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Screening and biochemical analysis for Fusarium wilt resistance in chickpea (*Cicer arietinum* L.)

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Abstract
Chickpea (*Cicer arietinum*) is third most important grain legume crop grown in the arid and semi-arid regions of the world. *Fusarium* wilt (*Fusirium oxysporum* f. sp. *ciceri*) is a major yield limiting factor in chickpea. Screening of chickpea parents (ICC 506 EB and Vijay), 196 RIL's, F_2 and BC_1F_1 populations for *fusarium* wilt resistance were done by Pot culture method and wilt sick plot condition at pulses research unit, Dr. PDKV, Akola. JG-62 a higly succeptible genotype was used as a check. The susceptible parent ICC 506 EB, showed 83.33 percent wilting, whereas Vijay was resistant till maturity. F_2 progenies segregated in a ratio of 3 susceptible and 1 resistant. The RILs closely fit a 1:1 segregation ratio for resistance and susceptibility and the BC_1F_1 segregated in 1:1 ratio for resistance and susceptibility indicating that resistance to *fusarium* wilt was monogenic with the recessive allele conferring resistance to *fusarium* wilt in this population.
Biochemical studies are carried out on parents Vijay and ICC 506 EB and check variety JG-62 of chickpea in relation to its resistance to *fusarium* wilt caused by *Fusarium oxysporum.* The polyphenol oxidase activity and peroxidase activity was found increased after inoculation with the fungus. This increase was more in resistant genotype as compared to the susceptible genotypes.

Keywords: RIL's, F_2, *fusarium* wilt, BC_1F_1, peroxidase, polyphenol oxidase

Introduction
Chickpea (*Cicer arietinum*) is one of the important grain legume crop of India which plays an important role in food security and balanced diet. It is mainly used for human consumption, and is an essential constituent of the Mediterranean diet and a basic food in India and Pakistan. It serves as an important source of protein in human diet and plays an important role in the enrichment of soil fertility. Chickpea seeds containing 20–30% protein, about 40% carbohydrates, 3–6% oil, 6% crude fiber and 3% ash (Gil *et al*., 1996) [6]. Chickpea is a good source of essential amino acids such as tryptophan and lysine (Awasthi *et al*., 1991) [3]. Chickpea holds prestigious position among all legume crops because it plays an important role in food security and balanced diet. It is virtually an indispensable item in the kitchen and is considered as "king of pulses" (Bhatt and Patel, 2001) [5]. Two main types of chickpea cultivars are grown globally- kabuli and desi, representing two diverse gene pools (Pundir *et al*., 1985) [15].
Fusarium wilt is a soilborne disease that causes severe yield losses. The pathogen is both seed and soil borne, survives in the soil for more than six years in the absence of susceptible host plants (Haware *et al*., 1986) [8]. Eight physiological races of the pathogen (race 0, 1A, 1B/C, 2, 3, 4, 5 and 6) have been identified by reaction on set of differential chickpea cultivars (Jimenez-Diaz *et al.*, 1989) [9]. Races 0 and 1B/C induce yellowing symptoms, whereas remaining races inducing wilting. Among the biotic stresses that affect chickpea (*Cicer arietinum*), *Fusarium* wilt (*Fusirium oxysporum*) is a major yield-limiting factor.

Materials and Methods
The experimental chickpea seed material for the present investigation comprised of a mapping population in the form of 196 recombinant inbred lines (RILs) derived from a cross between Vijay (resistant to *fusarium* wilt) X ICC 506 EB (susceptible to *fusarium* wilt). The experimental material was kindly provided by Dr. H. C. Sharma, Principle Scientist, and Entomology from ICRISAT.

Collection of diseased samples
Chickpea wilt infected samples were collected from the field of Pulses Research Unit, Dr. Panjabrao Deshmukh Krishi Vidyapeeth, Akola, and Maharashtra.

The samples were collected during chickpea growing season in the year 2012-2013.

