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# Changes in the Activity of Enzymes Phenylalanine Ammonia-Lyase, Polyphenol Oxidase, and Peroxidase in Some Wheat Genotypes against Take-All Disease

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## ABSTRACT

**Enzymes play a crucial role in plant-pathogen interactions and are very important to manage plant diseases. Take-all is a disease (*Gaeumannomyces graminis* var. *tritici*) affecting the crowns and roots in wheat. So far, the resistance mechanism of this disease has not been identified; therefore, this research was performed to identify the components of resistance to this disease in a number of wheat genotypes. In this study, 8 bread wheat genotypes were cultured, and the changes in "peroxidase, Polyphenol Oxidase (PPO), Phenylalanine Ammonia-Lyase (PAL), and total protein" was assessed in 0, 4, 7, 9, and 12 days after inoculation. The results showed that different genotypes of wheat had different pathogenicity reactions to the take-all disease. Based on the average disease intensity, the genotypes were divided into three groups: resistant, moderately resistant, and susceptible. The results indicated that the level of polyphenol oxidase and phenylalanine ammonia-lyase, and the total protein increased in the resistant and moderately resistant groups. Cluster analysis by K-means was performed to produce three clusters. Polyphenol oxidase activity, phenylalanine ammonia-lyase activity, and total protein content in the second (resistant) and third (moderately resistant) clusters were higher than the first cluster (susceptible). Multivariate analysis indicated that peroxidase enzyme might indirectly influence the resistance. The results have clarified the role of polyphenol oxidase enzymes and total protein in enhancing resistance to take-all disease.**

**Keywords:** Disease resistance, *Gaeumannomyces*, Host-pathogen interaction, Multivariate analysis.

## INTRODUCTION

Bread wheat is (*Triticum aestivum* L.) as one of the most important food sources of the people of the world that is widely cultivated worldwide. Wheat is constantly exposed to a variety of biotic and abiotic stresses that alter its growth and proliferation. Take-all (*Gaeumannomyces graminis* var. *tritici* (*Ggt*)) is one of the most important root diseases of winter wheat in all cropping areas around the world.

When plants are attacked by pathogens, some antimicrobial compounds, the cell wall defense mechanisms, and accumulate reactive oxygen species are activated (Ausubel, 2005). Disease resistance in plants is associated with the activation of a wide array of defense responses that slow down infection at certain stages of the host-pathogen interactions. One method of protection relies on inducible defense responses in the form of enzymes that are activated upon infection (Vanitha *et al.*, 2009). When a pathogen attacks the host, roots have a variety of defense mechanisms

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against fungal pathogens, such as pathogenesis-related proteins and cell wall strengthening. Infection with *Ggt* can lead to the synthesis of phytoalexins in wheat (Guilleroux and Osbourn, 2004).

The interaction between the pathogen and the host plant induces some changes in cell metabolism, primarily the activity of defense enzymes. Polyphenol Oxidase (PPO) is a nuclear-encoded enzyme that catalyzes the oxygen-dependent oxidation of phenols to quinines. Phenylalanine Ammonia-Lyase (PAL) is the primary enzyme in the phenylpropanoid pathway. It is the key enzyme in the synthesis of several defense-related secondary compounds such as phenols and lignin (Vanitha *et al.*, 2009).

In a study, it was revealed that the defense enzymes PAL and PPO were actively involved in tomato resistance to bacterial wilt (Vanitha *et al.*, 2009). Studies have shown that the interaction of the take-all pathogen with wheat has expressed about 1400 genes and oxidative reactions in the plant (Puga-Freitas *et al*., 2016). The use of multivariate statistical methods is a good way to identify disease resistant components in plants. In the analysis of a diversity of maize inbred lines, a positive genetic correlation between resistance to southern leaf blight, gray leaf spot, and northern leaf blight diseases was discovered (Wisser *et al*., 2011).

Evaluation of the enzymatic mechanism of resistant and susceptible wheat genotypes in response to a disease is one of the methods of identification of the plant defense system against take-all disease. However, very few studies have been performed on the defense system of wheat plants against take-all disease. The current study aimed to identify the most crucial defense enzyme and components resistant to take-all disease, using multivariate analysis.

## MATERIALS AND METHODS

### Fungus Resource and Fungus Inoculum Preparation

T-41 isolate ((*Gaeumannomyces graminis* var. *tritici*) was selected for our research. This isolate was obtained from the mycological collection of the Vali-e-Asr University of Rafsanjan (Gholizadeh Vazvani *et al*., 2017). This isolate has strong pathogenicity. Previously, this isolate had been proven to have pathogenicity to wheat seedlings. It was investigated in interaction with some bio-control agents (Lagzian *et al*., 2013; Alavi Nejad *et al*., 2014).

