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 the Pulses and Oil Seeds Research Station, Berhampore, West Bengal. Ten cultivars namely Soymax, JS.2, UPSM-19, Bragg, PK-327, Punjab1, PK-564, 17-PK-472, 18-PK-564, 19-PK-466, were used. Seeds were air dried and stored at room temperature (30±2°c) as well as at 20°c. 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 / 25cm. dia pot). Prior to sowing, seeds were disinfected with 95% ethyl alcohol (Orellana *et al.,* 1976) for 3-5 minutes to remove superficial contaminants, followed by several washings with sterile distilled water. The plants were grown in the Nursery of the Department of Botany, University of North Bengal under natural conditions of day light and temperature (26-35°C). The pots were watered daily with ordinary tap water. The plants were grown during March to October.

3.2. FUNGAL CULTURES

3.2.1. Source of Cultures

A virulent strain of *Fusarium oxysporum* Schlecht (ITCC NO. 1803)obtained from Division of Mycology and Plant Pathology, Indian Agricultural Research Institute, New Delhi was used throughout this investigation. The culture was maintained on PDA medium by regular subculturings.

A culture of *Trichoderma harzianum Rifai* (ITCC No. 1894) obtained from Indian Agricultural Research Institute, New Delhi, India, was also used for experimental purposes.

3.2.2. Completion of Koch's Postulate

Surface sterilized soybean seeds were sown in pots which were

previously infested with conidia and mycelia of *F. oxysporum.* Reisolation of pathogen was done from infected roots. These 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 10 days, the isolated organism was examined, compared with the original stock culture of F. *oxysporum* and its identity was confirmed.

3.2.3. Stock Cultures

The cultures were maintained on PDA slants and stored under 3 different conditions [5°C, 20°C and at room temperature 30±2°C). The culture was examined at a regular interval to test its viability and pathogencity of the fungus.

3.2.4. Assessment of mycelial growth

3.2.4.1. Solid media

To assess mycelial growth of *F. oxysporum* in solid media, the fungus was first grown in petridishes, each containing 20ml of PDA and incubated for 7 days at 30°C. Agar block (4mm dia) containing the mycelia was cut with a sterile cork borer from the advancing zone of mycelial mat and transferred to each petridish containing 20 ml of sterilized media. Following solid media were used for assessment of grwoth.

Potato dextrose agar (PDA)

Peeled Potato	-	40.00 g
Dextrose	-	2.00 g
Agar	-	2.00 g
Distilled water	-	100 ml.

Richards Agar (RM)

KNO_3	-	1.00 g
KH_2PO_4	-	0.50 g
$MgSO_4, 7H_2O$	-	0.25 g
$FeCl_3$	-	0.002 g
Sucrose	-	3.00 g
Agar	-	2.00 g
Distilled water	-	100 ml.

Yeast extract mannitol agar (YEMA)

Yeast extract	-	0.040 g
Mannitol	-	1.00 g
$MgSO_4$, $7H_2O$	-	0.02 g
K_2HPO_4	-	0.05 g
NaCl	-	0.01 g
Agar	-	2.00 g
Distilled water	-	100 ml.

Carrot juice Agar (CJA)

Grated Carrot	-	20.00 g
Agar	-	2.00 g
Distilled water	-	100 ml.

Czapek - dox agar (CDA)

$NaNO_3$	-	0.20 g
K_2HPO_4	-	0.10 g
$MgSO_4$, $7H_2O$	-	0.05 g
KCl	-	0.05 g
$FeSO_4$, $7H_2O$	-	0.001 g
Sucrose	-	3.00 g
Agar	-	2.00 g
Distilled water	-	100 ml

All these petridishes were then incubated at 30°C for the desired period. Finally the mycelia were strained through muslin cloth, collected in aluminium fail of known weight, dried at 60°C for 96 h, cooled in a desicator and weighed.

3.2.4.2. Liquid media

To assess the mycelial growth of F. *oxysporum* in liquid media, the fungus, was first allowed to grow in petridishes containing 20ml of PDA and were incubated at 30°C for 7 days. From the advancing zone of the mycelial mat, agar block (4 mm-dia) was cut with a sterilized cork borer and transferred to each Ehrlenmeyer flask (250ml) containing 50ml of sterilized liquid medium for the desired period at 30°C. Finally, the mycelia were strained through muslin cloth, collected in aluminum foil cup of known weight, dried at 60°C for 96 h, cooled in a desiccator and weighed.

