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# Effect of Ascochyta blight disease on antioxidant enzymes activities, amount of proline and carbohydrate in some chickpea genotypes

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**Abstract**

Germplasm evaluation of crop plants as a repository of useful genes to cope with biological and abiotic stresses has always been the focus of plant breeders. The fungus causing Ascochyta blight is one of the most important biological factors limiting chickpea cultivation and production in most parts of the world, including Iran. The present study was conducted to identify the genetic sources of resistance of 20 chickpea genotypes in seedling, flowering, and podding stages in greenhouse conditions. Damages caused by the disease was recorded using a 9-degree scale after observing complete death in the sensitive control genotypes. Analysis of variance of the studied traits of chickpea genotypes was conducted via factorial experiment in a completely randomized design at two levels for factor A (disease-free and disease-contaminated conditions) and 18 levels (genotypes) for factor B (genotypes 13 and 15 were lost due to high susceptibility to the disease in the first stage of growth, samples were taken from 18 genotypes). The results showed that the resistant and susceptible genotypes were more accurately distinguished from each other in the podding stage. At this stage, 9 genotypes with a degree of damage 1, 2, and 3 (less than five) showed high resistance to the causative agent of Ascochyta blight. Physiological and biochemical traits involved in disease resistance were measured. The results showed that all traits except chlorophyll a, chlorophyll b and polyphenol oxidase had significant differences at 1% probability level in terms of disease stress. Chlorophyll a, chlorophyll b contents and polyphenol oxidase activity were significantly different at 5% probability level. In interaction of disease×genotype, only catalase was significantly different among all studied traits. The amount of peroxidase and polyphenol oxidase were affected by the disease and their rates increased. A positive relationship was observed between the level of polyphenol oxidase enzyme and pathogen resistance. Generally speaking, crops' reactions to harsh environmental conditions seems impossible to predict without the analysis of the relevant mechanisms.

***Key words*:** Ascochyta blight, Biochemical trait, Chickpea (*Cicer Arietinum*), Disease damage, Resistance.

## INTRODUCTION

Chickpea (*Cicer arietinum* L.) is one of the main sources for human nutrition. The cultivated area of this crop in Iran is 550,000 ha, with an average yield

of 536 kg/ha, its total production is 295.000 kg in the country. Worldwide, chickpea with cultivated area of over 12 million and the average yield of 930 kg/ha is the third most important crop of the legume group (FAO, 2017). Chickpea is cultivated mainly in arid and semi-arid areas of more than 50 countries across Asia, Africa, Europe, Australia, North America and South America (Chandora *et al*., 2020). It is the primary source of high quality protein, carbohydrates and minerals in human food throughout the chickpea growing regions around the globe (Sohrabi *et al.,* 2019). Due to its low production cost, high climatic adaptation, use of crop rotation and the ability to stabilize atmospheric nitrogen, it is served as the most important legume plants in sustainable farming system (Anonymous, 2000). Chickpea (*Cicer arietinum L*.) is the 3rd most imperative cool-season grain legume crop after the common bean (*Phaseolus vulgaris L.*) and field pea (*Pisum sativum L.*), and is the second most grown crop by poor farmers, primarily in the arid and semi-arid regions of Pakistan (Jan *et al.,* 2020; Rafiq *et al.,* 2020). It is commonly classified into two different market classes; the desi type (small seed size, dark in color) and the kabuli type (larger seed size, light color) and covers about 85% and 15% of the global chickpea production area, respectively (Ugandhar *et al.,* 2018). Chickpea is an excellent drought-tolerant grain legume (Merga and Haji, 2019). It plays a vital role in fulfilling the nitrogen requirement through symbiotic $N_2$ fixation and increasing soil fertility (Girma *et al.,* 2017).

Since chickpea is often cultivated in marginal and low-input soils, its grain yield is generally low, unstable, and less than potential. However, the yield potential of chickpea has been reported to be more than 6 t/ha (Toker, 2005). Chickpea breeding goals are to obtain high-yielding genotypes that are resistant to disease and can tolerate non-biological stresses well (Rao *et al*., 2007). Understanding the factors and processes that improve its yield in the face of environmental stresses seems necessary. However, production is seriously constrained by the fungal disease Ascochyta blight (AB), which is the most frequent and devastating disease of chickpea crops worldwide (Sagi *et al*., 2017). The fungus *Ascochyta rabiei* (syn. *Phoma rabiei*), can infect all parts of the plant above ground, and at any growth stage (Sharma and Ghosh, 2016). Comparison between different genotypes of this plant and how a genotype has higher tolerance and consequently higher yield stability than other genotypes lead us to understand the mechanisms and factors affecting chickpea tolerance to stress and to adopt more effective methods to improve tolerance. Pathogenic microorganisms are biological stressors that attack plants. Among the pathogens, viruses, viroids, bacteria, and fungi are the most affecting stresses on plant physiology. Phytopathogens or plant pathogens interact with the host plant depending on the degree of susceptibility of the host and environmental abnormal factors such as light and temperature, and can enter any part of the plant. Plant pathogens enter a plant after contact with it through abnormal mechanisms such as scarring on the branches and leaves or through natural pores such as stomata and lentils (Miri, 2009).

