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Induced Temporal and Spatial Variation in Pathogenesis Related Proteins in Cicer Arietinum Inoculated with *Fusarium Oxyporum F.SP. Ciceri*

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Abstract

Pathogenesis-related proteins (PRs) are a class of proteins that accumulate in response to biotic and abiotic stresses to protect plants from damage. The present study examined the defense response of Chickpea (Cicer arietinum L.) wilt resistant and susceptible genotypes inoculated with wilt pathogen Fusarium oxyporum f.sp. ciceri (Foc). Evaluation of pre-induced and pathogen-induced defense at 3 stages i.e. 7 (S1), 15 (S2) and 30 (S3) days showed that the PRs (i.e. β-1,3-glucanase and chitinase) differed not only among the root, stem and leaves but also among susceptible and resistant genotypes and increased after inoculation with Foc. Foc inoculation induced β-1,3-glucanase and chitinase activity in all the test cultivars. Maximum induction of chitinase was observed at S2 in roots of resistant cultivars whereas un-inoculated plants showed much less conspicuous changes. β-1,3-glucanase activity was high in stem tissues. Both control and Foc inoculated plants had higher level of β-1,3-glucanase activity at S2 and S3, but the resistant cultivars recorded much higher proportionate increase.. The activity/expression pattern of these PR proteins could be used as established resistance markers and for manipulating their expression towards development of superior wilt-resistant chickpea genotypes.

Keywords: Chickpea; Cicer arietinum; Fusarium oxyporum f.sp. ciceri; Pathogenesis; PR proteins, chitinase, β-1,3-glucanase.

Introduction

Chickpea (*Cicer arietinum* L.) is a self-pollinated diploid (2n=2x=16 with genome size of 738 Mb) leguminous crop, cultivated mainly in semiarid environments of the world and ranks second in area and third in production among the pulses worldwide. Around 65% of the total global area and around 68% of total global production of chickpea falls in India. Although much progress has been made in developing chickpea lines with resistance to biotic constraints and tolerance to abiotic stresses, yield loss in this crop is very high due to the high incidence of diseases and insect pests. Chickpea wilt caused by *Fusarium oxysporum Schl*.Emend.Snyd and Hans f.sp. *ciceri* (Padwick) is widespread in several chickpea growing regions of the world, especially the Indian subcontinent, Ethiopia, Mexico, Spain and Tunisia (Joshi *et al*., 2001). The wilt pathogen 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. *Fusarium* wilt epidemics can be devastating to individual crops and cause up to

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100% loss under favourable conditions.

Effector-triggered immunity (ETI) is an important part of plant innate immunity that protects against pathogen infections and is established via the recognition of virulence effectors by the corresponding receptor resistance (R) proteins in a specific gene-to-gene manner (Zhang *et al.*, 2018). These PRs have diverse functions, contributing to cell wall rigidification, signal transduction, and antimicrobial activities, and are mainly expressed in plants as chitinases, glucanases, and thaumatin-like proteins (Farrakh *et al.*, 2018). The PRs are usually small (10–40 kDa) and mostly acidic. Many PRs are distributed in plant cell gaps and vacuoles, and their distribution is related to their isoelectric point and the exposure to stress (Jo et al., 2020). Therefore, they are able to accumulate in intracellular and intercellular spaces. During a pathogen attack, the ETI induction mechanism in plants is activated, leading to the accumulation of pathogenesis related proteins (PRs) (Mazumder *et al.*, 2013). The accumulated PRs help plants prevent reinfections, resulting in the development of systemic acquired resistance (Zhang *et al.*, 2013).

Developing effective strategy for management of wilt diseases demands deep understanding of the molecular basis of pathogenesis and resistance mechanism. Plants activate a large array of defence mechanisms in response to pathogen attack and host pathogen cross talk. A crucial factor determining the success of these mechanisms is the speed of their activation which needs thorough understanding of how host plants recognize pathogen attack and control expression of defence mechanisms. The hypersensitive response of plants is accompanied by a combination of a number of host enzymes involved in immune systems. Defence-related enzymes form an important immune system that fights pathogen attacks as PAL is a key enzyme in phenylpropanoid metabolism and plays an important role in the synthesis of secondary defense-related chemicals called phytoalexins (Tahsili *et al.*, 2014). Elucidation of host defensive responses to pathogen invasion have determined "pathogenesis related" proteins (PRs) viz. chitinase and β-1,3-glucanase as vital defense related enzymes of plants against phytopathogens (Dehgahi et al., 2015). The use of β-1, 3-glucanase is suggested as a biomarker for genotypes that are resistant to downy mildew in pearl millet (Pennisetum typhoides) due to the activity of enzyme differentiation between resistant and genotypes (Ramachandra *et al.*, 2000). Giri *et al.* (1998) recorded differential induction of chitinase in susceptible and resistant genotypes of chickpea and decrease in β-1,3-glucanase in the resistant cultivars in response to the pathogen infection. Significant decrease in β-1,3-glucanase in cultivars resists the pathogen invasion due to a protective response. Expression of a number of enzymes involved in a participatory immune system may be constitutive while others are induced upon the attack of pathogen such as enzymes involved in phytoalexin biosynthesis or antioxidant supplementation and its enzymatic-related system.

