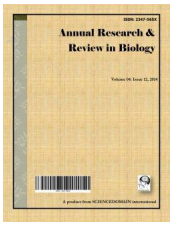

Annual Research & Review in Biology

12(5): 1-9, 2017; Article no.ARRB.32888
ISSN: 2347-565X, NLM ID: 101632869

Antioxidative Enzyme Responses against *Fusarium* wilt (*Fusarium oxysporum* f. sp. *ciceris*) in Chickpea Genotypes

U. S. Dalvi[1*], R. M. Naik[1] and A. A. Kale[1]

[1]Department of Biochemistry, Mahatma Phule Krishi Vidyapeeth, Rahuri 413 722, India.

Authors' contributions

This work was carried out in collaboration between all authors. Author RMN designed the study, Author USD managed analyses of the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Author AAK managed the literature searches. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/ARRB/2017/32888
Editor(s):
(1) Paola Angelini, Department of Applied Biology, University of Perugia, Perugia, Italy.
(2) George Perry, Dean and Professor of Biology, University of Texas at San Antonio, USA.
Reviewers:
(1) Vishal Chugh, College of Horticulture, Banda University of Agriculture and Technology, Punjab Agricultural University, India.
(2) Montaser Fawzy Abdel-Monaim, Plant Pathology Research Institute, Agricultural Research Center, Giza, Egypt.
Complete Peer review History: http://www.sciencedomain.org/review-history/19045

Original Research Article

Received 21st March 2017
Accepted 2nd May 2017
Published 13th May 2017

ABSTRACT

Three wilt resistant chickpea genotypes *viz*., JG 24, BCP-2010-1 and GJG 0919 confirmed by field screening and also by activity profile of defense related enzymes were screened in normal and wilt sick soil for antioxidative enzymes at pre and post infection stages along with four wilt resistant and three wilt susceptible checks. The evaluation of antioxidative enzyme profile in normal and wilt sick soil of these chickpea genotypes exhibited a differential response. The activity of three antioxidative enzymes *viz*., APX (ascorbate peroxidase), guaiacol peroxidase (GPX) and superoxide dismutase (SOD) increased significantly in wilt susceptible checks *viz*., JG 62, Vikas and at post infection stage. The non compatible interaction between wilt resistant genotype and pathogen demonstrated lesser increase. At preinfection stage mean APX activity did not vary significantly from normal soil to wilt sick soil, however significant increase was observed at post infection stage with 1.08 µmoles ascorbate oxidized mg^{-1} protein min^{-1} in normal soil to 1.75 µmoles ascorbate oxidized mg^{-1} protein min^{-1} in wilt sick soil. At the post infection stage the root APX activity increased significantly in wilt

**Corresponding author: E-mail: usdalvi2008@gmail.com;*

susceptible group of genotypes from 2.07 to 2.38 µmoles ascorbate oxidized mg^{-1} protein min^{-1} with mean percent increase of 97.94%. Though APX activity increased at post infection stage in wilt sick soil in the wilt resistant genotypes percent increase was from 33.03 to 57.64 with a mean percent increase of 40.4%. Same trend was recorded in GPX and SOD activity.

Keywords: Antioxidative enzymes; chickpea; genotype; post infection stage; wilt.

1. INTRODUCTION

Chickpea (*Cicer arietinum* L.) is a self pollinated leguminous crop, diploid (2n=16) grown since 7000 BC in different area of the world but its cultivation is mainly concentrated in semiarid environments [1]. Several diseases are known to limit worldwide production of chickpeas, of which *Fusarium oxysporum* f. sp. *ciceris* (*Fusarium* wilt) is one of the most important. Management of *Fusarium* wilt has been primarily through development of resistant cultivars as part of an integrated management approach. However, the high pathogenic variability in populations of *F. oxysporum* f. sp. *ciceris* presents problems for sustainability of resistant cultivars. Two pathotypes and eight races of the pathogen have been identified. The reliance on resistant cultivars for disease management of *Fusarium* wilt therefore places significant importance on the confident and efficient identification of pathogenic races of *F. oxysporum* f. sp. *ciceris*.

