PLANT SCIENCES

EFFECT OF SALICYLIC ACID ON ENZYME ACTIVITY IN WHEAT IN IMMEDIATE EARLY TIME AFTER INFECTION WITH *MYCOSPHAERELLA GRAMINICOLA*[*]

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The effect of salicylic acid (SA) on antioxidant enzymes activities in wheat infected with *Mycosphaerella graminicola* was investigated. Different concentrations of SA (0 and 2mM) were sprayed on susceptible and tolerant cultivars of wheat at a two-leaf stage. Enhanced activities of peroxidase, catalase, phenylalanine ammonia lyase, and polyphenoloxidase were determined in two wheat SA-treated cultivars in the presence or absence of pathogen. The results showed that the application of SA was more effective on antioxidant activities than pathogen. However, the highest activities of all tested enzymes were detected in cultivars treated both with SA and pathogen. Although in the earliest time of infection the antioxidant enzymes activities in susceptible cultivar were weaker than in the tolerant cultivar, the enzymes activity enhancement by SA in susceptible cultivar was observable, too. These results suggest SA as plant defense inducer could be an effective agent against *M. graminicola* in wheat.

antioxidant enzymes, salicylic acid, *Triticum aestivum* L.

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INTRODUCTION

Salicylic acid (SA) is a phenolic derivative distributed in a wide range of plant species. It is a natural product of phenylpropanoid metabolism. SA results from decarboxylation of *trans*-cinnamic acid to benzoic acid and its subsequent 2-hydroxylation (Lee et al., 2000). SA belongs to endogenous compounds of phenol nature with plant hormone characteristics (Alvarez, 2003). SA has direct involvement in plant thermogenesis, growth, flower induction, and exchange of ions (O'Donnell el al., 2001). It affects ethylene biosynthesis, stomatal movement, and also reverses the effects of ABA (Abscisic Acid) on leaf abscission (Van Wees, Glazebrook, 2003). In small quantities SA is present in tissues of all plants. Most often the SA present in plant organism is in the form of conjugates, rarely in free. SA induces the development of systemic acquired resistance (SAR) by reactive oxygen species (ROS) production (Kawano, Muto, 2000). Subsequently, SA changes catalase (CAT) and peroxidase (POX) activities (increases or reduces depending on H_2O_2 concentration) (Guan, Scandalios, 2006). CAT and POX are known as a defensive team, targeted at protecting cells from oxidative damage (Mittler, 2002).

POX also has a role in the biosynthesis of lignin and defense against biotic stresses by consuming H_2O_2 in the cytosol, vacuole, and cell wall as well as in extracellular space (Karuppanapandian et al., 2011). The phenolic compounds produced in plants due to pathogen attack are often converted into

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more reactive species by POX and polyphenol oxidase (PPO) (Gómez-Vásquez et al., 2004). They are involved in the oxidation of phenolic compounds in cell wall, suberization and lignifications of host plant cells during the defense reaction against pathogen agents (Welinder, 1992). PPO catalyzing the oxygen-dependent oxidation of phenols to quinines is ubiquitous among angiosperms and is assumed to be active in plant defense against pests and pathogens (Li, Steffens, 2002).

Phenylalanine ammonia lyase (PAL) is involved in the biosynthesis of SA and plays an important role in plant defense (Nugroho et al., 2002; Chaman et al., 2003). In addition, PAL is the primary enzyme in the phenylpropanoid pathway and is the key enzyme in the synthesis of several defense-related secondary compounds such as phenols and lignin (Ngadze et al., 2012).

Zymoseptoria tritici blotch is currently one of the most important foliar diseases of wheat (*Triticum aestivum* L.) worldwide (Bearchell et al., 2005). The causal pathogen *Mycosphaerella graminicola* (Fuckel) J. Schroeter in Cohn (anamorph: *Septoria tritici* Roberge in Desmaz) attacks wheat leaves, causing necrotic blotches which can result in significant yield losses (Forrer, Zadoks, 1983). Recently, fungicides have been largely applied to reduce this disease and associated yield losses; however the recent emergence of resistance/reduced sensitivity among *M. graminicola* populations to the fungicides (Leroux et al., 2007) highlights the urgent need for improved integrated control strategies. Such practices should incorporate cultural control measures, host resistance, chemical application, and biological control.

