3. Materials and Methods

3.1. Plant materials

3.1.1. Host plants

3.1.1.1. Collection and selection of niger plants

Seeds of different niger varieties (GA-5, GA-10, RCR-18, BN-1, BN-5, Otcmond, NRS 69-1, JNC-6) were collected from All India Coordinated Research project on sesame and niger (Indian Council of Agricultural Research) Jawaharal Nehru Agricultural University campus, Jabalpur-482004, India. One local seed variety was collected from Jalpaiguri, India. Five different niger varieties (GA-5, GA-10, RCR-18, JNC-6, NRS 69-1) and one local variety widely growing in North Bengal (sub-Himalayan West Bengal) were selected for present study. Selection was done on the basis of the seed germination and growing suitability in the environmental and field condition of North Bengal.

3.1.1.2. Cultivation and maintenance of niger seed varieties

Selected niger varieties were cultivated in pots (Plate 3.1). Some of the plants were also cultivated in fields. Earthen pots of 30 cm in diameter were filled with 4 kg of soil mixture. Soil mixture was prepared by mixing 3.5 kg of fine dry soil and 0.5 kg of green manure. In field also same soil mixture was used for growth of the plants. Seeds were surface sterilised with 0.1 % sodium hypochlorite solution and then washed thrice with sterile distilled water. Sterilised seeds were kept overnight in pre-soaked cotton wool for soaking of water. Water soaked seeds were then broadcasted on prepared soil of pots or field. Finally the seeds were covered with 1 cm thick fine moist soil layer. Watering was done as and when required to maintain the moist condition of the soil. Weeds were removed after 15 days. To provide adequate spacing among the test plants, some niger plants were also removed from the pots or fields.

3.1.2. Collection of plants for extraction of botanicals

On the basis of easy availability in the growing areas of sub-Himalayan West Bengal several plant materials were collected from forests and adjoining areas of the region. Different parts of these plants (leaf, bark, root, rhizome as applicable) were extracted and screened for their fungi toxic properties against

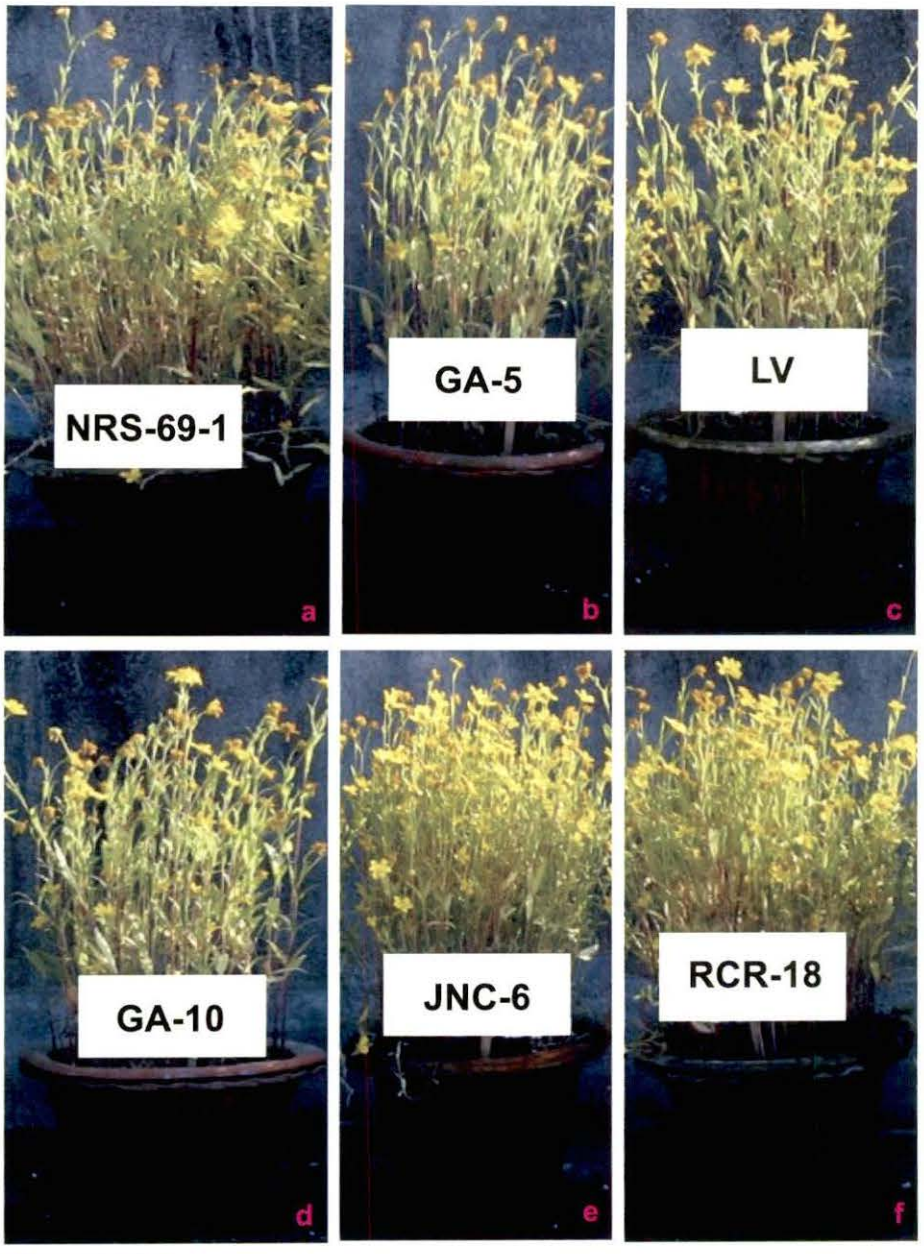

PLATE 3.1

Different varieties of niger in flowering condition

fig. a : NRS-69-1

fig. b : GA-5

fig. c : LV

ig. d : GA-10

fig. e : JNC-6

fig. f : RCR-18

fungal pathogen *Alternaria alternata*. Collected Plants were identified and voucher specimens were deposited in the NBU herbarium, Department of Botany, University of North Bengal.

3.2. Fungal and bacterial cultures used

3.2.1. Source of fungal culture

Three fungal cultures were isolated from the naturally infected leaves of niger plant grown in cultivated fields of North Bengal. Following isolation, the three isolates were subjected to Koch's postulates and have been listed in table 3.1.

Table 3.1: List of fungal cultures isolated from infected niger leaves in North Bengal

Fungal culture	Source	Identified by	Identification no
Alternaria alternata (Fr.) Keissier	Naturally infected niger plant	IARI, New Delhi.	6250.05
Alternaria porri	Naturally infected niger plant	Dr. A. Saha, Dept. of Botany, NBU	-
Aspergillus sp.	Naturally infected niger plant	Dr. A. Saha, Dept. of Botany, NBU	-

**Alternaria alternata* (Fr.) Keissier was used as test pathogen throughout the present study.

3.2.2. Source of microorganisms tested for antagonistic activity

For the study of antagonistic activity two different bacteria and one fungus were isolated from different soil. Three different fungal cultures were procured from Indian type culture collection, IARI, Pusa, New Delhi. One culture of fungal antagonist was collected from Dr. Apurba Choudhury, Uttar Banga Krishi Viswavidyalaya, West Bengal, India. The details of the source of the strains used in the present study are given in Table 3.2.

