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Induced Resistance against *Puccinia triticina*, the Causal Agent of Wheat Leaf Rust by Chemical Inducers

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ABSTRACT

The role of six inducing resistance chemicals (IRCs), *i.e.* ascorbic acid, oxalic acid, sodium salicylate, di-basic potassium phosphate, salicylic acid and benzothiadiazole (BTH) compared to the fungicide (Tilt) was tested against the wheat leaf rust caused by *Puccinia triticina* in two wheat cultivars Giza 139 and Giza 168 under greenhouse and field conditions in 2011/12 growing season in Sharkia Governorate, Egypt. All treatments and the fungicide significantly decreased disease severity (%), electrolyte leakage and suppressed the disease symptoms as compared to the control treatment in both cultivars. Reactive oxygen species (ROS) such as superoxide ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2) levels were decreased significantly as a result of chemical inducers treatments as compared to the control and fungicide treatments. Consequently, antioxidant enzymes, catalase (CAT) and dehydroascorbate reductase (DHAR) activities were significantly increased in the infected wheat leaves treated with IRCs as compared to the control and fungicides treatments. The fungicide was not affecting significantly on ROS levels and antioxidant activities, indicating its direct toxic effect on the pathogen. These results suggest that IRCs up-regulated the antioxidant enzymes, CAT and DHAR which can play an important role in suppressing wheat leaf rust pathogen. This was confirmed by the low levels of ROS such as $O_2^{\cdot-}$ and H_2O_2.

Key words: Wheat leaf rust, induced resistance, superoxide, H_2O_2, Chemical inducers.

INTRODUCTION

Wheat leaf rust, caused by *Puccinia triticina*, is the most common rust disease of wheat (*Triticum aestivum* L.). The fungus is an obligate parasite capable of producing infectious urediniospores as long as infected leaf tissue remains alive. The urediniospores can be wind-disseminated and infect host plants hundreds of kilometers from their source plant, which can result in wheat leaf rust epidemics on a continental scale. The pathogen was associated with wheat cultivation in the early 17th century, but was often overlooked as an important disease of wheat such as stem rust (Leonard and Szabo, 2005) and *Fusarium* head blight (Goswami and Kistler, 2004). Yield losses in wheat infected by *P. triticina* are usually the result of decreased number of kernels per head and lower kernels weight. *P. triticina* is now recognized as an important pathogen in wheat production worldwide, causing significant yield losses over large geographical areas (Kolmer, 2005). Plants have evolved sophisticated inducible defense mechanisms in which the signal molecules salicylic acid, jasmonic acid and ethylene often play crucial roles. Elucidation of signalling pathways of induced disease resistance is a major objective in researches of plant-pathogen interactions.

Control of cereal diseases is carried out by fungicide treatment. However, the application of fungicides is limited because of the development of pathogenic strains with fungicide resistance, the action on human health and the environmental pollution (Wilson *et al.*, 1994). In some cases the use of alternative control treatments such as benzothiadiazole (BTH) and other chemical inducers of resistance seems important (Bayoumi and Hafez, 2006; Hafez *et al*., 2008; Hafez, 2013 and Hafez and El-Baghdady, 2013). BTH is a chemical inducer of resistance and a functional analogue to SA (Görlach *et al.*, 1996). It induces systemic acquired resistance (SAR) during the activation of signal transduction pathway, while it has no anti-microbial properties (Görlach *et al.*, 1996). Investigators showed that BTH and other chemical inducers protected several plant species against viral, bacterial and fungal pathogens (Bán *et al.*, 2004; Bayoumi and Hafez 2006 and Körösi *et al.*, 2009). It was shown that BTH suppressed the grey mould caused by *B. cinerea* in strawberry (Terry and Joyce, 2000), induced resistance against *Penicillium expansum* in peach (Liu *et al.*, 2005) and pear fruits (Cao et *al.*, 2005) during postharvest storage. BTH protected rock melons, hami melons and passion fruits (Huang *et al.*, 2000; Willingham *et al.*, 2002) from decay caused by fungal pathogens during postharvest storage and protected white pepper fruits from *B. cinerea* (Hafez, 2010). Salicylic acid (SA), which exists in many plant organs, is an endogenous signal molecule inducing plant defence response and reducing population of pathogens (Vlot *et al*., 2009). Exogenous application of SA in non-toxic concentrations was effective in the regulation of biotic and abiotic stresses (Eraslan *et al.*, 2007 and Xu and Tian, 2008). Some other chemical compounds, *i.e.* salicylic acid (SA); di-basic potassium phosphate (K_2HPO_4); oxalic acid, ascorbic acid and sodium salicylate have been shown to induce resistance in plants (Ata *et al.*, 2008 and Ragab *et al.*, 2009).

Interestingly, that not too many experiments have been carried out in Egypt to study the relation of controlling wheat rust through those chemical inducers of resistance and its relation to the ROS levels exogenously. Therefore, the aim of this research was to induce resistance defense against *P. triticina* and the ROS levels and antioxidant activities pathways endogenously.

