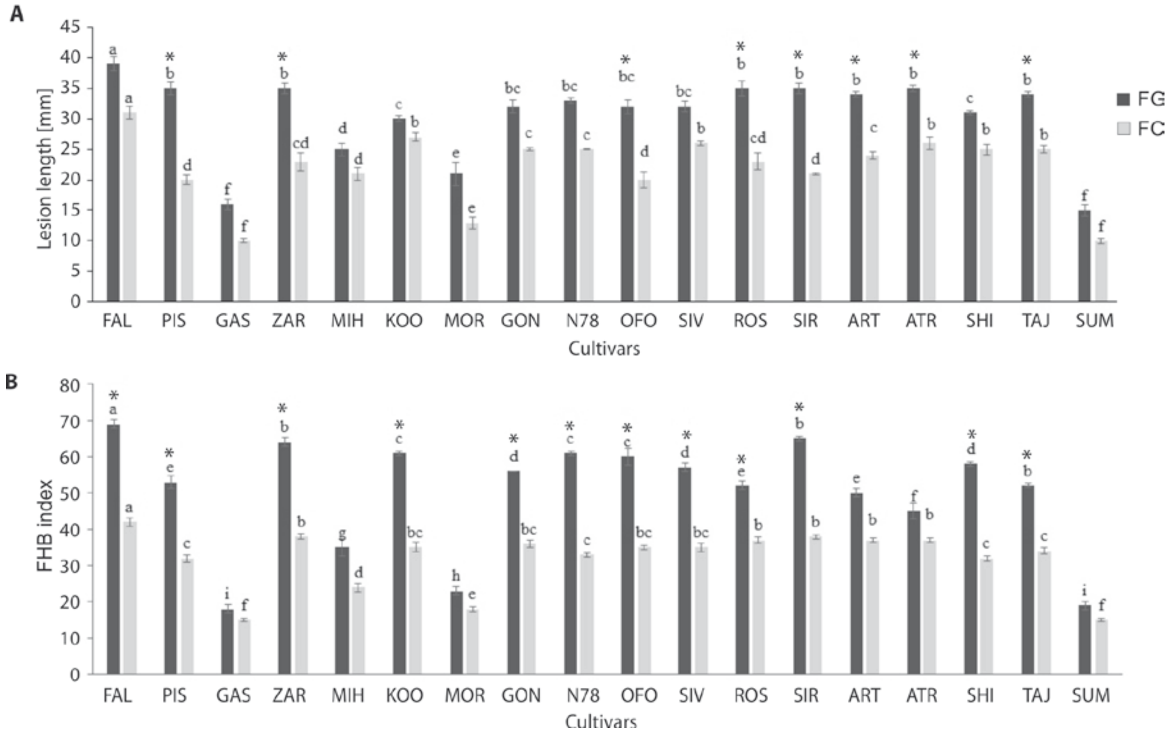


Fig . 1. Leaf lesion length (A) and FHB index (B) calculated for various cultivars of wheat (FAL – Falat, PIS – Pishtaz, GAS – Gaskozhen, ZAR – Zare, MIH – Mihan, KOO – Kouhdasht, MOR – Morvarid, GON – Gonbad, N78 – N87-20, OFO – Ofogh, SIV – Sivand, ROS – Roshan, SIR – Sirvan, ART – Arta, ATR – Atrak, SHI – Shiroodi, TAJ – Tajan, SUM – Sumai3) inoculated with isolates FH1 of *F. graminearum* (FG) and FH9 of *F. culmorum* (FC) is presented. Different letters indicate significant differences according to Duncan analysis using SPSS software ($p < 0.05$). The bars indicate standard errors (SE). Columns with an asterisk are statistically different from the control, within each treatment, according to Duncan's test ($p < 0.05$)

the highest disease severity with an average FHB index of 55.5±1.19 and mean lesion length of 35.3±2.13, which was significantly higher than that of other cultivars (Table 1). Therefore, two cultivars as partially resistant (Gaskozhen) and susceptible (Falat) were used to investigate defense mechanisms. Other wheat cultivars tested fell between Gaskozhen and Falat with various levels of susceptibility to different *Fusarium* isolates. No complete resistance to the pathogens was observed in any of the cultivars. The *Fusarium* isolates used in this study revealed differences in their virulence on wheat cultivars (Fig. 1). Overall, isolate FH1 of *F. graminearum* caused the highest disease progress with an average FHB index of 52.8±1.22 and leaf lesion length of 32.3±0.89, which were higher than those of the FH9 isolate belonging to *F. culmorum* (33.7±0.88 and 23.2±0.77, respectively) (Table 1).