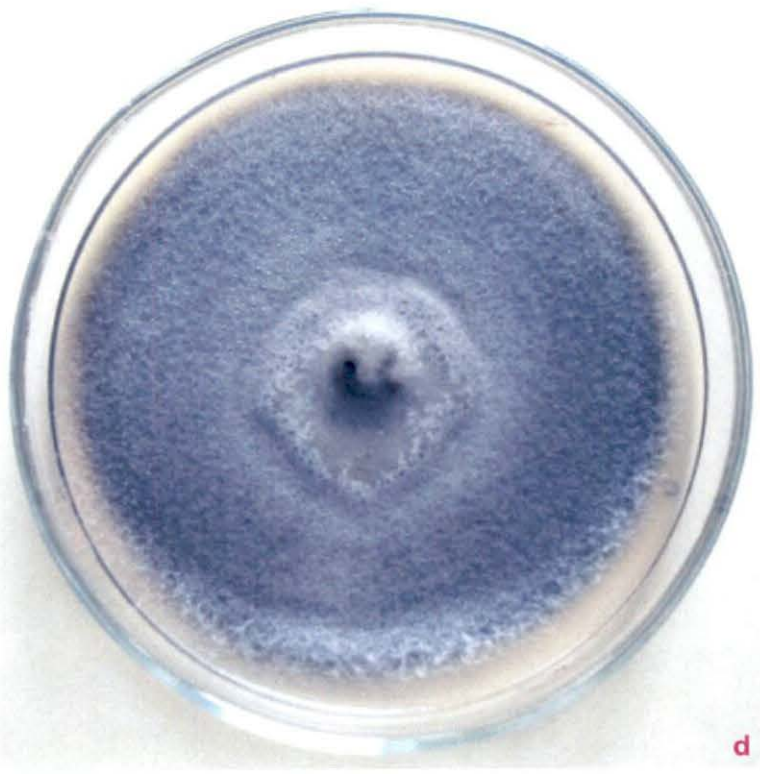

PLATE 3.2

fig. a : Healthy plant.

fig. b : Infected plant.

fig. c : *A. alternata* culture in test tube .

fig. d : *A. alternata* culture in PDA plate.

PDA slants. The inoculated slants were incubated at 28°C and were observed till sporulation. Sporulated cultures were used for microscopic studies. The organisms were confirmed after comparing them with the respective stock cultures. If an organism was consistently re-isolated then it was treated as a pathogen and was identified in the laboratory or elsewhere as mentioned in table 3.1 & 3.2.

3.2.4. Maintenance of stock cultures

Freshly prepared sterile PDA slants were used for the maintenance of the fungal cultures. The bacterial cultures were maintained in nutrient agar as well as in nutrient broth. Pathogens grown on sterile PDA media were stored in two different conditions, viz. at low temperature in refrigerator (at 5°C) and at room temperature. At the interval of two weeks subculture was done for preparation of inoculum for different experiments. Cultures of other fungal and bacterial antagonists were maintained at 5°C and also at room temperature.

3.3. Major chemicals used

In addition to the common laboratory reagents, several chemicals were used during the work. Some of the major chemicals have been enlisted (Table 3.3)

3.4. Composition of media and solutions used

A number of culture media and solutions were used during the present study. The name and compositions of these media and solutions are given below.

POTATO DEXTROSE BROTH (PDB)

Peeled potato	:	40 g
Dextrose	:	2 g
Distilled water	:	100 ml

Peeled potato in required amount was boiled in distilled water. The potato broth was collected by straining through cheese cloth and then required amount of dextrose was added. Finally, the medium was sterilized at 15 lb p.s.i. for 15 minutes.

POTATO DEXTROSE AGAR (PDA)

2% agar powder was added to the final potato dextrose broth solution to prepare potato dextrose agar. The agar was melted by heating the media before sterilization.

Table 3.3: Major chemicals used.

Chemical	Company
3,5- Dinitrosalicylic acid	Hi Media, India
2,1,3-Benzothiodiazole	Fluca,Switzerland.
4- Hydroxybenzoic acid	SRL, India
Acetic acid glacial	E. Merck, India
Acrylamide	SRL, India
Freund's Incomplete Adjuvant	Bangalore Genie
Agarose	SRL, India
Antirabbit IgG	Bangalore Genei
Bromophenol blue	Hi Media, India
Congo red	Hi Media, India
Coomassie brilliant blue	Hi Media, India
di-Sodium tetraborate (Borax)	Merck, India
DL-DOPA	Hi Media, India
Folin and Ciocalteu's phenol reagent	SRL, India
Freund's Incomplete Adjuvant	Difco Lab., USA
Goat anti-rabbit IgG horseradish peroxidase conjugate	Bangalore Genei
2,3-Dihydroxybenzoic acid	Fluca,Switzerland
Glutaraldehyde	Sigma, USA
Glycerol	Merck, India
Glycine GR	Merck, India
Guaiacol	SRL, India
Hydrogen peroxide	Merck, India
L-Alanine	Hi Media, India
Laminarin	Sigma, USA
L-Phenylalanine	SRL, India

Contd...Table 3.3

Table 3.3: (Contd...) Major chemicals used.

Chemical	Company
TEMED	SRL, India
N-Acetyl-D-glucosamine	Hi Media, India
Bisacrylamide	Hi Media, India
O-Dianisidine dihydrochloride	Hi Media, India
p-dimethyl amino benzaldehyde (DMAB)	Hi Media, India
Phenol reagent	Qualigens, India
Polyethelene glycol (PEG)	SRL, India
Polyvinyl pyrrolidone (PVP)	Hi Media, India
Potassium tetraborate	Hi Media, India
Riboflavin	Hi Media, India
Salicylic acid	Qualigens, India
Hydrogen peroxide	Hi Media, India
Sodium tetraborate	SRL, India
Sorbitol powder	Hi Media, India
TMB/H_2O_2	Bangalore Genei,
Triton-X-100	Hi Media, India
Tween 20	Hi Media, India
Immunogold reagent [Affinity isolated aqueous glycerol suspension of antirabbit IgG (Whole molecule)-gold (5nm) from goat]	Sigma, USA
Fluorescent brightener	Sigma, USA
Glycol chitosan	Sigma, USA
Bovine serum albumin	Sigma, USA
2- Aminobutyric acid	Fluca,Switzerland

OAT MEAL AGAR (OMA)

Oat meal	:	40 g
Agar agar	:	15 g
Distilled water	:	1000 ml

Required amount of powdered oat was boiled in distilled water in a water bath, stirred occasionally and strained through cheese cloth. Then agar powder was added to it and melted by heating before the medium was sterilized at 15 lb p.s.i. for 15 minutes.

MALT EXTRACT AGAR (MEA)

Malt extract	:	20 g
Agar	:	20 g
Distilled water	:	1000 ml

Malt extract was dissolved in distilled water by boiling. Then, required amount of agar powder was added. Finally the solution was boiled with constant shaking till the agar was dissolved. Sterilization was done at 15 lb p.s.i. for 15 minutes.

