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ESTIMATION OF GENETIC VARIABILITY AND ASSOCIATION AMONG DIFFERENT PHYSIOLOGICAL TRAITS RELATED TO BIOTIC STRESS (*FUSARIUM OXYSPORUM* L.) IN CHICKPEA

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ABSTRACT

Experiment was conducted to assess genetic differences among chickpea genotypes under normal and biotic stress (inoculated with *Fusarium oxysporum* L.) conditions, to estimate the relationship among different biotic stress related traits and to identify suitable parental material to be used in breeding programs for the development of genotypes resistant to fusarium wilt. The responses of 70 different chickpea genotypes were evaluated on the basis of different physiological traits by following principal component analysis (PCA). According to PCA, under normal and stress conditions out of eight only 4 PCs had more than one eigen value in each case and collectively contributed 71.50% and 75.2% variability under normal and stress conditions respectively. Biplot analysis depicted that under normal condition the genotypes 1007, 60101, 7008, 405, PB91, B3008, 3013, 7002, 7012, 1217, 6011, BITAL98, 6028 and CH-8 were highly diversified, whereas under stress condition, genotypes 7059, 4004, 6001, 7046, 1288 and 1143 proved resistant and presented the greatest diversity. Genotypes 6028, 7027, BRC236, 5028, 7056-1, 6010, 6003, 1217, 3013, 4028¸ 6017, 7010, 3019, PB2000, 5006, 6255, 4046, PB2008, B3008, 1159, 2009-1, and 6011 were found with least diversity and declared as susceptible because of poor performance.

Key words: Chickpea, Fusarium wilt, Biplot, PCA and Correlation.

INTRODUCTION

Plant genetic resources are actually the guarantee of world food security. Genetic resources possess genetic material variability contained in traditional varieties, cultivars, wild crop relatives and other wild species. It is necessary to exploit genetic resources to meet global food requirement (Farshadfar and Farshadfar, 2008).

Chickpea (*Cicer arietinum* L.) is an important crop possessing high variability for different qualitative and quantitative traits with 17-24% protein, 41-50.8% carbohydrates, high percentage of other mineral nutrients and unsaturated linoleic and oleic acid in seed. It is grown throughout the world with different names i.e. Chickpea in UK, Bengal gram in India, Garbanzo in Latin America, Hommes or Hamaz in Arabic world, Nohud or Loblebi in Turkey and Shimbra in Ethopia. Chickpea restores and retains the soil fertility by its nitrogen fixing ability predominantly in dry areas (Ahmad *et al*, 2010), and fit very well in different cropping patterns. Its yield is mostly concentrated in rainfed areas of Punjab (910.7 thousand hectares) followed by Sindh (55.9 thousand hectares) and Khyber Pakhtunkhwa (KPK) (49.0 thousand hectares), whereas in Balochistan it is cultivated on 36.7 thousand hectares (Bokhari *et al*., 2011). Globally, over 90% of the total chickpea is produced and used in Asia. It contains high protein contents and rich in zinc, dietary fiber, calcium, magnesium, phosphorus, potassium, iron and vitamins (Peksen and Artık, 2005; Kayan and Adak, 2012).

Fusarium wilt is a severe ailment of chickpea in India, Iran, Pakistan, Nepal, Burma, Spain and Mexico. In Pakistan *Fusarium oxysporum* is the second most severe problem of chickpea after blight in the districts of Jhang, Layyah, Khushab, Bhakkar and Mianwali (Shah *et al*., 2009). Areas with low rainfall alongwith favorable environmental conditions are prone to wilt (Nene *et al*., 1996). *Fusarium oxysporum f. sp. ciceri* is a soil borne, root pathogen which colonizes the xylem vessels and blocks them entirely (Singh *et al*., 2006). An expected loss of 12 million rupees annually was stated from Pakistan due to this disease (Shah *et al*., 2009). Wilting at earlier growth stage causes more loss than at lateral phase of growth and seeds harvested from wilted plants looks lighter and duller than those from healthy plants (Ahmad *et al*. 2010). In a highly susceptible cultivar, wilt symptoms can be observed within 25 days after sowing in the field. Yellowing of leaves, flaccidity, chlorosis and wilting (drooping of rachis, leaflets and petioles) are among the critical effects of fusarium wilt on chickpea plant (Haware, and Nene, 1982; Jiménez-Díaz *et al.*, 1993). It may appear at vegetative and reproductive growth stages accompanied with yield losses in both cases (Navas-Cortes *et al*., 2000). In Pakistan 10 -50% losses have been reported in chickpea due to fusarium