Antioxidant enzymes play an important role in defense responses of plants to pathogen (Lebeda et al., 2001). The enzymes patterns are intensively studied for cultivar identification in breeding, seed marketing, and in the other fields of agriculture (Samec et al., 1998).

In the present study, the effects of SA and *M. graminicolla* on antioxidant enzymes (CAT, POX, PAL, and PPO) were investigated separately and in combination. The obtained results provide convincing evidence for finding out the possibilities of enhancing the defense mechanism of tolerant and susceptible cultivars of wheat plants in immediate early after pathogen incubation.

MATERIAL AND METHODS

Plant material

In the present study two cultivars of bread wheat (*Triticum aestivum*) were used, one tolerant (Zagros) and the other susceptible (Atrak), based on their pathogenicity testing against *M. graminicolla* by Eslahi et al. (2013).

Preparation of plant extract

Wheat seeds (*Triticum aestivum* L. cvs Zagros and Atrak) were surface-disinfected with 70% alcohol for 5 s and washed twice with sterile distilled water. Then the seeds were sown in 10 cm pots containing a sterilized mixture of field perlite, soil, and leaf compost at a rate of 1 : 1 : 1. The seedlings were grown in a greenhouse under natural condition at 22/18°C (day/night). The pots were irrigated every two days. The pathogen isolate (S1) was received from the Department of Plant Pathology of the Tarbiat Modares University, Iran. S1 was grown on yeast malt extract agar (YMDA) for 5–7 days; spore concentration obtained from the media was adjusted to 10^7 ml^{-1} and supplemented with 0.5% Tween 20. The 12-day-old seedlings were sprayed with various concentrations of SA (0.1, 0.2, 0.5, and 1mM). Each pot was sprayed with 50 ml of the solutions (He, Wolyn, 2005). After 24 h of SA incubation, the plants were inoculated with a suspension of spores of *M. graminicola*. The control treatment was sprayed with sterile distilled water.

Enzyme activities

For measuring enzyme activities of CAT, POX, PAL, and PPO, treated wheat seedlings were used for protein extraction. The plant samples were collected at 0, 3, 6, 12, and 24 h after inoculation. The samples were homogenized in sodium phosphate buffer (0.1 mol l^{-1}, pH 6) and centrifuged at 12 000 *g* for 20 min at 4°C. The supernatants of each fraction were used for the enzymatic activity assay (Sahebani, Hadavi, 2008). For PAL enzyme assay, leaf samples were homogenized in an ice-cooled solution containing 50mM Tris-HCl buffer (pH 8.8) and 15mM β-mercaptoethanol (SIGMA, Germany). The homogenate was centrifuged at 10 000 *g* for 10 min, and the supernatant was collected for enzyme assay. Protein concentration was determined according to Bradford method (Bradford, 1976) using bovine serum albumin as standard protein.

CAT enzyme assay. The activity of CAT was quantified based on the rate of disappearance of the substrate H_2O_2 from the reaction medium containing the protein extract. The H_2O_2 concentration in the reaction medium was quantified by the change in absorbance at 240 nm. The reaction mixture (1 ml) contained 3% H_2O_2 and 50mM phosphate buffer (pH 7), and 0.035 ml was used to measure the decrease in absorbance at 240 nm (Rahnama, Ebrahimzadeh, 2006). Enzyme activity was calculated based on the changes in absorbance at 240 nm (ΔOD) per min per mg of total protein.

