Chapter 3
Materials and Methods

3.1. Plant material

3.1.1. Collection of seeds

Sorghum seeds were collected from Indian Institute of Millets Research, Hyderabad, India and local market of siliguri, West Bengal. Seeds of CSV 30F, Sorghum sudan grass, CSV 15, CSV 23, CSV 17, CSV 22 R, SPV 462, CSV 20, CSV 27 and CSV 29 F were selected for initial morphological screening and comparative analysis of spot blotch disease (Table.3.1, Fig.3.1).

3.1.2. Viability test

To check the viability of the sorghum seeds they were initially sterilized with 0.1% (w/v) $HgCl_2$ for 3-4 minutes and then washed with sterile distilled water and transferred to sterile Petri plates under aseptic conditions. The seeds were allowed to germinate for one week and percentage of germination was observed for each sorghum

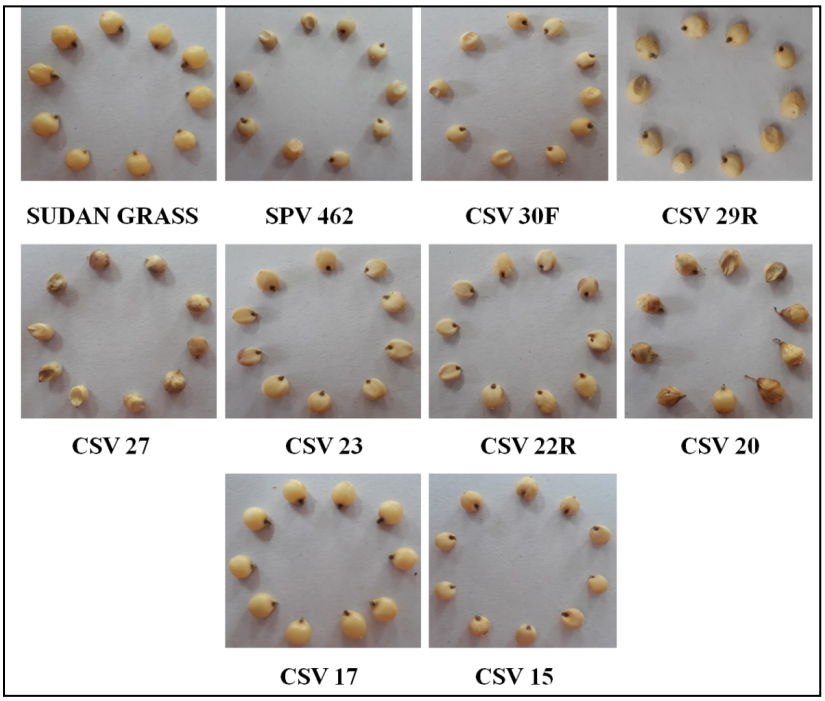

Figure 3.1: Ten different sorghum varieties showing morphology

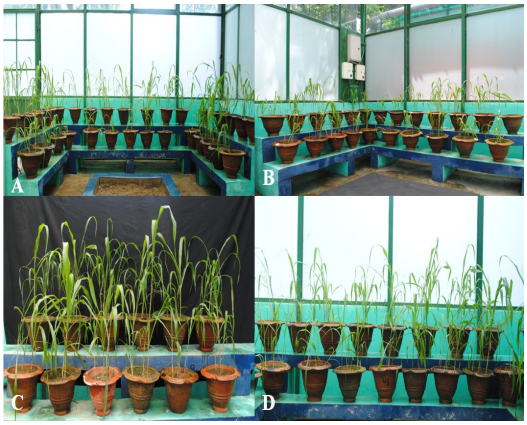

Figure 3.2. Maintenance of Sorghum bicolor varieties in glass house condition

variety. The seeds were stored in air tight bags at -4°C for further use.

Germination percentage was found to be highly variable in all the ten sorghum varieties. CSV 20 variety showed highest percentage of germination (89%) while CSV 22 R showed lowest germination percentage (35%). Similarly the variety Sudan grass showed 53%, SPV 462: 62.3%, CSV 15: 82.3%, CSV 17: 85.3%, CSV 23: 43%, CSV 27:47.6%, CSV 29 R:80% and CSV 30 F:67%. The varieties which showed high germination percentage can be useful for plant breeding programs.

3.1.3. Growth rate

Growth rate in terms of height of all the ten studied sorghum varieties was observed after every 15, 45, 75 and 105 days keeping a gap of 30 days time interval (Table. ST 4.1). Maximum height was observed in the variety SPV 462 (81cm) followed by CSV 29 R (78 cm), CSV 23 (76.5 cm), CSV 27 (74 cm), CSV 22 R (73 cm), CSV 15 (70 cm), CSV 30 F (68 cm), CSV 17 (66 cm), CSV 20 (60 cm) and Sudan grass showed the minimum growth of 58 cm. it is evident that the growth of local variety is quite slow in comparison with other agriculturally approved varieties.

3.1.4. Maintenance of sorghum seedlings in glass house

Ten different sorghum varieties were given for germination and maintained in glass house of Immuno-phytopathology Laboratory, Department of Botany, university of North Bengal prior to its transfer to the experimental field. They were initially given for germination in earthen pots (12" dia) containing soil and farmyard manure in the proportion of 2:1 by

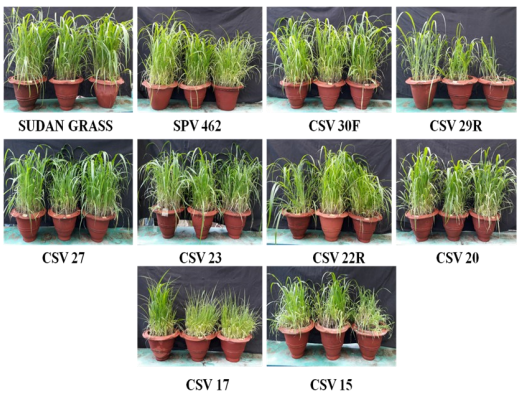

Figure 3.3: Different varieties of *Sorghum bicolor* in pot condition

Figure 3.4: Maintenance of Sorghum bicolor in field condition

weight (Fig. 3.2 & 3.3).

3.1.5. Maintenance in field condition

One month old seedling of different sorghum varieties were also sown in the experimental field of Department of Botany, University of North Bengal where all suitable management practices were taken into consideration for proper growth of the plants. Three different varieties (CSV 30 F, Sorghum sudan grass, CSV 15) that showed more susceptibility towards the spot blotch pathogen under glass house and field condition were transferred to experimental field of Immuno-Phytopathology Laboratory, NBU for close monitoring and further experimentation (Fig. 3.4). The plants were grown during last week of June to first week of July. Plants were watered regularly twice in a day and weeding was done once a week.

3.2. Fungal culture

3.2.1. Isolation and maintenance

The fungal pathogens which they are present deep seated tissue of infected leaves were isolated by culturing pieces of internal tissues. Infected tissues were thoroughly washed in sterile water and swabbed with cotton wool dipped into 80% ethanol, followed by exposure to an alcohol flame for a few seconds. The outer layer of tissues was quickly removed by a flame sterilized scalpel. Small pieces from the central core of tissue in the area of the advancing margin of infection were removed by a sterilized

Table 3.1: Different sorghum variety and area of collection

Sl.#	Sorghum varieties	Area of collection	Kernel colour
1.	SUDAN GRASS	Local market, Siliguri	Golden yellow
2.	SPV462	Millets Research, Hyderabad	Golden yellow
3.	CSV 15	Millets Research, Hyderabad	Golden yellow
4.	CSV 17	Millets Research, Hyderabad	Golden yellow
5.	CSV 20	Millets Research, Hyderabad	Brown
6.	CSV 22 R	Millets Research, Hyderabad	Off white
7.	CSV 23	Millets Research, Hyderabad	Off white
8.	CSV 27	Millets Research, Hyderabad	Brown
9.	CSV 29 R	Millets Research, Hyderabad	Golden yellow
10.	CSV 30 F	Millets Research, Hyderabad	Off white

scalpel and sterilized by dipping into 90% alcohol then flamed for a few seconds. The sterilized tissues were transferred to potato dextrose agar in Petri dishes and incubated at 28°C for 1 week. The grown fungal mycelium was transferred to PDA slants and kept for further identification.

3.2.2. Morphological and microscopic observation

The isolated fungi were then allowed to grow in Petri plates (7 cm.) containing sterile PDA medium for 7 days, then nature of mycelia growth, rate of growth and time of sporulation were observed. For identification, spore suspension was prepared from individual culture. Drops of spore suspensions were placed on clean, grease free glass slides, mounted with lacto phenol-cotton blue, covered with cover slip and sealed with wax. The slides were then observed under the microscope, following which spore characteristics were determined and size of spore measured.

