INTERNATIONAL JOURNAL OF PLANT PROTECTION
VOLUME 8 | ISSUE 1 | APRIL, 2015 | 86-92

● e ISSN-0976-6855 | Visit us : *www.researchjournal.co.in*

IJPP

RESEARCH PAPER

DOI : 10.15740/HAS/IJPP/8.1/86-92

# Ascorbic acid and ascorbate peroxidase based defence system induced by *Pseudomonas fluorescens* against wilt pathogen in chickpea

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ARITCLE INFO

**Received** : 27.10.2014
**Revised** : 03.02.2015
**Accepted** : 19.02.2015

KEY WORDS :
Ascorbic acid, Ascorbate peroxidase, Chickpea, *Fusarium oxysporum* f.sp. *ciceri*, *Pseudomonas fluorescens* isolates

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ABSTRACT

An induction of defense mechanisms against pathogens along with induction of antioxidant system is the mechanisms by which PGPR promote plant growth promotion is well known. So, the present experiment was conducted to study the plant defense system induced by PGPR bacteria *Pseudomonas fluorescens* against *Fusarium* wilt in chickpea. The results suggest that seed treatment of *Pseudomonas fluorescens* effective elicits activity of defense-related antioxidant system such as ascorbic acid and APX leading to improved plant resistance and reduces the mortality of chickpea plant against soil borne disease like wilt.

**How to view point the article :** Kandoliya, U.K. and Vakharia, D.N. (2015). Ascorbic acid and ascorbate peroxidase based defence system induced by *Pseudomonas fluorescens* against wilt pathogen in chickpea. *Internat. J. Plant Protec.*, **8**(1) : 86-92.

## INTRODUCTION

Wilt is one of the major factors amongst biotic stress limiting chickpea (*Cicer arietinum* L.) production in arid and semi arid regions. Antioxidant defense system plays vital role in plant's tolerance to this stress conditions by detoxification to efficiently scavenge for either the ROS themselves generated due to stress or their secondary reaction products. Number of ROS scavenging enzymes including ascorbate peroxidase (APX) are produced to avoid cellular disintegration by ROS (Mittler *et al.*, 2004). Moreover, ascorbic acid, small, water soluble, reductone sugar acid found in the majority of plant cell types, is also one of the most powerful antioxidants. The ability to donate electrons in a wide range of enzymatic and non-enzymatic reactions makes ascorbic acid as main ROS-detoxifying compound in the aqueous phase (Arrigoni and De Tullio, 2000). It acts as a primary substrate in the cyclic pathway for enzymatic detoxification of a number of reactive oxygen species (ROS) such as $H_2O_2$, and many other, harmful to normal functioning of plant metabolism. In addition, it acts directly to neutralize superoxide radicals ($O_2$·-), singlet oxygen (O·-) or hydroxyl radical (OH·-) simply by acting as a secondary antioxidant during reductive recycling of the oxidized form of α-tocopherol (Noctor and Foyer, 1998). An induction of defense mechanisms against pathogens along with induction of antioxidant system is the mechanisms by which PGPR promote plant growth promotion is also well known. Thus, the present experiment was conducted to study the plant defense induced by PGPR bacteria *Pseudomonas fluorescens* against *Fusarium* wilt in chickpea.

## MATERIAL AND METHODS

Chickpea seeds of five varieties (WR-315, JCP-27, GG-1, Saki and JG-62) differing in their susceptibility to wilt disease were obtained from Main Pulse Research Station, Junagadh

Agricultural University, Junagadh.

**Preparation of mass inoculums :**

*Pseudomonas fluorescens* isolates used in present experiment were isolated by from chickpea rhizosphere of ten different chickpea growing areas of Gujarat (India) using selective Kings B media. The best isolate (*Pf-3*) selected on the basis of *in vitro* inhibition of *Fusarium oxysporum* f.sp. *ciceri* was utilized for talk powder based seed treatment (Kandoliya and Vakharia, 2013a).

**Seed sowing and seed treatment :**

Earthen pots were washed thoroughly with tap water, followed by washed with 5 per cent formaldehyde solution and allowed to dry before use. Pots were filled with either normal black soil or with inoculated soil (10 kg. soil/pot). Prior to treatment, all the seed of chickpea varieties differed in susceptibility to wilt *i.e.* WR-315, JCP-27($V_2$), GG-1($V_3$), Saki ($V_4$) and JG-62 ($V_5$) were moistened with water, so, talc formulations retained to the seeds.

