copious amounts of appendages produced at the cell surface, such as curli (Figure 1 *d*, *e*) could concentrate ionic molecules from the bulk phase as the biofilm develops[15].

Occasionally, for purely mechanical reasons, some bacteria are shed from the colony, or some bacteria stop producing EPS and are thus 'released' into the surrounding environment (Figure 1 *h*). Biofilm cells may be dispersed either by shedding of daughter cells from actively growing cells, or detachment as a result of nutrient levels or quorum sensing, or shearing of biofilm aggregates (continuous removal of small portions of the biofilm) because of flow effects[21]. As the thickness of EPS increases, anaerobic condition develops within the biofilm with loci of biofilm consisting of anaerobic bacteria. As a result of the combination of film thickness and activity of anaerobic species, the film detaches and sloughs-off from the surface of the substrate[22]. Polysaccharidase enzymes specific for EPS of different organisms, may possibly be produced during different phases of biofilm growth and contribute towards the detachment[23].

Hence to mimic *in vivo* conditions, *in vitro*, *S.* Gallinarum was grown in liquid medium providing bentonite clay as inert surface, and depleting nutrients and growing for longer periods, which leads to slow growth of bacteria to form biofilms. Biofilm formation was under the process of phase variation, possessing vastly different phenotypic traits, by upregulation of EPS and curli, while flagellin and pilin production was down-regulated.

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Detection of pathogenesis-related proteins–chitinase and β-1,3-glucanase in induced chickpea

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Pathogenesis-related (PR) proteins, chitinase and β-1,3-glucanase were extracted from induced chickpea plant and purified by gel filtration. Time-course accumulation of these PR-proteins in induced chickpea plants was significantly ($P = 0.05$) higher than the control. Maximum activities of these PR-proteins were recorded after three days of inoculation in all induced plants. Thereafter, the activity decreased progressively. Two chitinases and three β-1,3-glucanases were detected in induced chickpea plants. The molecular mass of the purified chitinases was 31 and 62 kDa and β-1,3-glucanases was 23, 27 and 39 kDa. Purified chitinases

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and β-1,3-glucanases also inhibited growth of *Fusarium oxysporum* f. sp. *ciceri* and other phytopathogenic fungi.

Keywords: Chickpea, chitinase, β-1,3-glucanase, pathogenesis-related proteins, phytopathogenic fungi.

INTERACTION between a plant and a pathogen results in the induction of numerous host-specific biochemical responses, so that the plant could withstand attack from pathogens[1]. Chitin and glucan are cell-wall components of many pathogenic fungi and depolymerization of cell-wall by the combined activity of chitinases and glucanases could kill fungi *in vitro*[2]. Chitin is a naturally abundant polymer of β-1,4-linked *N*-acetylglucosamine (GlcNac). Chitin hydrolysate can be used as a carbon and nitrogen source in the production of single-cell proteins[3]. Production of lytic enzymes, chitinase and β-1,3-glucanase by several PGPR strains is considered as a major antagonistic property of these strains[4–6]. It has been reported that chitinase and β-1,3-glucanase can function in defence against many fungal pathogens[7]. These lytic enzymes have hydrolytic action and degrade the cell-wall of many pathogenic fungi[4,8]. Inhibition of growth of several fungi requires the presence of combinations of chitinase and β-1,3-glucanase activities. Activities of these enzymes are known to be induced in many plants in response to infection with fungal pathogens[9] and also correlated with induced resistance[10,11]. Moreover, these two enzymes act synergistically in the partial degradation of fungal cell-walls. It was also suggested that combinations of the two enzymes could strongly inhibit growth of many fungi, including those that could not be inhibited by chitinase or β-1,3-glucanase alone[12]. A parallel increase in the activities of these enzymes is important for their optimal function in plant defence[13]. It is also essential to analyse and distinguish different isozymes to elucidate their roles in host–pathogen interaction.

