MATERIALS AND METHODS

(A) PLANT MATERIALS :

(i) **Source of seeds** : The seeds of 10 cultivars (viz., JS-2, UPSM-19, Pusa-16, PK-327, R-184, KU-254, EC-2575, EC-55865, EC-95287 and Soymax) of soybean (Glycine max (L) Merrill) were obtained from the Pulses and Oil seeds Research Station, Berhampore, West Bengal. These seeds were stored at 20°C and also at room temperature (30 ± 2°C). The seeds were disinfected with 'Agrosan-GN' in order to avoid microbial decomposition during storage. For the varietal resistance test aforesaid soybean cultivars were used and finally on the basis of their maximum and minimum resistance to root rot disease of soybean, cvs. UPSM-19, EC-55865, R-184, Soymax, JS-2 and KU-254 respectively were selected for further study. It is necessasry to mention that about 80% of the seeds of JS-2 and UPSM-19 and 60% of that of Soymax and R-184 lost their viability after one year so, seeds were procured from the said research station every year.

(ii) **Growth of plants** : Healthy seeds of soybean were treated with 0.1% $HgCl_2$ for 5 minutes to remove superficial contaminants, washed several times with sterile distilled water and sown in earthen pots (10 seeds/pot of 25 cm. diam.) containing non-infested sandy soil (soil : sand 1:1). The plants were grown in the Phyto-pathological Experimental Garden, Department of Botany of the University of North Bengal under natural conditions of day light and temperature (26-34°C). The pots were watered daily with ordinary tap water. The plants were grown during March to October. Usually 15-day-old plants were used for inoculation throughout the investigation except otherwise stated.

B. Fungal culture:

i) **Source of culture:** A virulent strain of Fusarium graminearum Schwabe(Fg.1) obtained from Division of Mycology

and Plant Pathology, Indian Agricultural Research Institute, New Delhi, was used throughout this investigation (except otherwise stated) after completion of Koch's Postulate. Another isolate of F.graminearum (Fig 22) was obtained from the stock culture maintained in the Plant Pathology Laboratory, Department of Botany, University of North Bengal which was originally isolated from naturally infected roots of soybean (Cv.JS-2) grown in the field of Pulses and Oil Seeds Research Station, Berhampore. Some other fungal species which were used in this study are enlisted in Table No. 1.

(ii) Completion of Koch's postulates : Soybean seeds were surface sterilized with 0.1% $HgCl_2$ solution for 5 minutes, washed with sterile distilled water and sown in pots containing sandy soil previously infested with conidia & mycelia of F.graminearum.

The pathogen was reisolated from infected roots after 20 days of inoculation into potato-dextrose-agar(PDA) slants, examined after 15 days of incubation (at 28°C) and the identity of the organism was confirmed after comparing it with the stock culture already made available for the purpose.

(iii) Maintenance of stock cultures:

The fungi listed in Table-1 were grown on PDA slants. After 15 days, the cultures were stored under three different conditions (5°C, 20°C and 30°C) in sterile liquid paraffin. Apart from weekly transfer for experimental work, the cultures of F.graminearum isolates were also examined at a regular interval to test its pathogenicity.

(iv) Assessment of mycelial growth in liquid media :

F.graminearum was first grown in petridishes, each containing 20 ml of PDA medium and incubated for 4 days at

Table-1 : List of Fungal isolates used

Species with isolate code	Host of origin	Source of isolate
Fusarium graminearum Schwabe Fg1 (ITCC-1805)	Glycine max	Indian Agricultural Research Institute, New Delhi.
Fusarium graminearum Schwabe Fg2	G.max	Naturally infected roots of soybean, Cv.JS-2. Pulses and oil seeds Research Station, Berhampore, West Bengal.
Glomerella cingulata (Stoneman) Spauld & Schrenk GC-1(IMI-356806)	Camellia sinensis	Tocklai Experimental Station Jorhat, Assam.
Pestalotiopsis theae (Saw.) Stey. Pt1 (IMI-356807)	C.sinensis	Tocklai Experimental Station, Jorhat, Assam.
Drechslera oryzae(Breda de Haan) Subram, & Jain HO-1(ITCC-2537)	Oryza sativa	Indian Agricultural Research Institute, New Delhi.
Bipolaris carbonum Nelson. BC-1 (IMI-298762)	C. sinensis	Naturally infested leaves of Gayabari Tea Estate, Darjeeling.
Fusarium Solani (Martives) Saccaris. FS-1 (ITCC-1804)	Glycine max	Indian Agricultural Research Institute, New Delhi.
Fusarium Oxysporum Schlecht. FO-1 (ITCC -1803)	Glycine max	Indian Agricultural Research Institute, New Delhi.