3.2.3. Completion of Koch's Postulate

Fresh young sorghum leaves were collected from experimental field of Immuno-Phytopathology lab and inoculated with conidial suspension of the isolated fungal pathogen following detached leaf inoculation technique. After 96h of inoculation, the infected sorghum leaves were washed thoroughly, cut into small pieces, disinfected with 0.1% $HgCl_2$ solution for 3-5 min washed several times with sterile distilled water and transferred aseptically into Potato Dextrose Agar (PDA) slants. These isolates were examined 15 days of inoculation at 30°C and identity of the organism was confirmed by comparing with the stock culture.

3.2.4. Assessment of mycelia growth

Mycelia growth of the fungal cultures was assessed in both solid media and liquid media to study and evaluate their cultural characteristics.

3.2.4.1. Solid media

To assess the growth of fungal culture in solid media, the fungus was first grown on Petri dishes, each containing 20 ml. of PDA followed by incubation for 7 days at 30°C. Agar blocks (6 mm. diameter) containing the mycelium was cut with sterile cork borer from the actively growing region of mycelia mat and transferred to each Petri dish containing 20 ml. of different sterilized solid media. The colony diameter was studied at regular interval of time. The media were as follows:

A.Potato Dextrose Agar

Peeled potato-40.00 gm, dextrose-2.00 gm, Distilled water-100ml.

B. Richard's Medium (RMA)

KNO_3-1.00gm, KH_2PO_4-50gm, $MgSO_4$, $7H_2O$-0.25gm, $FeCl_3$-0.002gm, Sucrose-3.00gm,

Agar-2.00gm, Distilled water-100ml.

C. Oat Meal Agar (OMA)

Oat meal-3.00 gm, Agar-2.00gm, Distilled water-100ml

3.2.4.2. Liquid Media

To assess the mycelia growth in liquid media the fungus was first grown on Petri plates, each containing 20 ml. of PDA and incubated 5-8 days at 20^{O}C. the mycelia block (5mm.) from the actively growing region of the fungus in the Petri plate was cut with sterilized cork borer and transferred to Erlenmeyer flask (250 ml.) containing 50 ml. of sterilized Potato Dextrose Broth (PDB) and Richard's medium and incubated for 6-8 days with constant stirring at room temperature. After incubation the mycelia were harvested through muslin cloth, collected in aluminium foil cup of known weight and dried at 60^{O}C for 96 h, cooled in desiccators and weighed.

3.3. Soluble proteins

3.3.1. Extraction of soluble protein

3.3.1.1. Fungal mycelia

Mycelia protein was prepared following the protocol as outlined by Chakraborty and Saha (1994). The fungal mycelia were grown in 250 ml. Erlenmeyer flask containing 50 ml. of potato dextrose broth (PDB) and incubated for 10 days at 30±2^{O}C for extraction of antigen, mycelial mats were harvested, washed with 0.2% NaCl and rewashed with sterile distilled water. Mycelia were crushed with sea sand using a chilled morter and pestle and homogenized with cold 0.05 m. sodium phosphate buffer ($_{P}$H-7.2) supplemented with 0.8% NaCl, 10 mM sodium metabisulphate, PVPP (Polyvinyl pyrollidine Phosphate) and 0.5 mM magnesium chloride in ice bath. The homogenated mixture was kept for 2 h or overnight at 4^{O}C and then centrifuged at 10,000 rpm for 30 min, at 4^{O}C to eliminate cell debris. The supernatant was equilibrated to 100% saturated ammonium sulphate under constant stirring in ice bath and kept overnight at 4^{O}C. after this period, the mixture was centrifuged (10,000 rpm) for 30 minute at 4^{O}C, the precipitate was dissolved in the same

buffer. The precipitation was dialysed for 72 h through cellulose tubing (Sigma chemical co., USA) against 1L of 0.05 M sodium phosphate buffer (pH 7.2) with six changes. The dislysate was stored at -20^{O}C and used as antigen from the preparation of antiserum and other experiment.

3.3.1.2. Leaf

Soluble protein was extracted from sorghum leaves following the method of Chakraborty *et al.,* (1995). Leaf tissues were frozen in liquid nitrogen and ground in 0.05 mM sodium phosphate buffer (pH 7.2) containing 10 mM $Na_2S_2O_2$, 0.5 mM $MgCl_2$ and 2 mM PVP was added during crushing and centrifuged at 4^{O}C for 20 min at 12000 rpm. The supernatant was used as crude protein extract.

3.3.2. Estimation of soluble protein

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 (1 ml of 1% $CuSO_4$ and 1 ml. of 2% sodium potassium tartarate, added to 100 ml. of 2% Na_2CO_3 in 0.1 N NaOH) was added. This was incubated for 15 min at room temperature and then 0.5 ml. of 1 N Folin Ciocalteau reagent was added and again incubated for further 15 min following which optical density was measured at 720 nm. Quantity of protein was estimated from the standard curve made with bovine serum albumin (BSA).

3.4. SDS-PAGE analysis of soluble proteins

Sodium dodecyl sulphate polyacrylamide gel electrophoresis was performed for detailed analysis of protein profile following the method of Laemmli, 1970. For the preparation of gel the following stock solution were prepared.

3.4.1. Preparation of stock solution

Following stock solution were prepared

A.Acrylamide and N'N'-methylene bis acrylamide

Stock solution of 29% acrylamide and 1% bis-acrylamide was prepared in warm water. The pH of the solution was kept below 7.0 and the stock solution was then filtered through Whatman No. 1 filter paper and kept in brown bottle, stored at 4^{O}C.

B. Sodium Dodecyl Su;phate (SDS)

A 10% stock solution of SDS was prepared in warm water stored at room temperature.

C. Tris Buffer

i) 1.5 M Tris buffer was prepared for resolving gel. The pH of the buffer was adjusted to 8.8 with concentrated HCl and stored at $4^{O}C$ for further use.

ii) 1.0 M Tris buffer was prepared for use in the stacking and loading buffer. The pH of this buffer was adjusted to 6.8 with conc. HCl and stored at $4^{O}C$ for use.

D. Ammonium Persulphate (APS)

Fresh 10% APS solution was prepared with distilled water each time before use.

E. Tris-Glycine electrophoresis buffer

Tris running buffer consists of 25 mM Tris base, 250 mM Glycine (pH 8.3) and 0.1% SDS. A1X solution was made by dissolving 3.02 g Tris base, 18.8 Glycine and 10 ml. 0f 10% SDS in 1L distilled water

F. SDS gel loading buffer

This buffer contains 50 Mm Tris-HCL (pH 6.8), 10 mM β-mercaptoethanol, 2% SDS, 0.1% bromophenol blue, 10% glycerol. A 1x solution was prepared by dissolving 0.5 ml of 1 M Tris buffer (pH 6.8), 0.5 ml 0f 14.4 M β-mercaptoethanol, 2 ml of 10% SDS, 10 mg bromophenol blue, 1 ml glycerol in 6.8 ml of distilled water.

3.4.2 Preparation of gel

Mini slab gel (plate size 8cm x 10cm) was prepared for analysis of proteins patterns through SDS-PAGE. For gel preparation, two glass plates (8cm x 10cm) were washed with dehydrated alcohol and dried to remove any traces of grease. Then 1.5 mm thick spacers were placed between the glass plates at the two edges and the three sides of the glass plates were sealed with gel sealing tape or wax, clipped tightly to prevent any leakage and kept in the gel casting unit. Resolving and stacking gels were prepared by mixing compounds in the following order and poured by pipette leaving sufficient space for comb in the stacking gel (comb + 1cm). After pouring the resolving gel solution it was immediately over layered with isobutanol and kept for polymerization for 1h. After polymerization of the resolving gel was complete overlay was poured off and washed with water to remove any unpolymerized acrylamide. Stacking gel solution was poured over the resolving gel and the comb was inserted immediately and over layered with water. Finally the gel

was kept for polymerization for 30-45 minutes. After polymerization of the stacking gel the comb was removed and the walls were washed thoroughly. The gel was then finally mounted in the electrophoresis apparatus. Tris-Glycine buffer was added sufficiently in both upper and lower reservoir. Any bubble trapped at the bottom of the gel, was removed carefully with a bent syringe.

3.4.3. Sample preparation

Sample (50 µl) was prepared by mixing the protein extract (35 µl) with 1xSDS gel loading buffer (16 µl) in cyclomixer. All the samples were floated in a boiling water bath for 30 minutes to denature the proteins samples. After boiling, the sample was loaded in a predetermined order into the bottom of the well with T-100 micropipette syringe. Along with the protein samples, a marker protein consisting of a mixture of six proteins ranging from high to low molecular mass (Phosphorylase b-97, 4000; Bovine Serum Albumin-68,000; ovalbumin-43,000: Carbolic Anhydrase-29,000; Soybean Trypsin inhibitor-20,000; Lysozyme-14,300) was treated as the other samples and loaded in separate well.

3.4.4. Electrophoresis

Electrophoresis was performed at 18 mA current for a period of two to three hours or until the dye reached the bottom of the gel.