POTATO CARROT AGAR (PCA)

Grated Potato	:	20 g
Grated Carrot	:	20 g
Agar agar	:	20 g
Distilled water	:	1000 ml

Required amount of grated potato and grated carrot were mixed and boiled with distilled water. The broth was strained through cheese cloth and agar powder was added to the filtered broth. Finally, the medium was boiled to dissolve agar before sterilization at 15 lb p.s.i. for 15 minutes.

RICHARD'S SOLUTION / MEDIUM (RM)

Potassium nitrate (KNO_3)	:	10 g
Potassium Dihydrogen Phosphate (KH_2PO_4)	:	5 g
Magnesium sulfate ($MgSO_4$, $7H_2O$)	:	2.5 g
Ferric chloride ($FeCl_3$)	:	0.02 g
Sucrose	:	50 g
Distilled water	:	1000 ml

All the constituents were mixed with required amount of distilled water. Constituents were then dissolved by stirring and sterilized at 15 lb p.s.i. for 15 minutes.

RICHARD'S AGAR (RMA)

2% agar powder was added to the final Richard's solution to prepare Richard's agar. The agar was melted by heating the media before sterilization at 15 lb p.s.i. for 15 minutes.

CZAPEK DOX AGAR (CDA)

Sodium Nitrate ($NaNO_3$)	:	3 g
Potassium hydrogen phosphate (K_2HPO_4)	:	1 g
Potassium Chloride (KCl)	:	0.5 g
Magnesium sulfate ($MgSO_4$, $7H_2O$)	:	0.5 g
Ferrous Sulphate ($FeSO_4$)	:	0.01 g
Sucrose	:	30 g
Agar agar	:	15 g
Distilled water	:	1000 ml

All the ingredients except agar and K_2HPO_4 were dissolved. Then agar was added and dissolved by boiling. Finally K_2HPO_4 was added to the molten solution, mixed thoroughly and sterilized at 15 lb p.s.i. for 15 minutes.

YEAST EXTRACT MANNITOL AGAR (YEMA)

Yeast extract	:	2 g
Mannitol	:	10 g
Potassium Dihydrogen Phosphate (KH_2PO_4)	:	0.5 g
Magnesium sulfate ($MgSO_4$, $7H_2O$)	:	0.2 g
Sodium Chloride (NaCl)	:	0.1 g
Agar agar	:	20 g

All the ingredients except agar were dissolved in distilled water. Finally, agar was added and dissolved by boiling before the medium was sterilized at 15 lb p.s.i. for 15 minutes.

NUTRIENT BROTH

Beef extract	: 3g
Peptone	:10g
NaCl	: 5 g
Distilled water	1000ml

Required amount of beef extract and peptone were dissolved in distilled water by heating. Then NaCl was added. The pH of the solution was adjusted to 7.2 with 10 (N) NaOH. Finally the medium was sterilized at 15 lb p.s.i. for 15 minutes.

NUTRIENT AGAR (NA)

2% agar powder was added to the final nutrient broth solution to prepare nutrient agar. The agar was melted by heating the media before sterilization.

3.5. Inoculation technique

Pathogenecity test was done by artificial inoculation of different varieties of niger plants with the fungal pathogen following the inoculation technique of Dhingra and Sinclair (1995). Inoculation was done by spraying spore suspension bearing $1x10^5$ conidia / ml prepared from 10 d old culture of the pathogen grown on sterile potato carrot agar (PCA) slants. Conidial suspension was sprayed in experimental plants but sterile distilled water was sprayed in control plants by separate automizers. For each treatment ten plants of each variety were taken in experimental pots and were kept for 48 hours in transparent polythene chamber (to maintain high humidity) under normal conditions of light and temperature. The entire experiment was repeated thrice.

3.6. Disease assessment

Numbers of lesions produced on the leaves were counted and diameter of each lesion was measured after 2, 4, 6, 8, and 10 days of inoculation. The results were assessed following Sinha and Das (1972). Lesions were grouped into four categories and value was assigned to each category on the basis of visual observations. The categories are as follows:

- Minute restricted lesions (1-2 mm dia..... 0.10
- Small lesions with sharply defined margins (2-4 mm diameter) 0.25
- Medium slow spreading lesions (4-6 mm diameter) 0.50
- Large spreading lesions of various size with diffused margin (Beyond 6 mm diameter) 1.00

Number of lesions in each category was multiplied by the value assigned to it and the disease index for a plant was calculated as the sum total of such values for all the eaves. Results were computed as the mean of observations of three replications (10 plants in each treatment).

3.7. Morphology and physiology of the pathogen *Alternaria alternata*

3.7.1. Microscopy

Alternaria alternata was sub-cultured in PCA and PDA for ten days. A bit of fungal mycelia was taken from PDA slants, placed on a clean grease free slide and stained with lacto phenol and cotton blue. For study of spores, brown/gray masses of spores distinctly produced on the surface of the PCA slants were carefully taken out with the inoculating needle, placed on a slide and stained with lacto phenol and cotton blue. The slides were observed under light microscope (Olympus, India). Length and breadth of spores, breadth of mycelia etc. were measured by ocular micrometer standardized by stage micrometer. The details of the morphology of the fungus were noted.

3.7.2. Assessment of mycelial growth

Initially, Petri plates with sterile PDA or other media were inoculated with the test fungus (*Alternaria alternata*) and incubated for 7 days at 28 ± 1^{0}C for growth of fungus in the form of mycelia mat. The mycelia mats thus grown in Petri plates were used as inoculum. Mycelia block (4 mm) was cut from the advancing zone of hyphae and was placed one in each Petri plate of 90 mm diameter. The inoculated Petri plates were incubated for 10 days at 28 ± 1^{0}C. Radial growths of mycelia were measured at 2 days intervals to assess the mycelial growth in different solid media.

To assess the mycelial growth in liquid media, mycelia blocks (4 mm diameter) were obtained similarly as mentioned above and were transferred to conical flasks of 250 ml, each containing 50 ml of sterilized liquid medium and incubated at 28 ± 1^{0}C or stated otherwise. The growing fungal mycelia were strained through double-layered cheese cloth after 5, 10, 15, 20 and 25 days of incubation, then blotted on a blotting paper and dried in a hot-air oven at 60^{0}C. The dried mycelia mats were allowed to cool and then dry weight was taken in a precision balance.

3.7.3. Assessment of spore germination

3.7.3.1. Preparation of spore suspension

The fungus was initially cultured in PCA for 14 days at 28±2^{0}C for adequate sporulation. For preparation of spore suspension 4-6 ml (approx.) sterile distilled water was added aseptically and the surface of the fungal culture was gently scrapped using a sterile inoculation needle to loosen the spores only. The tubes were gently shaken for appropriate mixing of the spores with distilled water. The resultant mixture was strained through a muslin cloth and the filtrate was used as spore suspension. Sterile distilled water was added to the suspension to adjust the concentration of the spores following haemocytometer count.

