Induction of resistance in chickpea by cell wall protein of *Fusarium oxysporum* f. sp. *ciceri* and *Macrophomina phaseolina*

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In order to elucidate the elicitor properties of cell-wall protein (CWP) of *Fusarium oxysporum* f. sp. *ciceri* and *Macrophomina phaseolina*, CWPs were extracted and tested against chickpea. The chickpea seedlings exposed to CWPs showed enhanced synthesis of phenol, pathogenesis-related protein and activities of phenylalanine ammonia lyase and peroxidase relative to water-treated controls. Both *Fusarium* wilt and charcoal rot diseases of chickpea were significantly reduced in seedlings following CWP treatment. However, the same CWPs failed to antagonise the same pathogens in petri dish assays. The results suggest that CWPs of *Fusarium* and *Macrophomina* have elicitor properties and effectively induced resistance in chickpea.

Keywords: Cell-wall, chickpea, *Fusarium oxysporum*, induction of resistance, *Macrophomina phaseolina*.

ONE of the major hurdles in the production of high quality and yield of food crops is the difficulty in control of plant diseases; an aspect that concerns the producer and consumer as well. This is because many fungal pathogens have developed resistance against the active ingredients of a wide spectrum of fungicides, and there is a common perception that pesticides are undesirable. It is in this context that the application of biotechnology would be a better choice to minimize the incidence of disease in agricultural crops[1]. One such approach would be through the induction and enhancement of the plant's own defence mechanisms rather than the application of toxic compounds. The induction of such a plant defence strategy seems logical as the induced disease resistance in plants is likely to offer protection against different pathogens; the attractive alternative which is natural, safe, effective and sustainable in controlling plant diseases[2]. This study can unravel many of the intricate biochemical interactions between the plant and the pathogen. The induced resistance mechanism may involve not only some pre-formed components but also a 'cascade' of induced responses[3,4]. These include novel antimicrobial compounds (phytoalexins), proteins and physical barriers to penetration. This cascade of resistance factors is induced only when a plant recognizes the presence of a potential pathogen, and the compounds capable of triggering such responses are termed elicitors[3].

Plants can defend themselves against different pathogens through a wide array of mechanisms that may be local, systemic, inducible or constitutive[3–6]. There are several mechanisms like hypersensitive reactions (HR), production of phytoalexins and pathogenesis-related (PR) proteins, and simple physical barriers to penetration through deposition of lignin, etc. It is anticipated that molecules with elicitor properties from CWPs of *Fusarium oxysporum* and *Macrophomina phaseolina* would form the basis for further evaluation of their potential as a biocontrol agent against the two major chickpea pathogens inflicting considerable yield loss[7]. Already, a number of fungal elicitors have been extracted and characterized[1,8]. Cultured plant cells and fungal elicitors, fungal cell-wall fragments that elicit defence-related gene expression, have been extensively used to detect defence-related gene products in plant–pathogen interactions[9]. A number of fungal elicitors have been isolated and characterized from culture medium of fungi grown *in vitro* and from intracellular washing fluids of infected plants[10]. Some examples are increase in the activity of the defence gene phenylalanine ammonia-lyase (PAL) in tobacco, following treatment with proteinaceous elicitors of *Phytophthora* spp.[11]; in β-1,3-glucanase activity in suspension cultured bean cells with elicitors of *Colletotrichum lindemuthianum*[12], and in peroxidase activity of cultured tomato cells with elicitor of *Cladosporium fulvum*[13] in phenolic content in *Musa acuminate* roots cells with elicitors of *F. oxysporum* f. sp. *cubense*[14]. Picard *et al.*[15] purified a 10 kDa elicitin-like protein (oligandrin) from the culture filtrate of *Pythium oligandrum* that induced plant defence reactions in tomato plants against *Phytophthora parasitica*. Therefore, in the present study, we attempted to extract CWPs from two major chickpea pathogens, *F. oxysporum* f. sp. *ciceri* and *M. phaseolina*, and to examine their potential for induction of resistance against the two major diseases of chickpea.

