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Role of PAL and PPO Enzyme Activity in Host Pathogen interaction of Chickpea (Cicer Arietinum L) Root Tissue Infected with Fusarium Wilt

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Abstract— *In this study, changes in the activities of Phenlylalanine ammonialyase (PAL) and polyphenol oxidase (PPO) during development and stages of wilt (Fusarium oxysporum Schlechtend.:Fr. f.sp. ciceri (Padwick) Matuo & K. Sato) disease infection in chickpea(Cicer arietinum L) were investigated. During the early stages of disease development, at pre-infectional stage (S_1), cultivars did not show any significant change in PAL activity. The activity was significantly increased at infectional stage (S_2) as compared with pre infectional stage (S_1). Susceptible cultivars had the lower value of PAL at infectional stage (S_2). However, at S_2 stage cultivars GG-4 and JCP-27 were at par. At post infectional stage (S_3), the activity was found to be increased in the all cultivars as compared to infectional or mid growth stage and the resistant and tolerant cultivars were at par. Susceptible cultivars (JG-62 and GG-4) had the lower activity as compared to the resistant and tolerant cultivars. The resistant cultivars WR-315 and JCP-27 revealed higher level of activity, the level of activity significantly increased marginally during infection. In the present experiment significantly higher activity in infected plants grown in sick plot also suggested that polyphenol oxidase might be involved in oxidation of phenolics in susceptible cultivar (JG-62). These observations suggest that the increase in PAL and PPO activities has an important role in disease resistance mechanism.*

Keyword— chickpea, Polyphenols oxidase (PPO), phenylalanine ammonia lyase(PAL).

I. INTRODUCTION

Chickpea (*Cicer arietinum* L.) is the second most important pulse crop of the world. India is the world's largest chickpea growing country having a cultivation area of 6.5 Million hectares and an output of 5.77 million tonnes with an average yield 888 kg/ha (Deshmukh, 2005a) and contributes about 63 percent to the global production of chickpea. Gujarat having cultivation area of 0.17 lakh hectares and an output of 0.09 metric tonnes with yield 530 kg/ha in 2000-01(Anon, 2003). A number of pathogens affecting chickpea have shown a three fold increase during last 15 years (Nene *et al.*, 1996). The main fungi that affect chickpea are *Fusarium oxysporum* Schlechtend.:Fr. f.sp. *ciceri* (Padwick) Matuo & K. Sato, causing the plant to wilt. Wilt of chickpea (*Cicer arietinum*), caused by *Fusarium oxysporum* f. sp. *ciceris* is a major limiting factor of chickpea production in the Mediterranean Basin and the Indian Subcontinent (Jalali and Chand, 1992). Annual yield losses due to *Fusarium oxysporum*. f.sp. *ciceri* have been estimated to range from 10 to 15% but fusarium wilt epidemics can be devastating to individual crops and cause 100% loss under favorable conditions (Halila and Strange, 1996; Chaube and Pundhir, 2005). Disease resistance appears to be the rule rather than the exception in nature. The ability of a host plant to defend itself against a pathogen is governed by its genetic constitution and the environmental conditions under which the genes operate. As reported by many researcher and which are expressed through that all natural resistance is under genetic control and genes are expressed through biological products such as secondary metabolites viz. phenolics, lignin, callose, suberin, phytoalexins, alkaloids, terpenes, glycosides and pathogenesis-related proteins all of which contribute to disease resistance.

Enzymes secreted by pathogen and host cells play important roles in disease resistance mechanism. Peroxidase and polyphenol oxidase are involved in synthesis of toxic phenolics. Phenylalanine ammonia lyase, cinnamic acid-4-hydroxylase and 4-coumaryl CoA ligase are the key enzymes of phenylpropanoid pathway for production of phytoalexins, phenolics and their derivatives. The Phenolic compounds are a group of important plant secondary

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metabolites that have been suggested to play a variety of roles in defense mechanisms against pathogens. They were reported as phytoanticipins, phytoalexins, structural barriers, modulators of pathogenicity, and/or activators of plant defense genes (Mansfield, 2000; Ramos *et al*., 1997) The aim of the present work was to examine changes in the accumulation of PAL and polyphenol oxidase activity at different stages in diseased and healthy tissues, in order to associated patterns and role in defense mechanisms.

