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INVESTIGATION OF SUSCEPTIBILITY AND RESISTANCE MECHANISMS OF SOME EGYPTIAN WHEAT CULTIVARS (*Triticum aestivum* L.) INOCULATED WITH *Blumeria graminis* f.sp. *tritici* USING CERTAIN BIOCHEMICAL, MOLECULAR CHARACTERIZATION AND SEM

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ABSTRACT

Two greenhouse experiments were conducted in the greenhouse of Agricultural Botany Department, Faculty of Agriculture, Kafrelsheikh University, Egypt, during two seasons (2012-2013 and 2013-2014) to study biochemical, molecular characterization and SEM investigation of susceptibility and resistance mechanisms of ten Egyptian wheat cultivars (Sakha 93, Sakha 94, Sids 12, Sids 13, Giza 160, Giza 168, Giza 171, Misr 1, Misr 2 and Gemmiza 11) inoculated with *Blumeria graminis* f. sp. *tritici* fungus the causal organism of wheat powdery mildew. This disease one of most important diseases of wheat worldwide. Chemical and biological control methods are used routinely to control the disease. However, resistant cultivars are still the best control strategy. Consequently, characterization of susceptibility and resistance mechanisms is very important and required essentially. In wheat susceptible cultivars (Sakha 93, Sakha 94, Sids 12 and Sids 13), moderately susceptible (Giza 160), moderately resistant (Giza 168, Giza 171, Misr 1), resistant (Misr 2) and highly resistant cultivars Gemmiza 11) when inoculated with powdery mildew fungus, the percent of disease severity were significantly decreased in resistant cultivars compared with susceptible ones. Indeed the disease symptoms and electrolyte leakage were also significantly decreased in resistant cultivars. As well as levels of reactive oxygen species (ROS), such as superoxide (O_2^-) and hydrogen peroxide (H_2O_2) significantly accumulated early 6 and 12 hours after inoculation (hai) in the resistant cultivars. Consequently, catalase (CAT), superoxide dismutase (SOD) and peroxidase (POX) enzyme activities were significantly stimulated at 24-72 hai. Significant increase of chlorophyll a and b concentrations was found. However, in the susceptible inoculated cultivars, the fungus was intensively colonized with huge quantity of conidia spores compared with the moderately resistant, resistant and highly resistant cultivars using scanning electron microscope (SEM). Molecular investigations using PCR (SSR) technique proved that the resistant gene *Pm38* over expressed and accumulated in resistant cultivars not in susceptible ones. Expression of *Pm38* gene was correlated with the resistance degrees. It can be recommended giving more attention to these new mechanisms of resistance to improve and find out new resistant cultivars which over expressed new resistant-mediated-ROS genes.

Keywords: *Triticum aestivum* L., *Blumeria graminis* f.sp. *tritici*, Scanning Electron Microscope (SEM) , Antioxidants, Resistant *Pm38* gene, ROS

INTORODUCTION

It is well known that wheat (*Triticum aestivum* L.) crop is one of the most vital cereal crops all over the world (Peng *et al*., 2000; Wang *et al*., 2002; Chen *et al*., 2003; Abdelaal *et al.,* 2014).

Powdery mildew disease caused by the fungus *Blumeria graminis* f. sp. *tritici* (*Bgt*) which is a common pathogenic disease distributed in various wheat production countries, causing significant loss of yield (Leath *et al.,* 1989; Griffey, 1993). Fungicide treatment is regularly carried out to control cereal diseases. However, the development of fungicide resistant strains limited the fungicides usage. In addition, fungicides act as a hazard effects on human health and environmental pollution (Wilson *et al*., 1994; Hafez *et al*., 2014a; Abdelaal *et al.,* 2014). Resistant cultivars are the most real and economical method in controlling plant diseases (Röbbelen and Sharp, 1978; Line and Chen, 1995).

Using traditional breeding, certain resistant genes to powdery mildew pathogen in wild relatives of wheat have been transferred to the susceptible common wheat generating a series of isogeniclines showing varying degrees of resistance to a number of powdery mildew types. These powdery mildew-resistant lines have been suggested as the most economical and environmentally safe cultivars for disease control. It could be used as natural materials in the laboratory for a better characterization of the molecular mechanisms of the host-pathogen interaction and defense response in plants (Bhullar *et al.,* 2009; Brunner *et al.,* 2011). Advanced studies were conducted to clarify structural components and molecular mechanisms of resistance response in wheat (Wang *et al*., 2010; Hafez *et al.,* 2014b; Abdelaal *et al.,* 2014). Resistant host plant is the greatest real means to control the powdery mildew disease, then, planting resistant cultivars are recommended. However, different races of the fungus can occur from one year to the next and might overcome resistance. Different methods have been done to classify the pathogen-resistant genes in wheat which responding to several types of crop diseases (Bernardo *et al.,* 2007; Coram *et al.,* 2008). It was shown that 44 genes are differentially expressed at 72 hours after inoculation (hai) against *Fusarium* spp., and cause head blight resistant and/or susceptible (Bernardo *et al.,* 2007).

Infections of plants with phytopathogens (mostly fungi, bacteria and viruses) in many cases are accompanying with the accumulation of ROS which induces oxidative stress in plants (Hafez *et al.,* 2012). Under natural conditions, up-regulation of antioxidant defense systems seems to be a general response to oxidative stress (Halliwell and Gutteridge, 1999; Hafez and El-Baghdady 2013). Hydrogen peroxide (H_2O_2) and superoxide ($O_2^{\cdot-}$) are the most important ROS associated with oxidative stress, which up-regulate antioxidant systems even at very low concentrations (Gechev *et al*., 2002; Hafez *et al*., 2012). ROS are produced by all aerobic organisms as by-products of several metabolic pathways, including electron flows in mitochondria and chloroplasts, lipid catabolism and photorespiration in glyoxysomes and peroxisomes as well as enzymatic oxygenase reactions having a different cellular localization. In order to avoid ROS toxicity, aerobic cells are provided with a flexible set of enzymes and metabolites involved in ROS catabolism, which often acts at the site of ROS production. Although much metabolic energy is spent on ROS removal by plant cells, ROS are also actively produced by cell metabolism under optimal growth conditions (Heath, 2000). When ROS over accumulated early after the infection either in

resistant wheat cultivars against wheat stripe rust (Abdelaal *et al.,* 2014) or ROS stimulated in susceptible barley cultivars treated with resistant inducers (Hafez *et al*., 2014b) the fungus inhibited or killed early after infection. Fortunately, cells make a variety of antioxidant enzymes to fight the dangerous side-effects of life with oxygen. Two important players are superoxide dismutase, which converts $O_2^{\cdot-}$ into H_2O_2, and catalase, which converts H_2O_2 into water and oxygen gas (Apel and Hirt, 2004).

The present investigation aimed to clarify the biochemical, histochemical and molecular mechanisms associated with susceptibility and resistance of wheat cultivars inoculated with *Blumeria graminis* f. sp. *tritici* the powdery mildew fungus. The activities of antioxidant enzymes, ROS levels, disease severity, electrolyte leakage, chlorophyll a/b concentrations, scanning electron microscope (SEM) examination and accumulation of resistance gene *Pm38* were determined.

