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Induced resistance and phenolic acid accumulation in biological control of chickpea wilt by *Pseudomonas fluorescens*

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HPLC analysis of chickpea *(Cicer arietinum)* plant at various wilt disease developmental stages challenged with *Fusarium oxysporum* f.sp *ciceri* indicates the plants contain high amount of phenolics when the seed was treated with *Pseudomonas fluorescens* local isolates Pf-3. This biocontrol bacterial isolate induced the synthesis of specific phenolic acids like salicylic acid, chlorogenic acid etc. with varied amounts at different disease development stages of wilt in chickpea. Vannilic, chlorogenic and hydroquinone were also found higher in plants treated with *Fusarium oxysporum* f.sp. *ciceri*.All the above mentioned phenolics was found higher amount in resistant variety than susceptible variety. The induction of antifungal phenolic acids in chickpea plant in the present investigation due to the application of the local isolate Pf-3 provides a biochemical basis of induced resistance in chickpea against wilt disease.

Key words : Chickpea, *Fusarium oxysporum* f.sp. *ciceri*, HPLC, Phenolics, *Pseudomonas fluorescens*, Salicylic acid

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INTRODUCTION

Chickpea is a major source of human and domestic animal food, in India. The low productivity in chickpea is due to the several biotic and abiotic factors. Among biotic factors, wilt caused by *Fusarium oxysporum* f.sp. *ciceri* is the major problem. This pathogen is difficult to control with cultural practices and chemical control is one of the most used approaches. Biological control and plant resistance, however, provide an environmentally and economically appropriate means for disease control that can be easily included within an integrated disease management strategy (Mohamed *et al*., 2007). Different studies showed that induced resistance, through the accumulation of various phenolic compounds and phytoalexins may play a crucial role in the biological control and resistance of chickpea to pathogenic attacks. Phenolics are well known anti-fungal, anti-bacterial and anti-viral compounds occurring in plants (Sarma and Singh, 2003). The first step of the defense mechanism in plants involves a rapid accumulation of phenols at the infection site (Matern and Kneusal, 1988). Phenols by their simple structure penetrate the cell of microorganisms cause considerable damage to the cell metabolisms. Looking into the antifungal activity of phenolic acids, the present study was conducted to investigate the biochemical bases for systemic induction of phenolic compounds in chickpea plant following seed treatment of *Pseudomonas fluorescens.*

RESEARCH METHODOLOGY

Chickpea (*Cicer arietinum* L.) 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, Gujarat, India.

Preparation of mass inoculums :

Pseudomonas fluorescens isolates used in 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 *F. oxysporum* f.sp. *ciceri* by *P. fluorescens in vitro* 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 performing isolate (Pf-3) was utilized for talk powder based seed treatment.Talc based powder for both pathogen- *F. oxysporum* f.sp. *ciceri* as well as biocontrol agent Pf-3 were prepared as methods out lined by Singh *et al.* (2001). *F. oxysporum* f.sp. *ciceri* load on their talc based formulation was 2.5x10^7 *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 3x10^8 *cfu*/g talc powder which was used for seed treatment purpose.

Seed sowing and seed treatment :

Chickpea seed of (*Cicer arietinum* L.) differed in susceptibility to wilt *i.e.* WR-315(V_1), JCP-27(V_2), GG-1(V_3), Saki (V_4) and JG-62 (V_5) were grown in earthen pots. 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 moistened with water, so, talc formulations retained to the seeds.

T_1 = Seeds were treated with talc based powder only and sown in pots having normal soil 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 X 10^8 *cfu*/g talc powder) and sown in sick (*F. 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 x 10^8 *cfu*/g talc powder) and sown in pots having normal soil.

Recommended package of practices were followed to raise a plants in pots.

Sampling for HPLC analysis of phenolics :

Plants were excavated from each pots at three different stages *viz.*, disease initiation (20 DAS- S_1), moderate disease (24 DAS-S_2) and severe disease (28 DAS-S_3) based on wilt symptoms development in susceptible variety JG-62 (Stevenson and Veitch, 1998). Phenolic extraction was done using whole plant according to methods developed by Sharma and Singh (2002).