wilt during last few decades (Ikramul and Farhat, 1992; Mukhtar, 2007).

The cheapest, cost-efficient and the most ideal way to manage chickpea wilt, is the use of resistant cultivars. Chemical control of wilt is not reasonable and economical because of the soil as well as seed-borne nature of the pathogen. This pathogen can stay alive in soil for numerous years by means of chlamydospores (Ahmad *et al*. 2010). Fungal chlamydospores can stay alive in soil up to 6 years in the absence of the host plants (Iqbal *et al*., 2010). Therefore, this disease can be managed principally by the use of resistant cultivars (Ahmad *et al*. 2010). Estimation of genetic variability based on physiological and morphological standards is the main step in breeding programs as improvement depends upon the magnitude of variability and this leads towards selection of parents. High level magnitude of variability enables the researcher to use appropriate gene pool for improvement. There is a need to identify the resistant sources against different isolates of *Fusarium oxysporum* (Shah *et al*., 2009).

Present study was planned to assess the newly developed germplasm of chickpea for resistance against local isolates of wilt fungus on the basis of different physiological standards by using multivariate technique. There is dire need for continuous screening of chickpea germplasm for resistant against fusarium wilt because resistant varieties become susceptible with the passage of time. This conversion from resistance to susceptibility might be either due to resistance breakdown or evolutionary changes in pathogenic variability (Nikam *et al*., 2007).

MATERIALS AND METHODS

Germplasm of variable origin used in this study (70 genotypes) was collected from chickpea germplasm resources of the department of Plant Breeding and Genetics, University of Agriculture, Faisalabad. Pathogen (*Fusarium oxysporum*) was collected from Nuclear Institute of Agriculture and Biology (NIAB) Faisalabad, Pakistan in petri plates filled with potato dextrose media.

Experiment was divided in to two subunits. Each unit comprised of 10 seedlings per genotype per replication. One subunit was treated with inoculum and named as inoculated and second as standard / normal in which no inoculum was applied. Two factor factorial triplicated complete randomized design (70 genotypes and 2 treatments) was followed to plant the seeds of all the genotypes in polythene bags (7"× 4"). Initially two seeds per polythene bag were sown and thinned up to one seedling per bag after the establishment of seedlings.

Pathogen was multiplied in petri plates filled with potato dextrose media for two weeks at room temperature. Inoculum solution was prepared by mixing pathogen with distilled water and was applied at seedling stage with shower in root zone. Totally dry condition was maintained in order to provide favorable environment to pathogen for its dissemination. To maintain uniform intensity of pathogen and to increase the severity, inoculation in the inoculated set was repeated twice with the uniform interval of one week. Data were recorded after 25 days of inoculum application for leaf rolling (LR), survival rate (SR), ascorbic acid (AA), β-carotenoids, chlorophyll *a*, chlorophyll *b*, root/shoot ratio (RS) and root density (RD). Ascorbic acid contents in the plant samples were estimated by using Kampfenkel method (Kampfenkel *et al*., 1995). Chlorophyll contents were estimated by following formulae designed by Nagata and Yamashita (1992).

The level of resistance/susceptibility of each genotype was determined by using the rating scale (Iqbal *et al.* 2010; Table 2).

Table-2. Scale for evaluation of genotypes under stress condition (Inoculum applied).