MATERIALS AND METHODS

Plant materials

The research experiments were conducted in the greenhouse of Agricultural Botany Department, Faculty of Agriculture, Kafrelsheikh University, Egypt, during two growing seasons (2012-2013 and 2013-2014). Ten Egyptian wheat cultivars (Sakha 93, Sakha 94, Sids 12, Sids 13, Giza 160, Giza 168, Giza 171, Misr 1, Misr 2 and Gemmiza 11) were used. Ten grains of each cultivar were sown/plastic pot (7cm diam.) in a formalin sterilized soil mixture (5:2:1 v/v/v) of clay, sand and peat moss. Pots were placed in spore-proof greenhouse cabins under 16 hours light (17^{o}C) and 8 hours darkness (12^{o}C). An artificial light of 50–100 μEm^{-2} s^{-1} was applied when daylight was less than 10000 lux. Wheat seedlings were inoculated at 7-8 days (Awad *et al.,* 2015).

Artificial inoculation

Tables 1 and 2 show that artificial inoculation was carried out using rubbing technique at the flag leaf according to the method of Large (1954). Spores were harvested from infected seedlings of the susceptible cultivar (Sakha 93). Infected samples were kept in living susceptible wheat cultivar to be used in further studies and kept in per gamin envelops in fridge at 5-10 C˚. Each sample was picked and inoculated on 7-8 days seedlings of wheat cultivars (Awad *et al.,* 2015). Three replicates were inoculated for each cultivar. Then inoculated seedlings were placed under trays, sprayed with water and covered to keep 100% fresh during incubation at 10^{o}C for 22 hours. After that pots were transferred to the greenhouse and randomized in a spore-proof cabin (Chris, 2012).

Disease severity:

Infection types were scored 2 and 12 weeks after inoculation in seedling stage and at adult stage, respectively according to the methods of Shi *et al*., (1987) and Leath and Heun, (1990), respectively, as shown in Tables (1 and 2).

Table (1): Infection types of inoculated wheat at seedling stage

Host	Response (class)	Infection types	Disease symptoms
Resistant	Immune	Low 0	No visible symptoms
	Nearly immune	Low 0	Hypersensitive necrotic flecks
	Very resistant	Low 1	Minute colonies with few conidia produced
	Moderately resistant	Low 2	Colonies with moderately developed hyphae, but few conidia
Susceptible	Moderately susceptible	High 3	Colonies with well-developed hyphae and abundant conidia, but colonies not joined together few conidia
	Very susceptible	High 4	Colonies with well-developed hyphae and abundant conidia, and colonies mostly joined together

Table (2): Infection types of inoculated wheat at adult stage

Infection type	Host response	Symptoms
0	Immune	No visible sings or symptoms
1	Highly resistant	Small flecks only
2	Resistant	Chlorosis flecks evident
3	Moderately resistant	Large flecks with chlorosis and necrosis
4	Intermediate	Mycelium and conidia detectable
5	Moderately susceptible	Small to moderate sized pustules and conidia present
6	Moderately susceptible	Predominance of moderate sized pustules and conidia present
7	Susceptible	At the least 50% of the large pustules and conidia are visual
8	Susceptible	75 to 80% of the leaf segment were covered with large pustules and conidia
9	Highly susceptible	100% of the leaf segment curved with large pustules and conidia

Histochemical detection of $O_2^{\cdot-}$ and H_2O_2

Detection of $O_2^{\cdot-}$and H_2O_2 were visualized as a purple coloration of nitro blue tetrazolium (NBT) and a reddish-brown coloration of 3,3-diaminobenzidine (DAB), respectively. Wheat leaves were vacuum infiltrated with 10 mM potassium salicylate buffer (pH 7.8) containing 0.1 w/v % NBT (Sigma–Aldrich, Steinheim, Germany) or 0.1 w/v % DAB (Fluka, Buchs, Switzerland). NBT- and DAB-treated samples were incubated under daylight for 20 min and 2 hours, respectively and subsequently cleared in 0.15 w/v % trichloroacetic acid in ethanol: chloroform 4:1 v/v for 1 day (Hückelhoven *et al.,* 1999). Cleared samples were washed with water and placed in 50% glycerol prior to be ready for evaluation. Discoloration of leaves quantified using nicked eyes or a ChemiImager 4000 digital imaging system (Alpha Innotech Corp., San Leandro, USA).

Antioxidant enzyme activities

For enzyme assays in plants, 0.5 g fresh treated wheat leaf material of each particular treatment was homogenized at 0-4˚C in 3 ml of 50 mM Tris buffer (pH 7.8), containing 1 mM EDTA-Na2 and 7.5% polyvinyl pyrrolidone. The homogenates were centrifuged (12,000 rpm, 20 min, 4˚C) and the total soluble enzyme activities were measured spectrophotometrically in the supernatant (Hafez, *et al*., 2014b). All measurements were carried out at 25˚C, using the model UV-160A spectrophotometer (Shimadzu, Japan).

Superoxide dismutase (SOD; EC 1.15.1.1) activity

Activity of SOD was measured in a plate reader with modifications of Mishra *et al*., (1993). 290 µL of a mixture containing 100 mM potassium phosphate buffer (pH 7.8), 0.1 mM EDTA, 11 µM cytochrome-c, 11 µM xanthine, and 0.002 Units of xanthine oxidase to 20 µg of protein extracts. Xanthine oxidase controls produce an increase in the absorbance due to the reduction of cytochrome-c in the range of 0.025 ± 0.005 min^{-1}. Activity of SOD was expressed in units as described by McCord and Fridovich(1969).

Catalase activity (CAT; E.C. 1.11.1.6)

Activity of CAT was determined according to Aebi (1984). Decomposition of H_2O_2 by catalase results in the decrease of the ultraviolet absorption of hydrogen peroxide at 240 nm. Enzyme activity can be calculated from this decrease. The reaction mixture contained, in a final volume of 2.15 ml, 2 ml 0.1 M Na-phosphate buffer (pH 6.5), 100 µl hydrogen peroxide and 50 µl leaf extract supernatant. The solution is mixed, and then the absorption change is registered for 3 min at 240 nm using a quartz cuvette.

Peroxidase activity (POX; EC 1.11.1.7)

Activity of POX was determined according to procedure proposed by Hammerschmidt *et al*., (1982). The reaction mixture consisted of 2.9 ml of a 100 mM sodium phosphate buffer (pH 6.0) containing 0.25 % (v/v) guaiacol (2-methoxy phenol) and 100 mM H_2O_2. The reaction was started by adding 100 µl of crude enzyme extract. Changes in absorbance at 470 nm were recorded every 30 sec intervals for 3 min. Enzyme activity was expressed as increase in absorbance min^{-1} g^{-1} fresh weight.