Amongst these wilt of chickpea caused by *F. oxysporum f.* sp. *ciceris* is a major limiting factor in the Indian subcontinent and causes 10 to 15% annual yield losses [2], but the disease can completely destroy the crop under specific conditions. Previous studies have demonstrated that, in *Fusarium* wilt of chickpea, roots are most susceptible to inoculation. The most common site of penetration of the fungus is at or near the root tip. In initially attacked cell(s), rapid responses may ultimately lead to cell death within few hours of pathogen contact [3]. This host cell death has been classically termed as hypersensitive response (HR). The HR has been proposed to play a causal role in disease resistance against bacterial and fungal pathogens [4].

One of the earliest observable responses of plant cells in many incompatible interactions is oxidative burst. Oxidative burst is generally defined as rapidly stimulated production of reactive O_2 species (ROS) including superoxide anion (O_2), hydroxyl radical (OH) and hydrogen peroxide (H_2O_2). Doke and colleagues [5] were the first to report that superoxide anions were produced in incompatible interactions, initially between potato and *Phytophthora infestans* and then between tobacco and tobacco mosaic virus. Later it was recognized that oxidative burst is employed in many plant microbe interactions [6]. The production of reactive oxygen species (ROS) is the first response detected within minutes of an attack by virulent or avirulent pathogen [7]. Weak and transient ROS generation is due to a biologically non-specific reaction. After some hours, a massive and prolonged ROS production, called oxidative burst, occurs in cells attacked by avirulent pathogens. This two-phase kinetics of ROS production is typical of incompatible plant–pathogen interactions that are characterized by HR [8]. The present research work has therefore been undertaken to study the interaction between antioxidative enzymes in relation to wilt disease in chickpea.

2. MATERIALS AND METHODS

2.1 Growth Conditions and Plant Material for Analysis

Ten chickpea genotypes *viz*., wilt resistant [7] namely GJG 0919, BCP-2010-1, JG 24,Vijay, Digvijay, WR 315, ICC 4958 and wilt susceptible [3] namely JG 62, SAKI 9516 and Vikas were grown in normal and wilt sick soil contains inoculum load of $2x10^7$ cfu /g soil in pots in triplicate. After proper and uniform germination the root tissues of these genotypes were evaluated for activity of antioxidative enzymes both at preinfection and post infection stages of growth.

2.2 Enzyme Extraction and Activity Assays

Antioxidative enzymes such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) and guaiacol peroxidase (GPX) were extracted from leaf tissue by using the method of Costa et al. [9]. For assays of SOD, CAT, APX and GPX, 200 mg cleaned root samples were homogenized in a chilled mortar and pestle with 2 ml of an ice-cold 0.1 mM potassium phosphate buffer (pH 7.5) containing 1 mM EDTA, 1 mM PMSF and 5% (w/v) PVP.

The homogenates were filtered through four layers of cheesecloth and then centrifuged at 4°C for 20 min at 15,000xg. The supernatant fraction was used as crude extract for enzyme activity assays.

2.2.1 Superoxide dismutase

Superoxide dismutase activity was determined by measuring its ability to inhibit the photochemical reduction of nitroblue tetrazolium using the method described by Dhindsa et al. [10]. Three ml enzyme reaction mixture contained: 50 mM phosphate buffer (pH 7.8) (1.5 ml of 100 mM), 13.33 mM methionine (0.2 ml of 200 mM), 75 µM NBT (0.1 ml of 2.25 mM), 0.1mM EDTA (0.1 ml of 3 mM), 50 mM sodium carbonate (0.1 ml of 1.5 M), 100 µl enzyme extract, 0.8 ml of distilled water and 2 µM riboflavin (0.1 ml of 60 µM). The reaction was started by adding 2 µM riboflavin and placing the tubes under two 15 w fluorescent bulbs for 15 min. A complete reaction mixture without enzyme extract, which gave the maximal colour, served as irradiated control. After 15 min, the reaction was terminated by switching off light and covering the tubes with black cloth. A complete reaction mixture without enzyme extract kept in dark served as non-irradiated blank. The absorbance of the reaction mixture was red at 560 nm. One unit of SOD was defined as the amount of enzyme required to cause 50 per cent inhibition of NBT reduction per min at 560 nm.

