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Research Article

INVOLVEMENT LYTIC ENZYMES AND HYDROXY PROLINE RICH-GLYCOPROTEIN/GLYCINE-RICH PROTEIN IN THE RESISTANCE OF PEARL MILLET TO DOWNY MILDEW

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ABSTRACT
The biomolecular difference in the structural and functional components of resistant and susceptible varieties of pearl millet which is greatly affected by downy mildew disease caused by *Sclerospora graminicola* were analyzed. Mesocotyl region has shown to be the most susceptible region for attach of pathogen. Both resistant and susceptible varieties expressed differential level of lytic activity in 2 day old pearl millet seed and seedlings of resistant and susceptible verities. Specific induction of lytic activity and small molecular weight protein in the resistant seedlings only after inoculation, which was not observed either in uninoculated resistant or inoculated susceptible varieties. Resistant varieties showed the increased levels of hydroxy proline rich-glycoprotein and cell wall components than the susceptible verities. Upon inoculation decreased levels of hydroxy proline rich-glycoprotein in the resistance varieties suggest the possible formation of oxidative cross-linking which forms a strong barrier against the entry of the downy mildew pathogen into the host.

Key Words: *Pennisetum glaucum, Pearl millet, Downy mildew, Resistant, Susceptible, Seed, Seedlings, Lytic Activity, Hydroxy proline rich- glycoprotein*

INTRODUCTION
Pearl millet (*Pennisetum glaucum* (L.) R.Br.) is an important crop cultivated for food and fodder in the semi-arid tropics including India. This crop is known to be affected by a variety of fungal diseases in the field, resulting in the great yield losses. Among them downy mildew caused by different pathotypes of *Sclerospora graminicola* (Sacc.) Schroter is the most widespread serious disease in India and West Africa (Shetty, 1990). In order to regulate the invasion of the pathogen, plants exhibit resistance through the production of inhibitors, phytoalexins, deposition of lignin and accumulation of cell wall hydrolytic enzymes. The responses are also known to occur due to the presence of glycoprotein and lipid elicitors present in the fungal cell walls (Vidyasekaran, 1988). The dynamic nature and functions of plant cell walls in terms of growth and development, environmental sensing and signaling, plant defense, intercellular communication and selective exchange interfaces are reflected in the variation in cell wall components (Bowles, 1990). Five classes of cell wall proteins viz., (a) extensions; a family of hydroxy proline-rich glycoproteins (HRGPs) (b) Glycine-rich proteins (GRPs) (c) Proline-rich proteins (PRPs) (d) Solanacious lectine and (e) Arabinogalactan proteins (AGPs) have been depicted in the literature are involved in development , regulation of expression of wounding, fungal infection, viral infections and during drought stress etc. During the interaction between host and pathogen the composition in cell walls have been encountered and their regulated expression shown to be depended on specific gene activation. Within the specific host-pathogen interaction there are specific alterations in cell wall components which account for resistance in a particular host cultivar (Showalter, 1993). Increased production of hydroxy proline-rich glycoproteins and an associated arabinosyl transferase enzyme, specific for HRGP synthesis is well documented during interactions (Roby *et al*., 1985). However, the precise structure and functional relationship of HRGPs in pearl millet and their specific role in downy mildew disease resistance in not clearly understood. In the present study, considering all these parameters, emphasis has been made

regarding the understanding of basic mechanisms of resistance with special reference to lytic activity, hydroxy proline-rich glycoproteins and glycine-rich proteins in seeds and seedlings of mildew resistant varieties of pearl millet.

MATERIALS AND METHODS

Collection of Seeds

The seeds of pearl millet of resistant varieties viz., SDN-503R, IP 18292R, IP 18293, IP 18294, IP 18295, IP 18296 and susceptible verities namely 7042S, 843B, 852B, 23D_2BS and 841B were collected from the Project Co-ordinator, All India Co-ordinated Pearl Millet Improvement Project (AICPMIP), Pune, and the International Crops Research Institute for Semi-Arid Tropics (ICRISAT), Hyderabad, India. The seeds were sown in the downy mildew sick plot, which is being maintained at the department, and their reaction to the disease was tested and confirmed by adopting the procedure of Williams *et al.*, (1981).

Collection of Downy Mildew Pathogen — S. graminicola

Pearl millet leaves infected with *S. graminicola* pathotype 1 were collected from the greenhouse of the Applied Botany Department, University of Mysore, India. Leaves of infected pearl millet plants, showing symptoms of downy mildew disease, were collected in the evening, washed in running tap water to remove pre-existing sporulation, blot-dried and placed in a moist chamber for sporulation (Safeeulla, 1976).

Fresh sporangia were collected in distilled water, and washed with20 mM phosphate buffer saline (PBS), pH 7.4 Washings were discarded after centrifugation at 3000 rpm for 15 min. Fresh sporangia, free from other possible contaminants, were collected in PBS and were allowed to release zoospores. The pathogen suspension, pathogen suspension, phores, sporangia and zoospores, was used for the studies.

