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RESEARCH ARTICLE

Detoxification of oxalic acid by *Pseudomonas fluorescens* during wilt disease condition in chickpea plant

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ABSTRACT

Oxalic acid is a simple metabolite produced by many fungi including *Fusarium oxysporum* f.sp. *ciceri*. Its phytotoxic activity has been known for years and it has been implicated as a virulence factor for several phytopathogenic fungi. It was also reported that effective reduction of oxalic acid by *Pseudomonas fluorescens* in a culture medium. So the present experiment has been designed to prove the ability of *Pseudomonas fluorescens* isolate *(Pf-3)* as detoxification agent of oxalic acid produced by fungal pathogen in wilt disease condition at various stages of seedling growth in different varieties of chickpea. The result showed the higher oxalic acid content in susceptible variety as compared to resistant one which was positively corresponded to mortality of the seedling due to wilt in wilt sick soil compared to control one. The oxalic acid content was significantly high in a plant grown in wilt sick soil condition in general compared to control one which also reflected higher mortality rate of chickpea seedling particularly in susceptible variety JG-62 compared to rest of the varieties. The significantly lowest value of oxalic acid was observed in *Pseudomonas fluorescent Pf-3* seed treated chickpea plants grown even in wilt sick soil also. It indicated effective detoxification of oxalic acid by *Pf-3* which induced by *Fusarium oxysporum* f.sp. *ciceri* in chickpea seedling. It was also reflected in less mortality of seedlings due to wilt in respective treatment.

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INTRODUCTION

The phytotoxic activity of pathogenic fungi may reside in the structurally simple secondary metabolites, such as organic acids and their derivatives like oxalic acids (Berestetskiy, 2008). The oxalic acid content was found to be an indicator for development of wilt in chickpea. It proved the pathogenicity of *Fusarium oxysporum* f.sp. *ciceri* which caused wilt in chickpea. Its role in pathogenicity is for lowering the pH at the infection site to allow for optimal cell wall degradation by fungal enzymes to weaken the plant cell wall barrier (Bakkeren and Gold, 2004). Its phytotoxic activity has been known for years and it has been implicated as a virulent factor for several phytopathogenic fungi.In contrast to this, induction of systemic acquired resistance by oxalic acid in chickpea and other crops against fungal pathogen was also reported. Induced systemic resistance (ISR) of plants against pathogens is a widespread phenomenon that has been intensively investigated with respect to the underlying signalling pathways as well as to its potential use in plant protection (Heil and Bostock, 2002). It has also been reported the effective reduction of oxalic acid by *Pseudomonas fluorescens* in a culture medium. So, the present experiment was designed to prove the ability of *Pseudomonas fluorescens* isolate, *(Pf-3)* as detoxification agent of oxalic acid produced by fungal pathogen in wilt disease condition at various stages of seedling growth in different varieties of chickpea.

MATERIAL AND METHODS

Preparation of mass inoculums :

Pseudomonas fluorescens isolates used in the present experiment were isolated by following the method of Vlassak *et al.* (1992) from chickpea rhizosphere of ten different chickpea growing areas of Gujarat (India) using selective Kings B media (Simon and Ridge, 1974). Isolation of the pathogenic fungus *Fusarium oxysporum* f.sp. *ciceri* was made by tissue isolation technique using solidified PDA media in Petri plates (Subramanian, 1954). Per cent growth inhibition of *Fusarium oxysporum f.sp. ciceri.* by *Pseudomonas fluorescens* was measured using 20 ml of King's B+PDA medium (1:1) by dual culture techniques (Reddy *et al.* 2008) with some modification and optimization of media. All the inoculated plates were incubated at 30 ±1 ^{0}C. Index of antagonism was determined after six days of incubation (DAI) as described by Zarrin *et al.* (2009) and best isolate (*Pf-3*) was utilized for talk powder based seed treatment. Talc based powder for both pathogen, *Fusarium oxysporum* f.sp. *ciceri* as well as biocontrol agent *Pf-3* were prepared as methods out lined by Singh *et al.* (2001). *Fusarium oxysporum* f.sp. *ciceri* load on their talc based formulation was 2.5 x 10^7 *cfu* / g talc powder which was used for preparation of sick soil for further study. Similarly, *Pf-3* microbes on their talc based formulations had 3 × 10^8 *cfu* / g talc powder which was used for seed treatment purpose.

Seed sowing and seed treatment :

Earthen pots were washed thoroughly with tap water, followed by washing 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 seeds of chickpea varieties differed in susceptibility to wilt *i.e.* WR-315, JCP-27(V2), GG-1(V3), Saki (V4) and JG-62 (V5) 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 of biocontrol agents *Pf-3* (microbial load 3 × 10^8 *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 × 10^8 *cfu*/g talc powder) and sown in normal soil pots.