3.4.5. Fixing and staining

After completion of electrophoresis, the stacking gel was cut off from the resolving gel and finally fixed in GAA: methanol: water (10:20:70) for overnight. The staining solution was prepared by dissolving 250 mg of Coomassie brilliant blue (Sigma R 250) in 45 ml of methanol. When the stain was completely dissolved, 45 ml of water and 10 ml of glacial acetic acid were added. The stain was filtered through Whatman No.1 filter paper. The gel was removed from the fixer and stained in this stain solution for 4 h at 37^{O}C with constant shaking at low speed. After staining the gel; was finally destained with destaining solution containing methanol, water and acetic acid (4:5:1) at 40^{O}C with constant shaking until the background become clear.

3.5. Preparation of antigen

3.5.1. Fungal antigen

Mycelia protein was prepared following the method as outlined by

(Chakraborty and Saha 1994). Mycelial mats were harvested from 7-10 days old culture and washed with 0.2% NaCl then again rewashed with sterile distilled water. Washed mycelia were crushed with sea sand using a chilled mortar and pestle and homogenized with cold 0.05 M sodium phosphate buffer (pH 7.2) supplemented with 0.85% NaCl, 10 mM sodium metabisulphate and 0.5 mM $MgCl_2$ in ice bath. The homogenate mixture was kept for 2h or overnight at 4^OC and then centrifuged at 10,000 rpm for 30 minutes at 4^OC to eliminate cell debris. The supernatant was stored in 020OC and used for as antigen for the preparation of antiserum.

3.5.2. Leaf antigen

3.5.2.1. Healthy leaf

Antigens from healthy leaves were prepared following the method of Chakraborty and Saha (1994). Fresh, young healthy leaves were collected from the experimental garden and kept at 4^OC. then the leaves were weighed and crushed in mortar and pestle with 0.05 M sodium phosphate buffer supplemented with 10 mM sodium metabisulphate, 2mM PVPP 10,000 (soluble) and 0.5 mM $MgCl_2$ (pH 7.2). At the time of crushing with sea-sand, soluble PVPP of equal weight was used. The leaf slurry was strained through a muslin cloth and then centrifuged (15,000 rpm) for 30 minutes at 4^OC. the supernatant was used as healthy antigen and was kept at -20^OC until required.

3.5.2.2. Artificially inoculated leaf

Antigen from *B. sorokiniana* inoculated leaves were extracted following the method Alba & DeVay (1985) with modification. Fresh, young leaves were collected from experimental garden and kept in plastic trays as described in detached leaf inoculation technique. Leaves were inoculated with conidial suspension of both the pathogens separately. Control sets were prepared by mounting the leaves with drops of sterile distilled water. Antigens were prepared from inoculated leaves as well as control leaves as described earlier. The prepared antigens were stored at -20^OC until further experimental purpose.

3.5.2.3. Naturally infected leaf

For the extraction of naturally infected leaf antigens, the infected leaves were collected from the experimental garden and kept at 4^OC. Then the infected portion of leaf was cut into small

pieces, weighed and antigens were prepared as before.

3.6. Serology

3.6.1. Rabbits and their maintenance

Polyclonal antibodies were prepared against fungal and bacterial antigens in New Zealand white male rabbits of approximately 2kg of body weight. Before immunization, the body weights of rabbits were recorded and observed for at least one week inside the cages. Rabbits were maintained in Antisera reserves for plant pathogens, Immuno-Phytopathology Laboratory, Department of Botany, NBU. They were regularly fed with green grass, soaked gram, green vegetables and carrots etc. twice a day. After each bleeding they were given saline for three consecutive days and kept in proper hygienic conditions.

3.6.2. Immunization

Before immunization, normal sera were collected from each rabbits. For developing antisera, intramuscular injections of 1ml antigen (protein extracted) mixed with 1ml of Freund's complete adjuvant (Genei) were given into each rabbit for 7 days after pre-immunization bleeding and repeating the doses at 7 days intervals for consecutive week followed by Freund's incomplete adjuvant (Genei) at 7 days intervals upto 12-14 consecutive weeks as required. Method of Alba and Devay, 1985 and Chakraborty and Saha, 1994 were followed for immunization.

3.6.3. Bleeding

Bleeding was performed by marginal ear vein puncture, three days after the first six injections, and then every fourth injection. In order to handle rabbits during bleeding, they were placed on their back on a wooden bored fixed at an angle of 60^{O}, and held the rabbits tight so that it could not move during the bleeding. The hairs from the upper side of the ear was removed with the help of a razor and disinfected with alcohol. The ear vein was irritated by the application of xylene and an incision was made with the help of a sharp sterile blade and 5-10 ml of blood samples were collected in sterile graduated glass tube. The blood samples were incubated at $37^{O}C$ for 1h for clotting. After clotting; the clot was loosened with a sterile needle. Finally, the serum was classified by centrifugation (2000 rpm for 10 minutes at room temperature) and distributed in 1 ml of vials and stored at $-20^{O}C$ as crude antisera. The serum was used for double diffusion analysis,

dot blot analysis and Enzyme linked Immunosorbent Assay (ELISA), Western blot analysis and FITC.

3.6.4. Purification of IgG

3.6.4.1. Precipitation

IgG was purified as described by Clusen, 1988. Crude antiserum (2 ml) was diluted with two volume of distilles water and an equal volume of 4M (NH_4SO_4) ammonium sulphate was taken and pH was adjusted to 6.8, stirring the mixture for 16 h at 20^{O}C in magnetic stirrer. The precipitation thus formed was collected by centrifugation at 12,000 rpm for 1h at 22^{O}C. supernatant was discarded and pellet was used for further steps.

3.6.4.2. Column preparation

Eight gram of DEAE cellulose (Sigma Co. USA) was suspended in distilled water for overnight. The water was poured off and the DEAE cellulose was suspended in 0.005 M phosphate buffer (pH 8.0), and the washing was repeated for 5 times. The gel was then suspended in 0.02 M phosphate buffer (pH 8.0) and was transferred to a column (2.6 cm in diameter and 30 cm height) and allowed to settle for 2h. after the column material had settled 25 ml of buffer (0.02 M sodium phosphate, pH 8.0) washing was given to the column material.

3.6.4.3. 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 0.03 M. The initial elution buffer (1) was 0.02 sodium phosphate (pH 8.0). The buffer was applied in the flask on which rubber connection from its bottom was supplying column. Another connection above the surface of buffer (1) was connected to another flask with buffer (2). The buffer (2) had also connection to the open air. During the draining of buffer (1) to column buffer (2) was soaked into buffer (1) thereby producing a continuous raise in molarity. Ultimately, 40 fractions each of 5 ml were collected and the optical density values were recorded at 280 nm using UV-VIS spectrophotometer (DIGISPEC-200GL).

3.7. Immunological assays

3.7.1. Agar gel double diffusion

3.7.1.1. Preparation of agarose slides

The glass slides (6cm x 6cm) were degreased using ethanol 90% v/v: diethyl ether (1:1 v/v) and ether, then

dried in hot air oven. After drying the plates were sterilized inside the Petri plate each containing one plate. Conical flask with Tris-Barbiturate buffer (pH 8.6) is placed in boiling water bath. Agar/agarose (0.9%) was boiled over water bath to dissolve the agar at 90^{O}C for next 15 minutes. The pinch of 0.1% (w/v) sodium azide was added and mixed well. For the preparation of agarose gel, the molten agarose is poured (6 to 10 ml) on the grease free sterilized slide with the help of a sterile pipette in laminar air flow chamber and allow it to solidify, after solidification cut 3-7 wells (6mm diameter) with sterilized cork borer distance of 1.5 to 2 cm away from central well and 2.0 to 2.5 cm from well to well.

3.7.1.2. Diffusion

Agar gel double diffusion tests were carried out using antigen and antiserum following the method of Ouchterlony (1967). Antigen plus undiluted antisera appropriately diluted were poured into wells with micropipette (50 µl/well) antisera in the middle. Slides were kept in moist chamber at 25^{O}C for 72h. Precipitation reaction was observed in the agar gel only in cases where common antigen was present.

3.7.1.3. 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 1% NaN_3) for 72h with 6 hourly changes to remove unreacted antigens and antisera widely dispersed in the agarose gel. Then the slides were stained with Coomassie brilliant blue (R250, Sigma: 0.25g Coomassie blue, 45 ml methanol, 45 ml distilled water and 10 ml glacial acetic acid) for 10 minutes at room temperature. After staining, the slides were washed in destaining solution (methanol: distilled water: acetic acid in 45:45:10 ratios) with changes until background become clear. Finally slides were washed with distilled water and dried in hot air oven for 3h at 50^{O}C.