Electrolyte leakage assay

Twenty leaf discs (1 cm^2) of wheat leaves were placed individually into flasks each contained 25 ml deionized water (Milli-Q 50, Millipore, Bedford, Mass., USA). Flasks were shaken for 20 hr at ambient temperature to facilitate electrolyte leakage from injured tissues. Initial electrical conductivity measurements were recorded for each vial using an Acromet AR20 electrical conductivity meter (Fisher Scientific, Chicago, IL). Flasks were then immersed in a hot water bath (Fisher Isotemp, Indiana, PA) at 80°C (176°F) for 1 hr to induce cell rupture. The vials were again placed on the Innova 2100 platform shaker for 20 hr at 21°C (70°F). Final conductivity was measured for each flask. Electrolyte leakage Percentage for each bud was calculated as: initial conductivity/final conductivity × 100 M according to Szalai*et al*. (1996).

Concentration of chlorophyll a and b

Chlorophyll (Chl.) concentration as mg/g fresh weight of one gram fresh leaves was extracted with 5 ml N,N-dimethyl-formamid for overnight at 5°c then estimated Chl. a and b spectrophotometerically at 663 and 647 nm as described by Moran and Porath (1982). The concentrations were calculated in the following equations: Chl. a = 12.76 A_{663} – 2.79 A_{647} (mg/g fresh weight), Chl. b = 20.76 A_{647} – 4.62 A_{663} (mg/g fresh weight).

Scanning Electron Microscopic examination

Anatomical structure of susceptible and resistant wheat leaves was investigated with scanning electron microscope (SEM). Wheat leaves were taken (4mm²) from susceptible and resistant leaves and immediately fixed in glutraldhyde (2.5%) for 24 hrs at 4°C, then post-fixed in osmium tetraoxide (1% OS04) for one hour at room temperature (Harley and Fergusen, 1990). Samples were dehydrated with passing through ascending concentrations of acetone (30-100%). Samples were dried till the critical point finally, leaf was sputter coated with gold. The examination and photographing were done through a Jeol Scanning Electron Microscopy (T.330 A).

Detection of *Pm38* resistant gene

DNA extraction

Total DNA of wheat seedlings was extracted from 60 mg of fresh leaves which initially were mashed in liquid nitrogen with a mortar and pestle using Invisorb® Spin Plant Mini Kit (STRATEC Molecular, Germany) according to manufacturer's instructions.

Conditions and amplification of PCR:

Amplification of *Pm*regions were conducted in an automated thermal cycler (C1000^{TM} Thermal Cycler, Bio-RAD) using the primer sequences of *Pm38* gene (L34DINT9-R11), primers are listed as follows as mentioned by Lagudah *et al*., (2009). (F5'TTGATGAAACCAGTTTTTTTTCTA3' and R5'GCCATTTAACATAATCATGATGGA 3') with one cycle pre-denaturation at 94ºC for 3 min. Amplification step: (30 cycles): 94^{0}C, 300sec for denaturation., 48^{0}C, 60 sec for annealing, 72^{0}C, 60 sec for extension & final extension at 72^{0}C, 5min. Each PCR mixture for *Pm* genes detection (25 µl) was prepared as follow, (1 µl) of 25 ng nucleic acid, 1 µl of each primer (10 pmol), (12.5 µl) of GoTag® Colorless Master Mix (Promega Corporation, USA) and 9.5 µl of Nuclease free water (Promega). 15 µl of all PCR products were analyzed by electrophoresis through a 1.5% agarose gel, stained with ethidium bromide, and DNA bands were visualized using a UV trans-illuminator.

Statistical analysis

Two experiments were conducted during two seasons in a complete randomized design with three replicates for each treatment. Data represent the mean ± SD. Student's t-test was used to determine whether significant difference ($P<0.05$) existed between mean values according to O'Mahony (1986).

RESULTS AND DISCUSSION

Infection types of inoculated wheat cultivars with *B. graminis* f. sp. *tritici*

According to the results during the two successive growing seasons (2012-2013 and 2013-2014) presented in table 2, the infection types of wheat powdery mildew on wheat cultivars inoculated with *B. graminis* f. sp. *tritici* showed great differences between all tested cultivars. Sakha 93, Sakha 94, Sids 12 and Sids 13 cultivars revealed the infection types of the susceptibility (S). Giza 160 cultivar was moderately susceptible (MS). Giza 168, Giza171 and Misr 1 were moderately resistant (MR). Misr1 and Gemmiza 11 cultivars exhibited resistance (R) and highly resistance infection types, respectively (Table 3). These results agreed with the results obtained by Abdelaal *et al.,* (2014) which showed a series types of infection in wheat cultivars infected with *Puccinia striiformis*.

Table (3): Infection types of *B. graminis* f. sp. *tritici* on wheat cultivars

No.	Wheat cultivars	2012-2013 season		2013-2014 season	
		Seedling	Adult	Seedling	Adult
1	Sakha -93 (S)	4	9	3	9
2	Sakha -94 (S)	3	7	4	6
3	Sids 12 (S)	4	7	3	6
4	Sids 13 (S) 13	4	7	3	6
5	Giza 160 (MS)	3	4	2	3
6	Giza -168 (MR)	1	3	2	2
7	Giza -171 (MR)	1	3	2	2
8	Misr 1 (MR)	1	12	1	2
9	Misr 2 (R)	1	1	1	2
10	Gem. 11 (HR)	0	0	0	1

Disease severity of inoculated wheat cultivars

When, susceptible and resistant wheat cultivars inoculated with wheat powdery pathogen *B. graminis* f. sp. *tritici*, the disease severity percentage (Fig. 1) was highly significantly reduced in Gemmiza 11 and Misr 2 compared to Sakha 93, Sakha 94, Sids 12 and Sids 13 cultivars which showed the susceptibility to *B. graminis* f.sp. *tritici*. Disease severity percentage (Fig. 1) was significantly reduced also in Giza 160, Giza 168, Giza 171 and Misr 1 compared to the susceptible cultivars. It seems that the disease severity percentage was concomitant with the degree of infection types during the first season and the second season. Similar results were obtained when susceptible and resistant wheat cultivars infected with *Puccinia striiformis* fungus (Abdelaal *et al.,* 2014).