Disease incidence	Response	Scale	Response
0-10 percent	Highly resistant	1	No leaf rolling
11-20 percent	Resistant	2	1/4 leaf rolling
21-30 percent	Moderately resistant/ Tolerant	3	1/2 leaf rolling
31-50 percent	Susceptible	4	3/4 leaf rolling
51-100 percent	Highly susceptible	5	Complete leaf rolling

Statistical Analysis: Data were analyzed for the significance of differences using factorial analysis of variance (ANOVA) devised by Steel and Torrie (1980). Survival rate was recorded as the percentage of survived plants. The principal component analysis is a multivariate statistical procedure for investigation and simplifying complex data sets. The ability of this method to transform several possibly associated variables into a smaller number of variables called principal components. This method has been established by Everitt and Dunn (1992) and followed by Kayan and Adak (2012). Biplot analysis was used to study the diversity among genotypes. Correlation coefficient reflects how stronger or weaker association is present among variables and provides the basis for the selection standards to be used in selection procedure.

RESULTS

Analysis of variance (ANOVA) presented highly significant differences among genotypes and treatments for all the traits (Table-1). Under normal condition there was slight or no leaf rolling in all genotypes except 60101 and 6028 which showed high level of leaf rolling. However, under stress condition, a range of leaf rolling from 1 to 5 was observed. Genotypes PB2000, 5006, 6255, 4046, PB2008, B3008, 1159, 2009-1, 6011, 6028, 7027, BRC236, 5028, 7056-1, 6010, 6003, 1217, 3013, 4028¸ 6017, 7010, and 3019 were ranked 5 on the basis of leaf rolling strength. Genotypes 7059, 4004, 6001, 7046, 1288 and 1143 showed from no to very slight leaf rolling. Analysis exhibited that genotypes with high level of leaf rolling showed very low survival rate i.e. upto 10%, whereas, genotypes with little or no leaf rolling showed high survival rate upto 50% (data not shown).

Under normal condition out of eight, four PCs had more than one eigen-value and collectively contributed 71.50% of the total variability (Table-3). These four PCs were given due consideration for further interpretation. PC1, PC2, PC3 and PC4 contributed 29.6%, 15.4%, 14.0% and 12.5% of the total variability respectively among the characters under study (Table-3). The PC1 showed that all the traits contributed positively towards variability (29.6%) except leaf rolling (Table-3). However in PC2 leaf rolling, chlorophyll contents (*a* & *b*) and beta carotenoids contributed positively while, survival rate, ascorbic acid, root shoot ratio and root density contributed negatively (Table-3). Ascorbic acid, beta carotenoids and root density reflected positive and increasing effects while rest of the traits showed negative and decreasing effects in PC3. According to results of PC4, leaf rolling, survival rate, ascorbic acid, chlorophyll *a* and beta carotenoids were decreasing and contributed negatively whereas chlorophyll *b*, root shoot ratio and root density were increasing and contributed positively (Table-3).

Under inoculated conditions, out of eight, four PCs had more than one eigen value and cumulatively contributed 75.2% of the total variability. PC1 contributed 27.7% of the total variability while contributions of PC2, PC3 and PC4 were 19%, 16.1% and 12.4%, respectively for all parameters under study. Results of PC1 showed that leaf rolling was not increasing positively whereas all other parameters were positively increasing. In PC2 the contribution of survival rate, ascorbic acid contents and beta carotenoids was positive towards variability and rest of the traits contributed negatively. In case of PC3, the positive contribution was only of survival rate, ascorbic acid contents, root shoot ratio and root density. However in case of PC4 leaf rolling, ascorbic acid and chlorophyll *b* contents contributed positively and the contribution of all other parameters was negative (Table-3).