HPLC analysis of phenolic acids :

Analysis of the samples was performed as per the method of Sharma *et al.* (2002). The HPLC system (Shimadzu Corporation, Kyoto, Japan) was equipped with two Shimadzu LC-10 ATVP reciprocating pumps, a variable Shimadzu SPD-10 Rheodyne Model 7725 injector with a loop size of 20 µl. Reverse phase chromatographic analysis was carried out in isocratic conditions using C-18 reverse phase column (250 ×4.6 mm id, particle size 5 µm Luna 5µ C-18 (2), Phenomenex, USA) at 25^{o}C. Running conditions included injection volume 20 µl, mobile phase methanol: 0.4 per cent acetic acid (80: 20 v/v), flow rate 0.75 ml/min and detection at 290 nm. Samples were filtered through membrane filter (pore size 0.45 µm, E-Merck, Germany) prior to injection in sample loop. Phenolic acids present in the samples were identified by comparing chromatographic peaks with the retention time (Rt) of individual standards and further confirmed by co-injection with isolated standards.

RESEARCH FINDINGS AND ANALYSIS

Individual phenolics of chickpea varieties grown in control (T_1), wilt sick (T_2), wilt sick (*Foc*)+ *Pseudomonas fluorescens* Pf-3 seed treated (T_3) and *P. fluorescens* Pf-3 seed treated alone (T_4) pots at different disease developmental stages were analyzed through HPLC. After separation of phenolic acids, individual phenolics were detected with corresponding retention time (Rt) of standard phenolic compounds. Total 8 phenolic acids were identified using HPLC and data are presented in Fig. 1 to 4.

Salicylic acid (SA) was higher in healthy tissues (T_1) at disease initiation stage S_1 and it declined with disease advancement (Fig.1). All the chickpea varieties showed higher amount of SA in plants grown in normal soil *i.e.* control

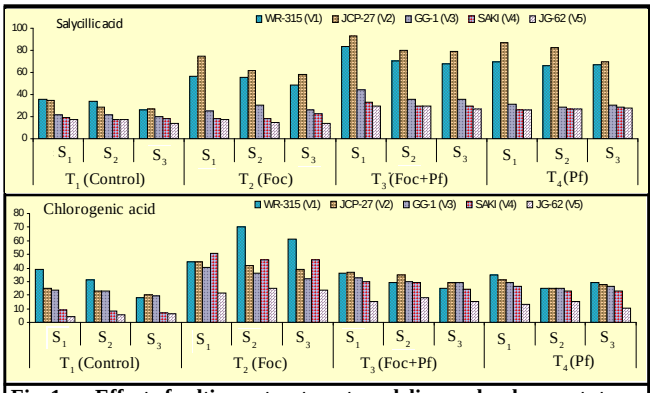

Fig. 1: Effect of cultivars, treatments and disease development stages on salycillic acid and chlorogenic acid content («g.g^{-1} Fr.Wt.) in chickpea seedling

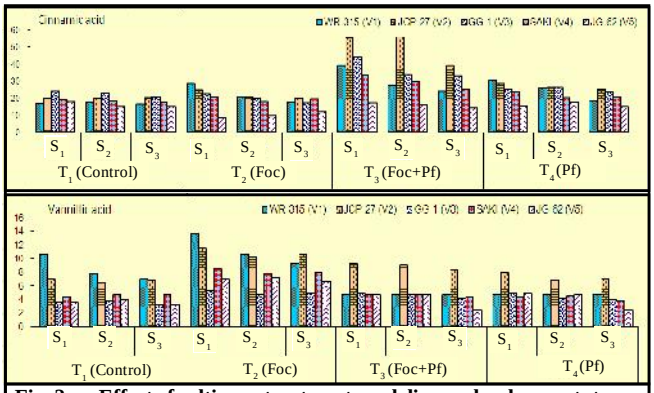

Fig. 2: Effect of cultivars, treatments and disease development stages on cinnamic acid and vanillic acid content («g.g^{-1} Fr.Wt.) in chickpea seedling

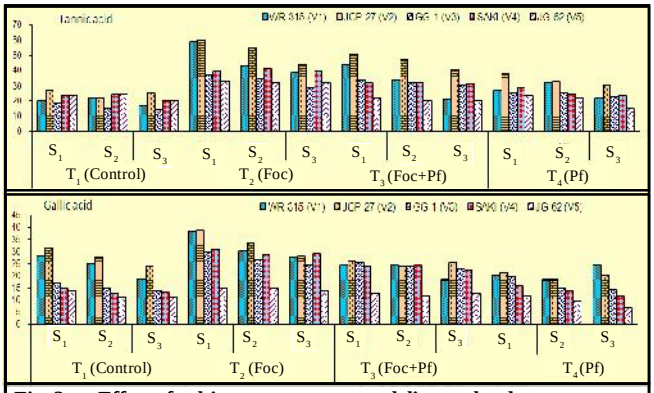

Fig. 3: Effect of cultivars, treatments and disease development stages on tannic acid and gallic acid content («g.g^{-1} Fr.Wt.) in chickpea seedling

