Materials and Methods

3.1 Plant material

3.1.1. Source of seeds

Seeds of different cultivars of soybean [*Glycine max* (L.) Merrill] were collected from National Research Centre for Soybean , Indore , Madhya Pradesh , India . Six cultivars namely Macs-58 , J-80 , NRC-7 , Bragg , PK-262 , Pusa-16 were used. Seeds were air dried and stored at room temperature (28± 2^0C) as well as 20^0C . Since the seeds lost their viability after one year , it was necessary to procure seeds every year .

3.1.2. Growth of plants

Soybean seeds were sown in sandy soil (Soil : Sand – 1 : 1) contained in earthenware pots (10 plants / 25 cm diameter pot) . Prior to sowing , seeds were treated with 0.1% $HgCl_2$ for 5 minutes to remove superficial contaminants , followed by several washing with sterile distilled water .The plants were grown in two research station viz., Phytopathological Experimental Garden , Department of Botany, University of North Bengal and Bidhan Chandra Krishi Viswavidyalaya, North Bengal Campus , Cooch Behar under natural conditions of day light and temperature (26-35^{0}C) . The pots were watered daily with ordinary tap water. The plants were grown during March to October .

3.2 Fungal culture

3.2.1. Source of culture

A virulent strain of *Sclerotium rolfsii* Sacc . obtained from culture collection centre , Department of Plant Pathology, Bidhan Chandra Krishi Viswavidyalaya, North Bengal Campus, Cooch Behar. The culture was maintained on Potato Dextrose Agar medium by regular subculturings .

3.2.2. Completion of Koch's Postulate

Soybean seeds were surface sterilized with 0.1% $HgCl_2$ solution for 5 minutes, washed with sterile distilled water and sown in earthenware pots containing sandy soil. Seedlings (14 days old) were inoculated with *S. rolfsii.* Infected roots were collected, washed, cut into small pieces , treated with 0.1% $HgCl_2$ for 3-5 minutes , rewashed with sterile distilled water and transferred to PDA slants . After 7 days, the isolated organism was examined, compared with the original stock culture of *S. rolfsii* and its identity was confirmed.

3.2.3. Maintenance of Stock Cultures

The fungi listed in (Table –1) were grown on PDA slants and stored under 3 different conditions [5^0C, 20^0C and at room temperature 28^0C±2^0C]. Apart from weekly transfer for experimental work at a regular interval culture of *S. rolfsii* was examined in order to test its pathogenicity.

3.3.Test Chemicals

The following chemicals of diverse nature were used for seed treatment in different experiments. Most of the chemicals used were Sigma products and all were of analytical grade.

3.3.1. Metal Salts

a) Cupric chloride
b) Lithium sulphate
c) Ferric chloriole
d) Magnesium sulphate
e) Sodium molybdate
f) Manganese sulphate
g) Zinc chloride
h) Barium sulphate

3.3.2.Growth regulators

a) 2,4, - Dichlorophenoxy acetic acid (2,4,-D)
b) 2,4,5 – Trichlorophenoxy acetic acid (2,4,5-T)
c) Indole – 3 – acetic acid (IAA)
d) Cycocel [(2 chloroethyl) trimethyl ammonium chloride].

3.3.3.Other Compound

Chitosan (a polymer of β – 1,4 linked glucosamine) : The deacetylated derivative of natural biopolymar,chitin. It is the product of Bentech Laboratories Inc. USA.

Initially three concentrations ranging between 10^{-3}M to 10^{-5}M or 10^{-5}M to 10^{-7}M for metal salts and growth regulators while six concentrations ranging from 0.01% to 1.0% for chitosan were selected for *in-vitro* and *in-vivo* test and finally effective concentrations were chosen for further experiments.

3.4. Process of chemical treatment

Most of the chemicals were dissolved in distilled water. Chitosan was dissolved in 1% glacial acetic acid solution in distilled water and pH of the solution was then adjusted to 5.9 by adding 1 N NaOH. Indole-3-acetic acid, 2,4 – dichlorophenoxy acetic acid and 2,4,5 – trichlorophenoxy acetic acid were dissolved initially in a few drops of ethanol and then distilled water was added to make the require volume.

Seeds (100g) were surface sterilized by dipping them in 0.1% $HgCl_2$ solution for two minutes, thoroughly washed with distilled water and soaked separately in experimental chemical solution (200ml) for 24 hours. For chitosan treatment, surface sterilized seeds were treated with chitosan solution (2ml / 100 g of seed) followed by thorough shaking to spread the solution to seed surface as a thin film and then drying them before sowing in pots or field plots.

3.5 Inoculation technique

3.5.1. Sand maize meal culture

S. rolfsii was initially grown on sterilized sand maize meal medium (Sand:Maize meal-3:1) for six days at 28^0C. Finally the inoculum was mixed with sterile soil at the ratio 1:8. Fungus-soil mixture (50gm) were mixed with top soil of each pot containing 14 day- old soybean seedlings (10 plants/pot) and kept in glass house for observation of disease reaction.