POX enzyme assay: POX activity was determined by measuring the appearance of pink/brown colour resulting from guaiacol oxidation in the presence of hydrogen peroxide according to the modified method

Table 1. Effect of treatment with SA and *M. graminicolla* infection agent on the activity of catalase (CAT) in tolerant and susceptible wheat

Treatments	Time after pathogen incubation				
	0 h	3 h	6 h	12 h	24 h
Tolerant wheat					
F	3.2^{bc}	3.9^{b}	4.9^{ab}	4.8^{ab}	5.1^{a}
F+SA	5.7^{c}	7.2^{b}	7.8^{b}	8^{b}	11.3^{a}
N	3.5^{a}	3.8^{a}	3.6^{a}	3.8^{a}	3.54^{a}
N+SA	5.6^{c}	6.35^{bc}	7.1^{b}	7.8^{ab}	8.3^{a}
Susceptible wheat					
F	2.9^{b}	3.1^{b}	3.3^{ab}	3.6^{ab}	3.90^{a}
F+SA	3.52^{c}	4.34^{b}	4.94^{ab}	5.14^{ab}	6.02^{a}
N	3.01^{a}	3.3^{a}	3.2^{a}	3.25^{a}	3.28^{a}
N+SA	3.64^{d}	4.16^{c}	5.06^{b}	5.43^{ab}	5.97^{a}

F = *M. graminicolla*, SA = 2mM salicylic acid, N = Control values are the mean of three replications of changes in absorbance (ΔOD) per min per mg of total protein

$^{a–d}$values in the same row followed by the same letter are not statistically different in Duncan's Multiple Range Test ($P < 0.05$)

of Bania, Mahanta (2012). The reaction mixture contained 5mM guaiacol, 2 ml of phosphate buffer (pH 7.0), 0.2 ml of leaves extract and was initiated with 3% H_2O_2. The changes in absorbance were read at 470 nm in 10-s intervals up to 2 min. Enzyme activity was calculated based on the changes in absorbance at 470 nm (ΔOD) per min per mg of total protein.

Phenylalanine ammonia lyase (PAL) enzyme assay: PAL activity was determined based on the rate of cinnamic acid production as described by Wang et al. (2006). Briefly, 1 ml of the extraction buffer, 0.5 ml of 10mM L-phenylalanine, 0.4 ml of double distilled water, and 0.1 ml of enzyme extract were incubated at 37°C for 1 h. The reaction was terminated by adding 0.5 ml of 6M HCl, and the product was extracted with 15 ml ethyl acetate, followed by evaporation to remove the extracting solvent. The solid residue was suspended in 3 ml of 0.05 ml NaOH and the cinnamic acid concentration was quantified with the absorbance measured at 290 nm. One unit of PAL activity equals to 1mM of cinnamic acid produced per min and expressed as U per mg protein.

Polyphenoloxidase (PPO) enzyme assay: PPO activity was determined according to the method of Shi et al. (2001). The reaction mixture (1 ml) containing 40 µg of protein extract and 10mM phosphate buffer (pH 7.0) was aerated for 2 min in a small test tube, then 100 mm of catechol was added as the substrate. The PPO activity was expressed as a change in absorbance of the reaction mixture at 420 (ΔOD) per min per mg of total protein.

Statistical analysis

All assays were carried out in a completely randomized design. Each treatment consisted of three replicates, and each replicate contained 3 plants for all enzyme activities. Statistical significance was assessed at the level $P < 0.05$. When the analysis was statistically significant, Duncan's Multiple Range Test (SSR Test) was used to test mean separations among mean values of each treatment.

RESULTS

The application of SA resulted in enhancing the activity of all tested enzymes in both infected and uninfected wheat cultivars. The enzyme activity in plants treated with fungus, SA, and their combination increased with time.

CAT: The CAT activity increased through timeline in 0.5 to 2mM SA treatments, reaching the maximum at 24 h after inoculation with pathogen and SA in both cultivars. The activity in 2mM SA treatment was higher than that in other treatments in either the presence or absence of *M. graminicola* inoculation and showed a significant difference. The highest enzyme activity of CAT was observed in the wheat inoculated with pathogen and SA at 24 h after infection in the tolerant and susceptible cultivar with values of 11.3 and 7.23 ΔOD per min per mg protein, respectively. SA and *M. graminicola* increased CAT activity during the entire experiment. In the absence of SA the fungal infection could increase the enzyme activity, but not as effectively as SA. Combination of SA and *M. graminicola* resulted in the sharp increase in CAT activity in tolerant cultivar within 12–24 h and displayed a significant difference to other treatments. On the other hand, the increased activity of the enzyme in sensitive cultivar did not change (Table 1).