Collection of Infected and Non-infected Seedlings

Seeds of both resistant and susceptible cultivars were plated on sterile wet blotters in Petri dishes, and germinated according to the standard blotter method (ISTA, 1993). Seeds were germinated at 25±1°C, under near ultraviolet light, with alternating periods of 12:12 h light and darkness. In order to collect infected seedlings, 2-day-old seedlings were dipped in a fresh suspension of pathogen zoospores (50 000ml ¯1). After 24 h of infection, the seedlings were used for further study. Coleoptile (mesocotyl), root, and shoot portions were separated from infected and non-infected seedlings, and used for the study.

Preparation of Seed Extracts

500 mg each of resistant and susceptible seeds were ground to a fine powder in a mortar and pestle and extracted using 0.2 M using sodium phosphate buffer (pH 7.4) containing 0.1 % triton X-100. The homogenate(w/v) was centrifuged at 1500 rpm for 5 min to remove the debris and the supernatant was dialysed against 20 mM sodium phosphate buffer, pH 7.4 using 6000–12 000 cut-off dialysis tubing (Sigma Chemical Co., St. Louis, MO; USA), over night at 4°C, the dilution was noted down and stored at 5^0C for further studies.

Isolation of Cell Wall Components from Resistant and Susceptible Seeds

500 g of resistant and susceptible seeds were powdered and extracted in three parts of ethanol to one part of 1.25 N HCL (v/v). The mixture was centrifuged at 1500 rpm for 15min and the supernatant was precipitated with 20% TCA. The TCA precipitate was collected by centrifugation at 3000 rpm for 10 min. the supernatant was separated and precipitated with cold acetone for 3 h. acetone precipitate was collected by centrifugation at 10000 rpm for 10 min. both acetone and TCA precipitates were washed with cold acetone 3 times by centrifugation at 10,000 rpm for 10 min each. The precipitate was dried in the hood and was designed as cell wall extracts.

Preparation of Seedling Extracts

For the purpose of inoculation, *S. graminicola* infected leaves of pearl millet were obtained from downy mildew plot in Mysore, India washed in running tap water to remove sporangia, blotted and incubated in a moist chamber and encourage new sporulation. Fresh sporangia were collected in distilled water and washed in 20 nM phosphate buffer saline (pH 7.4) by centrifugation at 3000 rpm for 15 min. the

sporangia were collected and allowed to release zoospores. The zoospores suspension was used for inoculation of young seedlings of pearl millet of both susceptible and resistant varieties.

Three day old seedlings raised on damp blotting papers were inoculated by dipping seedling roots into the zoospore suspension for 30, 60, 90, 120 min. the inoculated seedlings were incubated for 24 h on wet blotters. From each treatment the mesocotyl, shoot and coleoptiles portions of seedling were separated and extracted separately in buffer for further experiments. Uninoculated seedling components were maintained as corresponding controls.

Collection of Red Cells

Fresh blood was collected from local slaughter house in a bottle containing 5% sodium citrate sodium and stored at 4^0 C. red blood cells (RBC) were isolated by centrifugation at 5000 rpm for 5 min at room temperature. Isolated cells were washed in phosphate buffered saline (pH 7.4) for 4 - 5 times and used for further studies.

Effect of Temperature on Lytic Enzyme Activity

The resistant and susceptible seeds extracts were boiled in a boiling water bath for 5 min. the precipitate was removed by centrifugation and the supernatants were examined for lytic activity. Comparative activity was determined before and after boiling crude resistant and susceptible seed extracts.

Quantitative and qualitative analysis of protein and carbohydrate through colorimetric and spectrophotometric methods

Proteins were estimated quantitatively using Lowry's method (Lowry *et al*., 1951). To 1 ml of standard and test sample, 3.0 ml of copper reagent was added and incubated at 28 ± 2^0C for 30 min.300ul of 1N folin –Coicaltaeu reagent was added and vortexed gently. The incubated samples were anlysed spectrophotometrically for optical density at 660nm (UV-Spectrophotometer U-2000, Hitachi, Japan). The protein concentrations of test samples were determined using a standard curve for BSA (Bovin Serum Albumin) at 0 to 100ug concentrations.

Crude extracts of the mesocotyl portion of inoculated 30, 60, 90, 120 min and uninoculated resistant and susceptible seedlings samples were loaded on to columns of Sephadex G-100 and G-15 separately. The 2.0 ml fractions were collected were monitored for protein at 280nm.

Sephadex G-15 column chromatography was performed for the cell wall components isolated from resistant and susceptible seeds and mesocotyl portions of resistant and susceptible, inoculated and uninoculated samples. 500ul fractions were collected and evaluated for protein and carbohydrate spectrophotometrically and the elution profiles were compared.

Total carbohydrate content was estimated by a phenol sulphuric acid method (Dubois *et al*., 1956). Concentrations range of 0 to 50ug of D-glucose was used as standard. To 1.0ml of test sample, 300 ul of 0.5% phenol and 2.0 ml of Con. H_2SO_4 were added. After cooling, the orange coloured complexes of the samples were estimated for carbohydrates spectrophotometrically at 490 nm.