Previously, we reported that different *Pseudomonas* spp. induced systemic resistance against charcoal rot[14] and *Fusarium* wilt of chickpea[15], as well as sheath blight of rice (data not published). Our previous studies indicated that *P. fluorescens* could produce SA and induced systemic resistance to chickpea against *Fusarium oxysporum* f. sp. *ciceri*. In continuation with our previous work, the present study aims (i) to extract and purify lytic enzymes (chitinase and β-1,3-glucanses) from induced chickpea plants, (ii) to analyse the isozymes of these PR-proteins, and (iii) to observe the antifungal properties of these proteins produced by induced chickpea.

Pseudomonas aeruginosa RsB29 (*Pa*RsB29) and *F. o.* f. sp. *ciceri* Rs1 (*Foc*Rs1) used in this investigation, was obtained from Laboratory of Applied Mycology, Department of Botany, Banaras Hindu University, Varanasi. The isolate *Pa*RsB29 was grown in King's B (KB) medium in 250 ml Erlenmeyer flasks on a rotary shaker (100 rpm) for 48 h at 28°C and cell concentration was adjusted at 10^8 cell ml^{-1}. Chickpea cv JG-62 cultivar susceptible to *Foc*Rs1 was used in this investigation. The seeds were surface-sterilized as described earlier[15]. Seeds were then allowed to germinate in glass tubes (1 seed per tube). The tubes were incubated in an environmental chamber under 16 h light and 8 h dark photoperiod at 28 ± 2°C. Seven-day-old seedlings were transplanted to earthen pot (radius 18 cm, three seedlings per pot) and incubated. After ten days, soil was treated with either 5 ml *Foc*Rs1 (ca. 10^3 conidia ml^{-1}) or *Pa*RsB29 (ca. 10^8 cells ml^{-1}) alone. One day after bacterial application, one set of bacterial treated plants were challenge-inoculated with 5 ml *Foc*Rs1. Activities of chitinase and β-1,3-glucanase were estimated after 0, 1, 2, 3, 4, 5, 6 and 7 days of inoculation and plants treated with sterile distilled water (SDW) served as control.

Colloidal chitin was prepared as described by Berger and Reynolds[16]. One gram of the above chickpea plant material was homogenized with 5 ml sodium citrate buffer (SCB; 0.1 M), pH 5. The homogenate was centrifuged for 20 min at 12,000 rpm. Supernatant was used as enzyme source. The assay mixture consisted of 10 µl SCB (1 M, pH 4), 0.4 ml, enzyme solution and 0.1 ml colloidal chitin (1 mg). After 2 h of incubation at 37°C, the reaction was stopped by centrifugation. An aliquot of the supernatant (0.3 ml) was pipetted into a glass reagent tube containing 30 µl potassium phosphate buffer (1 M, pH 7), and incubated with 20 µl (w/v) desalt snail gut enzyme (3%). After 1 h, pH of the reaction mixture was brought to 8.9 by addition of sodium borate buffer (1 M), pH 9.8. The mixture was incubated in boiling water for 3 min and then rapidly cooled in an ice water bath. Two ml of *p*-dimethylaminobenzaldehyde (DMAB) reagent was added and the mixture was incubated for 20 min at 37°C. The absorbance value was read at 280 nm using a spectrophotometer (Thermospectronic, USA). *N*-acetylglucosamine (GlcNac) was used as a standard. The enzyme activity was expressed as µmol GlcNac min^{-1} g^{-1} fresh tissues.

Total β-1,3-glucanase activity was colorimetrically assayed by the laminaria-dinitrosalicyclate method[13]. One gram of chickpea plant was extracted with 5 ml sodium acetate buffer (SAB; 0.05 M), pH 5. The extract was then centrifuged at 12,000 rpm for 15 min at 4°C. The supernatant was then used as crude enzyme extract. The extract (62.5 µl) was added to laminarin (4%, 62.5 µl) and incubated at 40°C for 10 min. The reaction was stopped by adding 375 µl of dinitrosalicylic acid reagent and heated for 5 min in boiling water bath. The resulting coloured solution was diluted with 4.5 ml water, vortexed and absorbance at 500 nm was determined. The blank was the crude enzyme preparation mixed with laminarin with zero time incubation. The enzyme activity was expressed as µmol equivalent glucose release min^{-1} g^{-1} fresh tissue.