The selective medium for fungal culturing was Potato Dextrose Agar (PDA) containing streptomycin (0.03 gram in 1,000 cc PDA). The fungus was purified once every 20 days; the border was growing in a Petri-dish, and the fully developed fungus was stored in the refrigerator at 4°C. Because of a high colonization rate and the uniformity of prop gules, millet was chosen for the prepared fungus inoculums. A mixture of 100 grams of cooked millet seed and 100 grams of wet sand was poured into a flask and autoclaved twice at 120°C for 20 minutes. For fungus propagation, a few circles of mycelia with one centimeter in diameter from the edge of the growing colonies were inoculated into each of the flasks and incubated at 20-25°C for 15 days. The flasks were then removed and incubated for 15 days at a temperature of 20-28°C in a laboratory environment under natural and fluorescent light. The flasks were shaken several times for aeration and were avoided from being shot. They were then refrigerated until time of use.

### Wheat Genotypes Resources

The Genetic resource was eight genotypes of wheat collected and received from different locations of Iran and other countries (Table 1). At first, these genotypes were planted in one line in the field of Vali-e-Asr University and a single plant was selected from each line, and their seeds were used in screening for resistance and susceptibility to take-all in the greenhouse (Gholizadeh Vazvani *et al*., 2016; 2017).

### Greenhouse Experiment

A suitable sieved soil (EC= 1.2-2 ds/m, pH= 7.5-8) was autoclaved at 121°C for one





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Table 1. The genotypes of studied in this research.

| Genotype | Reaction to the fungus *Gaeumannomyces graminis* var. *tritici* isolation T-41 |
|---|---|
| 1510 | Moderately Resistant, Spring |
| 1528 | Resistant, Winter |
| 1526 | Susceptible, Winter |
| 1879 | Resistant, Winter |
| 1530 | Resistant, Winter |
| 2167 | Resistant, Winter |
| *Aegilops tauschii* | Resistant, genotype wild type, Spring |
| Chinese Spring | Susceptible, Spring (Kim *et al*., 2003) |

hour. Seeds (four seeds in each pot) were disinfected (with hypochlorite sodium 1% and ethanol 70%), and then planted in pots containing 800 grams of soil in the greenhouse (20-25°C). Inoculation was performed 14 days after planting 0.5% of the pot weight was dumped close to the crown of the plant and covered with sand. The leaves from both inoculated and non-inoculated plants were sampled at 0, 4, 7, 9, and 12 days after inoculation and examined in six replications. Seven weeks after inoculation, the percentage of the blackened crowns, shoot dry weight and root dry weight were measured and recorded.

Contamination levels based on the percentage of necrosis in the roots and crowns were scored based on 0 to 5 as follows (Ownley *et al*., 2003). Disease Intensity (DI) was calculated according to the Equation (1):

DI: [(Sum of scores of each pot/5)×Number of plants]×100 (1)

Scoring was as follows: 0= Roots and crowns without necrotic spots; 1= Roots with one or more necrotic spots and crowns without symptoms; 2= Roots with continuous necrotic spots (more than 25% and less than 50% necrosis of roots) and crowns without symptoms; 3= More than 50% necrosis of the roots and blackened crowns; 4= Roots approximately black with 75% blackened crowns; 5= Blackened and dried roots and crowns.

### Biochemical Studies

To study the biochemical parameters, 0.5 grams of leaves were homogenized using 50 mM potassium phosphate buffer (pH 7.5 )containing 1mM Ethylene Diaminetetraacetic Acid (EDTA), 1% (w/v) Polyvinylpyrrolidone (PVP). After centrifugation (12,000×g, 20 minutes), the supernatant was used for the determination of Phenylalanine Ammonia-Lyase (PAL), Polyphenol Oxidase (PPO), Peroxidase (PO) activities and the total protein content. Activity of PAL, PPO, PO enzymes and total protein content was determined according to the method of D´cunha *et al*. (1996), Nicoli *et al*. (1991), Gichner *et al*. (1994), and Bradford (1976), respectively.

### Experimental Design and Statistical Analysis

Factorial analysis was conducted for eight genotypes and infection on two levels (infected and control) in the completely randomized design. Regarding growth traits, there were four replications (two replications as infected and two replications as control). As for biochemical characteristics, there were six replications (three replications as infected and three replications as control). Statistical analyses were performed according to the MSTATC and MINITAB software. The mean comparison was conducted by the Least Significant Difference (LSD) at 5% level.

## RESULTS AND DISCUSSION

### Results of Analysis of Variance

The analysis of variance on disease intensity and score disease (infected





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environment) showed that there was a significant difference between genotypes concerning disease intensity (results not shown). The Chinese spring cultivar with an average disease intensity of 100% was found to be most susceptible, followed by the genotype 1526 with an average disease intensity of 73.33%. Genotypes 2167, 1530, 1879, and 1528 had an average disease intensity of 20, 20, 13.33, and 0%, respectively. The average disease intensity was 53.33% for the *Aegilops tauschii*, and 46.66% for 1510, which were the moderately resistant genotypes to disease (result not shown). The difference between the cultivars in terms of disease intensity and disease score is due to potential differences between the penetration and the development of the pathogen. Figures 1, 2, 3 and 4 show the root system of infected and susceptible genotypes.