Lytic activity assay by spectrophotometric method by using red blood cells

RBC lysis and pathogen lysis assays were carried out by spectrophotometric method Studies were conducted in quadruplicates and experiments were repeated in five different batch seeds, which were obtained during different harvest time (Umesha, 1993). Washed RBC were mixed with equal proportions of 20 M phosphate buffer saline (pH 7.4) and incubated with various doses of crude extracts of resistant and susceptible seedlings. The reaction mixture was incubated at 37^0C for 45 min, centrifuged at 5000rpm for 5 min and the supernatant separated and diluted to 2.5 ml. the release of haemoglobin into the supernatant by lysis of RBC was measured quantitatively at 415 nm using a uv-spectrophotometer. The release of haemoglobin pigment by distilled water was considered as positive control with 100% lytic activity. The relative absorbance was measured for all samples, the relative percent lytic activity is calculated and presented.

Lytic activity assay by spectrophotometric method by Using S. graminicola zoospore suspension

Washed zoospores of *S. graminicola* were tested as potential substrates for lytic activity of resistant pearl millet, since lysis of the pathogen observed previously *S. graminicola* zoospore suspensions were

incubated with host extracts at 37°C for 30 min. At the end of the incubation period, the reaction mixture was centrifuged at 1500 rpm for 10 min, to separate sedimented zoospore from the supernatant, which contained amino acids and sugars released from zoospores The quantities of amino acids and sugars released from the zoospores were estimated, by means of spectrophotometric method, and the phenol–sulphuric acid method respectively, and the results were compared. The quantitative release of amino acids and sugars were calculated using the calibration curves obtained from aromatic amino acid at 280 nm (5–300 ug:ul) and D-glucose (0–100 ug:ul) by phenol –sulphuric acid method at 490 nm respectively. The release of amino acids and sugars from zoospores by susceptible and resistant host extracts indicates the comparative levels of lytic activity in them.

SDS-PAGE for hydroxy proline rich-glycoproteins and other cell wall components

15% SDS-PAGE was performed for hydroxy proline rich-glycoproteins and other cell wall components in resistant and susceptible seeds as described by Oswald (Lammeli, 1970).

RESULTS AND DISCUSSION

Resistant (SDN503 R, IP18292) and susceptible ($23D_2BS$, 852B) varieties were analysed for the expression of lytic activity before and after *S. graminicola* infection. Results indicated in figure 1 revealed the differential levels of lytic activity expressed among resistant and susceptible samples. IP 18292 although did not differ much from $23D_2B$ and 852B, showed remarkable increase in the lytic activity. Biochemical differences have been observed in the seed, seedling, mesocotyl extracts and cell wall components of resistant and susceptible varieties. In the inoculated resistant varieties, interestingly, remarkable changes were noticed in the expression of lytic activity than that of inoculated susceptible varieties, hence, it suggest the occurrence of at least two types of regulation of lytic activity. Among the seedlings of 30, 60, 90 and 120 min of incubation, 30 min incubated seedlings showed the remarkably increased lytic activity. 20% reduction in the activity was observed from 30 to 60 min, however no change was observed in the susceptible variety. Initial raised level of lytic enzymes in resistant varieties might have lysed the pathogen which has entered the host tissues.

Resistant and susceptible seeds samples were boiled for 3 min at 100^0C. The precipitate thus obtained was removed. The susceptible were examined for lytic activity compared with unboiled extract. > 80% reduction in enzyme activities was noted after boiling (Figure 2). Further, studies were undertaken to understand whether the lytic activity is due to the presence of probable chemical component or lytic factors. Because lytic enzymes have the potential chemical higher order of specificity to lyse the pathogen can be obtain with lytic enzymes rather than the chemical lysing component. The seed (Figure2) and seedling extract were subjected to heat treatment which potentially damages the lytic enzyme activity and not the lytic chemical component. It is clearly indicated in figure that activity is completely lost upon heat denaturation suggesting the presence of lytic enzymes with resistant pearl millet.

Lytic activity was expressed at higher levels only in the mesocotyl extracts of resistant varieties. Compared to that of susceptible varieties there was higher level of activity in the extracts of resistant varieties irrespective of the components of the seedlings used (Figures 3 and 4). In fact it is true the mesocotyl region is the most susceptible sight of the attack by the pathogen. In the present study, higher lytic enzyme activity in the mesocotyl extracts confirms the higher level of susceptibility to the pathogen. At least five fold increases in lytic activity was observed in resistant variety than that of susceptible variety (Table 1). Mesocotyl extracts fractions obtained through (Sephadex G-100) column chromatography were evaluated for protein content at 280 nm. Major peaks 1 and 2 were found in uninoculated samples. In the fungus inoculated samples there was a shift in the peaks towards void. On the contrary in the samples of susceptible variety lower levels of peak was observed. At the same time peak 1 was not observed (Figure 5 and 6). Specific induction of 15K protein was observed in resistant mesocotyl after inoculation with the pathogen. However, in susceptible variety such induction were not encountered (Figure 7) indicating the occurrence of differential profiles of high molecular weight components in the inoculated resistant plant extracts may be attributed to the lytic enzymes. As per the