$T_1$ = Seeds were treated with talc based powder only and sown in normal soil pots as a control.

$T_2$ = Seeds were treated with talc based powder and sown in sick (*Fusarium oxysporum* f.sp. *ciceri* infected) soil pots.

$T_3$ = Seeds were treated with talc powder based formulation contained biocontrol agents *Pf-3* (microbial load $3\times10^8$ *cfu*/g talc powder) and sown in sick (*Fusarium oxysporum* f.sp. *ciceri* infected) soil pots.

$T_4$ = Seeds were treated with talc powder based formulation of bio-control agents *Pf-3* (microbial load $3\times10^8$ *cfu*/g talc powder) and sown in normal soil pots.

**Ascorbate peroxidase (APx, EC 1.11.1.11) :**

Extract for determination of ascorbate peroxidase (APX) activities were prepared from 500 mg of fresh chickpea seedlings homogenized with a pre-chilled mortar and pestle under ice cold condition in 5 ml of extraction buffer, containing 50 mM sodium phosphate buffer (pH 7.4) with the addition of 1 mM EDTA and 1 per cent (W/V) polyvinylpyrolidone (PVP). The homogenates were centrifuged at 10,000 rpm for 20 minutes and the supernatant was used for the assay (Costa *et al*., 2002). Protein from the extract was measured by Folin lowly method which was used for expression of specific activities of the enzymes. APx activity was measured immediately in fresh extract and was assayed as described by Nakano and Asada (1981). 3 ml reaction mixture containing 50 mM sodium phosphate buffer pH 7.0, 0.1 mM $H_2O_2$, 0.5 mM ascorbic acid, 0.1 mM EDTA and 0.1 ml enzyme extract. The hydrogen peroxide dependent oxidation of ascorbate was followed by a decrease in the absorbance at 290 nm ($\varepsilon$ = 2.8 $mM^{-1}cm^{-1}$). Blank was carried out without substrate ($H_2O_2$). The specific activity was expressed as $U.mg^{-1}$ protein and unit activity was defined as $\Delta$ OD.$min.^{-1}g.^{-1}$ Fr.Wt. tissues.

**Ascorbic acid content :**

Ascorbic acid was extracted from chickpea seedlings (0.500 g) by adding 5 ml m-phosphoric acid–acetic acid solution and ascorbic acid was estimated as per Malik and Singh (1980).

**Per cent disease incidence :**

The incidence of wilt in each treatment was recorded based on the germination at 28 days after sowing (DAS) using following formula. The observations were based on 25 seeds sown in each pot (Rao and Sitaramaih, 2000).

$$\text{Per cent disease incidence} = \frac{\text{No. of seedlings survived in normal pots} - \text{No. of seedlings survived in infected pots}}{\text{No. of germinated seedlings in normal pots}} \times 100$$

## RESULTS AND DISCUSSION

In present experiment, specific activity of ascorbat peroxidase(APX) and its substrate ascorbic acid level in seedlings of five chickpea varieties grown in control pots ($T_1$), wilt sick pots ($T_2$), wilt sick soil + *Pseudomonas* treated seed pots culture($T_3$) and only *Pseudomonas* treated seed ($T_4$) plants at different crop growth and disease developmental stages was measured.

The results for mean varietals differences were found statistically significant for their APX specific activity as well as ascorbic acid content in chickpea seedling (Fig. 1A). Among them, resistant varieties JCP-27 (3.434 $U.mg^{-1}$ protein) showed maximum activity of APX which followed by WR-315 (3.098 $U.mg^{-1}$protein), while susceptible varieties JG-62 showed the lowest values (1.883 $U.mg^{-1}$protein). Similar results were observed in pearl millet leaves (Yadav *et al*., 1998) and groundnut seedlings (Gajera, 2009) revealed the presence of higher ascorbic acid content in tolerant varieties. The highest content of ascorbic acid was recorded in variety GG-1 (8.29 $mg.100\ g^{-1}$ Fr.Wt.) and lowest value was obtained with JG-62 (Fig. 1B). The trend of APX activity and ascorbic acid content was also reflects variety vise mortality due to wilt (Table 1). Wilt disease incidence was higher in susceptible (JG-62; 100%) varieties and lower in tolerant (WR-315 and JCP-27 *i.e.* 10.7 and 6.7 %, respectively) and moderately tolerant variety (GG-1 *i.e.* 22.7%) of chickpea in *Foc* infected pot ($T_2$) at 28 DAS ($S_7$). This indicates the special adaptation of resistant variety against biotic stress by induction of antioxidant and peroxide scavenging system (Rathod, 2008).