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 (Rao and Sitaramaih, 2000). The observations were based on 25 seeds sown in each pot.

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

Estimation of oxalic acid content :

The oxalic acid content was estimated as per Mahadevan and Sridhar (1986) from whole chickpea plant collected at different stages *viz.*, 0 (S_0), 4 (S_1), 8 (S_2), 12 (S_3), 16 (S_4), 20(S_5), 24 (S_6), and 28 Days after sowing (DAS) (S_7).

RESULTS AND DISCUSSION

The findings of the present study as well as relevant discussion have been presented under following heads :

Per cent disease incidence :

Chickpea varieties were grown in control (T_1) , wilt sick pot (T_2), wilt sick pot+ *Pf-3* seed treated (T_3) and *Pf-3* seed treated pots alone (T_4). The per cent disease incidence was based on wilting of chickpea plants up to 28 days after sowing

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.Em ±			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.

(DAS) in a pot was recorded and showed statistically significant difference. Wilt disease incidence was higher in susceptible (JG-62; 100%) varieties ($V_5T_2S_7$) 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). Seed treatment of *Pseudomonas fluorescens 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) Vidhyasekaran 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 % reduction in wilt disease in chickpea crop due to use of biocontrol agent, *Pseudomonas fluorescens.*

Oxalic acid content in chickpea plant:

Chickpea varieties grown in control (T_1), wilt sick (T_2), wilt sick + seed treated with *Pseudomonas fluorescent Pf-3*(T_3) and seed treated with *Pseudomonas fluorescens Pf-3* alone (T_4) pots have shown significant difference in oxalic acid content (Fig. 1 and Table 2). The mean value of oxalic acid content in varieties WR-315 (V_1) was lower (0.423 mg.g^{-1} Fr. Wt.) and it significantly increased and reached the maximum level (0.695 mg.g^{-1} Fr. Wt.) in susceptible variety JG-62 (V_5) which was 64 per cent and 40 per cent higher than the resistant variety WR-315 and JCP-27, respectively (Fig. 1 A). This also reflected higher mortality rate in susceptible variety (Table 1) due to infection with pathogen. Among the treatments, T_2 showed highest oxalic acid content (0.806 mg.g^{-1} Fr. Wt.) which may be induced by the pathogen, *F. oxysporum* f. sp. *ciceri*. The significantly lowest value (0.384 mg.g^{-1} Fr. Wt.) was observed in T_4 (*Pseudomonas fluorescent Pf-3* treated alone) (Fig.1 B) reflects effective detoxification of oxalic acid and less mortality of seedlings. The oxalic acid content was significantly increased due to process of infections with the advancement of disease (Fig. 1 C).

Mean interaction effects of V x T significantly differed for oxalic acid content (Fig.2 A). The different varieties of chickpea grown in wilt sick pots (T_2) had maximum oxalic acid content in the range of 0.593 to 1.164 mg.g^{-1} Fr. Wt., however, in the plants received from treatment T_4 and control pots (T_1) had minimum oxalic acid content in the range of 0.313 to 0.458 and 0.394 to 0.565 mg.g-1 Fr. Wt., respectively. In case of wilt sick + *Pf-3* treated seeds (T_3) significantly reduced the oxalic acid contents compared to the T_2. Maximum oxalic acid content in V_5T_2 (1.164 mg.g^{-1} Fr. Wt.) was recorded in wilt susceptible variety (JG-62) which was significantly reduced to 50% and 41% in V_5T_3 treatment (wilt sick+seed treated with *Pf-3*) and