Isolation of *Fusarium oxysporum* f. sp. *ciceri* from diseased part

The plants showing typical wilt symptoms were collected from different locations of Maharashtra during *rabi* season. The plants were gently uprooted, the root system was washed to remove the adhesive soil and the samples were kept 24 hours in laboratory. The infected root preferably the tap root and secondary root were used for isolation of pathogen.

Purification of *Fusarium oxysporum* f. sp. *ciceri*

In order to obtain the pure cultures of *Fusarium oxysporum* f. sp. *ciceri* single spore culture technique was used with some modification under aseptic condition. The spore suspension of the respective isolates was prepared in sterilized distilled water so as to obtain 10-12 spores per microscopic field (10x). The suspension was spread over the surface of sterilized 2 per cent plain agar medium in Petri dishes and incubated at 27 ± 2 °C. After six hours, the single germinating spore was observed under low power objective and cut through dummy objective. Such pieces containing germinating spores were transferred separately on potato dextrose agar slants with the help of an inoculating needle and incubated at 27 ± 2 °C for seven days. These cultures were observed under microscope and the stock cultures were kept in refrigerator for further studies.

Identification of isolated pathogen

The identification of *Fusarium oxysporum* f. sp. *ciceri* isolates was done on basis of morphological characters described by Booth, 1977 [6].

Preparation of mass inoculums

Purified cultures of six isolates of *Fusarium oxysporum* f. sp. *ciceri* were mass multiplied separately on sorghum sand medium (1 part partially broken sorghum grain + 3 part sand + distilled water to moisten the media). The media was prepared by mixing broken sorghum grains with clean sand in plastic tub followed by moistening with distilled water. About 500 g mixture was transferred in 2000 ml Erlenmayer flask plugged using nonabsorbent cotton and sterilized in an autoclave at 15 p.s.i. for 30 minutes consequently for two days. It was allowed to cool and the flasks containing the sterilized media were inoculated with mycelial disc of pure culture of *F. oxysporum* f. sp. *ciceri* (5 mm diameter) and incubated at 27± 2 ^{0}C for 15 days. Sufficient quantity of inoculum was prepared and used for preparing sick pots required in pot experiments.

Preparation of sick soil

Soil was collected in gunny bags and sterilized in autoclave at 1.05 kg/cm^2 for one hour consequently for three days. Sand was added to the soil to facilitate proper drainage and aeration in pots. Finally the mass multiplied fungus inoculum was added in 1: 10 proportion to soil and thoroughly mixed, thus the soil was made sick.

Pathogenicity test

Plastic pots of size 10 cm diameter were taken and surface sterilized with 0.1% $HgCl_2$. The sick soil was filled in sterilized pots 1/4th of its capacity. The pots were given water lightly and incubated for 4 days. Five seeds of susceptible chickpea cultivar JG-62 were surface disinfected with 4% sodium hypochlorite solution for 30 seconds and sown in pots each isolate in 3 replications. The seedlings maintained in sterilized soil without inoculums were served as control. Plants were observed periodically upto 30 days after sowing (DAS) for wilt symptoms and disease incidence (%) and total mortality was calculated. Different isolates of *F. oxysporum* were tested by sick soil method for their virulence on susceptible variety JG-62. The percent wilting was recorded on the basis of healthy and wilted plants. Wilt incidence was calculated by using formula,

The isolates of *Fusarium oxysporum* f. sp. *ciceri* were tentatively divided into three groups on the basis of virulence as Nonpathogenic isolates (0-10 percent), Moderately pathogenic isolates(10.1-30 percent), Highly pathogenic isolates(>30 percent).