Mean data for different treatments resulted significant difference in mean value of APX activity irrespective of variety

and stages. The minimum specific activity of APX (1.973 U.mg$^{-1}$ protein) was recorded in control pots treatment $T_1$ (Fig. 2A). The mean activity significantly increased (2.679 U.mg$^{-1}$ protein) in a plants obtained from wilt sick soil pots ($T_2$) as well as wilt sick soil + *Pf-3* treated seed pots $T_3$ (2.834 U.mg$^{-1}$ protein) and recorded maximum mean activity for *Pf-3* seed treated pots alone $T_4$ (3.034 U.mg$^{-1}$protein). So far as ascorbic acid was concerned, $T_4$ showed the highest ascorbic acid content (6.98 mg.100 g$^{-1}$ Fr.Wt.) followed by control $T_1$ (6.81 mg.100 g$^{-1}$Fr.Wt.) and $T_3$ (6.21 mg.100 g$^{-1}$Fr.Wt.). The significantly lowest

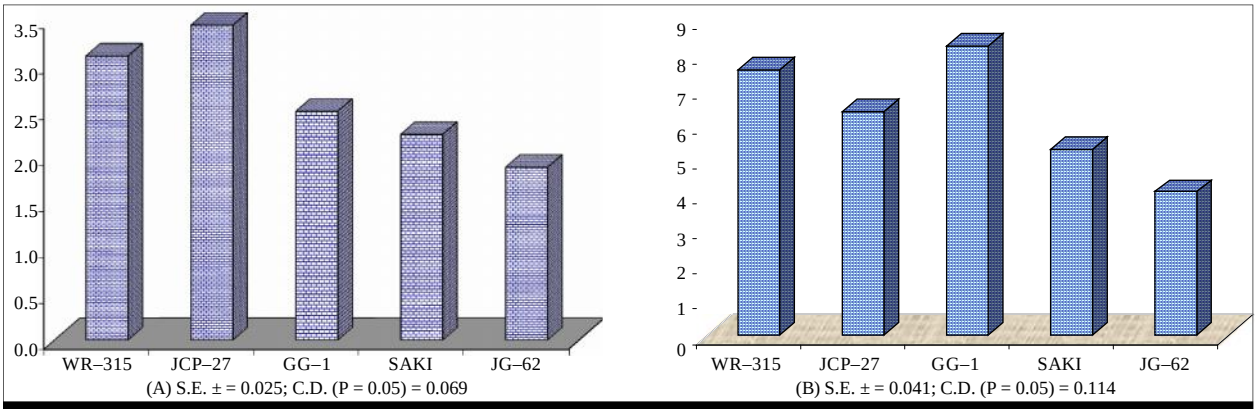



**Fig. 1 : Mean effect of varieties on (A) ascorbate peroxidase activity (change in U.mg$^{-1}$ protein) and (B)Ascorbic acid content (mg.100g$^{-1}$ Fr.Wt.) in chickpea seedlings**