3.7.3.2. Spore germination assay

Assessment of spore germination of the test pathogen was done under various conditions but the basic methodology is essentially the same and is described here. Spore suspension (30 µl) of fungus was placed on slides and the slides were incubated in a humid chamber (in Petri plates) at 28±1^{0}C. The slides were stained with cotton blue-lacto phenol after the desired period of incubation and observed under light microscope. Finally, the percent spore germination [(No. of germinated spores / no. of spores counted) x 100], average germ tube length in each case were calculated for each slide. Percent spore germination was calculated on the basis of 300 spores and average germ tube length was calculated based on 60 germ tubes.

3.8. Collection and maintenance of rabbits for raising antisera

Three New Zealand male white rabbits were used for raising polyclonal antibody in the study (Plate 3.3, fig a). The rabbits were purchased from the laboratory animal supplier and brought to the laboratory at least a month before starting any immunization programme for proper acclimatization. The body weights of rabbits were approximately 1.5 kg and their age was around 9 months. The rabbits were kept in separate cages (75 cm x 75 cm x 90 cm) attached with plastic trays at the bottom and placed in a well-ventilated room. The room was cleaned regularly with permitted room freshening solutions. Rabbits were fed with sufficient quantities of vegetables like soaked gram (*Cicer arietinum* L.), carrot (*Daucus carrota* L.), common grass [*Cynodon dactylon* (L.) Pers.], cabbage

leaves (*Brassica oleraceae* L. var. *capitata*), cauliflower leaves (*B. oleraceae* L. var. *botrytis*), lettuce leaves (*Lactuca sativa* L.) etc. and clean water. The quantity of food was adjusted after a consultation with local veterinary doctor. Routine healths checking of rabbits were also done. The immunization program was started after one month after ascertaining complete body fitness of rabbits.

3.9. Antigen preparation

3.9.1. Antigen preparation from niger leaf

For preparing leaf antigens from young niger leaves, the protein extraction procedure of Alba and DeVay (1985) and Chakraborty and Saha (1994) was followed. For this, fresh young leaves of the each niger varieties were collected from the experimental garden, washed thoroughly with cold water and kept for 2 hours at -20^{0}C. The Frozen leaves (20 g fresh weight in each case) were grounded in prechilled mortar at 4^{0}C with 10 g insoluble polyvinyl pyrrollidone (PVP). The leaf paste was suspended in cold 0.05 M sodium phosphate buffer (pH 7.0) containing 0.85% sodium chloride and 0.02 M ascorbic acid. Then the leaf slurry was strained through cheese cloth and centrifuged at 4^{0}C for 30 min at 12,000 g. The supernatants were collected and ammonium sulphate was added at 4^{0}C to 100% saturation under constant stirring. It was allowed to stand overnight at 4^{0}C and finally centrifuged at 4^{0}C for 15 min at 12, 000 g. The precipitate obtained was dissolved in cold 0.05 sodium phosphate buffer (pH 7.0) and was dialyzed against 0.005 M sodium phosphate buffer (pH 7.0) for 24 hours at 4^{0}C with 16 changes. Finally, the preparation was centrifuged at 4^{0}C for 15 minutes at 12,000 g and supernatant was stored at -20^{0}C until required.

3.9.2. Antigen preparation from fungal mycelia

Antigens from fungal mycelia were prepared following the method as described by Chakraborty and Saha (1994) with some modifications. Mycelial discs (4 mm diameter) from 7 d old PDA culture plates were transferred to 10 conical flasks of 250 ml capacity, each containing 50 ml of sterilized PDB medium and incubated at 28$\pm$1^{0}C. The fungal mycelia were harvested after 15 days, washed with 0.2% NaCl and rewashed with sterile distilled water. Mycelia (50 g fresh weight) were homogenized in 0.05 M sodium phosphate buffer (pH 7.4) containing 0.85% NaCl in a mortar and pestle with sea sand and kept overnight at 4^{0}C. Centrifugation of the homogenates was done at 4^{0}C for 30 minutes at

12,000 g. The supernatants were collected and equilibrated to 100% saturated $(NH_4)_2SO_4$ under constant stirring and again kept overnight at 4^0C. Then, the mixtures were centrifuged at 4^0C for 30 minutes at 12,000 g. Precipitates were dissolved in 5 ml cold sodium phosphate buffer (0.05 M, pH 7.4) after discarding the supernatants. The preparations were dialyzed for 24 hours at 4^0C against 0.005 M sodium phosphate buffer (pH 7.4) with 8 to 10 changes. Following dialysis, the preparations were centrifuged at 4^0C for 15 minutes at 12, 000 g and resultant supernatant were stored at -20^0C until further use. Protein content of both the plant and fungal antigens were determined by the methods of Lowry *et al.* (1951) using bovine serum albumin (BSA) as standard.

3.10. Raising of polyclonal antisera

3.10.1. Immunization of rabbits

Normal sera were collected from each rabbit before immunization. Antisera against antigens of resistant and susceptible host varieties of niger and pathogen (*A. alternata*) were raised in separate rabbits by giving intramuscular injections (Plate 3.3, fig b) 1 ml of antigens emulsified with equal volume of Freund's complete adjuvant. The doses were repeated at 7 days intervals with Freund's incomplete adjuvant for 6 consecutive weeks. The final protein concentration was 5 mg/ml in the emulsion. Blood samples were collected on the 4^{th} day after the last injection and kept at 37^0C for one hour. After incubation antisera were separated from the blood coagulates by the process as mentioned in the following paragraph.

3.10.2. Collection of normal sera and antisera

Blood from rabbits was taken by marginal ear vein puncture and blood was collected in tubes (Plate 3.3, fig c & d). For this, the rabbits were first taken out from the cage, placed on a table and the hairs from the margin of an ear were removed with a sterilized blade. The marginal ear vein was rubbed with xylene-soaked cotton wool to excite blood flow in the veins. The excited ear vein then disinfected with rectified sprit. An incision was made with a sharp sterilized blade on the vein of the ear and about 10 ml blood was collected in a sterile centrifuge tube. Precautions (by using surgical tapes etc.) were taken to stop the flow of the blood from the punctured area of the ear after taking blood. The blood samples were kept undisturbed for an hour at 37^0C for clotting. In order to avoid the loss of

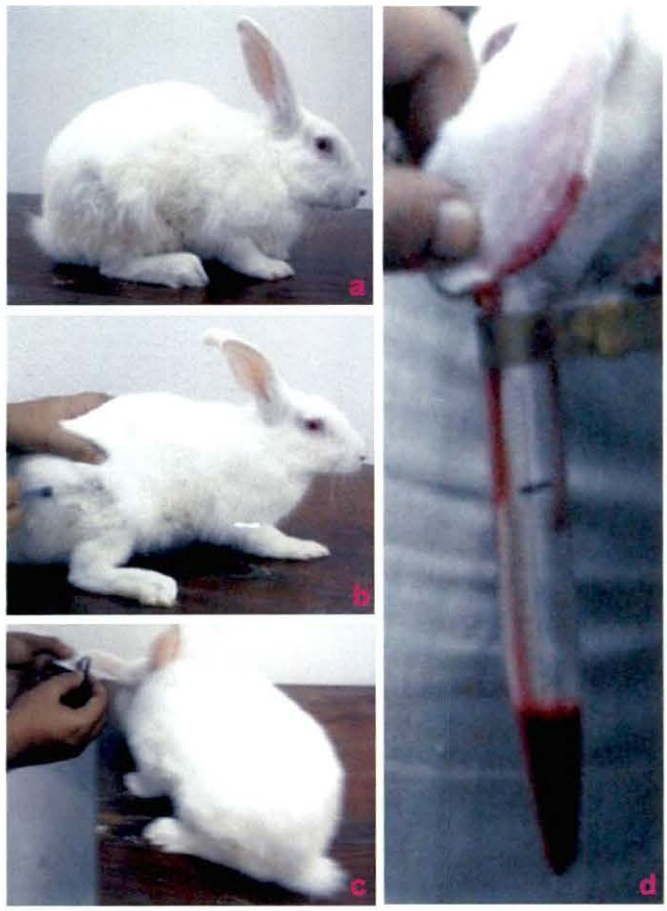

PLATE 3.3

fig. a : Newzealand white male rabbit (approximately 9 month old and 1.2 kg body weight).