MATERIALS AND METHODS

Plant materials

Ten wheat grains of (*Triticum aestivum* L.) cultivars; Giza 139 (highly susceptible) and Giza 168 (moderately susceptible) were sown under greenhouse conditions in plastic pots (10 cm in diameter) containing clay soil and received normal irrigation and fertilization. Three pots were used for each particular treatment. Fourteen day old seedlings from both cultivars were sprayed separately at each treatment. Under field conditions, wheat grains of both cultivars and spreader cultivars (*Triticum, Spleta, saharences* and *Morroco*) were sown in randomly plots (2×2.5m) at the rate of 40 g/plot.

Chemical inducers

Six inducing resistance chemicals, at two concentrations 200 and 250 ppm of each of ascorbic acid, oxalic acid, sodium salicylate, di-basic potassium phosphate, salicylic acid as well as 0.6 and 0.9 mM benzothiadiazole (BTH) compared with the systemic fungicide, 250 ppm propiconazole (Tilt) 25% EC were individually tested for their effects on the infection type of wheat leaf rust caused by artificial inoculation with *P. triticina* urediospores under greenhouse conditions.

Fungal inoculation

In the greenhouse, 5 days after plant treatment with the chemical inducers, plants were uniformly artificially inoculated with fresh urediospores of *P. triticina* race TTT (obtained from Wheat Disease Research Dept., Plant Pathol. Res. Instit., ARC, Egypt) according to the method approved by Tervet and Cassel (1951) and directly the pots were transferred under conditions of high humidity for 24 hours. These experiments were carried out in Wheat Disease Research Dept., Plant Pathol. Res. Instit., ARC., Egypt. In the field, mixed urediospores of *P. triticina* were prepared by adding 1 gm of urediospores to 20 gm of talc powder. The suspension of spores was prepared by adding 1 gm of urediospores to 20 ml of distilled water and trace of mineral oil. 70 days after sowing, plant spreaders were uniformly inoculated with freshly collected urediospores by injected leg with previously prepared spore suspensions as mentioned before and also by dusting with the mixed spores. Seven days after inoculation the spreader wheat plants were sprayed separately with each treatment. Treatments were repeated 3 times every 15 days. Disease severity was assessed every 10 days.

Disease severity assessment

Artificially inoculated plants were carefully examined to estimate disease severity % of infected leaves with rust and infection type depending on a modified scale (0-4) for seedling stage reactions where; (0); (1–2) = Resistant and (3- 4) = susceptible. While, the plant reactions (infection types) were expressed by five types in adult stage; immune = (0), resistant = (R), moderately resistant = (MR), moderately susceptible = (MS) and susceptible = (S) as described by Roelf *et al.*, (1992) in table (1).

Electrolyte leakage

Measurements were carried out as described by Szalai *et al.* (1996) with some modifications. 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). The flasks were shaken for 20 hr at ambient temperature to facilitate electrolyte leakage from injured tissues. Initial electrical conductivity measurements were recorded for each flask, using an Acromet AR20 electrical conductivity meter (Fisher Scientific, Chicago, IL). Flasks were then immersed

Table (1): Scale of disease severity assessments

Reaction	Description	Observation	R-Value
No Disease	No visible infection	0	0.0
Resistant	Visible chlorosis or necrosis, no uredia	R	0.2
Moderately resistant	Small uredia surrounded by chlorosis or necrosis areas	MR	0.4
Moderately susceptible	Uredia medium size with no necrotic margins but possibly some distinct chlorosis	MS	0.8
Susceptible	Large uredia with no necrosis and little or no chlorosis	S	1.0

in a hot water bath (Fisher Isotemp, Indiana, PA) at 80°C (176°F) for 1 hr to induce cell rupture. The flasks were placed again 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**.**

Detection of $O_2^{\cdot-}$ and H_2O_2

$O_2^{\cdot-}$ 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 (2 cm pieces) 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 by water and placed in 50% glycerol prior to be ready for evaluation. Discoloration of leaves resulted by NBT or DAB staining was quantified using a ChemiImager 4000 digital imaging system (Alpha Innotech Corp., San Leandro, USA).

Biochemical assays of antioxidant enzymes

For enzyme assays in plants, 0.5 g fresh treated wheat leaf material was homogenized at 0-4˚C in 3 ml of 50 mM TRIS buffer (pH 7.8), containing 1 mM EDTA-Na_2 and 7.5% polyvinylpyrrolidone. The homogenates were centrifuged (12,000 rpm, 20 min, 4˚C) and the total soluble enzyme activities were measured spectrophotometrically in the supernatant. All measurements were carried out at 25˚C, using the model UV-160A spectrophotometer (Shimadzu, Japan). Activity of CAT was determined according to Aebi (1984) and activity of DHAR was determined according to Asada (1984).