3.7.2. Plate trapped antigen coated (PTA)-ELISA

Plate trapped antigen coated (PTA)-ELISA performed following the method of Chakraborty *et al.*(1995) with modifications. Antigen were diluted with coating buffer and were loaded (200 µl per well) in ELISA plate (Coaster EIA/RIA, strip plate, USA) arranged in 12 rows in a

(Cassette) ELISA plate. After loading, the plate was incubated at 25^{O}C for 4h. Then the plate was washed four times under running tap water and twice with PBS-Tween and each time shaken well to dry. Subsequently, 200 µl blocking reagent was added to each well for blocking the unbound sites and plate was incubated at 25^{O}C for 1h after incubation, the plate was washed as mentioned earlier. Purified polyspecific IgG was diluted in antisera dilution buffer and loaded (200 µl per well) and incubated at 4^{O}C overnight. After a further washing, antirabbit IgG goat antiserum labeled with alkaline phosphate diluted 10000 times in PBS, was added to each well (100 µl per well) and incubated at 37^{O}C for 2h. The plate was loaded with 200 µl of ρ-nittrophenyl phosphate substrate in each well and kept in dark for 1h. Colour development was stopped by adding 50 µg per well of 3 N NaOH solution and the absorbance was determined in an Multiscan Ex (Thermo Electron) ELISA Reader (Multiskan, Thermo Labsystems) at 450 nm.

3.7.3. Dot immunobinding assay

Dot blot was performed following the method suggested by Lange *et al.* (1989) with modifications. Following buffers were used for dot immunobinding assay.

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

B. Tris buffer saline (10 mM pH 7.4) with 0.9% NaCl and 0.5% Tween 20 for washing.

C. Blocking solutiuons 10% (w/v) skimmed milk powder (casein hydrolysate, SRL) in TBST (0.05 M Tris-HCL, 0.5 M NaCl) 5% v/v Tween 20, pH 10.3.

D. Alkaline phosphatase buffer (100 mM Tris-HCL, 100 mM NaCl, 5 mM $MgCl_2$)

Nitrocellulose membrane (Milipore, 7cm x 10cm, Lot No. H5SMO 5255, pore size 0.45 µm. Milipore corporation, Bedford) was first cut carefully into the required size and fix between the template with filter paper at the bottom. 0.5 M carbonate-bicarbonate buffer (pH 9.6), 4µl, was loaded in each well and allowed to dry for 30 minutes at room temperature. Antigen (5 µl) was loaded on NCM allowed to dry for 30 minutes at room temperature. Template was removed and blocking of NCM was done with 19% non fat dry milk (casein hydrolysate, SRL) prepared in TBST for 30-60 minutes on a shaker.

Respective polyclonal antibody (IgG 1:500) prepared against the antigen was added directly in the blocking solution and further incubated at $4^{O}C$ for overnight. The membrane was then washed gently in running tap water for three min, thrice followed by washing in TBST (pH 7.4), (Wakeham and White, 1996). The membrane was then incubated in alkaline phosphatase conjugated goat antirabbit IgG (diluted 1:10,000 in alkaline phosphatase) for 2h at $37^{O}C$. the membrane was washed as before. 10 ml of NBT/BCIP susbstrate (Genei) was added next and colour development was stopped by washing the NCM with distilled water and colour development was categorized with the intensity of dots.

3.7.4. Western blot analysis

Protein samples were electrophoresed on 10% SDS-PAGE gels suggested by Laemmli (1970) and electro transferred to NCM using semi dry Trans-blot unit (BioRad) and probed with PAbs of the pathogen (*B. sorokiniana*) following the method of Wakeham and White (1996). Hybridization was done using alkaline phosphatase conjugate and 5-bromo-4-chloro-3-indolylphosphate (NBT-BCIP) as substrate. Immunireactivity of the proteins was visualized as violet coloured bands on the NCM.

3.7.5. Fluorescence antibody staining and microscopy

Indirect fluorescence staining of fungal mycelia, cross-section of sorghum leaves were done using FITC labeled goat antirabbit IgG following the method of Chakraborty and Saha (1994)

3.7.5.1. Fungal mycelia

Fungal mycelia were grown in liquid potato dextrose medium as described earlier. After five days of inoculation young mycelia were taken out from flask and taken in eppendorf tube and washed with PBS (pH 7.2) by centrifugation at slow speed. Then mycelia was treated with normal sera or antisera diluted (1:50) in PBS and incubated for 1h at room temperature. The mycelia was washed thrice with PBS-Tween (pH 7.2) as mentioned above and treated with Goat antirabbit IgG conjugated with fluorescein isothiocyanate (FITC) (Sigma chemicals) diluted 1:40 with PBS (7.2) and incubated in dark for 45 minutes at room temperature. After incubation mycelia was washed thrice in PBS and mounted in 10% glycerol. A cover slip was placed and sealed. The slides were

observed and photographed under both phase contrast and UV fluorescence condition using Leica Leitz Biomed microscopy with fluorescence optics equipped in (UV) filter set 1-3.

3.7.5.2. Cross section of sorghum leaves

Initially, cross section of healthy sorghum leaves were cut and immersed in PBS (pH 7.2). These section were treated with normal serum or antiserum diluted (1:50) in PBS and incubated for 1h at room temperature. After incubation, cross sections were washed thrice with PBS-Tween (pH 7.2) for 15 minutes and transferred to 40 µl of diluted (1:40) goat antirabbit IgG conjugated with fluorescein isothiocyanate (FITC). The sections were incubated for 45 minutes in dark. After that sections were washed thrice with PBS-Tween as mentioned above and then mounted on a grease free slide with 10% glycerol. Fluorescence of the leaf sections were observed using Leica Leitz Biomed Microscope with Fluorescence optics equipped with UV-filter set 1-3 and photograph was taken.

3.8. Isolation of genomic DNA

Isolation of fungal genomic DNA was done by growing the fungi for 3-4 days. Liquid nitrogen was used for crushing the cell mass for both cases.

3.8.1. Preparation of genomic DNA extraction buffer

The following buffer for DNA extraction were prepared by mixing appropriate amount of desired chemicals with distilled water and adjusted the desired pH.

DNA Extraction buffer

1M Tris0HCL pH 8.0

5M NaCl

0.5 mM EDTA, pH 8.0

10% SDS

3.8.2. Genomic DNA extraction

The fungal mycelia was grown in PDB for 6-7 days and then harvested. Total genomic DNA was extracted as described by Kuramae-Izioka (1997). The mycelium was ground into the fine powder under liquid nitrogen and suspended in 700 µl extraction buffer. Upon homogenization, the tubes were incubated for 30 minutes at 65^{O}C. DNA samples were purified with equal volumes of chloroform: isoamyl alcohol (24:1) mixture (1X), and precipitated with isopropanol. The tubes were centrifuged at 15400 rpm for 10 minutes and DNA pellets were rinsed with 70% ethanol, air dried,

suspended in TE buffer (pH 8.0) and stored at 4^{O}C till further use.

3.8.3. Purification of genomic DNA

The extraction of total genomic DNA from the isolated microorganisms as per the above procedure was followed by RNAse treatment. Genomic DNA was resuspended in 100 µl 1 X TE buffer and incubated at 37^{O}C for 30 minutes with RNAse (60 µg). after incubation the sample was re-extracted with PCI (Phenol: Chloroform: Isoamylalcohol 25:24:1) solution and RNA free DNA was precipitated with chilled ethanol as described earlier. The quality and quantity of DNA was analyzed both spectrophotometrically and in 0.8% agarose gel. The DNA from all isolates produced clear sharp bands, indicating good quality of DNA.

3.8.4. Agarose gel electrophoresis to check DNA quality

Gel electrophoresis is an important molecular biology tool. Gel electrophoresis enables us to study DNA. It can be used to determine the sequence of nitrogen bases, the size of an insertion or deletion or the presence of a point mutation: it can also be used to distinguish between variable sized alleles at a single locus and to assess the quality and quantity of DNA present in a sample.

3.8.4.1. Preparation of DNA samples for electrophoresis

Agarose (0.8%) in 1X TBE buffer was melted, cooled and poured into the gel casting tray with ethidium bromide. Gel solidify in 15-20 minutes.

3.8.4.2. Run gel for electrophoresis for DNA fraction

15 µl of sample and 5 µl of DNA loading dye mixed properly was loaded in each well of agarose gel (1%). The electrical head of the gel tank was attached firmly and electric supply was applied at constant current 90 mA and voltage 75 volt (BioRAD power Pac 3000) at least for 90 minutes. The DNA migrated from cathode to anode. Run was continued until the bromophenol blue had migrated an appropriate distance through the gel. Then electric current was turned off and gel was removed from the tank and examined on UV transilluminator and photographed for analysis

3.9. ITS PCR analysis

All isolates of *Bipolaris* were taken up for ITS-PCR amplification. Genomic DNA was amplified by mixing the template DNA (50 ng), with the polymerase reaction buffer, dBTP mix,

primers and Taq polymerase. Polymerase Chain Reaction was performed in a total volume of 100 µl, containing 78 µl deionized water, 10 µl of 10X Taq pol buffer, 1 µl of 1 U Taq polymerase enzyme, 6 µl of 2mM dNTPs, 1.5 µl of 100 mM reverse and forward primers and 1 µl of 50 ng template DNA. PCR was programmed with an initial denaturing at 94^{O}C for 2 minutes. Followed by 30 cycles of denaturation at 94^{O}C for 50 seconds, annealing at 52^{O}C for 40 seconds and extension at 72^{O}C for 1 minute 30 seconds and the final extension at 72^{O}c for 6 minutes in a Primus 96 advanced gradient Thermocycler. PCR product (20 µl) was mixed with loading buffer (8 µl) containing 0.25.5 bromophenol blue, 40% w/v sucrose in water and then loaded in 2% agarose gel with 0.1% ethidium bromide for examination with horizontal electrophoresis.