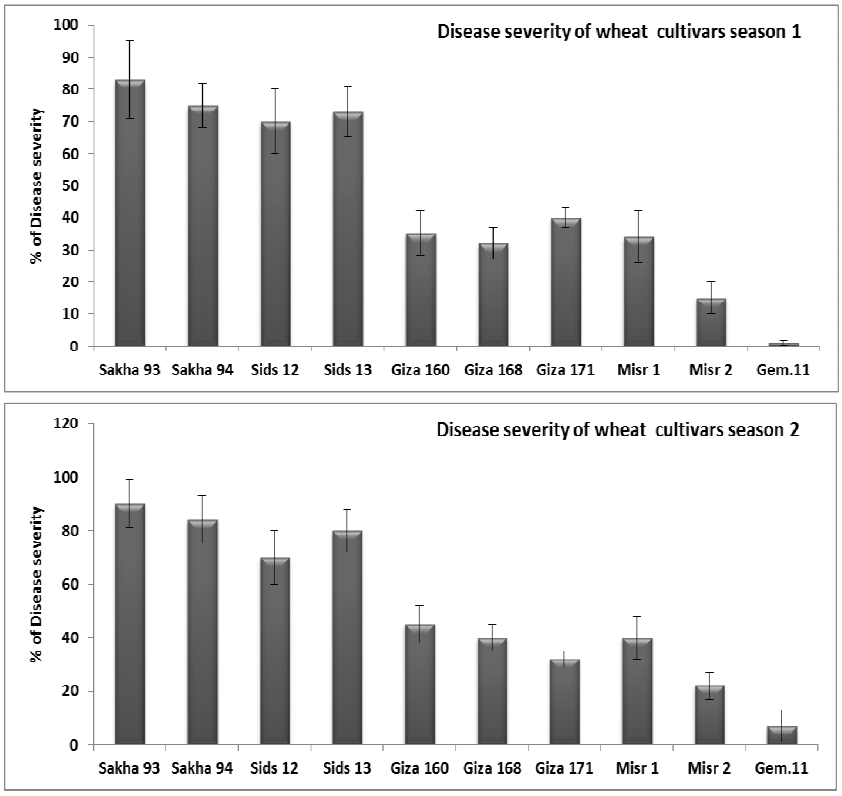

Fig. (1): Disease severity percentage of susceptible and resistant wheat cultivars inoculated with *Blumeria graminis* f. sp. *tritici* during 2012-2013 (season 1) and 2013-2014 (season 2) seasons. Sakha 93, Sakha 94, Sids 12 and Sids 13: susceptible wheat cultivars. Giza 160: moderately susceptible cultivar. Giza168, Giza 171 and Misr 1: moderately resistance cultivars. Misr 2: resistant cultivar. Gem. 11: Gemmiza 11, highly resistant cultivar.

Disease symptoms of inoculated wheat cultivars

As a result of inoculated susceptible and resistant wheat cultivars with *B. graminis* f. sp. *tritici* fungus, the disease symptoms significantly appeared on Sakha 93, Sakha 94, Sids 12 and Sids 13 cultivars which showed the mycelium growth of the fungus compared with Gemmiza 11 and Misr 2 resistant cultivars (Fig. 2). Weak disease symptoms also were appeared on Giza 160, Giza 168, Giza 171 and Misr 1 compared to susceptible cultivars. The disease symptoms seem also correlated with the degree of susceptibility and resistance. These results can be supported with the results obtained by Hafez *et al.,* (2014a) which conducted with wheat cultivars infected with wheat leaf rust *Puccinia triticina*.

Levels of reactive oxygen species (ROS) in inoculated wheat cultivars

Histochemical staining of ROS mainly hydrogen peroxide (H_2O_2) and superoxide ($O_2^{\cdot-}$) were visualized as brown and purple discoloration respectively. Accumulation of H_2O_2 and $O_2^{\cdot-}$ were significantly increased in resistant and moderately resistant wheat cultivars early 6 hours after inoculation (hai) compared to the susceptible cultivars (Fig. 3). The strong brown and purple discoloration intensity indicated high levels of H_2O_2 and $O_2^{\cdot-}$, respectively (Fig 3).

Quantification of discoloration of wheat leaves resulted by DAB or NBT staining indicated that levels of ROS was significantly elevated in resistant and moderately resistant cultivars compared to susceptible cultivars 6, 12 and 18 hai then decreased at 24 and 48 hai (Fig. 4).

Enhanced ROS production in plants is termed 'oxidative burst' (Wojtaszek, 1997). ROS appeared continuously in the chloroplasts by partial reduction of O_2 molecules or energy transfer to them during the duration of photosynthesis. The production of ROS is an inevitable consequence of aerobic respiration. When the terminal oxidases-cytochrome oxidase and the alternative oxidase react with O_2, four electrons are transferred and H_2O is released. It has been noted that $O_2^{\cdot-}$ is usually the first ROS to be generated.

Fig. (2): Disease symptoms on susceptible and resistant wheat cultivars inoculated with wheat powdery mildew *B. graminis* f. sp. *tritici* 5 days after inoculation.

In plant tissues, about 1-2% of O_2 consumption leads to the generation of $O_2^{\cdot-}$. ROS derived from the oxidative burst can directly damage bacteria (Molina-Cruz *et al*., 2008) and can also function as signaling molecules (Atia *et al*., 2005). In plant cells, for example, ROS derived from the stress-induced oxidative burst activate a variety of defense responses including synthesis of phytoalexins, synthesis of pathogenesis-related proteins, and suppression of pathogen growth by programmed cell death (Lamb and Dixon, 1997). It has been well established that excess of H_2O_2 in plant cells leads to the occurrence of oxidative stress (Atia *et al*., 2005, Hafez *et al*., 2012; Hafez *et al*., 2014a).

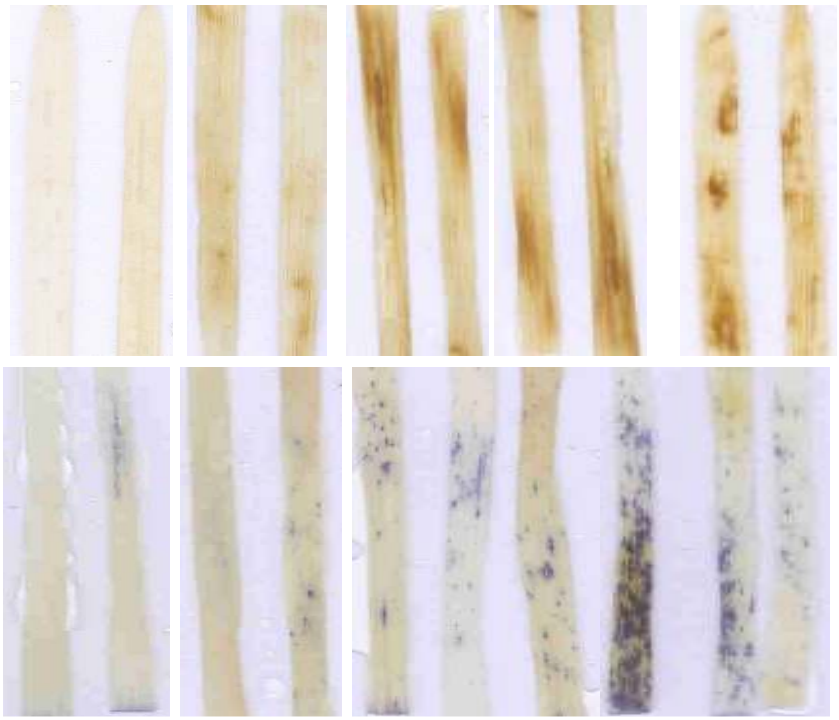

Fig. (3): Accumulation of reactive oxygen species (ROS), purple discoloration of superoxide (lower row) and brown discoloration of hydrogen peroxide (upper row) in susceptible and resistant wheat cultivars 6 hours after inoculated with wheat powdery mildew *B. graminis* f. sp. *tritici*.