3.5.2. Water culture

Fifteen surface sterilized (with 0.1% $HgCl_2$ solution for 5minutes) soybean seeds were sown in each pot containing autoclaved sandy soil. The seedlings were grown in the glass house conditions. *S. rolfsii* was grown in potato dextrose broth (100 ml broth/250 ml flask) at 30^0C for 10 days. Mycelial suspension containing sclerotia were prepared by homogenizer. Subsequently 15 day-old seedlings were uprooted from the experimental pots, the root system was washed thoroughly in running tap water, then rinsed twice in sterile distilled water and finally 10 seedlings were placed in each Erlenmeyer flasks (250 ml) containing 200 ml mycelial suspension and plugged with cotton. Control plants were kept in sterile distilled water.

3.6 Disease assessment

The external symptoms were assessed thrice (4,7 and 14 days) after inoculation with *S. rolfsii* . The plants were carefully uprooted, washed in tapwater and lesions at the colar region were intially examined and graded as follows : 0 = Plant is healthy ;1 = Incipient lesions at the collar region, 2 – 7 mm in length ; 2 = Large lesion, 8–12 mm in length, loss of turgor at the top i.e. drooping of tips; 3 = Extensive rotting at the collar region, wilting and drying of many leaves, drooping of the stem; 4 = Plant completely wilted, dead and dry.

Total number of plants per treatment ranges from 60-70 and mean disease index for a plant and percent disease index (PDI) were calculated as follows:

$$\text{Mean disease index} = \frac{\Sigma \text{ Disease index}}{\text{No. plants observed}}$$

$$\text{Percent Disease Index} = \frac{\Sigma \text{ Disease index} \times 100}{\text{No. plants observed} \times \text{maximum rating}}$$

In addition, percentage mortality of the plants were also recorded at the last date of sampling .

3.7. Fungitoxicity assay of chemicals

The fungitoxicity of test chemicals were assayed as follows. Filter papers were soaked in test chemical solutions, 25 sclerotia of *S. rolfsii* were placed on each filter paper kept in sterile petridishes (90mm diam). There were four replicates for each treatment. The petridishes were incubated at 28^0C for 48 hours. Then the percentage of germination of sclerotia were determined under a binocular microscope.

3.8. Extraction of enzymes

Four enzymes viz., Pectolytic enzyme, Polyphenol oxidase, Peroxidase and Phenylalanine ammonia lyase were extracted from healthy and *S. rolfsii* infected soybean plants. For *S. rolfsii* infected soybean plant, 2-3 cm collar region were collected and while collecting tissue samples from inoculated plants, every attempt has been made to keep the unaffected green portion to a minimum.

3.8.1. Pectolytic enzyme

Five grams of infected plant tissue was crushed with 5ml of 0.05M citrate phosphate buffer at pH 5.0 and centrifuged at 5000 r.p.m. for 20 min and the supernatant was taken .Four ml of 0.3% polygalacturonic acid in 0.05M citrate phosphate buffer (pH 5.0) was taken with 1ml of tissue extract and this reaction mixture was kept at 30^0C for 1 hour and then placed in ice to stop the enzyme activity and measured the amount of galacturonic acid using this mixture as enzyme source .

3.8.2 Polyphenoloxidase

For the extraction of polyphenoloxidase the method of Jennings *et al.* (1969) was followed. Two grams of fresh tissue was taken in a pre-cooled glass mortar with 0.05M Tris HCl buffer pH 7.4 (5 ml/g) and ground with pestle at 0^0C with a pinch of neutral sand to facilitate good grinding . The homogenate was then centrifuged at 15,000 r.p.m. for 30 minutes at 0^0C . The supernatant liquid was then used as the source of enzyme.

3.8.3 Peroxidase :

Two grams of fresh tissue was taken in a pre-cooled glass mortar with phosphate buffer pH 6.0 (5ml/g) and a pinch of neutral sand and ground with pestle at 0^0C. The homogenate was then centrifuged at 15,000 r.p.m. for 30 minutes at 0^0C and the supernatant was used as the source of enzyme.

3.8.4 Phenylalanine ammonia lyase

One gram tissue was ground with a mortar and pestle with 5 ml/g sodium borate buffer in 2 mM mercaptoethanol [0.1 (M), pH – 8.8] . Slurry centrifuged at 15000g for 4 minutes and supernatant was collected and enzyme activity was assayed by measuring the production of cinnamic acid from L–phenyl alanine spectrophotometrically (Bhattacharya and Ward, 1987).

3.9 Assay of enzyme activity

3.9.1 Pectolytic enzyme

Pectolytic enzyme activity was measured following the method as described by Miller (1972). Initially the following reagents were prepared.

A. Sodium potassium tartarate (300g of this salt was mixed in 500ml of distilled water).

B. 3,5- Dinitrosalicylic acid (10g of this reagent was dissolved in 200ml of 2N sodium hydroxide).

C. DNS reagent : This reagent was prepared freshly by mixing solution (A) and (B) and making the volume upto 1 liter by addition of water.

One ml aliquot of the reaction mixture was taken in a test tube and 3ml of DNS reagent was added to it and the mixture was kept in a boiling water bath for 5 minutes, cooled and the volume was made upto 25ml with distilled water. The optical density was measured at 540 nm in Bausch and Lomb Spectronic-20 colorimeter. D-galacturonic acid was used as the standard (1 mg/ml) for the purpose of estimation.