IMI - International Mycological Institute, ITCC - Indian Type Culture Collection.

30°C. From the advancing zone of the mycelial mat, agar block (4mm diameter) containing the mycelia, was cut with a sterilized cork borer and transferred to each Ehrlenmeyer flask(250 ml) containing 50 ml of sterilized Richard's medium (KNO_3, 10.0 g; K_2HPO_4, 5.0 g; $MgSO_4,7H_2O$, 2.5 g; $FeCl_3$, 0.02g; Sucrose, 30.0 g; Distilled water 1 lt.) for a desired period at 30°C. Finally, the mycelia were strained through muslin, collected in aluminium foil cup of known weight, dried at 60°C for 96 h cooled in a desicator and weighed.

(C) Chemicals :

In addition to ordinary laboratory reagents, the following chemicals were used.

Acrylamide	- Merck, German
Adjuvant complete(Freund)	- Difco Laboratories, Detroit Michigan, USA.
Adjuvant incomplete(Freund)	- Difco Laboratories, Detroit Michigan, USA.
Amido black	- Hi-Media Laboratories, Bombay, India.
Ammonium peroxodisulphate	- E.Merck(India) Ltd., Bombay,
Barbitone sodium GR	- Loba Chemicals Co., Bombay, India.
Barium chloride	- BDH
Bis acrylamide	- Merck, German.
Bovine serum albumin	- Sigma Chemical Co. USA.
Cadmium chloride	- SDS
Brilliant blue R 250	- Sigma Chemical Co. USA.
Ferric Chloride	- Sarabhai M Chemicals
Folin-Ciocaltea u's reagent	- Glaxo Laboratories, Bombay, India.
Goat antirabbit-IgG(whole molecule) fluorescein isothiocyanate conjugate.	- Sigma Chemicals Co. USA.

Goat antirabbit-IgG(whole molecule) Horse radish peroxidase conjugate.	- Sigma Chemical Co. USA.
Glycine	- Merck, German
Mercury(II) chloride	- Merck
Nickel nitrate	- E.Merck(India) Ltd. Bombay
p-Nitroaniline	- Loba Chemical Co. USA.
Riboflavin	- Sigma Chemical Co. USA.
Sea-Sand	-Ricdel-De Haen Ag Seelza-Hannover
Silica gel G	- E.Merck(India) Ltd. Bombay.
Sodium acetate	- E.Merck(India) Ltd. Bombay.
Sodium azide	- E.Merck(India) Ltd. Bombay
Sodium malonate	- E.Merck(India) Ltd. Bombay
Sodium molybdate	- E.Merck(India) Ltd. Bombay
Sodium selenite	- E.Merck(India) Ltd. Bombay
Tris	- Merck, German.

(D) Preparation of inoculum and inoculation technique :

(i) Sick pot : Sick pot method as described by Nene et.al. (1981) was adopted with modification. Pots (size 9" diam.) were filled with sandy soil (1:1). Naturally infected as well as artificially inoculated (with F.graminearum) soybean plants were chopped into small pieces and these were incorporated uniformly in the surface soil of those pots and kept for one month. After the said period of incubation, 10 seeds each of the different soybean cultivars were separately sown in each pot. Control sets were maintained by sowing soybean seeds in non infested sandy soil.

On the other hand, sand maize meal medium was prepared by mixing riverbed sand and maize meal in the ratio of 9:1 respectively and taken in 100 ml Ehrlenmeyer flasks (40 g in each flask), 10 ml distilled water was added and the medium was autoclaved at 15 lbs pressure for 20 minutes. After

cooling, each flask was inoculated with F.graminearum and incubated at 28°C for 10 days. The sand maize meal culture was throughly mixed with non-infested sandy soil in each pot. Pots were watered and kept as such for 15 days. Surface sterilized soybean seeds were sown in each pot and disease intensity was assessed.

(ii) Water culture : Fifteen surface sterilized (with 0.1% $HgCl_2$ solution for 5 min.) soybean seeds of each cultivar were sown in each pot containing autoclaved sandy soil. The seedlings were nursed until their further transfer. F.graminearum was grown in potato dextrose broth (100 ml broth/250 ml) at 30°C on a shaker (8 h . each day) for 10 days. Entire contents of a flask was dilute with sterile distilled water to get the final inoculum dilution of 2.5% (approx. 6.5×10^5 spores/ml.). In each sterilized glass tube (150 x 15 mm) 20 ml. of inoculum was poured. Subsequently, 15 days old seedlings were uprooted from the experimental garden. The root system was washed throughly in running tap water, then rinsed twice in sterilized distilled water. One seedling was transferred into each tube and plugged with cotton. After every two days sterilized distilled water was added to the tubes in order to make up the loss. In control tubes one seedling in each tube was transferred.

