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BIOCHEMICAL STUDIES DURING HOST-PATHOGEN INTERACTION BETWEEN SCLEROSPORA GRAMINICOLA AND PEARL MILLET

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ABSTRACT

Sclerospora graminicola is a plant pathogen that causes downy mildew in pearl millet, foxtail millet, and maize worldwide. A high level of variation is observed among the isolates for downy mildew incidence. In our study we have localized the protein responsible for host pathogen recognition. Based on the interaction with pearl millet, the pathogen specificity has been evaluated. Biochemical analysis of enzymes involved in virulence indicated that variation in localization of the test enzymes in sporangia, protoplasts and cell wall of S. graminicola indicating the variation in virulence among propagules was largely due to these differences.

Key words: Sclerospora graminicola, protoplasts, sporangia, host-pathogen interaction

1. INTRODUCTION

Until now, information on fungal protoplasts has been scattered throughout various sources. Protoplasts offer an alternative method for genetic manipulation. The cell wall composition matters a lot for deciding the interaction pattern with the host or with pathogen [1]. Investigating the cell wall assembly as an indirect method for identification of cell wall assembly is emerging as a popular tool. The ability to recognize self and non-self is a general attribute of all the organisms and is the basis for many fundamental activities. In infective stage of a plant by a pathogen, recognition of a compatible host is the first event. According to Lippincott and Lippincott [2], recognition mechanism seems to have evolved to enhance the success of microbe-host interaction once they are in close proximity. In succession, this could involve tactic responses of the microorganism, specific adherence of substances produced by both microbe and host, sensing or activating mechanisms that promote microbe and host responses.

The recognition of pathogen products, such as surface located or released polysaccharides or glycoproteins, by receptor molecules located on host cell walls or membranes has been suggested to be the primary event leading to either susceptibility or resistance expression [3]. In many fungal cell walls, alkali insoluble chitins are embedded in fibrils in the β-glucan matrix. These molecules have shown to be elicitors or protection factors in some host-pathogen combinations. Lectins are known to interact with fungi by agglutinating germinated cystospores and also mediate binding of germinated cystospores to protoplast membrane of

potato tubers. Enzymes produced by the protoplasts also play a vital role in interaction with the invading host as well as defense interaction in fungi [4]. Fungus has been successfully isolated by aseptic techniques and extract from the cultures fungus and their cell walls was analyzed by liquid chromatography coupled to mass spectrometry and found to produce taxol and taxol-related compounds [5]. Consequently, the objectives of this study were to analyse fro presence of enzymes on the cell wall and to localise the protein involved in host pathogen interaction.

2. MATERIALS AND METHODS

Pathogen used: A virulent pathotype of S. graminicola isolated from and maintained on its susceptible host (cultivar HB3 of pearl millet) under green house conditions at 22±2°C was used for the study.

Sporangia: Isolation of sporangia was as per the method of Safeeulla [6]. Infected leaves of pearl millet were collected in the evening and the remnants of previous crop of sporangia removed by flushing in running tap water. The leaves were blot dried, cut into 1cm long and incubated for sporulation at 25±2°C under 60-70% relative humidity. Sporangia were harvested in the following morning into cold sterile distilled water. Precaution was taken not to take sporangiophore and other parts with sporangia.

Antibody production: The 90 kDa protein obtained from the sporangia of a virulent pathotype of S. graminicola [7] was used. Intramuscular injection of the antigen (500 mg protein) in Tris buffer saline (TBS; 0.01 M, pH 7.2, 0.85 %) mixed with equal volumes of Freund's complete adjuvant was administered on 0, 14, 28 and 42 days. Antiserum was separated from the bleeds one week after the last injection and subjected to ammonium sulphate precipitation for purification of immunoglobulin. The immunoglobulin dissolved in TBS were dialysed extensively against TBS and preserved at -20°C with 0.1 % sodium azide until use.