Screening of chickpea parents, RIL's, F_2 and BC_1F_1 populations by Pot culture method

Screening of chickpea parents (ICC-506 and Vijay), 196 RIL's (Obtained from ICRISAT, Hyderabad), F_2 and BC_1F_1 populations for wilt resistance were done by Pot culture method in green house. Plastic pots of size 10 cm diameter were taken and surface sterilized with 0.1% $HgCl_2$. The sick soil was filled in sterilized pots upto 1/4th of its capacity. The pots were watered lightly and incubated for 4 days. Chickpea seeds of parents (ICC 506 and Vijay) and 196 RIL's of susceptible chickpea cultivar JG-62 were surface disinfected with 4% sodium hypochlorite solution for 30 seconds and sown in pots in 3 replications (10 seeds per pot). The seedlings maintained in sterilized soil without inoculums were served as control. Plants were observed periodically upto 30 days after sowing (DAS) for wilt symptoms and disease incidence (percent) and total mortality was calculated. Reactions were graded as resistant (0-10 percent wilt), moderately resistant (10.1 to 30 percent wilt) and susceptible (> 30 percent wilt) (Anonymous, 2016) [1].

Screening of chickpea genotypes in Field

Chickpea parents (ICC 506 and Vijay), 196 RIL's (Obtained from ICRISAT, Hyderabad), F_2 and BC_1F_1 populations were screened in wilt sick plot condition at Pulses Research Unit, Dr. PDKV, Akola. A field screening technique for wilt screening developed at ICRISAT was adopted in the present studies (Nene *et al*., 1980). In this screening technique a wilt susceptible check (JG-62) was sown intermittently after every five test entries so as to monitor the disease pressure. Sowing of chickpea germplasm was completed in November, 2012 with two replications of row length 3 m at 30x10 cm spacing. The seed emergence was recorded 18 days after sowing. Observation on number of plants wilted was recorded at 30 days and 60 days after sowing. The percent wilt incidence was calculated on the basis of initial plant count and total number of wilted plants in each genotype and graded as follows (Anonymous, 2016) [1].

Crossing of selected genotypes

Crossing chickpea is tedious and time consuming and a crossed pod generally produces only one seed. Emasculation is required for artificial hybridization in chickpea. The crossing programme was carried out at experimental field, Pulses Research Unit, Dr. Panjabrao Deshmukh Krishi Vidyapeeth, Akola during rabi 2012-2013 to 2014-2015. The crosses were made during rabi 2012-2013, to obtain first filial (F_1) generation. The F_1 was grown to produce F_2 population. The F_1's were crossed with above female to produce BC_1F_1

backcross populations during rabi 2013-2014. All these populations viz., P1, P2, F1, $BC1F_1$ were sown in rabi 2014-2015.

Biochemical analysis

Biochemical constituents such as peroxidase and polyphenol oxidase from different genotypes were estimated from roots at different disease rating healthy and diseased samples of roots of chickpea plants which were collected from the experimental greenhouse of Department of Botany, biotechnology centre Dr. PDKV, Akola and sick plot at Pulse Research Unit, Akola All the samples were dried in shade for 48 hrs and then oven dried at 45 °C for 12 hrs. Dried samples were finely powdered using mortar and pestle and preserved in refrigerator at 4 °C for further studies.

Quantitative estimation of enzymes Peroxidase

About one gm of sample was taken and crushed in 7ml phosphate buffer saline and the suspension was centrifuged at 8000 rpm for 20 min at 4 °C. The supernatant was used for analysis of peroxidiase. The reaction mixture contain 4 ml of 0.2 M phosphate buffer, 0.1 ml of guaiacol, 1 ml of H_2O_2 and 500 µg enzymes into a test tube. Blank was prepared in the same manner excluding H_2O_2 but add water to make volume and incubate at 30 °C. Finally the absorbance was measured at 470 nm after 15 and 90 sec. of enzyme addition (Retig, 1974).