fig. b : Intramuscular injection of rabbit

fig. c : Marginal ear vein puncture

fig. d : Collection of blood

serum included within the clot, it was carefully loosened from the glass surface by turning a sterile wooden stick around the clot. Normal sera as well as antisera were clarified by centrifugation at 4^0C for 10 minutes at 3,000 g and were distributed in sterile cryo-vials and stored at -20^0C until required.

3.11. Determination of titre value

Titres 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 antisera (diluted with normal saline with ratios 1:1, 1:2, 1:4, 1:8, 1:16, 1:32, 1:64 and 1:128 respectively) were pipetted into the outer well. Diffusion was allowed for 48-72 h at 25^0C in a humid chamber. The highest dilution of antiserum or antigen that reacted with antigen or antiserum giving precipitin lines was determined as titre value.

3.12. Immunotechniques

3.12.1. Immunodiffusion (ID)

Agar gel double diffusion test was performed following the method of Ouchterlony (1958).

3.12.1.1. Agarose coated slide preparation

For preparation of agarose coated slide, barbital buffer (50 ml, 0.05 M, pH 8.6) was taken in a 100 ml Erlenmeyer flask and was placed in a boiling water bath. Agarose (0.4 g) was mixed with hot barbital buffer. The buffer-agarose mixture was carefully placed on water bath. Finally a clear molten agarose solution was prepared. To this, 0.1% (w/v) sodium azide (a bacteriostatic agent) was mixed and the agarose solution was dispensed carefully in clean, dry square glass plates of 6 cm x 6 cm so that no air bubble remained trapped in the agarose medium to avoid asymmetrical diffusion. Before dispensing the molten agarose solution, the glass plates were washed with extran solution and water and then the glass plates were serially dipped in 90% ethanol, ethanol: ethyl ether (1:1, v/v) and ether for removal of grease.

3.12.1.2. Diffusion

In order to perform the experiment, glass plates were placed inside sterile Petri dishes. Four to six wells of 6 mm diameter were cut by a sterile cork borer. The distances of the peripheral wells from the central wells were approximately 5 mm. The antigens, normal sera and undiluted antisera were placed with a micropipette directly into the appropriate wells and diffusion was allowed to continue in humid chamber at 25^{0}C for 48-72 hours. After proper staining of the slides, the precipitation reaction was observed only in cases where common antigens were present.

3.12.2. Immunoelectrophoresis (EI)

3.12.2.1. Slide preparation

Rectangular glass pieces (8 cm x 3.0 cm) were made grease free to perform immunoelectrophoresis. The slides were dried and placed on a clean surface. Thin and uniform layers (2 mm thick) of molten agarose medium (0.8%), containing 0.1% sodium azide in 0.05 M barbital buffer (pH 8.6) were dispensed on each slide, taking care so that no air bubbles were trapped in the agarose medium. This was necessary in order to avoid irregularity that may cause asymmetrical migration and diffusion during later stages. The slides were stored at 4^{0}C in Petri dishes until use.

3.12.2.2. Electrophoresis

Two central wells of 4 mm diameter were dug out from each agarose-coated slide before they were placed in the middle compartment of the electrophoretic platform. The anode and cathode chambers were filled with 0.05 M barbital buffer (pH 8.6). Different antigens were placed into separate wells. To trace the electrophoretic movement of the antigens, bromophenol blue was used as marker. Filter paper (Whatman no-1) strips were soaked in buffer and placed on both ends of the slides, which connected the buffer solution of anode and cathode compartments with the agarose surface of the slides. An electric current of 2.5 mAmp / slide; 10 V/cm was passed through the slides for approximately two hours at 4^{0}C. The current was discontinued and the glass slides were taken out when the bromophenol blue marker reached near the short edge of the glass slides.

3.12.2.3. Diffusion

After electrophoresis, a longitudinal trough parallel to the long edge of the slides was cut between the two central wells of the agarose surface and undiluted antiserum was placed into the trough. Diffusion was allowed to continue up to 72 h in a moist chamber at 25^{0}C. Precipitation arcs if formed were recorded.

3.12.2.4. Staining of slides

After immunodiffusion and immunoelectrophoresis the glass slides were washed with 0.9% aqueous NaCl carefully for 48 hours to remove all the unreacted antigens and antisera widely dispersed in the agarose surface. Next, the slides were washed with distilled water for three hours to remove the NaCl and dried at 40^{0}C for 30 min. Then, the slides were stained either with 0.5% coomassie blue or 0.5% amido black (0.5 g coomassie blue/amido black, 5 g $HgCl_2$ and 5 ml glacial acetic acid dissolved in 95 ml distilled water) for 30 minutes at room temperature. Following that, the slides were washed thrice with 2% v/v acetic acid for 3 h (one hour each time) to remove the excess stain. Finally the slides were washed with distilled water and dried at 40^{0}C for 30 min. The plates were then photographed using a cannon digital camera (Canon, A310).

3.13. Indirect Enzyme Linked Immunosorbent Assay (Indirect ELISA)

Indirect ELISA was performed after combining the methods of Koenig and Paul (1982) and Dasgupta *et al* (2005). At first, antigens were diluted with coating buffer [carbonate buffer (0.1 M), pH 9.6] and 100 µl of each diluted antigens was placed on the wells of a flat bottomed micro titre ELISA plate (Tarsons), except one well which was considered as blank. The plate was incubated for 6 hours at 4^{0}C in refrigerator. After incubation, the plate was taken out and each well of the plate was flooded with phosphate buffer saline (PBS) -Tween (0.15 M PBS + 0.8% NaCl + 0.02% KCl + 0.05% Tween 20) and washed thoroughly four to five times. The plate was dried in air after washing. Following this, 100 µl of PBS-BSA (0.15 M PBS containing 1% BSA) was added to each well to coat all the unbound sites and incubated for 2 h at room temperature. The plate was again washed with PBS-Tween, air-dired and 100 µl of diluted antisera (diluted with PBS-Tween) was added to each well except the blank and the control wells where normal sera was added (serially diluted with PBS-Tween containing 0.5% BSA).