3.9.2 Polyphenoloxidase

Polyphenoloxidase activities were assayed following the method of Jennings *et al* (1969). The reaction mixture consisted of 0.05 ml of crude extract, 3.0 ml of 0.02M citrate phosphate buffer (pH 6.0), 1.0 ml of proline (5.0 mg/ml) and 1.0 ml of catechol (2.0 mg/ml). The mixture was aerated, using glass capillary for two minutes, before addition of catechol which initiated the reaction and absorbance was measured at 420nm in a Bausch and Lomb Spectronic–20 colorimeter. Enzyme activity was expressed as the change in absorbance/0.05ml of extract (10mg of extracted tissue) per minute but final change of optical density after 30 minutes at 420nm was taken into consideration. Enzyme extract autoclaved for 20 minutes at 121^0C was used as the time control in all cases.

3.9.3 Peroxidase

Peroxidase activity was measured following the method of Addy and Goodman (1972).To 3ml of 0.05 M pyrogallol reagent in a colorimeter tube, 0.05ml of homogenate was added and thoroughly mixed. The tube was then inserted into Bauch and Lomb Spectronic –20 colorimeter at 420nm. After the colorimeter galvanometer had been adjusted to '0' optical density, 0.5ml of 1 percent H_2O_2 was quickly added to the tube which was then inverted once and immediately reinserted into the colorimeter. The change in optical density between 40 and 160 sec at 420nm was used to plot peroxidase activity. A change in the absorption by 0.01 per minute was accepted as a unit of activity. Results were expressed as unit of activity g/ tissue.

3.9.4 Phenylalanine ammonia lyase

The reaction mixture was prepared with 0.3 ml of 300 µm sodium borate buffer (pH – 8.8), 0.3ml of 30µm L- phenylalanine, 0.5ml of supernatant and 1.9 ml distilled water was added to make the total volume of 3.0ml. The reaction mixture was incubated for 1h at 40^0C and finally the absorbance at 290nm was noted in UV-spectrophotometer. The enzyme activity was expressed as µg cinnamic acid produced in one minute/g fresh weight of tissue.

3.10. Total phenol

3.10.1 Extraction

Tissues freshly collected from plants, washed with distilled water and then cut into 1-2 cm pieces. Such pieces were used to extract phenol following the procedure of Biehn *et al.* (1968) with minor modifications.

Five grams of fresh tissue were put into boiling 80% ethanol (5ml/g tissue) in a water bath and kept for 10 minutes. The extract was cooled in a pan of cold water and then the tissue was crushed in a mortar with pestle for 5-10 minutes and the extract passed through two layers of cheese cloth .The ground tissue was re-extracted for three minutes in boiling 80% ethanol (using 3ml of alcohol for every g of tissue). The two ethanol fraction were pooled together and the mixture then evaporated in vacuum (at 40^0C) and the residue was suspended in glass distilled water, acidified to pH 4.5 with (N) HCl, and extracted three times with equal volume of ethyl acetate. The ethyl acetate fractions were then taken together and evaporated to dryness in vaccum at 40^0C. The dry material was then taken in 10 ml of ethanol.

3.10.2 Estimation

The total phenol content was estimated using Folin – ciocalteau reagent following the method of Mahadevan and Sridhar (1982).

One ml of extract was pipetted into a graduated test tube, to which 1ml of Folin – ciocalteu reagent was added followed by 2ml of Na_2CO_3 (20%) solution. The tube was shaken and heated on a boiling water bath for 1min. and then cooled under running tap water. The resulting blue solution was diluted to 25 ml with distilled

water and its absorbance was measured at 650 nm in a Bausch and Lomb Spectronic – 20 colorimeter. For comparison a blank containing ethanol and reagents was used. Total phenol was determined as catechol equivalent after comparing with the standard curue prepared from distilled catechol, obtained by using the same reagents. Total phenol was expressed as mg/g fresh weight of tissue.

3.11. Ortho – dihydroxy phenol

3.11.1 Extraction

Plant tissues (2g) were cut into pieces and immediately immersed in 20ml of boiling alcohol. After 15 minutes of boiling it was cooled and then crushed in a mortar with neutral sand using ethanol. The slurry was centrifuged at 3000 r.p.m. for 20 minutes and the supernatant was taken for O-dihydroxyphenol estimation using Arnow's reagent.

3.11.2 Estimation

The O-dihydroxyphenol was estimated following the method of Mahadevan and Sridhar (1982). One ml of alcoholic tissue extract was taken in a test tube to which 2 ml of 0.5N HCl, 1 ml of Arnow's reagent ($NaNO_2$-10g ; Na_2MoO_4-10g, Distilled water – 100 ml.), 2ml of 1 N NaOH were added and mixed thoroughly in room temperature following which the volume of the reaction mixture was raised to 10ml. Optical density was recorded in a Baush and Lomb Spectronic-20 colorimeter at 515 nm. A blank was prepared for comparison by adding 1ml of alcohol instead of tissue extract with other reagents. Standard curve was prepared with different concentrations of catechol. Results were expressed as mg/g fresh weight of tissue.