Since details on pre-induced and induced biochemical mechanisms of resistance are pre-requisite to understand interaction between pathogens and host and provide basis for better disease management, the present research work attempted to correlate and analyse the biochemical basis of wilt disease resistance in resistant and susceptible cultivars of chickpea by measuring spatial and temporal levels of chitinase and β-1,3-glucanase.

Material and Methods

Plant material, pathogen inoculation and sample collection for biochemical analyses

Two each of wilt resistant (WR 315, ICC 4958) and wilt susceptible (JG 62, BG 256) cultivars of chickpea (*Cicer arietinum* L.) were used for the present study. The *Fusarium oxysporum* f. sp. ciceri (Foc) pathogens were isolated from fourth node stem sections taken from wilted chickpea plants according to the procedure described by Tullu *et al.* (1998) 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. Single spore culture of fungus was obtained by serial dilution method. Isolated fungus was identified as *F. oxysporum* f. sp. ciceri and its pathogenicity test was conducted in pot experiments on chickpea cultivar, JG 62. Plastic pots of 30 cm diameter, surface sterilized with 0.1% w/V mercuric chloride ($HgCl_2$), 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 and, 10 g maize meal

and 20 ml distilled, sterilized water) @ 50 g kg–1 soil. Ten seeds (surface-sterilized using 2% sodium hypochlorite for 3 min, and rinsed in sterile water) of each cultivar were sown in each pot for disease scoring.

The root, shoot and leaf tissues were collected separately at 7 (S1), 15 (S2) and 30 (S3) days after sowing (DAS) and were frozen immediately in liquid nitrogen to store at -20°C. Biochemical basis of disease resistance was studied (temporal and spatial accumulation and activity) for PR-proteins (chitinase, and β-1,3-glucanase) in *Fusarium* wilt resistant and susceptible chickpea genotypes.

Isolation and activity assay of chitinase (EC 3.2.1.14)

Defatted and depigmented tissue powder was stirred with extraction buffer (1:6 w/V in 0.1 M phosphate buffer, pH 6.9 containing 0.05 M NaCl) at 4°C for 12 hrs. The mixture was centrifuged at 10,000 X g for 20 min and the proteins form the supernatants were precipitated by adding ammonium sulphate $[(NH_4)_2SO_4]$ to 90% saturation (60 g ammonium sulfate per 100 ml extract). The precipitated protein were collected by centrifugation, resuspended and dialyzed against the extraction buffer (Giri *et al.*, 1998).

Chitinase activity was determined as described by Chen *et al.* (1982) and Tsukomoto *et al.* (1984). The reaction mixture contained 1.0 ml of colloidal chitin solution (7 mg), 1.0 ml of sodium acetate buffer (50 mM, pH 5.2) and 1.0 ml of suitably diluted enzyme. After incubation at 50°C for 1 hr, the released reducing sugar was measured as N-acetyl glucosamine (NAG) equivalents by the method of Reissig *et al.* (1955). One unit (U) of chitinase activity is defined as the amount of enzyme that produces 1 mg of NAG per hour, under the given assay conditions and expressed as U per gram fresh weight (U gfw-1).

Isolation and activity assay of glucanase (EC 3.2.1.39)

β-1, 3-glucanase activity was estimated using the procedure of Koga *et al.* (1988). The assay mixture contained 1.0 ml of suitably diluted enzyme and 1.0 ml of 1% laminarin solution in sodium acetate buffer (50 mM, pH 5.2).The mixture was incubated at 40°C for 30 min and the released reducing sugar was measured as glucose equivalents (Somogyi, 1952). One unit (U) of β-1, 3-glucanase activity is defined as the amount of enzyme that produces 1 mM of glucose ($C_6H_{12}O_6$) per hour, under the given assay conditions.