POX: The POX activity increased in extracts from SA sprayed wheat at 3 h after pathogen infection and continued for the next 24 h after pathogen challenge.

Table 2. Effect of treatment with SA and *M. graminicolla* infection agent on the activity of peroxydase (POX) in tolerant and susceptible wheat

Treatments	Time after pathogen incubation				
	0 h	3 h	6 h	12 h	24 h
Tolerant wheat					
F	0.63^{c}	0.61^{c}	0.77^{b}	0.86^{ab}	0.91^{a}
F+SA	0.72^{d}	0.89^{c}	1.14^{b}	1.33^{ab}	1.42^{a}
N	0.61^{b}	0.58^{c}	0.69^{a}	0.71^{a}	0.7^{a}
N+SA	0.71^{d}	0.83^{c}	0.97^{bc}	1.07^{b}	1.18^{a}
Susceptible wheat					
F	0.63^{c}	0.61^{c}	0.77^{b}	0.86^{ab}	0.91^{a}
F+SA	0.72^{d}	0.89^{c}	1.14^{b}	1.33^{ab}	1.42^{a}
N	0.61^{a}	0.58^{ab}	0.69^{a}	0.71^{a}	0.7^{a}
N+SA	0.71^{c}	0.83^{c}	0.97^{b}	1.07^{ab}	1.18^{a}

F = *M. graminicolla*, SA = 2mM salicylic acid, N = Control values are the mean of three replications of changes in absorbance (ΔOD) per min per mg of total protein

$^{a–d}$values in the same row followed by the same letter are not statistically different in Duncan's Multiple Range Test ($P < 0.05$)

Table 3. Effect of treatment with SA and *M. graminicolla* infection agent on the activity of phenylalanine ammonia lyase (PAL) in tolerant and susceptible wheat

Treatments	Time after pathogen incubation				
	0 h	3 h	6 h	12 h	24 h
Tolerant wheat					
F	3.4^{e}	5.2^{d}	7.37^{c}	9.6^{b}	12.4^{a}
F+SA	6.85^{e}	9.61^{d}	12.28^{c}	15.57^{b}	18.8^{a}
N	3.25^{a}	3.5^{a}	3.52^{a}	3.35^{a}	3.28^{a}
N+SA	7.02^{e}	8.56^{d}	10.81^{c}	13.45^{b}	15.05^{a}
Susceptible wheat					
F	1.69^{c}	1.58^{c}	3.76^{b}	4.19^{ab}	5.15^{a}
F+SA	2.84^{c}	5.28^{b}	6.03^{ab}	6.97^{ab}	7.96^{a}
N	2.98^{a}	3.12^{a}	3.15^{a}	3.06^{a}	3.01^{a}
N+SA	2.97^{d}	3.96^{cd}	5.26^{b}	6.69^{ab}	7.15^{a}

F = *M. graminicolla*, SA = 2mM salicylic acid, N = Control alues are the mean of three replications of changes in absorbance (ΔOD) per min per mg of total protein

$^{a–e}$values in the same row followed by the same letter are not statistically different in Duncan's Multiple Range Test ($P < 0.05$)

The activities were more intensive in tolerant cultivar compared to susceptible cultivar and significantly differed. The maximum POX activity was observed in the inoculated wheat with pathogen and SA at 24 h after infection in the tolerant and susceptible cultivar with the value of 2.74 and 1.78 ΔOD per min per mg protein, respectively (Table 2).

PAL: The enzyme activity of PAL had the same pattern as that of POX. PAL activity increased in the SA sprayed wheat at 3 h after pathogen infection. Activity of PAL in both SA sprayed and infected plants increased significantly compared to control plants. However, in combination treatment the PAL activity was more enhanced than in the other treatments and showed a significant difference. In general, the activity of PAL was increased in the SA treatment and pathogen inoculation and the peak appeared 24 h after pathogen challenge (Table 3).