3.3. AGGLUTINATION RESPONSE OF CONIDIA

The agglutination response of condia was determined following the method of Lis and Sharon (1986) and Cristimzio *et al* (1988). Concanavalin A (Con A), *Helix pomatia* agglutinin (HPA), *Ulex europaeus* agglutin -I (UEA - I) and wheat germ agglutinin (WGA) of Sigma Chemical, USA, were diluted (1mg/ ml) with 50 mM phosphate buffered saline (PBS), pH 7.2 and were used for agglutination reactions. Con A solution contained 1mM each of $CaCl_2$, $MgCl_2$ and $MnCl_2$.

3.3.1. Preparation of conidial suspension

Agglutination tests were done with ungerminated spores. At first thefungus was allowed to grow on PDA slant for 6 days at 30°C. Conidial suspension was prepared by adding 3-5 ml of sterile distilled water. The resulting suspension was centrifuged at 3500g for 15 min at 4°C. The pellet was washed thrice with cold PBS and resuspended in the PBS to a concentration of approximately $6x10^6$/ml. The conidial suspensions were used immediately after preparation.

3.3.2. Agglutination tests

Ungerminated conidial suspension (10μl) was taken in a slide and incubated with diluted lectin solution (10μl) in a moist chamber at room temperature for various incubation times (upto 2h). During incubation, slides were gently swirled several times to ensure maximal cellular contact. Agglutination of conidia was observed under Leica Leitz Biomed microscope in bright field and arbitrarily scaled from '0' (no agglutination) to '4' (maximum agglutination).

3.4. BACTERIAL CULTURE

3.4.1. Source of culture

An isolate of *Bradyrhizobium japonicum* (SB119) was obtained from the Indian Agricultural Research Institute, New Delhi. The culture of *B. japonicum* was maintained in Yeast Extract Mannitol Agar (YEMA) medium during the investigation and sub-culturing were done at a regular interval.

3.4.2. Inoculation, re-isolation and identification of the bacterium

Seeds of soybean after disinfection with 95% ethanol were inoculated with *B. japonicum,* kept overnight and sown. Following nodulation, firm large nodules were selected for isolation of bacterium. They were first detached from the root, washed, disinfected with 95% ethanol and rewashed with sterile distilled water. Each nodule was placed in the centre of a sterile petridish, 1 or 2 drops of sterile distilled water was added to it and crushed with a sterile scalpel (Peltier *et al., 1961).* Sterilized YEMA medium was poured into petridishes and inoculated with bacteria (using the crushed nodule as inoculum) and incubated at 37°C. After 6 days raised, moist, and glistening colonies were observed in petridishes. Typical colonies were selected for Gram staining and some were transferred aseptically to YEMA slants. After 7 days of incubation, cultures were compared with the original stock culture of B. *japonicum* and its identity was confirmed.

3.4.3. Assessment of Bacterial growth

B. *japonicum* was grown on YEMA slants and bacterial suspension of known concentration ($1x10^6$ bacteria / ml) was prepared from 6-8 day old culture. YEMA medium was prepared, dispensed in Ehrlenmeyer flasks (50ml / 250 ml flask), sterilized at 15 lbs. p.s.i pressure for 15 minutes and inoculated with the bacterial suspension (0.5ml/flask). After a desired period of incubation, growth was measured by colorimetry (Eklund and Lankford, 1967). The concentration of bacterial suspension was determined (bacteria / ml of medium) by noting its OD value and compared with a standard value. The standard value was determined by cell counting method.

3.5. PRODUCTION OF INOCULA AND INOCULATION TECHNIQUE

3.5.1. *Fusarium oxysporum*

i) Pot screening - *F. oxysporum* was grown on sterilized sand - maize meal medium (sand maize meal -1:1) and incubate for 10 days at room temperature. After 10 days, fungus soil mixture (100 gm medium / 12" pot) was prepared by hand mixing, watered and kept for 3-4 days. Then surface sterilized seeds (20 seeds / pot) were sown in each pot at 2-3 cm depth.