In biplot graph the lengths of environment/parameter vectors show the discriminating nature of environment/parameter (Yan and tinker, 2006). Longer the vector length greater the parameter is informative about the performance of genotypes in biplot graph. Chlorophyll *a*, chlorophyll *b*, ascorbic acid, root shoot ratio, root density and survival rate % were more discriminating as compared to other parameters in present studies. These discriminating parameters were important for selection of adapted genotypes (Yan and Tinker, 2006). Under normal condition, significant variability was observed among genotypes for the desired characters. Genotypes 1007, 60101, 7008, 405, PB91, B3008, 3013, 7002, 7012, 1217, 6011, BITAL98, 6028 and CH-8 exhibited the highest level of variability for the parameters under study. This variability indicated the wide dispersion of genotypes on biplot graph (Figure-1). Genotypes 1288, 7046, 4004, 6001, 7059, 1017, 504, 6010, 2052, 405, 1143 and 5006-1 performed well and exhibited the highest level of variability under inoculated condition. These genotypes showed wide dispersion from the origin of biplot graph. Genotypes which were concentrated towards the origin the graph had low variability for the studied parameters under stress environment (Figure-2).

Under normal condition, ascorbic acid (AA) was non-significantly and positively correlated with beta-carotenoids (β-C), chlorophyll-*a* (Ch. *a*), root shoot ratio (R/S), root density (RD) and survival rate (SR) and negatively correlated with chlorophyll *b* (Ch. *b*) and leaf rolling (LR). Beta carotenoid (β-C) had positive correlation with Ch. *a*, RD and SR whereas negatively correlated with Ch. *b*, LR, and R/S. Chlorophyll *a* was positively correlated with Ch. *b*, RD and SR. The correlation between chlorophyll *a* and *b* was positive and strongest among all the parameters (0.8414). Chlorophyll a and *b* were negatively correlated with LR and R/S, whereas chlorophyll *b* was positively associated with RD and SR. Leaf rolling showed negative correlation with all the parameters. R/S showed positive association with RD and SR (Table-4).

Under inoculated environment, AA positively and non-significantly correlated with all parameters except β-C and LR. β-C showed negative association with all parameters except leaf rolling (Table-4). Chlorophyll *a* exhibited non-significant and negative association with SR and LR. Chlorophyll *b* positively correlated with R/S and SR while negatively correlated with RD. LR showed negative correlation with all parameters except β-C. R/S exhibited negative correlation with SR, β-C and LR but positively associated with all the parameters. RD had negative association with R/S, β-C, chlorophyll *b* and LR but positive with rest of the parameters.

Table-1. Analysis of variance for various chickpea genotypes under stress conditions

Source	DF	LR	SR	A.A (µgm/ml)	Ch. *a* (mg/100ml)	Ch. *b* (mg/100ml)	ß-C (mg/100ml)	R/S	RD
Treatment(T)	1	432.086**	112374**	318.055**	57.1301**	91.6394**	92.3752**	3.20723**	1.64062**
Genotype (G)	69	3.29317**	1169.72**	7.03929**	1.38297**	2.46396**	129.353**	0.70156**	0.13129**
T*G	69	3.86832**	1090.96**	7.93955**	1.30291**	1.93889**	126.214**	0.76956**	0.11056**
Error	280	0.00082	0.00124	0.00727	0.01306	0.000222	0.000926	0.00486	0.00144
Total	419								

Where * = Significant, ** = Highly Significant and NS= non-significant

Table-3. PCA under normal and stress conditions:
Eigen-analysis of the Correlation Matrix

Eigenvalue	**2.3694,** 2.2141	**1.2344,** 1.5221	**1.1161,** 1.2873	**1.0070,** 1.0037	**0.8478,** 0.7326	**0.7410,** 0.6290	**0.5925,** 0.3472	**0.1017,** 0.2741
Proportion	**0.296,** 0.277	**0.154,** 0.190	**0.140,** 0.161	**0.125,** 0.124	**0.106,** 0.092	**0.093,** 0.079	**0.074,** 0.043	**0.013,** 0.034
Cumulative	**0.296,** 0.277	**0.450,** 0.467	**0.590,** 0.628	**0.715,** 0.752	**0.821,** 0.844	**0.913,** 0.922	**0.987,** 0.966	**1.000,** 1.000

Note: Bold values represent normal treatment and non-bold values represent data of inoculated set.