Estimation of Polyphenol oxidase

About one gm of sample was taken and crushed in 7 ml phosphate buffer saline and the suspension was centrifuged at 8000 rpm for 20 min. at 4 °C. The supernatant was used for analysis of polyphenol oxidase was taken 200 µl of enzymes and 1.5 ml of 0.1 M sodium phosphate buffer in a test tube. Initiate the reaction by adding 200 µl of 0.01 M catechol. Measure the rate of increase in absorbance at 495 nm against blank (prepared in absence of enzyme) at every 30 s interval up to 3 min. Plot the changes in the absorbance between 30 to 180 seconds of incubation and calculate the enzyme activity from linear part of the curve (Mayer *et al.*, 1965) [11].

Results and Discussion

Chickpea wilt infected samples were collected from the field of Pulses Research Unit, Dr. Panjabrao Deshmukh Krishi Vidyapeeth, Akola, Maharashtra during chickpea growing season in the year 2012-2013. The tissue isolation method was used for isolation of *Fusarium oxysporum* f. sp. *ciceri* from infected plants showing typical wilt symptoms. The pure culture thus obtained was identified as *Fusarium oxysporum* f. sp. *ciceri* on the basis of morphological characters reported by Booth (1977) [6] (Plate 1).

Plate 1: Isolation and morphology of Fusarium oxysporum f. sp. ciceri

The Pathogenicity test of isolates of *F. oxysporum* f. sp. *ciceri* isolated was tested by using susceptible cultivar JG-62. The samples of *Fusarium oxysporum* f. sp. *ciceri*, proved to be pathogenic to susceptible cultivar JG-62 (64.28%). The isolate from Pulses Research Unit, Dr. Panjabrao Deshmukh Krishi Vidyapeeth, Akola, Maharashtra were used for further screnning of parents and RIL population (Table 1).

Table 1: Pathogenicity test of *Fusarium oxysporum* f.sp. *Cicero* against susceptible variety JG-62

Sr. No.	Place	Total plants	Germina-tion	Wilted plants	% Wilting
1	Akola	15	14	9	64.28
2	Control	15	13	00	00

Screening of chickpea parents (ICC 506 EB and Vijay) and 196 RIL's (Obtained from ICRISAT, Hyderabad) for wilt resistance were done by Pot culture method (Fig.1) in green house. JG-62 a highly susceptible genotype was used as a check. Among the 196 RILs, 22 RILs were resistant, 55 RILs were moderately resistant and 119 were susceptible. The RILs also segregated in 1:1 ratio for resistance and susceptibility, indicating that resistance to *fusarium* wilt was monogenic in this population. The experiment was analysed statistically to confirm the significance of the findings. The details of the experiment are given in Table 2. (Plate 2, 3).

Table 2: Screening of Parents and RIL populations in wilt sick pot

% Mean wilt incidence	
Mean of RIL's	38.60 (37.93)*
Vijay	10 (18.43)
ICC 506 EB	83.33 (66.14)
JG-62 (check)	90 (90.00)
SE ±	3.62
C. D. @ 5%	10.08