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Sephadex G15 and electrophoretic studies there was a remarkable increase in the low molecular weight components after inoculation in the resistant variety compared to that of resistant uninoculated and susceptible varieties. It is remarkable to note that these changes were not observed in the susceptible variety. Peak 2 recorded in higher levels in susceptible inoculated samples which may be responsible for favoring the establishment of the pathogen. After inoculation in the resistant variety 20 K component was increased extensively over resistant uninoculated and susceptible samples. This suggests that the induced 20 K components might have involved in offering protection against the downy mildew pathogen. Gel filtration profile on Sephadex G-100 and G15 were compared among inoculated and uninoculated mesocotyl extracts of resistant and susceptible varieties. The differential elution profile was tabulated in table 2. The initial raised levels of lytic activity in resistant variety might be due to lysis of the pathogen which has entered into the plant tissues. These studies are supported by earlier histological observations made by Umesha (1993), Nagarathna (1993), Sharada (1995), where in, the coleoptile of resistant pearl millet after 24 h of inoculation, the lysed fungal hyphae was observed, while in the coleoptile of susceptible variety establishment of fungal hyphae and mycelia were observed were predicted to be the lysis of fungal cell wall is due to lytic enzymes.

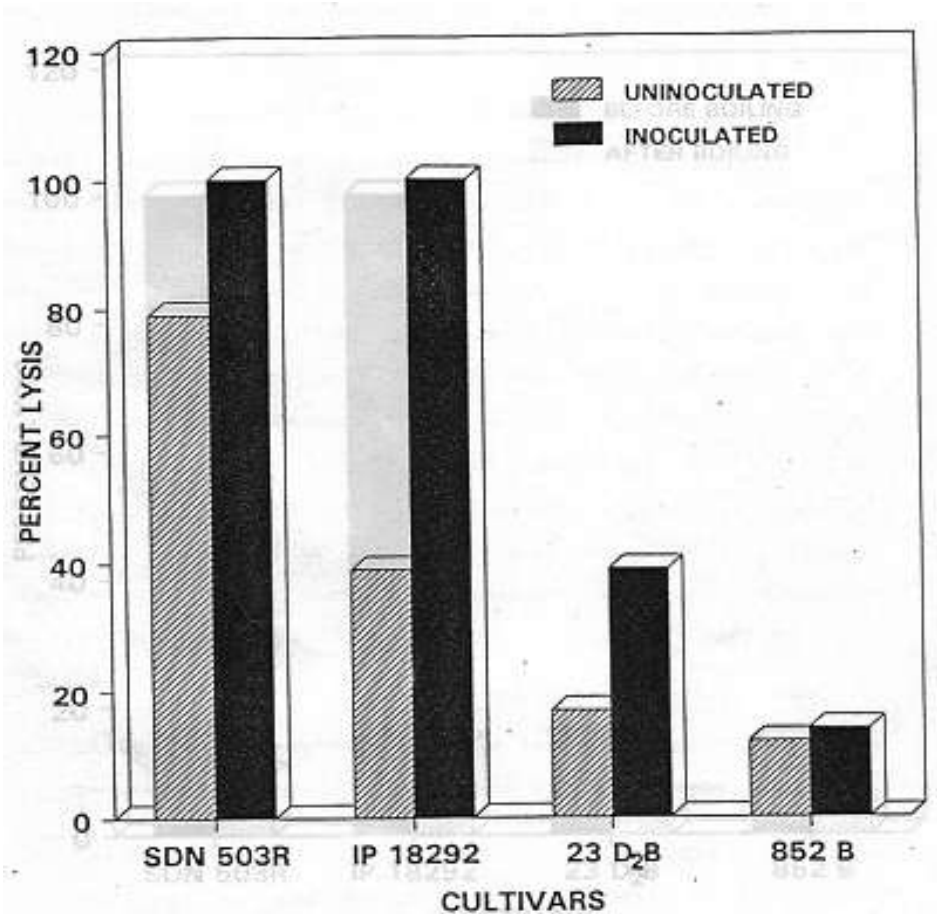

Figure 1: Expression of lytic activity in resistant and susceptible pearl millet seedlings. Two day old resistant and susceptible seedlings were inoculated with *S.graminicola* Zoospores with respective controls. Seedlings were homogenized in 20 mM phosphate buffer saline containing 0.1% tween. The supernatant obtained after centrifugation at 1500 rpm for 5 min was estimated for lytic activity. Percent lysis was calculated.

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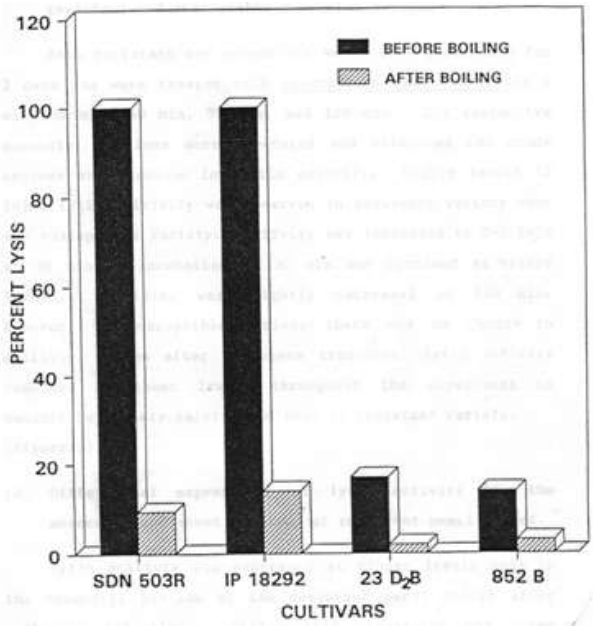

Figure 2: Effect of temperature on lytic enzyme activity. Resistant and susceptible seeds were homogenized in 20 mM phosphate buffer saline containing 0.1% tween. The supernatant obtained after centrifugation at 1500 rpm was boiled in boiling water bath for 5 min. the precipitate was removed by centrifugation and the supernatant was collected. Lytic enzymes assays were performed between boiled and unboiled extracts.