Variable	PC1	PC2	PC3	PC4	PC5	PC6	PC7	PC8
L.R	-0.434, -0.549	0.179, -0.131	-0.056, -0.201	-0.081, 0.114	-0.175, -0.246	-0.572, -0.184	0.628, -0.632	-0.130, 0.366
S.R (%age)	0.365, 0.346	-0.355, 0.498	-0.237, 0.267	-0.273, -0.033	-0.133, 0.438	0.423, 0.092	0.644 , -0.534	-0.004, 0.274
A.A (µgm/ml)	0.089, 0.323	-0.510, 0.219	0.444, 0.399	-0.103, 0.061	0.642, -0.793	-0.309, -0.177	0.124, 0.008	-0.034, 0.157
Ch. *a* (mg/100ml)	0.556, 0.457	0.227, -0.293	-0.052, -0.448	-0.116, -0.072	-0.060, -0.017	-0.437, 0.121	0.020, 0.158	0.655, 0.678
Ch. *a* (mg/100ml)	0.577, 0.493	0.222, -0.159	-0.143, -0.426	0.163, 0.074	0.066 , -0.117	-0.250, -0.202	-0.014, -0.452	-0.710, -0.535
ß-C (mg/100ml)	0.103, -0.072	0.116, 0.120	0.651, 0.104	-0.561, -0.970	-0.435, -0.106	0.034, -0.113	-0.106, -0.055	-0.190, -0.041
R/S	0.008, 0.118	-0.681, -0.501	-0.221, 0.424	0.051, -0.073	-0.504, 0.288	-0.368 , -0.676	-0.302, -0.002	-0.062, 0.104
R.D.	0.137, 0.063	-0.060, -0.557	0.498, 0.397	0.742, -0.158	-0.298, -0.102	0.087, 0.634	0.269, -0.289	0.099, -0.087

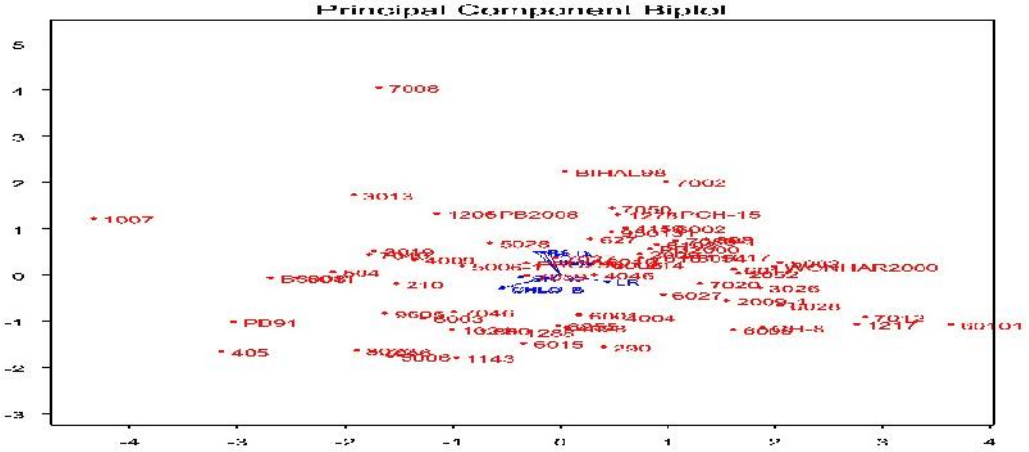

Fig-1: Principle Component Biplot graph of seventy genotypes under normal environment.

Based on the results of leaf rolling scale and biplot analysis it was concluded that genotypes 7059, 4004, 6001, 7046, 1288 and 1143 had tolerance against fusarium wilt whereas, PB2000, 5006, 6255, 4046, PB2008, B3008, 1159, 2009-1, 6011, 6028, 7027, BRC236, 5028, 7056-1, 6010, 6003, 1217, 3013, 4028, 6017, 7010, and 3019 proved as susceptible among all studied chickpea genotypes.