Statistical analysis

Three experiments were conducted in a complete randomized design, with five replicates/ treatment. Data represented with mean±SD. Student's t-test was used to determine significant differences ($P<0.05$) among mean values according to O'Mahony (1986).

RESULTS AND DISCUSSION

Effect of chemical resistance inducers on infection type (IT) and symptom expression of wheat leaf rust under greenhouse conditions during seedling stage

Obtained results indicated that all treatments with chemical resistance inducers changed obviously the infection type from high to low level. Most of the treatments showed clear differences as compared with the untreated control (infection type No. 4). Each of Tilt (fungicide) and oxalic acid at 250 ppm concentration, as well as benzothiadiazole **(**0.6 and 0.9 mM BTH) changed the IT from susceptible (4) to resistant (0 and 1), as compared with the untreated control. Consequently, other treatments changed the infection type from susceptible (4) in the untreated control to IT 1, 2 and 3 as well (Table 2). The tested chemical inducers of resistance suppressed significantly the disease's symptoms compared with the control (Fig. 1).

Effect of resistance inducers on disease severity (%) of wheat leaf rust under field conditions

All chemical inducers of resistance at the tested concentrations significantly decreased disease's severity and symptoms as well. Treatments showed significant differences compared with the untreated infected control in Sharkia Governorate, Egypt. Efficacy of these inducers to reduce wheat leaf rust severity was increased by increasing the concentration of the tested chemical. Chemical fungicide (Tilt) showed the highest effect against *P. triticina* on both used cultivars. In Sharkia, 250 ppm ascorbic acid and 0.9 mM BTH showed the highest reduction of wheat leaf rust disease severity in Giza 139, however, 0.6 and 0.9 mM BTH and 200 and 250 ppm concentration of sodium salicylate showed the highest reduction in Giza 168 (Fig. 3). Obtained results are in agreement with those obtained by El-Salamony (2002); Ata *et al.* (2008) and Mersha *et al.* (2012).

Effect of IRCs on electrolyte leakage

The electrolytes leakage (EL) constitutes as an indicator of the membrane permeability. All IRCs resulted in a significant reduction in electrolyte leakage in both wheat cultivars (Fig. 4). Tilt

Table (2): Effect of treatments with resistance inducers on infection type of wheat leaf rust

Chemical Inducers	Concentration (ppm or mM)	Infection Type	
		Giza 139	Giza 168
Control	0.0	4	4
Tilt (fungicide)	250	0	0
Ascorbic acid	200	3	2
	250	2	1
Oxalic acid	200	2	1
	250	1	0
Sodium salicylate	200	3	2
	250	2	2
Di-basic potassium phosphate	200	4	3
	250	3	3
Salicylic acid	200	3	2
	250	2	2
Benzothiadiazole (BTH)	0.6 mM	1	0
	0.9 mM	1	0