Monitoring callose deposition

Histochemical analyses revealed higher levels of callose formation in the leaves of partially resistant Gaskozhen wheat cultivar at various time points after inoculation with isolates tested (Fig. 2A). Callose deposition was investigated in the epidermal cells of Gaskozhen and Falat leaves at various time points after inoculation with *Fusarium* isolates. At 24 hpi, a high level of callose deposition was observed in the leaves of Gaskozhen compared to the Falat cultivar. Callose deposition in the Falat cultivar, increased to a lesser extent and was later than in Gaskozhen plants and reached its maximum level at 48 hpi. After these time, callose deposition remained approximately the same (Fig. 2B). According to the results of determining the callose content in wheat spikes, high levels of callose deposition at milk and dough stages were observed in both cultivars (Fig. 2C). Overall, higher amount of more callose was formed in the partially resistant Gaskozhen than in the susceptible Falat cultivar. In both cultivars, a higher level of callose was detected in response to *F. culmorum* than to *F. graminearum* in both leaf and spike bioassays.

Investigating OH^- accumulation

A higher level of OH^- accumulation was observed in Falat than in Gaskozhen cultivar, under infected conditions. A higher level of OH^- accumulation was observed in the leaves and spikes of wheat in Falat – *F. graminearum* interaction at 120 hpi and flowering stage, respectively (Fig. 3A and B).

Analysis of lipid peroxidation

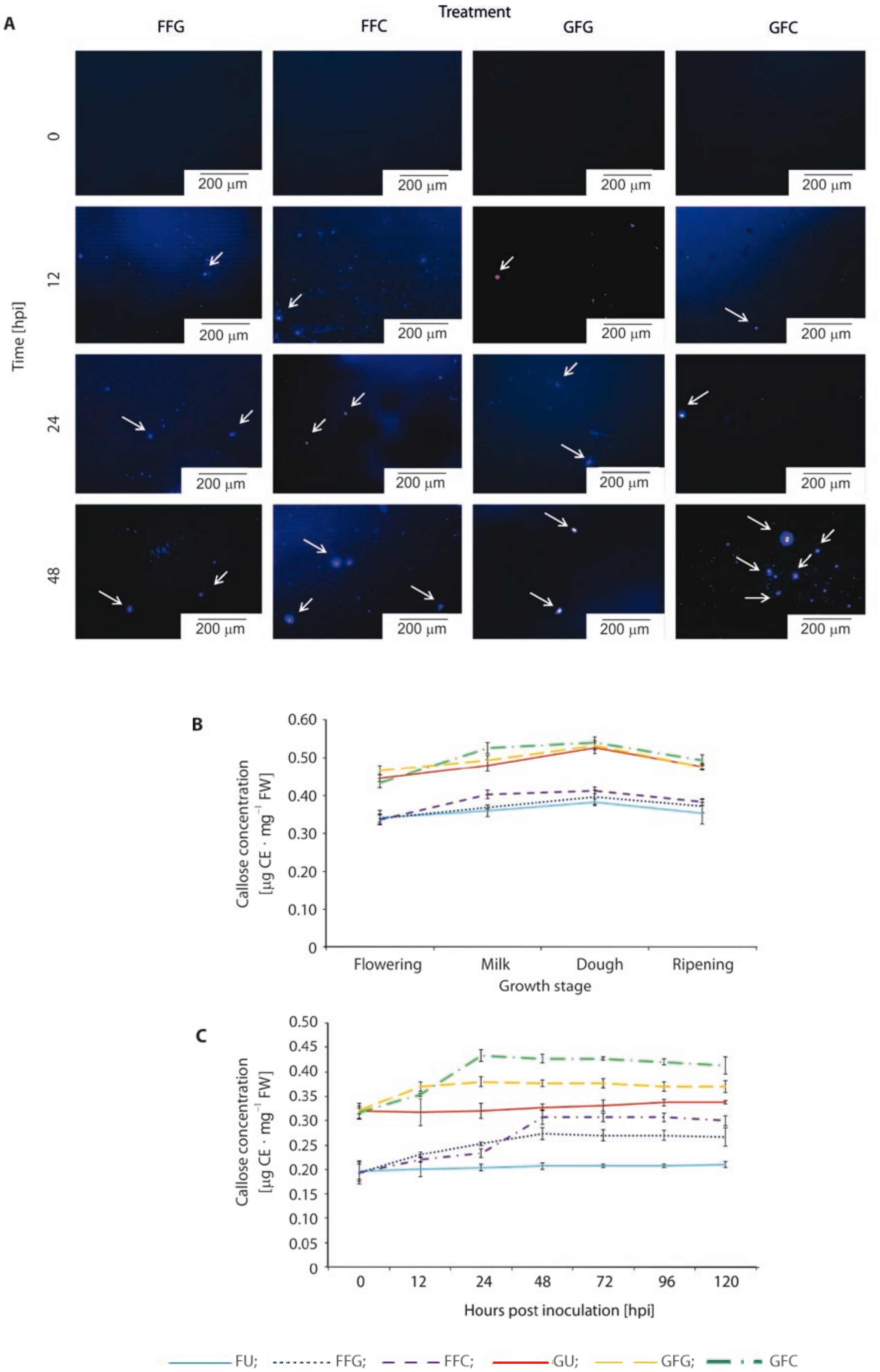
We investigated lipid peroxidation, as the main destruction mechanism of oxidative stress, in the leaves and spikes of wheat cultivars inoculated with *Fusarium* species. In infected leaves of Falat plants, MDA contents increased until 48 hpi, as the first peak. Afterward, a decreasing rate of MDA content was observed until 72 hpi followed by the second peak at 96 hpi. In infected leaves of Gaskozhen plants, MDA contents increased until 72 hpi, and decreased afterwards (Fig. 4A). In the wheat spikes, MDA content increased after infection by *Fusarium* spp. isolates until milk stage, and decreased afterwards (Fig. 4B). MDA content in the leaves and spikes of wheat in Fala – *F. graminearum* interaction was higher than wheat cultivars – *F. culmorum* interaction at various time points tested.