* - transformed values

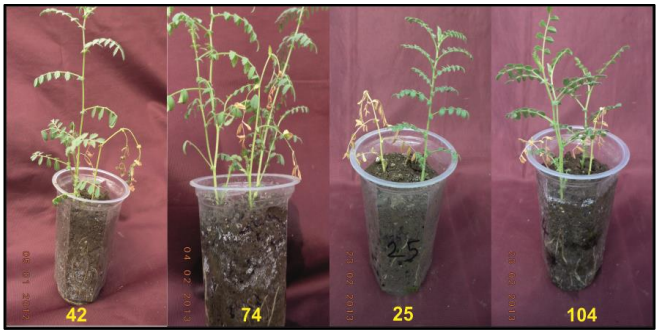

Plate 2: Screening of chickpea by Pot culture method

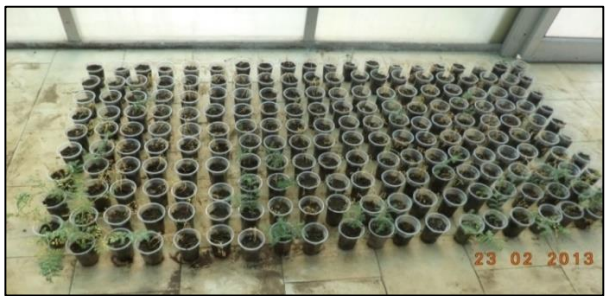

Plate 3: Screening of RIL's against *fusarium* wilt by Pot culture method

Chickpea parents (ICC 506 EB and Vijay) and 196 RIL's (Obtained from ICRISAT, Hyderabad) were screened in wilt sick plot condition at Pulses Research Unit, Dr. PDKV, Akola. A field screening technique for wilt screening developed at ICRISAT was adopted in the present studies (Nene *et. al*., 1980). The susceptible parent ICC 506 EB, showed 80% wilting in 30 to 60 days after sowing, whereas Vijay was resistant with 6.66% wilting and develop very few wilting symptoms till maturity. The check genotype, completely wilted (90%) in 30 days after sowing (Plate 4).

Plant 4: Field views of wilt sick plot at pulses Research until, Dr. P.D.K.V. Akola

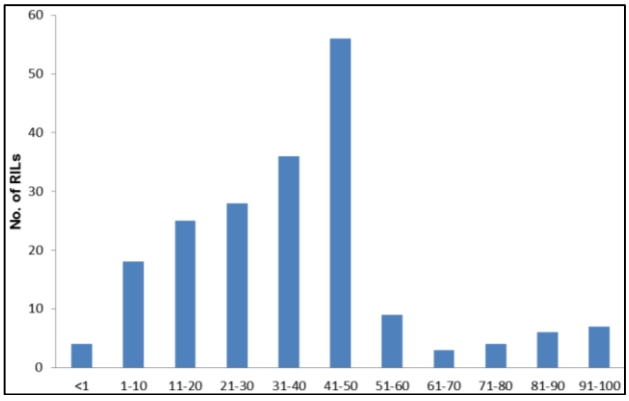

Fig 1: Frequency distribution of disease scores for Fusarium wilt under Pot conditions; RILs

Table 3: Screening of RIL populations in wilt sick plot

	% Mean wilt incidence
RIL's	28.83 (30.79)*
Vijay	6.66 (12.28)
ICC 506 EB	80.00 (63.43)
JG-62 (check)	90.00 (71.56)
SE ±	2.79
C. D. @ 5%	7.78

* - transformed values

Among the 196 RILs, 22 RILs were resistant, 55 RILs were moderately resistant and 119 were susceptible (Table 3, Fig.2). The susceptible RIL's took 25 to 30 days for wilting. Wilting symptoms also observed at flowering and podding stage. The experiment was analysed statistically to confirm the significance of the findings.