II. MATERIALS AND METHODS

Chickpea cultivars viz., GG-1(V_1, Resistant), GG-2 (Resistant), WR-315, (V_3, Susceptible), JG-62 (V_4, Highly susceptible) and JCP-27, (V_5,Highly Resistant) GG-4 (V_6)were grown under field condition in two plots of Junagadh Agricultural University, at Pulse Research Farm ,Junagadh. One plot was *i.e* normal plot without diseased while other was kept free for infection of wilt disease in chickpea plants in sick plot that is maintained since 20 years for *F.oxysporium f.sp.ciceri* at JAU Junagadh. Root tissues were collected at pre-infectional (12 Days after sowing), Infectional (21 days after sowing) and post infectional (26 Days after sowing) stage from both plots and Extraction and estimation of PAL and PPO enzyme from root tissues were done as per the methods described by Malik and Singh (1980).

Extraction and Assay of Phenylalanine ammonialyase (PAL) activity

100mg acetone powder/ml of root tissue with 1mM polyvinylpyrrolidine in 0.1M sodium borate pH 8.8 were extracted and than centrifuge with 10000 rpm, 10 min, -4^{0}C. The assay mixture of PAL contained substrate as 0.1M Phenylalanine in 0.1M sodium borate buffer pH 8.8 at 4°C. The reaction mixture (3.6ml) consisted of 0.1M sodium borate buffer pH 8.8 with 0.5ml substrate and addition of 100uL of crude enzyme extract to initiate the reaction with hour's incubation 37^{0}C, which was measured SpectroPhotometrical at 290 nm. Enzyme activity expressed per nmole cinnamic acid released/h/g acetone powder. Product reaction colour shown brown to oxidized (Systronics spectrophotometer, Ahmedabad, India).

Preparation of Acetone Powder from Plant Tissues: Acetone powder from root tissues was prepared for the assay of PAL (phenylalanine ammonia lyase) enzyme. Tissues (2 to 3g) were ground using a mortar and pestle at 4 ^{0}C with 4 volumes of pre chilled acetone (-20 ^{0}C). Crushed material was filtered and again ground with acetone. The procedure was repeated till white powder of tissue was obtained. Powder was dried in desiccator and stored at -4 ^{0}C till assay of the enzymes was carried out.

Extraction and Assay of polyphenoloxidase (PPO) activity

Sample weight 500mg/ml in 1mM PVP with 0.1M Phosphate pH 7.2 of tissues were grind with chilled mortal pestle. Extract were centrifuged to10000 rpm, 10 min, -4^{0}C . The assay mixture of Polyphenol oxidase (PPO) contained 3.1mL of 0.1 M phosphate buffer (pH 6.0) at 4°C. The reaction mixture consisted of 3.0 ml 0.01M Catechol in 0.1M Phosphate buffer pH 6.0. The addition of 100ul of crude enzyme extract initiated the reaction, which was measured spectrophotometrically at 420 nm at 3min interval for 15 min , Change in O.D/min/g fresh tissues.. Product released showed Oxidized catechol light green colour