PPO: The PPO activity increased in the SA sprayed wheat and the increase continued 24 h after pathogen challenge in tolerant more than in susceptible cultivar. Although in pathogen inoculated plants the PPO activity was increased significantly compared to control plants, the increase was lower if compared to activities of infected and SA sprayed wheat. The PPO activity in tolerant cultivar sharply increased

Table 4. Effect of treatment with SA and *M. graminicolla* infection agent on the activity of polyphenol oxidase (PPO) in tolerant and susceptible wheat

Treatments	Time after pathogen incubation				
	0 h	3 h	6 h	12 h	24 h
Tolerant wheat					
F	3.4^{e}	5.2^{d}	7.37^{c}	9.6^{b}	12.4^{a}
F+SA	6.85^{e}	9.61^{d}	12.28^{c}	15.57^{b}	18.8^{a}
N	3.25^{a}	3.5^{a}	3.52^{a}	3.35^{a}	3.28^{a}
N+SA	7.02^{e}	8.56^{d}	10.81^{c}	13.45^{b}	15.05^{a}
Susceptible wheat					
F	0.2^{c}	0.23^{bc}	0.27^{b}	0.32^{ab}	0.4^{a}
F+SA	0.24^{c}	0.42^{b}	0.46^{ab}	0.51^{a}	0.56^{a}
N	0.25^{b}	0.27^{ab}	0.32^{a}	0.33^{a}	0.31^{a}
N+SA	0.26^{c}	0.39^{b}	0.43^{ab}	0.46^{a}	0.48^{a}

F = *M. graminicolla*, SA = 2mM salicylic acid, N = Control values are the mean of three replications of changes in absorbance (ΔOD) per min per mg of total protein

$^{a–e}$values in the same row followed by the same letter are not statistically different in Duncan's Multiple Range Test ($P < 0.05$)

3 h after the SA treatment and this enhancing was observed in both infected and uninfected plants with a significant differerence (Table 4).

DISCUSSION

In this study two wheat cultivars with a different level of tolerance against *M. graminicola* – Atrak (as susceptible) and Zagros (as tolerant) – were used. The aim of this study was considering defense strategies in wheat against *M. graminicola* in the early time after infection with pathogen in the presence and absence of SA.

The increase in enzyme activities was reported in many plant–abiotic and plant–microorganism interactions (Chutia et al., 2012; Cass et al., 2015). It is regarded as a general defense response of the organism to the effect of stress (Dikilitas, 2003).

Phenylpropanoids belong to the largest group of secondary metabolites (including lignin, phytoalexins, tannins etc.) produced by plants in response to biotic and abiotic stresses (Vogt, 2010). During compatible and incompatible interaction between pathogen and plants, the shikimic acid pathway is involved in plant defense due to breakdown products such as lignin, phenolics, and phytoalexins (Somssich, Hahlbrock, 1998; von Forell et al., 2015). PAL and POX play important roles in biosynthesis of phenolics, phytoalexins, and lignin, the three key components responsible for disease resistance. POX decomposes indole-3-acetic acid (IAA) and has a role in the biosynthesis of lignin and defense against biotic stresses by consuming H_2O_2 in the cytosol, vacuole, and cell wall as well as in extracellular space (Gill, Tuteja, 2010; Karuppanapandian et al., 2011). PAL catalyzes the conversion of phenylalanine to *trans*-cinnamic acid, a key intermediate in the synthesis of salicylic acid (Ramamoorthy et al., 2002).

There are numerous studies that investigate the strategy of over activation of antioxidant enzymes with the ultimate aim of enhancing stress resistance in plants.

Maksimov et al. (2013) showed that the wheat infection with *S. nodorum* strain caused the enhanced transcriptional activity of the CAT gene and the increased synthesis of its product. Our results showed that the maximum activity of CAT (11.3) was observed after 24 h of pathogen infection in Zagros (tolerant cultivar) when sprayed with 2mM of SA. Anahid et al. (2013) showed that the amount of CAT and superoxide dismutase activity increased significantly in tolerant cultivars, and also in susceptible wheat cultivars infected with *Puccinia striiformis* f.sp. *tritici* and this amount in susceptible cultivars was lower than in tolerant cultivars.