Different stresses reduce yield in chickpea. These stresses can be divided into two groups: biotic and abiotic stresses. Biotic and abiotic stresses result in a severe decline in agricultural yields worldwide. Meanwhile, the role of non-biotic stresses is estimated at about 70% and the effect of biotic stresses is about 30%. Annually, about 6.4 million tons of global production of chickpeas is reduced due to abiotic stresses and about 4.8 million tons of the production is reduced due to bio-stresses (Singh and Saxena, 1993). In the case of bio-stresses reducing the yield of this product, the role of AB and Fusarium has been reported to be more important than others (Aslam *et al*., 2018). Among the biotic stresses affecting chickpea, AB is the most destructive chickpea disease in the world (Pandey *et al.,* 2017) caused by *Ascochyta rabiei* (pass.) Lab. The disease was first detected at the beginning of the 20th century by the identification of the pathogen according to Butler's report (1918) in Northwestern India, now part of Pakistan, but later reported from most countries in the world. This disease is the first in terms of damage to chickpeas and was reported for the first time in Iran in 1953 from Qazvin farms. Then it has been reported in Azerbaijan, Fars, Khuzestan, Kerman, Khorasan, Gorgan, Mazandaran and Zanjan provinces (Parsa and Bagheri, 2008).

The disease starts from distal plant portion which may result in wilting with eventual death of the infected plant (Mahmood *et al.,* 2019). It can be effectively managed through chemical practices such as seed and foliar applications of synthetic fungicides (Namriboi *et al.,* 2018). However, due to ill effects of fungicides on environment, use of natural plant-based products against this pathogen is being explored (Shuping and Eloff, 2017). Many of the plants are rich in secondary metabolites that have inhibitory effects against the fungal pathogens (Akhtar *et al.,* 2020; Javaid *et al.,* 2020).

AB can be effectively controlled via intensive fungicide application, implementation of crop rotation strategies and seed treatment; however, using varieties with improved resistance remains one of the most cost-effective ways to manage AB in chickpea. The first Australian cultivar with improved resistance to AB compared to current varieties at the time, was Howzat released in 2001, followed by, Flipper, Yorker, and the most significant improvement with Genesis090 in 2005. As a result of selective breeding for AB resistance in chickpea, current varieties that make up the majority of annual chickpea production in Australia are rated as moderately resistant or resistant although loss of resistance was observed in a number of cultivars in 2016 (SA Sowing Guide, 2017). The genetic basis of AB resistance in chickpea has been previously investigated and QTL explaining resistance identified in bi-parental mapping populations have been reported (Li *et al.,* 2015).

AB has been reported in almost all chickpea cultivating regions across the world and is deemed to be the most devastating biotic factor resulting in significant loss of yield and degradation of seed quality (Khan *et al.,* 2018). The AB disease has become a major limiting factor for yield enhancement in chickpea in all chickpea growing areas. *Ascochyta rabiei* is the causal organism for the blight, which is known to be a highly variable fungus (Baite and Dubey, 2018). Attempts to identify genetic sources of resistance to pathogens are one of the most important steps in the implementation of chickpea breeding programs. Improving the resistance of genetic stocks in plants is a suitable way to deal with plant diseases. In reaction to the pathogen attack, plants activate complex defense mechanisms that cause the plants to be resistant to the pathogen. These mechanisms are hypersensitivity reactions, local accumulation of phytoalexins, cell wall thickening of cellulose, pectin, lignin, and activation of the phenyl-propanoid pathway (Kavousi *et al*., 2009). Improvement for resistance to this disease has not been improved due to the lack of high resistance in the primary gene pool and the pathogenic diversity of the causative fungus (Dey and Singh, 1993). However, breeders have made numerous varieties with moderate levels of resistance using the relative strengths available in genetic stock collections (Malhotra *et al.*, 2003; Warkentin *et al*., 2005). In many of these cultivars, resistance has been broken by the emergence of new pathotypes or by increasing the invasion power of existing pathotypes. Therefore, to diversify the genetic basis of resistance to AB and to promote resistance durability by gene pyramidization in breeding programs more sources of resistance in chickpea genetic stocks are required. Although resistant varieties have been used to fight the disease since the early 1960s, lack of complete information on the genetic diversity of fungal isolates has always hampered breeding programs so that the resistance of breeding varieties has been broken after sometimes (Jayakumar *et al*., 2005). Studies have shown that biotic stresses, such as fungi and bacteria, affect plant growth and development. Plant responses are associated with many changes in complex gene networks, and therefore, it is necessary to study the expression pattern of genes involved in adaptation or tolerance to these stresses. Given the focus of disease management on employing resistant cultivars as an effective and practical method for controlling the disease, achieving resistant lines to AB is an important step in this regard because identifying sources of disease resistance can be used in breeding programs for establishing sustainable resistance in high yielding but susceptible cultivars. The clear understanding of chickpea defense mechanism against *A. rabiei* is very important for breeding of resistant cultivars and better management of this fungal disease. Induced resistance to pathogen is one of the ways which plants use against biotic stresses. The objective of this research was to study the effect of AB disease on antioxidant enzymes activities, proline and carbohydrate contents in some chickpea genotypes.