III. RESULTS AND DISCUSSIONS

Phenylalanine ammonia lyase: Chickpea cultivars grown in normal and sick plots showed significant difference in root phenylalanine ammonia lyase activity. (Fig.1.) the root tissues obtained from sick plot showed higher activity as compared to the tissues collected from sick plot. Cultivars differed significantly in their phenylalanine ammonia lyase activity. Among the cultivars, tolerant cultivars GG-1 and GG-2 revealed higher activity than the resistant cultivars JCP-27 and WR-315. Susceptible cultivars JG-62 and GG-4 showed significantly lower level of activity. (Fig.1). Among the different infectional stages, the activity increased from 4.62 to 113.42 nmole cinnamic acid released. $h^{-1}.g^{-1}$ acetone powders with the advancement of disease i.e. pre-infectional stage to post infectional stage but the activity drastically increased at infectional stage (S_2). Plants grown in sick plot, the enzyme phenylalanine ammonia lyase activity in root tissues of chickpea obtained from the different cultivars were varied between 67.90 to 81.63 nmole cinnamic acid released.$h^{-1}.g^{-1}$ acetone powder (Table.1). Susceptible cultivar JG-62 had the highest value of enzyme activity followed by moderately susceptible cultivar GG-4. However the lowest activity was found in resistant cultivars WR-315 and JCP-27. In general, plants grown in sick plot had significantly higher values than the plants grown in normal plot. In contrast to this, plants grown in normal plot the PAL activity was varied from 41.56 to 64.83 nmole cinnamic acid release. $h^{-1}.g^{-1}$ acetone powders. A comparison was made between sick and normal plot showed that the activity of PAL was almost doubled in susceptible cultivars grown in sick plot. Irrespective of treatments, at pre-infectional stage (S_1), cultivars did not show any significant change in PAL activity. The activity was significantly increased at infectional stage (S_2) as compared with pre infectional stage (S_1). Susceptible cultivars had the lower value of PAL at infectional stage (S_2). However, at S_2 stage cultivars GG-4 and JCP-27 were

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at par. At post infectional stage (S_3), the activity found to be increased in the all cultivars as compared to infectional or mid growth stage and the resistant and tolerant cultivars were at par. Susceptible cultivars (JG-62 and GG-4) had the lower activity as compared to the resistant and tolerant cultivars. Nighat (2001) studied on phytoalexin accumulation and PAL activity in chickpea cultivars viz resistant and susceptible after inoculation with *Ascochyta rabiei.* This accumulation was preceded by a transient rise in activity of PAL. Maximum PAL activity was observed 12 to 24 hours after inoculation. Phenylalanine ammonia-lyase activity in susceptible cultivars was higher than in resistant cultivars of chickpea infected with ascochyta blight (Sindhu *et al*.,1995). Thus the data from the present experiments are in agreement with Sindhu *et al*.(1995) and Nighat, (2001). Combined effect of treatment X stage was found to be significant. Chickpea cultivars grown in sick plot resulted into higher phenylalanine ammonia lyase activity (5.47 to 121.53 n. mole cinnamic acid release. $h^{-1}.g^{-1}$ acetone powder) in root tissues of chickpea as compared to the activity recorded in the normal plot (3.78-105.32 Δ O.D. $h^{-1}.g^{-1}$.fr.wt.acetone powder) at all the stages, in general. Interaction effect of treatments x varieties x stages showed significant difference for phenylalanine ammonia lyase activity in root tissues. (Fig.2) at pre infectional stage (S_1) the lowest activity of PAL was recorded in cultivar JG-62 grown in sick plot. However, the rest of the cultivars did not show any significant change in their activity. All the cultivars resulted drastically rise in their enzyme activity at infectional stage (S_2). The resistant cultivars showed significantly lower activity as compared to tolerant (GG-1 and GG-2) and susceptible cultivars (JG-62 and GG-4) with the advancement of disease. i.e. at S_3 stage. The PAL activity continuously increased in all the cultivars. Changes among the severity of diseases development and activity correlate at this stage showed significant changes in cultivars root tissues. Resistant cultivars WR-315 and JCP-27 revealed significantly lowest phenylalanine ammonia lyase activity followed by tolerant cultivars, and highest activity found in JG-62 at infectional stage (S_2). At post infectional stage (S_3) JG-62 exhibited significantly highest phenylalanine ammonia lyase in all the cultivars. The enzyme activity increased with the advancement of growth/ infectional stages.).