(E) The assessment of disease intensity :

Plants were examined after 10, 20 and 30 days of inoculation. Disease intensity was assessed on the basis of percentage loss in dry weight of roots as described by Chakraborty and Shil (1989). After desired period of incubation the plants were uprooted, washed,dried at 60°C for 96 h and weighed. Root rot index was calculated in the following way : - on the basis of percentage loss in dry weight of root in relation to control, they were graded into 5 groups and a value was assigned to each group (viz., 1-10% loss

in weight = 1, 11-25% = 2, 26-50% = 3, 51-75% = 4, 76-100%=5). The root rot index in each case was quotient of the total values of the replicate roots and the number of roots (i.e., number of plants).

(F) Method of application of chemicals :

Soybean (CV. Soymax) seeds were soaked for 24 h. either in different dilutions (10^{-3}M to 10^{-8}M) of nine chemicals or water separately before sowing them in earthen pots as described by Chakraborty and Sinha (1984).

(G) Method of extraction and separation of glyceollin from infected host roots

To extract glyceollin, the method of Keen et.al., (1971) was followed with modifications. Fifteen day-old plants were inoculated with F.graminearum following water culture method as stated earlier. After 24, 48, 72 and 96 h. of inoculation, the plants were uprooted from earthen pots, washed and roots were used for extraction of glyceollin. Thirty grams of roots were homogenised with 120 ml of 95% ethanol in an electric blendor. The extracts were filtered through filter paper and the residues were re-extracted with an equal volume of 80% ethanol and filtered. The filtrates were combined and the residues discarded. The combined filtrates were reduced in volume in a rotary evaporator at 45°C. The concentrated filtrates were extracted once with 30 ml hexane and then thrice with equal volume (30 ml) of ethyl acetate. The hexane fraction was discarded and the ethyl acetate fraction was dried at 45°C. The residues was disolved in 3 ml ethyl acetate and the aliquots were used for separation on the plates and chemical detection of glyceollin. TLC plate bioassay as well as for petridish bioassay test.

Thin layer chromatograms were prepared with Silica gel G (0.2 mm thickness) and activated for 1 h . at 80°C. Aliquots (10 µl) of ethyl acetate extract of roots, as well

as authentic glyceollin were separately spotted on the activated chromatograms and developed separately in four different solvent systems in order to select the best one (showing better separation of glyceollin). The following 4 solvent systems were used.

(i) Hexane : ethyl acetate : methanol (60:40:1)
(ii) Hexane : ethyl acetate : acetic acid (80:20:4)
(iii)Chloroform : acetone : 28% NH_3(65:35:1) &
(iv) Chloroform : acetone : acetic acid (90:10:0.5)

The solvent front was first marked in the chromatogram and after drying the plate was observed under UV light or sprayed with Diazotized, P-nitroaniline (5 ml of 0.5%) P-nitroaniline solution in 2N HCl + 0.5 ml of 5% aquous sodium nitrite solution + 15 ml of 20% aquous sodium acetate solution).

Rf value of glyceollin (obtained from root extract) was compared with authentic glyceollin (obtained from Professor N.T.Keen, University of Californea, Riverside, USA) for confirmation.

(H) Ultraviolet spectrophotometry and quantification of glyceollin :

Ultraviolet spectrophotometry - Aliquots (50 µl) of ethyl acetate extract were spotted on the plates as described and developed in hexane : ethylacetate : methanol (60:40:1) solvent system and allowed to dry. Silica gel from unsprayed chromatogram corresponding to reacting zones (reacting with reagent) were scraped off, eluted in 5 ml absolute ethanol and stored for overnight at 5°C. The eluates were centrifuged to remove the silica gel particles and the volume of supernastant was made upto 10 ml and used for UV spectrophotometric studies. UV absorption spectrum of glyceollin was determined by UV spectrophotometer (Shimadzu-model 160).

Quantification : Quantity of glyceollin ($C_{20}H_{19}O_5$) was estimated from UV-spectrophotometric curve by assuming molar extinction co-efficient of 10,800 at 286 nm as described by Bhattacharyya and Ward (1985).

$$\text{Molar extinction co-efficient} = \frac{\text{O.D. of the tested solution}}{\text{Concentration}^{(a)} \text{ of the tested solution(moles/litre)} \times \text{Path length of the cell (cm)}}$$

a = moles/litre converted to g/litre by multiplying moles with molecular weight of glyceollin. Results have been expressed in /ug/g fresh weight of roots.