## MATERIALS AND METHODS

### Plant material

For the purpose of the study, 20 chickpea genotypes were investigated for screening resistance to the fungal disease (Table 1). Seeds were obtained from Gachsaran Agricultural Research Center. The experiment was conducted as a factorial experiment in a completely randomized design with 3 replications. Disease damage was recorded using a 9-degree scale after observing complete death in the sensitive control genotype. Analysis of variance of the studied traits of chickpea genotypes was conducted via factorial experiment in a completely randomized design at two levels for factor A (disease-free and disease-contaminated conditions) and 18 levels (genotypes) for factor B (genotypes 13 and 15 were lost due to high susceptibility to disease in the first stage of growth, samples were taken from 18 genotypes). To ensure contamination, Kc-217854 genotype (native Bionij cultivar), which is susceptible to AB (ICARDA, 2000), was used as the standard cultivar.

**Table 1.** Average degree of disease damage and reactions of advanced *Cicer arietinum* lines to Ascochyta blight.

| Genotype code | Genotypes | Origin | Average degree of disease damage | | | Reaction |
|---|---|---|---|---|---|---|
| | | | Podding | Flowering | Seedling | |
| G1 | FLIP 03-71C | ICARDA | 1 | 2 | 3 | R |
| G2 | FLIP 03-64C | ICARDA | 1 | 1 | 2 | R |
| G3 | FLIP 98-106C | ICARDA | 1 | 2 | 3 | R |
| G4 | FLIP 00-40C | ICARDA | 1 | 1 | 1 | R |
| G5 | FLIP 99-66C | ICARDA | 1 | 3 | 5 | M |
| G6 | FLIP 00-21C | ICARDA | 1 | 1 | 2 | R |
| G7 | FLIP 99-34C | ICARDA | 1 | 1 | 1 | R |
| G8 | FLIP 01-32C | ICARDA | 6 | 7 | 8 | S |
| G9 | FLIP 01-50C | ICARDA | 1 | 1 | 2 | R |
| G10 | FLIP 01-52C | ICARDA | 1 | 3 | 5 | M |
| G11 | FLIP 97-120C | ICARDA | 3 | 5 | 6 | S |
| G12 | FLIP 03-71C | ICARDA | 5 | 6 | 7 | S |
| G13 | FLIP 03-135C | ICARDA | 8 | 8 | 9 | S |
| G14 | FLIP 03-152C | ICARDA | 1 | 1 | 2 | R |
| G15 | FLIP 04-18C | ICARDA | 7 | 8 | 9 | S |
| G16 | FLIP 82-150C | ICARDA | 2 | 4 | 6 | S |
| G17 | FLIP 88-85C | ICARDA | 4 | 5 | 7 | S |
| G18 | FLIP 93-93C | ICARDA | 1 | 1 | 2 | R |
| G19 | ARMAN | IRAN | 1 | 5 | 6 | S |
| G20 | AZAD | IRAN | 2 | 4 | 5 | M |

R: Resistant, M: Moderately Resistant, S: Susceptible.

**Preparation of spore suspension**

Assessing the reaction of genotypes to the AB was done using Pathotype III isolate 13 (Udupa *et al*., 1998). *Ascochyta rabiei* (chickpea AB fungus) was cultured in Chickpea Dextrose Agar (CDA) and stored at 18 °C for 14 h. After 7 to 10 days, 0.5 $cm^2$ of the fungus sample was transferred to a vial containing Chickpea Dextrose Broth (CDB) to produce spores. After a few days, the spores were proliferated and the suspension culture was passed through the two-dimensional membrane and the number of spores in the transient solution was measured using a hemocytometer (Santra *et al*., 2000).