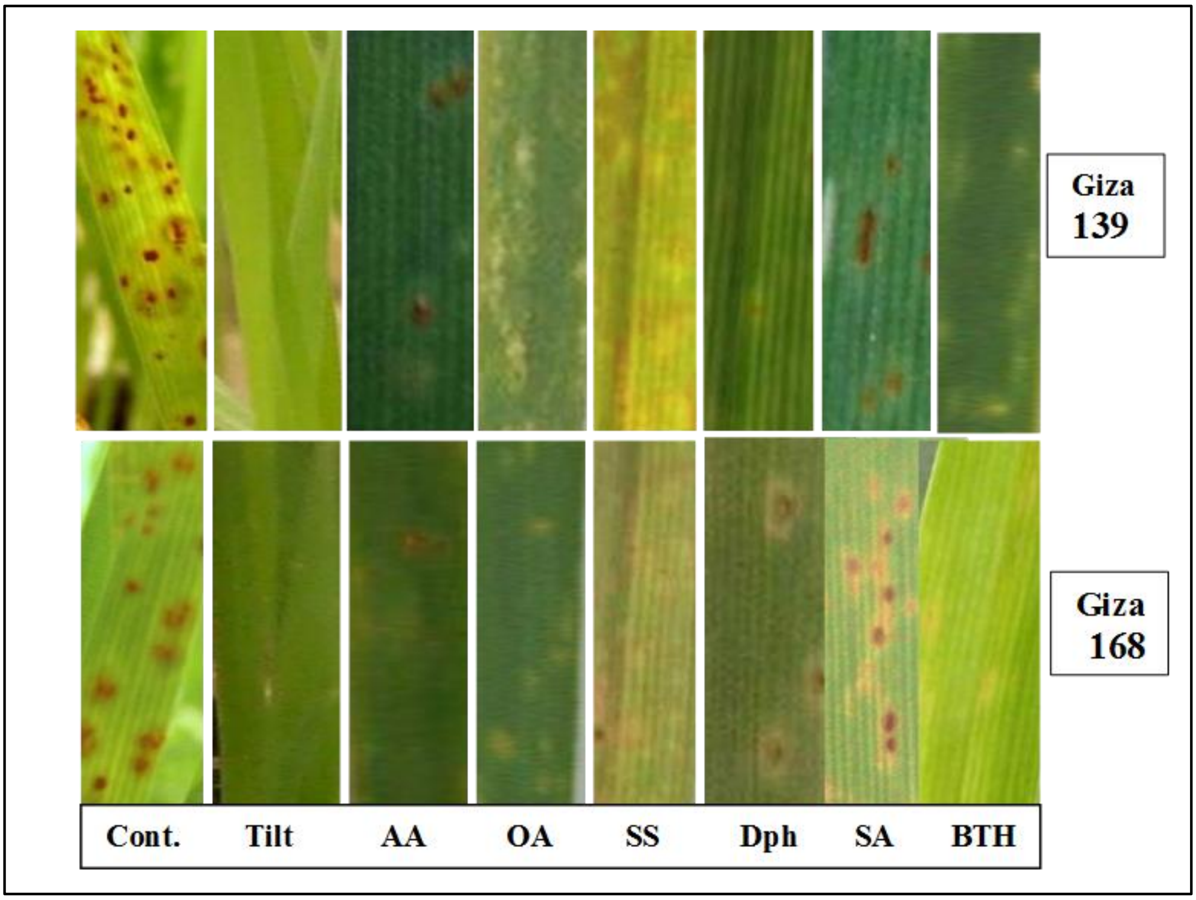

Fig. (1): Effect of IRCs and fungicide (Tilt) on disease symptoms of wheat infected with *P. triticina* in Giza 139 and Giza 168 cultivars under greenhouse conditions. **Control:** leaves sprayed with water. **Tilt:** leaves sprayed with 250 ppm fungicide Tilt. **AA:** leaves sprayed with 250 ppm ascorbic acid**. OA:** leaves sprayed with 250 ppm oxalic acid. **SS:** leaves sprayed with 250 ppm sodium salicylate**. Dph:** leaves sprayed with 250 ppm di-basic potassium phosphate. **SA:** leaves sprayed with 250 ppm salicylic acid. **BTH:** leaves sprayed with 0.9 mM benzothiadiazole**.**

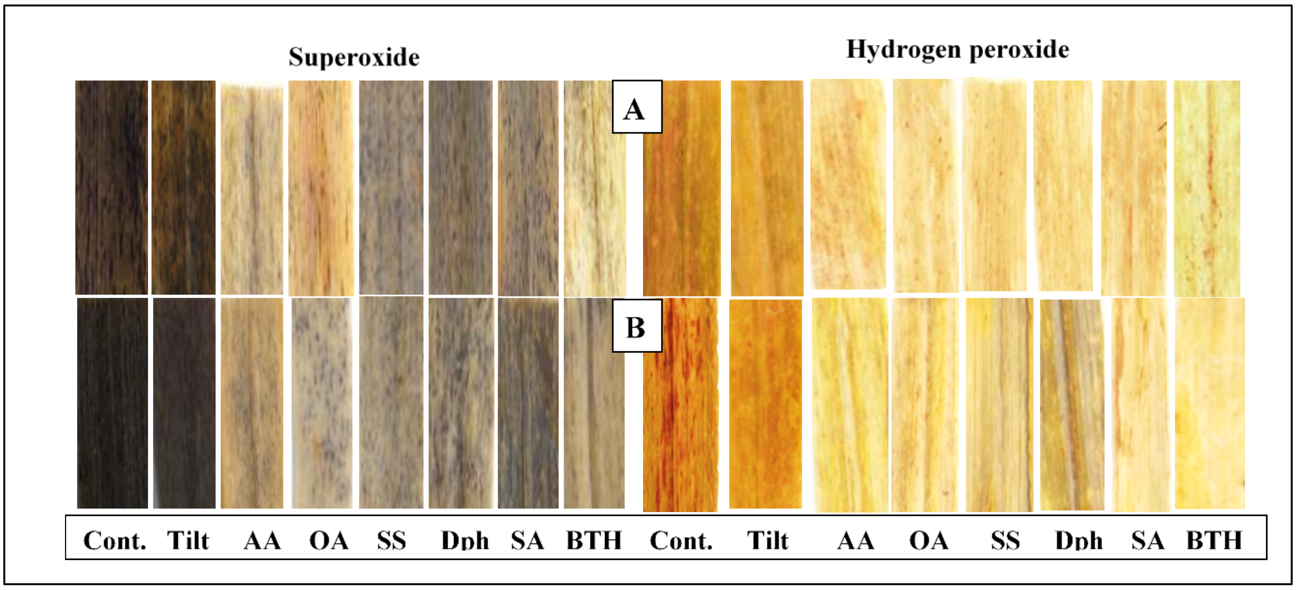

Fig. (2): Purple discoloration of superoxide and brown discoloration of hydrogen peroxide in wheat leaves infected by wheat leaf rust *P. triticina* on cvs. Giza 139 (upper row) and Giza 168 (lower row) treated with IRCs and the fungicide (Tilt) compared with control non-treated leaves.