F. oxysporum f. sp. *ciceri* Rs1 (*Foc*Rs1) and *M. phaseolina* Rs420 (*Mph*Rs420) obtained from the culture collection repository of National Bureau of Agriculturally Important Microorganisms (NBAIM), India were grown in potato-dextrose agar (PDA). A susceptible cultivar of chickpea, cv JG-62 was used as the target. Chickpea seeds were washed with 2% soap solution (phosphate-free), soaked under running water for 30 min, surface sterilized in 2% NaOCl (10 min), and rinsed with double sterile distilled water (SDW). Seeds were sown in earthen pots (radius 18 cm, three seeds pot^{-1}) and maintained in a greenhouse.

CWP was extracted from *Foc*Rs1 as described by Takenaka and Kawasaki[16]. To assay for induction of resistance, 15-day-old seedlings were treated aseptically with 50 µl solution containing 75 µg of CWP at the base of the

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stem. Following three days of treatment, soil in pots is inoculated with mycelial suspension of *Foc*Rs1/*Mph*Rs420 (10^3 CFU ml^{-1}, 500 μl^{-1} pot). The experiments were designed as complete randomized block design (CRBD), and disease severity examined for the next 27 days[16].

A split root technique was used to observe whether CWP-induced resistance occurs systemically. For this, Y-shaped PVC tubes were used in gnotobiotic system in the greenhouse. The roots of 15-day-old plants were split with a razor and transplanted into PVC tube, such that the root system spread into two parts in the tube. After 5 days, one side of the root system was treated with 25 µl CWP of the above concentration or with SDW (control). After 3 days, the other side of the root system was challenged with either 100 µl of conidial suspension of *Foc*Rs1 (ca. 10^3 conidia ml^{-1}) or 100 µl of mycelial suspension (10^3 CFU ml^{-1}) of *Mph*Rs420. Tubes were covered with plastic bags to prevent pathogen contamination. Seedlings were watered daily with sterile water. Appearance of disease symptoms (wilting/charcoal rot) was checked routinely for 27 days.

A petri plate assay was carried out to check for any direct effect of CWP on the growth of *Foc*Rs1 and *Mph*Rs420 by agar diffusion test on PDA medium. Sterilized paper disc (5 mm) containing 20 µl of CWP was put at the centre of the petri plates containing sterilized PDA medium and mycelial disc (5 mm) of the actively growing pathogens, viz. *Foc*Rs1 and *Mph*Rs420 were inoculated individually at four points of the petri plate equidistant from the centre. The plates were incubated for 7 days (28 ± 2°C) and inhibition zone recorded.

In enzyme assay for induction of resistance, 15-day-old seedlings in earthen pot (as mentioned earlier) were sprayed with CWP (100 µl $plant^{-1}$) of the two pathogens and accumulation of phenol and activities of PAL and peroxidase estimated following 0, 1, 2, 3, 4 or 5 days of inoculation.

For phenolic content, 1 g fresh plant sample was homogenized in 10 ml of 80% methanol and agitated for 15 min at 70°C. Next, 1 ml of the extract was added to 5 ml of distilled water and 250 µl of 1 N Folin–Ciocalteau reagent and kept at 25°C. The absorbance (725 nm) was measured spectrophotometrically (Thermospectronic, USA) with Catechol (Sigma, USA) as the standard. The amount of phenol is expressed in term of µg g^{-1} fresh weight.

PAL activity in CWP-treated plants was measured as the rate of conversion of L-phenylalanine to trans-cinnamic acid at 290 nm[17]. One gram of plant material was homogenized with 5 ml of sodium phosphate buffer (0.1 M, pH 7.0) containing 0.1 g of polyvinyl pyrrolidone (PVP, Sigma). The extract was filtered through a cheese cloth, the filtrate centrifuged at 20,000 *g* (30 min) and the supernatant used for enzyme activity. Enzyme extract (0.4 ml) was incubated with 0.5 ml of 0.1 M borate buffer (pH, 8.8) and 0.5 ml of L-phenylalanine (12 mM) for 30 min (30°C). In reference, 0.4 ml of enzyme extract was taken in 1.0 ml borate buffer. The amount of trans-cinnamic acid synthesized was calculated using extinction coefficient of 9630 M^{-1} cm^{-1}. The enzyme activity is expressed as the amount of trans-cinnamic acid (nmol min^{-1} g^{-1} fresh weight).