(I) Bioassays of glyceollin :

(i) TLC - plate Bioassay :

For bioassay of glyceollin 10 /u1 ethyl acetate extract of infected roots were spotted on TLC plates as described and developed in hexane : ethyl acetate : methanol (60:40:1) solvent system. After drying, spore suspension of Bipolaris carbonum Nelson (Spores suspended in nutrient solution - KNO_3, 1% ; KH_2PO_4, 0.5% ; $MgSO_4$, $7H_2O$, 0.25%) and incubated for 96 h. in a moist, closed glass chamber at 25°C. Finally, inhibition zone was marked on the chromatogram.

(ii) Petridish Bioassay of Phytoalexin :

For petridish bioassay of phytoalexin (glyceollin) the method of Keen(1971) was followed. Ethyl acetate extract(0.2 ml) of infected roots containing glyceollin was taken in each of the 3 sterilized petridishes and allowed to evaporate. Subsequently 0.2 ml ethanol was poured in each petridish, agitated and 20 ml sterilized PDA medium was added to it. Each petridish was inoculated with an agar block (4mm

diam.) containing 4-day-old mycelia of F. graminearum and incubated at 30 ± 1°C. Diameter of the hyphal growth was measured and compared with control.

(iii) Slide germination

Partially purified glyceollin was assayed by measuring its effects on the spore germination of F.graminearum following the method of Rouxel et.al., (1989). Glyceollin was added to spore suspension (10^6 spores/ml) in water and drops of the suspension was placed on microscopic slides and allowed to germinate in a humid chamber. The germination rate and germ tube length was measured using a light microscope after 24 h incubation at 25°C and compared with control.

(J) Extraction of total soluble protein

(i) Root protein

Soluble protein were extracted from healthy and F.graminearum infected roots of soybean cultivars as well as from the mycelia of F.graminearum following the method as described by Chakraborty and Saha (1994). Seeds of soybean cultivars were grown in earthen pots containing Fusarium infested soil as well as in sterilized soil separately. Healthy and infected plants were uprooted after two week intervals, washed with cold water and kept at -15°C for 1 h. Finally, roots (25 g fresh weight) were crushed with sea-sand in mortar and pestle in cold (4°C) and stored at -15°C for 1 h and homogenized with 25 ml of 0.05 M Tris-HCl buffer (pH 7.4) at 4°C. Homogenate was strained through cheese cloth and then was centrifuged(12,100 g) at 4°C for 1 h and known quantity of ammonium sulphate was added to it for 100% precipitation (Green & Hughes, 1965), kept at 4°C for overnight and centrifuged (10,000g) for 15 min. at 4°C. Precipitate was dissolved in the same extractive buffer (pH 7.4) and dialysed against 0.005 M Tris solution for 24 h at

4°C. During this period 10 changes were given. The dialysate (i.e., soluble protein) was used for gel electrophoretic study.

(ii) Mycelial protein

To extract soluble mycelial protein, F.graminearum was grown in sterilized liquid medium (g/L. distilled water, sucrose, 50g; KNO_3, 10g ; KH_2PO_4, 5g; $MgSO_4$, $7H_2O$, 2.5g and $FeCl_3$, 0.02g) 12 days at 30±1°C. Mycelia (50g fresh wt.) were collected, washed with 0.2% NaCl solution, rewashed with sterile distilled water and crushed in cold (4°C), stored at -15°C for 2 h. Rest of the procedure was as described for root protein preparation. The soluble proteins were used for gel electrophoretic study.

(K) Protein estimation :

The soluble proteins were estimated following the method of Lowry et.al. (1951). Initially an alkaline mixture was prepared by mixing of 0.5 ml of 1% $CuSO_4$, 0.5 ml of 2% sodium potasium tartarate, 50 ml of 2% Na_2CO_3 dissolved in 0.1N NaOH. Finally, reaction mixture was prepared by mixing 0.1 ml of the protein sample, 0.9 ml water and 5 ml of Folin-phenol solution (Folin-phenol : water= 1:1) was added and again incubated for 15 min. In case of blank, water was used instead of protein sample. At the end of the incubation period O.D. value of each sample was determined by systronics photoelectric colorimeter 101 at 710 nm. Quantity of protein was estimated following the standard curve made with bovine serum albumin (BSA).