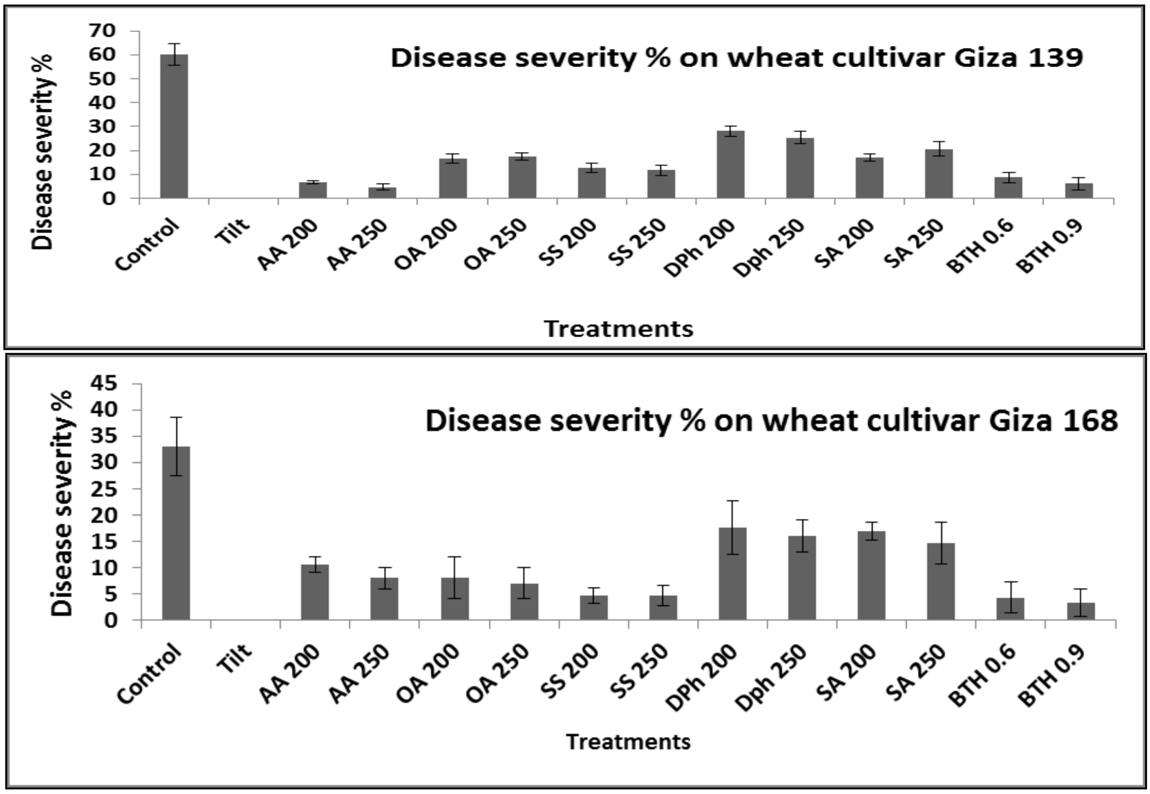

Fig. (3): Effect of IRCs and the fungicide Tilt treatments on the disease severity % of wheat infected with wheat leaf rust *P. triticina* under field conditions in Giza 139 and Giza 168 cultivars during 2011/12 growing season. **Control:** plants sprayed with water. **Tilt:** plants sprayed with 250 ppm. **AA:** plants sprayed with 200 and 250 ppm ascorbic acid**. OA:** plants sprayed with 200 and 250 ppm oxalic acid. **SS:** plants sprayed with 200 and 250 ppm sodium salicylate**. Dph:** plants sprayed with 200 and 250 ppm di-basic potassium phosphate. **SA:** plants sprayed with 200 and 250 ppm salicylic acid. **BTH:** plants sprayed with 0.6 and 0.9 mM benzothiadiazole**.**