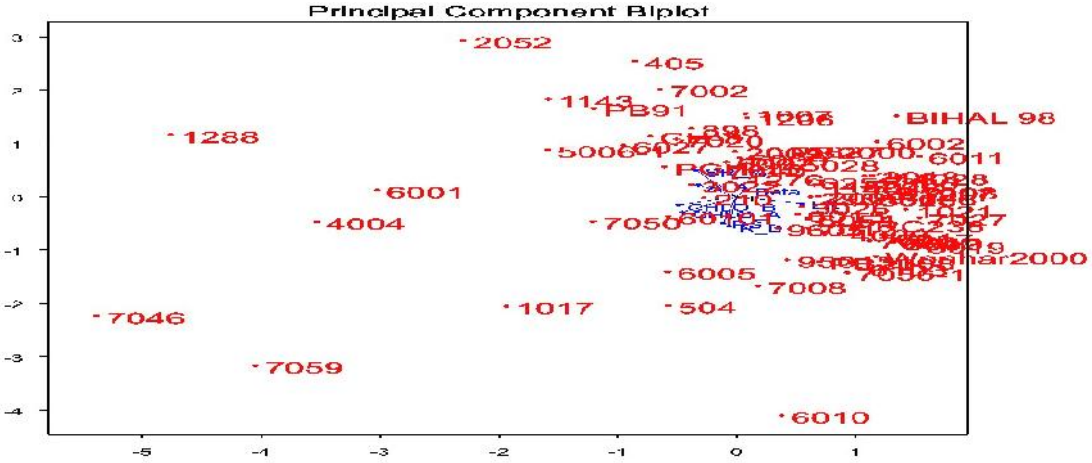

Fig-2: Principle Component Biplot graph of seventy genotypes under stress environment.

Table-4. Correlation coefficients among different studied parameters under normal and stress environments.

ß –C	**0.0769ns,** -0.0508**						
Chl. *A*	**0.0271ns,** 0.0207**	**0.1931**,** -0.0148**					
Chl. *B*	**-0.0112ns,** 0.1520**	**-0.0377ns,** -0.0835ns	**0.8414**,** 0.6710ns				
L.R	**-0.1413*,** -0.3563**	**-0.0625ns,** 0.0199ns	**-0.3152**,** -0.3661*	**-0.4475**,** -0.3589**			
R/S	**0.1041ns,** 0.0424ns	**-0.0878ns,** -0.0718ns	**-0.0364ns,** 0.0681ns	**-0.0855**,** 0.0585ns	**-0.0297*,** -0.1242ns		
R.D	**0.0753ns,** 0.0380*	**0.0647ns,** -0.0425ns	**0.0451ns,** 0.1130ns	**0.1697ns,** -0.0390ns	**-0.1393ns,** -0.0867ns	**0.0223ns,** 0.3760ns	
S.R	**0.0887ns,** 0.2939ns	**0.0383ns,** -0.0017**	**0.3032**,** -0.0010ns	**0.3036**,** 0.1026ns	**-0.3362**,** -0.5377ns	**0.1759ns,** -0.0801ns	**-0.0255**,** -0.1810**
	A.A	**ß-C**	**Chl. *a***	**Chl. *B***	**L. R**	**R/ S**	**R. D**

Note: Bold values represent correlation under normal condition while non-bold values represent data under stress condition.

DISCUSSION

Multivariate analysis such as clustering, metroglyph and principle component analysis (PCA) are used to determine the genetic variability for various traits. Many researchers evaluated chickpea germplasm by following PCA to search out resistance against different types of abiotic stresses. Hasan *et al*. (2007) evaluated 11 chickpea genotypes to find suitable genotypes for Isparta, Turkey local climatic conditions using principle component analysis. Naseer *et al.* (2011) assessed the genetic diversity among the Iranian north-western chickpea genotypes with the help of RAPD marker. Nazari and Pakniyat (2010) used biplot analyses for the assessment of tolerance against abiotic stress in barley genotypes.

Correlation coefficient helps the researcher to set different standards for selection and use best of them. Farshadfar and Farshadfar (2008) analyzed the data of chickpea by using correlation coefficient analysis.