3.5.2. *Trichoderma Sp.*

To study the effect of*Trichoderma* sp. on disease development first the inocula were prepared. *Trichoderma* sp. was grown on sterilized soil : wheat bran (1:1) medium. After 7 days incubation, the cultures of *Trichoderma* sp. was added to the soil where already F.*oxysporum* was infested before 3-4 days (Raghuchander *et. al. 1993).* Then seeds were sown.

3.5.3. *Bradyrhizobium japonicum*

6-8 days old cultures of *Bradyrhizobium japonicum* was selected for seed bacterization. At first bacterial suspension was prepared and concentration was determined by noting its OD (no. of bacteria / ml of medium) and compared with a standard value. Seeds were surface sterilized and then soaked in a bacterial suspension overnight before sowing.

3.6. ASSESSMENT OF DISEASE INTENSITY

Plants were examined after 7, 14, 21 and 28 days of inoculation. Disease intensity was assessed on the basis of percentage loss in dry weight of roots and colour intensity of the infected roots as described by Chakraborty and Shil (1989). After a desired period of incubation, plants were uprooted, washed, dried at 60°C for 96 h and weighed. For determination of root rot index roots were graded into 5 groups on the basis of percentage loss in dry weight of root in relation to control, and a value was assigned to each group (vix. 1-25% loss in weight =0.25, 26-50% = 0.50, 51-75% = 0.75, 76-100%=1). The root rot index in each case was the quotient of the total values of roots of replicate plants and the number of plants. Colour intensity of roots was expressed as light brown (+); dark brown (++) blackish brown (+++) and black (++++).

The number of nodules in each case was counted.

3.7. EXTRACTION OF ENZYMES FROM HEALTHY & INFECTED ROOTS

3.7.1. Phenylalanine ammonia lyase

For the extraction of phenylalanine ammonia lyase (PAL), the method of

Bhattacharya & Ward (1987) was followed. Seeds of two soybean cultivars (Soymax & JS-2) were surface sterilized, and half were inoculated with *B. japonicum.* Seeds were then sown in soil. Fifteen day old plants were uprooted and dipped in sterile distilled water as well as in mycelial and spore suspension of *F. oxysporum.* At a regular 4 h intervals, roots of each treatment (1g) were excised and crushed with a mortar and pestle with 0.1M sodium borate buffer. pH-8.8 containing 2 mM β-mercaptoethanol. The slurry was centrifuged in a microcentrifuge at 15,000 g for 20 minutes. The supernatant was collected and after recording its volume, was immediately stored at - 20°C until required.

3.7.2. Peroxidase

To extract peroxidase, the method of Chakraborty & Kapoor (1993) was followed with modifications. Both bacterized and non-bacterized fifteen day old plants were uprooted and dipped in sterile distilled water as well as in mycelial and spore suspension of *F. oxysporum.* At a regular 4 h interval, roots of each treatement (1g) were exiced and crushed with a mortar and pestle with 0.1 M sodium borate buffer, pH 8.8. The homogenate was centrifuged in a microcentrifuge at 15,000 g for 30 minutes and the resulting supernatants were assayed for peroxidase activity.

3.8. ASSAY OF ENZYME ACTIVITIES

3.8.1. Phenylalanine ammonia lyase

PAL activity in the supernatant was determined by measuring the production of cinnamic acid from L-phenyl alanine spectrophotometrically. The reaction mixture contained 300 μM sodium borate, pH 8.8, 30 μM/L-phenylalanine and 0.5 ml of supernatant in a total volume of 3 ml. Following incubation for 1 h at 40°C the absorbance at 290 nm was read against a blank without the enzyme in the assay mixture. The enzyme activity was expressed as μg cinnamic acid produced in one minute / gm fresh weight of tissue.

3.8.2. Peroxidase

For determination of peroxidase activity, 100 μl of crude extract was

added to the reaction mixture consisting of 1 ml of 0.2 M sodium phosphate buffer (pH-5.4), 100 µl of 4mM/L H_2O_2, 100 µl of 0- dianisidine (5 mg/ml) and 1.7 ml of distilled water. Peroxidase activity was assayed spectrophotometrically at 460nm by monitoring the oxidation of O-dianisidine in presence of H_20_2. Specific activity was expressed as the increase in absorbance of 460 nm/gm tissue / minute.