3.12 Soluble protein

3.12.1 Extraction

Soluble protein were extracted from soybean root tissue (collar region) following the method of Chakraborty *et al.* (1995). Root tissue (1g) were homogenized with 0.05M Sodium phosphate buffer (pH 7.2) containing 10mM $Na_2S_2O_5$, 0.5 mM $MgCl_2$, 2mM polymethyl sulphonyl fluoride (PMSF) in mortar with pestle at 4^0C with sea sand. The homogenate was centrifuged at 4^0C for 20 minutes at 10,000 r.p.m. and the supernatant was used as crude protein and immediately stored at -20^0C for further use.

3.12.2 Estimation

Soluble proteins were estimated following the method as described by Lowry *et al.* (1951). To 1ml of protein sample 5ml of alkaline reagent (0.5ml of 1% $CuSO_4$ and 0.5 ml of 2% potassium sodium tartarate, dissolved in 50ml of 2% Na_2C0_3 in 0.1N NaOH) was added. This was incubated for 15min at room temperature and then 0.5ml of Folin ciocalteau reagent (diluted 1 : 1 with distilled water) was added and again incubated for 15 min for colour development following which optical density (O.D.) was measured at 720 nm. Quantity of protein was estimated from the standard curve made with bovine serum albumin (BSA).

3.13 Calcium and magnesium

3.13.1 Extraction

Plant samples were first air dried for 2 days and then oven dried at 40^0C for 24 hr. Then 0.5g of such dried plant samples were digested with 10ml of Tri–acid mixture (Conc. $HN0_3$. Conc H_2S0_4 and Conc. $HClO_4$ at a ratio of 10 : 1 : 4). After digestion the volume was made up to 50ml with distilled water and filtered through Whatman 42 filter paper.

3.13.2 Estimation

The calcium and magnesium content in plant tissue was determined following the method of Black (1965).

Five ml of above filtrate was taken in a 100ml conical flask for estimation of ca^{++} and Mg^{++} and 2.5 ml of NH_4Cl- NH_4OH buffer was added to it. A few drop of NaOH solution was added to raise the pH to 11.0. Then few drops of Eriochrome black T indicator was added and titrated against standard EDTA solution (0.01M), and the colour changed from wine-red to pink.

For estimation of calcium, 5ml of aliquot and 2.5ml of NH_4Cl – NH_4OH buffer was taken in 100ml conical flask to which a few drops of NaOH solution was added to raise the pH to 12.0 and then it was titrated with standard EDTA solution (0.01M) using calcon indicator, the colour changed from pink to blue.

3.14 Lignin

3.14.1 Extraction

Stem tissue (1g) was cut into 0.5 cm pieces and ground in 5ml of cold 0.01M phosphate buffer, pH 7.0. The solids were collected by centrifugation (1000 g) for 10 min. and washed with 10ml cold buffer thrice and cold water twice. The residue was further extracted with 10ml hot ethanol thrice and then successively with ethanol, acetone and diethyl ether. After air drying, lignin was extracted with 5ml 0.5M NaOH at 70^0C for 16 h. The residue was removed by centrifugation (1000g) for 5min and the supernatant was adjusted to pH 8.0 with 2.5 M HCl and the extract was dialyzed against water for 24h.

3.14.2 Estimation

Lignin was estimated following the method of Stafford (1960) as modified by Ride (1975). The spectrum of absorbance was recorded from 230 to 380nm in a Backman UV – spectrophotometer by diluting 0.5 ml of extract with 2.5ml of 0.06 M phosphate buffer, reading the sample at pH 12.0 directly against at pH 7.0.

3.15 Extraction and separation of glyceollin

To extract glyceollin, the method of Keen *et.al* (1971) was followed with modifications. Fifteen day old plants were inoculated with *S.rolfsii* following water culture method. In this case, *S. rolfsii* was grown in potato dextrose broth at 30^0C for 10 days. Mycelial suspension containing sclerotia were prepared by homogenization. Subsequently plants uprooted from experimental plot, the root system washed thoroughly in running tap water following sterile distilled water and transferred in 500ml flasks. After 24, 48 and 72h. of inoculation, roots were used for extraction of glyceollin. Infected as well as healthy roots (30g) were homogenised with 120ml of 95% ethanol in an electrical blender. The extracts were filtered through filter paper and the residues were re-extracted with an equal volume of 80% ethanol and filtered. The ethanol extracts were combined and reduced in volume in a rotary film evaporator at 45^0C.The residue was dissolved in ethyl acetate (0.1ml/g fresh wt. of roots) and was used in subsequent experiments.

For separation and chemical detection of glyceollin, thin layer chromatograms were prepared with silica gel G (0.2 mm thickness) and activated for 1h at 80^0C. Aliquots of ethyl acetate extracts of roots were spotted and developed in a solvent system (Benzene : Methanol 95 : 5), dried and examined under UV-light or sprayed with Diazotized, P-nitroaniline (5ml of 0.5% P-nitroaniline solution in 2N HCl + 0.5ml of 5% aqueous sodium nitrite solution + 15ml. of 20% aqueous sodium acetate solution). Rf value was determined in each case.

