Hydroxyproline-rich glycoproteins accumulate in pearl millet after seed treatment with elicitors of defense responses against Sclerospora graminicola

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Abbreviations: HRGPs, hydroxyproline-rich glycoproteins; Hyp, Hydroxyproline; hai, hours after inoculation.

1. Introduction

Success of a plant defense response depends on the speed by which the plant recognizes the attacking pathogen and the intensity by which appropriate defense responses are activated. The basal resistance response in plants to restrict the colonization of the pathogen can be enhanced by specific biotic and abiotic stimuli in the form of elicitors [1–4]. Protection of pearl millet [Pennisetum glaucum (L.) R. Br] against the downy mildew causing oomycete Sclerospora graminicola (Sacc.) Schroet is possible by application of abiotic elicitors such as β-amino butyric acid (BABA) [5], proline [6], chitosan [7], Trichoshield [8] and 2,6-dichloroisonicotinic acid (DCINA) [9]. It has also been shown that microorganisms like Pseudomonas fluorescens [10] and plant extracts of Datura metel [11,12] have the potential to control S. graminicola.

The effect of abiotic and biotic elicitors involves biochemical changes in the host metabolism that may play a role in limiting plant infection by S. graminicola. Cell wall reinforcements due to accumulation and cross-linking of hydroxyproline-rich glycoproteins (HRGPs) as a response to S. graminicola has been reported [13]. HRGPs are important plant cell wall structural components, which during the course of pathogen invasion are induced in several plant pathogen interactions [13–16]. The involvement of HRGPs in systemic acquired resistance (SAR) has been established recently using transformed tobacco cultivars having the nahG gene for salicylate hydroxylase. The transformed plants that were insensitive to salicylic acid signaling showed poor HRGP accumulations [15]. Also a highly co-ordinated localized alteration to plant cell walls with HRGP accumulation was show at the challenge sites of pathogen infection using monoclonal antibodies specific to HRGPs [13,17]. This represents a rapid defense mechanism to strengthen
the cell wall as a barrier to pathogen ingress prior to the development of transcription dependent defenses [18]. The possible mechanism by which HRGP accumulation contributes to disease resistance involves cross-linking between HRGP monomers catalyzed by peroxidase and hydrogen peroxide to form a network, which might provide anchorage for lignifications and creates a barrier impenetrable to fungal hyphae [16,18]. The current study was carried out to investigate the role of HRGPs during the induction of resistance in pearl millet against S. graminicola by seed treatment with selected biotic and abiotic elicitors.

2. Materials and methods

2.1. Plant material

Pearl millet cultivars 7042S (highly susceptible, HS) with >25% downy mildew disease incidence (DMDI) and IP18296 (highly resistant, HR) with 0% DMDI after inoculation with S. graminicola under field conditions were used in the study. The seeds were obtained from the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Hyderabad, India. The seeds of each line were sown in the downy mildew disease plot of the Department of Studies in Biotechnology, University of Mysore, Manasagangotri, Mysore 570006, Karnataka, India, for testing their reaction to the disease following the procedure of Williams et al. [19].

2.2. Pathogen and preparation of inoculum

S. graminicola was isolated from pearl millet cv. 7042S and maintained on the same cultivar under greenhouse conditions and was used for all inoculation experiments. Leaves of infected plants showing symptoms of downy mildew were collected in the evening, washed in running tap water to remove the remnants of previous sporulation, blotted dried, cut to pieces about 2 inches in length and placed in a moist chamber for sporulation. Fresh sporangia were collected the next morning and zoospores released by them used as inoculum [20].

2.3. Test seedlings used for the study

Seeds of resistant cv. IP18296 and susceptible cv. 7042S cultivars of pearl millet were surface sterilized in 0.1% sodium hypochlorite for 15 min and washed thoroughly with sterile distilled water. Seeds of the susceptible cv. 7042S were treated with the biotic and abiotic elicitors. The concentrations of elicitors used and duration of treatments were chosen based on earlier studies (Table 1). For each elicitor treatment, one hundred seeds were used. Simultaneously, seeds of the resistant and susceptible cultivars were treated with distilled water under similar conditions to serve as a standard control of resistance.

The treated and the untreated/control seeds were further germinated on moist filter paper under aseptic conditions at 25 ± 2 °C in darkness for two days. The two-day-old seedlings were inoculated by the root dip technique with a 4 × 10⁷ zoospores ml⁻¹ suspension of S. graminicola [20]. Seedlings dipped in sterile distilled water served as an uninoculated control. The seedlings were harvested at 8/9 h after inoculation for further experiments.