3.9. ESTABLISHMENT OF CALLUS

3.9.1. Culture media

For the callus induction, MS basal media (Murashige and Skoog, 1962) was used. Following stock solutions were prepared :

MS-1 (20x)

a)	KNO_3	-	38.0 g
	NH_4NO_3	-	33.0 g
	$MgSO_4,7H_2O$	-	7.4 g
	KH_2PO_4	-	3.4 g
	Double distilled water	-	1L
b)	$CaCl_2$, $2H_2O$	-	8.82 g
	Double distilled water	-	500 ml

Solution (a) and (b) were mixed and the volume was adjusted to 2L with double distilled water and stored at 4^0C.

MS-II (100x)

$MnSO_4$	-	2.23 g
$ZnSO_4$, $7H_2O$	-	860 mg.
H_3BO_3	-	620 mg
KI	-	83 mg
Na_2Mo_4, $2H_2O$	-	25 mg
$CuSO_4$, $5H_2O$	-	2.5 mg
$CaCl_2$, $6\ H_2O$	-	2.5 mg
Double distilled water	-	1L

MS -II (20x)

$Na_2EDTA, 2H_2O$	-	746 mg
Boiling double distilled water	-	80 ml
$Fe_2SO_4, 7H_2O$	-	556 g
Double distilled water	-	80 ml

$FeSO_4, 7H_2O$ solution was added to Na_2EDTA solution with vigorous stirring and volume was adjusted to 200 ml with double distilled water.

MS-IV (100x)

Myo - inositol	-	100 mg
Thiamine HCl	-	0.5 mg
Nicotinic acid	-	0.5 mg
Pyridoxin HCl	-	0.5 ng
Glycine	-	2 mg
Double distilled water		100ml.

MS - V

For 10 ml

Glycine	-	10 mg.
KI	-	8.3 mg in 20 ml

MS-VI

MSI, II, III, IV, Vand VI were mixed in the following ratio.

MS - I	-	100 ml
MS - II	-	50 ml
MS - III	-	5 ml
MS - IV	-	10 ml
MS - V	-	2 ml
MS - VI	-	2 ml

The medium was supplemented with 3% sucrose, 0.8% -1% agar, 1g/L 2,4.D (1mg / L) and .625mg/ L BAP (Kato 1989). Finally volume was made 1L by double distilled water and sterilized.

3.9.2. Callus induction

Seeds of different soybean cultivars were taken and surface sterilized with 90% ethanol for 5 min. and washed five times with sterile distilled water

for the removal of ethanol. After final washing, seeds were transfered into semi-soild media contained in flasks for germination. After 7-10 days, seedlings were cut in a sterile condition and finally one piece was transferred to each culture tube. They were incubated under 16 h photoperiod at 26°C and observed regularly.

3.10. EXTRACTION AND SEPARATION OF GLYCEOLLIN

3.10.1. Roots

To extract glyceollin, the method of Keen *et al.* (1971) was followed with modifications. Fifteen day. old plants were inoculated with F *oxysporum* following water culture method as stated earlier. After 24, 48 and 72 h. of inoculation roots were used for extraction of glyceollin. Infected as well as healthy roots (30g) were homogenised with 120 ml of 95% ethanol in an electric 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°C. The concentrate was extracted thrice with equal volume of ethyl acetate. The ethyl acetate fraction was dehydrated with sodium sulphate and dried at 45°C. The residue was dissolved in ethyl acetate (0.1 ml g^{-1} 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 (0.2mm thickness) and activated for 1 h at 80°C. 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 + 0.5 ml of 5% aqueous sodium nitrite solution + 15 ml of 20% aqueous sodium acetate solution).

RF value was determined in each case.

3.10.2. Callus

For extraction of glyceollin from callus, spore suspension of *F oxysporum* was placed on each callus and incubated for 24-72h. Extraction of glyceollin was done as described above

3.11. ULTRAVIOLET SPECTROPHOTOMETRY AND QUANTIFICATION 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 scraped off and eluted in spec methanol. The eluates were stored at 5^0C for overnight and centrifuged to remove the silica gel. These eluates were examined by UV-spectro. photometry (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 286nm as described by Bhattacharyya and Ward (1985).