3.9.1. Amplification conditions

Temperature profile, 94^{O}C for 2minutes followed by 30 cycles of denaturation at 94^{O}C for 50 seconds, annealing at 52^{O}C for 40 seconds and extension at 72^{O}C for 1 minute 40 seconds and the final extension at 72^{O}C for 6 minutes in a Primus 96 advanced gradient Thermocycler.

3.9.2. Sequencing of rDNA gene

The rDNA was used for sequencing purpose. DNA sequencing was done bidirectionally using the ITS primer pairs by Credora Lifesciences, Bangalore. DNA sequence information was analyzed using bioinformatics algorithms tools e.g. Bioedit, MEGA 4, NTSYSpc as well as the few online softwares. The chromatogram of the DNA sequence was analysed by the software Chromus. All the DNA sequences was edited by using software BioEdit and aligned with Clustal W algorithms.

3.10. BLAST analysis

The DNA sequences were analyzed using the alignment software of BLAST algorithm (http:// ingene2.upm.edu.my/Blast,Altschul *et al.,* 1997) for the different characteristics of DNA sequence for the identification of microorganism. Identification of microorganism was done on the basis of homology of sequence.

3.11. Submission to NCBI GenBank

The DNA sequences were deposited to NCBI GenBank through BankIt procedure and approved as the ITS sequence after complete annotation and given accession numbers.

3.12. Multiple sequence alignment and Phylogenetic analysis

The sequenced PCR product was aligned with ex-type strain sequence from NCBI GenBank and established fungal taxonomy for identification. Sequences were aligned following the Clustal W algorithm (Thompson *et al.,* 1994), included in the Megalign module (DNASTAR Inc.). multiple alignment parameters used were gap = 10. Both of these values are aimed to prevent lengthy or excessive numbers of gaps. The default parameters were used for pairwise alignment. The use of Clustal W determines that, once a gap is inserted, it can only be removed by editing. Therefore, final alignment adjustments were done manually. Phylogenetic analyses were completed using the MEGA package (version 4.01). Neither gaps (due to insertion-deletion events) nor equivocal sites were considered phylogenetically informative. Hence, complete deletion prevented the use of any of these sites in further analyses. Phylogenetic interference was performed by the UPGMA method. Bootstrap tests with 1000 replications were conducted to examine the reliability of the interior branches and the validity of these trees obtained. Phylogenetic analyses were conducted in MEGA 4 as described by Tamura *et al.,* 2007.

3.13. RAPD PCR analysis

For RAPD, random primers were selected. PCR was programmed with an initial denaturing at 94^{o}C for 4 min. followed by 35cycles of denaturation at 94^{o}C for 1 min, annealing at 36^{o}C for 1 min and extension at 70^{o}C for 90 s and the final extension at 72oC for 7 min. in a Primus 96 advanced gradient Thermocycler. PCR product (20 µl) was mixed with loading buffer (8 µl) containing 0.25 % bromophenol blue, 40 % w/v sucrose in water, and then loaded in 2% Agarose gel with 0.1% ethidium bromide for examination by horizontal electrophoresis.

3.13.1. Amplification conditions

Temperature profile, 94^{O}C for 4 minutes followed by 35 cycles of denaturation at 94^{O}C for 1 minute, annealing at 36^{O}C for 1 minute and extension at 70^{O}C for 90 second and the final extension at 72^{O}C for 7 minutes in a Primus advanced gradient Thermocycler.

3.13.2. Analysis of RAPD band patterns

RAPD band patterns were initially assessed by eye and isolates were

grouped according to their shared band patterns.

3.13.3. Scoring of individual bands

Two methods of scoring bands were assessed. The first method involved scoring bands using the computer programme NTSYSPC and the second method was to score the number of shared bands (i.e. bands of equal size) on a gel bye eye. For both methods, photographs of the gels were scanned into a computer and saved as graphic files.

3.13.4. Reconstruction of the phylogenetic tree

As with sequence data, RAPD data can be analysed in a number of different ways. The simplest form of analysis is to group isolates with identical band patterns for a given primer. More complex analysis involve cladistic analysis of data and reconstruction of the phylogenetic tree.

3.13.4.1. UPGMA method

The image of the gel electrophoresis was documented through Bio-Profil Bio-1D gen documentation system and analysis software. All reproducible polymorphic bands were scored and analysed following UPGMA cluster analysis protocol and computed InSilico into similarity matrix using NTSYSpc (Numerical Taxonomy System Biostatistics, version 2.11 W). The SIMQUAL programme was used to calculate the Jaccard's coefficients. The RAPD patterns of each isolate was evaluated, assigning character state "1" to indicate the presence of band in the gel and "0" for its absence in the gel. Thus a data matrix was created which was used to calculate the Jaccard similarity coefficient for each pair wise comparison. Jaccard coefficients were clustered to generate dendograms using the SHAN clustering programme, selecting the unweighted pair-group methods with arithmetic average (UPGMA) algorithm in NTSYSpc.

3.14. Denaturing Gradient Gel Electrophoresis (DGGE)

3.14.1. PCR amplification of genomic DNA of the isolates for DGGE analysis

Denaturing Gradient Gel Electrophoresis was performed according to the method of Zhao *et al.,*2006. 18S DNA (200bp with GC clamp) was amplified with the forward primer containing GC clamp at 5' end) F352T: 5'-CGCCCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GAC TCC TAC GGG TGG C-3' AND 519r: 5'-ACC GCG

GCT GCT GGC AC-3') in 25µl of reaction mixture containing 1X PCR buffer, 2.5 mM $MgCl_2$ (Bangalore Genei, India), 100ng of the template DNA, 25.0 pmol each of the forward and reverse primers, 250 µM each of the dNTPS and 1U of Taq DNA polymerase (Bangalore Genei, India). The touchdown PCR program was performed which consisted of an initial denaturation of 95^{O}C for 5 minutes, followed by 6 cycles of 95^{O}C for 1 minute, 65^{O}C for 1 minute and 72^{O}C for 1 minute, in which the annealing temperature was reduced by 0.5^{O}C per cycle from the preceding cycle and the 24 cycles of 95^{O}C. Perpendicular DGGE was performed with "the decode universal mutation detection system" (Bio Rad laboratories, USA).

A uniform gradient gel of 0% to 100% denaturant was prepared which was changed several times so as to optimize suitable concentration and finally 20% to 60% denaturant was found optimal for the best result.

3.14.2. DGGE of the PCR products

3.14.2.1. Creating the gel sandwich (DCode Syatem BioRad)

Large glass plates were cleaned with soap and a soft sponge and rinsed with tap water. After drying, they were cleaned with 96% ethanol. Both 1mm spacers were also cleaned with 90% ethanol and placed on the large glass plates. The clamps were screwed to the sides of the sandwich, in order to be sure that the spacers, 2 glass plates and especially the glass plates were aligned at the bottom side of the sandwich and placed in the holder. The clamps were unscrewed and the alignment of the glass plates was checked. Then the sandwich was placed on the top of the rubber gasket and the handles pressed down.

3.14.2.2. Preparation of the gel

One tube of APS 10% per gel was prepared. The tubing needle was replaced with a new one, the screw between the compartments was opened and the compartments rinsed with water using pump at a uniform speed. The system was completely drained and flushed with compressed air. The gel solutions were prepared as required. Stacking gel was also prepared according to the following:

APS and TEMED was added to the high and low solutions according to the table, stirred gently by hand and proceeded immediately for pouring the high concentration solution in the compartment closet to the outlet of the

gradient mixer and the low concentration solution in the other compartment by the delivery system. The whole system was kept for polymerization.

3.14.2.3. Running a gel

Fresh 0.5X TAE buffer was added to the buffer tank to the mark "Fill". The DCode$_{TM}$ Universal Mutation Detection System (Bio-Rad) was switched on at least 60 minutes before electrophoresis, so that the buffer can heat up to 60 minutes before electrophoresis, so that the buffer can heat up to 60^{O}C. after 2-3 hours of polymerization the comb was removed carefully and the bottom of the sandwich was rinsed with tap water to remove non-polymerized gel. The sandwich was set in the sandwich-holder. A dummy sandwich was also set at the order side to get a closed upper buffer compartment (a dummy consists of a large and small glass plate stuck together with no spacers in between). The DCode$_{TM}$ was then switched off and the lid taken off after 1 min. The sandwich holder was then slided into the buffer tank, with the red dot of the cathode at the right side. The DCode$_{TM}$ pump and the stirrer underneath the tank were switched on (300 rpm) until the samples were loaded.

3.14.2.4. Staining of gels and photography

Before taking DGGE units out from the tank, the run-evaporated H_2O was replaced up to the marked level. Carefully the DGGE unit was dismantled. The ethidium bromide stain was added into a tray with 50X TAE buffer and the gel was stained for 5 minutes and destained with running buffer. The gel was photographed under UV-transilluminator.