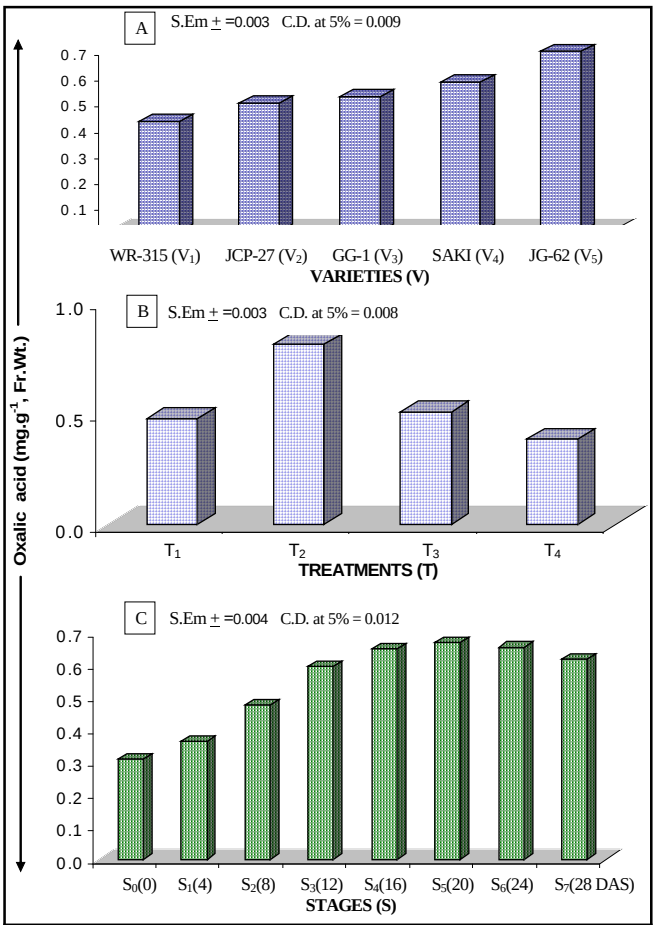

Fig. 1 : Mean effect of (A) varieties, (B) treatments and (C) stages on oxalic acid content (mg.g^{-1} Fr.Wt.) in chickpea seedlings

V_5T_4 treatment (Seed treated with *Pf-3* alone). However, resistant varieties JCP-27 and WR-315 had lower (0.665 and 0.593 mg.g-1 Fr. Wt., respectively) oxalic acid content in T_2 and it was also further significantly reduced to 66-70% in T_3 treatment and 52-57% in T_4 treatment. Other two moderately tolerant varieties GG-1 and SAKI revealed higher values of oxalic acid content than tolerant varieties but lower than the susceptible variety JG-62. Combined effects of V x S were found to be significant (Fig. 2 B). During disease developmental stages (S_0 to S_7), oxalic acid content was increased significantly from 0.240 (V_1S_0) to 0.521 mg.g^{-1} Fr. Wt. (V_1S_4) in tolerant variety WR-315 followed by significantly declined up to S_7. Incase of JCP-27, oxalic acid content was increased significantly from 0.310 (V_2S_0) to 0.599 mg.g^{-1} Fr. Wt. (V_2S_5) followed by significantly declined up to S_7. However, the difference between S_4 and S_5 was at par. The same trend was followed by other two varieties *i.e.* GG-1 and SAKI. Whereas, the content was gradually increased up to 24 DAS (V_5S_7) in susceptible variety JG-62. Overall, tolerant varieties had lower oxalic acid content followed by

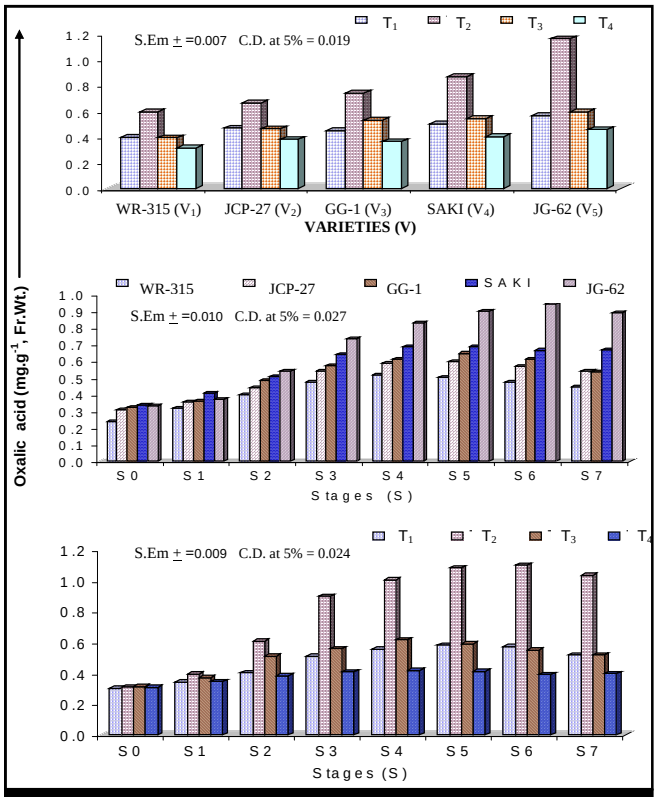

Fig. 2 : Interaction effect of (A) V x T, (B) V x S and (C) S x T on oxalic acid content (mg.g^{-1} Fr.Wt.) in chickpea seedlings

moderately susceptible and susceptible variety during disease developmental stages. Interaction effects of T x S for oxalic acid content were significant (Fig. 2 C). Chickpea varieties sown in sick soil (T_2) had the highest oxalic acid content followed by T_3, T_1 and T_4. In wilt sick pot (T_2) grown seedlings, the content was significantly increased with progress of disease developmental stages upto S_6 followed by decreased at S_7 however, treatment T_3 and T_4 showed rise up to S_4 stage (16 DAS) only. In case of T_1, it showed same trend up to T_5 and then declined up to T_7. Seeds sown in wilt sick soil (T_2) recorded 1.103 mg.g^{-1} Fr. Wt. oxalic acid content at S_6 stage while the same was 50% and 35% lower in T3 and T_4 treatment at same stage.