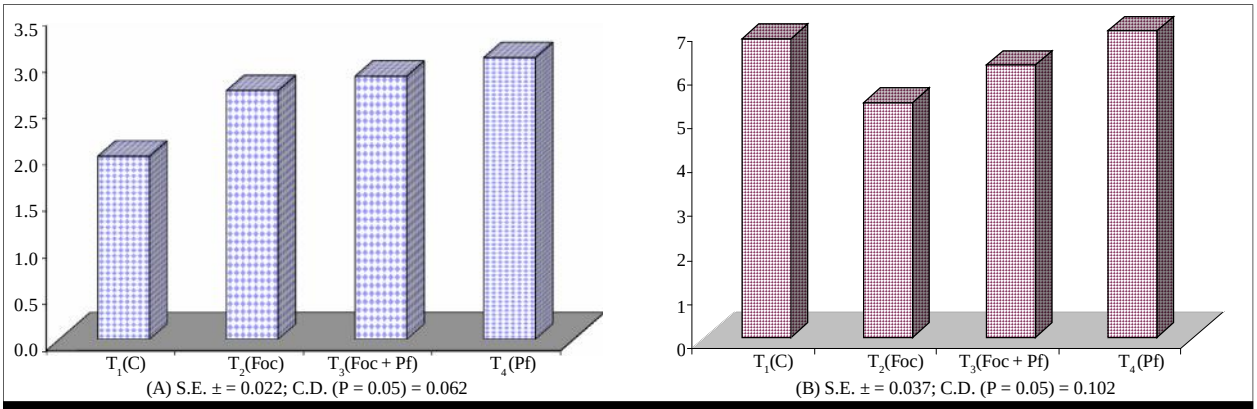



**Fig. 2 : Mean effect of treatments on (A) ascorbate peroxidase activity (change in U.mg$^{-1}$ protein) and (B)Ascorbic acid content (mg.100g$^{-1}$ Fr.Wt.) in chickpea seedlings**

**Table 1 : Per cent disease incidence of wilt in chickpea seedlings at 28 DAS**

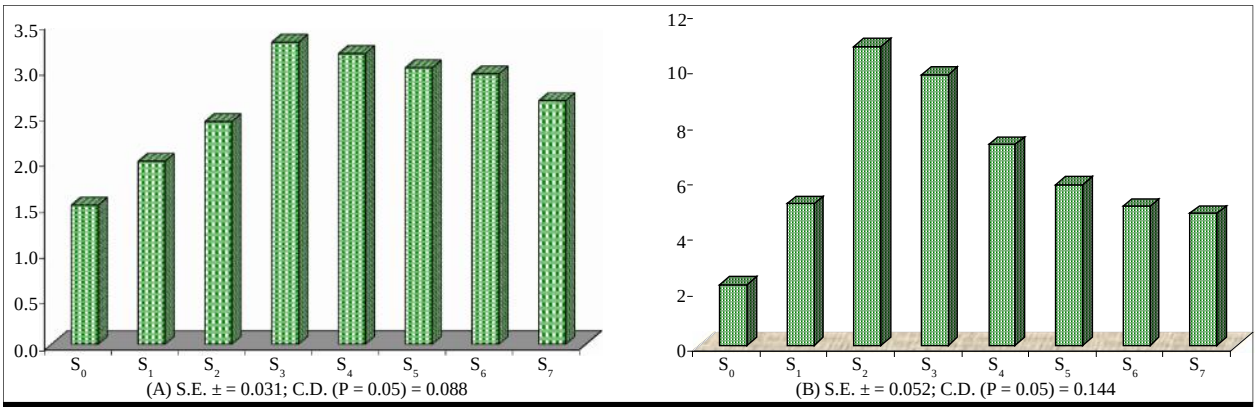
| Treatments | Varieties | | | | |
|---|---|---|---|---|---|
| | WR-315 ($V_1$) | JCP-27 ($V_2$) | GG-1 ($V_3$) | SAKI ($V_4$) | JG-62 ($V_5$) |
| | Per cent disease incidence | | | | |
| C* ($T_1$) | 1.28 (0.0)** | 1.28 (0.0) | 1.28 (0.0) | 1.28 (0.0) | 4.70 (1.3) |
| F ($T_2$) | 18.99 (10.7) | 14.80 (6.7) | 28.41 (22.7) | 70.07 (84.0) | 88.72 (100.0) |
| F + Pf ($T_3$) | 1.28 (0.0) | 1.28 (0.0) | 1.28 (0.0) | 13.17 (5.3) | 23.47 (16.0) |
| Pf ($T_4$) | 1.28 (0.0) | 1.28 (0.0) | 1.28 (0.0) | 1.28 (0.0) | 4.70 (1.3) |
| S.E. ± | | | 1.64 | | |
| C.D. (P = 0.05) | | | 4.62 | | |
| C.V. (%) | | | 29.08 | | |

* C=Control ($T_1$), F = *Fusarium oxyspoum* f.sp. *ciceri* sick soil ($T_2$), F + Pf = *Fusarium oxyspoum* f.sp. *ciceri* sick soil + *Pseudomonas fluorescen* seed treatment ($T_3$) and Pf = *Pseudomonas fluorescens* seed treatment ($T_4$); ** Figures in parenthesis indicate retransformed (original) value of arc sin transformation

(5.34 mg. 100 $g^{-1}$ Fr.Wt.) content was found in chickpea plants grown in sick soil inoculated with *Fusarium oxysporum* f.sp. *ciceri* ($T_2$) (Fig. 2B).