Localization of host-recognition protein (HRP): In vivo localization of the antigen was done by the indirect fluorescent antibody tracing method. For this, intact sporangia, protoplasts isolated from sporangia, cell walls of sporangia, zoospores and germinating zoospores of S. graminicola were taken in 1.5 ml microfuge tubes, washed in 0.01 M phosphate buffer saline (PBS, pH 7.0, 0.85%) and incubated with anti serum against 90 kDA pathogen surface protein (1:1000 dilution) at 37°C for 1 h. Unreacted antiserum was removed by repeated washings in PBS by centrifugation at 1500 g. The bound antibody in the samples was detected indirectly using anti-rabbit goat IgG conjugated to flourescein isothiocyanate (FITC; Sigma 1:100 dilution). In order to identify the bound antibodies, the samples were incubated in the FITC tagged conjugate for 2h at 37°C. Again the unbound anti-antibodies were removed by repeated washing in PBS. Finally, the samples were suspended in 1 % glycerol and mounted on glass slides. Controls consisted of cells treated with preimmune serum (obtained from uninjected rabbits) and processed as above. Samples mounted on glass slides were observed for fluorescence using Leitz epiflourescence microscope attached with photoautomat using UV filter (360-400 nm).

Microscopy: Samples of protoplast and immunolocalisation of HRP in the intact sporangia, protoplasts from sporangia and cell wall of S. graminicola were examined with compound

light Zeiss microscope with fluorescence attached at various magnifications (40x10X & 100x10X). Photographs were taken in Konica film, 400 ASA.

Sample for Biochemical studies: Sporangia at concentration of 8x 10^5 spores/ml in 1 M KCl treated with 4% v/v of NOVOZYME incubated for 75 min for release of protoplasts. The protoplasts and cell walls were isolated by centrifugation and used for biochemical studies.

Enzyme extraction: The sporangia as well as the sporangial protoplasts and the cell walls were subjected to biochemical studies. Enzyme activities were determined by extracting the samples in different buffers for various enzymes.

Chitinase: For chitinase activity, 1x10^7 sporangia/sporangial protoplast/cell walls were macerated to a fine paste in minimum buffer consisting 0.05 M sodium acetate buffer pH 5.2 at 4°C. The homogenate was centrifuged at 20,000 g for 20 min. at 4°C. The supernatant was used directly for enzyme assay.

Proteinase: Proteinase in samples was extracted by grinding 1x10^7 sporangia/sporangial protoplast/cell wall and macerated to a fine paste in minimum buffer consisting 0.05 M sodium acetate buffer pH 5.8 at 4°C. The homogenate was centrifuged at 20,000 g for 20 min. at 4°C. The supernatant was used directly for enzyme assay.

β-l,3-glucanase: For β-1,3-glucanase, one gram each of sample 1x10^7 sporangia/ sporangial protoplast/cell walls was macerated in 0.05 M sodium acetate (pH 5.2). The homogenate was centrifuged at 10,000 rpm for 15 min. and the supernatant used as the source of enzyme.

Enzyme assay:

β-l,3-glucanase: Activity of β-1,3-glucanase in the extract was assayed following the method of Isaac and Ghokale [8]. 0.05% of Laminarin, (Sigma, USA) in 0.05 M sodium acetate buffer (pH 5.2) was used as substrate. After incubation at 37°C for 15 min, the hydrolysis product was treated with 2, 5-dinitro salicylic acid and the color developed measured at 540 nm using a spectrophotometer (Hitachi, 2000). The enzyme activity was expressed as µmoles of glucose released min^{-1} mg^{-1} protein using standard prepared with glucose.

Proteinase: Enzyme activity was determined spectrophotometrically based on the procedure of Isaac and Gokale [8]. 0.25% of casein in 0.05 M sodium acetate buffer was used for the assay. Equal volume of substrate and enzyme were incubated for 30 min at 37°C. To this reaction mixture, 1.5 ml cold Trichloroacetic acid was added and centrifuged for 10 min at 7,500 rpm at 4°C. The absorbance of the supernatant was read at 280 nm against distilled water. The enzyme activity was expressed as absorbance mg 1 protein min^{-1}.

