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TEMPORAL AND SPATIAL CHANGES IN PHENOLIC COMPOUNDS IN RESPONSE TO *FUSARIUM* WILT IN CHICKPEA AND PIGEONPEA

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Abstract

Plant phenolic compounds are known to play an important role in innate plant defense and are reported to show temporal and spatial changes in response to abiotic and biotic stress including invading pathogens. In the present study, spatial and temporal variations in phenolic compounds in response to infection by wilt pathogen, *Fusarium oxysporum* f. sp. *ciceri* (*Foc*) and *Fusarium udum* (*Fud*) were studied in wilt resistant and wilt susceptible cultivars of chickpea (*Cicer arietinum* L.) and pigeonpea (*Cajanus cajan* L. Millspaugh) (i) before the onset of wilt infection (S1 stage; 7 Days after sowing (DAS)), (ii) after the onset of wilt infection (S2 stage; 15 DAS) and (iii) at severe disease stage (S3 stage; 30 DAS), respectively and analyzed for association of total phenol with disease reaction. Under un-inoculated condition, maximum phenol content (21.8 mg gdw^{-1}) was found in wilt resistant cultivars and minimum (16.5 mg gdw^{-1}) in susceptible lines of chickpea. Wilt resistant cultivars of chickpea showed two fold increase in total phenolic content at the onset of infection. In case of pigeonpea, roots of resistant cultivars showed 2.27 fold increase in phenolics, but the increase was marginal in susceptible cultivars. In the present study, interaction between *Fusarium* and host plants was found to enhance defense responses against wilt disease in resistant cultivars of chickpea and pigeonpea.

Key words: Chickpea, *Fusarium*, Phenolics, Phenyl propanoid, Pigeonpea, Susceptibility, Wilt.

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INTRODUCTION

Chickpea (*Cicer arietinum* L.) and pigeonpea (*Cajanus cajan* (L.) Millspaugh) are among the world's most important pulse crops. Chickpea is the third most important pulse crop in the world, ranks first in Indian subcontinent and the Mediterranean base. Around 65% of the total global area and around 68% of total global production of chickpea falls in India. The annual growth rate of chickpea growing area has been slowed down by 1.9% and yields have risen at the rate of only 0.6% annually. Similarly, pigeonpea is grown in about 50 countries in Asia, Africa and America for multiple uses (food, fodder and firewood). Around 76% of the total global area and around 73% of total global production of pigeonpea falls in India (1). Although much progress has been made in developing chickpea and pigeonpea lines with resistance to biotic constraints and tolerance to abiotic stresses (26), yield losses in these crops are very high due to high incidence of diseases and insect pests.

Among biotic stresses, fungal diseases, especially, wilt diseases caused by *Fusarium oxysporum* f. sp. *ciceri* (*foc*) and *Fusarium udum* (*fud*) cause maximum damage. The wilt pathogen, *Fusarium* is both soil and seed borne and difficult to eradicate as fungal chlamydospores survive in soil up to six years even in the absence of host plant (14, 25) Plants activate a large array of defense mechanisms in response to pathogen attack. A crucial factor determining the success is the speed of activation of defense mechanisms. Consequently, there is a considerable interest in understanding how plants recognize pathogen attack and control expression of defense mechanisms. Biochemistry and physiology of the *Fusarium*-plant interaction have been characterized extensively, but definitive enquiry into identification of individual molecules essential for *Fusarium* pathogenesis to plants did not begin until molecular genetic technology became available for filamentous fungi (4, 11). To develop effective strategy for management of wilt diseases, understanding of the molecular basis of pathogenesis and resistance mechanism is very important.