To estimate PR-protein accumulation and peroxidase in CWP-treated chickpea plants, 1 g of the plant sample was homogenized with 2 ml of sodium phosphate buffer (0.01 M; pH 6.5) at 4°C. The homogenate was filtered through four-layers of muslin cloth and the filtrate centrifuged at 6000 *g* at 4°C (20 min) in order to procure the supernatant for enzyme assay.

Peroxidase activity was determined according to the procedure reported earlier[18]. To a spectrophotometer sample cuvette, 1.5 ml of pyrogallol (0.05 M) and 100 µl of enzyme extract were taken, while the reference cuvette contained and equal volume of the inactivated enzyme (by boiling) and pyrogallol. Absorbance was measured at 420 nm. To initiate the reaction, 100 µl of hydrogen peroxide (1%, v/v) was added to the sample cuvette. The enzyme activity is expressed as change in absorbance $min^{-1}g^{-1}$ fresh weight of the sample.

Peroxidase was purified by fractionating the crude enzyme extract over sephadex G-25 column (1.5 × 30 cm) using 0.01 M sodium phosphate elution buffer (pH 6.0). Fractions of 5 ml each were collected and assayed for peroxidase activity. Enzyme activity was estimated spectrophotometically, and fractions showing peroxidase activity were pooled and applied to a DEAE-Sephadex (Sigma) column (1.5 × 30 cm) equilibrated with 0.05 M Tris-HCl, pH 8.0. Peroxidase was eluted from the column with a linear salt gradient of 0, 0.2 M NaCl in a total volume of 150 ml. Fractions with peroxidase activity were dialysed four times against distilled water and lyophilized, and the molecular weight of purified enzyme determined by SDS–PAGE with protein standards of known molecular weight.

The CWP extracted from both the pathogens induced resistance against *Fusarium* wilt and charcoal rot in

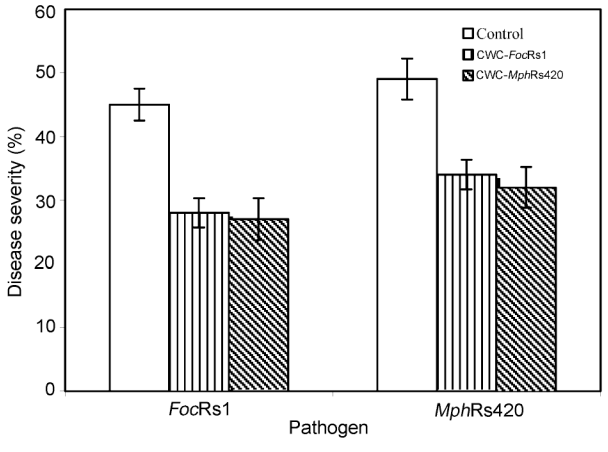

Figure 1. Disease severity (%) in chickpea by treatment with cell wall protein.

chickpea. The results demonstrate that CWP of *Foc*Rs1 reduced wilt disease by 37% and charcoal rot by 40% relative to control and CWP of *Mph*Rs420 also reduced both wilt and charcoal rot by 31 and 35% respectively (Figure 1).

CWP-induced resistance in chickpea against *F. oxysporum* f. sp. *ciceri* and *M. phaseolina* developed systemically. In split root experiments, one side of the root system was dosed with the CWP and then was challenged with pathogens, *F. oxysporum* f. sp. *ciceri* and *M. phaseolina*. There was significant decrease in disease severity relative to control, as the range was 36–38% and 29–33% with regard to *Fusarium* wilt and charcoal rot respectively (Figure 2).

CWP did not directly affect the growth of both the pathogens as observed by *in vitro* experiments on their growth in the PDA medium. Both the pathogens grew optimally as evident from comparable colony diameters on CWP-treated PDA plates (Figure 3).