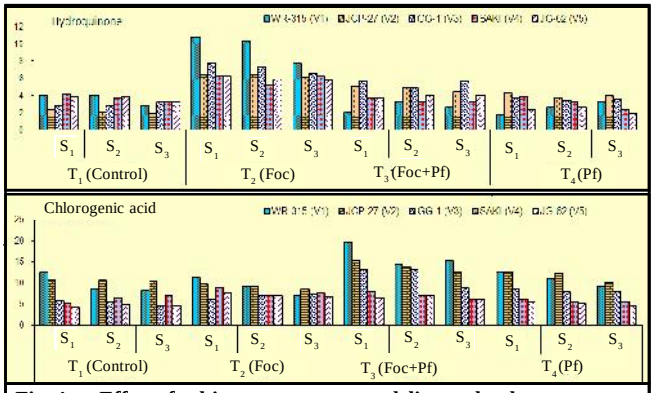

Fig. 4: Effect of cultivars, treatments and disease development stages on hydroquinone and ellagic acid content («g.g^{-1} Fr.Wt.) in chickpea seedling

T_1. The content increased in plants raised in Foc inoculated soil (T_2) and the greater accumulation was recorded with the tolerant varieties *i.e.* WR-315 and JCP-27. The SA was further induced by the Pf-3 treatment *i.e.* T_3 (Foc+Pf-3) and T_4 (Pf-3 alone). The highest value (13.7 μg.g^{-1} Fr. Wt.) was noted for variety JCP-27 in T_3 treatment at disease initiation stage (S_1). In general, salicylic acid (SA) was found higher in wilt sick+ *P. fluorescens* (Pf-3) treatment indicates possible induced systemic resistance in response to both of the microbs. Several workers reported that hypersensitive plant responses induced the accumulation of salicylic acid which is a signal transduction molecule participating in local as well as systemic acquired resistance (Malamy *et al.*, 1990; Metraux *et al.*, 1990). Ribincky *et al.* (1998) reported that the increased concentration of salicylic acid induced the accumulation of a pathogenesis-related protein (PR-1) and increased resistance in tobacco to TMV.

Similar pattern was observed for the chlorogenic acid at moderate diseased stage S_2 and severe diseased stage S_3 in all the varieties of chickpea plants obtained from T_1. The chlorogenic acid in tolerant varieties WR-315 (38.7 μg.g^{-1} Fr. Wt.) and JCP-27 (24.9 μg.g^{-1} Fr. Wt.) was found higher amount compared to susceptible variety JG-62 (4.2 μg.g^{-1} Fr. Wt.) in control treatment (Fig.1). In contrast to salicylic acid, chlorogenic acid content was found higher in T_2, followed by treatment T_3. The higher amount chlorogenic acid was maintained in *P. fluorescens* Pf-3 seed treated (T_3) seedlings through out the disease developmental stages. In general, tolerant varieties had higher amount of chlorogenic acid content than susceptible varieties by these stages. Raskar *et al.* (2005) also found higher levels of total phenols and chlorogenic acid in more tolerant to Foc than the susceptible cultivar of chickpea plant parts.

In control treatment T_1, cinnamic acid was found higher in variety GG-1 (Fig. 2). The content was increased with the infection (*i.e.* in T_2) however; the rate of increase was higher in resistant variety as compared to the other varieties. The content further showed increasing trends in T_3 and followed the same trend as salicylic acid. The highest cinnamic acid (55.9 μg.g^{-1} Fr. Wt.) was recorded in JCP-27 variety at S_6 stage in T_3 treatment (sick soil+ *Pf-3* seed treated). Cinnamic acid is synthesized from phenylalanine through catalysis by PAL and plays a key role in host resistance under pathogenic stress (Sharma *et al.*, 2002).