## Results of Analysis of Variance on Growth Traits

There was a significant difference between genotypes ($P< 0.001$) in terms of characteristics of dry root weight and dry shoot weight (Table 2). The effect of genotype×environment was significant for shoot and root dry weights. Thus, it is noteworthy that different genotypes react differently in various environments. Previous reports suggested that there were complex interactions between wheat genotype, environmental conditions, and take-all inoculum (McMillan *et al*., 2018).

Genotypes 1530, 1879, 2167, and *Aegilops*, with a significant and negative difference from the control, had a reduction in root dry weight. Also, genotypes 1526 and 1528, with a significant and positive difference from the control, had an increase in dry root weight. The infected environment of *Aegilops* cultivar with a negative and significant difference from the control had the lowest shoot dry weight, while infected environments of 1526, 1528, 1530, 1510, and 1879 genotypes, with significant and


**Figure 1.** Root system of control (A) and Chinese Spring wheat cultivar (B) infected with *Gaeumannomyces graminis* var. *tritici*.

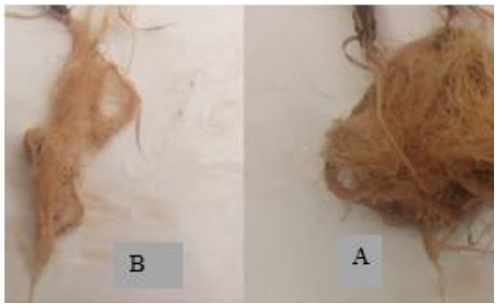


**Figure. 2.** Root system of infected **(A)** and control (B**)** genotype 1528 (Resistance) against to *Gaeumannomyces graminis* var. *tritici*.

**Figure 3**. Root system of genotype 1510 infected with *Gaeumannomyces gramin*is var. *tritici*

**Figure 4.** Root system of genotype 1879 infected with *Gaeumannomyces graminis* var. *tritici*





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**Table 2.** Analysis of variance of root and shoot dry weights in genotypes and infected and non-infected environments.

| | | Mean square | |
|---|---|---|---|
| SOV | df | Root dry weight | Shoot dry weight |
| Genotype | 7 | 0.0311$^{**}$ | 0.0046$^{**}$ |
| Environment | 1 | 0.0010$^{ns}$ | 0.0456$^{**}$ |
| Genotype × Environment | 7 | 0.0184$^{**}$ | 0.0093$^{**}$ |
| Error | 32 | 0.0008 | 0.0005 |
| Total | 47 | | |
| CV (%) | | 22.90 | 19.91 |

$^{**}$ and ns: Significant at 0.01 and not significant, receptivity.

positive differences from the control, had the highest weight of shoot dry weight. When wheat is attacked by *Gaeumannomyces graminis* var. *tritici*, the expression of a series of compounds in the plant increases, which may indirectly or directly affect the plant's growth. In addition, previous researches had shown that when fungi *Gaeumannomyces graminis* var. *tritici* attacks the roots, some of the wheat cultivars produce secondary metabolite compounds (Gordon-Weeks *et al*., 2010). These secondary metabolites can provide protection against take-all disease (McMillan *et al*., 2014) and, probably, increase the plant's root system. However, it should be noted that the type of reaction to the disease depends on the type of genotype and the defense structure of the plant.

In the field study, it was determined that the resistant genotype 1528 in the infected environment of *Gaeumannomyces graminis* var. *tritici* showed a higher amount of ferric in the seeds, chlorophyll content, leaf area, and height than the control environment (Gholizadeh Vazvani *et al*., 2016). The ferric element regulates several metabolic activities in the plant. Thus, the increase in dry root weight in some genotypes may be related to the interaction of the fungus with the plant (genotype) and the activation of the plant's defense mechanisms, such as an increase in ferric and chlorophyll content, leading to enhanced metabolic activity in the plant. This, in turn, affects further growth and physiological activity of the plant.

### Analysis of Variance on Biochemical Traits

The results showed that all effects, including bilateral and tripartite interactions, became significant in infected and control environments. No significant difference was observed in the peroxidase enzyme between the infected and control environments (result not shown).

### The Effect of Genotype×Environment

Genotypes 1526, 1530, 1528, and *Aegilops* in the infected environment had more enzymes (phenylalanine ammonia-lyase (unit/mg protein)) than the control environment, 0.2118$^{*}$, 0.1345$^{*}$, 0.0818$^{*}$ and 0.1108$^{*}$, receptivity.