The activity of POX and PAL enzymes was enhanced in pepper roots during interactions with *Verticillium dahliae* (Idoia et al., 2006). Koche, Choudhary (2012) showed that the activities of chitinases, 1,3-glucanases, and PAL were higher in tolerant cultivars of mungbean seedlings when challenged with *Cercospora canescens*. This induced activity of defense related enzymes was found to be associated with the tolerance status of mungbean cultivars. In the present research, PAL activity was significantly higher in all treatments of the tolerant cultivar than of the susceptible one. PAL activity showed increasing trend from the first time points (at the time of infection) to 24 h after inoculation, in tolerant and susceptible cultivars.

Nawar, Kuti (2003) showed that there are positive relationships between the POX isozymes and resistance development in plants. Furthermore, Caruso et al. (2001) experimentally supported the

idea that POX plays a defense role against invading pathogens of wheat kernels. They planned to determine changes in POX activity and newly developed POX isozymes in plants pre-sprayed with salicylic, benzoic, citric, and oxalic acids, by using ribavirin as the inducer. The involvement of POXs in plant defense responses against pathogens has repeatedly been reported. For wheat POXs, it was observed that the POXs could bind to chitins and germinating spores of the fungus *T. caries*. Specific chitin-binding isozymes of POX play an important role in pathogenesis of plant diseases caused by fungi (K h a i r u l l i n et al., 2000).

A direct evidence for the role of PPO in inhibiting pathogen ingress or growth comes from challenging transgenic tomato plants with enhanced or suppressed PPO levels, by the bacterial pathogen *Pseudomonas syringae* pv *tomato*. During the infection process, in PPO over-expressing plants a reduction in bacterial growth was detected, whereas PPO anti-sense-suppressed lines supported greater bacterial numbers (T h i p y a p o n g et al., 2004). The pathogen-induced PPO activity continues to be reported for a variety of plant taxa, including monocots and dicots (e.g. C h e n et al., 2000; D e b o r a h et al., 2001). Similarly, studies describing correlations of high PPO levels in cultivars or lines with high pathogen resistance continue to provide support for the pathogen defense role of PPO (R a j et al., 2007). Our results showed that the PPO activity of tolerant cultivar was significantly higher than that of susceptible cultivar in all time points. It was also reported that the activity of PPO and POX increases in roots of wheat when infected with *Ustilago tritici*, in early and later stage of the disease development (A n j u m et al., 2012).

In our study, treatment with SA enhanced the activity of all four enzymes (CAT, POX, PAL, and PPO) during 24 h after the plants infection with *M. graminicola*. The specific SA action on the defense enzymes activity in different plants is due to different isoforms of the given enzyme that are activated by SA in different ways (G u a n , S c a n d a l i o s , 2006). In the present research, SA treatment was more effective in increasing enzymes activity compared with the use of *M. graminicola* during 24 h after inoculation. However, treatment with SA combined with pathogen induced the highest activity of antioxidant enzymes. When citric and benzoic acids were used as inducer against faba bean chocolate spot disease, these components were very effective and resulted in the lowest percentages of disease severity and the highest levels of POX activity (Hassan et al., 2007). This increase reached up to two-folds of the control treatment.

According to our results, the SA treatment increased the activity of all tested enzymes in both the tolerant and susceptible cultivars. From the beginning of the experiments, the enzymes activity was more intensive in the tolerant cultivar than in the susceptible one. SA had greater impact on the increase of enzymes activity than pathogen in the susceptible cultivar. Consequently, even in susceptible cultivars SA can be used as a resistance inducer.

Overall, in this paper, four enzymes activity related to the defense system was performed in the inoculated wheat with pathogen as well as the induction agent defense system SA in the early hours. The main objective was to study the effect of SA on the activity of these enzymes in tolerant and susceptible cultivars in the immediate early hours after incubation with pathogen.

CONCLUSION

The application of SA resulted in enhancement of enzymes activity in both resistant and susceptible plants during examination periods. The role of oxidative enzymes such as CAT, POX, and PPO could be explained as an oxidation process of phenol compounds to oxidized products (quinines) which may limit the fungal growth. Finally, our results showed that induction of resistance with SA affected the defense enzymes activity. Due to the deleterious effects of chemical control on human health and environment, using such alternative substances to manage *M. graminicola* and other pathogens is very promising in the future.

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