Infected seedling (T_2) in all chickpea varieties had higher amount of vanillic acid (Fig.2). The concentration of vanillic acid (13.7 μg.g^{-1} Fr.Wt.) was higher in WR-315 variety at S_1. However, the amount of vanillic acid remained high in JCP-27 in Pf-3 seed treated (T_3 and T_4) seedlings. Overall, tolerant variety particularly JCP-27 had higher vannilic acid than susceptible during disease developmental stages.

Tannic acid was higher in T_2 treatment (sick) at all stages and in all the chickpea varieties (Fig. 3). The highest tannic acid was recorded in JCP-27 (59.49 μg.g^{-1} Fr. Wt.) followed by declined in rest of the treatments. However, the concentration of tannic acid was maintained higher in T_2, T_3, and T_4 as compared to T_1.

The concentration of gallic acid was increased in infected (T_2) and at higher rate as compared to the control (Fig. 3). The gallic acid was higher in resistant variety in T_3 (Foc + Pf-3) treatment for all chickpea varieties. The highest (36.6 $\mu g.g^{-1}$ Fr. Wt.) gallic acid was recorded in JCP-27 variety in T_2 (*Foc*) treatment. In general, resistant varieties (JCP-27 and WR-315) had higher gallic acid than susceptible variety JG-62. Gallic acid not being antifungal, is converted into gallotannins, which along with other tannins, is also known to provide protection to the hosts from bacterial and fungal infections (Salisbury and Ross, 1986). Singh *et al.* (2000) have shown successful control of powdery mildew of pea through the application of *P. fluorescens* (strains Pf1, Pf3, Pf5) and *P.aeruginosa* (Pag). Hence, the report of the induction of antifungal phenolic acids in chickpea plant in the present investigation due to the application of the local isolate Pf-3 provides a biochemical basis of resistance in chickpea against wilt disease.

The higher amount of hydroquinone (HQ) was recorded at all stages in infected (T_2) seedlings of all chickpea varieties and the content reduced with advancement of disease, except GG-1 infected with *Fusarium oxysporum* (T_2) (Fig. 4). The level of HQ declined in all chickpea varieties in T_3 followed by T_4 *i.e.* in a *Pf-3* treated seed plants as compared to T_2. The highest level of HQ was recorded in JCP-27 (10.8 $\mu g.g^{-1}$ Fr. Wt.) of T_2 treatment after 20 DAS (at S_1 stage).

Higher amount of ellagic acid was detected in healthy seedling (T_1) at either S_2 or S_3 in resistant variety JCP-27 and WR-316 as compared to susceptible variety JG-62. The content showed slight rise under inflectional stage of treatment T_2. Again the rise was found in a seedling of Pf-3 treated seed (T_3) pots (Fig.4). The highest content of ellagic acid (19.6 $\mu g.g^{-1}$Fr.Wt.) was detected in WR-315 variety at S_1 in treatment T_3. Varieties JCP-27 and GG-1 had comparatively higher ellagic acid than Saki and, JG-62 in T_3 and T_4 treatment. Maximum induction of phenolics like ellagic acids was also found in PGPR *S. marcescens* NBRI1213–treated betelvine plants infected with *Phytophthora nicotianae* (Lavania *et al.*, 2006).

In general, Individual phenolic acids, particularly salicylic acid, cinnamic acid and ellagic acid was more accumulated in seedlings of a chickpea seed treated with *Pseudomonas fluorescens* grown in wilt sick pots (T_3). Whereas vannilic, chlorogenic and hydroquinone found higher in T_2. All the above mentioned phenolics was found higher amount in resistant variety than susceptible variety. Singh *et al*. (2003) reported plants growth promoting rhizobacteria (PGPR) strains induced the synthesis of specific phenolic acids, salicylic acid (SA) with varied amounts at different growth stages of chickpea seedlings against *Sclerotium rolfsii* infection and gallic, ferulic, chlorogenic and cinnamic acids were the major phenolic acids detected by HPLC analysis which support our findings. Sarma and Singh, (2003) also identified three major peaks that appeared consistently as gallic, vanillic and ferulic acids by in different parts of *Sclerotium rolfsii* infected and healthy seedlings of chickpea. They also found antifungal activity of ferulic acid *in vitro* against *S. rolfsii*. Present findings also showed increase in ellagic, salicylic, cinnamic acids in *Pseudomonas fluorescens* (biocontrol agents) treated chickpea seedlings infected with *Fusarium oxysporum*.

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