**Inoculation of chickpea plants**

In this experiment, the seeds of 20 chickpea genotypes were transferred to a Petri dish for germination and washed at 25 °C for 48 hours after washing and surface disinfection with sodium hypochlorite, five seeds of each genotype were sown in pots containing a mixture of sterile soil, sand and peat at 2: 1: 1 ratio in 2.5 cm depth. Spore suspension ($2\times10^5$ spores/ml) was sprayed uniformly on 14-day-old chickpea seedlings. The spraying process continued until the first drop from the leaf surface. The pots were kept under plastic for five days in order to maintain 90% moisture content and sprayed three times daily under plastic and then the plastic cover was removed. The greenhouse moisture content was adjusted to 70%. After one week, specific symptoms of the disease became apparent, and two weeks after inoculation, the plants of the completely susceptible control cultivar died. After observing the condition of the cultivars, the reaction of the genotypes to the disease was recorded. Symptoms of the disease and the extent of damage were determined at three stages including seedling, flowering and podding stages on a scale ranging from 1 to 9 (Singh and Reddy, 1993):

1. No observation of scars
2. Small and finely scattered spots on the leaves
3. Limited and elliptical scars on the stem
4. Elongated and inscribed scars around the stem
5. Fragmentation of the stem at the scar site
6. Fracture of the stem at the scar site
7. The top-down spread of scars towards the stem
8. Relative plant death
9. Complete plant death

Based on the degree of damage in the podding stage, the genotypes were divided into resistant (damage degree from 1 to 4), tolerant (damage degree 5), and susceptible (damage degree from 6 to 9) groups (Udupa and Weigand, 1997).

**Measurement of physiological and biochemical traits**

Two weeks after inoculation, in order to evaluate the

physiological and biochemical traits, leaf samples of control and diseased plants were taken and immediately frozen in liquid nitrogen and transferred into -80 °C freezer.

**Measurement of photosynthetic pigments**

Fresh leaves were used to measure photosynthetic pigments, including chlorophyll a and b and carotenoids. For this purpose, 0.1 g of fresh plant tissue was purged inside a porcelain mortar with 10 ml of 80% acetone and the resulting solution was completely transferred to centrifuge tubes. The tubes were centrifuged for 10 minutes at 6,000 rpm and the amount of chlorophyll in the supernatant was determined according to the method reported by Lichtenthaler (1987), using a spectrophotometer at at 470, 645 and 663 nm. The amount of chlorophyll a and b was calculated based on the following equations.

(1) Chlorophyll a=(19.3*A663-0.86*A645) V/100W

(2) Chlorophyll b=(19.3*A645-3.6*A663) V/100W

(3) ChlT=Chla+Chlb

(4) Car=(1000 A470-3.27[Chla]-104 [Chlb])/227

The A663, A645, and A470 are the absorbance reads at 663, 645, and 470 nm wavelengths, respectively.

**Proline**

Proline was extracted from the youngest leaves and roots using the method of Bates *et al*. (1973). About 0.1 g of leaf and root tissues were washed in 10 ml 3.3% sulfosalicylic acid and the homogenate was filtered off and centrifuged at 4000 rpm at 4°C for 10 min. In a separate tube, 2 ml of the extract was added to 2 ml of ninhydrin reagent and 2 ml of pure glacial acetic acid. The tubes were placed in a bain-marie for 1 h and then vortexed for 15 to 20 seconds after adding 4 ml of toluene to each tube. After forming two separate phases, the upper colored phase was carefully separated and measured in a spectrophotometer at 520 nm.

**Soluble sugars**

The method reported by Iriguen *et al*. (1992) was used to measure soluble sugars. About 0.2 g of green plant tissue was placed in the test tubes with 10 ml of 95% ethanol and heated in a binarium bath at 80 °C for 1 hour. After cooling, 1 ml of the samples were combined with 1 ml of phenol 0.5% and 5 ml of 98% sulfuric acid. Finally, absorbance was measured using UV-160 spectrophotometer at 483 nm and the soluble sugars content of the samples was calculated based on mg/g leaf fresh weight.

**Protein content**

Total protein was extracted from the youngest leaves using the method of Bradford (1976). About 0.1 g of leaf sample was pelleted with 1 ml phosphate buffer in a porcelain mortar and was centrifuged at 12000 rpm for 15 minutes at 4 °C after transfer to a microtube. 100 µL of transparent supernatant was taken and added to test tubes that had been poured into each of 5 ml of Bradford reagent. Five min after the soluble dye fixation, the measurements were made at 595 nm and room temperature using a spectrophotometer.

**Catalase enzyme assay**

The kinetic activity of the catalase enzyme was measured using the method of Chance and Mahley (1955) with modifications. For this purpose, 2.5 ml Tris buffer with 3.0 ml oxygenated water were mixed with 60 ml enzymatic extract in ice bath. The absorbance changes were measured at 240 nm using a spectrophotometer.

**Peroxidase enzyme assay**

To measure the quantitative peroxidase activity, the method of Kar and Mishra (1976), albeit with some modifications, was employed. For this purpose, 1 M Tris buffer solution, 50 mM oxygenated water, and 100 mM pyrogallol were prepared. Then, 10 ml of each solution was taken and the resulting solution was brought to 100 ml. Finally, 2.5 M of the above solution was mixed with 50 µl of the enzyme extract. The absorbance was measured at 425 nm using a spectrophotometer.

**Polyphenol oxidase assay**

The activity of polyphenol oxidase was evaluated by Kar and Mishra method (1976), with some modifications. For this purpose, 1.5 ml Tris buffer was mixed with 0.4 ml pyrogalel and 0.1 ml Good enzymatic extract and incubated at 25 °C for 5 minutes. The absorbance was measured at 420 nm with a spectrophotometer.