Results and Discussion

Spatial and temporal activity of chitinase

Induction of chitinase in chickpea was significantly high in leaves. Chitinase activity showed a marked increase in different tissues upon inoculation with *Fusarium*. In general the maximum chitinase activity was observed at S2 stage in resistant cultivars. The chitinase activity increased progressively from S1 to S2 stage and thereafter showed decrease with progression of the disease and was very low at S3 stage (Table 1).

Table 1: Chitinase activity (U gfw-1) in different tissues of chickpea genotypes differing in susceptibility to *Fusarium* wilt.

Genotype	Treatments	Root			Stem			Leaf		
		S1	S2	S3	S1	S2	S3	S1	S2	S3
WR 315	Control	3.21 ± 0.01	3.23 ± 0.01	3.19 ± 0.02	3.66 ± 0.04	4.06 ± 0.10	3.93± 0.01	2.63 ± 0.02	3.22 ± 0.02	3.16 ± 0.02
	Foc	3.49 ± 0.03	9.96 ± 0.10	8.77 ± 0.01	4.00 ± 0.02	9.95 ± 0.04	9.71 ± 0.01	3.29 ± 0.00	10.15 ± 0.05	9.96 ± 0.04
ICC 4958	Control	3.16 ± 0.01	4.45 ± 0.01	4.41 ± 0.01	2.67 ± 0.01	3.09 ± 0.01	3.07 ± 0.00	3.95 ± 0.02	3.35 ± 0.02	3.36 ± 0.01
	Foc	3.42 ± 0.03	10.40 ± 0.05	10.41 ± 0.05	3.17 ± 0.08	10.62 ± 0.01	10.23 ± 0.00	4.13 ± 0.01	11.72 ± 0.08	11.81 ± 0.00
BG 256	Control	1.51 ± 0.08	4.68 ± 0.00	4.51 ± 0.01	3.19 ± 0.00	5.84 ± 0.05	5.46 ± 0.00	3.29 ± 0.05	4.68 ± 0.00	4.62 ± 0.00
	Foc	4.70 ± 0.04	8.95 ± 0.08	5.28 ± 0.03	4.90 ± 0.04	9.10 ± 0.05	6.54 ± 0.04	5.65 ± 0.05	8.84 ± 0.05	4.74 ± 0.01
JG 62	Control	4.34 ± 0.03	4.57 ± 0.04	4.50 ± 0.00	4.17 ± 0.00	4.39 ± 0.03	4.42 ± 0.03	3.99 ± 0.02	4.24 ± 0.05	4.20 ± 0.00
	Foc	5.22 ± 0.01	9.12 ± 0.15	6.86 ± 0.01	5.63 ± 0.15	8.80 ± 0.00	7.48 ± 0.00	5.94 ± 0.02	9.50 ± 0.05	7.94 ± 0.01

S1: Pre infection stage (7 DAS), S2: Disease initiation stage (15 DAS), S3, Severe disease stage (30 DAS). All values are mean of three replications ± SD.

On the contrary, these changes were much less conspicuous in case of un inoculated plants, where the increase or decrease was only marginal.

At S1 stage, maximum constitutive level of chitinase activity was observed in leaf, stem and root tissue of susceptible cv. JG 62. The enzyme activity in these tissues was 4.34, 4.17 and 3.99 U gfw-1, respectively (Table 1). Maximum induction of chitinase activity was observed at S2 stage in the root tissue. In resistant cultivar WR 315 this increase was 3.08 fold and in cv. ICC 4958 increase was 2.3 fold. This increase in activity was lower in susceptible cultivars (1.9 fold only). The enzyme activity significantly increased in comparison to control in the stem tissue of resistant genotypes. More than three-fold increase in enzyme activity was observed in resistant plants compared to respective controls but in susceptible cultivars this increase was only 1.18 and 2.24 fold. At S3, in both control and inoculated plants the increase in activity was less pronounced but resistant cultivars showed higher activity of chitinase than susceptible cultivars.