Phenolic compounds are plant secondary metabolites that constitute one of the most common and widespread groups of substances in plants. There are several thousand (among them over 8,150 flavonoids) different compounds identified with a large range of structures: monomeric, dimeric and polymeric phenolics. Phenolic compounds have been suggested to play a variety of roles in defense mechanism against predators and microbial pathogens on the basis of their toxic nature and repellence to insect herbivores and microbes. They are reported as phytoanticipins, phytoalexins, structural barriers, modulator of pathogenicity, and activators of plant defense genes (2, 21). Medicarpin and maackiain, the major isoflavonoids found in chickpea as phytoalexins, are known for their antimicrobial activities and produced via the phenyl propanoid pathway.

Plant cell walls are known to be a barrier to the entry of many microorganisms and contain many components, including phenolic compounds that consist of phenyl propanoid units, found as both conjugated acids and more commonly, lignin alcohols. Phenolic acids are precursors for the synthesis of lignin (17). Deposition of phenolics into the cell walls during pathogen infection is an important defense mechanism, either because of hypersensitive reaction of entire cell or for local wall reinforcement due to deposition of papillae (3,8,10,12). The accumulation of the phenolic acids in infected tissues was responsible for the response of soybean seedlings towards *Phytopthora sojae* (5,13,23,24). The fungi toxicity of phenolics to the mycellial growth or to zoospore germination of *P. sojae* was further determined *in vitro* (6). Phytoalexins accumulated in soybean cell suspension cultures infected with *Pseudomonas syringae* pv. *glycinea* harboring an

avirulence gene (16) or exposed to *P. sojae* culture filtrate. Glyceollin also accumulated in soybean roots inoculated with the soybean cyst nematode (15). Lozovaya *et al*. (18) reported that *F. solani* f. sp. *glyciens* infection resulted in marked difference in phenolics content between the upper roots of susceptible and partially resistant lines. The role of phenols in disease resistance is evident from other studies also, though the mechanism may not be similar. For example in *Leucaena* plants, the uninoculated plants had lower amounts while the inoculated ones had higher level of protein and phenols in their root exudates (15). Changes in phenolic compounds in relation to fungal challenge in chickpea have been studied (2) wherein HPLC analyses revealed a very high accumulation of isoflavones and their glycoside conjugates in chickpea roots. Mehta *et al.* (22) reported that in case of cowpea cultivars moderately susceptible to *Rhizoctonia* had higher levels of total phenols and orthohydroxyphenols in their root exudates compared to highly susceptible cultivars. In their studies in chickpea, Stevenson *et al.* (30) had reported that root exudates of wilt resistant genotypes had significant inhibitory effect on fungal spore germination. Mandavia *et al*. (20) analyzed resistant and susceptible chickpea varieties for total phenols and different phenolic compounds in stem and leaf tissues collected at pre-infectional, disease initiation and severe disease stages. This study has shown significant correlation of the phenol content with wilt resistance thereby confirming the role of phenol compounds in disease resistance. In the present study, spatial and temporal changes in phenolics have been investigated in *Fusarium* infected and un-inoculated plants of chickpea and pigeonpea to observe differences among wilt resistant and susceptible genotypes and association of total phenolics with disease reaction.

MATERIALS AND METHODS

Plant Material

Two each of wilt resistant and wilt susceptible cultivars of chickpea (*Cicer arietinum* L.) and pigeonpea (*Cajanus cajan* L. Millspaugh) were used for studying variation in phenolic compounds in response to *Fusarium* infection. The chickpea cultivars were WR 315, ICC 4958 (resistant) and JG 62, BG 256 (susceptible); whereas the pigeonpea cultivars comprised of Asha (ICPL 87119), Maruthi (ICP 8863) in resistant group and Bahar and Type 7 as susceptible.