In the infected environment, total protein content (mg/g) of genotypes 1526, 1528, 1530, and 2167 had a positive and significant difference from the control, 117.544$^{*}$, 45.344$^{*}$, 74.68$^{*}$, 18.654$^{*}$, receptivity. Regarding the peroxidase enzyme (unit/mg protein), the infected environments of genotypes 1526, 1879, 2167, 1510 with a positive and significant difference from the control environment showed a higher level of this enzyme, 0.041$^{*}$, 0.026$^{*}$, 0.0225$^{*}$ and 0.036$^{*}$, receptivity. Concerning the polyphenol oxidase enzyme (unit/mg protein), the





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infected environments of genotypes 1510 and 1879 had a positive and significant difference from the control environment, 0.0037* and 0.0104* receptivity (result not shown). The increase in enzymes in the susceptible genotypes (1526) can be attributed to the plant's response to the pathogen, increased peroxidase activity has been observed in infected plants (Ward, 1986). According to Figures 2, 3, 4, and 5, an increase in defense enzymes in early infection has been observed in susceptible genotypes, which is probably due to plant-pathogen interaction. Decreases in enzyme levels in the following days in susceptible genotypes confirm the plant's unsuccessful attempt to combat disease and reduce ROS production during stress (Chenarani *et al*., 2014). *Gaeumannomyces graminis* var. *tritici* could induce a Hypersensitive Response (HR), the defense mechanism that relies on the production of reactive oxygen species (Puga-Freitas *et al*., 2016). The infection of plants by *Gaeumannomyces graminis* var. *tritici* and the subsequent establishment of the disease involves substantial changes in the biochemistry and physiology of plants. The amount of defense enzymes and total protein content in the infected environment at 4, 7, and 9 days after inoculation with the pathogen was significantly higher than the control environment. In almost all infected environments, the total protein content of the genotypes increased significantly compared to the control environment. In some genotypes, the total protein content in the plant is high at the beginning of growth. This rate gradually decreases as the plant ages, but infected environments have more protein than the control (result not shown). At the beginning of growth, the plant uses grain storage protein so that the protein content is high at the beginning of plant growth, however, the total protein content also depends on the type of wheat genotype. Hard wheat contains different protein levels depending on the variety and growing conditions (Wellington, 2014).

Genotypes react differently in different environments, and the defense systems of different genotypes are different depending on the disease. Previously, genotypes were classified by take-all disease (T-41 isolate). Scores (Sc) were as follows: Sc= 0 (highly resistant), 0< Sc≤ 1 (resistant), 1< Sc≤ 2 (moderately resistant), 2< Sc≤ 3 (moderately susceptible), 3< Sc≤ 4 (susceptible) and 4< Sc≤ 5 (highly susceptible) (Gholizadeh Vazvani *et al*., 2016, 2017).

In this study, to obtain an appropriate result, genotypes were grouped. Genotypes 1528, 2167, 1879 and 1530 with an average disease intensity of 13.33% were placed in the resistant group, *Aegilops* cultivar and genotype 1510 with an average disease intensity of 49.99% fell into the moderately resistant group, and genotype 1526 and Chinese spring cultivar, with an average disease intensity of 86.66%, were in the susceptible group. Group analysis of variance and sampling time (4, 7, 9, and 12 days after inoculation) were performed as a two-factor factorial experiment in biochemical traits (we did not consider zero time since the purpose of this section was to change the defense enzymes in the reaction of wheat to take-all disease) (result not shown). The activity of the enzyme PAL was the highest on 4 and 7 days after inoculation in moderately resistant and resistant groups; on 7 days after inoculation in the moderately resistant and susceptible groups; and 12 days after inoculation in the resistant and susceptible groups (Figure 5-a). The activity of peroxidase was the highest in 4 days after inoculation in the moderately resistant and susceptible groups and 7, 9, and 12 days after inoculation in the moderately resistant group (Figure 5-b). The activity of the PPO enzyme (Figure 5-c) and total protein content (Figure 5-d) was highest in all days of sampling in resistant and moderately resistant groups. In the resistant group, the enzyme content of PAL, PPO, and total protein content increased simultaneously 12 days after inoculation. The activity of these two enzymes and total protein content and genes expressed in this