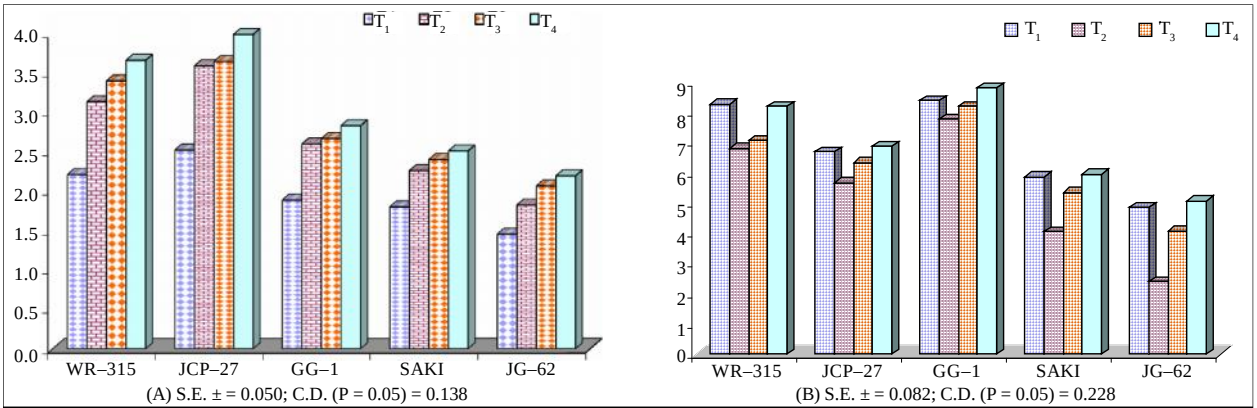
Among the different stages of chickpea seedlings, mean specific activity of APX enzyme significantly varied between 1.519 and 3.296 U.$mg^{-1}$ protein . The activity was significantly raised from 0 DAS-$S_0$ (1.519 U.$mg^{-1}$ protein) to 12 DAS-$S_3$ (3.296 U.$mg^{-1}$ protein) followed by declined with advancement of crop growth of chickpea but remain higher compared to initial stage (Fig. 3A). Earlier results on also depicted similar pattern. Garcia *et al*. (2002) reported higher level of APX activity in chickpea plants parts as the disease progress. De Meyer *et al*. (1999) also stated that rhizosphere colonization by *Pseudomonas aeruginosa* activated APX activity in bean roots. In case of, ascorbic acid content, it significantly increased from 2.19 ($S_0$) to 10.77 mg.100 $g^{-1}$ Fr.Wt. ($S_2$) showed 4.9 times increased (Fig. 3B) with the advancement of crop growth stages. Then it was declined gradually to lower value (4.76 mg.100 $g^{-1}$Fr.Wt.).

So far as interaction effect of varieties and treatment (V×T) was concerned, the APX activity in resistant varieties WR-315 significantly increased about 42, 54 and 66 per cent in treatment, *Foc* + *Pf-3* treatment ($T_3$) and plants of *Pseudomonas* seed treatment only ($T_4$), respectively, as compared to the control ($T_1$). The same was 42, 45 and 58 per cent for the JCP-27(Fig. 4A). In case of GG-1 and SAKI, follows the same trend for $T_2$, $T_3$ and $T_4$. In case of susceptible variety (JG-62), the activity remained low for all the respective treatments as compared to resistant varieties. The minimum ascorbic acid content in $T_2$ for seedlings of JG-62 (Fig. 4B). However, seeds sown with *P. fluorescens* treatment ($T_4$) had maximum ascorbic acid content and it was varied between 5.01 (JG-62) to 8.80 mg.$g^{-1}$ Fr.Wt. (GG-1). The content was significantly higher in treatment $T_3$ (*P. fluorescens* along with *F. oxysporum* f.sp. *ciceri*) as compared to the treatment $T_2$ (*Foc* inoculated soil) however, it was less than the $T_1$ and $T_4$. However, the content declined in susceptible variety in $T_3$ as compared to resistant one but increase in ascorbic acid content in $T_3$ as compared to $T_2$ was almost 69 per cent for the same variety (JG-62). Seed treatment of *Pseudomonas fluorescens*