3.14.2.5. Data Analysis

3.14.2.5.1. Scoring of individual band

Two methods of scoring bands were assessed. The first method involved scoring bands using the computer programme BioProfil were 1D and the second method was to score the number of shared bands (i.e. bands of equal size) on a gel by eye. For both methods photograph of the gels were scanned into a computer and saved as graphic files.

3.14.2.5.2. UPGMA analysis of the DGGE bands

Variability among the different groups of isolates were detected on the basis of the banding pattern obtained on

denature gradient gel. All reproducible polymorphic bands were scored and analysed following UPGMA cluster analysis protocol and computed in silico into similarity matrix using NTSYSpc (Version 2.11 W) as in case of RAPD analysis. However, a more complex analysis involved cladistic analysis of data and reconstruction of phylogenetic tree. A two (2-D) and three dimension (3-D) principal component analysis was constructed to provide another means and test the relationship among different tested group using EIGEN programme (NTSYS-PC).

3.15. Tubulin Gene specific diversity analysis

Total genomic DNA from the fungi was isolated by N- Cetyl- N, N, N-trimethyl- ammonium bromide (CTAB) method.

Chemicals and reagents

Extraction buffer

1 M Tris HCl (100 mM Tris HCl)

1M EDTA (100 mM EDTA)

4 M NaCl (1.4 M NaCl)

1% CTAB (Proteinase K -0.03µg/ µl)

SDS 20% w/v

Chloroform: isoamyl alcohol (24:1)

Isopropanol

Ethyl alcohol 70% v/v

3.15.1. DNA isolation protocol

0.5 g Fungal Mycelium was taken and grinded with 25 mg PVPP using mini grinder and then centrifuge at 10000 rpm 2 min. at 4^{o}C. The pellet was washed with sterile distilled water and centrifuge at 1000 rpm 20 min. at 4^{o}C). 675 µl of extraction buffer was added and incubated at 37°C for 30 min. 75µl of SDS (20%) was added and incubated at 65°C for 2 hours. Centrifuged at 10000 rpm for 10 min at 4°C Clear solution was collected in a sterile microcentrifuge tube. Equal volume of Phenol: chloroform:isoamyl alcohol (25:24:1).Centrifuged at 10000 rpm for 10 min. at 4°C. Equal volumes of Chloroform: Isoamyl alcohol (24:1) was added. Centrifuged at 10000 rpm for 10 min. at 4°C. The aqueous phase was removed and taken in a sterile microcentrifuge tube.0.6 volumes of isopropyl alcohol was added and incubated at room temperature for 1hour. Centrifuged at 10000 rpm for 10 min. Pellet was washed in 500µl of 70% ethanol. Centrifuged at 10000 rpm for 10 min at room temperature. Pellet was dried and dissolved in 20 µl sterile

distilled water.

3.15.2. Quantification of Isolated DNA

The quantity of the isolated DNA was checked in UV-VIS spectrophotometer (Vivaspec Biophotometer, Germany). From the stock 1µl DNA was mixed with 49-µl sterile distilled water to get 50 times dilution. The A260/A280 ratio was recorded to check the purity of DNA preparation.

3.15.3. PCR Amplification

PCR amplification of ITS region was done in 20 µl of reaction mixture containing PCR buffer, 1X (Kappa, SA); MgCl2, 3 mM; dNTP mix, 0.25 mM; *Taq* DNA polymerase, 0.05 U; primer, 1 picomol and template DNA, 50 ng. Sterile nuclease free water is used as negative control.

3.15.4. Sequencing of β-tubulin gene

The rDNA was used for sequencing purpose. DNA sequencing was done bidirectionally using the β-tubulin specific pairs by Credora Lifesciences, Bangalore. DNA sequence information was analyzed using bioinformatics algorithms tools e.g. Bioedit, MEGA 4, NTSYSpc as well as the few online softwares. The chromatogram of the DNA sequence was analysed by the software Chromus. All the DNA sequences was edited by using software BioEdit and aligned with Clustal W algorithms.

The DNA sequences were analyzed using the alignment software of BLAST algorithm for the different characteristics of DNA sequence for the identification of microorganism. Identification of microorganism was done on the basis of homology of sequence.

The DNA sequences were deposited to NCBI GenBank through BankIt procedure and approved as the tubulin gene sequence after complete annotation and given accession numbers.

The sequenced PCR product was aligned with ex-type strain sequence from NCBI GenBank and established fungal taxonomy for identification. Sequences were aligned following the Clustal W algorithm (Thompson *et al.,* 1994), included in the Megalign module (DNASTAR Inc.). Multiple alignment parameters used were gap = 10. Both of these values are aimed to prevent lengthy or excessive numbers of gaps. The default parameters were used for pairwise alignment. The use of Clustal W determines that, once a gap is inserted, it can only be removed by

editing. Therefore, final alignment adjustments were done manually in order to artificial gaps. Phylogenetic analyses were completed using the MEGA package (version 4.01). Neither gaps (due to insertion-deletion events) nor equivocal sites were considered phylogenetically informative. Hence, complete deletion prevented the use of any of these sites in further analyses. Phylogenetic interference was performed by the UPGMA method. Bootstrap tests with 1000 replications were conducted to examine the reliability of the interior branches and the validity of these trees obtained. Phylogenetic analyses were conducted in MEGA 4 as described by Tamura *et al.,* 2007.

3.16. Inoculation techniques and disease assesment

3.16.1. Inoculum preparation

B. sorokiniana was grown in 100 ml PDA medium for 7-10 days till sporulation occurred. The spores were then scraped off from the surface of the media with the help of inoculating needle and the spores were collected in sterile distilled water. The spore suspension containing $3X10^4$ spores/ml with 0.01% Tween 20 was sprayed on to the treated as well untreated potted plants and covered with plastic bags for 48 h.

3.16.2. Detached leaf inoculation

A detached leaf inoculation technique as described by Dickens and Cook (1989) was followed for artificial inoculation of leaves. Fresh, young, fully expanded sorghum leaved detached from plants were placed in trays lined with moist blotting paper. Wounds were made on adaxial surface of each leaf with 26 G ½ needle and inoculated with 20 µl droplets of spore suspension ($1.2X\ 10^6$ conidial ml^{-1}) of the fungus (prepared from 14 days old culture in PDA). In control sets, drops of sterile distilled water were placed on the wounded leaves. Each tray was covered with a glass lid and sealed with petroleum jelly in order to minimize drying of the drops during incubation. Percent drops that resulted in lesion were calculated after 48, 72 and 96 hours of incubation as described by Chakraborty and Saha (1994). Observations were made based on 50 inoculated leaves for each treatment in average on three separate trials.

3.16.3. Whole Plant inoculation

Whole plant inoculation was carried out easily as described by Mathur *et al* (2000) with minor modifications. The

fungus was grown in PDA for 14 days at $30^{O}\pm2^{O}C$ and spore suspension was prepared (1.2X 10^{6} conidia ml^{-1}). Tween-20 was added @ $2ml^{-1}$ to facilitate adhering of the spores to leaf surface. 1 month old plants were spray-inoculated with as atomizer @ 100 ml per plant so as to wet both ventral and dorsal surfaces. The plants were immediately covered with ploythene bags so as to maintain high relative humidity and kept overnight. Next day, the polythene bags were removed and transferred to glass house benches and maintained at $30^{O}\pm2^{O}C$.

3.16.4. Assessment of disease

The disease severity on plant leaves was recorded using a 0-5 rating scale (Lakshmi *et al*., 2011), where 0 = no infection; 1 = up to 5%; 2 = 6-10%; 3 = 11-20%; 4 = 21-50% and 5 = > 50% leaf area affected by the disease. Based on these numerical ratings Percent Disease Index (PDI) was calculated using the formula:

PDI = [total numerical ratings/ (number of leaves examined x max rating scale)] x 100

Results were always computed as the mean of observations of 25 well-established and branched 1 month old sorghum plants in average of three separate experiments.

3.17. *In vitro* testing for antagonism to fungal pathogens

3.17.1. Antifungal test of PGPR

The obtained PGPR isolates were evaluated against leaf pathogen *B. sorokiniana* in dual culture using NA medium. The bacteria were streaked on one side of the Petri plate and 4 mm fungal pathogen block was placed at the other side of the plate, incubation was undertaken for 5-7 days at $28^{O}\pm2^{O}C$ and inhibition zone towards the fungal colony in individual plate was quantified. Results were expresses as mean of percentage of inhibition of the growth of the pathogen in presence of the bacterial isolates. For each test three replicate plates were used. Those bacteria, which were antagonistic to *B. sorokiniana* were selected for further evaluation.

3.17.2. Antifungal test of PGPF

The efficacy of PGPF (*Trichoderma* sp.) isolates was tested for *in vitro* for inhibiting growth of the pathogen (*B. sorokiniana*) in dual culture using PDA. Each fungal isolate was placed at one side of the agar plate about 1 cm away from the edge and a 4 mm

diameter block of the pathogen, taken from growing edge replicate plates were used. The plates were incubated for 7 days (depending upon the growth of the pathogen) at 28^{O}C and inhibition zone towards the fungus colony in individual plate was quantified. Results were expressed as mean % of inhibition in presence of the fungal isolate.