3.16 Ultraviolet spectrophotometry and quantifcation of glyceollin

For UV- spectrophotometric studies, ethyl acetate extract (50µl) was spotted on TLC plates and developed in benzene : methanol (95 : 5) solvent system and allowed to dry. The silica gel from unsprayed reacting zones was scrapped off and eluted in spec methanol. The elutes were stored at 5^0C for overnight and centrifuged to remove the silica gel. These elutes were examined by UV- spectrophotometry (Sico, Model Digispec 200 GL) and the absorbance at 285 nm were determined.

Quantification : Quantity of glyceollin ($C_{20}H_{19}O_5$) was estimated from UV-spectrophotometric curve by assuming molar extinction co-efficient of 10800 at 286 nm as described by Bhattacharya and Ward (1985).

$$\text{Molar extinction co-efficient (E)} = \frac{\text{OD of the tested solution}}{\text{Concentration (x)* of the tested solution (moles/litre) x Path length of the cell (cm).}}$$

X* = moles / liter converted to g/liter by multiplying with molecular weight of glyceollin ($C_{20}H_{19}O_5$). Results have been expressed in µg/g fresh weight of roots.

3.17 Petridish bioassay of phytoalexin :

In case of petridish bioassay, 0.2 ml of ethyl acetate extracts of infected roots were taken in sterilized petridish (9 cm dia.) and allowed to dry. Then, 0.2 ml of ethanol was added to it , followed by 20ml of sterilized PDA (20ml / petridish) and mixed well. Each petridish was inoculated with an agar block (4 mm.dia.) containing 4 day old mycelia of *Sclerotium rolfsii* and incubated at 30 ± 1^0 C. Diameter of mycelial mat was measured after 24,48 and 72 hours of inoculation and compared with the controls.

3.18 Preparation of antigen

3.18.1. Root antigen

Root antigens were extracted from healthy and *Sclerotium rolfsii* infected soybean roots following the method of Chakraborty and Saha (1994) . Seeds of soybean cultivars were grown in earthen pots containing sterilized soil. When the seedlings were at the age of 14 days they were inoculated with fungal inocula . Healthy and infected plants were uprooted after two-week intervals, washed with cold water and kept at 15^0 C for 1 hour. Finally, roots (20 gm fresh weight) were crushed with sea sand in mortar and pestle in cold (4^0C) and stored at -15^0C for 1 hour and homogenized with 20ml of 0.05M sodium phosphate buffer supplemented with 10 mM sodium metabisulphite and 0.5 mM magnesium chloride . Homogenate was strained through cheese cloth and then centrifuged (12,000g) at 4^0C for 1 hour and known quantity of ammonium sulphate was added to it for 100% precipitation (Green and Hughes, 1995), kept at 4^0C. Precipitate was dissolved in the same extractive buffer (pH-7.4) and dialysed against 0.005M phosphate buffer for 24 hour at 4^0C. During this period 10 changes were given. The dialysate (i.e. soluble protein) was used for antisera production and for gel electrophoretic study.

3.18.2. Mycelial antigen.

Mycelial antigen was prepared following the method of Chakraborty and Saha (1994). Initially fungal mycelia (4mm disc) were transferred to 250ml Erlenmeyer flasks each containing 50ml of sterilized liquid Richards medium (g / 1 distilled water, sucrose, 30; KNO_3, 10; KH_2PO_4, 5; $MgSO_4$, $7H_2O$, 2.5 and $FeCl_3$, 0.02) and incubated for 10 days at 30 ± 1^0C. For extraction of soluble antigens, mycelial mats were harvested, washed with 0.2% NaCl and rewashed with sterile distilled water. Washed mycelia (30g fresh wt.) were homogenized with 0.05M sodium phosphate buffer (pH-7.2) supplemented with 10mM sodium metabisulphite and 0.5mM magnesium chloride and 0.85% NaCl in mortar and pestle in presence of sea sand. Cell homogenates were kept overnight at 4^0C and then centrifuged (15000g) for 30 min at 4^0C. The supernatant was equilibrated to 100% saturated ammonium sulphate under constant stirring and kept overnight at 4^0C. After this period the mixture was

centrifuged (15000g) for 30min at 4^0C, the precipitate was dissolved in 10ml 0.05M sodium phosphate buffer (pH-7.2). The preparation was dialysed for 72h through cellulose tubing (Sigma Chemical Co. USA) against 1 L of 0.005 M sodium phosphate pH-7.2 with 10 changes. Then the dialysed material was stored at -20^0C and used as antigen for the preparation of antiserum and other equipments.

3.19 SDS-polyacralamyde gel electrophoresis of total soluble protein

3.19.1 Preparation of Slab Gel

3.19.1.1 Stock solutions

For the preparation of gel, the following stock solutions were initially prepared as described by Laemmli (1970).

(A) Acrylamide and N, N'- methylenebisacrylamide :

Acrylamide	29 g
N,N'-methylenebisacrylamide	1 g
Distilled water	100 ml

Solution was filtered, and pH adjusted to 7.

(B) Sodium dodecyl sulphate

SDS	10 g
Distilled water	100 ml.