The plate was incubated overnight at 4^0C. Next day, thorough washing of the plate was done with PBS-Tween. After washing and drying, 100 µl (1:10000) goat-anti rabbit IgG-Horse radish peroxidase conjugate was added to each well except the blank and the plate was incubated for 2 h at 30 ± 1^0C. After incubation, the plate was again washed with PBS-Tween and shaken to dryness. Then 100 µl (1:20) tetramethyl benzidine / hydrogen peroxide (TMB/H_2O_2), a chromogenic substrate was added to each well except the blank. A blue colour was produced due to the reaction between the enzyme and the substrate. Finally, the reaction was terminated after 30 min by adding 100 µl of 1(N) H_2SO_4 to each well except blank. Absorbance values were recorded in an ELISA reader (Mios Junior, Merck) at 492 nm.

3.14. Immunogold labelling followed by silver enhancement

Fresh healthy leaves of niger varieties were collected and washed thoroughly. The plant parts were kept at 4^0C before use. Thin cross sections of leaves were cut and placed on clean grease free slides. In case of fungus, mycelia as well as spores were taken by a needle and placed on slides containing Mayer's albumin for proper fixing. Water drops (100 µl) were mounted on each section. The slides were incubated at 2-5^0C for 30 min and excess water surrounding the sections was blotted off. Thirty micro litre of blocking buffer (0.15 M PBS pH 7.2 containing 5% normal sera of goat) was placed on the cross sections and incubated for 10 min. Excess solution was wiped off and primary polyclonal antibody (1:50 dilution) raised in rabbit against target antigens were applied on the sections and incubated overnight at 25^0C. After incubation, the sections were carefully rinsed in 0.15 M PBS (pH 7.2) for 4 min. Excess buffer was poured off by tilting the slide slightly. Next, 100 µl of diluted (1:50) immunogold reagent containing 0.5 nm gold particle (Sigma, USA) was applied on the sections. Following incubation for 1 h the sections were again rinsed for 4 minutes with PBS. Excess buffer was poured off and the sections were fixed in 200 µl of PBS-glutaraldehyde (2.5% glutaraldehyde solution in PBS) for 15 min. The sections were rinsed in distilled water and placed on slides for silver enhancement. For this, silver enhancement kit of Sigma (Product No. SE-100) were used. Initially solution A (silver salt) and solution B (an initiator) were mixed (1:1) according to the manufacturer's instruction. Mixed solution (100 µl) was used to flood each section. After 5 min of incubation, the cross sections were washed with distilled water. Distilled water was poured off and 100 µl of sodium

thiosulphate solution (2.5 % aqueous) was placed on the sections and allowed to incubate for 3 minutes. The sections were again washed in distilled water and mounted on slides with a drop of distilled water. Immediately after the staining, photographs were taken in a binocular light microscope (Leica, Germany) using digital camera (Canon, A310) with appropriate attachment system.

3.15. Preparation and application of chemical and botanical inducers

3. 15.1. Preparation of plant extracts as inducers

Several plant materials were collected from the nearby forests and hills. Freshly harvested leaves (50g) were crushed in 100 ml distilled water using mortar and pestle at 4^0C. The crushed slurry was strained through muslin cloth and then centrifuged at 3,000 g at room temperature. Finally the supernatant was stored at 4^0C until used.

3.15.2. Application of chemicals and botanicals as elicitors on niger plants

Niger plants were taken in pots of 30 cm diameter. Aqueous leaf extracts were applied on lower leaves of niger plants of 2 months old using hand sprayer leaving 3-4 topmost leaves. The treated plants were labelled accordingly. In addition, Four different chemicals (salicylic acid, 2, 3-dihydroxy benzoic acid, 2, 1, 3-benzothiadiazole, and 2-amino butyric acid) at a concentration of 10^{-3}M, were also used in the similar way as mentioned for leaf extracts. To ensure adhering both the chemicals and botanical elicitors were supplemented with Tween-20 before spraying. After treatment, treated lower leaves were inoculated with conidial suspension ($1x10^5$conidia/ml) of *Alternaria alternata*. Two control sets (treated-uninoculated and untreated-uninoculated) were also maintained for each treatment to compare. The whole experiment was performed in a sterile environment to avoid contamination. Untreated and uninoculated upper leaves were harvested (for experimental purpose) after 0d, 2d, 4d, 6d and 8d following inoculation by *Alternaria alternata*.

3.16. Extraction and estimation of defence related enzymes

3.16.1. Peroxidase (PO): Peroxidase activity was determined according to the procedure given by Hammerschmidt *et al.* (1982). Freshly harvested niger leaves (1 g) were instantly dipped in liquid nitrogen and after 10 min the frozen leaves were crushed in 0.1M sodium phosphate buffer (pH 6.5) at 4^0C. The homogenate

was then filtered through four-layered muslin cloth and the filtrate was centrifuged at 6,000 g at 4^0 C for 15 min. Supernatant obtained was considered as crude enzyme. For estimation of the enzyme activity, 1.5 ml of 0.05M guaiacol and 200 µl of extracted crude enzyme was mixed in a cuvette. The cuvette was then placed in a UV-VIS Spectrophotometer (Model no.118, Systronics, India) and the initial reading adjusted to zero at 420 nm. Then 100 µl H_2O_2 (1% v/v) was added to the cuvette and the changes in absorbance values were recorded for 5 min at 1min intervals. The enzyme activity was expressed in unit enzyme activity. Change in absorbance [Δ A_{420} $min^{-1}g^{-1}$ fresh weight tissue] of 0.001 were considered as unit of enzyme activity.

3.16.2. Polyphenol oxidase (PPO): The method of Sadasivam and Manickam (1996) was followed for determination of Polyphenol oxidase activity. Freshly harvested niger leaves (1 g) were instantly dipped in liquid nitrogen and after 10 min the frozen leaves were crushed in 5 ml of 50 mM Tris-HCl buffer (pH 7.2) containing 0.4 M sorbitol and 1.0 mM NaCl. The homogenate was centrifuged at 12,000 g at 4^0 C for 10 min and supernatant was considered as crude enzyme. For estimation, 2.5 ml of 0.1 M sodium phosphate buffer (pH 6.5) and 0.2 ml crude enzyme was mixed in a cuvette. Then the cuvette was placed in a UV-VIS Spectrophotometer (Model no.118, Systronics, India) and the initial reading was adjusted to zero at 495 nm. Following that, 0.3 ml of 0.01M catechol was added to the reaction mixture cuvette and the changes in absorbance were recorded at 1 min intervals up to 5 min. The enzyme activity was expressed as change in absorbance [Enzyme activity = K X (ΔA min^{-1}) µMol min^{-1} g^{-1} fresh weight tissue (K= 0.272 for polyphenol oxidase)].

3.16.3. β-1, 3-glucanase: Colorimetric method of Pan et al., 1991 was followed for the determination of the activity of β-1,3-glucanase. The method is also called as laminarin-dinitrosalicylate method. Freshly harvested niger leaves (1 g) were instantly dipped in liquid nitrogen and after 10 min the frozen leaves were crushed in 5 ml of 0.05 M Sodium acetate buffer (pH 5.0) at 4^0C and filtered through four-layered muslin cloth. The filtrate was then centrifuged at 10,000 g at 4^0 C for 15 min. The supernatant was used as crude enzyme. To estimate the enzyme activity, 15.6 µl crude enzyme extract was added to 15.6 µl of 4% Laminarin (Sigma, USA) and was incubated at 40^0C for 10 min. The reaction was stopped by addition of 94 µl of dinitrosalicylic acid reagent followed by heating for 5 minutes on a boiling water bath. The final colour of the solution was diluted with

1 ml distilled water and absorbance values were recorded at 500 nm in a UV-VIS Spectrophotometer (Systronics, Model no.118, India). Enzyme activity was expressed on fresh weight basis (nmol min^{-1} mg^{-1}) using D-Glucose as standard.