3.18. Extraction of antifungal compounds from bacteria

3.18.1. Preparation of cell free culture filtrate

The antagonistic isolate was grown in NB medium for 96 h at 37^{O}C in shaking condition. Centrifugation was done at 15,000 rpm for 20 minutes and supernatant was collected and passed through the micro filter (0.22 µm pore size). Portion of this was further used for further bioassays. The cell free filtrate (200 ml) was extracted separately with equal volume of acetone, benzene, chloroform, ethyl acetate and diethyl ether. The culture filtrate was mixed with equal volume of organic solvents and taken in separating funnel. The mixture was shaken vigorously and allows to stand for few minutes and observed for the separation of two liquid phase. The organic as well as aqueous fraction was collected in beakers. The extraction procedure was repeated thrice with each solvent. The organic fraction and corresponding aqueous fractions were evaporated to complete dryness in a rotary evaporator at room temperature and residue in each case was dissolved in 2 ml of respective solvent used for the assessment of antifungal activity. A control was maintained with the solvent only at the time of assessment of antifungal activity.

3.18.2. Partial characterization of active principle

3.18.2.1. GC-MS analysis of crude cell free extract

Identification of the antimicrobial metabolites was done by Gas Chromatography Mass Spectrometry (GCMS) analysis with JEOL GC MATE II GC System (Agilent Technologies 6890N Network GC system for gas chromatography). 1 µl of sample solution was injected into the GC system provided with HP 5 Ms column at 220^{O}C and high pure helium was used as a carrier gas at a flow rate of 1 ml/min. for GC-MS the GC oven was held at 50^{O}C and then ramped from 50^{O}C 250^{O}c at 10^{O}C/min. Temperature of ion chamber was held

at 250^{O}C. The chromatogram and mass spectra were recorded and analyzed by quadruple double focusing mass analyzer with a photon multiplier tube detector. The m/z peaks representing mass to charge ratio characteristics of the antimicrobial fractions were compared with those in the mass spectrum of NIST (National Institute for Standards and Technology) library of the corresponding organic compound.

3.18.3. Bioassay of antimicrobial compound

The test substance's inhibition was determined by using a method as described by a Poison Food Technique and the inhibition to the mycelia growth of fungi was assayed (Wang *et al*., 2011). PDA was used as the medium for the test fungus. The media incorporating test compounds at a serial concentration of 2~10% (v/v) was inoculated with agar discs of the test fungi (5 mm) at the centre. Replicate plates were incubated at 27±2^{o}C for all test fractions. Control plates containing media mixed with sterile water (10%, v/v) were included. After an incubation period of 2 to 6 days, the mycelia growth of fungi (mm) in both treated (T) and control (C) Petri dishes was measured diametrically in perpendicular directions until the fungi growth inhibition (I) was calculated using the formula:

I (%) X [(C-T)/C] X 100

The corrected inhibition (IC) was then calculated as follow:

IC (%) = [(C-T) / (C-C_0)] X 100

C_0 means the diameter of the test fungi agar discs (5 mm).

3.19. Sequencing of endochitinase gene of *Trichoderma*

Total genomic DNA from the PGPF, *Trichoderma harzianum* and *Trichoderma asperellum* was isolated by N- Cetyl- N, N, N-trimethyl-ammonium bromide (CTAB) method.

Chemicals and reagents

Extraction buffer

1 M Tris HCl (100 mM Tris HCl)

1M EDTA (100 mM EDTA)

4 M NaCl (1.4 M NaCl)

1% CTAB (Proteinase K -0.03µg/ µl)

SDS 20% w/v

Chloroform: isoamyl alcohol (24:1)

Isopropanol

Ethyl alcohol 70% v/v

3.19.1. DNA isolation protocol

0.5 g Fungal Mycelium was taken and grinded with 25 mg PVPP using mini grinder and then centrifuge at 10000 rpm 2 min. at 4°C. The pellet was washed with sterile distilled water and centrifuge at 1000rpm 20 min. at 4°C). 675 µl of extraction buffer was added and incubated at 37°C for 30 min. 75µl of SDS (20%) was added and incubated at 65°C for 2 hours. Centrifuged at 10000 rpm for 10 min at 4°C Clear solution was collected in a sterile microcentrifuge tube. Equal volume of Phenol: chloroform:isoamyl alcohol (25:24:1).Centrifuged at 10000 rpm for 10 min. at 4°C. Equal volumes of Chloroform: Isoamyl alcohol (24:1) was added. Centrifuged at 10000 rpm for 10 min. at 4°C. The aqueous phase was removed and taken in a sterile microcentrifuge tube.0.6 volumes of isopropyl alcohol was added and incubated at room temperature for 1hour. Centrifuged at 10000 rpm for 10 min. Pellet was washed in 500µl of 70% ethanol. Centrifuged at 10000 rpm for 10 min at room temperature. Pellet was dried and dissolved in 20 µl sterile distilled water.

3.19.2. Quantification of Isolated DNA

The quantity of the isolated DNA was checked in UV-VIS spectrophotometer (Vivaspec Biophotometer, Germany). From the stock 1µl DNA was mixed with 49-µl sterile distilled water to get 50 times dilution. The A260/A280 ratio was recorded to check the purity of DNA preparation.

3.19.3. PCR Amplification

PCR amplification of ITS region was done in 20 µl of reaction mixture containing PCR buffer, 1X (Kappa, SA); MgCl2, 3 mM; dNTP mix, 0.25 mM; *Taq* DNA polymerase, 0.05 U; primer, 1 picomol and template DNA, 50 ng. Sterile nuclease free water is used as negative control.

3.19.4. Sequencing of endochitinase gene

The rDNA was used for sequencing purpose. DNA sequencing was done bidirectionally using the *Trichoderma* specific endochitinase specific pairs by Credora Lifesciences, Bangalore. DNA sequence information was analyzed using bioinformatics algorithms tools e.g. Bioedit, MEGA 4, NTSYSpc as well as the few online softwares. The chromatogram of the DNA sequence was analysed by the software Chromus.

All the DNA sequences was edited by using software BioEdit and aligned with Clustal W algorithms.

The DNA sequences were analyzed using the alignment software of BLAST algorithm for the different characteristics of DNA sequence for the identification of microorganism. Identification of microorganism was done on the basis of homology of sequence.

The DNA sequences were deposited to NCBI GenBank through BankIt procedure and approved as the *Trichoderma* endochitinase gene sequence after complete annotation and given accession numbers.

The sequenced PCR product was aligned with ex-type strain sequence from NCBI GenBank and established fungal taxonomy for identification. Sequences were aligned following the Clustal W algorithm (Thompson *et al.,* 1994), included in the Megalign module (DNASTAR Inc.). Multiple alignment parameters used were gap = 10. Both of these values are aimed to prevent lengthy or excessive numbers of gaps. The default parameters were used for pairwise alignment. The use of Clustal W determines that, once a gap is inserted, it can only be removed by editing. Therefore, final alignment adjustments were done manually in order to artificial gaps. Phylogenetic analyses were completed using the MEGA package (version 4.01). Neither gaps (due to insertion-deletion events) nor equivocal sites were considered phylogenetically informative. Hence, complete deletion prevented the use of any of these sites in further analyses. Phylogenetic interference was performed by the UPGMA method. Bootstrap tests with 1000 replications were conducted to examine the reliability of the interior branches and the validity of these trees obtained. Phylogenetic analyses were conducted in MEGA 4 as described by Tamura *et al.,* 2007.

3.20. Mass multiplication and application of bioinoculants

3.20.1. Arbuscular Mycorrhizal Fungi (AMF)

3.20.1.1. Isolation of AMF spores

Spores of arbuscular mycorrhizal fungi were isolated from rhizosphere soil of sorghum plant by wet sieving and decanting method (Gerdemann and Nicolson, 1963). Approximately 250 gm of soil was suspended in 1 L water. Heavier particles were allowed to settle for a few seconds and the liquid was

decanted through sieve of decreasing size (BS 60, BS 80, BS 100, BS 150 and BS 200). Pores are fine enough to remove the larger particles of organic matter, but coarse enough to allow the desired spores to pass through. The suspension that passed through these sieves was saved and stirred to resuspend all particles. The heavier particles were allowed to settle for a few seconds and the liquid decanted again through the sieve and spores collected by fine brushes and were kept in different Petri plates according to their size and colours. Moreover for further observations of purification of AMF spores sucrose gradient centrifugation method was used. In sucrose gradient centrifugation (Daniels and Skipper, 1982) spores and minimal amount of organic particles were further purified by suspending sieving in 40% sucrose solution and centrifuging t 2000 rpm (approximate 370 x g) for 1 minute. The supernatant (with spores) was passed through a sieve of 400 mesh and rinsed with distilled water to remove sucrose residue.