(Stored at room temperature)

(C) Lower gel buffer (1.5 M Tris)

Tris	18.18 g
Distilled water	100 ml

pH was adjusted to 8.8

(D) Upper gel buffer (0.5 M Tris)

Tris	6.06 g
Distilled water	100 ml

pH was adjusted to 6.8

(E) Ammonium peroxidisulphate (APS)

Ammonium peroxidisulphate 0.1 g

Distilled water 1.0 ml

(freshly prepared each time)

(F) Tris-glycine electrophoresis buffer

(25 mM Tris Base ; 250 mM glycine)

5x Stock can be made;

Tris Base 15.1 g

Glycine 94 g

In 900ml of d. H_2O, pH was adjusted to 8.3. Then 50 ml of 10% SDS was added and volume made upto 1000 ml.

(G) 1x SDS gel loading buffer :

50 mM Tris Cl (pH-6.8)

10mM β-Mercaptoethanol

2% SDS

0.1% bromophenol blue

10% glycerol.

3.19.1.2 Slab gel preparation:

For slab gel preparation, two glass plates (17cm X 19cm) were washed with dehydrated alcohol and dried. Then 1 mm thick spacers were placed between the glass plates and the two edges and the 2 sides of glass plates were sealed with grease and gel sealing tape and kept in the gel casting unit. Resolving gel solution was prepared as follows:

H_2O	11.9 ml
30% Acrylamide mix	10.0 ml
1.5 M Tris (pH 8.8)	7.5 ml
10% SDS	0.3 ml
10% APS	0.3 ml
TEMED	0.012 ml

The gel solution was cast very slowly and carefully up to a height of 12 cm by a syringe. The gel was over layered with water and kept for 2-3 hrs for polymerization. Then stacking gel solution was prepared as follows:

H_2O	6.8 ml
30% acrylamide mix	1.7 ml
1 M Tris (pH-6.8)	1.25 ml
10% SDS	0.1 ml
10% APS	0.1 ml
TEMED	0.01 ml

After polymerization of resolving gel, water overlay was decanted off and a 13 well 1 mm thick comb was placed. Stacking gel solution was poured carefully upto a height of 4 cm over the resolving gel and overlayed with water. Finally the gel kept for 30 min for polymerization.

3.19.2 Sample preparation

Sample was prepared by mixing the sample protein with 1 x SDS gel loading buffer (final volume 80µl). All the samples were floated in boiling water bath for 3 min. After cooling, upto 80 µl of each sample was loaded in a predetermined order into the bottom of the wells with a micro liter syringe. Along with the samples, protein markers consisting of a mixture of six proteins ranging in molecular weight from 30 to 200 KD [carbonic anhydrase – 29,000 , Albumin (egg) – 45,000 , Albumin (bovine) – 66,000, Phosphorylase b – 97,400 , β- galactosidase – 116,000 and Myosin – 205,000] was treated as the other samples and loaded in a separate well.

3.19.3 Electrophoresis

Electrophoresis was performed at 25 mA for a period of 3h until the dye front reached the bottom of the gel.

3.19.4 Fixing and Staining

For fixing the fixer solution was prepared as follows-

Glacial Acetic Acid	- 10 ml
Methanol	- 20 ml
Distilled water	- 70 ml

The entire gel was removed from the glass plates and then the stacking portion was cut off from the resolving gel. After that gel was soaked for 20h in the fixer for fixing.

The staining solution was prepared as follows –

Coomassie Brilliant Blue R250	- 0.25 g
Methanol	- 45 ml
Distilled water	- 45 ml
Acetic Acid	- 40 ml

At first, gel were stained by staining solution for 2-3h and finally soaked with destaining solution (methanol : d H_2O : Acetic acid - 4.5 : 4.5 : 1) until the background become clear.

3.20 Antisera production

3.20.1 Rabbits and their maintenance

For the production of antisera against different fungal and root antigens, New Zealand White, male rabbits were used. Before immunization, the body weights of rabbits were recorded. They were regularly fed with 500g green grass. Besides, every alternate day they were also given 50-75g of chickpea seeds soaked in water. Beside this, they were given saline water after each bleeding for two consecutive days. Cages were cleaned with Phytofresh every day in the morning for better hygienic conditions.

3.20.2 Immunization

Polyclonal antibody against mycelial antigens of *S. rolfsii* was prepared by immunizing rabbits. Before immunization normal sera were collected from each rabbit. Each time antigen emulsified with an equal volume of Freund's complete/incomplete adjuvant (Difco) and was injected intramuscularly. Doses were repeated at 7 days intervals and continued for 9 consecutive weeks.

3.20.3 Bleeding

Blood was collected from the marginal ear vein puncture 3 days after sixth week of first immunization and subsequently seven times more every fortnight. During bleeding, rabbits were placed on their backs on a wooden board after taking them out from the cage. The board was fixed at a 60^0 angle. The neck of the rabbit was held tight in the triangular gap at the edge of the board, and the body was fixed in such a way that the rabbits could not move during the bleeding. The hairs were removed from the upper side of the ear with the help of a razor and disinfected with rectified spirit. Then the ear vein was irritated by xylene and an incision was made with the help of a sterile blade and blood samples (2ml) were collected in a sterile graduated glass tube. After collection, all precautionary measures were taken to stop the flow of the blood from the puncture. For clotting, the blood samples were kept at 30^0C for 1h and then the clot was loosened with a sterile needle and the antiserum was clarified by centrifugation at 2000g for 10 min. Finally, blood samples were distributed in 1ml vials and stored at -20^0C until required.