Results from present study, however, indicated that as disease infection to root tissues follows the increase in enzyme activity that might be due to rapid production of phytoalexins which is part of the defense mechanism of chickpea and PAL has a regulatory role in the biosynthesis of these secondary metabolites. Phenylalanine ammonia-lyase (PAL) activity in susceptible cultivars was higher than in resistant cultivars of chickpea infected with ascochyta blight (Sindhu et al., 1995). In case of plants grown in normal plot, the PAL activity increased with the advancement of growth stages. i.e. S_1 to S_3. In general, plants grown in normal plots had significantly lower enzyme activity at pre infectional (S_1) to post infectional stage (S_3) as compared to sick plot. The enzyme activity further increased in all the cultivars from S_2 to S_3 stage. Among the cultivars JG-62 and GG-4, resulted significantly lower value of enzyme activity at infectional (S_2) and post infectional stage (S_3) as compared to the sick plot. In root tissues of resistant cultivars grown in sick plot.i.e inoculated with *Fusarium oxysporum f.sp. ciceris*. PAL was actively associated during pre infection process as a part of disease resistant mechanism. As the fungi could not progress in root tissue of resistant and tolerant cultivars of normal plot grown, higher activity of PAL were not needed and may not be involved actively in phenyl propanoid pathway and therefore the level of activity significantly declined. In susceptible cultivars grown in sick plot showed the continuous progress of fungus occurred and hence level of PAL always beneficial part of natural host as chickpea plants. Thus, significantly higher activity of PAL in root tissue of susceptible and tolerant cultivars after pre infectional stage positively correlated with the progress of disease and active phenol metabolism. Higher activity in tissues suggested that the level of PAL would be insufficient in the root tissues to impart defense responses and therefore the activity remained higher. The results suggested that the enzyme is involved in lignin and phytoalexin synthesis and it is continuous presence in higher activity may be essential till chickpea plant become sure of strong structural and metabolic defense. The observations recorded here are also supported the findings made by the Mandavia, *et al*., (1999), Shukla, (2001) and Bhut (2005).

Polyphenol oxidase:

Chickpea cultivars grown in normal and sick plots resulted significant change in root polyphenol oxidase activity. The root tissue obtained from sick plot resulted higher polyphenol oxidase activity as compared to the tissues obtained from sick plot (Fig.3). Among the cultivars, the polyphenol oxidase activity was varied from 3.80 to 4.07 Δ $O.D.min^{-1}.g^{-1}$.fr.wt. Tolerant cultivars GG-1 and GG-2 consist of lower polyphenol oxidase activity as compared to resistant cultivars and the differences were found to be significant. However, the cultivar GG-4 hold the significantly higher activity. (Fig.4.24). Polyphenol oxidase

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activity among the different infectional stages increased from 4.30 to 5.35 Δ O.D.min^{-1}.g^{-1}.fr.wt with the advancement of disease i.e. pre-infectional stage (S1) to infectional stage (S2) but at later stage (i.e.S3) the enzyme activity drastically reduced to 2.08 Δ O.D.min^{-1}.g^{-1}.fr.wt at post infectional stage (S3). Data indicated fluctuating trend of activity of this enzyme in root tissues.

Plants grown in sick plot, the polyphenol oxidase activity in root tissues of chickpea obtained from the different cultivars varied between 4.87 to 5.38 Δ O.D.min^{-1}.g^{-1}.fr.wt.(Table.2). Resistant cultivar JCP-27 contained the highest value followed by moderately susceptible cultivar GG-4. Tolerant cultivars GG-1 and GG-2 possessed moderate level of polyphenol oxidase activity as compared to the activity found in susceptible cultivar JG-62. In contrast to this, plants grown in normal plot had lower activity of polyphenol oxidase as compared to sick plot but the trend remains same.

At pre-infectional stage (S_1), susceptible cultivars GG-4 and JG-62 resulted the highest polyphenol oxidase activities (Table.2). The resistant cultivars (WR-315 and JCP-27) contained significantly higher polyphenol oxidase activity as compared to the tolerant cultivars (GG-1 and GG-2). However, at infectional stage the activities showed fluctuating trend among all the six cultivars. Cultivar WR-315 had significantly higher value of polyphenol oxidase activity (i.e 5.45 Δ O.D.min^{-1}.g^{-1}.fr.wt) as compared to the susceptible cultivars (JG-62 and GG-4) and tolerant cultivars (GG-1 and GG-4) at infectional stage. At post infectional stage (S3), the activities drastically reduced in all the cultivars as compared to infectional / mid growth stage.