2.4. Analysis of hydroxyproline-rich glycoproteins (HRGPs)

2.4.1. Hydroxyproline (Hyp) content in cell walls of pearl millet coleoptiles

Test seedlings from resistant, susceptible and elicitor treated susceptible seeds were sampled at 9 hai (hours after inoculation) with S. graminicola. Seedlings dipped in sterile distilled water served as an uninoculated control. Cell walls from the coleoptiles regions of the test seedlings were isolated following the procedure of Shailasree et al. [13]. The coleoptiles of the seedlings were homogenized using pestle and mortar at 4 °C in 0.5 M potassium phosphate buffer, pH 7.0. The complete disruption of cells in the paste was examined by light microscopy. The homogenized suspension was centrifuged at 10,000g for 10 min. The pellet obtained was repeatedly washed with buffer followed by distilled water for five times. Washed cell walls were suspended by vigorous stirring in 5 volumes of 1:1 (v/v) chloroform–methanol. The organic solvent was carefully removed with out disturbing the cell wall pellet. Cell walls were washed three times with 5 volumes of acetone and then air-dried. The amount of HRGPs was determined by analyzing the Hyp content in the cell wall hydrolysate. Hydrolysis of the cell walls took place with 6 N HCl for 18 h at 110 °C in sealed tubes. Hydrolysates were evaporated to dryness. Hyp was then extracted in the minimum amount of distilled water from the dried hydrolyzed samples and the amount estimated following the spectrophotometric method of Prockop and Udenfriend [21]. Hyp content was expressed as µg Hyp mg⁻¹ cell wall (dry weight).

2.4.2. Hydroxyproline in suspension cells of pearl millet

The pearl millet cell culture was raised from the susceptible (7042S) cultivar by following the method of Vasil and Vasil [22]. The well-established suspension cells were regularly sub-cultured onto fresh medium at 1:5 dilution rates at 10-day intervals and after 10 sub-cultures the cells were used for the study. A cell culture (10⁹ cells ml⁻¹) at the mid-point of log phase of growth (16 day old) was used for the experiment. The suspension cells were treated with elicitors P. fluorescens (UOMSAR 14) at 10⁸ cfu/ml or Chitosan 4 A talc-based formulation containing 100 million spores per gram of Trichoderma harzianum, Gliocladium virens, and Bacillus subtilis, was obtained from Nutri-Tech Solution P/L, Queensland, Australia.

### Table 1

<table>
<thead>
<tr>
<th>Elicitor</th>
<th>Concentration</th>
<th>Time of seed treatments</th>
<th>Field protection observed</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chitosan (Sigma)</td>
<td>0.3% in distilled water</td>
<td>9 h</td>
<td>73%</td>
<td>Sharathchandra et al. [7]</td>
</tr>
<tr>
<td>2,6 dichloronicotinic acid (INA)</td>
<td>0.2 ml in distilled water</td>
<td>6 h</td>
<td>73%</td>
<td>Shivakumar et al. [9]</td>
</tr>
<tr>
<td>Pseudomonas fluorescens (UOMSAR – 14)</td>
<td>10⁶ cfu/ml⁻¹</td>
<td>6 h</td>
<td>70%</td>
<td>Raj et al. [10]</td>
</tr>
<tr>
<td>Trichoshielda</td>
<td>5% in distilled water</td>
<td>6 h</td>
<td>67%</td>
<td>Raj et al. [8]</td>
</tr>
<tr>
<td>Datura metel</td>
<td>2% leaf extract in distilled water</td>
<td>3 h</td>
<td>67%</td>
<td>Devaiah et al. [11]</td>
</tr>
<tr>
<td>Proline</td>
<td>15 mM in distilled water</td>
<td>3 h</td>
<td>67%</td>
<td>Shivakumar et al. [12]</td>
</tr>
</tbody>
</table>

Reference:

1. A talc-based formulation containing 100 million spores per gram of Trichoderma harzianum, Gliocladium virens, and Bacillus subtilis, was obtained from Nutri-Tech Solution P/L, Queensland, Australia.
(Sigma, St. Louis, USA), at 0.3% in distilled water for 1 h. After treatment the suspension cells were inoculated with zoospores of *S. graminicola* (4 × 10⁴ spores ml⁻¹) and harvested at different time intervals, viz., 0 h (before inoculation with the pathogen), 2, 4, 6, and 8 h after inoculation. After washing thoroughly in distilled water, cell walls were extracted from the suspension cells and HYP content was determined as described in subsection 2.4.1.