3.21 Purification of IgG

3.21.1 Precipitation

IgG was purified following the method of Clausen (1988). The antiserum (5ml) was diluted with two volumes of distilled water and an equal volume of 4.0 M ammoniumsulphate. The pH was adjusted to 6.8 and the mixture was stirred for 16h at 22^0C. Then it was centrifuged at 10,000g for 1h at 22^0C and the precipitate was dissolved in 5ml of 0.02M Sodium phosphate buffer, pH 8.0.

3.21.2 Column Preparation

Initially DEAE sephadex (Sigma Co.USA) was suspended in distilled water overnight after which the water was decanted off and the gel was suspended in 0.005 M phosphate buffer, pH 8.0. The buffer washing was repeated 5 times. The gel was next suspended in 0.02M phosphate buffer, pH 8.0 and was applied to a column (2.6cm in dia, 30 cm high) and allowed to settle for 2h. After that 25ml of 0.02M phosphate buffer (pH 8.0) was applied to the gel material.

3.21.3 Fraction collection

At the top of the column, 2ml of ammonium sulphate precipitate was applied and the elution was performed at a constant pH and a molarity continuously changing from 0.02M to 0.03M . The initial elution buffer (1) was 0.02M sodium phosphate buffer pH 8.0 (diluted from a 0.10M sodium phosphate buffer pH 8.0 containing 16.86g Na_2HPO_4 , $2H_2O$ + 0.731g NaH_2PO_4 , H_2O/L.).The final elution buffer (2) was 0.30M sodium phosphate buffer pH 8.0.

The buffer (1) was applied in a flask in which one rubber connection from its bottom was supplying the column. Another connection above the surface of buffer (1) was connected to another flask with buffer (2). During the draining of buffer (1) to column , buffer (2) was sucked into buffer (1) thereby producing a continuous rise in molarity. Finally, 40 x 5 ml fraction were collected and the optical density (OD) values were recorded by UV- spectrophotometer at 280 nm.

3.22 Immunodiffusion test

3.22.1 Preparation of agar slides

The agar slides (5cm x 5cm) were degreased successively in 90% (v/v) ethanol: di-ethyl ether (1:1 v/v) and ether, then dried in hot air oven and sterilized inside the Petri dish each containing one slide. A conical flask containing Trisbarbiturate buffer (pH 8.6) was placed in boiling water bath; when the buffer was hot, 0.9% agarose was mixed to it and boiled for the next 15 min. The flask was repetedly shaken in order to prepare absolutely clear molten agarose which was mixed with 0.1% (w/v) sodium azide (a bacteriostatic agent) . The molten agarose was poured on a glass slides (6 ml/slide) and allowed to solidify. After 3-7 wells were cut on the agar plate with a sterilized cork borer (4 mm dia.) at a distance of 5 mm from the central well .

3.22.2 Diffusion

Agar gel double diffusion test was performed following the method of Ouchterlony (1967) . The antigens and undiluted antisera (50 µl/well) were pipetted directly into the appropriate wells and diffusion was allowed to continue in a moist chamber for 48-72h at 25^{O}C.

3.22.3 Washing, staining and drying of slides

After immunodiffusion , the slides were initially washed with sterile distilled water and then with aqueous NaCl solution (0.9% NaCl and 0.1% NaN_2) for 72h with 6 hourly changes to remove unreacted antigen and antibody widely dispersed in the agarose. Then slides were stain with 0.5% coomassie blue (0.5g coomassie blue, 5g $HgCl_2$, 5ml glacial acetic acid, and 95ml distilled water) for 10min. at room temperature. After staining slides were washed thrice in destaining solution [2% (v/v) acetic acid] for 5h to remove excess stain. Finally, all slides were washed with distilled water and dried in hot air oven for 3h at 50^0C.

3.23 Immunoelectrophoresis

3.23.1 Preparation of agarose slides.

The slides (7.5 X 2.5 cm) were degreased, dried and sterilized as described earlier. Thin and uniform layer (2mm thick) of fluid agarose medium (0.97% agarose, 0.1% NaN_3 dissolved in 0.05 barbitol buffer (pH-8.6) was poured on each slide taking care that no air buble was present in the agarose medium. This was necessary in order to avoid any irregularity which may cause asymmetrical migration and diffusion during electrophoretic separation or the immunodiffusion. The slides were kept in petridishes and stored at 4^0C until use.

3.23.2 Electrophoresis

Two central wells (3 mm dia) were cut out from the agarose plate of each slide following the conventional method (Ouchterlony, 1967). Slides were placed in the middle compartment of the electrophoretic box. The anode and cathode chambers were filled with barbitol buffer (0.05M pH 8.6). Antigens (40µl) were introduced into the wells. Filter paper strips (Whatman) were soaked in buffer and laid on both ends of the slides which connected the buffer solution in the anode and cathode compartments with the agarose surfaces. An electric current (2.5 mA slide; 10v/cm) was passed through the slides for 3h at 4^0C. After electrophoresis the current was discontinued.