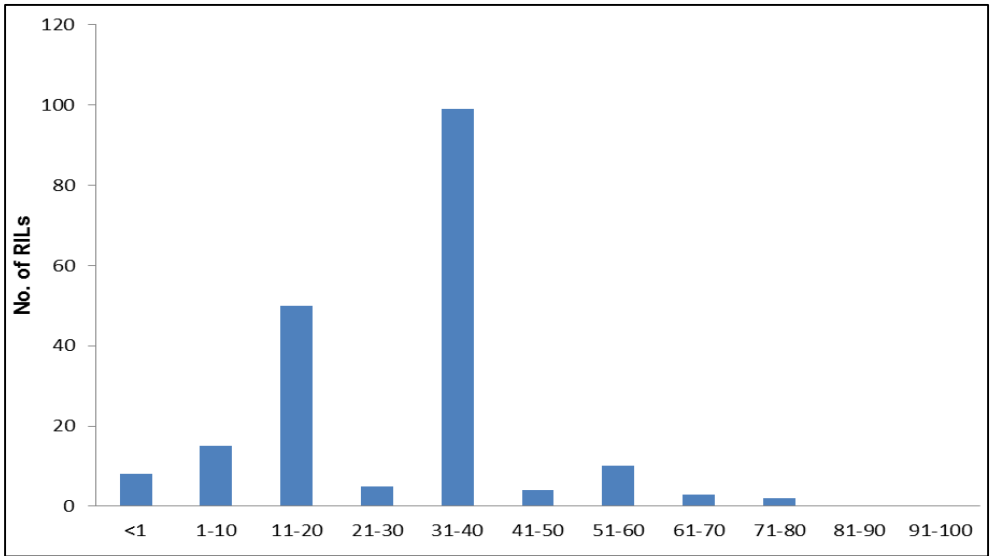

Fig 2: Frequency distribution of disease scores for Fusarium wilt under field conditions; RILs

The crossing programme was carried out at experimental field, Pulses Research Unit, Dr. Panjabrao Deshmukh Krishi Vidyapeeth, Akola. Total 310 flowers were pollinated to obtaine F_1 and 260 flowers were pollinated to obtaine BC_1F_1. Percent pod set observed for F_1 was 20.64% and for BC_1F_1, % pod set was 18.64% (Table 4).

Table 4: Observations for cross ICC-506 × Vijay

Crosses	ICC 506 EB × Vijay (F_1)	F_1 × ICC 506 EB (BC_1F_1)
No. of Pollinations	310	260
No. of Pod set	64	48
No. of seed set	69	52
% Pod set	20.64	18.64

Screening of parents (Vijay and ICC 506 EB), BC_1F_1 and F_2 generations for wilt resistance were done by Pot culture method in green house. JG-62 a highly susceptible genotype was used as a check. Among the 51 BC_1F_1 26 plants were resistant and 25 were susceptible. The susceptible parent ICC 506 EB, showed 83.33 percent wilting in 30 days after sowing, whereas Vijay was resistant till maturity. Among the 136 F_2, 107 were found resistant and 29 were susceptible. The BC_1F_1 segregated in 1:1 ratio for resistance and susceptibility and F_2 progenies segregated in a ratio of 3 susceptible and 1 resistant. The RILs also closely fit a 1:1 segregation ratio for resistance and susceptibility indicating that resistance to *fusarium* wilt was monogenic in this population. The data revealed segregation of a single gene with the recessive allele conferring resistance to *fusarium* wilt (Table 5).

Table 5: Inheritance of wilt resistance in a cross ICC-506 x Vijay

	Total plants	Wilted plants	Non wilted plants	Expected ratio	*df*	x^2	P-value
RIL's	196	119	77	1:1	1	9.433	0.0021
F_2	136	107	29	3:1	1	0.1985	0.655
BC_1F_1 (F_1× ICC 506 EB)	51	25	26	1:1	1	0.078	0.780

df= 1; P=0.05; x^2=3.841

From the table 6. it was observed that among all the germplasam of chickpea, the polyphenol oxidase activity at flowering stage was highest in Vijay at inoculated treatment (3.91 U/min/gm). The lowest activity of polyphenol oxidase was found in JG-62 (1.54 U/min/gm). At flowering stage activity of polyphenol oxidase was increased upto 40.14 percent in Vijay (Fig. 3).

Table 6: Polyphenol oxidase activity (U/min/mg)

Sr. No.	Variety	Treatment	At flowering stage		At podding stage		Reaction score
			Content	% increase/ Decrease	Content	%increase/ Decrease	
1	Vijay	Control	2.79	40.14	3.51	69.80	R
		Inoculated	3.91		5.96		
2	ICC 506 EB	Control	3.11	22.8	3.56	35.95	S

		Inoculated	3.80		4.84		
3	JG-62	Control	1.54	5.84	2.57	9.33	S
		Inoculated	1.63		2.81		

At podding stage, in control treatment the polyphenol oxidase activity was lowest in JG-62 in control (2.57 U/min/mg). In the same stage this enzyme showed highest polyphenol oxidase activity (5.96 U/min/mg) in Vijay. In pathogen inoculated sample the activity of enzyme was minimum (2.81 U/min/mg) in JG-62 and maximum in Vijay which was 5.96 U/min/mg. This activity was increased upto 69.80 percent in Vijay variety during podding stage.