2.5. Extraction of total cell wall proteins

Test seedlings from resistant, susceptible and chitosan/P. *fluorescens* treated susceptible seeds were sampled at 9 hai with *S. graminicola*. Seedlings dipped in sterile distilled water served as an uninoculated control. Cell wall proteins were extracted from coleoptiles of the seedlings as reported by Shailasree et al. [13]. All procedures were carried out at 4 °C. Coleoptiles were homogenized in 0.5 M potassium phosphate buffer, pH 7.0, followed by centrifugation at 10,000g for 10 min. Subsequently, the supernatant was washed five times with the same buffer followed by washing with distilled water. The pellet was suspended in three volumes of 3:1 (v/v) absolute ethanol: 1.25 N HCl and incubated at 4 °C. After two days, cellular debris was removed by centrifugation at 10,000g. Proteins were precipitated by adding 3 volumes of cold acetone followed by incubation at 4 °C overnight. The precipitated proteins were centrifuged at 10,000g for 15 min. Acetone was decanted and the pellet was air-dried.

2.6. Electrophoresis

Total protein from the cell wall extracts were separated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) following the method of Laemmli [23] in a 1 mm thick, 12% sodium dodecyl sulphate – polyacrylamide gel electrophoresis (SDS-PAGE) following the method of Laemmli [23] in a 1 mm thick, 12% polyacrylamide gel. The acetone precipitate was dissolved in 0.05 M sodium acetate buffer (pH 3.5). Fifty microgram protein equivalents of each sample were loaded into the gel. Following SDS-PAGE, separated proteins were stained with Coomassie blue. Glycoproteins in the total cell wall extract were identified by periodic acid Schiff (PAS) staining [13].

2.7. Western blot analysis

Immediately after SDS-PAGE, gels were blotted onto nitrocellulose membranes (Millipore) using a Multiphor II (LKB, Pharmacia) electrophoretic transfer apparatus according to the manufacturer’s protocol. The blots were blocked in 2% fat-free milk powder in Tris buffered saline (TBS: 10 mM Tris HCl, pH 8.0, 150 mM NaCl). The blots were incubated for 2 h at 37 °C with primary antibodies (MAC 265, a rat monoclonal antibody against pea HRGP [24], kind gift from Elizabeth A. Rathbun, John Innes Centre, England) diluted in TBS buffer. After washing three times with TBS, the blots were incubated with anti-rat IgG horseradish peroxidase-conjugate for 1 h at room temperature followed by three washes with TBS. Subsequently, the blots were stained for peroxidase activity with 1.33 mM 3,3’-diaminobenzidine (DAB, Sigma, MO, USA) and 10 mM hydrogen peroxide. The proteins on the blots were visualized by periodic acid-silver methenamine-Schiff (PAS) staining [13].

2.8. Tissue printing

Test seedlings from resistant, susceptible and chitosan treated susceptible seeds were sampled at 9 hai with *S. graminicola*. Tissue print was carried out as described by Cassab and Varner [25]. Coleoptile regions were separated and cross-sectioned, dried on a kim wipe, and pressed onto nitrocellulose membrane for 30 s. Nitrocellulose paper was pretreated with 0.2 M CaCl₂ for 30 min and dried before use. After printing the paper was air-dried for 10 min and subjected to immunolabeling. The blots were blocked in 3% BSA in Tris buffered saline (TBST: 10 mM tris (pH 7.2), 0.8% NaCl and 0.05% Tween 20) for 1 h. The blots were probed with MAC 265 monoclonal antibody as described above in the western blot analysis. The images were observed using a stereo binocular microscope (Wild Heerbrugg, Switzerland) with high magnification and recorded using a digital camera (Nikon coolpix 990) attached to the microscope.

2.9. Peroxidase activity and isoforms

Test seedlings from the resistant, susceptible and chitosan/P. *fluorescens* treated susceptible seeds were sampled at 8 hai with *S. graminicola*. Seedlings dipped in sterile distilled water served as an uninoculated control. The peroxidase activity and isoforms accumulation pattern was obtained and compared in these samples.