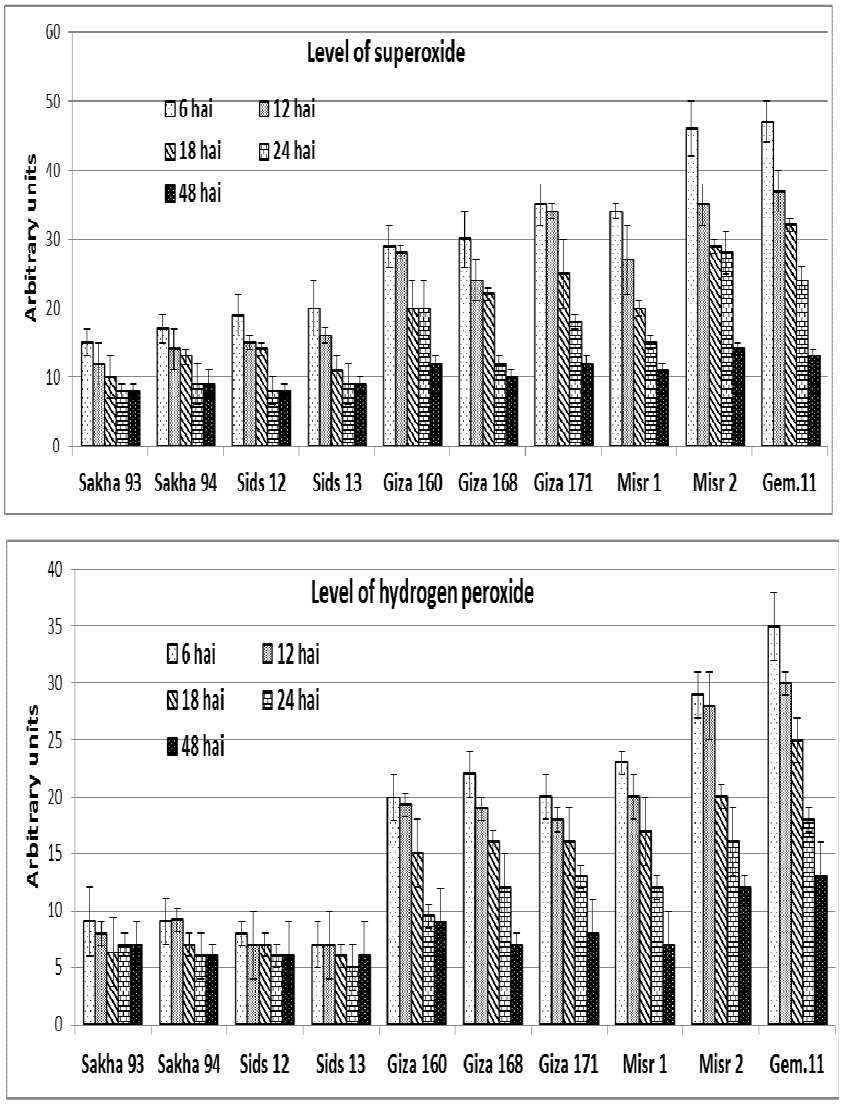

Fig. (4): Levels of superoxide (upper) and hydrogen peroxide (lower) in susceptible and resistant wheat cultivars inoculated with wheat powdery mildew *B. graminis* f. sp. *tritici* 6, 12, 18, 24 and 48 hours after inoculation.

These results showed early elevated levels of H_2O_2 and $O_2^{\cdot-}$ in the resistant wheat cultivars lead to less damage in the cells. Levels of ROS were increased when antioxidants were decreased. Similarly, in resistant Egyptian and other wheat cultivars inoculated with leaf rust (*Puccinia triticina*), H_2O_2 has a key role in resistance (Atia *et al*., 2005; Hafez *et al*., 2009). Similar results were obtained in susceptible and resistant wheat cultivars inoculated with *Puccinia striiformis* the causal agent of strip rust (Abdelaal *et al.,* 2014). ROS are elevated also when the host plant infected with phytoplasma (Musetti *et al.,* 2005; Sanchez-Rojo *et al.,* 2011) and plant treated with inducers of resistance substances (Atia *et al*., 2005). Consequently, it is important for bacterial pathogens to suppressing ROS by activation of antioxidant enzymes for their survival (Halliwell, 1974).

Activity of antioxidant enzymes in inoculated wheat cultivars

According to the results, up-down regulation of antioxidant enzymes activities were expected particularly, SOD, CAT and POX could be up-regulated. The activities of antioxidant enzymes in susceptible and resistant wheat cultivars inoculated with wheat powdery mildew fungus were undertaken. SOD plays a major role in the degradation and dismutation processes of superoxide in ROS detoxification pathways (Fridovich, 1995). Superoxide dismutase (SOD), catalase (CAT) and peroxidase (POX) activities in wheat susceptible and resistant cultivars were a little bit increased early 6, 12 and 18 hai with wheat powdery mildew pathogen (Fig. 5). However, the activities of these enzymes were significantly increased in resistant and moderately resistant wheat cultivars compared to susceptible cultivars 24, 48 and 72 hai (Fig. 5). It is clearly known that the first response following the oxidative burst is the stimulation of ROS scavenging enzymes. SOD is usually considered the first line of defense against oxidative stress (Dewir, 2001). Antioxidant defense systems are enzymatic and non-enzymatic antioxidants. Enzymatic antioxidants include superoxide dismutase (SOD), catalase (CAT), peroxidase (POX), ascorbate peroxidase (APX) and polyphenol oxidase (PPO). The non-enzymatic antioxidants are glutathione (GSH), carotenoids and tocopherols. The up-regulation of CAT, POX and PPO plays vital role during elevated the ROS levels. So,, protected resistant wheat cultivars from pathogen attack. Similarly, tobacco plants protected against viral, bacterial and fungal infections (Hafez *et al*., 2012; Abdelaal *et al.,* 2014; Hafez *et al*., 2014a; Hafez *et al*., 2014b).

H_2O_2 plays central role in the oxidative burst, acting as a signal for the localized death of challenged cells (Martinez *et al.,* 2000; Atia *et al.,* 2005) and as a diffusible signal for the induction of cellular protective genes in adjacent healthy cells and tissues (Levine *et al.,* 1994). H_2O_2 generated by SOD is further degraded and detoxified by other antioxidant enzymes such as POX, APX or CAT. Previous reports demonstrated that POX and PPO may participate in the responding defense reaction by inducing plant resistance against pathogenic agents (Ray *et al.,* 1998).