Spatial and temporal activity of β-1,3-glucanase

Glucanase activity was higher in stem and leaf tissues as compared to root tissue. Inoculation of the plants with *Fusarium* recorded a significan increase in glucanase activity in various tissues. In general the maximum enzymatic activity was observed in resistant cultivars at S2 stage and the same was more or less maintained until S3 stage. In case of chickpea, at S1 stage maximum (0.67 mM glucose $h^{-1}gfw^{-1}$) constitutive level of glucanase activity was observed in BG 256 roots. Minimum activity (0.30 mM glucose $h^{-1}gfw^{-1}$) was found in WR 315 (Table 2). At disease initiation stage (S2) maximum induction of enzymatic activity was in resistant chickpea genotype WR 315, where this increase was three times greater in root and stem compared to control plants of same stage. While, under similar condition, in wilt susceptible chickpea genotypes, the increase in enzymatic activity was only 1 to 1.5 fold.

Table 2: Glucanase activity (mMh-1 gfw-1) in different tissues of chickpea genotypes differing in susceptibility to Fusarium wilt.

Genotype	Treatments	Root			Stem			Leaf		
		S1	S2	S3	S1	S2	S3	S1	S2	S3
WR 315	Control	0.30 ± 0.00	0.43 ± 0.00	0.44 ± 0.01	0.52 ± 0.03	0.69 ± 0.00	0.40 ± 0.01	0.34 ± 0.01	0.62 ± 0.00	0.77 ± 0.01
	Foc	0.50 ± 0.01	1.34 ± 0.02	1.29 ± 0.01	1.25 ± 0.02	2.00 ± 0.07	2.29 ± 0.05	0.62 ± 0.01	1.47 ± 0.07	1.73 ± 0.03
ICC 4958	Control	0.46 ± 0.02	0.74 ± 0.03	0.59 ± 0.00	0.90 ± 0.04	0.97 ± 0.01	0.55 ± 0.00	0.95 ± 0.00	0.84 ± 0.01	0.82 ± 0.05
	Foc	1.43 ± 0.00	1.35 ± 0.01	0.96 ± 0.00	0.32 ± 0.02	1.85 ± 0.02	0.80 ± 0.14	0.59 ± 0.01	1.90 ± 0.00	1.88 ± 0.00
BG 256	Control	0.67 ± 0.00	0.74 ± 0.01	1.06 ± 0.01	0.76 ± 0.09	0.96 ± 0.03	0.9 6 ± 0.02	0.47 ± 0.02	0.80 ± 0.00	0.64 ± 0.02
	Foc	1.02 ± 0.00	0.92 ± 0.03	0.39 ± 0.02	0.81 ± 0.03	1.00 ± 0.01	0.96 ± 0.01	0.72 ± 0.17	0.96 ± 0.03	0.57 ± 0.02
JG 62	Control	0.46 ± 0.02	0.96 ± 0.04	1.03 ± 0.01	0.30 ± 0.01	0.79 ± 0.09	0.96 ± 0.01	2.18 ± 2.40	0.57 ± 0.05	1.42 ± 0.14
	Foc	0.50 ± 0.08	1.00 ± 0.00	0.71 ± 0.02	1.017 ± 0.01	0.83 ± 0.00	0.81 ± 0.00	1.14 ± 0.01	0.92 ± 0.00	0.92 ± 0.00

S1: Pre infection stage (7 DAS), S2: Disease initiation stage (15 DAS), S3, Severe disease stage (30 DAS).
All values are mean of three replications ± SD

Induction of plant protection against pathogen invasion is controlled by a complex network of different signals. In the present study, pathogen-host interaction improved immune responses in resistant cultivars of chickpea. Spatial and temporal changes in various enzymes investigated in *Fusarium* infected and uninoculated plants showed that induction of plants' own defence system program began only after the infection by respective pathogen, and subsequently led to hypersensitive reaction conferring resistance. Chickpea plants exposed to *Foc* showed enhanced synthesis of pathogenesis related proteins relative to their controls.

Inhibition of growth of several fungal pathogens requires the presence of chitinases and β-1,3-glucanase activities to reduce the polymerization of cell wall polymers (Saikia *et al.*, 2005). Ferraris *et al.* (1987) found that infection of susceptible and resistant tomato cultivars with *F. oxysporum* f. sp. *lycopersici* caused several fold increases in chitinases, β-1,3-glucanases, glucosidases and N acetyl glucosaminidases activities between 5 to 90 days after inoculation. The present study in chickpea proved that chitinase activity was increasing 3.08 and 2.33 fold in root tissue, 2.45 fold and 3.43 fold in stem tissue, 3.15 fold and 3.5 fold in leaf tissues of chickpea resistant cultivars while in susceptible cultivars it increased up to 2 fold upon infection. Similarly, in case of β-1,3-glucanases, the activity increased by 2-3 fold in resistant cultivars and 1-1.5 fold in susceptible varieties of chickpea. Giri *et al.*