(L) Polyacrylamide gel electrophoresis of soluble protein :

(i) Preparation of gel solution and gel column :

Protein patterns of healthy and infected roots of both resistant and susceptible varieties of soybean as well as

mycelia of F.graminearum were determined by polyacrylamide gel electrophoresis following the method of Davis(1964) with modifications, Clean gel tubes(120m. long, 4 mm. diam.) were kept vertically on the tube stand. One end of each tube was closed with rubber cap. Mixtures of working solutions were prepared as follows :

Lower gel
(solution I:II:III=1:1:1)

Solution I
Acrylamide - 30g
Bisacrylamide-0.8g
distilled water-100 ml

Solution II
Tris - 18.15g
(N)HCl - 24.0 ml
TEMED - 0.4 ml
Dist. water - 100 ml

Solution III
Ammonium peroxide -
Sulphate - 60 mg
Distilled water - 100ml.

Upper gel
(Solution IV:V:VI=2:1:1)

Solution IV
Acrylamide - 5 g
Bisacrylamide - 1.25 g
distilled water - 100 ml.

Solution V
Tris - 2.10 g
(N) HCl - 13 ml
TEMED - 0.2 ml
Dist. water - 86.8 ml.

Solution VI
Riboflavin - 2 mg
2M Sucrose -100 ml.

Lower gel solution was poured slowly into the tube up to a height of 9 cm. and drop of water was placed on the top of the gel solution with a view to have a plain surface instead of concave one. The gel tube was kept as such until it became solidified. Water drop was soaked with filter paper and the lower gel over layered with the upper gel solution. Again, a drop of water was placed on the top of the gel solution and kept in light until the gel became solidified. The rubber cap was removed and the gel tube with gel column was fitted on to the reservior.

(ii) Electrophoresis :

Soluble protein (0.01 ml = 150 µg) was added to an equal volume of 2M sucrose and trace of bromophenol blue and eventually loaded on to the gel column. Tris glycine buffer [Tris, 6g ; glycine, 28.8g. and distilled water upto 1 L. (total volume of the solution)], pH 8.4 was poured into both the upper and lower buffer reserviors. The whole set was incubated in cold room at 4°C supplying a constant current of 3 m.amp./tube approx. for 3-4 h until the dye front reached the bottom of the gel column. The gel was stained with Brilliant blue R 250 (0.25%) in methanol : acetic acid : water = 5:1:5) for 1h and then destained with the approximate solution (methanol : acetic acid : water=5:1:5) until the protein bands become clear. Rf values of individual bands were determined.

(M) Source and maintenance of rabbits for serological works :

Male rabbits used for immunological works were supplied by M/S Sujit Sarkar, Bagdogra, animal supplier. All of them were of Australian strain, and white in colour. (Plate 2 ; Fig. A). The initial weight of the rabbits varied from 1.2-1.8 kg and their age varied from 9-10 months, before experimentation. The rabbits were kept in separate cages (60cm x 45cm x 30cm) attached with metal trays at the bottom and placed in a well ventilated cleaned room. Each rabbit was supplied with 20 g carrot (Daucus carota); 50g ; soaked gram (Cicer arietinum), 50-70g. grass (Cynodon dactylon), 4-5 leaves of lettuce (Lactuca sativa) or Cabbage (Brassica oleracea L.var. Capitata) or Cauliflower (Brassica oleracea L.var. botrytis) daily. Along with the food, fresh water was supplied proportionately with the age of rabbits. Under new environmental conditions the rabbits were kept under close observation at least for a week before immunization.

(N) Preparation of antigens :

(i) Plant antigen

The seed and root antigens were extracted following the procedure as described by Chakraborty and Purkayastha (1983). Soybean seeds and roots of both resistant and susceptible varieties were selected for the preparation of antigens. Surface sterilized seeds were first soaked in sterile distilled water for 12 h and subsequently the seeds were kept for 2 h at -20°C. Roots after thorough washing with sterile distilled water were kept for 1h at -20°C. Then the seeds and roots were crushed separately in morter and pestle at 4°C with sea sand and homogenized with 0.5 M Tris-HCl buffer (pH-7.4), and centrifuged (12,100 g) for 1h at 4°C.Known quantity of $(NH_4)_2SO_4$ was added to these supernatant for 100% precipitation (Green and Hughes, 1965), kept for 12h at 4°C and centrifuged (10,000 g) for 15 min at 4°C. The precipitate was dissolved in the same extractive buffer (pH 7.4) and dialysed against 0.005 M tris-HCl solution for 24h at 4°C. During this period 10 changes were given. The dialysate (i.e., soluble protein) was used for gel electrophoretic study.