The activity of each enzyme was calculated in terms of unit changes of absorbance per minute for mg of protein.

**Data analysis**

Analysis of variance of experimental data was conducted via factorial analysis in a completely randomized design. Prior to analysis of variance, the normality test was performed for all traits. All physiological traits had normal distribution. Mean comparisons were made by Duncan's new multiple range test (MRT) at the 5% level of probability using Excel software. SPSS v.19 and SAS were employed for statistical calculations.

## RESULTS AND DISCUSSION

### Evaluation of genotypes reaction to Ascochyta blight

One week after inoculation, the early signs of the disease were well-visible on the sensitive genotype (Bionij cultivar) at the end of the leaves. Small dark spots on the leaves and stems showed that the inoculation was well done. Two weeks after inoculation, the disease developed in the petiole and stems of susceptible genotypes, leading to their complete death. At this time, in order to evaluate physiological and biochemical traits, leaf samples were taken from control and diseased plants.

### The process of disease development

Examination of the disease symptoms showed that the damage in leaves and petioles, stems, terminal buds, axillary buds and crowns differed in different genotypes. In a few genotypes, disease damage was only visible at the tip of the leaves. However, other organs, including stems and terminal buds were resistant to the disease. In a limited number of genotypes, although all shoots were damaged, the crowns were healthy and axillary stems were grown from the crown site and even entered the flowering and podding stage. The reaction of different organs is very important for identifying the effective factors in the expression of resistance genes. These factors can be identified via studying the protein content of these tissues under stress conditions (Dickinson and Beynon, 2000). Of the 20 genotypes evaluated in this study, 9 genotypes with a degree of damage less than 4 were highly resistant to the pathogen. In addition, 3 genotypes with the degree of damage 5 showed limited symptoms. Most of the resistant, tolerant and a limited number of disease susceptible genotypes entered the podding stage and were collected at the end of the seeding period. Although genotypes 5, 11, 19 showed good resistance in early stages of growth, but they were susceptible to the disease in the flowering and podding stages.

In the seedling stage, disease damage levels may rise from 1 to 4, a month after the first inoculation (Table 1). At this stage, most genotypes with damage levels of 1 and 2 are resistant to the disease, and disease damage occurred in susceptible control cultivar with ulcer, fracture and eventually complete death. Although susceptible genotypes were recognizable at the seedling stage, the symptoms were not sufficient to distinguish other levels of resistance. Genotypes identified at the end of the season as susceptible to the isease were not significantly different from resistant genotypes at the seedling stage. Most plants entered the flowering stage about one month after inoculation of the pathogen, about two months after planting. At this stage, the majority of the samples showed high resistance to disease with a degree of damage smaller than 4. At the podding stage, disease damage significantly increased and the number of genotypes with degree of damage smaller than 4 decreased. The average damage was about one degree higher than the previous stages. This increase may have been influenced by factors such as the time course and the provision of sufficient time for disease development and plant susceptibility at this stage. This result indicates that the initial resistance of genotypes at early stages is not very reliable. Thus, it is necessary to investigate the disease symptoms after the podding stage.

Comparison of the mean of the three study stages showed that only the podding stage had a significant difference in damage to the specimens compared to the first and second stages. Therefore, it seems that the difference between genotypes at seedling or flowering stage is not reliable, and it is better to compare the resistance or susceptibility of the samples at the podding stage.

The podding stage is a very suitable for differentiating the resistance levels of genotypes. In many reports, the podding stage has been mentioned as the most sensitive (Singh and Reddy, 1993), but other factors appear to include the elapsed time for disease development and the cumulative effect of infection on the maximum rate of disease damage at this stage. The resistant cultivars identified in this study are native to Iran. It seems that due to the particularity of genotypes in the population of *Ascochyta rabiei* in Iran, native cultivars have developed higher resistance to the disease. The sources of resistance identified among native populations can be used as suitable sources of resistance for this pathogen population in Iran for designing breeding projects.

### Analysis of variance and comparison of average physiological traits

The results of analysis of variance showed that all traits except chlorophyll a, chlorophyll b and polyphenol oxidase had significant differences at 1% probability level in terms of disease stress. Chlorophyll a, chlorophyll b and polyphenol oxidase traits were significantly different at 5% probability level. Genotypes were significantly different in terms of chlorophyll a and total chlorophyll traits. In interaction of disease×genotype, only catalase was significantly different among all studied traits (Tables 2,3).