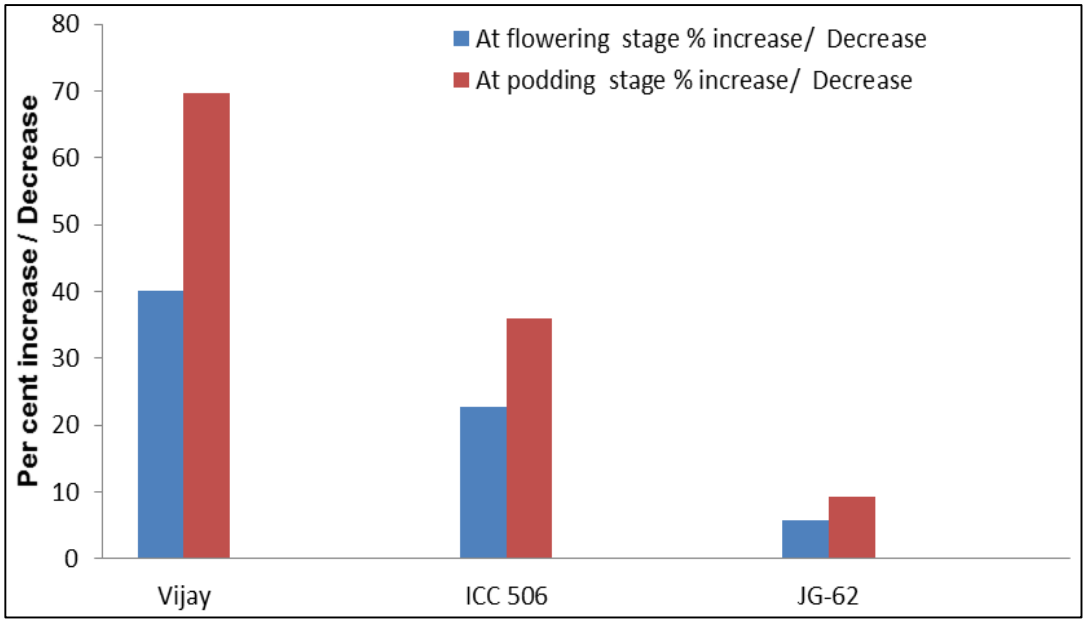

Fig 3: Polyphenol oxidase activity (U/min/mg) at flowering and podding stage

The polyphenol oxidase activity was found increased after inoculation with the fungus and showed similar pattern as total phenols with more increase after inoculation in case of resistant than susceptible. Similar trend were reported for different crops and pathogenic organisms by various scientist such as Matta and Daibong (1963) [10], Patel *et al*. (2001) [14] and Parmeshwaran (2004).

Table No.7. depicits the values about the activity of enzyme peroxidase at flowering and podding stage. The peroxidase activity at flowering stage was found maximum in Vijay (6.26 U/min/mg) when inoculated with pathogen inoculum and minimum activity was found in JG-62 which was 1.34 U/min/mg in control treatment. The 27.23 percent increase in activity was found in Vijay (Fig. 4).

Table 7: Peroxidase activity (U/min/mg) at flowering and podding stage and wilt reaction

Sr. No.	Variety	Treatment	At flowering stage		At podding stage		Reaction score
			Content	% increase/Decrease	Content	%increase/Decrease	
1	Vijay	Control	4.92	27.23	5.79	34.36	R
		Inoculated	6.26		7.77		
2	ICC 506 EB	Control	4.78	6.90	6.86	10.93	S
		Inoculated	5.11		7.61		
3	JG-62	Control	1.34	5.97	2.68	8.20	S
		Inoculated	2.02		2.89		

At podding stage the JG-62 showed lowest peroxidase activity at both control (2.68 U/min/mg) and inoculated (2.89 U/min/mg) treatment. The highest activity was observed in Vijay which was 5.79 U/min/mg at control and at inoculated treatment (7.7 U/min/gm). The maximum increase in percentage was observed in Vijay germplasm which was 34.36 percent.