Inoculation of plants with the fungus and sample preparation for analyses of phenols

Fusarium oxysporum f. sp. *ciceri* (*Foc*) race 2 and *Fusarium udum* (*Fud*) isolates maintained in the laboratory as stock cultures were reinoculated in susceptible cultivars of chickpea (JG 62) and pigeonpea (Bahar) respectively. The pathogens were reisolated from fourth-node stem sections taken from wilted chickpea and pigeonpea plants according to the procedure described by Brett and Waldronm (9) and were colonized on filter paper, dried in the transfer hood, and aseptically cut into small pieces. The colonized filter paper pieces were placed in potato-dextrose broth and incubated to produce liquid cultures of the pathogen. The liquid cultures were filtered through cheese cloth to remove mycelia. The spore suspension was pelleted by centrifugation. After discarding the supernatant, the conidia were washed with sterile water to adjust the spore suspension to 1 x 10^6 spores ml^{-1} with a haemocytometer.

Plastic pots of 30 cm diameter were surface-sterilized with 0.1% w/v mercuric chloride. Pots were filled with 2 kg sterilized soil (three subsequent sterilizations at 1.1 kg/ cm^2 for 1 h for 3 days). Seven days before sowing, pots were inoculated with the 14-day-old culture of the pathogen multiplied on sand maize meal water medium (90 g sand, 10 g maize meal and 20 ml distilled, sterilized water) @ 50 g kg^{-1} soil. Seeds were surface-sterilized using 2% sodium hypochlorite for 3 min, and rinsed in sterile water. Ten seeds of the selected cultivar were sown in each pot for disease scoring. The root, stem and leaf tissues were collected separately at 7, 15 and 30 days after sowing (DAS; S1, S2 and S3 stages respectively) and were frozen immediately in liquid nitrogen to store at -20°C until further used.

Isolation and quantification of total phenols

One gram of dry powdered sample was mixed with 50 ml 8% (v/v) hydrochloric acid containing one pinch animal charcoal. The mixture was slowly refluxed in a condenser for 2.5 hrs on heating mantle. The extract was cooled and filtered using Whatman No. 1 filter paper. The filtrate is extracted with 2 x 250 ml of ethyl acetate. Cold water was added using separating funnel to remove ethyl acetate. Water layer was rejected and ethyl acetate layers were pooled together. Excess moisture was removed using anhydrous sodium sulphate. Ethyl acetate was evaporated and the residue dissolved in HPLC grade methanol. The solution was filtered through 0.45 µm filter and the residues were rejected. Final volume of extract was made to 5 ml and this extract was used for quantification of total phenolics using modified phenol-reagent method as described by El-Khallal (2007). One ml of the methanolic extract was added to 5 ml of distilled water and 250 µL of Folin-Ciocalteu reagent, and the solution was kept at 25°C for 3 min. Then 1 ml of a saturated solution of sodium carbonate and 1 ml of distilled water were added, and the mixture was incubated for 1 h at 25^0C. The absorption of the developed blue colour was measured using spectrophotometer (Bio-Rad Smart-Spec Plus) at 725 nm. Differences in temporal and spatial accumulation of phenol were studied in *Fusarium* wilt resistant and susceptible genotypes were calculated by comparison with a standard curve obtained from a Folin reaction with phenol.

RESULTS AND DISCUSSION

Association of total phenol with disease reaction
Before the onset of wilt infection (S1 stage; 7 DAS)

Under un-inoculated condition, total phenol content was maximum (14.5 mg/ gram dry weight (gdw) of tissue) in root tissue of susceptible chickpea cv. JG 62 and minimum (10.3 mg gdw^{-1}) in ICC 4958 roots (Table 1). Phenolic contents marginally increased in *foc* inoculated plants over uninoculated ones in resistant as well as susceptible genotypes. The stem tissue although had more content of phenolics but the pattern was similar to roots. At same stage, in leaf tissue, maximum phenol content (21.8 mg gdw^{-1}) was in WR 315 (resistant) and minimum (16.5 mg gdw^{-1}) in JG 62 (susceptible). In case of pigeonpea, at S1stage, the roots had phenolic contents ranging from 11.9 mg gdw^{-1} (Asha) to 18.0 mg gdw^{-1} (Bahar) under uninoculated conditions. In case of stem tissue, highest phenol content

(29.8 mg gdw^{-1}) was in cv. Type 7 (resistant) and minimum (16.8 mg gdw^{-1}) in Asha (susceptible). In leaf tissue, maximum phenolic content (28.7 mg gdw^{-1}) was in Asha and minimum (21.5 mg gdw^{-1}) in Bahar (Table 2). Inoculation with *fud* caused marginal increase of phenolic content in root, stem and leaf tissues of pigeonpea.