(ii) Fungal antigen :

Discs (4 mm) of mycelium were transferred to Ehrlenmeyer flasks (250 ml) each containing 50 ml of sterilized liquid medium (g/L distilled water, sucrose, 30 g; KNO_3, 10 g : KH_2PO_4, 5g; $MgSO_4$, $7H_2O$, 2.5g and $FeCl_3$, 0.02 g) and incubated for 15 days at 30 $\pm$ 1°C. For extraction of soluble antigens, mycelial mats were harvested, washed with 0.2% NaCl and rewashed with sterile distilled water. Washed mycelia (50 g fresh wt.), were homogenized with 0.05 M sodium phosphate buffer (pH 7.4) containing 0.85% NaCl in a mortar and pestle in the presence of sea sand. Cell

homogenates were kept overnight at 4°C and then centrifuged (15000 g) for 30 min at 4°C. The supernatant was equilibrated to 100% saturated ammonium sulphate under constant stirring and kept overnight at 4°C. After this period the mixture was centrifuged (22,000 g) for 30 min at 4°C, the supernatant dissolved in 10 ml 0.05 M Phosphate buffer (pH 7.4). The preparation was dialysed for 48 h. through cellulose tubing (Sigma Chemical Co. U.S.A.) against 1 L. of 0.005 M phosphate buffer (pH 7.4) with ten changes. After dialysis the preparation was centrifuged (12,000 g) for 15 min at 4°C and supernatant was stored at -20°C until required.

The protein contents of both plants and fungal antigen preparations were determined following the method as described by Lowery et.al., (1951) using bovine serum albumin as the standard.

(O) Preparation of antisera :

(i) Immunization

Before immunization, normal sera were collected from each rabbit. Antisera against antigens of host (Glycine max) and pathogen (F.graminearum) were raised in separate rabbits (Plate 2, Fig. B) of antigen (1.5 mg/ml protein of either host or pathogen) emulsified in equal volume of Freund's complete adjuvant (Difco) and repeating the doses at 7 days intervals with Freund's incomplete adjuvant (Difco) for 7 consecutive weeks. Five days after the last injection the blood samples were collected.

(ii) Bleeding :

Bleeding of rabbit was performed by ear vein puncture. In order to bleed rabbit or to handle them during injection, the animal was taken out from the cage, placed on its back on the wooden boards (measuring 60 cm x 30 cm x 1 cm,

fixed in a 60° position) with the neck in the triangular gap and the head below the board ; legs were tied to the screws and thus the body was fixed. The hairs were removed from the vein on the ear with the help of a razor and disinfected with rectified spirit. After irritation of the ear with xylene an incision was made with a sharp sterilized blade on the border vein of the ear and about 10 ml of the blood samples were collected in a sterile glass graduated tube. (Plate 2 , Fig. C). After collection of desired quantity of blood all precautions were taken to stop the flow of blood from the punctured area of the ear. The blood samples were kept as such for 1 h at 30°C for clotting. In order to avoid loss of serum included in the clot, it was loosened from the glass surface by turning a sterile wooden stick around the glass near the glass wall. Finally, normal sera as well as antisera were clarified by centrifugation(2000 g for 10 min. at 4°C) and distributed in small amounts in sterile closed ampules and stored at -20°C for further use.

(P) Determination of titre value :

Titre of antisera against the homologous antigens and titres of antigens against homologous antisera were determined following immunodiffusion technique as described by Ouchterlony (1967) and Clausen(1969). A constant amount (5 µl) of undiluted antiserum or antigen was placed in the central well, while diluted antigens or antiserum (diluted with normal saline, 1:1, 1:2, 1:4, 1:8, 1:16, 1:32, 1:64 and 1:128) were pipetted into the outer well. Diffusion was allowed for 48-72 h. at 25°C in a humid chamber. Titre was expressed as the reciprocal of the highest dilution of antiserum or antigen which reacted with antigen or antiserum giving precipition lines.

Plate-2 Fig : (A) Male rabbit (9 month old); (B) Antigen preparation emulsified with adjuvant being injected intramuscularly in rabbit, (C) Blood collection by ear vein puncture.