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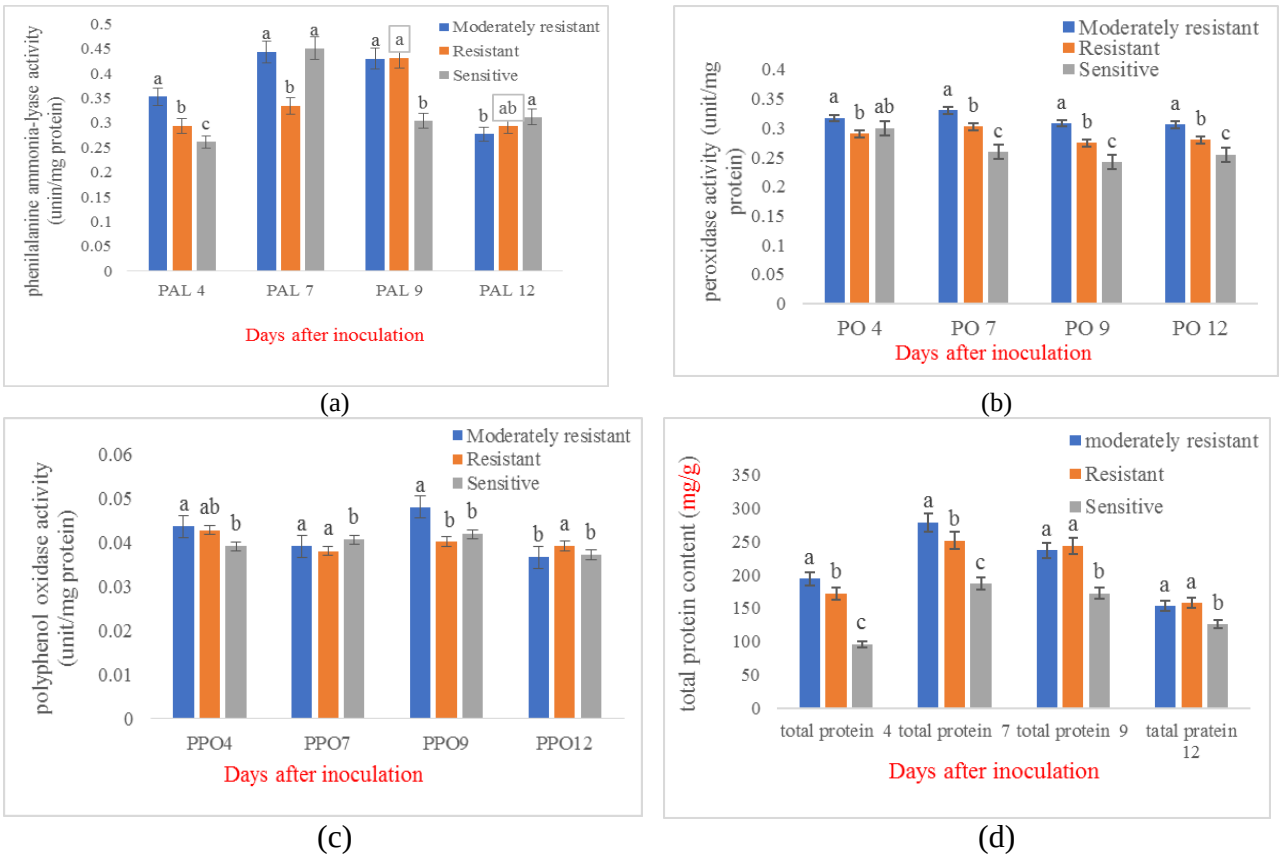


**Figure 5.** Mean comparisons of (a) phenylalanine ammonialyase enzyme (b) peroxidase enzyme (c) polyphenol oxidase enzyme and (d) total protein content on days 4, 7, 9 and 12 days after inoculation in groups. Different letters indicate significant differences at 5% level.

pathway may lead to the synthesis of several antifungal metabolites. Studies have shown that the level of the peroxidase enzyme increases in the infected environment by fungi (Gonçalves *et al*., 2013). Enzyme activities started by *Gaeumannomyces graminis* var. *tritici* in all the genotypes. However, the expression rhythm of activities varied among the genotypes. When the fungus *Gaeumannomyces graminis* var. *tritici* attacks wheat genotypes, in resistant and susceptible genotypes, a set of reactions are activated that result in increasing level of defense enzymes and proteins involved in resistance. Incompatible plant-pathogen interactions are characterized by a hypersensitive response (HR), which includes the production of ROS and activates defense pathways (including defense enzymes such as peroxidase, polyphenol oxidase, and phenylalanine ammonialyase) that lead to resistance.

## Results of Multivariate Analysis of Defense Enzymes on Disease Intensity

The resistance of a genotype depends on the pathogen and its various traits, and the correlation between the various traits makes it difficult to interpret and draw conclusions from each of the traits. In breeding programs for resistance to disease, the selection is based on a large number of traits, and multivariate analysis is very important.

## Correlation

The correlation results of traits (Table 3) showed that there was a positive and





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**Table 3.** Correlation of biochemical traits with disease intensity and growth factor in sampling days.