Detection of endogenous NO content

The results revealed that endogenous NO content could be induced in wheat by *Fusarium* spp. infection. In infected leaves of Gaskozhen plants, NO content increased until 48 hpi, as the first peak. Afterward, a decreasing rate of NO content was observed until 72 hpi followed by the second peak at 96 hpi. In infected leaves of Falat plants, two peaks of NO accumulation were observed at 48 and 72 hpi, with its maximum level at 48 hpi (Fig. 5A). In the wheat spikes, NO content increased after infection by *Fusarium* spp. isolates until milk stage, and decreased afterwards (Fig. 5B). The NO content in the leaves and spikes of wheat in Gaskozhen – *F. culmorum* interaction was higher than wheat cultivars – *Fusarium* species interaction at various time points tested (Fig. 5).

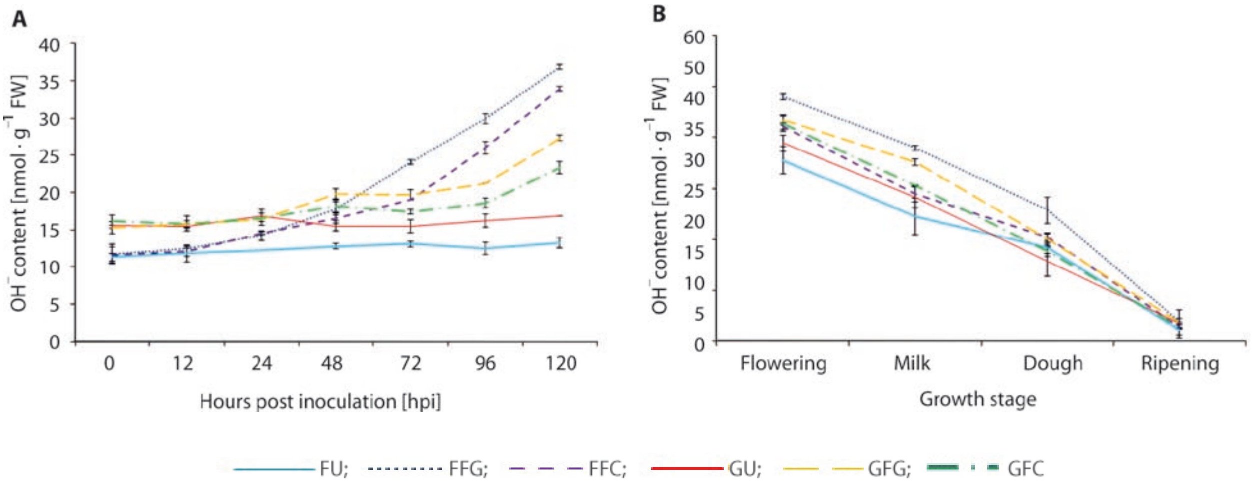
Total protein content

Total protein content was different between non-infected partially resistant and susceptible cultivars. In the Falat cultivar, a lower level of total protein was observed than in the Gaskozhen cultivar under non-infected condition. An increased protein content in the leaves and spikes of Gaskozhen cultivar infected with *Fusarium* spp. was observed. But in the Falat cultivar infected with *F. culmorum*, total protein decreased after 24 hpi (Fig. 6A). In Falat plants