Isozymes of chitinase and β-1,3-glucanase were analysed by native polyacrylamide gel electrophoresis[13]. For native anionic PAGE, 15% polyacrylamide resolving gels (1.5 mm thick) were prepared by mixing 5.3 ml 30% ac-

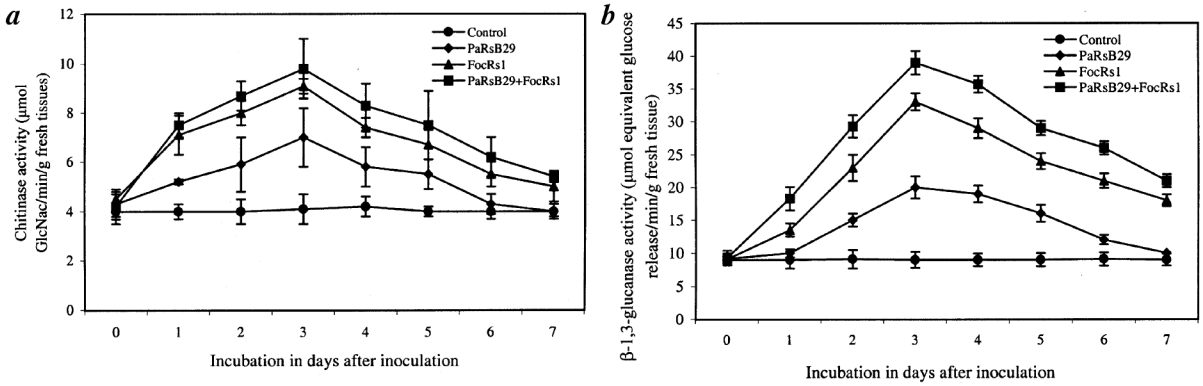

Figure 1. Change in PR-protein activities in chickpea by *P. aeruginosa* RsB29 against challenged inoculation with *F. o.* f. sp. *ciceri*; ***a***, chitinase; ***b***, β-1,3-glucanase.

rylamide (acrylamide and bis acrylamide; 30 : 0.8), 5.0 ml 1.5 M Tris-HCl (pH 8.8), 0.1 ml freshly prepared ammonium persulphate (10%), 9.3 ml of distilled water, and 10 µl of TEMED. The stacking gel contained 1.3 ml acrylamide (30%) and 2.5 ml 0.5 M Tris-HCl (pH 6.8); 50 µl freshly prepared 10% ammonium persulphate gel were poured between glass plates. The non-denaturing gel was run for 8 h at 10°C, 30 mA in an Electrophoresis Unit (Genei, Bangalore). Gels were stained with 0.2% coomassie brilliant blue.

Chitinase and β-1,3-glucanase were purified by fractionating the crude enzyme extract over a sephadex G-75 column (1.0 cm × 30 cm) using 0.01 M sodium citrate (0.1 M) or sodium acetate buffer (0.05 M; pH 5) as elution buffer. Next, 5 ml fractions were collected and analysed for PR-protein activity in each fraction. Fractions showing the PR-protein activity were pooled and applied to a DEAE-sephadex (Sigma, USA) column (1.0 cm × 30 cm) equilibrated with 0.05 M Tris-HCl (pH 8). Fractions having chitinase and glucanase activity were pooled, dialysed four times against SDW and lyophilized. Molecular weight of purified PR-proteins (chitinases and β-1,3-glucanase) was determined by SDS–PAGE with protein standards of known molecular weight.

Antifungal property of the purified chitinase and glucanase was determined by agar diffusion test on PDA medium. Sterilized paper disc (5 mm dia) containing 15 µl purified chitinase or glucanase was put at the centre of the petri plates containing sterilized PDA medium. Mycelial disc (5 mm) of some actively growing pathogens, viz. *F. o.* f. sp. *ciceri*, *F. udum* and *Macrophomina phaseolina* was inoculated individually at four points of the petri plates maintaining an equal distance from the centre. The plates were incubated for 7 days at 28 ± 2°C and zone of inhibition was recorded.