| Traits[a] | DI | PO4 | PO7 | PO9 | PO12 | PPO4 | PPO7 | PPO9 | PPO12 | PAL4 | PAL7 | PAL9 | PAL12 | Pr4 | Pr7 | Pr9 | Pr12 | Root | Shoot |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| DI | 1 | | | | | | | | | | | | | | | | | | |
| PO4 | 0.740*** | 1 | | | | | | | | | | | | | | | | | |
| PO7 | 0.177 | -0.050 | 1 | | | | | | | | | | | | | | | | |
| PO9 | 0.132 | -0.033 | 0.510** | 1 | | | | | | | | | | | | | | | |
| PO12 | 0.304 | -0.053 | 0.086 | -0.142 | 1 | | | | | | | | | | | | | | |
| PPO4 | -0.433* | -0.305 | 0.058 | 0.19 | -0.192 | 1 | | | | | | | | | | | | | |
| PPO7 | 0.083 | -0.146 | -0.014 | -0.342 | 0.561** | -0.007 | 1 | | | | | | | | | | | | |
| PPO9 | 0.247 | 0.513** | 0.018 | -0.105 | -0.252 | -0.137 | -0.305 | 1 | | | | | | | | | | | |
| PPO12 | -0.190 | -0.103 | 0.199 | -0.128 | -0.065 | 0.246 | 0.092 | -0.175 | 1 | | | | | | | | | | |
| PAL4 | -0.152 | 0.117 | -0.223 | -0.509* | -0.164 | -0.301 | 0.136 | 0.245 | -0.150 | 1 | | | | | | | | | |
| PAL7 | 0.239 | 0.672*** | -0.346 | -0.405* | -0.385 | -0.361 | -0.212 | 0.511* | -0.104 | 0.512* | 1 | | | | | | | | |
| PAL9 | -0.394* | 0.054 | -0.256 | -0.575** | -0.422* | -0.102 | -0.041 | 0.209 | 0.011 | 0.851*** | 0.581** | 1 | | | | | | | |
| PAL12 | -0.168 | 0.034 | -0.429* | -0.477* | -0.222 | -0.114 | -0.032 | 0.051 | 0.005 | 0.349 | 0.498* | 0.555** | 1 | | | | | | |
| Pr4 | -0.197 | 0.096 | -0.134 | -0.456* | -0.236 | -0.340 | 0.109 | 0.281 | -0.056 | 0.935*** | 0.468* | 0.841*** | 0.277 | 1 | | | | | |
| Pr7 | 0.261 | 0.622** | -0.410* | -0.435* | -0.250 | -0.405* | -0.103 | 0.497* | -0.167 | 0.587** | 0.962*** | 0.560** | 0.534** | 0.527** | 1 | | | | |
| Pr9 | -0.431* | -0.004 | -0.226 | -0.542* | -0.421* | -0.117 | -0.058 | 0.203 | 0.049 | 0.848*** | 0.576** | 0.978*** | 0.561** | 0.815*** | 0.565** | 1 | | | |
| Pr12 | -0.310 | -0.090 | -0.227 | -0.198 | -0.380 | -0.184 | 0.047 | 0.028 | 0.029 | 0.267 | 0.418* | 0.470* | 0.792*** | 0.346 | 0.470* | 0.478* | 1 | | |
| Root | -0.353 | -0.321 | 0.200 | -0.131 | -0.289 | 0.332 | 0.113 | -0.153 | 0.186 | 0.046 | 0.013 | 0.252 | 0.500* | 0.075 | 0.036 | 0.251 | 0.637*** | 1 | |
| Shoot | -0.654** | -0.698** | 0.288 | -0.058 | -0.241 | 0.330 | 0.112 | -0.422* | 0.310 | -0.009 | -0.386 | 0.252 | 0.268 | 0.070 | -0.409* | 0.269 | 0.0.390 | 0.681*** | 1 |

[a] DI: Disease Intensity; PPO4, PPO7, PPO9, PPO12: The Polyphenol Oxidase enzyme in 4, 7, 9, 12 days after inoculation; PO4, PO7, PO9, PO12: The Peroxidase enzyme in 4, 7, 9, 12 days after inoculation; PAL4, PAL7, PAL9, PAL12: The Phenylalanine Ammonia Lyase in 4, 7, 9, 12 days after inoculation; pr4, pr7, pr9, pr12: Total protein content in 4, 7, 9, 12 days after inoculation; Root: Dry Root weight, Shoot: Dry Shoot weight.

significant relationship between peroxidase time 4 and disease intensity. The correlation between the PPO enzymes in the fourth day after inoculation with the disease intensity is negative and significant. Increased peroxidase enzyme in susceptible genotypes (compatible relationship) leads to reduced plant resistance. However, increasing the PPO enzyme in some of the genotypes (incompatible interaction) leads to plant resistance. An increase in total protein content was seen in genotypes with low disease intensity, 9 days after inoculation (negative and significant correlation). The correlation between PAL and disease severity was negative (-0.394*), and its increase leads to increased resistance.

In a study on the resistance of cultivars to soft rot of potato disease, the resistance of the varieties was correlated with high PPO and PAL enzyme activity (Ngadze *et al*., 2012). The correlation between disease intensity and enzyme in different days after inoculation is negative and, in some cases positive (the correlation between PO4 with disease intensity is significant and positive). This means that increasing PO4 increases the disease intensity, which is somewhat unexpected compared to the above observations. Pathogens easily penetrate into susceptible genotypes due to their structure, a positive response to the pathogen, and the lack of defense mechanisms. Following the penetration of the pathogen, a series of compounds and changes in plant metabolism will occur, one of which is an increase in peroxidase. Perhaps, this is why the disease intensity is positively and significantly correlated with peroxidase time 4 (early infection). The researchers showed that increased activity of the peroxidase in the resistant cultivars to *Puccinia graminis* f.sp. *tritici* in wheat does not cause resistance, as it is a non-specific response (Vanderplank, 1978). The interaction among enzymes is significant in some cases. Previous reports on the resistance of cultivars to soft potato rot have shown a positive correlation between amounts of PPO, PO, and PAL (Ngadze *et al.,* 2012). It seems that enzymes have a positive or negative interaction with each other. Some enzymes may have either a positive or a negative effect on resistance, through other enzymes to have. These relationships can be clarified through multivariate regression and pathway analysis.