Combined effect of treatment x stage was found to be significant (Table.2). At all stages chickpea cultivars grown in sick plot resulted higher polyphenol oxidase i.e. activity 2.47 to 8.23 Δ O.D.min^{-1}.g^{-1}.fr.wt as compared to the tissue obtained from normal plot (1.68-3.90 Δ O.D.min^{-1}.g^{-1}.fr.wt). However in normal plot polyphenol oxidase activity of root tissues found to be gradually reduced as growth of the plants increased from S1 to S3.

Interaction effect of treatments x cultivars x stages revealed significant differences for polyphenol oxidase activity in root tissues (Fig.4). Plant grown in sick plot resulted differential response in polyphenol oxidase activity for the chickpea cultivars. Resistant cultivars JCP-27 and WR-315 had the highest polyphenol oxidase activities at pre-infectional and infectional stages. However, tolerant cultivars (GG-1 and GG-2) had the lowest peroxidase activity at pre-infectional stage. All cultivars showed remarkable rise in their enzyme activity at infectional stage and resistant cultivars WR-315 and JCP-27 had higher polyphenol oxidase activity followed by tolerant cultivars GG-1 and GG-2. At post infectional stage (S3).

As the fungi could not progress further, the high level of PPO was no longer necessary in resistant cultivars. In susceptible cultivars grown in sick plot showed continuous progress of fungi occurred towards the upper part of host pants from root external structures to the internal cell mechanism and hence high level of PPO always help to oxidize accumulated phenolics in response to wilt disease. In all the cultivars the level of polyphenol oxidase activity showed noticeable rise. In contrast to this, the plants grown in normal plot, the activity declined from pre-infectional to post-infectional stage in all the cultivars and the values were lower than the values recorded in sick plot.

Overall data recorded for polyphenol oxidase activity are in agreement with the findings of Shukla and Parameswaran (2004), who stated that changes in polyphenol oxidase activity during Fusarium wilt disease infection in chickpea cultivars grown in normal and inoculated soil had higher PPO activity in root tissue of both the cultivars and it increased in root with growth of plant.

The results obtained in the present experiment are supported with the findings made by Chowdhury and Sinha (2000), who reported that chickpea cultivars susceptible to wilt contained higher level of polyphenol oxidase activity that was usually associated with the defense responses of plants. The post-infection stage, the increase in PPO parameters was moderately higher than that of untreated plants and came closer to those of resistant plants. Similar results were also stated by Wang-ChangXian et al., (2005) in cucumber roots infected with *F. oxysporum* f.sp. *cucumerinum*.

DISCUSSIONS:

Phenolic acid metabolism is activated through phenyl propanoid pathway during infection which gives rise to suberin, lignin and wall bound phenolics as described below (Hahlbrock and Scheel, 1989). Amongst the secondary plant products, phenolic compounds are the most important group implicated in both constitutive and induced resistance. Presence of phenols and their oxidation products in plant tissues is considered to be potentially toxic to the growth and development of pathogens. Increase in phenolic content after elicitor treatments may be due to increase in PAL activity as PAL has been reported to be associated with the synthesis of phenolic compounds via phenylpropanoid pathway (Hahlbrock and Scheel, 1989).

In root tissues of resistant cultivars grown in sick plot i.e. inoculated with *Fusarium oxysporium f.sp. ciceris*, PAL was actively associated during pre infection process as a

part of disease resistant mechanism. The observations recorded here also supported the findings made by the Chakraborty and Gupta (2001); Mandavia, et al. (1999); Shukla, (2001) ; Bhut (2005); Rezazadeh and Dehaghi (2005) and Shou-SenYan et al. (2005). As the fungi could not progress in root tissues of resistant and tolerant cultivars of normal plot grown plants, higher activity of PAL were not needed and may not be involved actively in phenyl propanoid pathways and hence the level of activity significantly declined. In susceptible cultivars grown in sick plot showed the continuous progress of fungus occurred and hence level of PAL always beneficial part of natural host as chickpea plants. Thus, significantly higher activity of PAL in root tissues of susceptible and tolerant cultivars after pre infectional stage positively associated with the progress of wilt disease and active phenol metabolism. The results suggested that moreover as this enzyme is involved in lignin and phytoalexin synthesis its continuous presence in higher activity may be essential till chickpea plant become sure of strong structural and metabolic defense. (Hahlbrock and Scheel, 1989).