inoculated with *F. graminearum*, the levels of total protein were lower than those of non-inoculated plants of this cultivar at all time points. Higher levels of total soluble proteins were observed in the leaves of inoculated Gaskozhen plants after 48 hpi (Fig. 6A). In Gaskozhen plants, total protein of spikes slightly increased after infection by *Fusarium* isolates until milk stage and then decreased in consecutive growth stages, but it was still higher than that of non-infected plants. In the Falat cultivar, total protein of spikes decreased after infection by *Fusarium* isolates and was less than that of non-infected plants (Fig. 6B).



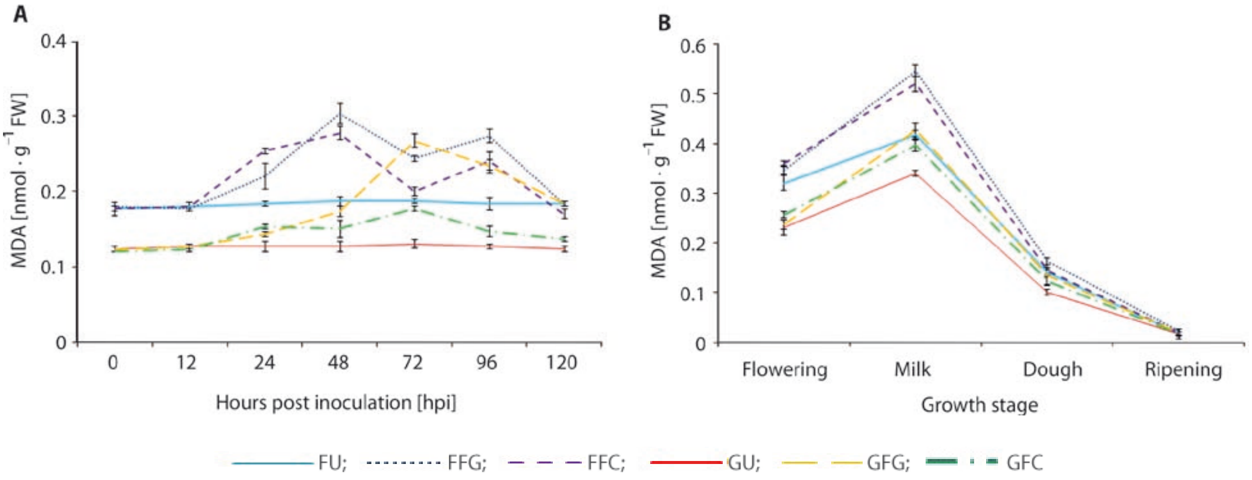
FU – Falat uninoculated (control), FFG – Falat inoculated by *Fusarium graminearum*, FFC – Falat inoculated by *F. culmorum*,
GU – Gaskozhen uninoculated (control), GFG – Gaskozhen inoculated by *F. graminearum*, GFP – Gaskozhen inoculated by *F. culmorum*

Fig. 2. Callose detection in the leaves of partially resistant (Gaskozhen) and susceptible (Falat) wheat cultivars at various time points after inoculation with *Fusarium* spp. Distribution and amount of callose depositions stained with aniline blue until 48 h after pathogens challenge (arrows) (A). Callose contents were quantitatively determined in the leaves (B) and spikes (C) of wheat cultivars