3.16.4. Phenylalanine ammonialyase (PAL): PAL activity was determined as the rate of conversion of L-phenylalanine to trans-cinnamic acid at 290 nm as described by Sadasivan and Manickam (1996). For that, freshly harvested niger leaves (1 g) were instantly dipped in liquid nitrogen and after 10 min the frozen leaves were crushed in 5 ml of 0.25 M borate buffer (pH 8.7) at 4^0C. The homogenate was then filtered through four-layered muslin cloth and centrifuged at 12,000 g at 4^0C for 15 min. The yellowish green supernatant was used as crude enzyme extract. For estimation of the enzyme activity, 0.5 ml borate buffer, 0.2 ml crude enzyme, 1.5 ml distilled water and 1 ml of 0.1 M L-phenylalanine were mixed and was then incubated for 30 min at 30^0 C. After incubation, the reaction was stopped by adding 0.5 ml of 1M Trichloroacetic acid. The absorbance values were recorded at 290 nm in a UV-VIS Spectrophotometer (Systronics, Model no.118, India). Enzyme activity was expressed as µMol $min^{-1}g^{-1}$ fresh weight tissue using trans-cinnamic acid as standard.

3.16.5. Chitinase: The colorimetric assay of chitinase was carried out according to the procedure developed by Mahadevan and Sridhar (1982) with some modifications. Freshly harvested niger leaves (1 g) were instantly dipped in liquid nitrogen and after 10 min the frozen leaves were crushed in 5 ml of 0.5 M sodium acetate buffer (pH 5.2) containing 700 mg of PVP using mortar and pestle at 4^0C. The homogenate was then filtered through four-layered muslin cloth, centrifuged at 10,000 g at 4^0C for 15 min and the supernatant was used as crude enzyme source. For estimation, the assay mixture consisted of 0.5 ml crude enzyme, 0.25 ml of 0.1 M Sodium acetate buffer (pH 5.2) and 1ml colloidal chitin (1.8 mg/ml) was incubated at 37^0 C for 2 h. One ml of reaction mixture was taken and 1ml of distilled water was added to it. The mixture was boiled for 10 min in a boiling water bath and centrifuged at 5,000 g for 3 min. from the mixture, 0.5 ml of the supernatant was taken and 0.1ml of 0.8 M Potassium tetraborate was added to it. This was then boiled exactly for 3 min on a water bath and cooled. After cooling, 3 ml of p-dimethyl amino benzaldehyde (DMAB) reagent was added and incubated at 37^0 C for 20 min. Immediately after incubation the mixture was cooled and absorbances were recorded at 585 nm in a UV-VIS

Spectrophotometer (Systronics, Model no.118, India). Enzyme activity was expressed as mg GlcNAc g^{-1} fresh weight tissue h^{-1}.

3.17. Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis (PAGE) was done following the method as described by Davis (1964) with some modifications. The compositions of the solutions used for preparing resolving and stacking gels were as follows:-

Solution A

Acrylamide = 30 g, Bis acrylamide = 0.8 g and Distilled water = 100 ml

Solution B

Tris = 18.15 g, 1(N) HCl = 24 ml. Temed = 0.4 ml. and Distilled water = 100 ml. (pH to be adjusted to 8.0)

Solution C

Ammonium persulphate = 60 mg and Distilled water = 100 ml. (to be prepared freshly every time)

Solution D

Acrylamide = 5 g, Bis acrylamide = 1.25 g and Distilled water = 100 ml

Solution E

Tris = 2.10 g, 1(N) HCl = 13 ml. Temed = 0.2 ml. and Distilled water = 86.8 ml. (pH to be adjusted to 6.7)

Solution F

Riboflavin = 2 mg and 2 (M) Sucrose solution = 100 ml.

Preparation of gel: A mini slab gel (8X5 cm) was prepared for isozyme analysis by PAGE. For preparation, two glass plates ware thoroughly cleaned with dehydrated alcohol to remove any traces of grease and air-dried. Spacers (1.5 mm) were placed between the two glass plates on three sides and sealed with 1% agar solution. The slabs were tightly clipped to prevent any leakage of the gel solution while casting the gel. A 10% resolving gel was prepared by mixing solutions A, B and C in the ratio 1:1:1 by a pipette leaving sufficient space (for

comb + 1 cm) for the stacking gel. After pouring the resolving gel solution, it was immediately over layered with water and allowed to polymerize for 1.5 - 2 hours. The stacking gel (4%) solution was prepared by mixing solutions D, E and F in the ratio 2:1:1. The over layered water from the resolving gel was decanted off and the stacking gel was poured on the resolving gel followed by immediate insertion of the comb. Again the gel was over layered with water. The gel was kept for 30 min in strong sunlight. After polymerization of the stacking gel, the comb was removed. The gel was then finally mounted into the electrophoresis apparatus. Chilled tris-glycine running buffer (pH 8.3) was added sufficiently in both upper and lower reservoirs. Any bubbles, trapped at the bottom of the gel, were carefully removed with a bent syringe.

Sample preparation: During sample preparation, ice-cold conditions were maintained throughout the entire process. The samples were prepared by mixing the enzyme extracts (40 µl) and gel loading dye (25 µl). The gel loading dye consisted of 40% sucrose and 1% bromophenol blue in double distilled water. The samples (30 µl) were immediately loaded in a pre-determined order in to the wells with a microlitre syringe.

Electrophoresis: The gel was incubated at 4^0C supplying a constant current of 2.5 m Amp per well continuously for 3-4 hours until the dye front reached the bottom of the gel.

3.18. Analysis of isozyme pattern

3.18.1. Peroxidase: Peroxidase activity was determined following peroxidase specific staining method as described by Patel and Anahosur (2001). After electrophoresis, gels were incubated for 30 min in 0.25% guiaicol solution. After incubation gels were transferred to 0.3% hydrogen peroxide solution and kept for 15 min. Radish brown bands appeared gradually on the gel.

3.18.2. Polyphenol oxidase: Polyphenol oxidase specific staining method as described by Mahadevan and Sridhar (1996) was followed to determine the enzyme activity on PAGE gel. After electrophoresis, the gels were incubated in 0.1M Phosphate-citrate buffer (pH 7.0) for 10 min. Then the gels were transferred in 0.01M DL-3,4-Dihydroxyphenylalanine (DOPA) in the same buffer and incubated under aeration until the bands developed.