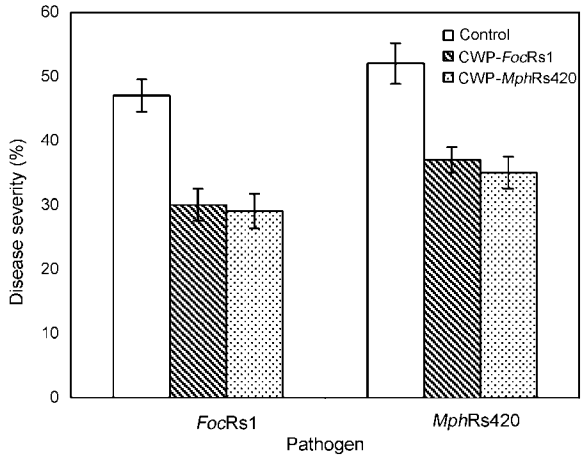

Figure 2. Disease severity (%) in chickpea (in split root system) by treatment with cell wall protein.

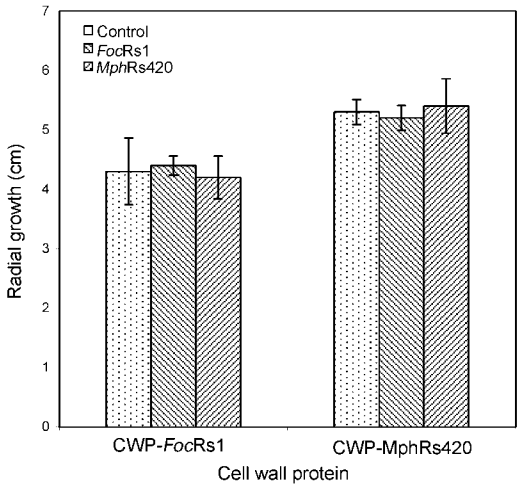

Figure 3. Effect of cell wall protein in radial growth of pathogens.

CWP induced accumulation of phenolic content, PAL and PR protein (peroxidase) in host plants (Figures 4 and 5). In general, SDW-treated plants did not show any accumulation of the above parameters or activity of both the enzymes. In contrast, CWP-treated plants exhibited rapid and significant increase in the activity and amount of the examined defence-related components. Maximum levels of phenol and PAL were recorded on the third and second day respectively, and decreased thereafter (Figure 4 *a* and *b*). Plants treated with SDW did not show such changes. CWP-treated plants significantly increased the PR-protein and peroxidase activity (Figure 5). Accumulation of PR-protein was observed following the first day of inoculation; it reached a maximum after the second day, and was maintained up to the third day, followed by a progressive decrease thereafter. SDS–PAGE of the purified peroxidase showed no change in the protein profile of chickpea control, whereas similar plants treated with CWPs of *Foc*Rs1 or *Mph*Rs420 showed similar banding pattern, e.g. 28, 50 and 54 kDa.

This study clearly demonstrates that CWPs from *Foc*Rs1 or *Mph*Rs420 induced resistance in chickpea against both the pathogens. However, CWP had no effect on the growth of *Foc*Rs1 or *Mph*Rs420 on *in vitro* assay, indicating the lack of fungicidal activity against the two pathogens. Takenaka *et al.*[10] also observed induction of defence reactions in sugar beet and wheat by treatment with CWP of *P. oligandum*.

The effect of CWP treatment on the activity of PAL, phenolic compounds and PR-protein (peroxidase) in chickpea seedlings was reflected as a significant increase in all the defence related enzymes. PAL is the initial entryway enzyme in the phenolic compound biosynthesis; therefore, it is ideal to determine the production rate of such compounds[10]. The present study also showed that cell wall-bound phenolic content accumulated significantly in chickpea seedlings. Earlier studies also demonstrated that rapid esterification of phenolic compounds into the plant cell wall is a common and early response in the expression of resistance[19]. Stadnik and Buchenauer[20] reported enhancement of PAL activity and accumulation of cell wall-bound phenolic compounds in wheat plants treated with BTH, a novel systemic acquired resistance (SAR) inducer in response to powdery mildew infection. Our study also indicates a rapid increase in the PAL activity in CWP-treated chickpea seedlings.