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

* x = moles / litre converted to g / litre by multiplying moles with molecular weightof glyceollin ($C_{20}H_{19}O_5$)

Results have been expressed in μg/g fresh weight of roots.

3.12. PETRIDISH BIOASSAY OF PHYTOALEXIN

In case of petridish bioassay, 0.2 ml of ethyl acetate extract of infected roots was taken in a 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 (20 ml / petridish) and mixed well. Each petridish was inoculated with an agar block (4 mm. diam.) containing 4-day old mycelia of F. *oxysporum* and incubated at 30$\pm$ 1^0C. Diameter of mycelial mat was measured after 24, 48, and 72 h of inoculation and compared with the controls.

3.13. PREPARATION OF ANTIGEN

3.13.1. Root Antigen

Root antigens were extracted from healthy and *F. oxsporum* infected soybean roots following the method of Chakraborty & Saha (1994). Seeds of soybean cultivars were grown in earthen pots containing *Fusarium* infested soil as well as in sterilized washed soil separately. Healthy and infected plants were uprooted after two week intervals, washed with cold water and kept at -15^{0}C for 1 h. Finally, roots (20 g fresh weight) were crushed with sea-sand in mortar and pestle in cold (4^{0}C) and stored at -15^{0}C for 1 h and homogenized with 20 ml. of 0.05M sodium phosphate buffer supplemented with 10 mM Sodium metabisulphite and 0.5mM magnesium chloride. Homogenate was strained through cheese cloth and then centrifuged (12,000g) at 4^{0}C for 1 h and known quantity of ammonium sulphate was added to it for 100% precipitation (Green & Hughes, 1965), kept at 4^{0}C. Precipitate was dissolved in the same extractive buffer (pH-7.4) and dialysed against 0.005 M phosphate buffer for 24 h at 4^{0}C. During this period 10 changes were given. The dialysate (i.e., soluble protein) was used for antisera production and for gel electrophoretic study.

3.13.2. Mycelial Antigen

Mycelial antigen was prepared following the method of Chakraborty & Saha (1994). Initially fungal mycelia (4mm disc) were transferred to 250 ml Ehrlenmeyer flasks each containing 50ml of sterilized liquid Richards medium (g/1 distilled water, sucrose, 30; KNO_3, 10; KH_2PO_4, 5; $MgSC_4$, $7H_2O$, 2.5 and $FeCl_3$, 0.02) and incubated for 10 days at 30 $\pm$ 1^{0}C. For extraction of soluble antigens, mycelial mats were harvested, washed with 0.2% NaCl and rewashed with sterile distilled water. Washed mycelia (30 g fresh wt.) were homogenized with 0.05 M sodium phosphate buffer (pH-7.2) supplemented with 10mM sodium metabisulphite and 0.5 mM magnesium chloride and 0.85% NaCl in mortar and pestle in presence of sea sand. Cell homogenates were kept overnight at 4^{0}c and then centrifuged (15000g) for 30 min at 4^{0}C. The supernatant was equilibrated to 100% saturated ammonium sulphate under constant stirring and kept overnight at 4^{0}C. After this period the mixture was centrifuged

(15000 g) for 30 min at 4^{0}C, the precipitate was dissolved in 10ml 0.05M sodium phosphate buffer (pH-7.2). The preparation was dialysed for 72 h 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 matarial was stored at -20^{0}C and used as antigen for the preparation of antiserum and other experiments.

3.13.3. Protein Estimation

Soluble proteins were estimated following the method as described by Lowry *et. al.* (1951). To 1 ml of protein sample 5 ml of alkaline reagent (0.5 ml of 1% $CuSO_4$ and 0.5 ml of 2% potassium sodium tartarate, dissolved in 50 ml of 2% Na_2CO_3 in 0.1N NaoH) was added. This was incubated for 15 min at room temperature and then 0.5 ml of Folin Ciocalteaus reagent (diluted 1:1 with distilled water) was added and again incubated for 15 min for colour development following which optical density (OD) was measured at 720 nm. Quantity of protein was estimated from the standard curve made with bovine serum albumim (BSA).