Results showed that inoculum application favored the disease proliferation and enabled us to study the differential responses of the genotypes under prevalence of fusarium wilt. Environmental conditions and concentration of inoculum was same for all genotypes so, different responses were due to differences in their genetic makeup. Evaluation for leaf rolling based on devised scale; revealed that great variation was present

in chickpea germplasm for leaf rolling. These findings are also in agreement with previous findings which declared leaf drooping as primary indicator for disease prevalence (Haware, and Nene, 1982; Jiménez-Díaz *et al.*, 1993; Navas-Cortes *et al*., 2000). Leaf rolling results in reduced leaf surface area exposed to sunlight and impaired photosynthetic activity. Leaf rolling is the result of closure of stomata due to loss of turgor which ultimately affect gaseous exchange.

Variation for survival rate was studied on percentage basis. Higher survival rate was corroborated with lower mortality and lower survival rate with higher mortality. Mortality effects have also been revealed by previous experiments and they remarked that drooping and chlorosis lead towards plant mortality (Haware, and Nene, 1982; Jiménez-Díaz *et al.*, 1993; Navas-Cortes *et al*., 2000).

Chlorophyll contents showed variability among genotypes under fusarium prevalence. Tolerant genotypes were able to retain the higher chlorophyll and beta-carotenoid contents than susceptible genotypes. Reason for higher chlorophyll and beta-carotenoid contents might be due to strong defense mechanism of intended genotypes. It was previously reported that chlorophyll and beta-carotenoid contents were reduced by *Fusarium oxysporum* in Pea plant (Siddiqui *et al*, 1999). Reduced chlorophyll contents are responsible for chlorosis and impaired photosynthetic activity followed by yield reduction.

Root vascular tissues were adversely affected by *Fusarium oxysporum* up to blockage (Harveson, 2011), so reduced water uptake followed by loss of leaf turgor and increased leaf rolling. Leaf death and even death of the whole plant might be due to sever leaf rolling (Singh *et al*. 2007). It was reported that resistance against fusarium wilt was conferred by recessive gene and to get complete resistance, susceptible parents are needed to be used in breeding program (Kumar and Haware, 1982). It can be inferred from this statement that resistant genotypes in present studies are homozygous recessive for that particular locus.

Interaction between plant and pathogen results in the regulation of expression of genes related to either defense or pathogenicity. Relationship of these genes is responsible for either development of disease or resistance in plants. Defense mechanisms are backboned by array of genes which confer resistance either in oligogenic or polygenic form (Giri *et al*, 1998; Gurjar *et al*, 2012). In present study it was observed that there were differential responses of chickpea genotypes for fusarium wilt. Differential tolerance responses of chickpea genotypes might be due to differences in their defense mechanisms. It was reported that proteases, glucanases and chitinases were involved in defense of chickpea against *fusarium oxysporum.* It can be perceived that tolerant genotypes of present study are bestowed with these enzymatic defenders whereas; susceptible genotypes are lacking these enzymes. There are also several genes for causing pathogenicity which are regulated by certain regulators and have host specificity (Gurjar *et al*, 2012).

Conclusion: Leaf rolling, survival rate, chlorophyll and carotenoid contents, and root traits are suitable for selection of resistant genotypes against fusarium wilt. Pathogen effects reflected through all the studied traits of chickpea and genotypic responses were different which depicted the different liabilities of defense mechanisms. Genotypes 7059, 4004, 6001, 7046, 1288 and 1143 proved resistant against fusarium wilt whereas, PB2000, 5006, 6255, 4046, PB2008, B3008, 1159, 2009-1, 6011, 6028, 7027, BRC236, 5028, 7056-1, 6010, 6003, 1217, 3013, 4028¸ 6017, 7010, and 3019 proved as susceptible among all the chickpea genotypes. Resistant genotypes could be grown as such. Susceptible genotypes can be used as parent in breeding program for development of resistance because resistance is controlled by recessive genes.

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