**Table 2.** Analysis of variance of physiological traits in chickpea genotypes.

| Source of variation | df | Mean of square | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Content soluble protein (mg $g^{-1}$ DW) | Soluble sugars (mg $g^{-1}$) | Carotenoids (mg $g^{-1}$ FW) | Chlorophyll *a* (mg $g^{-1}$ FW) | Chlorophyll *b* (mg $g^{-1}$ FW) | Chlorophyll t (*a+b*) (mg $g^{-1}$ FW) | Proline (mg $l^{-1}$) | POX activity (U $\mu g^{-1}$ protein) | CAT activity (U $\mu g^{-1}$ protein) | PPO activity (U $\mu g^{-1}$ protein) |
| Stress (A) | 1 | 0.007** | 1206.32** | 0.185** | 1.034* | 0.131* | 1.90** | 10.92** | 17.07** | 4.80** | 0.427* |
| Genotype (B) | 17 | 0.001$^{ns}$ | 76.37$^{ns}$ | 0.027$^{ns}$ | 0.38** | 0.002$^{ns}$ | 0.53* | 0.67$^{ns}$ | 1.41$^{ns}$ | 0.48$^{ns}$ | 0.077$^{ns}$ |
| A*B | 17 | 0.001$^{ns}$ | 58.13$^{ns}$ | 0.018$^{ns}$ | 0.18$^{ns}$ | 0.023$^{ns}$ | 0.28$^{ns}$ | 0.46$^{ns}$ | 2.35$^{ns}$ | 0.86** | 0.116$^{ns}$ |
| Error | 72 | 0.001$^{ns}$ | 50.59$^{ns}$ | 0.017$^{ns}$ | 0.16$^{ns}$ | 0.026$^{ns}$ | 0.25$^{ns}$ | 1.07$^{ns}$ | 2.32$^{ns}$ | 0.29$^{ns}$ | 0.111$^{ns}$ |
| Coefficient of variation (%) | | 11.74 | 16.5 | 18.9 | 13.2 | 20.9 | 24.2 | 7.49 | 20.66 | 13.42 | 30.9 |

ns, * **: no significant and significant at 0.05 and 0.01 percentage, respectively.
POX: Peroxidase enzyme, CAT: Catalase enzyme, PPO: Poly phenol oxidase.

The highest coefficient of variation for peroxidase was 66.2 and the lowest was 16.5 for the soluble sugars. The significant interaction of genotype by stress showed that the trends of genotypes for traits under normal and stress conditions were not the same and superior genotypes under normal conditions were not necessarily recommended for disease stress conditions.

The results of mean comparison of the interaction effect analyzed with the Duncan multi-domain test method at 5% confidence level are given in Tables 4,5. Catalase activity increased with the application of stress. The increase in catalase enzyme in disease conditions is due to the fact that catalase is an antioxidant that removes and deactivates reactive oxygen species by converting hydrogen peroxide into water and oxygen, although this increase was not significant. Genotype number 16 in our study had the highest rate of catalase activity in the control and stress conditions. Genotype 9 had the lowest rate of catalase activity in the control, but its catalase activity increased in stress conditions compared to other genotypes, indicating resistance to the disease. Magbanua *et al*. (2007) measured the activity of catalase enzyme in corn under the influence of the disease caused by *Aspergillus flavus* and showed that increasing the activity of this enzyme is affected by the disease. The increase in the activity of antioxidant enzymes under stress conditions can be considered as an indicator for increasing the production of oxygen free radicals. Since catalase helps maintain active oxygen homeostasis during stress, its activity in the plant during stress is increased (Magbanua *et al*., 2007) and its synthesis is a kind of adaptive response to oxidative stress.

Chlorophyll a and b showed a significant decrease under disease conditions. Chlorophyll a is the predominant photosynthetic pigment, while chlorophyll b is a minor pigment and accounts for about one third of the total chlorophyll content of the leaf (Lefsrud *et al*., 2006). Rosaibarr and Maiti (1995) found that the decrease in chlorophyll content in stress is due to changes in nitrogen metabolism and the production of compounds such as proline that play a role in osmotic regulation. In fact, the decrease in leaf chlorophyll content and quantum efficiency of photosystem II in susceptible cultivars is due to the damage to the chloroplast membrane during stress (Yang *et al*., 2003). Chlorophyll b was more abundant at plant maturity. Chlorophyll depletion in some genotypes may be due to the increased production of reactive oxygen species in the presence of light in stress conditions because reactive oxygen species attack chlorophyll pigments and cause them to decompose. In addition, due to

**Table 3.** Comparison of mean effect of disease stress on physiological traits measured in chickpea genotypes using Duncan method at 5% probability level.

| Stress | PPO activity | POX activity | Content soluble protein | Carbohydrate | Proline | Carotenoid | Chlorophyll *b* |
|---|---|---|---|---|---|---|---|
| Disease-free | 1.0887$^{b}$ | 4.8742$^{b}$ | 96.32$^{a}$ | 15.1265$^{b}$ | 3.9850$^{b}$ | 0.5077$^{a}$ | 0.6192$^{a}$ |
| Disease-contaminated | 1.2144$^{a}$ | 5.6695$^{a}$ | 11.26$^{b}$ | 21.8107$^{a}$ | 4.6211$^{a}$ | 0.4249$^{b}$ | 0.5496$^{b}$ |