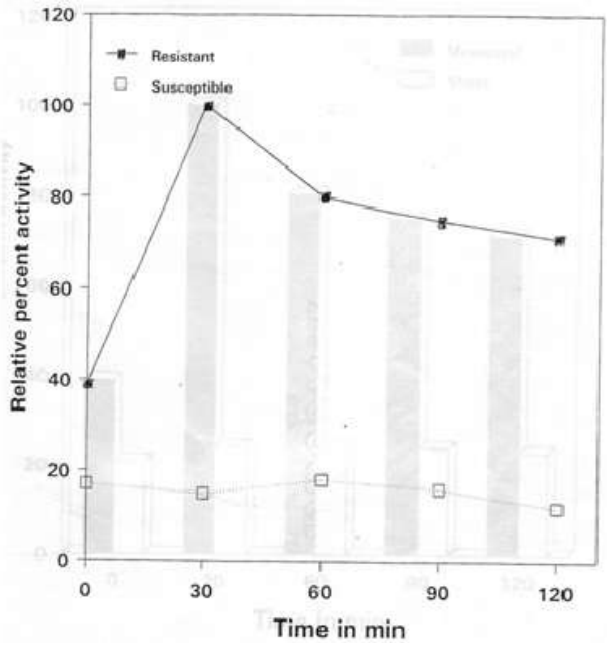

Figure 3: Kinetic expression of lytic activity in the resistant and susceptible mesocotyl portion of pearl millet. Two day old resistant and susceptible seedlings were inoculated with *S.graminicola* zoospores with respective controls. The seedlings were harvested at different intervals of 30 min., 60 min., 90 min and 120 min. the mesocotyl portions from each inoculated seedlings were separated, examined for lytic activity. Percent lysis was calculated and compared between resistant and susceptible pearl millet.