2.9.1. Extraction of protein

Seedlings were harvested 8 hai and coleoptiles of the seedlings homogenized in 2 ml of 0.05 M phosphate buffer, pH 7.0, at 4 °C and centrifuged at 12,000g for 15 min. The supernatant was used as crude enzyme for spectrophotometric assay of peroxidase and isoelectric focusing (IEF) analysis. The protein concentration was determined by the dye binding method of Bradford [26] using bovine serum albumin as standard (Sigma, St. Louis, USA).

2.9.2. Spectrophotometric analysis of peroxidase activity

Peroxidase assay was carried out as described by Hammerschmidt et al. [27]. The reaction mixture (3 ml) consisted of 0.25% (v/v) guaiacol and 10 mM hydrogen peroxide in 10 mM potassium phosphate buffer, pH 6.9. Addition of 5 µl of crude enzyme extract initiated the reaction, which was measured spectrophotometrically at absorbance (A₄₇₀) (Hitachi U 2000, Japan). Peroxidase activity was expressed in terms of change in A₄₇₀ for the linear phase of the slope (A₄₇₀ min⁻¹ mg⁻¹ protein). Results are presented from individual experiments, with 25 seeds per treatment. Three independent experiments were performed.

2.9.3. Isozyme analysis of peroxidase using isoelectric focusing (IEF)

IEF was performed on a 1.5 mm, 7.5% polyacrylamide gel containing 2% ampholyte (pH 3–10, Sigma, St. Louis, USA) using a Multiphor II (LKB) system according to the manufacturer’s protocol. pI markers (Sigma) ranging from pI 3.6 to 9.3 were co-electrophoresed to estimate the pI of the proteins. Forty micrograms of protein were loaded at the center of the horizontal gel maintained at 2 °C. IEF was performed at 2 °C for 3 h by stepwise increases in voltage: 200, 400, 600, and 800 V for 30 min each and lastly 1000 V for 1 h. After electrophoresis, gels were stained according to the method of Schrauwé [28]. The pI of the peroxidase isozymes were calculated using the Image Analysis System (Vilber Lourmat, France). The isoenzymes showing differential accumulation were quantified using the Bioprofile Image System (Vilber Lourmat, France). Results are presented in arbitrary units.

2.10. Localization of H₂O₂

Test seedlings from resistant, susceptible and chitosan treated susceptible seeds were sampled at 8 hai with *S. graminicola*. Seedlings dipped in sterile distilled water served as an uninoculated control. Coleoptile peelings from the test seedling were used for H₂O₂ localization following the method of Thordal-Christensen et al. [29]. The peelings were placed in freshly prepared solutions of 1 mg ml⁻¹ of 3,3’-diaminobenzidine (Sigma, St. Louis, USA), pH 3.
3.8 at 26 °C. After incubation for 30 min, the epidermal peeling were washed with 96% ethanol and mounted in 10% glycerol for light microscopy. H$_2$O$_2$ was seen as dark brown coloration in the cell walls. They could be classified into the following categories viz, 0) no accumulation; 1) light and confluent accumulation; 2) dark and patchy accumulation.

3. Results

3.1. Accumulation of HRGPs in pearl millet as a response to treatment with various biotic and abiotic elicitors

Several biotic and abiotic elicitors that are reported to protect pearl millet against *S. graminicola* infection (Table 1) were investigated for their ability to induce cell wall reinforcement through HRGPs. The accumulation of HRGPs as determined by Hyp content in the cell walls of pearl millet coleoptiles at 9 hai is presented in Fig. 1. Treatment of seeds with elicitors and further challenge inoculation with *S. graminicola* resulted in increased amounts of Hyp. The maximum level of Hyp in uninoculated plants was observed in the resistant cv. IP 18296 (0.28 μg Hyp mg$^{-1}$ cell wall, dry weight) and this increased after inoculation to 0.53 μg Hyp mg$^{-1}$. The Hyp content in the control of the susceptible cv. 7042S was significantly lower (0.16 μg Hyp mg$^{-1}$) and it did not change after inoculation. Treatment of the susceptible cultivar with chitosan or *P. fluorescens* resulted in increased constitutive Hyp content (0.21 μg Hyp mg$^{-1}$). Inoculation with *S. graminicola* increased the Hyp accumulation to 0.41 and 0.45 μg Hyp mg$^{-1}$ in chitosan and *P. fluorescens* treated plants, respectively. Seed treatment with proline, INA, *D. metel* and trichoshield did not result in significant increase in Hyp concentration compared to susceptible untreated control. However these treatments followed by further inoculation with *S. graminicola* resulted in increased Hyp content to 0.27, 0.36, 0.34 and 0.27 μg Hyp mg$^{-1}$ for proline, INA, *D. metel* and trichoshield treatment, respectively (Fig. 1).