3.14. SDS - POLYACRYLAMIDE GEL ELECTROPHORESIS OF PROTEIN

3.14.1. Preparation of Slab Gel

Stock solutions

For the preparation of gel, the following stock solutions were initially prepared as described by Laemmli (1970) & Sambrook *et. al.* (1989)

(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	- 100ml

(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 peroxidipsulphate	-	0.1 g
Distilled water	-	1.0 ml

(freshly prepared each time)

(F) Tris - glycine electrophoresis buffer

(25mM Tris Base ; 250 mM glycine)

5x stock can be made;

Tris Base	-	15.1 g
Glycine	-	94 g

in 900 ml of dH_2O, pH was adjusted to 8.3

Then 50 ml of 10% SDS was added and volume made upto 1000 ml.

(G) 1xSDS gel loading buffer :

50 mM Tris Cl (pH - 6.8)

100 mM dithiothreitol.

2% SDS

0.1% bromophenol blue

10% glycerol

Slab gel Preparation

For slab gel preparation, two glass plates (17cmx19cm) were washed with dehydrayted alcohol and dried. Then 1 mm thick spacers were placed between the glass plates at the two edges and the 2 sides of glass plates were

sealed with 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 (P^H 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 overlayered 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 (p^H-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 up to a height of 4 cm over the resolving gel and overlayered with water. Finally the gel kept for 30 min for polymerization.

3.14.2 Sample preparation

Sample was prepared by mixing the sample protein with 1x 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 microliter 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.

3.14.3. Electrophoresis

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

3.14.4. Fixing & 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 20 h in the fixer for fixing.

The staining solution was prepared as follows :

Coomassie Brilliant Blue R 250	- 0 .25 g
Methanol	- 45 ml
Distilled water	- 45 ml
Acetic Acid	- 10 ml

At first, gels were stained by staining solution for 2-3 h and finally soaked with destaining solution (methanol : dH_2O : Acetic acid - 4.5 : 4.5:1) until the background became clear.

3.15. ANTISERA PRODUCTION

3.15.1. 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 and were observed for at least one week inside the cages. They were regularly fed with 500 g green grass each time in the morning and evening. Every alternate day they were also given 50-75 g of

green seeds soaked in water. Besides this, they were given saline water after each bleeding for three consecutive days. Cages were cleaned everyday in the morning for better hygeinic conditions.

3.15.2. Immunization

Antisera were raised in separate rabbits against antigen preparations of mycelia of *F. oxysporum* as well as healthy root antigen of UPSM-19. Before immunization normal sera were collected from each rabbit. In each case 1ml of antigen emulsified with an equal volume of Freund's complete adjuvant (Difco) were injected intramascularly, repeating the doses at 7 days intervals with Freund's incomplete adjuvant (Difco) for 7 consecutive weeks.

3.15.3. 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 (2 ml) 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 1 h and then the clot was loosened with a sterile needle and the antiserum was clarified by centrifugation at 2000 g for 10 min. Finally, blood samples were distributed in 1 ml vials and stored at - 20^0C until required.

3.16. PURIFICATION OF Ig G

3.16.1. Precipitation

IgG was purified following the method of Clausen (1988). The antiseurm

(5ml) was diluted with two volumes of distilled water and an equal volume of 4.0 M ammonium sulphate. The pH was adjusted to 6.8 and the mixture was stirred for 16 h at 22^{0}C. Then it was centrifuged at 10,000g for 1 h at 22^{0}C and the precipitate was dissolved in 5 ml of 0.02 M sodium phosphate buffer, pH 8.0.

3.16.2. 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 0.02 M phosphate buffer, pH 8.0 and was applied to a column (2.6 cm in dia, 30 cm high) and allowed to settle for 2 h.After that 25 ml of 0.02 M phosphate buffer (pH 8.0) was applied to the gel material.

3.16.3. Fraction collection

At the top of the column, 2 ml of ammonium sulphate precipitate was applied and the elution was performed at a constant pH and a molarity continuously changing from 0.02 M to 0.3 M. The initial elution buffer (1) was 0.02 M sodium phosphate buffer p^H 8.0 (diluted from a 0.10 M sodium phosphate buffer pH 8.0 containing 16.86 g Na_2HPO_4, $2H_2O$ + 0.731 g NaH_2PO_4, H_2O/L.). The final elution buffer (2) was 0.30 M 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, 40x5 ml fractions were collected and the optical density (OD) values were recorded by UV-spectrophotometer at 280 nm.