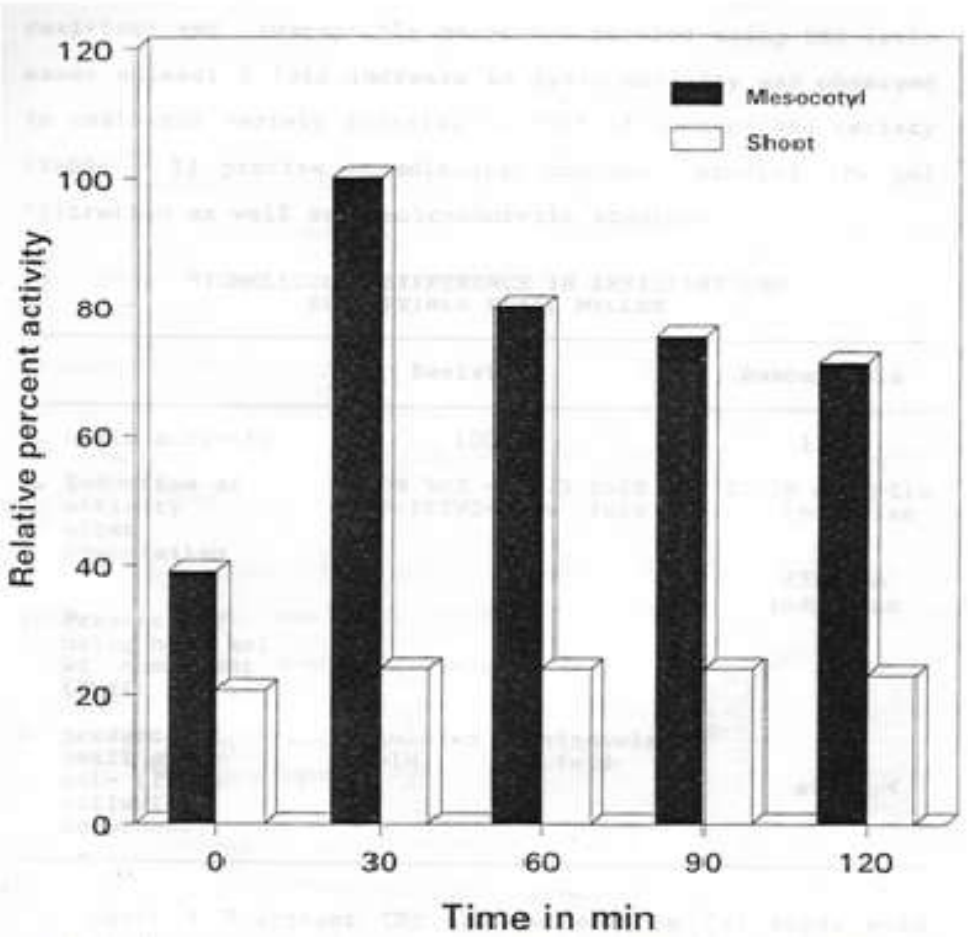

Figure 4: Comparative kinetic profile of lytic activity between the mesocotyl and shoot portions of resistant pearl millet. Two day old resistant and susceptible seedlings were inoculated with *S.graminicola* zoospores with respective controls. Seedlings were harvested at different time intervals of 30 min, 60 min and 120 min. The mesocotyl and shoot portions from inoculated seedlings were separated and extracted for lytic enzymes. Extracts were examined for lytic activity. Percent lysis was calculated between mesocotyl and shoot

Hydroxyproline- rich glycoproteins were specifically extracted using acid: ethanol and major cell wall components apart from HRGP proteins were precipitated using TCA. The supernatants were precipitated with acetone to obtain HRGP.

They were isolated both from resistant and susceptible variety and analysed using 15% SDS-PAGE. Figure 8 indicates the presence of < 15 K protein band specifically in the resistant variety (Lane 2) than susceptible variety (Lane 3). 40 K band was also observed in the cell wall extract (TCA precipitate) and was observed at higher levels in resistant (Lane 4) than the extracts of susceptible varieties (Lane5).

Such higher levels of cell wall pretein may be required to form a specific stronger protective coat around the host to resist the entry of the pathogen. In order to understand the broad spectrum differential arrangements the cell wall components of resistant and susceptible varieties (IP 18292R, IP 18293, IP 18294, IP 18295, IP 18296 7042S, 843B, 852B, $23D_2B$ and 841B) were compared by SDS-PAGE analysis. As they evident Figure 9 indicated the higher levels (Lane A, B) of < 15K HRGP in resistant varieties than that of susceptible varieties. The detailed mechanisms of action are to be elucidated.