Time-course activity of PR-proteins (chitinases and glucanases) was observed in induced plants (Figure 1 *a*, *b*). Increase in the activity of PR-proteins was started one day after inoculation in all the induced plants and reached maximum level after three days; thereafter it decreased progressively. In control plant no such activity was recorded.

When chickpea seedlings were treated with *Pa*RsB29, activity of chitinase increased significantly ($P = 0.05$) than control. However, in *Pa*RsB29 pre-treated seedlings challenge-inoculated with *F. o.* f. sp. *ciceri,* an additional increase in chitinases activity was observed after 48, 72 and 96 h of treatment; activity of the enzyme was significantly increased in comparison to control. In chickpea seedlings inoculated with the pathogen, *F. o.* f. sp. *ciceri*, more than two-fold increase in chitinase activity over control was also observed after 48 and 72 h of inoculation (Figure 1 *a*).

In all the treatments, activities of β-1,3-glucanase increased significantly ($P = 0.05$) than control. Rapid increase in β-1,3-glucanase activity was exhibited in induced plants from day 1 post-inoculation. After day-3, activity increased more than four-fold, which decreased rapidly after day-4 (Figure 1 *b*). In general, maximum accumulation of this enzyme was exhibited in *P. aeruginosa* RsB29 pre-treated plant challenge-inoculated with *Foc*Rs1. For example, more than four-fold activity of the enzyme was found at 72 h after induction of resistance. It was also observed that more than two-fold increase in enzyme activity was recorded after day-1 to day-7 in bacterized plants challenge-inoculated with the pathogen. However, in seedlings induced by the pathogen, more than two-fold increase in enzyme activity was shown only from 2 to 6 days after inoculation. However, no such activity was recorded in control plants (Figure 1 *b*).

Chitinase and β-1,3-glucanase isozymes analysed in induced chickpea seedlings showed induction of two PR-chitinases (Figure 2 *a*) and three PR-β-1,3-glucanase, detected in native PAGE (Figure 2 *b*). These isozymes were not detected in control plants. The highest expressions of these isozymes were exhibited in *P. aeruginosa* RsB29 pretreated plants challenge-inoculated with the pathogen, *Foc*Rs1. Purified enzymes showed similar pattern of migration in native and denaturing acrylamide. Molecular mass of purified chitinases was 31 and 62 kDa (Figure 3 *a*), and β-1,3-glucanases was 23, 27 and 39 kDa (Figure 3 *b*).

The purified proteins also exhibited antifungal activity by inhibiting the radial growth of different fungal species,

e.g. *F. o.* f. sp. *ciceri*, *F. udum* and *M. phaseolina* (Figure 4). Among these pathogens, *F. udum* produced more inhibition zone (4.9 mm) than the others. In general, proteins extracted from bacterized pretreated plants challenged with the pathogen showed the highest antifungal activity against these pathogens (Figure 5 *a*, *b*) and chitinase exhibited more antifungal activity in comparison to β-1,3-glucanase *in vitro*.

Accumulation of PR-proteins is related with SAR in plants[17]. Chitinase and β-1,3-glucanase have not only the potential to hydrolyse cell components like chitin and β-1,3-glucan respectively, but they also release elicitors from the walls of fungi, which in turn stimulate various defence responses in plants[18]. The results from this study demonstrate that *P. aeruginosa* RsB29 induced systemic resistance against *Fusarium* wilt of chickpea. Induced plants resulted in a significant increase in PR-proteins, chitinases and β-1,3-glucanase. *Pseudomonas* pretreated plants challenge-inoculated with the pathogen resulted in an additional increase in PR-proteins, whereas other treatments (e.g. with *Foc*Rs1 or *Pa*RsB29 alone) were least effective in inducing resistance by increasing the level of chitinase and β-1,3-glucanase.