2.2.2 Catalase

Catalase activity was measured immediately in fresh extract as described by Aebi [11]. Three ml enzyme reaction mixture contained: 50 mM potassium phosphate buffer (pH 7.0) (1.5 ml of 100 mM), 200 µl enzyme extract, 800 µl of distilled water and 12.5 mM hydrogen peroxide (0.5 ml of 75 mM).The reaction was initiated with addition of 0.5 ml of 75 mM H_2O_2. For measurement of catalase enzyme activity, the decline in absorbance was recorded at 240 nm for three min at an interval of 30 sec. The amount of hydrogen peroxide decomposed was determined from molar extinction co-efficient (ε 36 M^{-1} cm^{-1}). The enzyme activity was expressed as µmoles of H_2O_2 decomposed mg^{-1} protein min^{-1}.

2.2.3 Ascorbate peroxidase

Ascorbate peroxidase activity was measured immediately in fresh extract which was assayed as per the method described by Nakano and Asada [12]. Three milliliter of enzyme reaction mixture contained: 50 mM potassium phosphate buffer (pH 7.0) (1.5 ml of 100 mM), 0.5 mM ascorbic acid (0.5 ml of 3 mM), 0.1 mM EDTA (0.1 ml of 3 mM), 100 µl enzyme extract, 0.6 ml of distilled water and 0.1 mM hydrogen peroxide (0.1 ml of 3 mM). The reaction was initiated by the addition of 0.1 ml of 3 mM H_2O_2. The hydrogen peroxide dependent oxidation of ascorbic acid was followed by a decrease in the absorbance measured at 290 nm for three min at the interval of 30 sec. The amount of ascorbate oxidized was determined from molar extinction coefficient (ε 2.8 mM^{-1} cm^{-1}). The enzyme activity was expressed as nmoles of ascorbate oxidized mg^{-1}protein min^{-1}.

2.2.4 Guaiacol peroxidase

For GPX, the rate of decomposition of hydrogen peroxide by peroxidase, with guaiacol as a hydrogen donor was measured by the increase in absorbance at 436 nm per min as per the method described by Castillo et al. [13]. Three ml of enzyme reaction mixture contained: 50 mM phosphate buffer (pH 7.0) (1.5 ml of 100 mM), 16 mM guaiacol (0.5 ml of 96 mM) 100 µl enzyme extract, 0.4 ml of distilled water and 2 mM hydrogen peroxide (0.5 ml of 12 mM).

The above mixture was mixed properly by using a spinner for 3-5 seconds. The reaction was initiated with adding 0.5 ml of 12 mM H_2O_2. An increase in absorbance due to the formation of tetra-guaiacol was measured at 470 nm for three min at an interval of 30 sec. The enzyme activity was calculated as per molar extinction coefficient of its oxidization product, tetra-guaiacol ε = 26.6 mM^{-1} cm^{-1}. The enzyme activity was expressed as nmoles of tetra-guaiacol formed mg^{-1} protein min^{-1}.

2.2.5 Lipid peroxidation rate

The level of lipid peroxidation product was measured in terms of malondialdehyde as thiobutaric acid reactive substance [14]. Root samples 0.2 g was homogenized in 2 ml of 0.1% TCA. The homogenate was centrifuged at 15000 g for 15 min and the supernatant was used for the estimation of MDA content. Leaf extract, 0.2 ml was thoroughly mixed with 0.4 ml of TBA reagent. The mixtures were heated for fifteen min at 100°C, cooled and cleared by centrifugation at 1000 g for 10 min. The absorbance was taken at 535 nm on spectrophotometer. Results were expressed as A_{535} per gram of plant fresh weight.