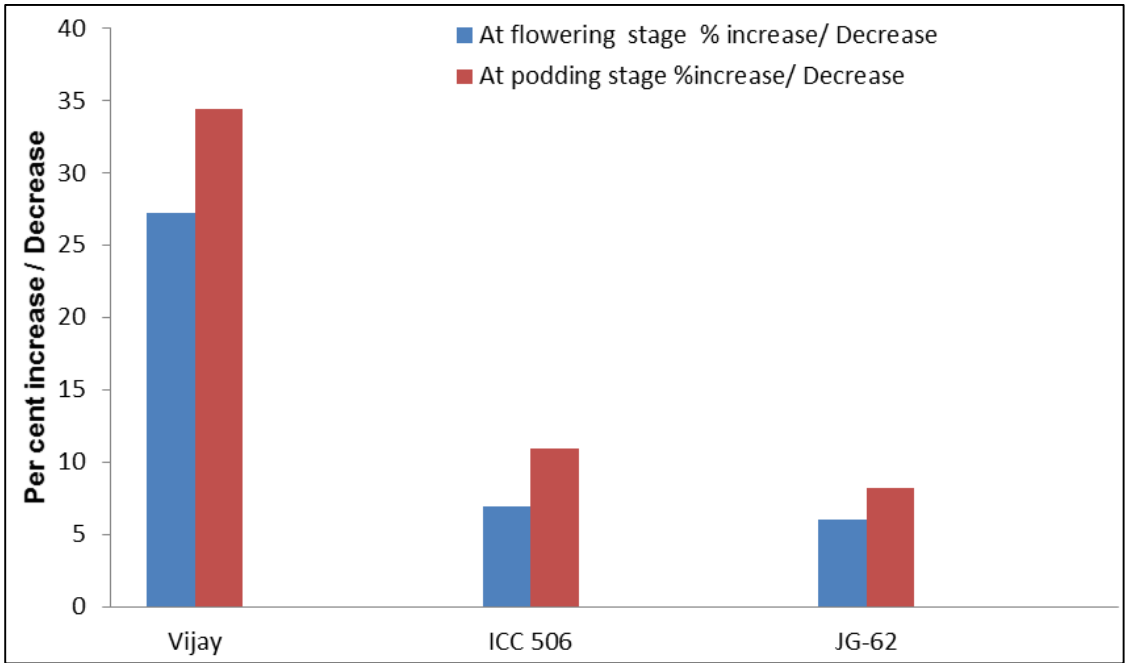

Fig 5: Peroxidase activity (U/min/mg) at flowering and podding stage

Conclusions

The polyphenol oxidase activity was found increased after inoculation with the fungus and showed similar pattern as total phenols with more increase after inoculation in case of resistant than susceptible. Similar trend were reported for different crops and pathogenic organisms by various scientist such as Matta and Daibong (1963) [10], Patel *et al*. (2001) [14] and Parmeshwaran (2004). Overall increase in peroxidase activity was observed in all the cases after inoculation and this increase was found more in case of resistant genotypes as reported previously by Bashan (1987) [4] and Ashraf *et al*. (2005) [2].

The BC_1F_1 segregated in 1:1 ratio for resistance and susceptibility and F_2 progenies segregated in a ratio of 3 susceptible and 1 resistant. The RILs also segregated in 1:1 ratio for resistance and susceptibility, indicating that resistance to *fusarium* wilt was monogenic in this population The wilt screening data from RIL's, BC_1F_1 and F_2 revealed segregation of a single gene with the recessive allele conferring resistance to *fusarium* wilt.

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