Interaction effects of V x T x S for oxalic acid content revealed significant differences (Table 2). Control (T_1) and 0 DAS (S_0) stage recorded minimum oxalic acid content, while maximum was in $V_5T_2S_7$ (1.855 mg.g^{-1} Fr. Wt.). For the JG-62 variety, oxalic acid content gradually increased and reached up to maximum level in wilt sick soil at 28 DAS (*i.e.* $V_5T_2S_7$), possibly due to the secretion of oxalic acid by fungal pathogen and progress of disease. Whereas, the same was raised up to 16-20 DAS (S_5 -S_6 stage) at lower rate for rest of the varieties as compared to the susceptible variety (JG-62) and rest of the treatments. The significantly lower values of oxalic acid were noted in all the varieties for T_3 treatment which indicated effective detoxification of oxalic acid by *Pf-3* that induced due to *Fusarium oxysporum* f.sp. *ciceri* in chickpea seedling.

Table 2 : Interaction effect of varieties, treatments and stages on oxalic acid content (mg.g^{-1}, Fr.Wt.) in chickpea seedlings

Variety (V)	Treatment* (T)	Disease development stages (DAS) (S)							
		S0 (0)	S1 (4)	S2 (8)	S3 (12)	S4 (16)	S5 (20)	S6 (24)	S7 (28**)
WR-315	C (T_1)	0.236	0.304	0.363	0.455	0.489	0.447	0.439	0.422
(V_1)	*F* (T_2)	0.245	0.363	0.489	0.641	0.810	0.784	0.751	0.666
	F+Pf (T_3)	0.245	0.320	0.413	0.439	0.447	0.472	0.413	0.388
	Pf (T_4)	0.236	0.295	0.337	0.363	0.337	0.312	0.312	0.312
JCP-27	C (T_1)	0.287	0.337	0.380	0.514	0.540	0.582	0.540	0.565
(V_2)	*F* (T_2)	0.320	0.388	0.540	0.767	0.860	0.877	0.852	0.717
	F+Pf (T_3)	0.312	0.363	0.447	0.472	0.531	0.548	0.514	0.514
	Pf (T_4)	0.320	0.337	0.405	0.422	0.422	0.388	0.371	0.380
GG-1	C (T_1)	0.329	0.337	0.405	0.422	0.472	0.548	0.565	0.489
(V_3)	*F* (T_2)	0.320	0.405	0.590	0.860	0.953	1.063	0.978	0.759
	F+Pf (T_3)	0.320	0.371	0.548	0.624	0.649	0.616	0.557	0.565
	Pf (T_4)	0.320	0.337	0.405	0.396	0.380	0.371	0.354	0.346
SAKI	C (T_1)	0.312	0.371	0.447	0.573	0.590	0.616	0.590	0.489
(V_4)	*F* (T_2)	0.337	0.447	0.666	0.987	1.071	1.113	1.130	1.181
	F+Pf (T_3)	0.354	0.447	0.548	0.607	0.666	0.624	0.565	0.523
	Pf (T_4)	0.354	0.380	0.371	0.405	0.439	0.405	0.388	0.489
JG-62	C (T_1)	0.346	0.371	0.422	0.590	0.692	0.725	0.742	0.633
(V_4)	*F* (T_2)	0.337	0.371	0.759	1.248	1.341	1.594	1.805	1.855
	F+Pf (T_3)	0.346	0.363	0.599	0.649	0.784	0.700	0.700	0.607
	Pf (T_4)	0.312	0.380	0.396	0.455	0.514	0.590	0.540	0.472
V x T x S		S.Em ± = 0.019			C.D.(p=0.05) =0.054			C.V.% = 6.14	

* 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 indicates Days after sowing (DAS)

This results are in agreement with Maurya *et al*. (2005) who reported the accumulation of oxalic acid in chickpea seedling infected by *Sclerotium rolfsii*. Nagrajkumar *et al*. (2005) reported that the virulent fungal isolates produced more oxalic acid. They also reported that effective reduction of oxalic acid by *Pseudomonas fluorescens* strain pfMDU2 in a culture medium proving the ability of *Pseudomonas fluorescens* as detoxification agent of oxalic acid produced by fungal pathogen in disease conditions.

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