Chitinase: Chitinase activity was determined according to Isaac and Ghokale [8] with N-acetyl glucosamine (Sigma, U.S.A.) as standard. Colloidal chitin was prepared from chitin (Sigma, U.S.A.) following the method of Skujins et al., [9]. Enzyme was assayed using 2% colloidal chitin in 0.05 M sodium acetate buffer (pH 5.2) as the substrate. Equal volume of the substrate and the enzyme were incubated for 2 h at 37°C in a shaking water bath and centrifuged at 10,000 rpm for 3 min at 37°C. 0.5 ml of the supernatant was added to O.lml of 0.8 M potassium tetra borate, pH 9.1 and boiled for 3 min and cooled to 4°C. To this, 3 ml of dimethyl amino benzaldehyde (DMAB) reagent was added and incubated at 37°C for 20 min. The absorbance of the reaction mixture was read at 585 nm. Chitinase activity was expressed in terms of nmol min/mg protein.

Protein estimation: Protein content in extracts was estimated by the dye binding method [10] using 0.01% Coomassie blue G-250 dissolved in 5% ethanol and 50% phosphoric acid. Standards prepared with Bovine Serum Albumin (Sigma) was used to quantitate the protein in the samples [10].

Statistical analysis: The data obtained were repeated thrice with three replicates and subjected to Fischer's least significant test.

3. RESULTS

Biochemical Studies using Isolated Protoplasts of Sclerospora graminicola

The cell surface compositions of fungal or bacterial pathogens have been known to decide their infective nature to host plants. Our previously reported study has identified distribution of the host-recognition protein to be responsible for the infective nature of the pathogen and thus leading to cultivar specific infection. We further extended our study to identify hydrolases in the cell wall and the protoplasts isolated from the pathogen to strengthen our hypothesis that the zoospores formed out of protoplasts are more infective than the sporangia themselves.

Results of the enzyme analysis in the test samples indicated higher activities of β-l, 3-glucanase in protoplasts compared to cell wall (Fig. 1).