2.3 Data Analysis

The data on biochemical constituents was statisticaly analyzed by using completely randomized block design [15].

3. RESULTS AND DISCUSSION

The transient production of AOS, in an oxidative burst, is frequently an early plant response in pathogen attack. Under wilt sick soil wilt susceptible chickpea genotypes have more pathogen attack in root tissues than wilt tolerant genotypes.

At preinfection stage mean APX activity did not vary significantly from normal soil to wilt sick soil, however significant increase was observed at post infection stage with 1.08 µmoles ascorbate oxidized mg^{-1} protein min^{-1} in normal soil to 1.75 µmoles ascorbate oxidized mg^{-1} protein min^{-1} in wilt sick soil (Table 1). At the post infection stage the root APX activity increased significantly in wilt susceptible group of genotypes from 2.07 to 2.38 µmoles ascorbate oxidized mg^{-1} protein min^{-1} with mean percent increase of 97.94%. Though APX activity increased at post infection stage in wilt sick soil in the wilt resistant genotypes percent increase was from 33.03 to 57.64 with a mean percent increase of 40.4%. Garcia et al. [16] reported remarkably increased root APX activity in wilt susceptible genotype JG 62 at post infection stage. APX activity was also increased but not significant in wilt immune genotype WR 315 at post infection stage. Maximal increase in root APX activity was recorded in JG 62 at post infection stage as compared to wilt resistant genotype JCP 27 by Joshi et al. [17].

At preinfection stage of growth the mean root GPX activity of chickpea genotypes did not vary significantly and increased from 0.44 to 0.45 µmoles of tetra guaiacol formed mg^{-1} protein min^{-1} in normal and wilt sick soil respectively, while the GPX activity increased significantly in root tissue at post infection stage from 0.83 to 1.01 µmoles of tetra guaiacol formed mg^{-1} protein min^{-1} in wilt sick soil. Wilt susceptible genotype JG 62 recorded maximum increase in root GPX activity at both pre and post infection stages. The GPX activity increased from 0.79 to 1.23 µmoles of tetra guaiacol formed mg^{-1} protein min^{-1} in JG 62 with 55.51 percent increase at post infection stage in wilt sick soil (Table 2). All the wilt resistant chickpea genotypes recorded minimum increase in GPX activity at both growth stages from normal to wilt sick soil. Levels of GPX activity increased in wilt susceptible genotypes at post infection stage in wilt sick soil to counter the oxidative burst due to more pathogen attack in the roots of susceptible genotypes.

Table 1. Levels of ascorbate peroxidase activity in roots of chickpea seedlings grown in normal and wilt sick soil

Sr. no.	Genotypes	Ascorbate peroxidase activity (µmoles ascorbate oxidized mg^{-1} protein min^{-1})					
		Pre infection stage			Post infection stage		
		Normal soil	Wilt sick soil	Per cent increase	Normal soil	Wilt sick soil	Per cent increase
1	GJG 0919	0.59	0.63	7.79	1.04	1.45	40.02
2	BCP-2010-1	0.49	0.52	6.50	1.12	1.77	57.64
3	JG 24	0.49	0.53	9.05	1.02	1.56	53.05
4	Vijay	0.53	0.55	4.56	1.12	1.69	50.53
5	Digvijay	0.56	0.60	6.42	1.10	1.57	43.61
6	WR 315	0.49	0.51	3.66	1.19	1.60	34.53
7	ICC 4958	0.42	0.44	3.77	0.82	1.09	33.03
8	JG62	0.47	0.55	17.49	1.11	2.38	115.01
9	SAKI9516	0.50	0.59	16.39	1.21	2.29	88.29
10	Vikas	0.49	0.58	18.67	1.09	2.07	90.52
	Mean	**0.50**	**0.55**		**1.08**	**1.75**	
	Comparison	S. E. ±	CD at 5 %		S. E. ±	CD at 5 %	
1	Genotypes	0.005	0.014		0.006	0.017	
2	Treatments	0.006	0.016		0.007	0.019	
3	Genotypes x Treatments	0.017	0.051		0.020	0.060	