Induced systemic resistance has been demonstrated by *Pseudomonas* spp. on several plants[19–21]. Different mechanisms of *Pseudomonas* spp. have reduced plant diseases such as accumulation of phenolic component, increasing activity of PAL and PR proteins and lysis of host structure by secretion of extracellular lytic enzymes[9,20,22]. ISR by *Pseudomonas* spp. involves increasing physical and mechanical strength of the host cell-wall and causing biochemical and physiological changes leading to synthesis of PR-proteins[23–25]. Our findings suggested that *P. aeruginosa* RsG18 could cause ISR against *F. o.* f. sp. *ciceri* via synthesis and accumulation of the PR-proteins chitinase and β-1,3-glucanase. Results of time–course study showed that at least one day is required between the initial inoculations with *P. aeruginosa* or *F. oxysporum* f. sp. *ciceri* and induction of resistance by accumulation of these proteins. These are in agreement with work of other workers also[25,26].

It is also reported that chitinase is produced by a number of bacteria and fungi living in chitin-containing habitats like soil, sediments and marine environment[27]. Ferraris *et al.*[28] suggested that infection with *F. oxysporum* f. sp. *lycopersici* caused several-fold increases in chitinases and β-1,3-glucanase activity in susceptible and resistance cultivars. These enzymes were originated from host plants and greater activities were consistently shown in susceptible cultivars during pathogen infection. Benhomou *et al.*[29] also found accumulation of these enzymes in incompatible interactions of tomato and *F. lycopersici* or *F. oxysporum radicis-lycopersici*. In plants, chitinases are present constitutively and are induced systematically also upon treatment with biotic as well as abiotic inducers[30]. Chitinases along with β-1,3-glucanases impart resistance against a wide array of plant pathogens[31]. The present study reveals that induction of resistance by *P. aeruginosa* on *F. oxysporum* f. sp.

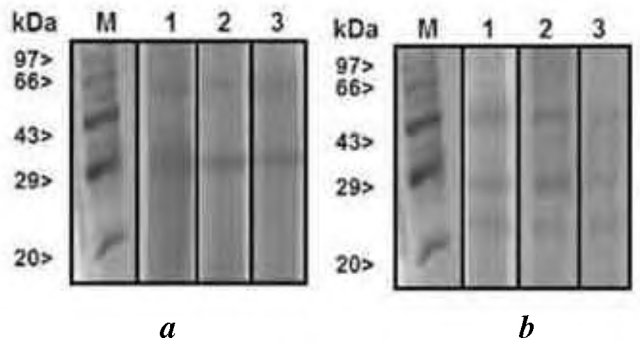

Figure 2. Native PAGE showing isozymes of PR-proteins in induced chickpea. ***a***, Chitinases; ***b***, β-1,3-glucanases. Lane M, Marker; lane 1, PR-protein from chickpea inoculated with *Foc*Rs1; lane 2, PR-protein from chickpea inoculated with *Pa*RsB29; lane 3, PR-protein from chickpea inoculated with *Pa*RsB29 + *Foc*Rs1.

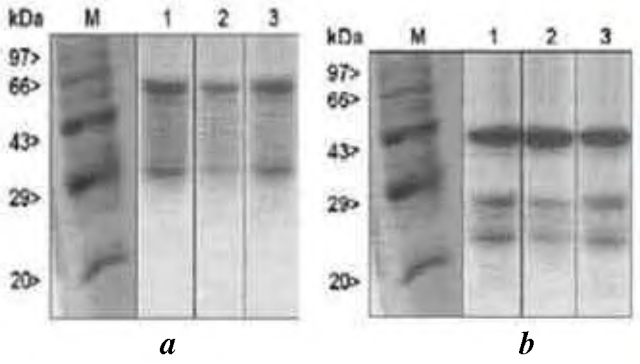

Figure 3. SDS–PAGE and molecular mass determination of purified chitinases (***a***) and β-1,3-glucanases (***b***). Lane M, Marker; lane 1, Purified PR-protein from chickpea inoculated with *Foc*Rs1; lane 2, Purified PR-protein from chickpea inoculated with *Pa*RsB29; lane 3, Purified PR-protein from chickpea inoculated with *Pa*RsB29 + *Foc*Rs1.