FU – Falat uninoculated (control), FFG – Falat inoculated by *Fusarium graminearum*, FFC – Falat inoculated by *F. culmorum*,
GU – Gaskozhen uninoculated (control), GFG – Gaskozhen inoculated by *F. graminearum*, GFP – Gaskozhen inoculated by *F. culmorum*

Fig. 3. OH^- content in the leaves (A) and spikes (B) of wheat cultivars at various time points after inoculation with *Fusarium* isolates



FU – Falat uninoculated (control), FFG – Falat inoculated by *Fusarium graminearum*, FFC – Falat inoculated by *F. culmorum*,
GU – Gaskozhen uninoculated (control), GFG – Gaskozhen inoculated by *F. graminearum*, GFP – Gaskozhen inoculated by *F. culmorum*

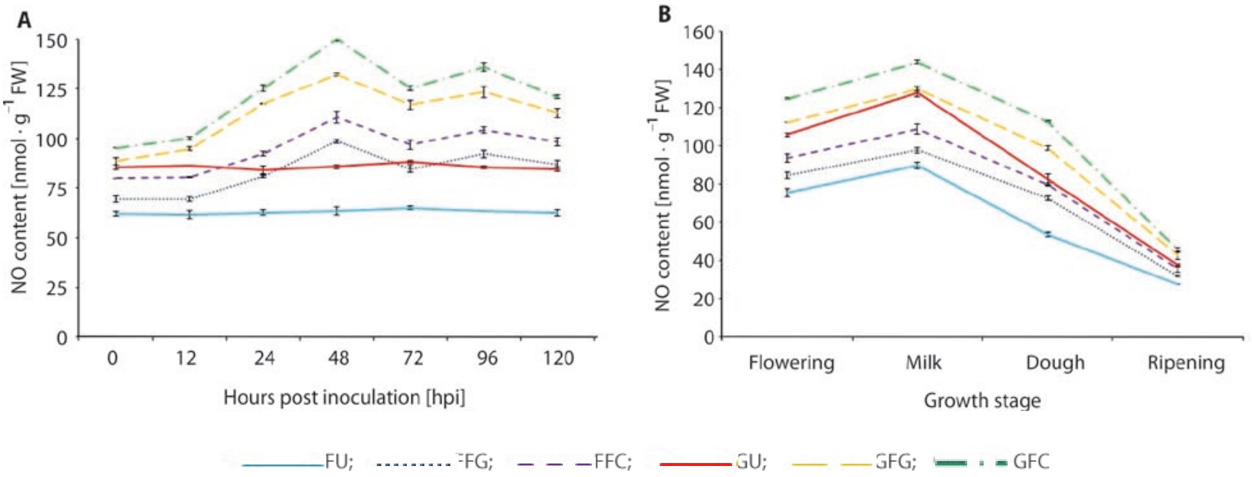
Fig. 4. Malondialdehyde (MDA) content in the leaves (A) and spikes (B) of wheat cultivars at various time points after inoculation with *Fusarium* isolates

Discussion

In this study, we screened the levels of resistance to FHB disease in Iranian wheat cultivars. Also, some of the defense mechanisms involved in basal resistance of wheat cultivars to the hemi-biotrophic *F. graminearum* and necrotrophic *F. culmorum* were investigated. The obtained results provided knowledge on the physiological and biochemical aspects as a part of wheat defense against mechanisms against *Fusarium* spp., causing FHB, which might be used as powerful markers for determining resistant wheat cultivars to this destructive disease.