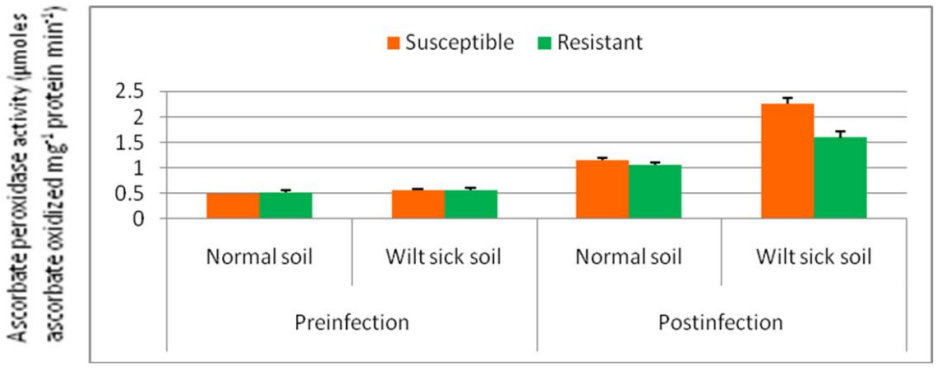

Fig. 1. Mean levels of ascorbate peroxidase activity in roots of wilt resistant and susceptible genotypes

Table 2. Levels of guaiacol peroxidase activity in roots of chickpea seedlings grown in normal and wilt sick soil

Sr. no.	Genotypes	Guaiacol peroxidase activity (µmoles of tetra guaiacol formed mg^{-1} protein min^{-1})					
		Pre infection stage			Post infection stage		
		Normal soil	Wilt sick soil	Per cent increase	Normal soil	Wilt sick soil	Per cent increase
1	GJG 0919	0.45	0.46	2.75	0.77	0.84	8.73
2	BCP-2010-1	0.45	0.48	4.87	0.99	1.14	15.43
3	JG 24	0.40	0.42	3.59	1.06	1.22	15.35
4	Vijay	0.46	0.48	3.79	0.74	0.85	14.50
5	Digvijay	0.45	0.46	1.20	0.69	0.75	7.90
6	WR 315	0.36	0.37	2.99	0.81	0.88	7.92
7	ICC 4958	0.45	0.47	3.28	0.85	0.93	8.80
8	JG62	0.39	0.42	8.57	0.79	1.23	55.51
9	SAKI9516	0.50	0.53	5.96	0.77	1.14	48.51
10	Vikas	0.44	0.47	5.88	0.78	1.12	43.37
	Mean	**0.44**	**0.45**		**0.83**	**1.01**	
	Comparison	S. E. ±	CD at 5 %		S. E. ±	CD at 5 %	
1	Genotypes	0.005	0.015		0.008	0.023	
2	Treatments	0.006	0.017		0.009	0.025	
3	Genotypes x Treatments	0.019	0.056		0.028	0.080	

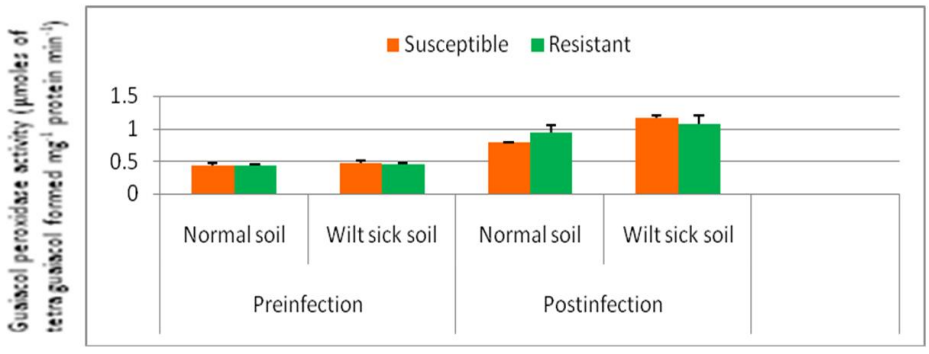

Fig. 2. Mean levels of guaiacol peroxidase activity in roots of wilt resistant and susceptible genotypes