3.17. IMMUNODIFFUSION TEST

3.17.1. Preparation of agar slides

The glass slides (5cmx5cm) were degreased successively in 90%(v/v) ethanol : di-ethyl ether (1:1v/v) and ether, then dried in hot air oven and sterilized inside the petridish each containing one slide. A conical flask containing Tris-barbiturate 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 repeatedly 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 the glass slides (6 ml / slide) and allowed to solidify. After that 3-7 wells were cut on the agar plate with a sterilized cork borer (4 mm dia.) at a distance of 5mm. from the central well.

3.17.2. 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 contiune in a moist chamber for 48-72 h at 25^{0}C.

3.17.3. 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 staine with 0.5% coomassie blue (0.5 g coomassie blue, 5 g $HgCl_2$, 5ml glacial acetic acid, 95 ml distilled water) for 10 min. at room temperature. After staining slides were washed thrice in destaining solution [2% (v/v) acetic acid) for 5 h to remove excess stain. Finally, all slides were washed with distilled water and dried in hot air oven for 3 h at 50°C

3.18. IMMUNOELECTROPHORESIS

3.18.1 Preparation of agarose slides

The slides (7.5x2.5cm) were degreased, dried and sterilized as described earlier. Thin and uniform layer (2mm thick) of fluid agarose medium (0.9% agarose, 0.1% NaN_3 dissolved in 0.05 M barbitol buffer (pH - 8.6) was poured on each slide taking care that no air bubble 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°C until use.

3.18.2. 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 (0.05 M 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 3 h at 4°C. After electrophoresis the current was discontinued.

3.18.3. Diffusion.

A longitudinal trough 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°C.

3.18.4. 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 3 h at 50°C.

3.19. ENZYME LINKED IMMUNOSORBENT ASSAY

3.19.1. Indirect ELISA

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

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

Stocks			
A	Sodium Carbonate	=	5.2995 g
	Distilled water	=	1000 ml
B	Sodium bicarbonate	=	4.2 g
	Distrilled water	=	1000 ml

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

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

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

With 280 ml of stock solution 'A', 720 ml 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)

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

Tris = 0.657 g

NaCl = 0.81 g

KCl = 0.223 g

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

5. Antisera dilution buffer (0.15 M 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

Sigma Fast PNPP substrate tablet sets were used. Each tablet set yields the following when dissolved in 20 ml of distilled water.

PNPP = 1.0 mg/ml

Tris buffer = 0.2 M

7. Stop Solution

0.3 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 (200 µl/ well) in a Nunc 96 well ELISA plate. After loading, plate was inculated at 25°C for 4 h. The plate was then washed four times under running tap water and once with PBS - Tween and each time, plate was shaken dry. Subsequently, 200 µl of blocking agent was added to each well for blocking the unbound sites and the plate was incubated at 25°C for 1h. After incubation, plate was washed as mentioned earlier. Purified antiserum (IgG) was diluted in antisera dilution buffer and loaded (200 µl / well) to each well and incubated at 4°C overnight. After a further washing 200 µl of antirabbit IgG goat antiserum labelled with alkaline phosphatase (Sigma

Chemicals, USA) was added and incubated at 37°C for 2 h. Plate was washed, dried and loaded with 200 µl of Pnitrophenyl phosphate substrate in each well and incubated in dark at room temparature for 30-45 min. Colour development was stopped by adding 50 µl / well of 0.3M NaOH solution and absorbance was determined in an ELISA reader (Cambridge Tech. Inc. USA) at 405 nm. Absorbance values in wells not coated with antigens were considered as blanks.

3.19.2. Dot -blot ELISA

For Dot ELISA following buffers were prepared as described by Hammond and Jordon (1990) with modifications.

1. Antisera dilution buffer (Carbonate - bicarbonate buffer 0.05M, pH9.6)

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

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

2. Tris HCl Buffer (10 mM Tris, pH - 7.4)

Tris	-	1.2114 g
Distilled water	-	1000 ml

pH was adjusted to 7.4, then 0.9% NaCl and 0.05% Tween 80 was added. (TBS - T80)

3. Blocking Reagent

(10mM TBS - T80, pH -7.4)

In 100 ml TBS - T80, 3 gm BSA was added.