**Table 4.** Comparison of mean genotype interaction effect in stress for catalase trait measured in chickpea genotypes using Duncan method at 5% probability level.

| Genotypes | Catalase | |
|---|---|---|
| | Disease-free | Disease-contaminated |
| G1 | 1.4188$^{cdef}$ | 1.1029$^{ef}$ |
| G2 | 1.7412$^{abcdef}$ | 1.7253$^{abcdef}$ |
| G3 | 1.4330$^{cdef}$ | 1.4714$^{cdef}$ |
| G4 | 0.8342$^{f}$ | 1.8341$^{abcdef}$ |
| G5 | 0.8339$^{f}$ | 1.7827$^{abcdef}$ |
| G6 | 1.5891$^{cdef}$ | 1.6513$^{bcdef}$ |
| G7 | 0.8080$^{f}$ | 1.9810$^{abcde}$ |
| G8 | 2.2931$^{abcd}$ | 1.5156$^{cdef}$ |
| G9 | 0.7702$^{f}$ | 2.7663$^{a}$ |
| G10 | 1.4851$^{cdef}$ | 1.0813$^{ef}$ |
| G11 | 1.4519$^{cdef}$ | 1.7736$^{abcdef}$ |
| G12 | 1.5769$^{cdef}$ | 1.2839$^{def}$ |
| G14 | 1.0072$^{ef}$ | 1.8357$^{abcdef}$ |
| G16 | 2.0578$^{abcde}$ | 2.2445$^{abcd}$ |
| G17 | 1.4707$^{cdef}$ | 1.4572$^{cdef}$ |
| G18 | 1.0117$^{ef}$ | 2.6578$^{ab}$ |
| G19 | 2.0850$^{abcde}$ | 2.2055$^{abcd}$ |
| G20 | 1.2807$^{def}$ | 2.3739$^{abc}$ |

**Table 5.** Comparison of the mean of chickpea genotypes for physiological traits measured in chickpea genotypes using Duncan method at 5% probability level.

| Genotypes | Chlorophyll a | Chlorophyll total |
|---|---|---|
| G1 | 1.4271$^{bcd}$ | 2.0211$^{bcd}$ |
| G2 | 1.4756$^{bcd}$ | 2.0707$^{bcd}$ |
| G3 | 1.4537$^{bcd}$ | 2.0269$^{bcd}$ |
| G4 | 1.4877$^{bcd}$ | 2.0660$^{bcd}$ |
| G5 | 1.4274$^{cd}$ | 1.9986$^{bcd}$ |
| G6 | 1.3189$^{cd}$ | 1.8360$^{abc}$ |
| G7 | 1.8779$^{abc}$ | 2.5188$^{abc}$ |
| G8 | 1.5775$^{bc}$ | 2.2052$^{abc}$ |
| G9 | 1.7977$^{abc}$ | 2.3989$^{abc}$ |
| G10 | 1.4631$^{bcd}$ | 2.0219$^{bcd}$ |
| G11 | 1.9101$^{ab}$ | 2.5721$^{ab}$ |
| G12 | 0.9720$^{d}$ | 1.4792$^{d}$ |
| G14 | 1.3180$^{cd}$ | 1.8361$^{cd}$ |
| G16 | 2.0786$^{a}$ | 2.7543$^{a}$ |
| G17 | 1.5277$^{bc}$ | 2.0379$^{bcd}$ |
| G18 | 1.4314$^{bcd}$ | 1.9825$^{bcd}$ |
| G19 | 1.5664$^{abc}$ | 2.1594$^{abcd}$ |
| G20 | 1.6035$^{abc}$ | 2.2479$^{abc}$ |

the disease, the intensity of sucrose loading from cytosol to the phloem is reduced and assembled. Sucrose accumulation results in the termination of the Calvin cycle. As a result of this action, the Cab gene (responsible for encoding a and b proteins) suppressed (Cakmac and Kirkby, 2008). This decreasing trend can be due to reduced cell division and the slowing down the plant growth due to stress, which increases the amount of chlorophyll per unit area. Therefore, when the chlorophyll content does not decrease under stress conditions it may indicate the plant's tolerance to the damage caused to chloroplasts by light (Yang *et al*., 2006). The comparing means of cultivars for chlorophyll a and total chlorophyll showed that cultivars 11 and 16 had the highest chlorophyll a contents among the studied cultivars, which had a significant difference with other genotypes, and genotype 12 showed the lowest chlorophyll a and total chlorophyll contents.

Carotenoid content was also affected by stress conditions and showed a decrease. Carotenoids can protect the photosynthetic apparatus from the scavenging of radical oxygen molecules. They can also be switched on or off directly with single oxygen or oxidized with single oxygen. Therefore, carotenoids indirectly reduce the production of oxygen species. Carotenoids also induce NADPH and protect chlorophyll from photosynthesis through a mechanism called the xanthophyll cycle (koyro, 2006).