Root catalase activity increased significantly at preinfection stage of growth with 17.47 to 26.75 µmoles H_2O_2 decomposed mg^{-1} protein min^{-1}. Wilt susceptible genotype JG 62 recorded maximum increase in CAT activity from 11.76 to 18.52 µmoles H_2O_2 decomposedmg^{-1} protein $min^{-1}$with 57.42 percent increase at post infection stage. Wilt resistant genotypes viz. Vijay, Digvijay and WR 315 showed minimum increase in catalase activity at post infection stage in wilt sick soil as compared to normal soil (Table 3). Among three genotypes evaluated for this study BCP 2010- 1 recorded maximum increase in CAT activity at post infection stage with 14.58 percent but which was not comparable with wilt susceptible genotypes. The SOD activity did not vary significantly at preinfection stage between wilt resistant and susceptible chickpea genotypes. At post infection stage wilt susceptible genotypes JG 62, SAKI 9516 and Vikas recorded maximum increase with 16.14, 15.87 and 11.47, respectively confirms that pathogen attack is more in roots of wilt susceptible genotypes (Table 4). The SOD activity increased in roots of chickpea genotypes JG62 and WR 315 in *Foc* 5 infected plants as compared to control plants at post infection stage of growth. In melon plants SOD, APX and CAT activities were increased remarkably when inoculated with *F. oxysporum* f. sp. *Melonis* Race 1.2 as compared to uninoculated plants [18]. Infection by *Alternaria sesami* significantly increased SOD, APX and CAT activities in leaves of sesamum plants at different stages of infection as compared with non-infected control [19].

Table 3. Levels of catalase activity in roots of chickpea seedlings grown in normal and wilt sick soil

Sr. no.	Genotypes	Catalase activity (µmoles H_2O_2 decomposed mg^{-1} protein min^{-1})					
		Pre infection stage			Post infection stage		
		Normal soil	Wilt sick soil	Per cent increase	Normal soil	Wilt sick soil	Per cent increase
1	GJG 0919	17.68	25.88	46.36	11.91	12.56	5.45
2	BCP-2010-1	21.73	34.00	56.47	12.46	14.28	14.58
3	JG 24	22.68	34.24	50.96	12.02	13.54	12.66
4	Vijay	21.49	33.23	54.60	15.22	16.10	5.76
5	Digvijay	14.60	21.20	45.21	14.04	15.20	8.30
6	WR 315	14.21	20.81	46.42	14.61	16.10	10.20
7	ICC 4958	18.23	29.63	62.60	11.44	13.13	14.77
8	JG62	13.35	22.34	67.35	11.76	18.52	57.42
9	SAKI9516	13.79	20.74	50.35	11.64	18.10	55.41
10	Vikas	16.93	25.43	50.18	11.98	17.59	46.89
	Mean	**17.47**	**26.75**		**12.71**	**16.37**	
	Comparison	S. E. ±	CD at 5 %		S. E. ±	CD at 5 %	
1	Genotypes	0.40	1.15		0.20	0.57	
2	Treatments	0.39	1.11		0.22	0.63	
3	G x T	0.78	2.99		0.70	1.99	