Since significant increase in HRGP accumulation was observed only for chitosan and *P. fluorescens* treatments, these were selected for further studies. Pearl millet suspension cells were established and a time course study on the accumulation of Hyp was carried out in susceptible and elicitor treated susceptible variety of pearl millet. The Hyp content remained constant in control samples. One hour treatment of suspension cells with chitosan and *P. fluorescens* resulted in the increased accumulation of Hyp in cell wall extracts of suspension cells. Maximum accumulation level was observed at 6 h after inoculation with *S. graminicola* in elicitor treated cells (Fig. 2A and B).

3.2. Analysis of acid-ethanol extracted proteins and identification of HRGPs

Cell wall proteins extracted from the coleoptiles of seedlings raised from seeds of resistant, susceptible and elicitor treated susceptible cultivars (9 hai with *S. graminicola*) were analyzed by electrophoresis. Distilled water treated seedlings were kept as a control check. Coomassie blue staining of SDS-PAGE separated proteins revealed several bands with molecular weights ranging from 45 to 14 kDa (Fig. 3A). To identify glycoproteins, PAS staining of the SDS-PAGE gel was carried out. A 17 kDa stained for PAS in all the samples (Fig. 3B). Western blot analysis using MAC 265 identified 27, 17 and 14 kDa HRGP in resistant cultivars (Fig. 3C). MAC 265 antibody revealed that the 14 kDa band absent in the uninoculated samples of susceptible cultivar was induced upon elicitor treatments (Fig. 3C). The two major proteins of 17 and 14 kDa reacted with higher intensity in resistant and elicitor treated susceptible (chitosan and *P. fluorescens*) samples upon inoculation with the pathogen.

3.3. Tissue printing

Tissue printing and immunolabeling with MAC 265 antibody showed differential localization of HRGPs in the cross sections of coleoptiles from all test samples of pearl millet seedlings (Fig. 4). An intense HRGP accumulation was observed in resistant cultivar of pearl millet specifically in the regions of vascular bundles which further increased upon challenge inoculation with *S. graminicola* at 9 hai (Fig. 4A). The susceptible cultivar did not show any intense banding pattern for HRGP during the same time interval (Fig. 4B). Interestingly, scattered and increased accumulation of HRGP was observed around the vascular bundles of chitosan treated susceptible (chitosan and *P. fluorescens*) samples upon inoculation with the pathogen.

3.4. Peroxidase assay

Peroxidase activity was determined in coleoptiles of resistant, susceptible, as well as in the chitosan/*P. fluorescens* treated pearl millet samples (Fig. 5). The maximum constitutive peroxidase activity was observed in IP18296 and this increased significantly 8 hai with *S. graminicola*. On the other hand, peroxidase activity was not significantly altered in the susceptible cultivar after inoculation. Treatment of susceptible seeds with chitosan and *P. fluorescens* resulted in marginal increased peroxidase activity of the seedlings.
compared to the control. This activity increased significantly after inoculation with the pathogen.

3.5. Peroxidase isoform analysis by isoelectric focusing (IEF)

Peroxidase isoforms were separated by IEF and detected by in-gel activity staining. Several basic and acidic isoforms were seen (Fig. 6A). Of these, the basic isoforms corresponding to pl 8.9, 8.7 and 8.5 stained with higher intensity in elicitor treated samples compared to their respective controls. Quantification of these bands also indicated higher accumulation of these isoforms in the samples treated with elicitors compared to the untreated ones (Fig. 6B).

3.6. Analysis of H$_2$O$_2$ localization

The accumulation of H$_2$O$_2$ was assessed by the appearance of brown coloration within the periplasmic space of seedling tissue after staining with DAB. The hypersensitive response (HR) lesions are visible microscopically as brownish-black spots. H$_2$O$_2$ accumulation was evaluated at 8 hai with the pathogen in the epidermal peelings of test seedling coleoptiles. The accumulation was observed in all test seedlings, but to varying degrees. In case of resistant cultivar HR like reaction showing the accumulation of H$_2$O$_2$ within cells close to the parasite (haustoria) was observed upon _S. graminicola_ inoculation at 2 h (Fig. 7A). With increase in time interval, a dark and confluent H$_2$O$_2$ deposition was observed.