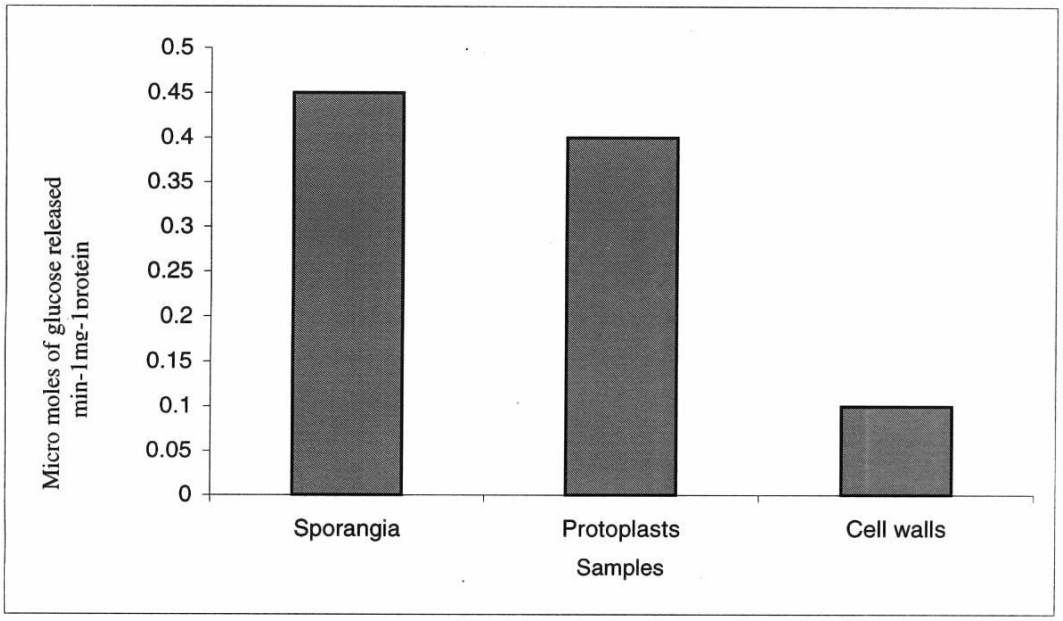

FIG. 1 BETA 1,3-GLUCANASE ACTIVITY IN SPORANGIA, SPORANGIAL PROTOPLASTS AND CELL WALLS OF SCLEROSPORA GRAMINICOLA

Least activity of the enzyme assayed was found in cell wall. 0.4 micromoles min^{-1} mg^{-1} protein of the enzyme activity was recorded in protoplasts against 0.45 micromoles min^{-1} mg^{-1} protein found in sporangia. In cell walls, the enzyme activity recorded only 0.1 micromoles min^{-1} mg^{-1} protein. Proteinase activity also showed the same trend. 0.35OD min^{-1} mg^{-1}protein of enzyme activity was recorded in protoplasts as against 0.43 OD min^{-1} mg^{-1} of protein in sporangia (Fig. 2).

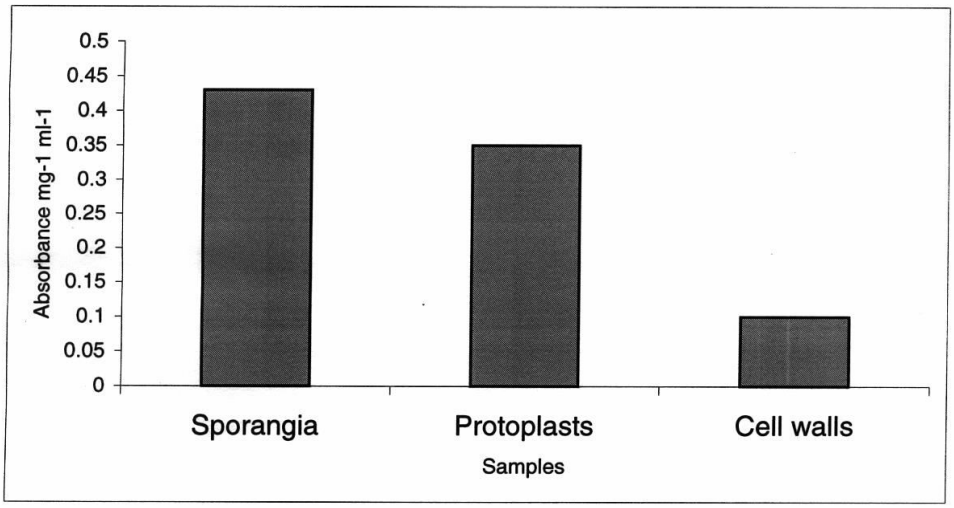

FIG. 2 PROTEINASE ACTIVITY IN SPORANGIA, SPORANGIAL PROTOPLASTS AND CELL WALLS OF SCLEROSPORA GRAMINICOLA

Cell wall showed the least enzyme activity with a record of 0.1 OD min^{-1} mg^{-1} protein. Though chitinase activity assay was performed in all the samples, the results indicated that the enzymes were not detected in any of the samples tested.

Immunoflourescent Tracing of Host-Recognition Protein in the Infective Propagules of Sclerospora graminicola.

Host recognition protein of 90 kDa responsible for recognition of host by the pathogen and cultivar specificity was traced for its distribution in sporangia, zoospores, sporangial protoplast and cell wall of S. graminicola. The results are represented in Fig. 3.