The amount of proline increased under stress conditions. Proline destroys oxygen free radicals, protects enzymes from degradation, osmotic regulation of the cell, and maintains the normal state of the membrane. In most plants, free proline accumulation occurs in response to biological and abiotic stresses. In some plants under prolonged stress conditions up to 100 times the normal proline concentration is increased (Matysik, 2002). Therefore, the increase in

proline may have been caused by the disease, since it has a protective property on proteins and enzymes. Proline is an amino acid that due to its hydrophilic and hydrophobic components, can affect the solubility of different proteins. This feature of proline is because there is an interaction between proline and hydrophobic proteins which increases their stability as a result of the increase in the total level of hydrophilic proteins. It should be noted that the process of proline oxidation is low in healthy plants therefore in stress conditions, high levels of proline cannot be justified. Thus, the increase in proline concentration under stress conditions in plants is often due to its spontaneous synthesis (Staden *et al*., 1999).

Another major change to increase stress resistance is the increase in soluble sugars. It is believed that soluble sugars in the cytoplasm protect the membrane structure during stress. Increased carbohydrates act as a metabolic signal under stress conditions and increase expression of defense genes and decreasing photosynthesis. Moreover, sugars play other ecological roles in protecting the plant against wounds, infections and detoxification of external compounds (Smekens, 2000). In many plants stress tolerance is associated with the accumulation of soluble sugars, especially sucrose in the cytosol.

The disease led to a decrease in total protein. The increase in the rate of protein degradation may be the result of stresse (Bolen and Baskakov, 2001). Changes in the expression, accumulation, and synthesis of proteins in response to environmental stresses are considered important mechanisms in plants to protect cell metabolism and induce nourishment. It seems that the process of adaptation of plants to adverse conditions, achieved through different biochemical and physiological mechanisms, minimizes the damage caused by stress. Proteins are metabolites that are expressed differently in response to stress. Proteins are involved in processes such as signaling transcription, RNA-related processes, translation, photosynthesis, optical respiration, carbon metabolism, nitrogen, sulfur and energy (Heidarvand and Maali amiri, 2010). The highest amount of soluble protein was obtained in genotype 2 with no significant difference with genotype 3, which was significantly higher than other genotypes.

The amount of peroxidase and polyphenol oxidase have been affected by the disease and their rates increased. These enzymes are the most important antioxidants that break down $H_2O_2$ into water and oxygen molecules (Janda, 2005; Yong, 2008). When the plant is attacked by pathogens, it uses defense mechanisms to counteract it. It is regulated through complex, interconnected networks of signaling paths. Signal transduction pathways lead to the strengthening and wooding of cell walls, the production of antimicrobial metabolites, and reactive oxygen species and reactive nitrogen species. One of the proteins induced during the defense of the host plant against the pathogen is the production of peroxidases. Peroxidases belong to a large family of multigens and interfere with a wide range of physiological processes such as lignin and sobrin formation, phytolexins synthesis, and ROS metabolism. Peroxidases have also been implicated in plant hypersensitivity (HR) response and host programmed cell death (PCD), at the site of infection, in relation to pathogen development (Almagro *et al*., 2009). Stress is oxidative and peroxidase isoenzymes play a key role in stress tolerance (Tale Ahmad and Haddad, 2010). Omranzadeh *et al*. (2011) investigated the induction of some defense compounds such as peroxidase and polyphenol oxidase enzymes against root nodule producing nematode *Meloidogyne javanica* in cucumber, showing that as a result of the disease, the activity of polyphenol oxidase enzyme significantly increased compared to the control and healthy plants. The polyphenol oxidase enzymes play an important role in chloroplast so that it may be involved in Mahler's cycle reactions and lead to the detoxification of reactive oxygen species (Sherman *et al*., 1995). Plants use a number of alternative defense mechanisms, including the Mahler's cycle, to counter the negative effects of the reduction of Calvin's cycle activity (Risky *et al*., 2003). Potassium deficiency appears to be a factor in the plant's resistance to disease, which may be due to its key role in activating various enzymes (Marshner, 1995). A positive relationship between polyphenol oxidase level and pathogen resistance was observed in the plants. There is ample evidence on the importance of polyphenol oxidase induction in plants, especially under stress and pathogen attack (Meyer, 2006). The amount of damage that stress inflicts on crops leads to further efforts to understand the effects of disease on different plant mechanisms and requires understanding of appropriate adaptive responses to this environmental factor. The chickpea breeding program has utilized the results of this study for resistance screening and breeding for AB resistance. Disease resistance screening has been simplified based on the results of this project as only one aggressive isolate is now used to determine resistance levels in indoor studies. The identification of genes involved in tolerance to these stresses and their required timing of expression shall greatly aid development of elite chickpea cultivars through molecular breeding or genetic manipulation.

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