Polyphenol oxidase has been found in number of plants but higher activity is marked during pathogenic condition. Polyphenol oxidase may indirectly influenced activity of peroxidase by the oxidation of phenol i.e. browning reaction as a part of defense mechanism. Many researchers have also reported similar result, in most of the cases the resistant cultivars WR-315 and JCP-27 revealed higher level of activity while in susceptible cultivars JG-62 and GG-4, the level of activity significantly increased marginally during infection. In the present experiment significantly higher activity in infected plants grown in sick plot also suggested that polyphenol oxidase might be involved in oxidation of phenolics in susceptible cultivar (JG-62). These observations further suggested that PPO activity is actively associated during pre infectional and infectional stages as a part of disease resistance mechanism in response to wilt diseases in root tissues of resistant cultivars grown in sick plot i.e. inoculated with *Fusarium oxysporium f.sp. ciceris*. Our data are in agreement with the findings made by Shukla and Parameswaran (2004); Chowdhury and Sinha (2000) and Shou-SenYan et al. (2005).

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Table.1: Combined effect of cultivars, treatments and stages on phenylalanineammonia lyase activity (n. mole cinnamic acid release.$h^{-1}.g^{-1}$ acetone powder) in root tissues of chickpea.

Treatments and stages	WR-315 (V_1)	JCP-27 (V_2)	GG-1 (V_3)	GG-2 (V_4)	GG-4 (V_5)	JG-62 (V_6)
Sick plot (T_1)	67.90	68.00	76.66	76.18	76.22	81.63
Normal plot (T_2)	64.83	64.83	63.58	63.42	45.33	41.56
Pre infectional stage (S_1)	4.62	4.65	4.42	4.40	5.28	4.38
Infectional stage (S_2)	77.39	75.22	90.18	89.42	70.99	71.94
Post infectional stage (S_3)	117.09	117.57	115.76	115.58	106.06	108.47
	S_1	S_2	S_3			
Sick plot (T_1)	5.47	96.31	121.53			
Normal plot (T_2)	3.78	62.07	105.32			
	VXT	VXS	TXS			
S.Em	1.44	1.57	0.90			
C.D. at 5%	4.23	4.46	2.57			

Table.2: Combined effect of cultivars, treatments and stages on polyphenol oxidase activity (Δ O.D. $min^{-1}.g^{-1}$.fr.wt.) in root tissues of chickpea.

Treatments and stages	WR-315 (V_1)	JCP-27 (V_2)	GG-1 (V_3)	GG-2 (V_4)	GG-4 (V_5)	JG-62 (V_6)
Sick plot (T_1)	5.160	5.380	5.080	5.060	5.230	4.870
Normal plot (T_2)	2.610	2.540	2.520	2.600	2.910	2.930
Pre infectional stage (S_1)	4.170	4.240	4.090	4.150	4.570	4.570
Infectional stage (S_2)	5.450	5.390	5.340	5.240	5.400	5.290
Post infectional stage (S_3)	2.030	2.250	1.970	2.110	2.260	1.840
	S_1	S_2	S_3			
Sick plot (T_1)	4.69	8.23	2.47			
Normal plot (T_2)	3.90	2.47	1.68			
	VXT	VXS	TXS			
S.Em	0.040	0.030	0.20			
C.D. at 5%	0.100	0.090	0.57			