(A) S.E. ± = 0.031; C.D. (P = 0.05) = 0.088 (B) S.E. ± = 0.052; C.D. (P = 0.05) = 0.144

**Fig. 3 : Mean effect of growth stages on (A) ascorbate peroxidase activity (change in U.$mg^{-1}$ protein) and (B)Ascorbic acid content (mg.100$g^{-1}$ Fr.Wt.) in chickpea seedlings**




(A) S.E. ± = 0.050; C.D. (P = 0.05) = 0.138 (B) S.E. ± = 0.082; C.D. (P = 0.05) = 0.228

**Fig. 4 : Interaction effect of V×T on (A) ascorbate peroxidase activity (change in U.$mg^{-1}$ protein) and (B)Ascorbic acid content (mg.100$g^{-1}$ Fr.Wt.) in chickpea seedlings**

*Pf-3* ($T_3$ and $T_4$) reduced disease incidence in all the varieties including susceptible variety JG-62 at same stage showed its efficacy against pathogen in pot culture (Table 1). Vidhyasekharan and Muthemilan (1995) was also reported that *Pseudomonas fluorescens* strains obtained from the rhizosphere of different crops showed inhibitory action against the wilt pathogen *Fusarium oxysporum* f. sp. *ciceri* of chickpea (*Cicer arietinum*) crop. Boer *et al.* (1998) reported the effectiveness of *Pseudomonas* against *Fusarium oxysporum.* Inam-ul-Haq *et al.* (2003) reported up to 96 per cent reduction in wilt disease in chickpea crop due to use of biocontrol agent *Pseudomonas fluorescens.*

In case of interaction effect between variety and stages (V×S), at initial stage ($S_0$), the APX activity and ascorbic acid content remains very low in different chickpea varieties. The specific activity of APX increased in a range of 128 to 134 per cent during $S_0$ to $S_3$ stages, respectively, in the resistant varieties WR-315 and JCP-27 followed by declines with advancement of crop growth stages progressed. In case of JG-62, the activity raised 105 per cent from to $S_0$ to $S_3$ stages. Overall, the APX activity was higher in resistant varieties as compared to the susceptible varieties during disease developmental stages. During disease developmental stages, ascorbic acid content was significantly increased from $S_0$ to $S_2$ stage in WR-315, JCP-27 and in GG-1 followed by continuous declined up to $S_7$ stage. In contrast to this, the same was also increased up to $S_3$ stage in Saki and JG-62 and then declined. Overall, tolerant varieties had higher ascorbic acid content followed by moderately susceptible and susceptible varieties during disease developmental stages. Wang *et al*. (2002) also found that the resistant cultivar maintained higher content of ascorbic acid than the susceptible cultivar against *F. oxysporum* f. sp. *niveum* at the seedling stage in water melon. Patykowski and Urbanek (2003) also showed that after inoculation with *Botrytis cinerea*, total ascorbic acid content decreased more in susceptible cultivars of tomato which supports our data.

So far as interaction effect of treatment and Stage (T×S) was concerned, the APX activity was higher in $T_4$ treatment followed by $T_3$, $T_2$ and $T_1$. The activity was increased only by

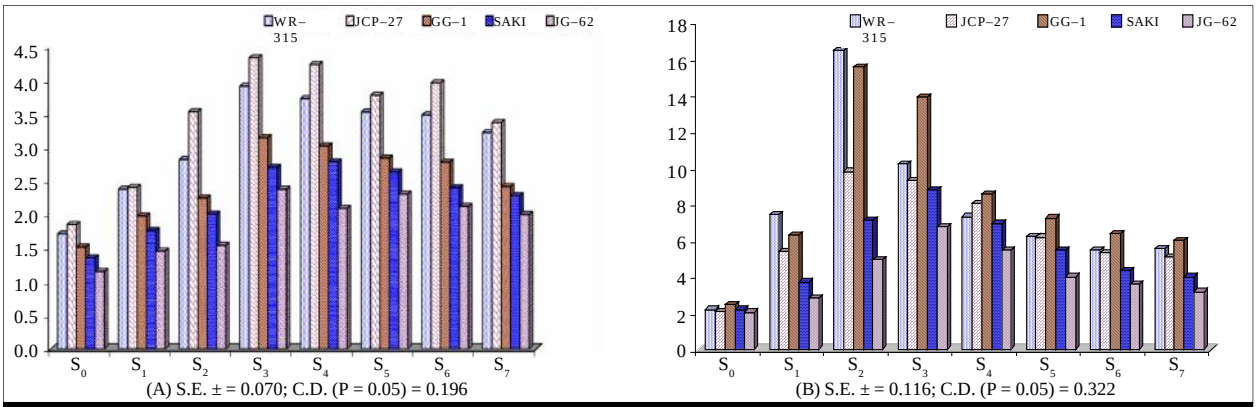



**Fig. 5 : Interaction effect of S×V on (A) ascorbate peroxidase activity (change in U.mg$^{-1}$ protein) and (B) Ascorbic acid content (mg.100g$^{-1}$ Fr.Wt.) in chickpea seedlings**