4. Antigen dilution buffer

(10mM TBS - T80, pH - 7.4)

In 10 mM TBS-T80, pH -7.4, 0.01 M Sodium diethyldithiocarbamate, 2%

pyrollidone (PVP), 1%, BSA and EDTA was added.

5. Substrate

Sigma Fast Red TR salt and Naphthol AS-Mx phosphate were used. Each Sigma Fast Red TR/Naphthol AS-MX tablet set contains the following when dissolved in 10 ml d H_2O.

Fast Red TR salt	-	1 mg / ml
Naphthol AS-MX	-	0.4 mg / ml
Levamisol	-	0.15 mg / ml
Tris buffer	-	0.1 M

For dot-blot ELISA, nitrocellulose membrane (NCM) filters (IMMOBILON-NC, HAHY 0.45 um, Sigma USA) first floated in distilled water for 5-10 second. Antiserum was diluted with carbonate bicarbonate buffer (pH - 9.6) and then NCM filters were dipped in this diluted antiserum. After 4 h incubation, NCM filters were rinsed 5 times with 10 mM TBS-T80 (pH - 7.4). After that NCM was blocked by putting it in blocking solution for 10-15 min (3% BSA in TBS-T80) following which it was rinsed as stated before. Antigen was diluted 1 : 1 with antigen dilution buffer and spotted (5-10μl) on to the dry antiserum coated NCM and incubated at 4^{0}C overnight. After washing, alkaline phosphatase (Sigma chemicals, USA) conjugated with IgG was added to each plastic dish and NCM was floated in the solution and incubated for 1-2 h at 27^{0}C. NCM was washed, dried and finally floated in the substrate developing solution. After 30-45 minutes colour dots were developed. NCM was rinsed with deionized water to stop the reaction.

3.20. FLUORESCENCE ANTIBODY STAINING AND MICROSCOPY

Indirect fluorescence staining of cross sections of soybean roots, fungal spores and mycelia were done using FITC labelled goat antirabbit IgG following the method of Chakraborty & Saha (1994).

3.21.1. Cross section of soybean roots

Initially, fresh cross sections of soybean roots were cut and immersed in

phosphate. buffer saline (PBS) pH 7.2 containing 0.8% NaCl and 0.02% KCl. Following this, sections were treated with normal serum or antiserum diluted (1:125) with PBS (pH 7.2) and incubated for 30 min at 27°C . After incubation, sections were washed thrice with PBS Tween (pH 7.2) for 15 min and transferred to 100 µl of diluted (1:40) goat antiserum specific to rabbit globulins and conjugated with fluorescein isothiocyanate (FITC). The sections were incubated for 30 min at 27°C. All prepations with FITC labelled antibodies were carried out in darkness or very low light. After that, sections were washed thrice with PBS Tween as mentioned above and then mounted on a grease free slide with 10% glycerol. A coverslip was placed on the section and sealed. Fluorescence of the root sections were observed using Leica Leitz Biomed microscope with fluorescence optics equipped with ultra violet (UV) fifter set I3. Tissue section were photographed under both phase contrast and UV fluorescent conditions for comparison of treatment.

3.21.2. Mycelia

Fungal mycelia were grown in PDB. 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 30 min at 27°C. 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°C. 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 as before.

3.21.3. Conidia and Chlamydospore

Fungal conidia and chlamydospores were collected from 15 day-old culture and a suspension of this was prepared with PBS (pH 7.2). Conidial suspensions were taken in micro centrifuge tube and centrifuged at 3000 g for 10 min and the PBS supernatant was discarded. Then 200 µl of diluted antiserum (1:125) was added into the microcentrifuge tube and incubated for 2h at 27°C.

After incubation, tubes were centrifuged at 3000 g for 10 min and the supernatant was discarded. Then the spores were rewashed 3 times with PBS-Tween by centrifugation as before and 200 μl of goat antirabbit IgG conjugated FITC (diluted 1:40 in PBS) was added and the tubes were incubated in dark at 26^{0}C for 1h. After the dark incubation excess FITC antisera was removed by repeated washing with PBS - Tween and the spores were mounted on glycerol jelly and observed under Leica microscope, equipped with I3 UV fluorescence filter and photographs were taken.