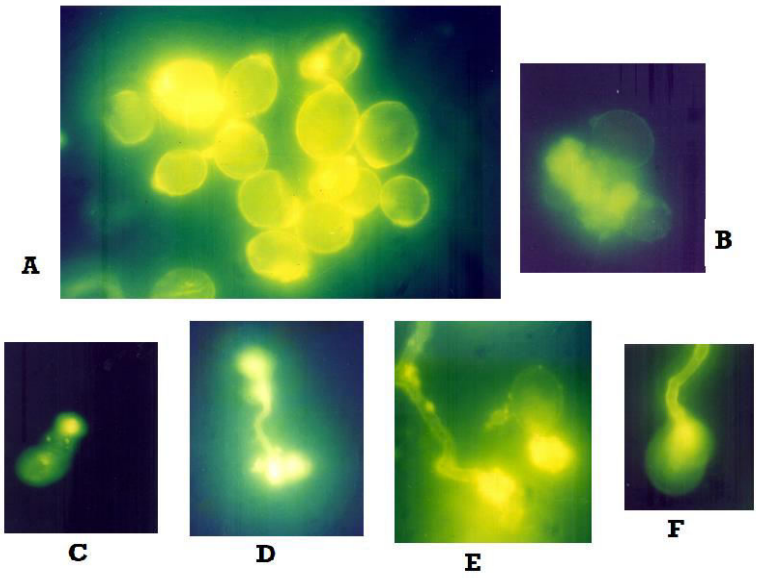

FIG. 3 IMMUNOLOCALIZATION OF HOST-RECOGNITION PROTEIN IN THE INFECTION PROPAGULES OF SCLEROSPORS GRAMINICOLA. THE PROTEIN WAS LOCALIZED WITH THE

ANTIBODY AND THE BOUND ANTIBODY TRACED INDIRECTLY USING FITC TAGGED ANTIRABBIT-GOAT IGG

A. Localization of whole sporangia; B. The protein localized in the released protoplasts of sporangia; C. In the initial phase of zoospore germination, the protein localized in the emerging germ tube tip; D, E & F. During extension of germ tube the host recognition protein was concentrated at the tips

Polyclonal antibodies were raised against the HRP and traced in the tested samples by indirect immunoflourescent technique using secondary antibody tagged with flourochrome-FITC at appropriate dilutions. The results indicated tracing of host-recognition protein in all the samples tested but to varying degrees (Fig. 3). In sporangia, more fluorescence was observed in the papillar region than in the remaining part. Tracing of host recognition protein in isolated protoplasts identified high intensity of fluorescence all over the surface of the protoplast, whereas in cell wall, there was very weak fluorescence observed throughout the surface.

The germinating zoospores also showed fluorescence but to varying degrees.

The just released zoospore showed fluorescence all over the surface, whereas as in the germ tube developed after encystment of zoospore, the fluorescence was observed in the tube surface, and finally it was identified only in the tip of the germ tube in the matured zoospore.

4. DISCUSSION

The ability to recognize self and non-self is a general attribute of all the organisms and is the basis for many fundamental activities. In infective stage of a plant by a pathogen, recognition of a compatible host is the first event. According to Lippincott and Lippincott [2], recognition mechanism seems to have evolved to enhance the success of microbe-host interaction once they are in close proximity. In succession, this could involve tactic responses of the microorganism, specific adherence of substances produced by both microbe and host, sensing or activating mechanisms that promote microbe and host responses. The recognition mechanism may function directly as a sensing device, initiating metabolic changes in the microorganism, host or both which contribute to the ultimate relationship. Recognition is mediated by complementary interactions between polysaccharide containing macromolecules leading to host-pathogen specificity. The recognition of pathogen products, such as surface-located or released polysaccharides or glycoproteins, by receptor molecules located on host cell walls or membranes has been suggested to be the primary event leading to either susceptibility or resistance expression. In many fungal cell walls, alkali insoluble chitins are embedded in fibrils in the β1, 3-glucan matrix. These molecules have shown to be elicitors or protection factors in some host-pathogen combinations. Lectins are known to interact with fungi by agglutinating germinated cystospores and also mediate binding of germinated cystospores to protoplast membrane of potato tubers. This observation indicates their possible role in fungal attachment to host cell membrane.

Reports on Phytophthora glycinea-soybean host-pathosystem have shown the involvement of lectin-ligand binding during the recognition by the fungus to the plant cell wall [11]. This reaction may be characterized by a lectin-like interaction between the surfaces of the host and pathogen [12], [13] and antibodies have been used to identify lectins of fungal cell walls [14]. β-1, 3-glucans present on the cell walls of Phytophthora species were also shown to be involved in plant microbe interaction [15]. Monacha and Chen [16] reported involvement of glyco-proteins of molecular weight 100 and 85 kDa in the recognition of Piptocephalis virginiana to its host.