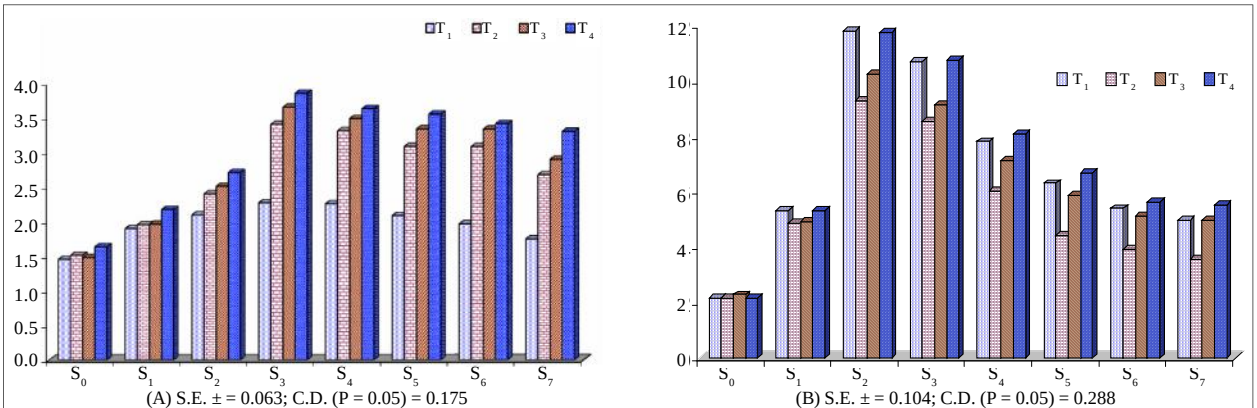



**Fig. 6 : Interaction effect of S×T on (A) ascorbate peroxidase activity (change in U.mg$^{-1}$ protein) and (B) Ascorbic acid content (mg.100g$^{-1}$ Fr.Wt.) in chickpea seedlings**

56 per cent during $S_0$ (1.453 U.mg$^{-1}$ protein) to $S_3$ stage (2.273 U.mg$^{-1}$ protein) in $T_1$ followed by gradual declined up to $S_7$ stage. In case of $T_3$ and $T_4$ the activity raised (147 and 136 %, respectively) at higher rate as compared to the rest of the treatments. Several studies have also shown that APX activity is induced in plants upon treatment with *Pseudomonas fluorescens* (Chen *et al.*, 2000; Sundaravadana, 2002 and Saravankumar *et al.*, 2003). Shooting up of APX activity in *Pseudomonas* treated chickpea plants in presence of fungus *S. sclerotiorum* suggested the role of PGPR as induced systemic resistance inducer (Basha *et al.*, 2006). In case of ascorbic acid, between the treatment-$T_4$ (*Pseudomonas* alone) and $T_1$ (control), did not show any significant differences for ascorbic acid content at all the stage observed except $S_5$ and $S_7$. However, the content remained higher as compared to $T_2$ and $T_3$ treatments for respective stages. The ascorbic acid content was significantly increased with progress of crop growth up to $S_2$ stage in all the other treatments followed by gradual decline (Kandoliya and Vakhalia, 2013 b and c). Lokesh *et al.* (2013) also worked on the efficacy of *Pseudomonas fluorescens* in relation to diseases incidence of soft rot in ginger in Uttara Kannada region of Karnataka.

**Conclusion :**

Overall, biocontrol agent *P. fluorescens* (*Pf-3*) can induced the ascorbic acid content and APX activity in a chickpea ($T_3$ and $T_4$) than the treatment $T_2$ and it was maintained through out the disease development stages. These results suggest that seed treatment of *Pseudomonas fluorescens* effective elicits activity of defense-related antioxidant system such as ascorbic acid and APX leading to improved plant resistance against soil borne plant disease.

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