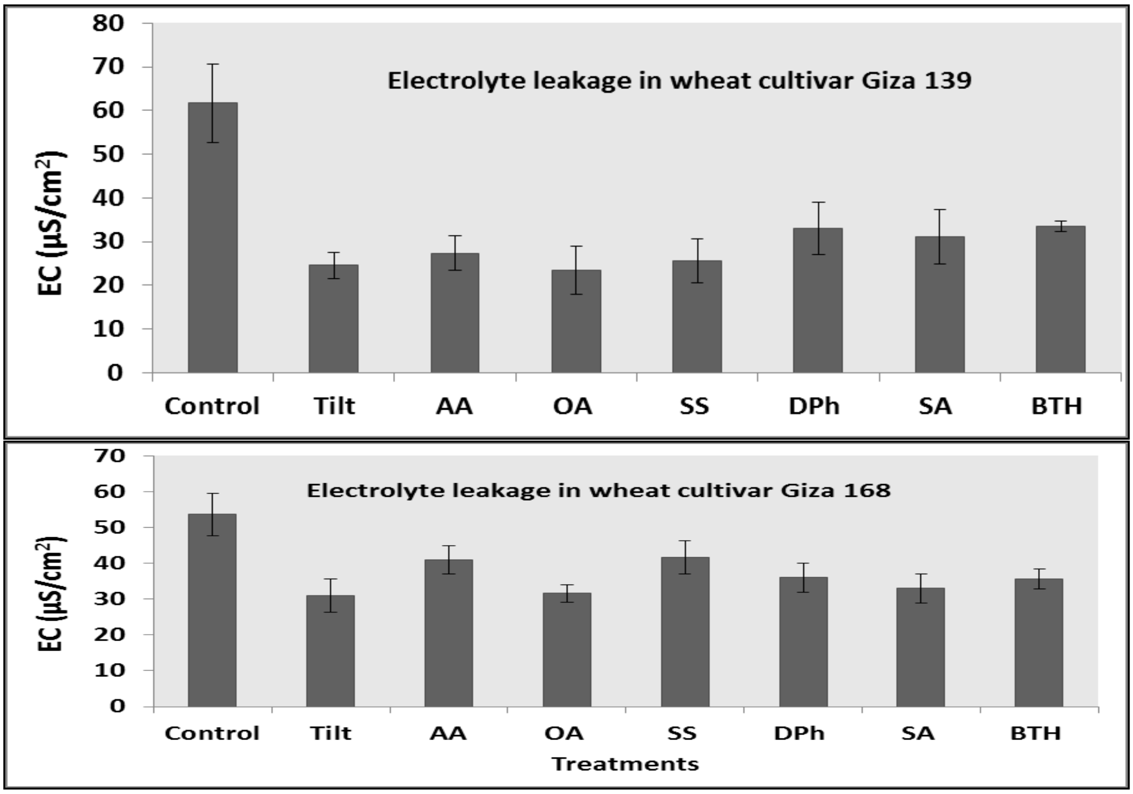

Fig. (4): Electrolyte leakage in infected wheat leaves with *P. triticina* (120 days old) of two wheat cultivars (Giza139 and Giza168) treated with IRCs and the fungicide Tilt compared with control non-treated leaves.

(fungicide) and oxalic acid treatments showed highest significant reduction in electrolyte leakage in both wheat cultivars Giza 139 and Giza168 as compared to other treatments (Fig. 4). Chemicals and other stresses such as high temperature could alter the resistance or susceptibility of plants to infection through their effects on membrane permeability. It is known that ethylene affects membrane permeability (Goodman *et al.,* 1986). Similarly, high temperature stress could induce susceptibility in maize through its effect on membrane permeability as measured by increased electrolyte leakage (Garraway *et al.,* 1989). This might result in the loss of host cells' constituents which may be used by the invading pathogen as a source of nutrients. These results indicated that the treatments protected cell membranes during the pathogen attack, while the cell membrane of the untreated wheat plants was affected by the pathogen infection and lost its constituents. Results of the present study are in agreement with those obtained by (Garraway *et al*., 1989 and Houimli *et al*., 2010).

Effect of resistance inducers on the levels of reactive oxygen species (ROS) in wheat leaves inoculated with *P. triticina*

Superoxide ($O_2^{.-}$) level was reduced significantly in wheat leaves treated with ascorbic acid, oxalic acid and benzothiadiazole **(**BTH) as compared with the control treatment. Meanwhile, moderate reduction was found in wheat leaves treated by sodium salicylate, di-basic potassium phosphate and salicylic acid as compared to the control on both wheat cultivars. No significant reduction of $O_2^{.-}$ level was found in the wheat leaves treated with Tilt (fungicide), as compared to the other treatments (Figs. 2 and 5). On the other hand, hydrogen peroxide (H_2O_2) level was significantly reduced in all treatments and in both cultivars as compared to the control, except sodium salicylate and di-basic potassium phosphate treatments that showed slight reduction in Giza 168 as compared to other treatments. Similarly, no significant reduction of H_2O_2 level was found in the wheat leaves treated with the fungicide as compared to other treatments (Figs. 2 and 5). ROS occurred continuously during photosynthesis in the chloroplasts by partial reduction of O_2 molecules or energy transfer to them. 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 were transferred and H_2O was released. It has been noted that $O_2^{.-}$was usually the first ROS to be generated. In plant tissues, about 1-2% of O_2 consumption led to the generation of $O_2^{.-}$. It has been well established that excess of H_2O_2 in plant cells led to the occurrence of oxidative stress (Hafez *et al.,* 2012). The results of this study showed high reduction on the level of H_2O_2 and $O_2^{.-}$ in the wheat plants treated with resistance inducers, and this might resulted in a less damage in the cell. Levels of H_2O_2 and $O_2^{.-}$ were reduced when antioxidants were elevated. BTH and oxalic acid showed highest reduction in the level of H_2O_2 and $O_2^{.-}$, while the treatment with the fungicide (Tilt) didn't reduce the level of H_2O_2 and $O_2^{.-}$, which was consistent with the inability of Tilt to activate antioxidant enzymes. Similar results were obtained by Hafez, (2010) and Jindrichova *et al.,* (2011).