Recognition of the host by the pathogen may be specific due to involvement of recognition molecules leading to race cultivar specificity or nonspecific due to direct contact between fungal wall and plant membrane [17] due to involvement of hydrophobic or hydrophilic interaction [13]. In downy mildew disease of pearl millet, Safeeulla and Thirumalachar [18] have observed that germ tubes of zoospores orient towards the roots of pearl millet seedlings. They reasoned this phenomenon as due to the chemotaxis of zoospores from root exudates, which has relationship with the process of infection. A pathogen surface 90kDa protein binding specifically to compatible host cell walls differentiated virulent from avirulent races. This suggested its possible role in the host recognition phenomenon [7]. The distribution of this protein on the pathogen propagules involved in host-infection further substantiated this observation.

β-1, 3-glucans with some β-1, 6-glucan linkages, chitin and protein are the principle cell wall components of most fungi with exception of oomycetes which are devoid of chitin [19], [20]. These enzymes decide the future trend of establishment of relationship with the host plant. Thus in our study, we have identified the occurrence of enzymes that are known to degrade the cell wall of the plant. They are responsible for the degradation of pearl millet cell wall and were found in increased quantity in the protoplasts than on cell wall. Enzyme activity was found in increasing amount in the sporangia also.

In S. graminicola-pearl millet interaction, the enzymes tested further activate the plants inherent defense systems by acting as elicitors-the molecules released from the plant's structural barriers due to attack of these enzymes. These elicitors activate the defense system of the host. Small amounts of β-1, 3-glucanases are known to be present in sieve tubes, primary cell walls and in papillae formed in response to wounding and pathogen attack [21]. The elicitor molecules released due to enzymatic action of the pathogen to degrade the cell wall of the host plant to gain entry further activates the accumulation of these enzymes. The timing and the quantity of the defense related enzymes released by the host decides the nature of the relationship viz., non- host, compatible or incompatible type that will be framed in future to subside or overtake the plants defense system.

In the downy mildew, secondary infections due to sporangia and zoospores contribute to maximum disease [22], [23], [24]. The sporangia are thick walled structures with a thin walled 'beak' for release of zoospores [6]. Through this site host infection by sporangia has also been described (6), suggesting direct involvement of this site in the recognition of the host. Immunolocalisation studies of the 90 kDa protein on the sporangia, identifying highest concentrations of this protein at this site (Fig. 3) supported the hypothesis that this protein is involved in host recognition.

Immunoflourescent tracing of the host recognition protein on the other infective propagules of S. graminicola further established the above observation. In S. graminicola sporangial germination, the protoplast is transformed into zoospores [6] and the released zoospores encyst and germinate on the root surfaces before causing infection [25]. Hence, for the protein to be involved in host recognition, it should be located on the membranes, rather than on the walls of the sporangia. Tracing of the protein with the antibody located highest concentrations of the protein on the isolated protoplast compared to the cell walls (Fig. 3B). The concentration being several folds more as evidenced by the fluorescence intensity (Fig 3). In germinating zoospores, the protein was localized at the tips of the infective hyphae (Figs. D, E & F). This is to be expected, since it is the tip that recognizes the host surface for penetration. Thus, the results describing localization of the protein at the regions of the fungal propagule involved in host recognition confirms its role in host recognition.

The sporangial protoplasts finally transform into zoospores, which are released from the papillar region of the sporangia. Thus in this study, the protoplasts represent the zoospores. Increased amounts of β-l, 3-glucanase and proteinase **(**Fig. 2**)** were found in the protoplasts compared to the cell wall thus evidencing that protoplasts are highly virulent and are responsible for infection. The enzymes are identified to the lesser extent in the isolated cell walls. The sporangia also showed increased amount of the enzymes since it is composed of both the protoplasts as well as the cell wall. For the attack of the host by the sporangia, the cell wall of the pathogen interacts with the cell wall of the host and the region involved is the papillar region of the cell wall [6], [7]. Since these protoplasts form the zoospores, these infective propagules are highly virulent in attacking the host plant. Thus this study further substantiates the observations which identified that the zoospores as more infective than the sporangia which also are known to infect but to lesser extent than the zoospores.

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