After the onset of wilt infection (S2 stage; 15 DAS)

At this stage, root tissue of both the wilt resistant cultivars of chickpea showed two fold increases in total phenols over un-inoculated controls and the susceptible cultivars (Table 1). Stem and leaf tissues did not show any significant difference in phenol content between resistant and susceptible cultivars. In case of pigeonpea, roots of resistant cv. Asha showed 2.27 fold increases in phenolics, but the increase was marginal in susceptible cultivars. The stem tissue of both the resistant cultivars of pigeonpea attained increase of 1.5 fold in total phenol content (Table 2). In case of pigeonpea resistant cultivar, Maruthi, the increase was 71.6% in root tissue at S2 stage while the susceptible cultivar, Type 7 did not show significant changes in the stem and leaf tissues in the disease initiation stage. Rate of increase of phenol accumulation was slightly higher in the susceptible cultivars as compared to the resistant ones in leaf tissue of pigeonpea.

At severe disease stage (S3 stage; 30 DAS)

Progression from S2 to S3 stage in chickpea caused decrease in phenolics in all the genotypes in control as well as infected plants. The magnitude of decrease was lesser in root and stem tissues followed by leaves. Comparison of phenolic content in roots, stem and leaves of *foc* inoculated plants showed that it increased rapidly in root and stem tissues where as its opposite was true for leaves (Table 1). In case of pigeonpea S3 stage recorded similar variation in phenolics. The decrease was marginal in control plants but was drastic and significant in *fud* inoculated plants. Among fud inoculated, Type 7 (resistant) genotype recorded highest phenolic content in root and stem tissues where as roots and stem of Asha (susceptible) genotype recorded minimum phenolics in pigeonpea (Table 2).

Induction of plant defence against pathogen attack is regulated by a complex network of different signals. In the present study, interaction between *Fusarium* and host plants was found to enhance defence responses against wilt disease in resistant cultivars of chickpea and pigeonpea. This study showed that induction of plant's own defence system started only after the infection by respective pathogen, and subsequently might have resulted in hypersensitive reaction conferring resistance.

In response to infection by the wilt fungus, the content of total phenols increased in the root, stem and leaf tissues of both the resistant and susceptible plants. The increase in total phenols in the tissues thus appears to be a general reaction common to both resistant and susceptible varieties exposed to the fungal pathogen. From Table 1, it is evident that phenol content was highest at disease initiation stage in leaf tissues of both the species.

The decline of total phenols during early (S2) to the late infection stages (S3) in both resistant and susceptible plants may be due to the cessation of hypersensitive reaction, although the reasons may be different. In the former, it may be due to the suppressed state of infection in tissues of wilt resistant plants compared to that in the susceptible plants. The changes during S1 to S2 stages were not consistent across tissues and genotypes.

These results clearly indicated that phenols content increased with the progression of the disease and rate of phenol accumulation was lower in susceptible than in resistant cultivars and hence may account for resistance expression. Studies in maize indicated higher levels of total phenols in maize inbred line resistant to leaf blight than in the susceptible lines (29). The resistant plant showed a tendency to accumulate higher amounts of total phenols than the susceptible ones following infection with fungi. Similar results have been reported in chilli (7) and rice (28). Relationship

Table 1. Changes in total phenols content (mg/g dry wt.) in different tissues of chickpea cultivars differing in susceptibility to *Fusarium* wilt at various disease stages.