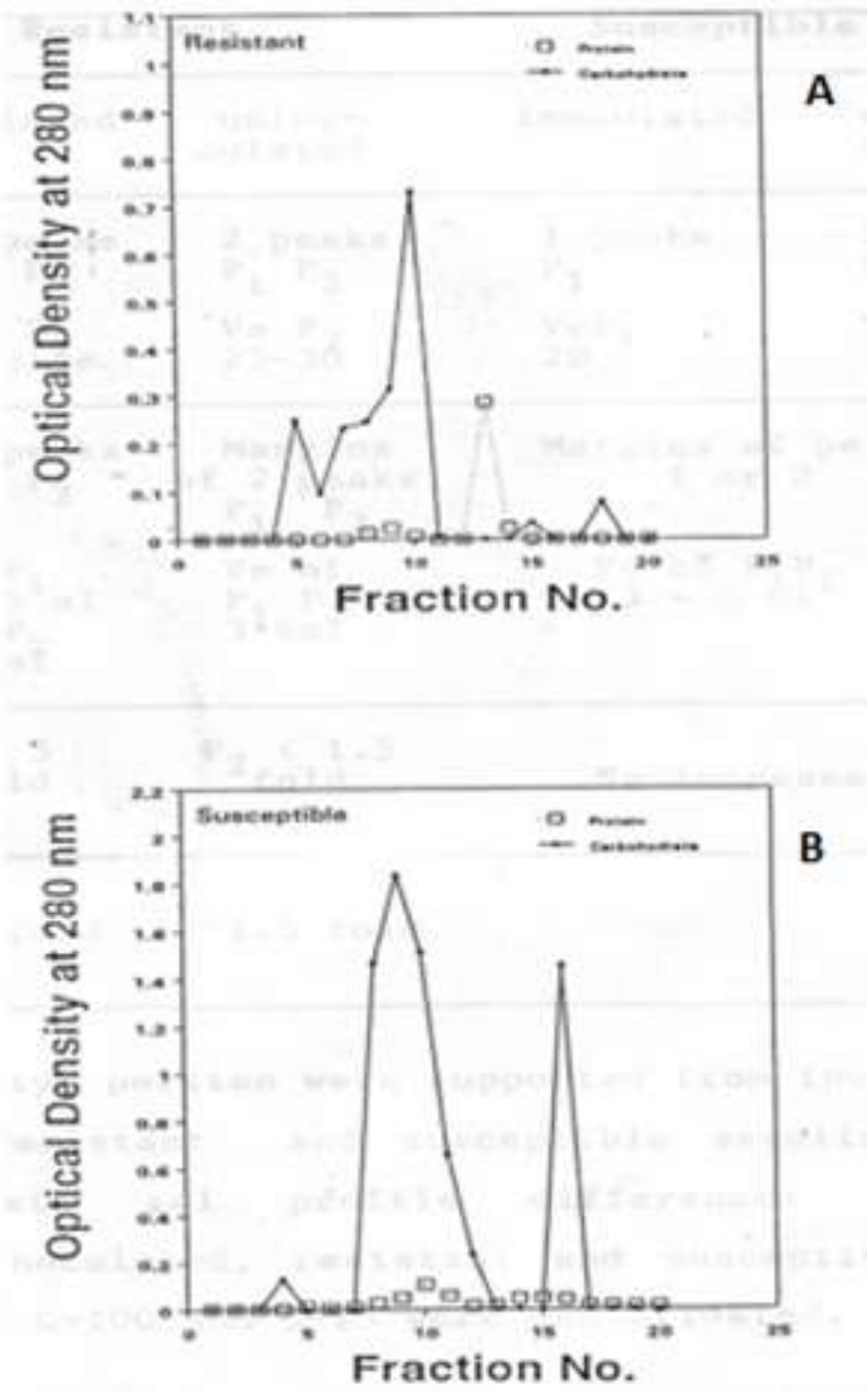

Figure 5: Sephadex G-15 profiles of cell wall extracts pf resistant and susceptible sees. Sephadex G-15 column chromatography was performed for cell wall extracts of (A) resistant and (B) susceptible seeds. Cell wall components were extracted from seeds using three parts of ethanol and one part of 1.25 NHCL (v/v). 500ml fractions were collected and monitored for proteins at 280nm in a spectrophotometer, and carbohydrates were analyzed by phenol-sulphuric acid method. Comparative protein and carbohydrate profiles of resistant seed cell wall extracts are presented.

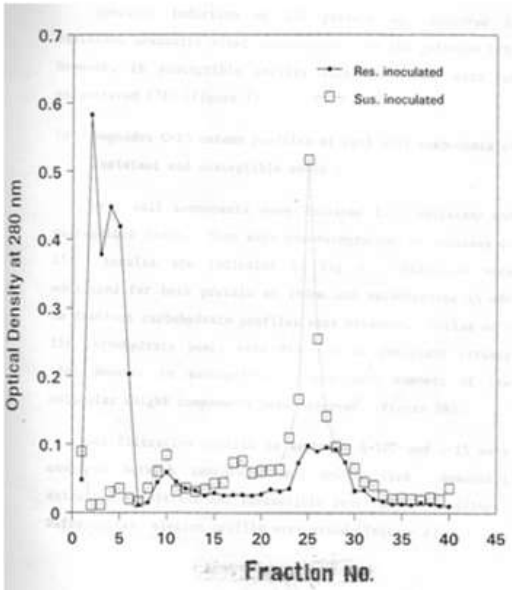

Figure 6: Comparative Sephadex G-100 profiles of mesocotyl portions of resistant and susceptible seedlings. Sephadex G-100 column chromatography was performed for two day old inoculated resistant and susceptible seedlings crude extracts of mesocotyl and monitored for proteins at 280nm in a spectrophotometer. Comparative profiles between inoculated resistant and susceptible samples were made

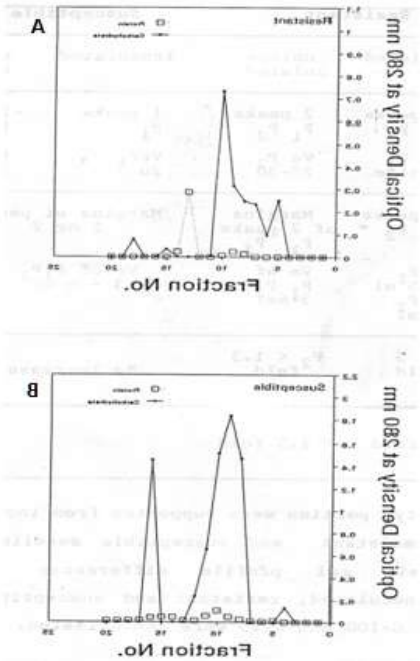

Figure 7: Sephadex G-15 profiles of cell wall extracts pf resistant and susceptible seeds. Sephadex G-15 column chromatography was performed for cell wall extracts of (A) resistant and (B) susceptible seeds. Cell wall components were extracted from seeds using three parts of ethanol and one part of 1.25 NHCL (v/v). 500ml fractions were collected and monitored for proteins at 280 nm in a spectrophotometer, and carbohydrates were analyzed by phenol-sulphuric acid method. Comparative protein and carbohydrate profiles of resistant seed cell wall extracts are presented.