3.18.3. β-1,3-glucanase: β-1,3-glucanase specific staining procedure described by Bargabus *et al.* (2002) was followed to detect the enzyme activity on PAGE gel. Following electrophoresis, gels were washed thoroughly with distilled water. After washing, gels were incubated in 0.1M citrate buffer (pH 4.8) containing 0.25% Laminarin at room temperature for 20 minutes. Then the gels were transferred to 0.1% Congo red solution and incubated overnight with constant shaking at room temperature. After proper staining, the gels were destained with 1M NaCl solution. β 1,3 glucanase activity was observed by the formation of yellow orange bands on a reddish purple background.

3.18.4. Chitinase:

Method 1: Chitinase activity was detected on the PAGE gel following the method of Trudel and Asselin (1989) with some modifications. After electrophoresis, gels were incubated in 150 mM sodium acetate buffer (pH 5.0) for 5 min and then in 100 mM sodium acetate buffer (pH 5.0), containing 0.01% glycol chitin for 30 min at 37^0C. The gels were finally transferred into 500 mM tris-HCl (pH 8.9) solution containing 0.01% (w/v) fluorescent brightener 28 (Sigma) and incubated for 5 min. After 5 min the brightener solution was removed and gels were rinsed with distilled water for 1 hour. Finally, lytic zones were observed under UV transilluminator. Results were recorded photographically and R_f of different bands were determined.

Method 2: Alternatively, 'Agar plate polyacrylamide gel assay' was also done to visualize the chitinase bands more prominently as suggested by Gohel *et al.* (2005). Chitin agar plates were prepared by 1% agar, 0.5% glycol chitin and 0.001% (w/v) Fluorescent brightener 28. After electrophoresis of the native PAGE gel slab was directly transferred onto a chitin agar plate. Thin layer of 0.2 M acetate buffer (pH 5.0) was put on each gel for diffusion of chitinase into the chitin agar plate. The plate was then incubated at 37^0C. Activity bands were visible by the formation of dark bands/zones against fluorescent background on chitin agar plate with Fluorescent brightener 28 after7 hours. The plate was observed under UV-transilluminator.

3.19. Evaluation of biocontrol agents

3.19.1. Dual culture test

Microbial antagonists were screened following the dual culture method of Johnson and Curl (1972) for their biocontrol potential. In this technique, both the

test pathogen and biocontrol agents were grown simultaneously in the same plate. Discs (4 mm) cut from 7 days old cultures of the pathogen and of a biocontrol agent were placed aseptically into the peripheral region of a sterile PDA Petri plate (90 mm) in a straight line but opposite to each other and incubated at 28±1 ^{0}C. Radial growth of the pathogen and the biocontrol agent were measured after the desired incubation period and percent inhibition (in comparison to control where no biocontrol agent was placed) was determined for each experiment.

3.19.2. Evaluation of crude culture filtrates of the antagonists

Culture filtrate of fungal antagonists were collected from 15 days old cultures grown in PDB in 100 ml Erlenmeyer flasks. Culture fluid was filtered through cheese cloth and finally centrifuged at 3,000 g and the supernatant was subjected to filter sterilization. One ml of filter sterilized culture supernatant was mixed with 9 ml of sterile molten PDA in a Petridish and allowed to solidify. One mycelial disc of 4 mm in diameter was cut from the advancing zone of a culture (maintained in petridishes) was placed on solidified PDA medium at the centre. In control set 1 ml of sterile distilled water and 9 ml of molten PDA media was poured. The control plates were also inoculated with mycelial discs as mentioned in case of culture fluid supplemented petridishes. All the petridishes were incubated at 28±1^{0}C.

3.19.3. *In vivo* evaluation on whole plants

Selected niger plants in pots (30 cm diameter) were sprayed with the crude culture filtrate supplemented with 0.05% tween 20 and kept in a transparent polyhouse under normal light and temperature conditions. Crude culture filtrates of the biocontrol agents were used for studying their potential to control the disease *in vivo*. After 24 h of spraying, plants were inoculated with the pathogen *A. alternata* following the method of whole plant inoculation technique as described by Dhingra and Sinclair (1995). In control sets, plants were inoculated with the pathogen after spraying with sterile distilled water. Mean disease index/plant was calculated following the method of Sinha and Das (1972) after 2, 4, 6, and 8 days of inoculation.

3.20. Evaluation of botanicals

3.20.1. Extraction

Extracts of the plant parts were done following the method of Mahadevan and Sridhar (1982) with some modifications. Fresh plant materials were collected and washed thoroughly with sterile distilled water and dried at room temperature. Plant materials were weighed, ground and extracted separately with sterile distilled water and 50% ethanol (0.5 g/ml) after drying. The extracts were filtered through double-layered muslin cloth and centrifuged at 5,000 g for 15 minutes. The supernatants of the extracts were sterilized by passing through a Millipore filter (0.2 µm). All extracts were stored at 4^0C. The extracts were screened for their antifungal activity through bioassays.

3.20.2. Spore germination bioassay

Inhibitory effects of botanicals against *A. alternata* were tested. The spores of the pathogen *A. alternata* were allowed to germinate in sterile distilled water drops mounted on sterile grease free slides kept in an humid chamber in case of control. In experimental sets plant extract (30 µl) was placed on the centre of a grease free microscopic slide and allowed to evaporate. After evaporation of the solvent (ethanol), spore suspension was mounted on the slides in the same place where the extract was applied. In solvent control set fresh solvent (50% ethanol) was placed and subsequently evaporated before application of spore suspension. The slides were then incubated at 28±1^{0}C in a humid chamber. Two small glass rods (60 mm in length) were placed in a 90 mm petridish and a slide was placed on the rods in a uniformly balanced position. Sterile distilled water was carefully poured in the petridish so that the bottom of the slide remained just above the water surface. The petridish was then covered and incubated at 28±1^{0}C. Following 12 h of incubation, the slides were stained with lacto phenol-cotton blue and observed under the microscope. Approximately, 200 spores were observed in each slide for germination. The entire experiment was repeated thrice.

3.20.3. Bioassay by poisoned food technique

One millilitre of plant extract was added to 9 ml of the molten PDA medium, mixed well and poured in sterile petridish (70 mm diameter) under aseptic condition and was allowed to solidify. In control sets 1 ml of sterile

distilled ware was added instead of plant extracts. The both experimental and control plates were inoculated with the pathogen and incubated for required period. Radial growth of the pathogen was measured.

3.20.4. *In vivo* bioassay on whole plants

To estimate the antifungal activity of different plant materials, fresh aquous extract (2 g in 10 ml distilled water) was prepared. Niger plants grown in pots (30 cm diameter) were sprayed with the crude extract and were kept for 24 hours in a transparent polyhouse under normal conditions of light and temperature. The plants were inoculated with the pathogen *A. alternata* following the method of whole plant inoculation technique as described by (Section 3.5). Control plants were sprayed with sterile distilled water and maintained under similar conditions of light and temperature. One set of control plants were also sprayed with the pathogen as mentioned. Mean disease index / plant were calculated following Sinha and Das (1972) after 2, 4, 6, and 8 days of inoculation.

3.21. Statistical analysis

Some of the statistical analysis was done using Smith's statistical package (version 2.5). The package was developed by Dr. Gray Smith, Pomona College, Claremont-91711, USA. In some other cases statistical Package for the Social Sciences (SPSS), version 11.0, SPSS Inc., Chicago, Illinois were also used.