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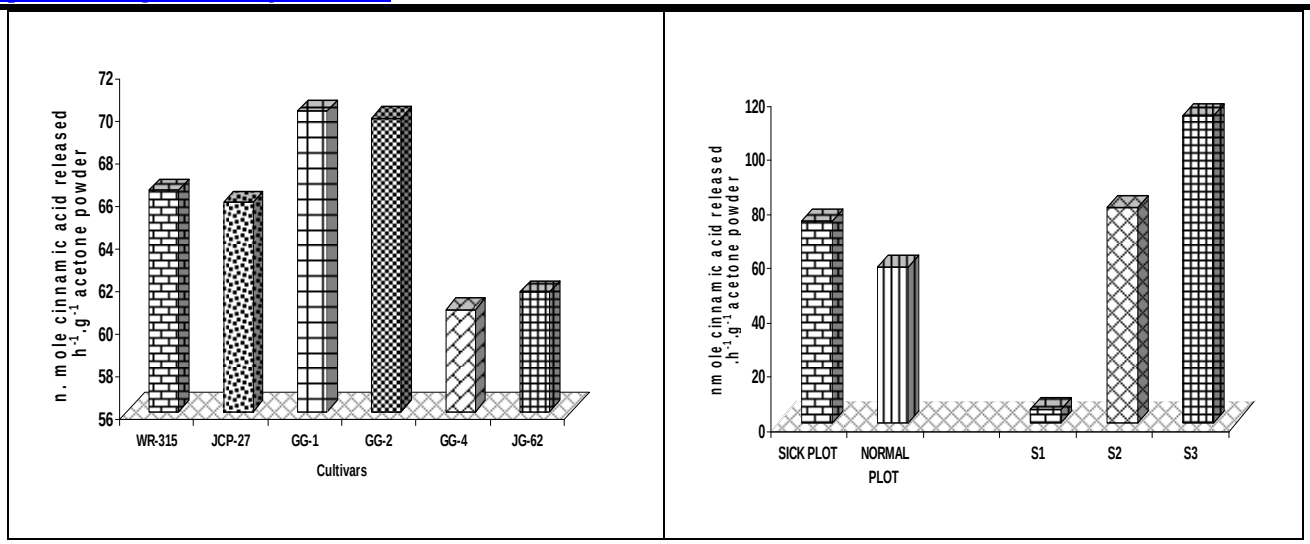

Fig.1: Mean effect of cultivars, treatments and stages on phenylalanine ammonialyase activity in chickpea root tissues. S_1-pre infectional stage; S_2-infectional stage; S_3-post infectional stage. S.Em± 1.02 (V), 0.59 (T), 0.64 (S);C.D. at 5%, 2.99V), 1.73 (T), 1.82 (S).

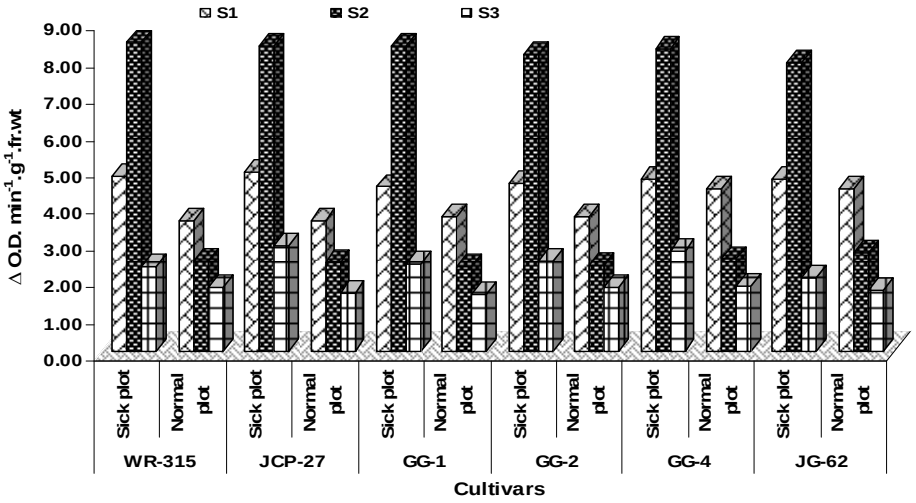

Fig.2: Interaction effect of TxVxS on phenylalanine ammonialyase activity in root tissues of chickpea cultivars. S_1- pre infectional stage; S_2-infectional stage; S_3-post infectional stage. S.Em±2.22, C.D. at 5%6.30.