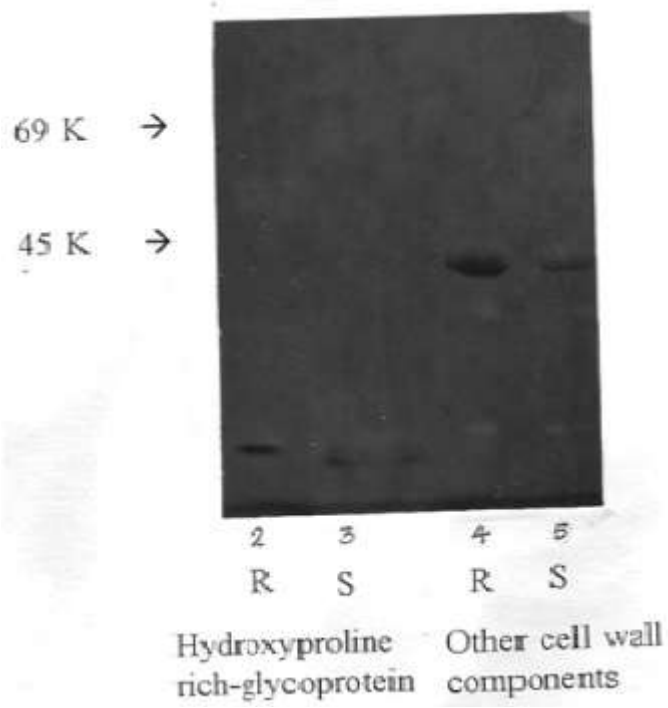

Figure 8: Comparative SDS-PAGE profiles of Hydroxyprolime-rich glycol proteins between resistant and susceptible pearl millet. 15% SDS-PAGE was performed for hydroxy prolime-rich glycoproteins (Lane 2 and 3) and other cell wall component (Lane 4 and 5), resistant (R-2 and 4) and susceptible (S-3 and 5) seeds were used for the study. Bands were visualized using silver nitrate staining. Molecular weight markers were load in the left time. R; P1892, S: $23D_2B$

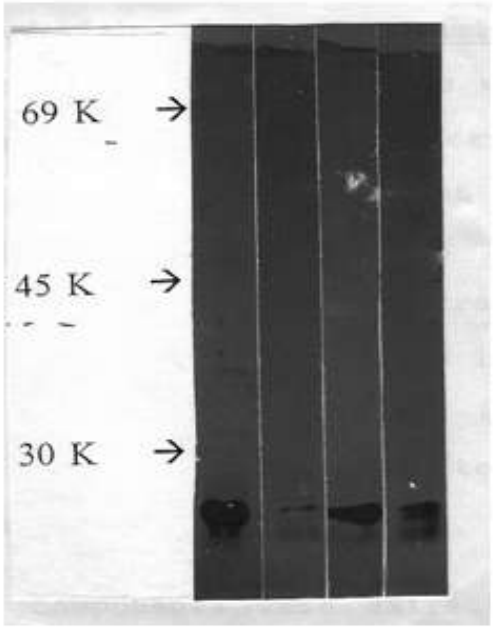

Figure 9: Hydroxy proline-rich glycoprotein profiles in resistant and susceptible seeds. Hydroxyproline-rich glycoproteins were isolated from resistant R_1 (IP 18292), R_2 (IP 18293) and susceptible S_1 (7042S), S_2 (843B) varieties. Levels were analysed by SDS-PAGE bands were detected using silver nitrate staining.

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Table 1: Biomolecular difference in resistant and susceptible pearl millet. Resistant (R) Susceptible (S) seeds were extracted for lytic enzymes and the lytic activity is compared induction of lytic activity was also studied in R and S samples before after inoculation with the pathogen. Effect of expression of <15K or Hydroxyprolime-rich glycoproteins were also studied using SDS-PAGE quantitative changes are noted and consolidated.

	Resistant		Susceptible
1. Lytic activity	100 %		17 %
2. Induction of activity after inoculation	SDN 503 - <1.5 fold IP-18292- > 4 fold		23D2B no lytic induction 852B No induction
3. Presence of using high mol wt component (lytic enzyme)	++++		---
4 presence of small mol col- (15 K) cellwall component	inoculated 1 fold	uninoculated 5 fold	not studied

Table 2: Gel filtration analysis of mesocotyl portion of resistant and susceptible seedlings. Mesocotyl portion were supported from inoculated and uninoculated resistant and susceptible seedlings were compared for their gel profile differences between uninoculated and susceptible.

	Resistant		Susceptible	
	inoculated	unino-culated	inoculated	union-culated
No.of peak on G-100	2 peaks P_1 P_2	2 peaks P_1 P_2	1 peaks P_1	1 peaks P_1
Elution volumes in ml	Ve P_1 Vvoide	Ve P_2 25-30	VeP_1 20	VeP_1 25
No.of peakl on sept G-15	2 peaks P_1 P_2	Margins of 2 peaks P_1 P_2	Margins of peaks 1 or 2	
Elution volumes in ml	VeP_1 4.5 ml veP_2 6 ml	Ve of P_1 P_2 3-6ml	Ve of P_1P_2 3 - 6 ml	
Fold increase after fold inoculation	P_1 5 fold	P_2 < 1.5 fold	No increase	
Fold increase of P_1 after inoculation	5 fold	< 1.5 fold		

Analysis cell wall proteins and dicots as suggested the possible role of structural protein and wall associated enzymes in growth, development and defense (Lamport, 1980; Showlater, 1993; Kieliszewski and Derek, 1987). The role of cell wall fractions in the infectivity of the pathogen was also reported by Nagarathna *et al.*, (1992). It is interesting to note that the changes were present in higher levels in inoculated samples and decreased significantly after inoculation. The significant reduction in the

Hydroxyproline rich-glycoprotein and cell wall components can be attributed to the oxidative cross linking (Joel, 1988; Bowles, 1990). The cross linking cell wall components in the resistant varieties are responsible for the prevention of entry of the pathogen. However similar studies with susceptible seeds as well as detailed kinetics of disappearance of the cell wall components would throw more light in including Hydroxyproline with glycoprotein may throw some light on the precise mechanism involve resistance and susceptibility.

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