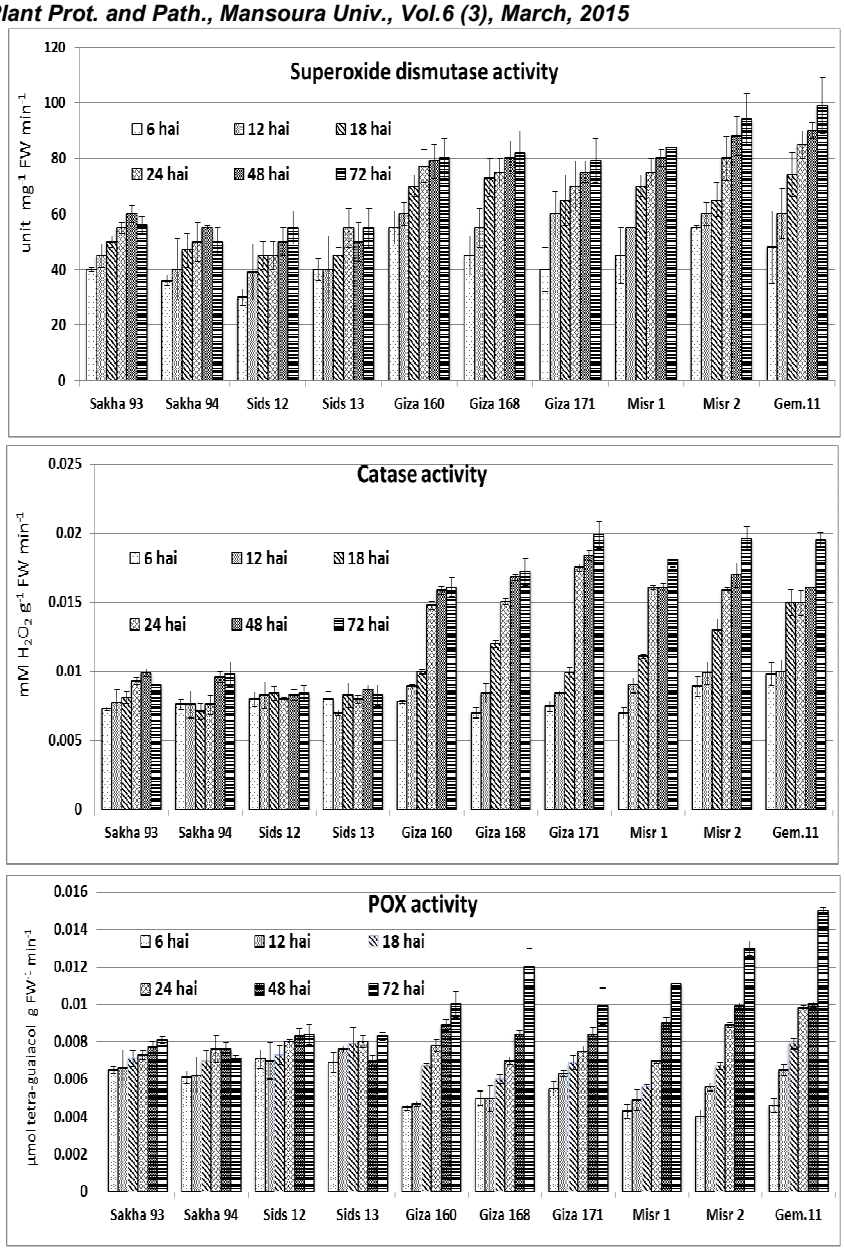

Fig. (5): Activities of antioxidant enzymes superoxide dismutase (SOD), catalase (CAT) and peroxidase (POX) in susceptible and resistant wheat cultivars inoculated with wheat powdery mildew fungus *B. graminis* f. sp. *tritici* 6, 12, 18, 24, 48 and 72 hours after inoculation.

Electrolyte leakage in inoculated wheat cultivars

Electrolyte leakage (EL) constitutes as an indicator of the membrane permeability. It was significantly increased in all susceptible wheat cultivars inoculated with *B. graminis* f. sp. *tritici* compared to the resistant or even the moderately resistant cultivars (Fig. 6). The cellular membrane dysfunction due to stress is well increased permeability and leakage of ions out, which can be readily measured by the efflux of electrolytes. Increase of cell permeability is one of the most common effects of pathogens on the plant cells. Electrolyte leakage has been used to quantify damages of cell membranes in response to biotic stresses (Adam *et al.,* 2000; Sriram *et al.,* 2000; Hafez *et al.,* 2014a; Abdelaal *et al.,* 2014) as well as a biotic stresses (Pearce, 2001; Zhou *et al.,* 2005; Abbas, 2012).

In the present study, inoculation *B. graminis* f. sp. *tritici* increased electrolyte leakage in susceptible wheat cultivars than the resistant cultivars because it is an obligatory parasite which depends heavily on their host cells for essential metabolic compounds, this could be because of pathogen-host compatibility. These results supported by results which chemical compounds and biotic or a biotic stresses could alter the resistance or susceptibility of plants to infection through their effects on membrane permeability (Hafez *et al*., 2014b). It is known that ethylene affects membrane permeability (Goodman *et al*., 1986). Similarly, high temperature stress could induce susceptibility in maize through its effects on membrane permeability as measured by increased electrolyte leakage (Garraway *et al*., 1989). This might results in the loss of host cells' constituents which may be used by the invading pathogen as a source of nutrients.

These results indicated that resistance of some wheat (cultivars) protected cell membranes during the pathogen attack, while the cell membrane of the susceptible cultivars was affected by the pathogen inoculation and lost its constituents. The present results are in agreement with those obtained by (Houimli *et al*., 2010; Hafez, *et al*., 2014b).

Chlorophyll a and b concentrations

Chlorophyll a and b concentrations increased in all the resistant (Gemmiza 11 and Misr 2), moderately resistant (Giza 168, Giza 171, Misr 1) and moderately susceptible (Giza 160) wheat cultivars compared to the susceptible cultivars (Sakha 93, Sakha 94, Sids 12, Sids 13) during both seasons (Fig. 7).

It seems that high concentrations of chlorophyll a and b correlated with the degrees of resistance (Fig. 7). Similar results obtained by Abdelaal *et al.,* (2014), Moriondo *et al*., (2005) and Lindenthal *et al*., (2005) in wheat cultivars inoculated with stripe rust *Puccinia striiformis* and squash plants infected with downy mildew On the other hand, chlorophyll a/b decreased in susceptible cultivars. This could be correlated with genes encoding chlorophyll a/b-binding proteins which responsive to powdery mildew infection (Xin *et al.,* 2012). It was found 32 genes encoding chlorophyll a/b-which responsive to powdery mildew infection, only two of them were up-regulated, while the others were repressed in susceptible than in resistant wheat genotype using microarray (Xin *et al.,* 2012). The decreased of chlorophyll concentration perhaps due to the spread of the pathogen hypha and its penetration in host

cells by haustoria are thought to destabilize the structural integrity, which reduces chlorophyll pigments in wheat infected with rust disease (Lindenthal *et al*., 2005 and Abdelaal *et al.,* 2014).

The reduction of chlorophyll is might be due to the decrease in the number and abnormal form of chloroplasts in the mesophyll tissue. Chlorophyll concentration was decreased gradually with the increase in disease severity (Mandal *et al*., 2009 and Abdelaal *et al*., 2014).