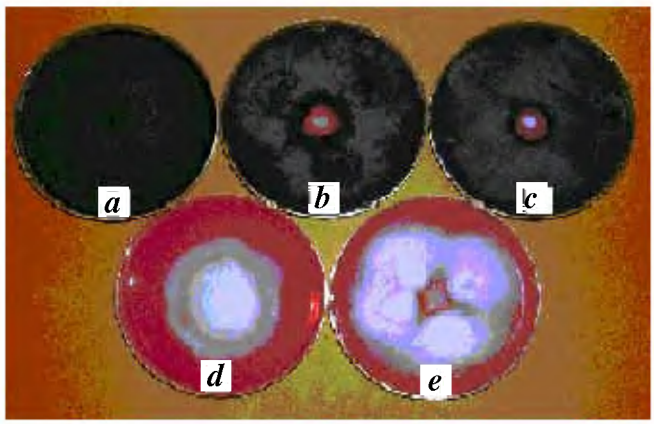

Figure 4. Growth inhibition zone produced by purified PR-proteins. *a*, *Macrophomina phaseolina* in control; ***b***, *M. phaseolina*-produced inhibition zone with chitinase; ***c***, *M. phaseolina* with β-1,3-glucanase; ***d***,. *Fusarium oxysporum* f. sp. *ciceri* Rs1 in control and ***e***, *F. oxysporum* f. sp. *ciceri* Rs1 produced inhibition zone with chitinase.

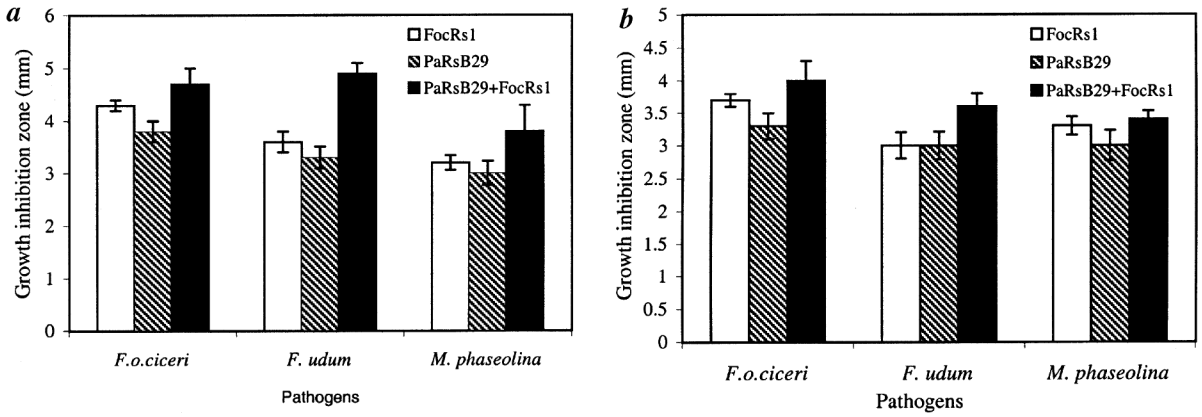

Figure 5. Antifungal activity of purified PR-proteins. *a*, chitinase; *b*, β-1,3-glucanase.

ciceri inoculated chickpea is caused by the accumulation of chitinases and β-1,3-glucanases.

In this study, we detected two chitinase and three β-1,3-glucanase isozymes in induced chickpea. An interesting finding is that purified chitinase and β-1,3-glucanase also exhibit antifungal activities against different phytopathogenic fungi *in vitro*. This is an indication of the direct effect of these PR-proteins on the pathogens. Mathivanan *et al.*[6] also reported that purified chitinase of *F. chlamydosporum* inhibited the germination of uredospores of *Puccinia arachidis* and lysed the walls of uredospores and germ tubes. Jr and Kuc[11] have also reported antifungal activity of cucumber β-1,3-glucanase and chitinase.

Overall, our findings suggest that (i) induced chickpea plants showed activities of defence related proteins – chitinase and β-1,3-glucanase; (ii) *P. aeruginosa* could cause ISR against *F. o.* f. sp. *ciceri* via synthesis and accumulation of these PR-proteins; (iii) induced chickpea plants produced two chitinases and three β-1,3-glucanases; (v) molecular mass of these purified chitinases was 31 and 62 and β-1,3-glucanases was 23, 27 and 39 kDa, and (vi) purified chitinases and β-1,3-glucanases also exhibited antifungal activity against phytopathogenic fungi.

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