(1998) analysed the levels of these two enzymes in resistant and susceptible cultivars of chickpea during *Fusarium* wilt development and found induction of chitinase activity in both resistant and susceptible cultivars; however induction in susceptible cultivar JG 62 was much lower than that of resistant cultivar. Further, the activities of these two enzymes increased several fold from S1 to S2 stage both in control and treated condition and declined thereafter. The induction was very low in the control condition. This fact is also supported by work of Saikia *et al.* (2005) where the radial growth of different fungal species, e.g. *F. oxysporum*. f. sp. ciceri, *F. udum* and *M. Phaseolina* was inhibited by purified proteins, thus exhibiting antifungal activity. Chitinase exhibited more antifungal activity in comparison to β-1, 3-glucanase *in vitro*. Rakshit *et al.* (2000) reported that increase in β-1, 3-glucanase activity was significantly higher and more severe in resistant genotypes as compared to the susceptible ones in pea after powdery mildew infection. Xue *et al.* (1998) observed significantly higher activity of chitinase in the cotyledons of bean plants than hypocotyls and a significant increase of peroxidase, β-1, 3-glucanases and chitinase in all fractions of diseased plant cells as compared to control, and β-1, 3-glucanases activity recorded increase up to 18 fold in induced bean hypocotyls tissues. Similar increases, but not as pronounced have been reported during ISR studies using incompatibility interactions between soybean and *Phytophthora megasperma* f. sp. *glyciensi* (Yi and Hwang, 1996) and between resistant bean cultivars and *C. lindemuthianum* (Daugrois *et al.*, 1990). In the present study, chitinase accumulated significantly in different tissues after the onset of disease and the same has been reported in other systems of host pathogen interactions where different types of chitinases are expressed depending on the type of plant tissue and developmental stages (Igratius *et al.*, 1994). Seed associated barley chitinases differ from those found in leaves infected with powdery mildew (Igratius *et al.*, 1994) while Anuratha *et al.* (1996) isolated and identified infection related chitinase transcripts that were only induced after infection of rice with the sheath blight pathogen *R. solani*.

The defence responses were induced in both pre induced and non induced plants infected by pathogens and the suppression of *Fusarium* wilt possibly involved an inhibitory effect on the pathogen of pre-induced plant defences, as well as induced defence. Chickpea resistant cv. ICC 4958 did not show significant increase in activity of β-1, 3-glucanase in root tissue at S2 stage as compared to susceptible cultivars. The increase in activities in susceptible hosts may have been a result of failure of containment of the pathogen and the colonization of ever increasing amounts in the vascular tissue. Benhamou *et al.* (1990) observed faster accumulation of defence related enzymes in incompatible interactions of tomato and *Fusarium oxysporum* f. sp. *lycopersici* or *Fusarium radicis lycoperscici* than in a compatible interaction. Beckman and Roberts (1995) in their model for host-pathogen interaction, suggested callose deposition and lignifications as one of the plants responses against wilt disease and rate of these processes determining the degree of reduction of pathogen invasion. The observed decrease in β-1, 3-glucanse activity in resistant cultivars in root and stem tissues may be associated with the higher rate of callose insertion. When expression of β-1, 3-glucanase is specifically blocked by the antisense mRNA technique, callose deposits are protected from degradation, leading in resistance to viral infection in tobacco (Beffa *et al.*, 1996). In the present study, levels of chitinase and β-1, 3-glucanases are reduced as the infection is lasted at S3 stage. This decrease may be associated with the reduction in the pathogen attack due to a protective response. In summary, this study shows that *Fusarium* isan inducer of these enzymes at both the local and systemic level in tissues of chickpea. Induced resistance is multi-component and it is neede to investigate further other mechanisms involved, either individually or collectively and focus on identification and transfer of disease resistance genes as part of an integrated wilt management strategy.

Conclusion

The present research study on temporal and spatial variation in PR proteins reveals that their expression pattern could be used as biomarkers to establish resistance. The host is protected against pathogen both by passive (physical barriers) and active (phytoalexins) mechanisms using defense enzymes. The isolation of pathogen induced defense enzyme genes bears scope for their cloning, manipulation of expression and development of transgenic crop plants resistant to various pathogens.

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