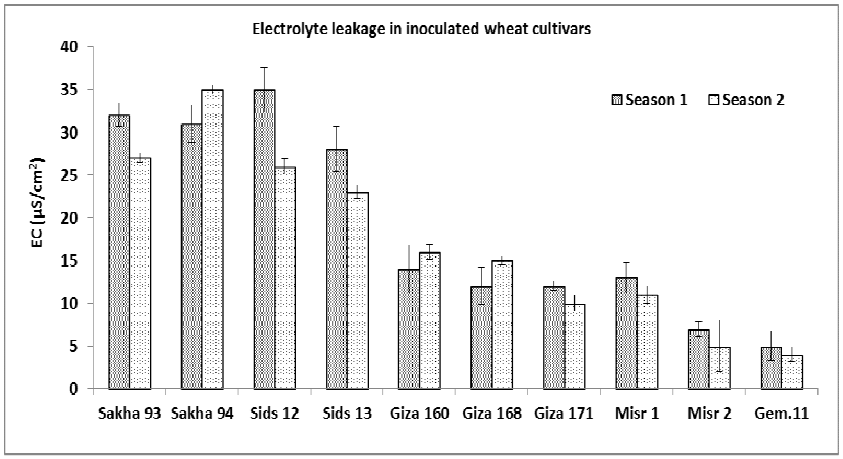

Fig. (6): Electrolyte leakage in susceptible and resistant wheat cultivars 72 hours after inoculation with wheat powdery mildew *B. graminis* f. sp. *tritici* during 2012-2013 (season 1) and 2013-2014 (season 2) seasons.

Histological alterations of wheat inoculated with *B. graminis* f. sp. *tritici*

The inoculated susceptible wheat cultivars Sakha -93, Sakha -94, Sids 12, Sids 13 were colonized extensively by *B. graminis* f. sp. *tritici* and producing much conidiophores and large amounts of conidia spores (Fig. 8 a, b, c and d). Similar results were recorded by Abdelaal *et al.,* (2014). On the other hand, in moderate susceptible cultivars such as Giza 160, Giza 168 (Fig.8 e and f) and in moderate resistant cultivars such as Giza 171 and Misr 1 (Fig.8 g and h) the fungus were colonized only in a limited extent, producing a very little number of conidia spores specially in moderate resistant cultivars (Fig.8 g and h). In the resistant cultivar such as Misr 2 and highly resistant cultivar such as Gemmiza11 (Fig.8 i and j) the fungus did not show colonies or spores on the leaves during this study.

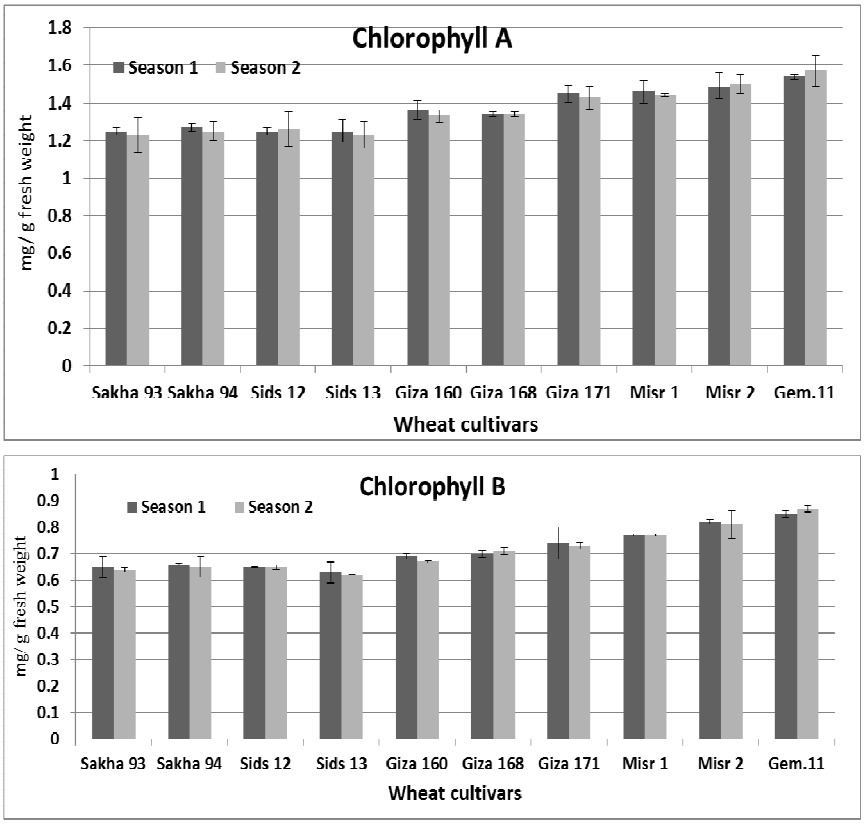

Fig. (7): Chlorophyll a (upper diagram) and chlorophyll b (lower diagram) concentrations in susceptible and resistant wheat cultivars 72 hours after inoculation with wheat powdery mildew *B. graminis* f. sp. *tritici* during 2012-2013 (season 1) and 2013-2014 (season 2) seasons.

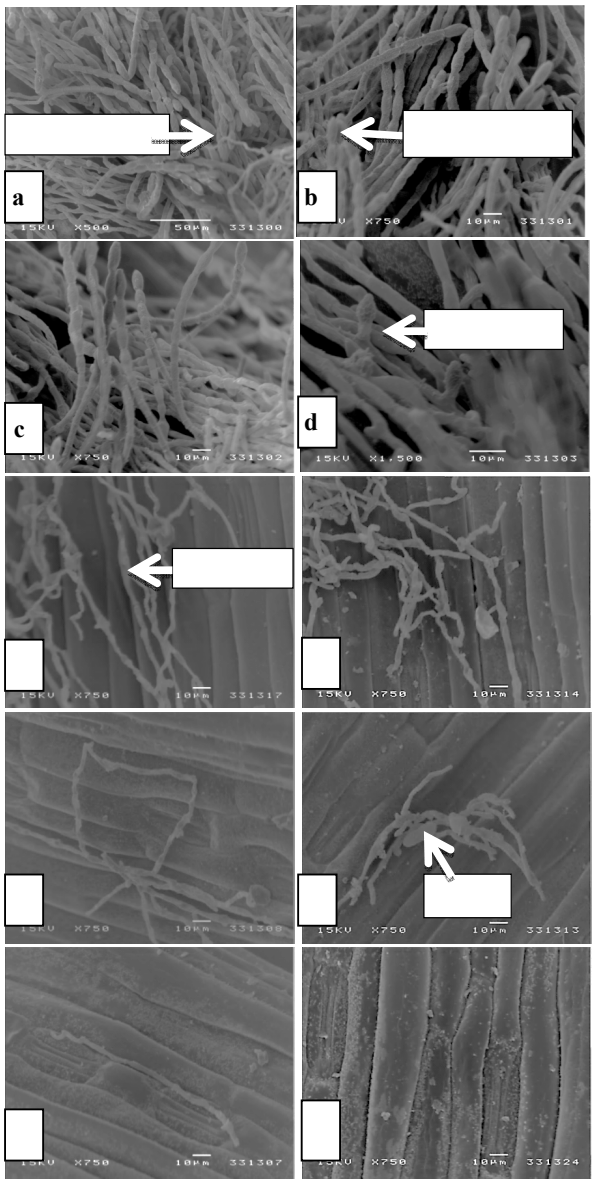

Fig. (8): Fungal development and host cell responses by SEM in susceptible and resistant wheat cultivars inoculated with *Blumeria gramins* f. sp. *tritici*. a. Sakha 93 susceptible cultivar, b. Sakha 94 susceptible cultivar, c. Sids 12 susceptible cultivar, d. Sids-13 susceptible, e. Giza 160 moderate susceptible f. Giza 168 moderate resistant, g. Giza 171 moderate resistant, h. Misr 1 moderate resistant, i. Misr 2 resistant cultivar, j. Gem.11 highly resistant cultivar; Bar = 10 µm., Bar = 50 µm.