## Multivariate Regression

In this study, stepwise regression was used to determine the traits that express a significant amount of changes in the disease intensity (result not shown). For this purpose, the Disease Intensity (DI) was compared as a response with biochemical traits, and the regression equation below was obtained (Equation 2) (Standard equation):

DI= 0.682PO–0.458PAL–0.271PPO (2)

The results showed that, among the studied biochemical traits, 3 traits entered into the model, namely, PO, PAL, and PPO, which together expressed 80.2% of the variations among the characters. According to the obtained regression equation, increasing the enzymes PAL and PPO lead to the disease intensity reduction, and an increase in the PO enzyme leads to increases in the disease intensity, which is in accordance with the correlation and mean comparisons. The PAL and PPO enzymes have a negative coefficient, and as they increase, the disease intensity declines (increasing resistance), providing that the other variables remain constant. The PO enzyme has a positive coefficient that appears to increase the disease intensity (susceptible). Chilling stress responses in tobacco growth rate and antioxidant enzymes of seedlings in 2 tobacco cultivars (susceptible and resistance) were studied by step wise regression. Result showed that regression equations containing catalase could be used in predicting seedling growth rate of tobacco under chilling stress condition (Xu *et al*., 2010). An increase in this enzyme may increase other enzymes reducing the disease intensity (resistance),





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therefore, to clarify this contradiction, path analysis was done.

## Path Analysis

Path analysis was used to identify the direct and indirect effects of entering traits into a regression model. Path coefficient analysis was conducted by considering peroxidase, phenylalanine ammonia-lyase, and polyphenol oxidase traits as predictor variables and disease intensity as the response variable. The comparison among the direct and indirect effects of disease intensity and some related traits was conducted. According to this result, the peroxidase enzyme has its high indirect and positive effect through polyphenol oxidase, having a negative effect on the disease intensity via phenylalanine ammonia-lyase. Polyphenol oxidase has its high indirect and negative effect on disease intensity through polyphenol oxidase (Table 4). In a study to find the relationship between resistance and resistance components to *Fusarium oxysporum* f. sp. *melonis*, biochemical and morphological characteristics were evaluated. The results of path analysis showed that superoxide dismutase due to the high direct effect and high indirect effects through other traits can be used as an indicator for increasing resistance to *fusarium oxysporum* f. sp. *melonis* (Hanifei *et al.*, 2016).

## Cluster Analysis

In screening genotypes or cultivars for resistance to disease, data clustering is a specific method used in the grouping. In cluster analysis, genotypes are classified into groups and subgroups based on similarity and non- similarity. K-means clustering is a centroid based algorithm, where K represents the number of clusters (Maity *et al*., 2018). A cluster analysis was performed to produce three clusters. In the first cluster, 1526 and spring Chinese genotypes

**Table 4.** Path analysis and direct and indirect effects of traits on disease intensity.

| Traits | correlation | Direct effects | Indirect effects | | |
|---|---|---|---|---|---|
| | | | Peroxidase | Phenylalanine ammonia-lyase | Polyphenol oxidase |
| Peroxidase | 0.740 | 0.681 | - | -0.025 | 0.082 |
| Phenylalanine ammonia-lyase | -0.394 | -0.458 | 0.036 | - | 0.027 |
| Polyphenol oxidase | -0.433 | -0.272 | -0.208 | 0.046 | - |
| Residual | 0.444 | | | | |

**Table 5.** Profiling of the cluster and the average vector of each cluster**.**

| Profiling of the clusters | Cluster 1 | Cluster 2 | Cluster 3 |
|---|---|---|---|
| Cluster members | 1526, Chinese spring | 1528, 1879, 2167 | 1530, 1510, *Aegilops* |
| Within cluster sum of squares | 12.438 | 8.753 | 10.222 |
| Average distance from centroid | 2.494 | 1.687 | 1.846 |
| Maximum distance from centroid | 2.494 | 1.986 | 1.895 |
| Traits | Cluster 1 | Cluster 2 | Cluster 3 |
| Disease intensity | 1.3525 | -0.8771 | -0.0246 |
| Root dry weight | 0.1360 | 0.5079 | -0.5986 |
| Shoot dry weight | -0.4872 | 0.7092 | -0.3844 |
| Polyphenol oxidase | -0.6146 | 0.4334 | -0.0237 |
| Total protein content | -0.2203 | -0.3473 | 0.5002 |
| Phenylalanine ammonia lyase | -0.1826 | -0.4081 | 0.5299 |
| Peroxidase | 1.0922 | -0.5366 | -0.1916 |





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(susceptible), in the second cluster, genotypes 1528, 1879, and 2167 (resistant), and in the third cluster genotypes 1530, 1510, and *Aegilops* (moderately resistant) were placed. The first cluster with a higher total square has more variety than the other two clusters (Table 5). In this cluster, there are genotypes susceptible to take-all disease, and a difference between these two genotypes was observed in reaction to the take-all disease, based on biochemical traits and growth factors. In research on the resistance of cultivars to soft rot of potato disease, cluster analysis grouped the varieties into three groups: resistant, intermediate, and susceptible. The resistant group had high enzyme activity (Ngadze *et al*., 2012). The results showed that the genotypes of the second cluster had a root and shoot dry weight higher than the other two clusters. Also, polyphenol oxidase activity, phenylalanine ammonia-lyase activity, and total protein content in the second and third clusters were higher than the first cluster.