Peroxidase is considered as one of the important PR-proteins[21] and plants express enzyme activity during host–pathogen interaction[22,23]. Peroxidases are involved in the defence of plants against pathogens either by their direct participation in the cell wall reinforcement or by their role as antioxidants in oxidative stress generated during plant–pathogen interaction[8,23,24]. It has also been implicated in phenol oxidation, IAA oxidation[25], lignifications[26] and plant defence[24]. Increase in peroxidase activity has been correlated with resistance in many plants, including rice

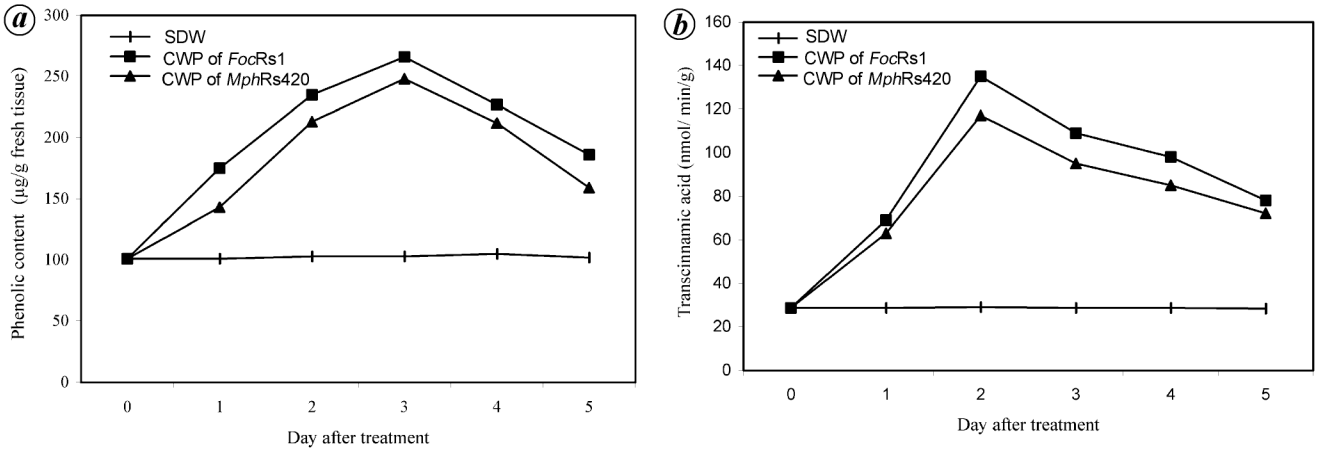

Figure 4. Accumulation of phenolic content (*a*) and PAL activity (*b*) in chickpea treated with cell wall protein.

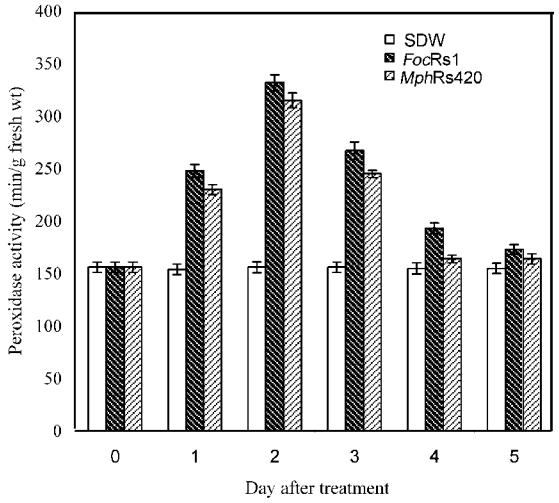

Figure 5. Changes in peroxidase activity in chickpea seedlings inoculated with cell wall protein.

and wheat[27]. In our study, significant increase in peroxidase activity in chickpea seedlings treated with CWP suggests that it may be one of the expressions of defence reactions in plants activated by the inducing agents. Overall our findings suggest that CWPs systemically induced resistance in chickpea plants accompanied by the accumulation of phenolic compounds, PR-protein and activities of PAL and peroxidase.

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