Effect of IRCs on activity of antioxidant enzymes

Activities of catalase (CAT) and dehydroascorbate reductase (DHAR) increased significantly in the infected wheat leaves treated with chemical inducers as compared to control. Sodium salicylate and oxalic acid treatments showed highest CAT activity, followed by other treatments as compared with the control and Tilt treatments in Giza 139. Benzothiadiazole (BTH) showed highest CAT activity, followed by salicylic acid, sodium salicylate, di-basic potassium and other treatments as compared with the control and Tilt treatments in Giza 168 (Fig. 6). Most of the treatments showed significant increase of dehydroascorbate reductase (DHAR) activity in both wheat cultivars (Fig. 6). Benzothiadiazole **(**BTH), ascorbic acid, oxalic acid and sodium salicylate increased significantly the DHAR activity, followed by other treatments as compared with the control and Tilt treatments on Giza 139 and Giza 168 cultivars. The fungicide (Tilt) in both cultivars did not increase significantly CAT and DHAR activities as compared to other treatments in which elevated significantly both enzymes (Fig. 6). Results of this study showed that all tested IRCs increased significantly CAT and DHAR activities in treated wheat plants as compared to the control. Similar results were obtained by Gill and Tuteja, (2010) and Kovács *et al.,* (2011). Components of antioxidant defense system are enzymatic and non-enzymatic antioxidants. Enzymatic antioxidants include superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), dehydroascorbate reductase (DHAR) and glutathione reductase (GR) and non-enzymatic antioxidants are glutathione (GSH), carotenoids and tocopherols. Up-regulation of CAT, DHAR and other enzymes protected tobacco plants against viral, bacterial and fungal infections (Hafez *et al.,* 2012).

DHAR regenerated ASH from the oxidized state and regulated the cellular ASH redox state which is crucial for tolerance to various abiotic stresses leads to the production of reactive oxygen species (ROS). Chen and Gallie, (2005) noted that over expression of DHAR in tobacco protected the plants against ozone toxicity. Ushimaru *et al.* (2006) reported that over expression of DHAR increased salt tolerance in Arabidopsis.