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**Fig. 2.** The Hyp accumulation in elicitor treated suspension cells of susceptible cv. 7042S. (2A) Chitosan treatment was carried out for 1 h. The samples were further inoculated with a suspension of _S. graminicola_ and collected at different time intervals. Distilled water treated suspension cells were kept as a control check. The samples are: (○) Susceptible control; (●) Susceptible inoculated; (▲) Chitosan treated control and (▼) Chitosan treated suspension cells inoculated with _S. graminicola_. (2B) _P. fluorescens_ treatment was carried out for 1h. The samples were further inoculated with suspension of _S. graminicola_ and collected at different time intervals. Distilled water treated suspension cells were kept as a control check. The samples are: (○) Susceptible control; (●) Susceptible inoculated; (▲) _P. fluorescens_ treated control and (▼) _P. fluorescens_ treated inoculated. The values are means of three independent experiments. Bars indicate ± SE.

**Fig. 3.** Analysis of acid-ethanol extracted proteins, HRGPs identification and induction pattern obtained (A) Coomassie blue; (B) Periodic acid Schiff staining and; (C) Western blot analysis using the MAC 265 antibody [24] of total cell wall proteins extracted from coleoptiles of the resistant (IP18296) and susceptible (7042S) and elicitor treated susceptible pearl millet cultivar. RC: Resistant control; RI: Resistant inoculated with _S. graminicola_; SC: Susceptible control, SI: Susceptible inoculated with _S. graminicola_; Pf C: _P. fluorescens_ treated susceptible control; Pf I: _P. fluorescens_ treated susceptible plants inoculated with _S. graminicola_; Chi-C: chitosan treated susceptible control; Chi-I: chitosan treated susceptible plants inoculated with _S. graminicola_; MW: low molecular weight markers. (For interpretation of the references to colour in figure legends, the reader is referred to the web version of this article.)
(Fig. 7B). In seedlings of the susceptible control, H$_2$O$_2$ accumulation was light and confluent (Fig. 7C) and this changed to small, dark and patchy spots 8 hai with _S. graminicola_ (Fig. 7D). When chitosan was used as an elicitor, H$_2$O$_2$ accumulation was induced as evidenced by its more pronounced dark and confluent appearance in susceptible cv. upon 8 hai with _S. graminicola_ (Fig. 7E).

### 4. Discussion

The present study investigated the induction pattern of HRGPs in a susceptible cultivar of pearl millet following treatment with several biotic and abiotic elicitors. The accumulation of HRGPs was determined by monitoring the Hyp content in the cell walls. The colorimetric estimation of Hyp is reported to be a sensitive indicator for the presence of HRGPs [30]. Results of the present study indicated a four fold increase in Hyp in the cell walls of resistant pearl millet cv. IP18296 upon _S. graminicola_ inoculation, when compared to susceptible cv.7042S. The analysis of Hyp among seedlings raised from susceptible seeds treated with abiotic and biotic elicitors indicated an increase in the wall-bound HRGP level upon elicitor treatment. Furthermore, when the Hyp accumulation (Fig. 1) was compared to the downy mildew protection data (Table 1), a higher amount of Hyp was recorded in those treatments where the protection against _S. graminicola_ exceeded 70% under field conditions. Among the various elicitors used in the study, induction of Hyp was observed more prominently in chitosan and _P. fluorescens_ treated samples. These treatments showed a further three fold increase in Hyp accumulation during challenge inoculation with _S. graminicola_ when compared to the susceptible controls. Higher accumulation was observed after 6 h of inoculation with _S. graminicola_ in the elicitor treated suspension cells of susceptible pearl millet cultivar. These results indicate that the seed treatment with...
elicitors triggers the defense reaction in pearl millet which includes the accumulation of HRGPs in the cell walls.