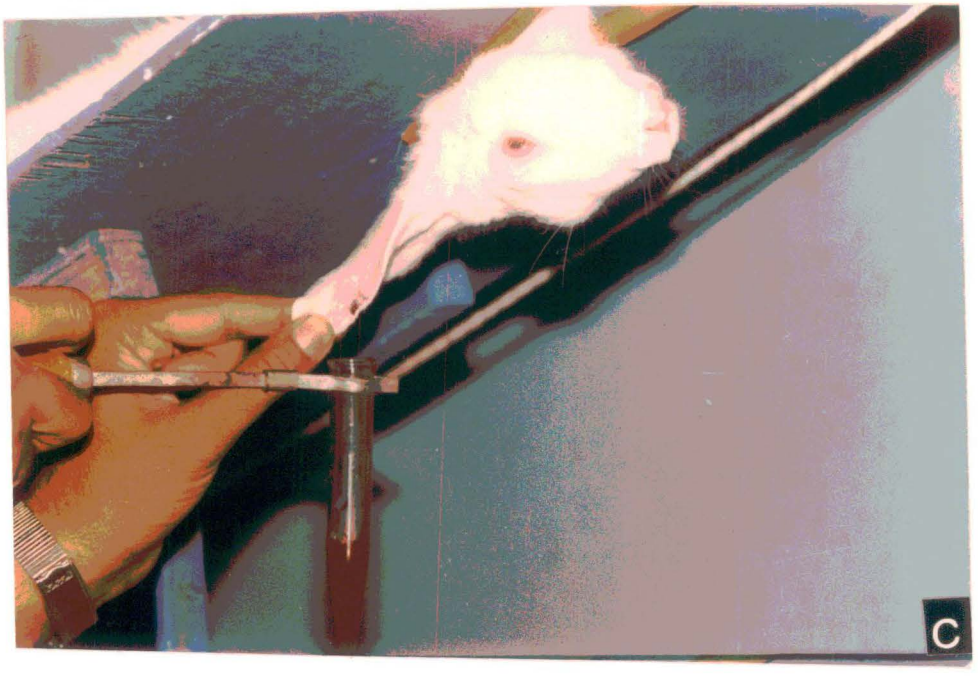

Plate- 2 .

(Q) Agar-Gel Double diffusion technique :

Agar-gel double diffusion test was performed following the method of Ouchterlony (1967). A conical flask (1 l) containing 500 ml barbitol buffer(0.05 M, pH-8.6) was placed in a boiling water bath, when the buffer was hot, it was mixed with 5 g. Difco agar during the next 40 min. The flask was repeatedly taken out and shaken thoroughly in order to prepare absolutely clear molten agar which was mixed with 0.1% (w/v) sodium azide (a bacterio static agent). The medium was dispensed in petridishes (5 ml/petridish, 5 cm. diam.) and 3-7 wells cut out with a sterilized cork borer at a distance of 5 mm. from the central well. The antigens and undiluted antisera were pipetted directly into the appropriate wells and diffusion was allowed to continue in a moist chamber for 24-28 h at 25°C. Precipitation reaction observed in the agar-gel only in cases where common antigens were present.

(R) Immunoelectrophoretic technique

(i) Preparation of agar slides :

The glass slides (7.5 x 2.5 cm) were degreased successively in 90%(v/v) ethanol; ethanol : ethyl ether (1:1 v/v) and ether. They were dried and numbered with glass marker and placed on horizontal wooden blocks. Thin and uniform layer (2 mm thickness) of fluid agar medium 0.8% Difco agar,0.1% NaN_3 dissolved in 0.05 M barbitol buffer pH-8.6) was drawn on each slide taking care that no air bubble was present in the agar medium. This was necessasry in order to avoid any irregularity which may cause asymmetrical migration and diffusion during electrophoretic separation or the immunodiffusion. The slides were placed in pairs in petridishes containing a few drops of water and stored at 4°C until use.

(ii) Electrophoretic step :

Two central wells (4 mm in diam.) were dug out from the agar plate of each slide following the conventional set up for comparison of two different antigens (Ouchterlony 1967). The slide was 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). Different antigens 10 /µl each were introduced into separate 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 agar surfaces. An electric current 2.5m-amp./slide ; 10V/cm was passed through the slides for 2h in cold (4°C). After electrophoresis the current was discontinued .

(iii) Diffusion :

A longitudinal trough parallel to the long edge of the slide was cut in the agar plates in between 2 wells and the undiluted antiserum(100 /µl) was pipetted into the trough. Diffusion was allowed to continue in a moist chamber for 24-48 h at 25°C. Precipitation arcs were formed only when the common antigens were present.

(iv) Washing, drying and staining of slides :

After immunoelectrophoresis, the slides were washed with 0.9% (w/v) aqueous NaCl carefully for 48 h to remove unreacted antigen and antibody widely dispersed in the agar subsequently NaCl was washed with distilled water for 3 h dried for 30 min. at 40°C and stained either with 0.5% commasie blue or 0.5% amido black(0.5 g commasie blue or amido black, 5 g $HgCl_2$, 5 ml glacial acetic acid, distilled water 95 ml) for 30 min at room temperature (28°C), washed thrice in 2% (v/v) acetic acid for 3 h.(1 h in each item) to remove excess stain, finally washed with distilled water and dried for 30 min at 40°C.