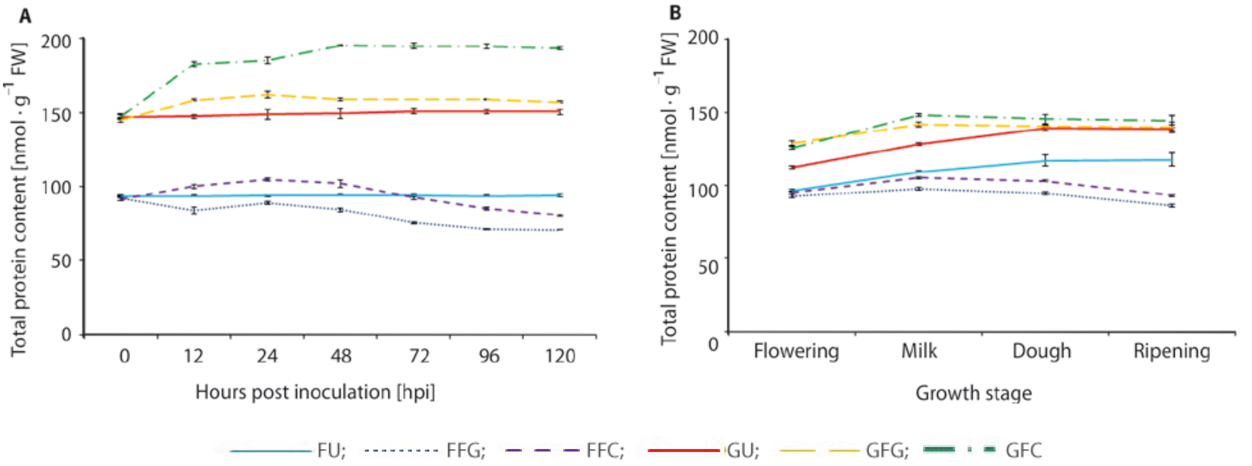
Based on the results of greenhouse infection assays, Gaskozhen and Falat plants were partially resistant and susceptible cultivars, respectively, among wheat cultivars tested.

We analyzed callose deposition, a common response by wheat to *Fusarium* attack, at the site of penetration. Our investigations revealed higher levels of callose deposition in Gaskozhen than in Falat cultivar. Motallebi *et al.* (2015) showed that callose content was higher in partially resistant wheat (Sumai3 cultivar) in response to *F. culmorum* than in the Falat cultivar. Also, they showed that the highest amount of callose content was seen in the Sumai3 cultivar. Similarly, Blümke *et al.* (2014) reported that linoleic and α-linolenic acid have a major function in the suppression of the innate immunity-related callose biosynthesis and, hence, progress of *F. graminearum* wheat infection.



FU – Falat uninoculated (control), FFG – Falat inoculated by *Fusarium graminearum*, FFC – Falat inoculated by *F. culmorum*,
GU – Gaskozhen uninoculated (control), GFG – Gaskozhen inoculated by *F. graminearum*, GFP – Gaskozhen inoculated by *F. culmorum*

Fig. 5. NO content in the leaves (A) and spikes (B) of wheat cultivars at various time points after inoculation with *Fusarium* isolates



FU – Falat uninoculated (control), FFG – Falat inoculated by *Fusarium graminearum*, FFC – Falat inoculated by *F. culmorum*,
GU – Gaskozhen uninoculated (control), GFG – Gaskozhen inoculated by *F. graminearum*, GFP – Gaskozhen inoculated by *F. culmorum*

Fig. 6. Total protein content in the leaves (A) and spikes (B) of wheat cultivars at various time points after inoculation with *Fusarium* isolates

In plant-pathogen interactions, an oxidative response is an early and complex host reaction to the phytopathogens, which occur in the attacked and neighboring cells of the infection site (Dmochowska-Boguta *et al.* 2013; Taheri *et al.* 2014). We observed increased OH^- content in the leaves and spikes of infected plants compared to non-infected samples. At most of the time points investigated, the OH^- content in the leaves changed depending on virulence of the *Fusarium* isolates. It was much higher in the leaves infected with the highly virulent *F. graminearum* isolate and, conversely, decreased in the leaves infected with the hypovirulent *F. culmorum* isolate. Briefly, the detailed characteristics of the process may vary depending on the particular plant, pathogen and type of interaction. The OH^- content increased in infected tissues compared to non-infected samples. Considerably higher OH^- content was observed in the Falat compared to the Gaskozhen cultivar. This finding is consistent with higher values of OH^- scavenging activity (41% higher) in FHB-resistant wheat than in FHB susceptible wheat group reported by Zhou *et al.* (2007). Increased OH^- accumulation in our assays provides support for the detrimental effect of trichothecene mycotoxins by promoting cell death through the induction of H_2O_2 and OH^- production thereby disturbing the balance between production and removal of ROS in cellular components (Desmond *et al.* 2008; Ponts *et al.* 2009).