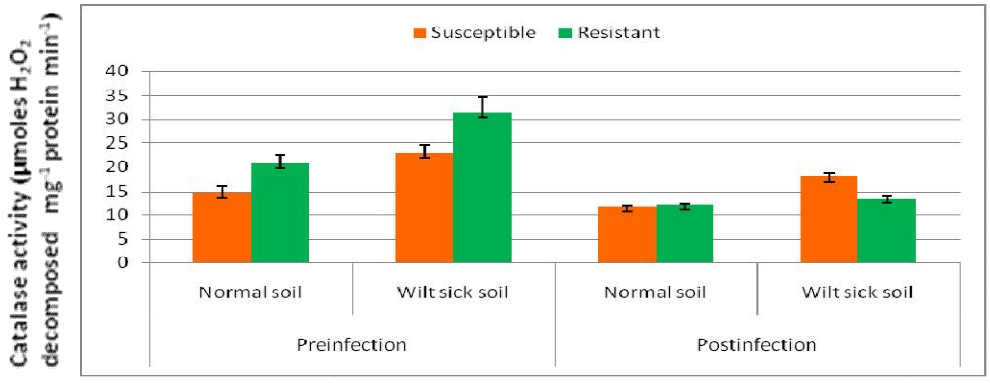

Fig. 3. Mean levels of catalase activity in roots of wilt resistant and susceptible genotypes

Table 4. Levels of superoxide dismutase activity in roots of chickpea seedlings grown in normal and wilt sick soil

Sr. no.	Genotypes	Superoxide dismutase activity (units mg^{-1} protein min^{-1})					
		Pre infection stage			Post infection stage		
		Normal soil	Wilt sick soil	Per cent increase	Normal soil	Wilt sick soil	Per cent increase
1	GJG 0919	16.32	17.07	4.63	12.08	12.3	1.82
2	BCP-2010-1	17.19	17.66	2.71	10.35	10.48	1.26
3	JG 24	16.22	16.65	2.66	10.14	10.34	1.97
4	Vijay	17.84	18.82	5.53	11.65	11.8	1.29
5	Digvijay	16.14	16.71	3.54	12.49	12.64	1.20
6	WR 315	15.86	16.11	1.56	12.59	12.76	1.35
7	ICC 4958	17.76	18.25	2.74	10.34	10.46	1.16
8	JG62	16.39	16.57	1.13	7.47	8.67	16.14
9	SAKI9516	16.27	16.78	3.15	7.60	8.80	15.87
10	Vikas	16.87	17.20	1.98	7.73	8.62	11.47
	Mean	**16.68**	**17.18**		**10.24**	**10.69**	
	Comparison	S. E. ±	CD at 5 %		S. E. ±	CD at 5 %	
1	Genotypes	0.068	0.196		0.0832	0.242	
2	Treatments	0.075	0.214		0.0760	0.217	
3	G x T	0.237	0.708		0.263	0.754	

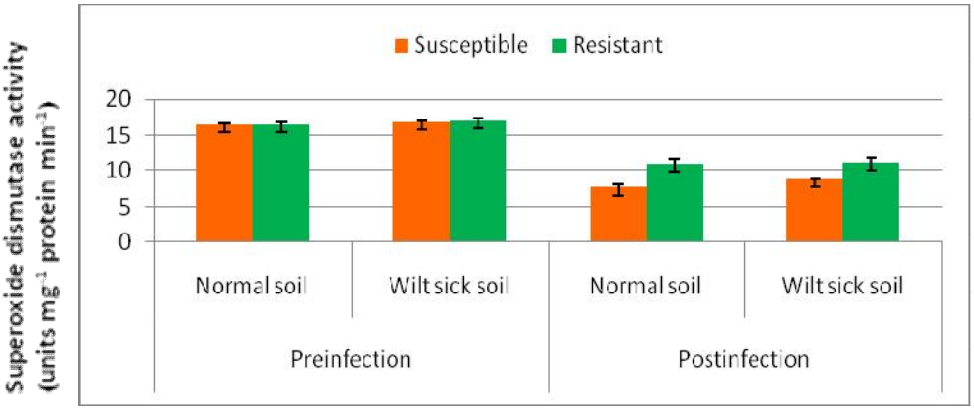

Fig. 4. Mean levels of superoxide dismutase activity in roots of wilt resistant and susceptible genotypes