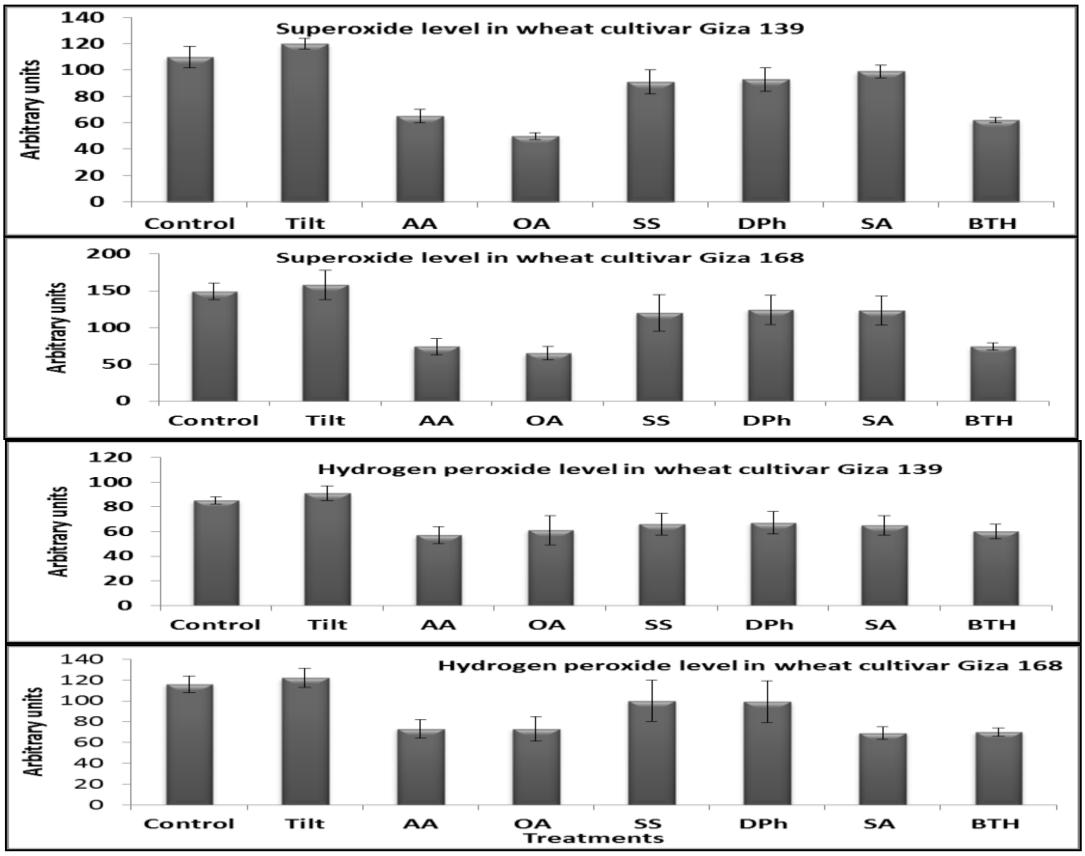

Fig. (5): Levels of superoxide and hydrogen peroxide in wheat leaves of the cultivars; Giza 139 and Giza 168 infected with *P. triticina* and treated with IRCs and the fungicide Tilt compared with non-treated leaves (control).

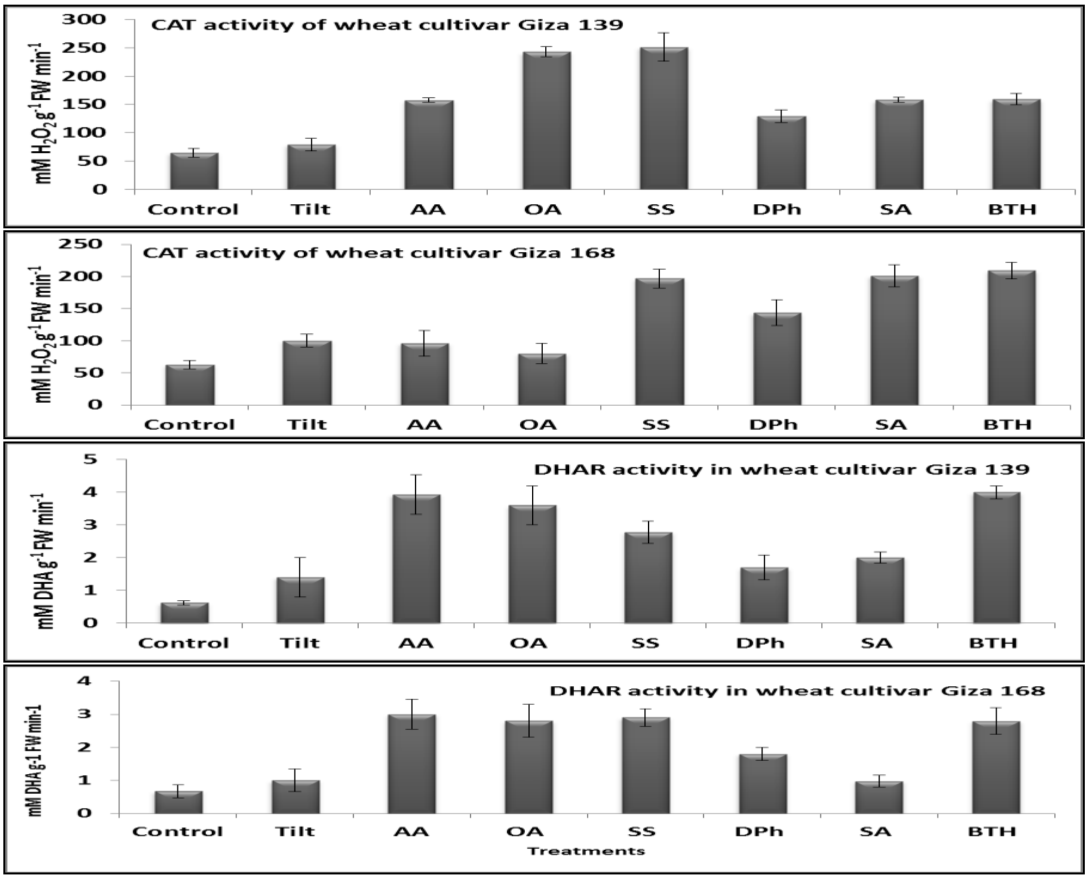

Fig. (6): Catalase (CAT) and dehydroascorbate reductase (DHAR) activities in infected wheat plants cvs. Giza139 and Giza168 by *P. triticina* treated with IRCs and the fungicide Tilt compared with control non-treated leaves.

It can be concluded that chemical inducers of resistance and the fungicides (Tilt) have a pivotal role in controlling wheat leaf rust. The fungicide has direct toxic effect on the pathogen however, all chemical inducers of resistance significantly down-regulated ROS levels, decreased electrolyte leakage and up-regulated the antioxidant enzymes CAT and DHAR in which perhaps play important role in suppressing wheat leaf rust *P. triticina*. It is important to give much attention to ROS levels and antioxidant enzyme activities in mechanisms of disease resistance during plant/ pathogens interactions.

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