(S) Indirect Enzyme Linked Immunosorbent Assay :-

Indirect ELISA technique described by Koening & Paul (1982) was adopted with modification. Plant and Fungal Antigens were serially diluted with loading buffer (0.05 M Carbonate Buffer. pH-9.6). Diluted antigens (100 /ul) were added to each well. The plates were incubated over night at 4°C and then washed thrice by flooding the wells with 0.15 M PBS, pH 7.2 containing 0.8% NaCl 0.02% KCl and 0.05% Tween 20 (PBS-Tween). After each washing the plates were shaken dry. Subsequently, 100 /ul PBS containing 1% BSA were added to each well in order to saturate all unbound sites (Sengupta et.al., 1989). The plates were further incubated ror 2 h at room temperature and washed thrice. Normal sera as well as plant and fungal antisera were serially diluted with PBS-tween containing 0.5% BSA and 100 /ul of diluted sera were added to each well. The plates were incubated overnight at 4°C following which the plates were washed thrice as before, shaken dry and 100 /ul of diluted (1:10,000) goat antirabbit IgG whole molecule) - Horse radish Peroxidase (HRPO, Sigma) conjugate were added to each well except the blank one & incubated for a further period of 2h at 30°C. After a further washing with PBS-tween, 100 /ul of the enzyme substrate [O-phenylene diamine (OPD), 1 /ug/ml in phosphate citrate buffer, pH-5.0, 25.7 ml of 0.2 M Dibasic Sodium phosphate (Na_2HPO_4), 24.3 ml 0.1 M Citric acid and 50 ml H_2O & 0.012% H_2O_2 was added just before use] was added to each well. Colour development was stopped by adding 50 /ul of 2M H_2SO_4 after 30 min. and absorbance was determined at 405 nm on a ELISa microplate reader, Model 700 (Cambridge Technology, USA).

(T) Fluorescent antibody staining and microscopy :

(a) Fusarium graminearum

Folowing the method of Merz et.al. (1969) with modifications mycelia of F.graminearum were grown on agar

squares (aproximately 1 cm^2) of Richards' medium (KNO_3, 10g; KH_2PO_4, 5g; $MgSO_4$; $7H_2O$, 2.5g; $FeCl_3$; 0.02g; Sucrose, 30 g; Agar, 20g; and 1 l. of distilled water). The inoculated agar blocks were placed on sterile microscopic slides, covered with sterile cover - glass, and incubated in sterile Petridishes containing filter paper for 5 days at 25°C. Finally, the agar blocks were removed, and the mycelium that had adhered to the glass slide was fixed in 95% ethanol-ethyl ether (1:1, v/v) for 10 min. at room temperature followed by 95% ethanol for 20 min. at 37°C. The fixed mycelium was flooded with appropriate antiserum, incubated in a moist chamber for 30 min. washed thrice (5 min. each) with PBS, pH-7.4. The slides were then air dried and goat anti-rabbit IgG (whole molecule) conjugated with fluorescein isothiocyanate (Sigma) diluted 1:10 with Carbonate-bicarbonate buffer, pH 9.6 was added for 30 min. The slides were then washed twice (5 min each), in PBS, pH-7.4 and once in distilled water. The slides were then air dried and mounted in a glycerol-based mounting medium, (Hardham et.al., 1986). A cover glass placed on the mycelium and sealed.

(b) Root of Glycine max. :

For fluorescence staining of root material, the technique of DeVay et.al., (1981) was followed.

Soybean seedlings grown in a sterile mixture of sand and soil (1:1) were harvested 10 days after sowing. Cross sections of the tap root at or just above the region of root hair formation were made and immediately immersed in 100 µl of normal serum, or antiserum diluted (10-fold) with phosphate buffer(0.01 M KH_2PO_4 - K_2HPO_4 containing 0.14 M NaCl, pH 7.0), and incubated for 30 min. at 27°C. The root sections were washed by shaking them in 4 ml of the buffer for 15 min and then transferring them to a 50 µl drop of goat antiserum specific to rabbit globulins and conjugated with fluorescein isothiocyanate(FITC). The sections were incubated for 30 min

in a moist chamber at 27°C. All operations with FITC labelled antibodies were made in darkness or very low light. The sections were then transferred to 4 ml of the buffer and incubated with shaking for 15 min at 27°C. This last step was repeated and then the sections were mounted on a slide in 100 /ul of the buffer diluted with 1 ml glycerol. A cover slip was placed on the tissue sections and sealed. Fluorescence of the root sections were observed using Leica Leitz Biomed microscope with fluorescence optics 020507 type equipped with ultra violet (UV) filter set I-3. Tissue sections were photographed under both phase-contrast and U.V. fluorescent conditions for comparison of treatments.