At pre infection stage of growth lipid peroxidation rate increased more in wilt resistant group than susceptible under wilt sick soil. Increase in lipid peroxidation rate in all chickpea genotypes was minimum at post infection stage. Among tested chickpea genotypes BCP 2010-1 recorded maximum increase in lipid peroxidation rate from 1.40 to 2.10 A_{535} g^{-1} fresh wt. with 50 percent increase in wilt infected plants over normal soil plants and comparable with wilt immune genotype WR 315. JG 62 recorded minimum increase in lipid peroxidation rate from 1.90 to 2.26 A_{535} g^{-1} fresh wt. with 19 percent increase in wilt sick soil over normal soil (Table 5). Lipid peroxidation measured in terms of malondialdehyde (MDA) content, which showed an increase in MDA content in all infected treatment compared to non-infected treatment. Large increase of MDA content after 4days of inoculation in both strains of bacteria infected leaves was recorded in lime plants [20]. In flax, powdery mildew resistant varieties recorded about 1.5 fold increases in MDA content as compared to susceptible varieties [21].

Table 5. Levels of malondialdehyde in roots of chickpea seedlings grown in normal and wilt sick soil

Sr. no.	Genotypes	Malondialdehyde content (A_{535} g^{-1} fresh wt.)					
		Pre infection stage			Post infection stage		
		Normal soil	Wilt sick soil	Per cent increase	Normal soil	Wilt sick soil	Per cent increase
1	GJG 0919	1.35	1.96	45	1.68	1.78	6
2	BCP-2010-1	1.40	2.1	50	1.74	1.84	6
3	JG24	1.46	2.14	47	1.62	1.70	5
4	Vijay	1.32	1.96	48	1.58	1.65	4
5	Digvijay	1.44	2.12	47	1.80	1.90	6
6	WR 315	1.42	2.2	55	1.74	1.80	3
7	ICC 4958	1.48	2.08	41	1.70	1.78	5
8	JG62	1.90	2.26	19	1.56	1.63	4
9	SAKI9516	1.82	2.3	26	1.60	1.66	4
10	Vikas	1.96	2.4	22	1.68	1.72	2
	Mean	**1.56**	**2.15**		**1.67**	**1.75**	
	Comparison	S. E. ±	CD at 5 %		S. E. ±	CD at 5 %	
1	Genotypes	0.006	0.016		0.005	0.015	
2	Treatments	0.008	0.022		0.006	0.018	
3	G x T	0.014	0.044		0.010	0.030	

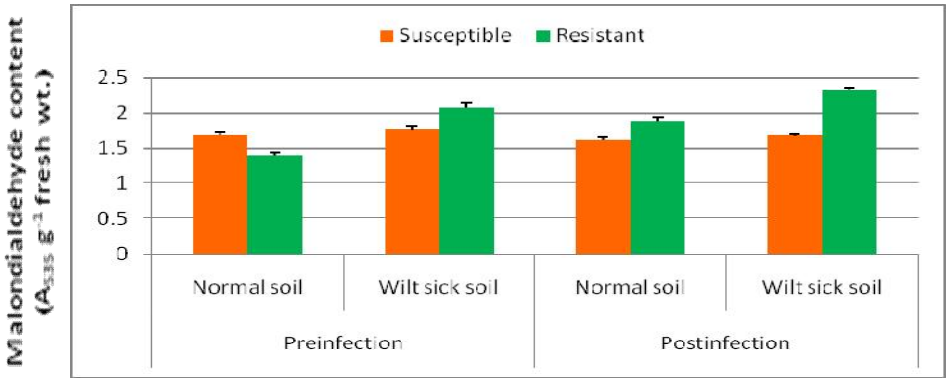

Fig. 5. Mean levels of malondialdehyde content in roots of wilt resistant and susceptible genotypes

4. CONCLUSIONS

The evaluation of antioxidative enzyme profile in normal and wilt sick soil of the three wilt resistant genotypes along with four wilt resistant and three wilt susceptible checks exhibited a differential response. The activity of three antioxidative enzymes *viz*., APX, GPX and SOD increased significantly in wilt susceptible checks at post infection stage. The non compatible interaction between wilt resistant genotype and pathogen demonstrated lesser increase.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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