In the present study, soluble proteins were removed by repeated washes with buffer and water and the insoluble cell wall proteins were extracted from the cell wall by using an acid and ethanol mixture. This process results in a protein preparation that is rich in HRGPs. SDS-PAGE of these acid-ethanol extracted cell wall proteins followed by Coomassie blue staining showed several proteins. Three proteins with molecular weights of 27, 17 and 14 kDa were extracted from the cell wall by using an acid and ethanol mixture. This process results in a protein preparation that is rich in HRGPs. SDS-PAGE of these acid-ethanol extracted cell wall proteins followed by Coomassie blue staining showed several proteins. Three proteins with molecular weights of 27, 17 and 14 kDa were extracted from the cell wall by using an acid and ethanol extraction and denaturing SDS-PAGE analysis. The 14 kDa HRGP was observed in highly susceptible pearl millet varieties only upon pathogen inoculation. Interestingly in the present study an induction of 14 kDa HRGP was observed upon treatment of susceptible cultivars with the elicitors of defense. Sensitizing a susceptible plant with a suitable elicitor has been reported to result in more rapid response of the plant against virulent pathogens. An increase in the 14 kDa HRGP was observed following oxidative burst after perceiving the presence of a pathogen, they cross-link with each other through covalent bridges to form an insoluble barrier. The possible mechanism by which HRGP accumulation contributes to disease resistance involves cross-linking between HRGP monomers to form a network which might provide anchorage for lignifications and create a barrier impenetrable to fungal hyphae. This might also lead to obstruction of haustoria production and nutrient shortage, which might be particularly unfavorable for biotrophic pathogens. It has also been proposed that HRGPs could act as microbial agglutinins.

The HRGP cross-linking is a peroxidase mediated process in the presence of H$_2$O$_2$. In our study on the pearl millet- downy mildew interaction, H$_2$O$_2$ accumulated to a higher extent in the highly resistant cultivar compared to the highly susceptible cultivar. We found clear indications of HR responses in the cell wall peelings of the resistant cultivar of pearl millet upon S. graminicola inoculation at 8 hai. It was observed that HRGPs in the hypersensitive response (HR) cells are cross-linked, a process fuelled by H$_2$O$_2$ which limit pathogen entry to other parts of the plant. Our findings of an intense accumulation pattern for H$_2$O$_2$ in cells close to the S. graminicola haustoria in the resistant variety undergoing HR reactions gives an indication of the possible HRGP cross-linking that can take place in those regions to stop the pathogen ingress.

In the present study, an early accumulation of H$_2$O$_2$ by 2 h following inoculation was recorded that continued reaching a peak by 8 hai. It was observed that maximum H$_2$O$_2$ accumulation was in the chitosan and P. fluorescens treated susceptible cultivar at 8 hai. This higher accumulation of H$_2$O$_2$ in elicitor treated pearl millet seedlings at 8 hai coincided with the onset of induction of HRGPs using the elicitors at the same time interval of 8–9 h. Peroxidase activity also followed a similar pattern Treatment with chitosan and P. fluorescens of the susceptible cultivar resulted in a marginal increase in peroxidase activity, which increased substantially after challenge inoculation with S. graminicola. In other host pathogen interactions for example in barley inoculated with Blumeria graminis f.sp. hordei, H$_2$O$_2$ accumulates several hours before cell death, first subcellularly, directly beneath fungal appressoria; then during a second H$_2$O$_2$ burst, filling the entire attacked epidermal cell. Similar results were obtained in case of wheat-powdery mildew interactions. HRGP cross-link in the presence of H$_2$O$_2$ and the peroxidase enzyme in-vitro. Similar kind of cross-linking is possible in vivo or inside the plant cell wall after oxidative burst or H$_2$O$_2$ accumulation to stop the pathogen ingress.
The class III plant peroxidases (Prxs) belonging to the basic isoforms of the superfamily of peroxidases helps in cell wall cross-linking in presence of H₂O₂ [40,41]. In our study, IEF analysis of peroxidase indicated that isoforms with pI 8.9, 8.7 and 8.5 were induced in resistant, chitosan and P. fluorescens treated seedlings infected with S. graminicola. These results corroborate earlier reports where basic isoforms of peroxidase were involved in HRGP cross-linking. A cationic peroxidase with cell wall cross-linking activity was also reported in rice plants infected by Xanthomonas oryzae pv. oryzae [42]. Jackson et al. [43] reported HRGP cross-linking activity induced and Industrial Research, New Delhi, India. The authors thank the research fellowship received from The Council of Scientific and Industrial Research, New Delhi, India. The authors thank Dr. Elizabeth A. Rathbun (John Innes Centre, England) for providing the MAC 265 monoclonal antibody.

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