Genotypes	Treatments	Root			Stem			Leaf		
		S1	S2	S3	S1	S2	S3	S1	S2	S3
WR 315	Control	11.0±0.36	12.1±0.14	10.4±0.17	13.6±.023	13.1±0.07	11.5±0.26	21.8±1.2	20.6±1.3	18.9±0.04
	Foc	11.2±0.24	23.0±0.06	12.5±0.09	14.0±0.12	21.3±1.5	15.2±0.21	22.6±1.13	25.1±0.02	14.9±0.21
ICC 4958	Control	10.3±0.15	11.5±0.14	9.5±0.10	14.8±0.08	15.2±0.23	12.4±0.07	19.2±0.02	21.4±0.36	20.4±0.8
	Foc	10.5±1.02	20.6±0.09	13.6±0.07	15.1±0.13	19.7±0.31	14.8±0.09	19.5±0.04	23.2±0.42	17.3±0.01
BG 256	Control	12.9±0.65	14.3±1.04	11.2±0.5	15.0±0.25	15.7±0.06	10.9±0.11	18.2±1.1	18.7±0.33	14.7±0.00
	Foc	13.1±0.28	16.7±1.12	11.2±0.03	15.4±0.41	20.4±0.00	19.3±0.01	19.0±0.27	20.5±0.51	11.2±0.50
JG 62	Control	14.5±0.05	14.8±0.32	12.6±0.15	15.9±0.20	16.4±0.30	13.4±0.04	16.5±0.22	15.6±0.31	14.7±0.14
	Foc	14.3±0.08	18.4±0.24	12.3±0.65	16.3±0.35	23.2±.033	18.7±0.55	17.3±0.15	19.7±1.3	09.7±0.01

Values are mean (n=3) ± SD, S1-Preinfection stage (7 DAS), S2-Disease initiation stage (15 DAS), S3-Severe disease stage (30 DAS).

Table 2. Changes in total phenols content (mg/g dry wt.) in different tissues of pigeonpea cultivars differing in susceptibility to *Fusarium* wilt at various disease stages.

Genotypes	Treatments	Root			Stem			Leaf		
		S1	S2	S3	S1	S2	S3	S1	S2	S3
Asha	Control	11.9±.8	12.3±0.04	13.1±0.05	16.8±0.03	18.2±0.05	14.2±0.65	28.7±0.39	32.8±0.55	23.5±0.31
	Fud	12.1±0.03	28.0±0.02	11.2±0.16	17.8±0.04	27.8±0.01	18.6±0.70	31.2±0.09	39.1±0.36	18.4±0.16
Maruthi	Control	14.8±0.03	16.2±0.23	12.1±0.03	18.3±1.1	21.0±0.06	16.2±0.32	26.7±0.08	33.4±0.01	29.5±0.31
	Fud	15.5±0.11	26.7±0.25	15.8±0.01	18.6±0.33	32.4±1.5	25.6±0.35	29.4±0.14	36.3±0.13	22.6±0.11
Type 7	Control	17.9±0.02	21.3±0.17	18.3±0.06	29.8±0.35	30.1±1.1	17.8±0.32	23.7±0.03	25.5±0.11	21.3±1.3
	Fud	18.1±0.13	22.4±0.10	13.6±0.09	31.2±0.23	38.1±1.6	26.4±0.15	24.5±0.77	33.1±0.20	25.1±0.37
Bahar	Control	18.0±0.05	19.3±0.14	14.5±0.07	29.6±0.06	31.5±0.33	24.6±0.13	21.5±0.21	25.6±0.04	23.1±0.02
	Fud	18.3±0.00	24.8±1.4	16.8±0.01	30.5±0.04	37.2±0.34	21.5±0.19	23.8±0.07	29.4±0.00	14.9±0.47

Values are mean (n=3) ± SD, S1-Preinfection stage (7 DAS), S2-Disease initiation stage (15 DAS), S3-Severe disease stage (30 DAS).