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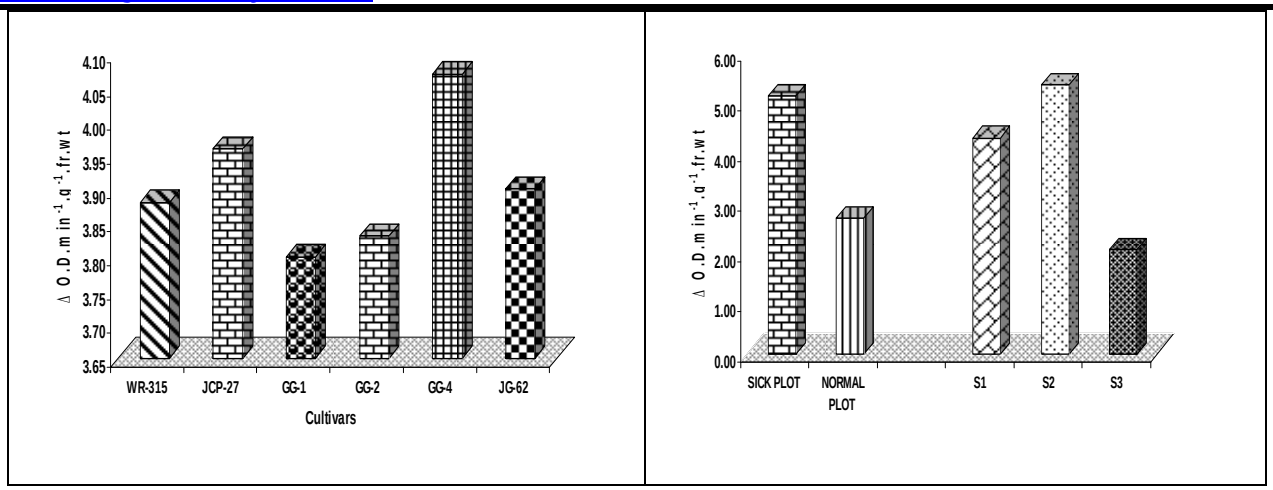

Fig. 3: Mean effect of cultivars, treatments and stages on polyphenol oxidase activity in chickpea root tissues. S_1- pre-infectional stage; S_2-infectional stage; S_3-post infectional stage. S.Em± 0.02 (V), 0.02 (T), 0.02 (S); C.D. at 5%, 0.04 (V), 0.06 (T), 0.04 (S).

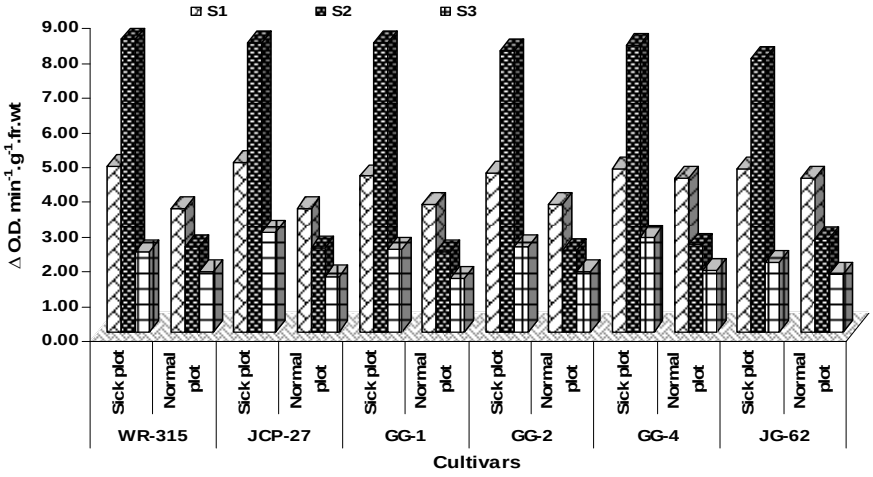

Fig.4: Interaction effect of TxVxS on polyphenol oxidase activity in root tissues of chickpea cultivars. S_1- pre infectional stage; S_2-infectional stage; S_3-post infectional stage. S.Em±0.02, C.D. at 5% 0.06.