3.23.3 Diffusion

A longitudinal through parallel to the long edge of the slide was cut in the agarose plates in between two wells and the undiluted antiserum (400µl) was pipetted

into the trough. Diffusion was allowed to continue in a moist chamber for 48 – 72h at 25^0C.

3.23.4 Washing, drying and staining of slides

After immunodiffusion, slides were washed, stained and destained as mentioned earlier. Then all slides were dried in hot air oven for 3h at 50^0C.

3.24 Enzyme linked immunosorbent assay

Following buffers were prepared for indirect ELISA following the method as described by Chakraborty et.al (1995).

1. Antigen coating buffer (Carbonate–bicarbonate buffer 0.05M, pH-9.6)

Stock	
A. Sodium carbonate	5.2995 g
Distilled water	1000 ml
B. Sodium bicarbonate	4.2 g
Distilled water	1000 ml.

160ml. of stock solution 'A' was mixed with 360ml. of stock solution "B" pH was adjusted to 9.6.

2. Phosphate Buffer saline (0.15 M PBS, pH – 7.2)

Stock	
A Sodium hydrogen phosphate	23.40 g
Distilled water	1000 ml
B Disodium hydrogen phosphate	21.2940 g
Distilled water	1000 ml.

With 280 ml of stock solution 'A', 720ml of stock solution 'B' was mixed and the pH of the mixed solution was adjusted to 7.2. Then 0.8%. NaCl and 0.02% KCl was added to the solution.

3. 0.15 M phosphate buffer saline –Tween (0.15M PBS-Tween, pH-7.2).

To 0.15 M phosphate buffer saline, 0.05% Tween 20 was added and the pH was adjusted to 7.2.

4. Blocking reagent (Tris buffer saline, pH-8.0).

(0.05M Tris, 0.135 M NaCl, 0.0027 M KCl).

Tris	0.657 g
NaCl	0.81 g
KCl	0.223 g

Distilled water was added to make up the volume upto 100ml. The pH was adjusted to 8.0 and 0.05% . Tween 20 and 1% bovine serum (BSA) were added.

5. Antisera dilution buffer (0.15M PBS – Tween, pH 7.2). In 0.15 M PBS Tween, pH 7.2, 0.2% BSA, 0.02% Polyvinyl polypyrrolidone, 10,000 (PVPP, 10,000) and 0.03% Sodium azide (NaN_2) was added.

6. Substrate:

The substrate P-nitrophenyl phosphate at 1.0 mg/ml in substrate buffer (1.0% [W/V] diethanolamine , 3mM NaN_3, pH 9.8) was added at 100µl per well.

7. Stop solution

3 M NaOH solution was used to stop the reaction. ELISA was performed following the method as described by Chakraborty et.al. (1995). Plant and fungal antigens were serially diluted with coating buffer and the diluted antigens were loaded (100µl / well) in Coster EIA/RIA 8 well flat bottom strip ELISA plate. After loading, plate was incubated at 25^0C for 4h. The plate was then washed three times under running tap water and once with PBS- Tween and each time, plate was shaken dry. Subsequently, 100µl of blocking agent was added to each well for blocking the unbound sites and the plate was incubated as 25^0C for 1h. After incubation, plate was washed as mentioned earlier. Purified antiserum (IgG) was diluted in antisera dilution buffer and loaded (100µl / well) to each well and incubated at 4^0C overnight. After a further washing 100µl of antirabbit IgG goat antiserum labelled with alkaline phosphatase (Sigma Chemicals, USA) was added and incubated at 37^0C for 2h. Plate was washed, dried and loaded with 100µl of Pnitrophenyl phosphate substrate in each well and incubated in dark at room temperature for 60 min. Colour development was stopped by adding 50µl / well of 3 M NaOH solution and absorbance was determined in an ELISA reader (Trans Asia Lisa-5 ELISA machine) at 405 nm. Absorbance values in wells not coated with antigens were considered as blanks.

3.25 Immunofluorescence

Indirect fluorescence staining of fungal mycelia were done using FITC labelled goat antirabbit IgG following the method of Chakraborty and Saha (1994).Fungal mycelia were grown in Richards solution. After 4 days of inoculation young mycelia were taken out from the flask and kept in grooved slide. After washing with PBS (pH – 7.2) mycelia were treated with normal sera or antisera diluted (1 :125) with PBS, and incubated for 3 min at 27^0C. Then mycelia were washed thrice with PBS tween as mentioned above and treated with goat antirabbit IgG (whole molecule) conjugated with fluorescein isothiocyanate (Sigma) diluted 1 : 40 with PBS (pH 7.2) and incubated in dark for 30 min at 27^0C. After incubation, mycelia were washed thrice in PBS and mounted in 10% glycerol. A cover glass was placed on mycelia and sealed. The slides were then observed using Leica Leitz Biomed microscope with fluorescence optics equipped with ultra violet (UV) filter set 13. Mycelia were photographed under both phase contrast and UV fluorescent conditions for comparison of treatment.