We observed a higher level of MDA accumulation in the Falat compared to the Gaskozhen cultivar, under non-infected and infected conditions. This result is in accordance with Motallebi *et al.* (2015), who reported higher levels of MDA in the Falat cultivar infected with *F. culmorum* compared to the partially resistant Pishtaz and Sumai3 cultivars. Sorahinobar *et al.* (2016) reported that seed treatment with *F. graminearum* extract significantly increased H_2O_2 and MDA contents in Sumai3 and Falat cultivars. Chakraborty and Pradhan (2012) have also reported that water stress increased accumulation of MDA in susceptible varieties compared to tolerant varieties. Tatar and Gevrek (2008) demonstrated increased MDA contents with an increasing degree of stress in wheat. Debona *et al.* (2012) observed increased ROS and MDA concentrations and electrolyte leakage in wheat after inoculation by *Pyricularia oryzae*. Also, abiotic stress such as chilling and ozone treatment increased levels of MDA and ROS (Esim *et al.* 2014). These findings suggest that wheat plants have similar defense strategies against various biotic and abiotic stress.

In this study, biphasic accumulation of NO was observed in partially resistant and susceptible wheat cultivars, following inoculation with *Fusarium* spp. isolates. Maximum rates of NO generation were observed in the leaves of Gaskozhen compared to Falat plants, under non-infected and infected conditions. However, NO contents increased in infected treatments compared to non-infected treatments. Nitric oxide generation in Gaskozhen was observed more rapidly than in the Falat cultivar. Accumulation of high NO levels in Gaskozhen at 48 hpi might be associated with a higher level of resistance in this cultivar. These results were similar to results reported by other investigators. Qiao *et al.* (2015) reported that NO production plays an important role in defense interactions between wheat and *Puccinia triticina* at 24 to 72 hpi. Guo *et al.* (2004) reported biphasic accumulation of NO in plant cells adjacent to the stomata and the cells surrounding an infection site of *P. striiformis* in wheat at 20 and 100 hpi, with its maximum level at 100 hpi. Also, they observed first peak of NO accumulation in the early infection stage whereas the second peak accumulated in the latent period. However, only a single peak of NO was observed in the latent period for the virulent isolate. RNS, such as NO and polyamines, and ROS can be directly involved in plant defense through hypersensitive response induction (Zaninotto *et al.* 2006). Nitric oxide acts as a signaling molecule in inducing gene expression of the enzymes such as superoxide dismutase, ascorbate peroxide and catalase (Chen *et al.* 2010). Wheat spikes at milk stage, showed considerably higher accumulation of NO in the Gaskozhen than in the Falat cultivar, under both non-infected and infected conditions. Our present results suggested that NO might have a protective effect on wheat cultivars – *Fusarium* spp. interaction.

The results showed that the total protein content of Gaskozhen was higher than the Falat cultivar at various time points investigated. The present results suggested that proteins which are induced rapidly in response to pathogen invasion might help the resistant wheat cultivar to limit infection by *Fusarium* species. Our data are in accordance with observations of Gherbawy *et al.* (2012) who reported that the content of soluble, insoluble and total protein increased in wheat inoculated with *Fusarium* species. On the other hand, total protein decreased after the increase in infected Falat plants. This confirms the results obtained by others (Collins *et al.* 2003; Haynes *et al.* 2004; Huang *et al.* 2011). The Iranian wheat cultivars with different genetic backgrounds showed different levels of resistance to FHB. A difference in defense responses under FHB inoculation was observed in partially resistant (Gaskozhen) and susceptible (Falat) cultivars, which agrees with Khaledi *et al.* (2016).

Conclusions

According to our results, rapid induction and high amounts of NO and callose deposition, and induction of protein in resistant cultivars may play an important role in Iranian wheat cultivar resistance as mechanisms of host-resistance to FHB. We showed that there are different physiological and biochemical response patterns between Gaskozhen and Falat in response to *Fusarium* species infection. There were higher levels of NO and protein contents in Gaskozhen compared to the Falat cultivar, but accumulation of OH^- and lipid peroxidation was higher in Falat than in Gaskozhen. These findings indicate the role of callose deposition, changes of protein, OH^- and NO as defense mechanisms of wheat cultivars in interaction with *Fusarium* spp. The major role of NO formation in wheat resistance to FHB can be interpreted in its regulatory function on defense mechanisms. This study showed that lipid peroxidation and OH^- accumulation play major roles in wheat susceptibility to FHB. Our knowledge on physiological and biochemical mechanisms involved in basal resistance could be useful in breeding programs leading to the introduction of wheat cultivars with high levels of resistance to FHB disease. Thus, the measurements of protein, OH^- and NO levels may be very helpful for breeding programs to screen and select FHB-resistant cultivars.

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