between resistance and phenolic content was explained by suggesting that, in the susceptible variety the fungus has enough time for its growth before phenol content reaches a level inhibitory to the fungus, whereas, in the resistant variety higher accumulation of phenols in initial stages restricts the growth of the fungus (27). Similarly, observed values in phenol content in present investigation are consistent with these earlier studies. In this study, it is the response of the plant to inoculation that led to the production of phenols to counter the *Fusarium* attack. In case of cowpea cultivars moderately susceptible to *Rhizoctonia* had higher level of total phenol and ortho-hydroxyl phenol in their root exudates, compared to highly susceptible cultivars which are also established in the present study (20). Significant correlation of phenol content with wilt resistance thus confirms the role of phenolic compounds in disease resistance. The rise in levels of phenolic compounds in infected plants may be due to their release from cell wall structures during their destruction.

We report for the first time that *Fud* inoculation of pigeonpea cultivars induces the phenyl propanoid pathway to synthesize phenolic compounds that have been suggested to play a variety of roles in defence mechanism against pathogens. These phenolic compounds possess biological activity against a wide range of pathogens and are potential bio-markers for the plant disease resistance

or tolerance.

Results of this study confirm that like in many plant species, phenols play an important role in chickpea and pigeonpea as well to defend themselves against plant pathogens. Antibiotic phenols have been found in all cultivars of chickpea and pigeonpea investigated. The basal level of phenol which is expressed constitutively is thought to function as pre-formed inhibitors. Others are found in response to the ingress of pathogen and their appearances are considered as part of an active defence response. The *de novo* synthesis and differential accumulation of anti fungal phytoalexins in incompatible and compatible plant pathogen interactions play crucial roles in the specificity of host resistance. Moreover, the accumulation of polymerized phenols cause lignifications in response to infection, acts as a barrier and prevents ingress of pathogen. It was observed that susceptible cultivars of chickpea and pigeonpea contained lower amount of phenolics. Therefore, they were unable to develop resistance, with the progression of wilt disease. On the contrary, the resistant cultivars of chickpea and pigeonpea contained higher amount of phenolics due to which the cultivars were able to exhibit resistance against *Fusarium.* Hence, the accumulation of phenolics in roots, stems and leaves of resistant cultivars might have collectively contributed to the resistance in the host plants against *Fusarium*.

Accumulation of phytoalexins is the most commonly observed defence reaction in plants in response to fungal infection. Chickpea phytoalexins derived from the phenyl propanoid pathway involve the activity of several enzymes such as Phenylalanine ammonia lyase (PAL), Chalcone synthase (CHS) and Isoflavone reductase (IFR). Priming resistance by inducing these genes could be an efficient and inexpensive way of achieving the control of *Fusarium* wilt in chickpea and pigeonpea. Several hypotheses can be formulated to explain the level of phenolics observed in the plants following *Foc* and *Fud* infection. This increase in accumulation of phenolics may also occur upon challenge by *Fusarium,* from the release of phytoalexins from their preformed conjugated forms. Furthermore, it is likely that these two mechanisms generating phytoalexins can act synergistically. The present findings confirm that the accumulation of phenolic compounds in resistant cultivars was induced only in the plants inoculated with *Foc* or *Fud.* It is also noteworthy that, overall both susceptible and resistant cultivars were responsive to the *Fusarium* inoculation in inducing the defence related genes. In conclusion, resistant cultivars upon infection activates genes involved in the phenyl propanoid pathway by enhancing the synthesis or accumulation of their transcripts and consequently by promoting the accumulation of phenolic compounds or phytoalexins that can restrict the disease development (2, 21). This is very important for future studies focussing on the isolation and transfer of disease resistance genes, as part of an integrated management strategy to protect the plants from wilt pathogen attack.

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Other articles in this theme issue include references (31-58).

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