Accumulation of *Pm38* gene in wheat cultivars:

In resistant wheat cultivars inoculated with *B. graminis* f. sp. *tritici, Pm38* gene was significantly accumulated compared to the susceptible cultivars (Fig. 9). Wheat plants have six chromosomal (A, B, D) in which could play partial role in increasing the DNA amount, therefore increasing *Pm38* copies in the resistant genome. The *Yr18/Lr34/Pm38* locus confers partial and durable adult plant resistance (APR) against leaf rust, stripe rust and powdery mildew of wheat (Lagudah *et al*., 2009). *Pm38* resistant gene over accumulated in resistant cultivars and perhaps play an important role together with the early accumulation of ROS mainly $O_2^{\cdot-}$ and H_2O_2 in which stimulate the activities of SOD, CAT and POX enzymes together. Similarly, Feng *et al*., (2014) and Abdelaal *et al.,* (2014) found similar results.

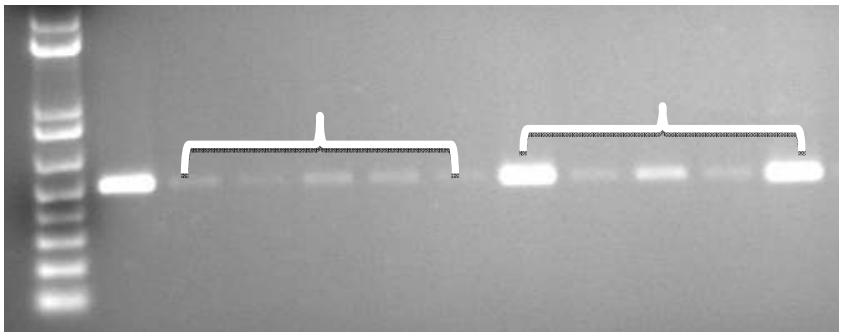

Fig. (9): Accumulation of *pm38* resistant gene in 10 wheat cultivars inoculated with fungus *B. graminis* f.sp. *tritici*. Ladder: 100 bp size ladder. *Pm38*: monogenic line. S: susceptible cultivars (Sakha 93, Sakha 94, Sids 12, Sids 13). MS: moderately susceptible cultivar (Giza 160). MR: moderately resistant cultivars (Giza 168, Giza 171, Misr 1). R: resistant cultivar (Misr 2). HR: highly resistant cultivar (Gemmiza 11).

It could be concluded that the disease severity of powdery mildew of wheat and symptoms were reduced and suppressed respectively, in the resistant wheat cultivars as well as high concentration of chlorophyll a/b correlated as a result of the *Pm38* resistant gene and ROS early accumulation which perhaps increased the enzyme activities and decreased electrolyte leakage compared to the susceptible cultivars.

Aknowledgement

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دراسة ميكانيكية القابلية للإصابة والمقاومة فى بعض أصناف القمح المصرية بفطر بلوميريا جرامينيز تريتيساي بيوكيميائياً وجزيئيا وبالميكرسكوب الالكترونى الماسح

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أجريت تجربتان داخل صوب قسم النبات الزراعى بكلية الزراعة، جامعة كفر الشيخ خلال موسمى 2012-2013 و 2013-2014 لدراسة الخصائص البيوكيميائة والجزيئية والفحص بالميكرسكوب الالكترونى الماسح لميكانيكيات القابلية للاصابة والمقاومة لعشرة أصناف من القمح المصرى (سخا ٩٣، سخا ٩٤، سدس ١٢، سدس ١٣، جيزه ١٦٠، جيزه ١٦٨، جيزه ١٧١، مصر ١، مصر ٢ و جميزه ١١) المصابة بفطر *Blumeria graminis* f. sp. *tritici* بلوميريا جرامينيز تريتيساي المسبب لمرض البياض الدقيقي فى القمح. يعتبر هذا المرض واحداً من أهم أمراض القمح على مستوى العالم، وتستخدم الطرق الكيماوية والبيولوجية لمكافحة المرض، ولكن تظل الاصناف المقاومة افضل استراتيجيات المقاومة، ولذلك يجب التعرف على ميكانيكيات القابلية للإصابة والمقاومة.

أوضحت النتائج أنه عندما تمت العدوى بالفطر المسبب للبياض الدقيقي على أصناف قمح قابلة للاصابة مثل سخا ٩٣ وسخا ٩٤ وجيزه وأصناف مقاومة، فقد انخفضت نسبة شدة الإصابة بشكل معنوى على الاصناف المقاومة بالمقارنة بالأصناف الحساسة، وكذلك انخفضت الأعراض المرضية ودرجة نفاذية الجدار الخلوي (تسرب الايونات) بشكل معنوى فى الأصناف المقاومة. وقد وجد أن مستوى مشتقات الاكسجين الحرة (ROS) مثل السوبراكساید ($O_2^{\cdot-}$) وفوق اكسيد الهيدروجين (H_2O_2) قد إرتفع بشكل معنوي مبكراً ٦ و ١٢ ساعة بعد عدوى الأصناف وبالتبعية تم استحثاث وزيادة معنوية لنشاط انزيمات الكتاليز (CAT) والسوبر اكساید ديسميوتيز (SOD) والبيروكسيديز (POX) فى الفترة من ٢٤ -٧٢ ساعة بعد العدوى، ونتيجة لذلك وجدت زيادة معنوية فى تركيز الكلوروفيل أ و ب. أما فى أصناف القمح الحساسة والمصابة بفطر البلوميريا جرامينيز تريتيساي فقد وجدت مستعمرات كثيفة وكمية كبيرة من الخيوط الفطرية (ميسيليوم) والجراثيم الكونيدية مقارنة بالأصناف المتوسطة المقاومة والعالية المقاومة والتى إنخفض فيها عدد المستعمرات والخيوط الفطرية أو انعدم تماما وخصوصا فى الاصناف عالية المقاومة وذلك باستخدام الميكروسكوب الالكترونى الماسح (SEM).

وباستخدام تكنيك تفاعل البلمرة المتسلسل (PCR) باستخدام برايمر (بادئ) متخصص (SSR) تم إثبات أن جين المقاومة (*Pm38*) قد تراكم وتم التعبير عنه بدرجة عالية فى ألاصناف المقاومة فقط دون الأصناف الحساسة، وأن درجة تراكم الجين كان مرتبطاً بدرجات المقاومة. ولذلك يمكن التوصية بمزيد من الدراسات الخاصة بميكانيكيات المقاومة وذلك لتحسين مقاومة الأصناف وإستنباط أصناف جديدة بها جينات المقاومة المرتبطة بمشتقات الأكسجين الحرة (ROS).