## CONCLUSIONS

The first step in identifying the response of different cultivars to the disease is to identify the most important enzymes in the host-pathogen interaction pathway. According to the results of this study, based on the average disease intensity, genotypes were divided into three groups: resistant, moderately resistant, and susceptible. The results showed that the levels of polyphenol oxidase, phenylalanine ammonia-lyase, and total protein content were higher in resistant and moderately resistant groups. There was a positive and significant correlation between the phenylalanine ammonia-lyase enzyme (in all four times after inoculation) and total protein content (in all four times after inoculation). The correlation between these pairs was positive and significant: peroxidase enzyme (4 days after inoculation) with polyphenol oxidase, phenylalanine ammonia-lyase enzyme (9 days after inoculation), with the total protein content (7 days after inoculation), as well as with disease intensity. It can be concluded that an increase in the peroxidase enzyme during 4 days after inoculation may lead to the expression of several secondary compounds and metabolites, which indirectly leads to an increase in polyphenol oxidase and phenylalanine-ammonia lyase. It may cause resistance in other times or following the active defense responses. Various genes are involved in the synthesis of these enzymes and the total protein content, so, it can be said that resistance is controlled by several genes, quantitative and probably polygenic resistance. An increase in defense enzymes in plants is dependent on the pathogen, the plant, the type of genotype, as well as the plant's defense structure. Based on the results, it can be stated that the polyphenol oxidase, phenylalanine ammonia-lyase, genes synthesized in this pathway, and pathogenesis-related protein were used as marker resistance. These enzymes are components of resistance to take-all disease. In the next steps, in order to determine the resistance mechanism to take-all disease, it is suggested that the expression pattern of the genes that synthesize the phenylalanine ammonia-lyase and polyphenol oxidase enzymes and pathogenesis-related proteins be examined during the inoculation of wheat plants with the pathogen. Further studies are underway.

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## مطالعه تغییرات فعالیت آنزیم‌های فنیل‌آلانین‌آمونیالیاز، پلی‌فنل‌اکسیداز و پراکسیداز در برخی از ژنوتیپ‌های گندم علیه بیماری پاخوره (Take-all Disease)

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### چکیده

آنزیم‌ها نقش مهمی در تعامل گیاه و پاتوژن دارند و از اجزای ضروری مدیریت بیماری‌های گیاهی هستند. بیماری پاخوره گندم (*Gaeumannomyces graminis* var. *tritici*) بیماری پوسیدگی ریشه و طوقه گندم است. تاکنون مکانیسم مقاومتی این بیماری شناسایی نشده است، بنابراین این پژوهش به منظور شناسایی اجزای مقاومت به بیماری در تعدادی از ژنوتیپ‌های گندم انجام شد. در این مطالعه ۸ ژنوتیپ گندم نان در برابر قارچ عامل بیماری پاخوره گندم مورد مطالعه قرار گرفتند و تغییرات آنزیم های پراکسیداز، پلی‌فنل‌اکسیداز و فنیل‌آلانین‌آمونیالیاز و محتوای پروتئین کل در ۰، ۴، ۷، ۹ و ۱۲ روز پس از تلقیح بررسی شد. نتایج این مطالعه نشان داد که ژنوتیپ‌های مختلف واکنش‌های متفاوتی





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نسبت به بیماری پاخوره دارند. براساس میانگین شدت بیماری، ژنوتیپ‌ها به سه گروه مقاوم، نسبتاً مقاوم و حساس تقسیم شدند. نتایج نشان داد که سطح پلی‌فنول‌اکسیداز و فنیل‌آلانین‌آمونیالیاز و محتوای پروتئین کل در گروه‌های مقاوم و نیمه‌مقاوم افزایش یافته است. تجزیه و تحلیل خوشه‌ای با استفاده از روش k-means به منظور تولید سه خوشه انجام شد. ژنوتیپ‌های گروه دوم (مقاوم) و سوم (نیمه‌مقاوم) دارای سطح بالایی از آنزیم‌های پلی‌فنل‌اکسیداز، فنیل‌الانین‌آمونیالیاز و محتوای پروتئین کل نسبت به گروه اول (حساس) هستند. نتایج تجزیه و تحلیل چندمتغیره نشان داد که آنزیم پراکسیداز ممکن است به طور غیر مستقیم بر مقاومت به بیماری پاخوره تأثیر بگذارد. نتایج به دست آمده نقش آنزیم پلی‌فنل‌اکسیداز و پروتئین کل را در ایجاد مقاومت به بیماری پاخوره گندم روشن نمود.



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