With the help of a simple microscope (20X) parasitized spores, plant debris etc were separated. Spores were sonicated at 30 Hz for two minutes to remove the debris adhered to the spores then clean spores were stained with Melzer's reagent (50% aqueous solution of chloral hydrate with 2.5-3.75% potassium iodide and 0.75-1.25% iodine) and studied microscopically. For further use, the AMF spores were isolated in Ringer's solution (8.6 g NaCl, 0.3 g KCl, 0.33 g $CaCl_2$ in 1 L of boiled distilled water) at -15^{O}C to -20^{O}C or in sterile distilled water. Identification of genera and species was done microscopically using the specific spore characters such as size, colour, shape, wall structure, surface ornamentation and bulbous suspensor by using identification manuals (Trappe, 1982; Schenck and Perez, 1990).

3.20.1.2. Histopathology of sorghum roots

Fungal association of AM fungi within the root tissues was observed according to Philips & Hayman (1970). Young roots from sorghum plants were dug out manually. Roots were cut into 1 cm or smaller pieces and washed in tap water gently to free them from soil particles. It was boiled in 2 % KOH in hot water bath for 1 hour. The KOH was decanted and the roots washed with water for 2-3 times. 1% HCL was

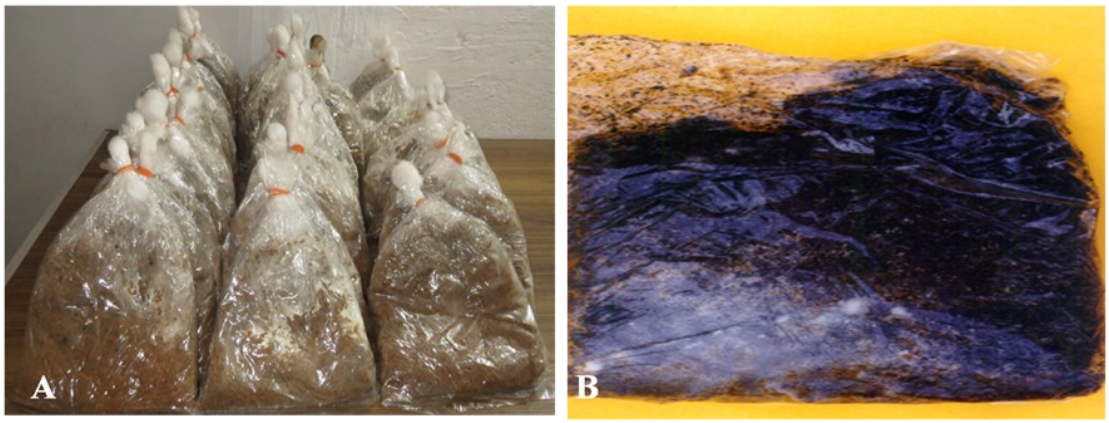

Figure 3.5: Mass multiplication of PGPF in (A) wheat bran and (B) rice straw

added and kept for 30 minutes. After decanting the HCL the sample was washed thrice in tap water and cotton blue, lactic acid and glycerol was added in the ratio 1:1:1 to stain the internal structures of AMF inside the root segments i.e. arbuscules, vesicles, auxiliary cells and boiled in water bath for 1 hour. The excess stain was decanted and sample placed in 50% glycerol for destaining. The roots were then crushed under pressure in slide and covered with cover slip for microscopic observation. Percent root colonization was determined following the method of Giovanetti and Mosse (1980).

3.20.1.3. Mass multiplication of AMF

AMF spores were isolates from rhizosphere of three variety of sorghum plant using decanting and sieving method as described earlier. The mass of spores were washed with distilled water several times to remove the adhered debris. Filter paper was cut into small bits about the size of 1 cm. with the help of fine tweezers, 45-60 AMF spores were placed in the filter paper bits. They were then carefully placed onto the roots of the 7-10 days old seedling in plastic pots (12 inch) having autoclaved soil to discard the presence of other fungal propagules. Maize plants were grown both in the field and pots. After 45 days, the presence of spores was verified and inocula were prepared by mixing the chopped roots of maize plants with the potted soil where extra radical spores of AMF were present. Approximately > 175 spores/ 100 gms could be considered as potent inocula for application.

3.20.2. Plant Growth Promoting Fungi (PGPF)

3.20.2.1. Selection of PGPF

Four isolates of *Trichoderma harzianum* [(NAIMCC-F-03287),

(NAIMCC-F-03288), (NAIMCC-F-03289) and (NAIMCC-F-03290)] and three isolates of *Trichoderma asperellum* [(NAIMCC-F-03291), (NAIMCC-F-03292),(NAIMCC-F-03293)] were selected as Plant Growth Promoting Fungi based on their performances as potential growth promoters as well as their biocontrol efficiency in field grown crops (Sunar *et al.,* 2014).

3.20.2.2. Mass multiplication

3.20.2.2.1. Wheat bran media

Inoculum of *Trichoderma harzianum* and *Trichoderma asperellum* were prepared by inoculating wheat bran (sterilized) with 5 mm disc of the fungus and incubating at 28^{O}C for 10 days (Fig. 3.5).

To each pot of soil (2000 g), 10 g of the wheat bran colonized by *T. harzianum* and *T. asperellum* was mixed to give a concentration of 105 cfu/g of soil as described by Chakraborty *et al* (2003).

3.20.2.2.2. Tricho-compost

Six layers of compost materials (each layer about 25 cm thick) was made. 3 parts cellulosic waste (rice straw, grass, corn stalk, spent mushroom substrate) and 1 part mixture of leguminous plant materials (Mungbean, Peanut, Soybean) and animal manure was mixed. Each layer of piled compost materials was sprinkled with 30 litres of Tricho inoculants solution. Additional water is sprinkled to keep the compost heap moist. It was covered with plastic sheet or sack to increase temperature and prevent too much water in case of rainfall. Compost heap was turned from top to bottom after two weeks. The Tricho compost was ready for harvest four weeks after preparation. The compost was stored in

Figure 3.6: Mass multiplication of PGPR: (A) Mass mixture machine, (B) mass multiplied PGPR in talcum

sacks or applied directly into soil (Fig. 3.5).

3.20.3. Plant Growth Promoting Rhizobacteria (PGPR)

3.20.3.1. Selection of PGPR

Six strain of Plant Growth Promoting Rhizobacteria [*Bacillus methylotropicus* (NAIMCC-B-01492), *B. symbiont* (NAIMCC-B-01489), *B. altitudinus* (NAIMCC-B-01484), *B. megaterium* (NAIMCC-B-01482), *B. pumilus* (NAIMCC-B-01483) and *P. polymyxa* (NAIMCC-B-01491)] based on their performances as potential growth promoters as well as their biocontrol efficiency in field grown crops (Sunar *et al.,* 2013)

3.20.3.2. Mass multiplication

3.20.3.2.1. Soil drench

The bacteria were grown in NB for 48 h at 28^{O}C and centrifuged at 12,000 rpm for 15 minute. The pellet obtained was suspended in sterile distilled water. The optical density of the suspension was adjusted using UV-VIS spectrophotometer following method to obtain a final density of 3 X 10^6 cfu ml^{-1}. The bacterial suspension was applied to the pots during transplantation of seedlings. Application was done @ 100 ml per pot at regular interval of one month for three months subsequently. The rhizosphere of two year's old potted plant was inoculated twice at an interval of 20-15 days.

3.20.3.2.2. Foliar spray

The bacterial pellet suspended in sterile distilled water at a concentration of 3 X 10^6 CFU ml^{-1} after the addition of a few drops of Tween-20 was sprayed until run off on the foliar part of the one month old sorghum plants. The spraying was done forth nightly till the new leaves started appearing.

3.20.3.2.3. Talc based formulation

10 gm of carboxy methyl cellulose sodium salt (Himedia) was mixed with 1 kg of talcum powder and pH was adjusted to 7.0 by adding calcium carbonate. It was then sterilized twice for 30 minutes each. The bacterium was first grown in nutrient broth and after 48 h the actively growing cells in log phase were harvested by centrifugation at 21000 rpm, and aqueous suspension was made to achieve a concentration of 3 X 10^9 CFU ml^{-1} which was determined spectrophotometrically. To 1 kg of sterilized talcum powder 400 ml of bacterial inoculums was added and mixed well under sterile condition. The talc mix was dried under stage to bring

moisture to less than 20%. The formulation was packed in milky white colour polythene nags to eliminate UV exposure, sealed and stored at room temperature for future use. The talcum based formulation was applied in the field at the rate to 100 gm per pot (12 X 10^{10} bacterial cells) (Fig. 3.6).

3.21. *In vivo* assessment of plant growth promotion

3.21.1 Assessment of plant growth following application of bioinoculants

Plant growth promotion was recorded after 30 and 60 days of application of bioinoculants in potted plants and in field grown plants. The growth parameters such as number of leaves and plant height was observed.

3.21.2 Assessment of disease severity

The disease severity on plant leaves was recorded using a 0-5 rating scale (Lakshmi *et al.,* 2011), where 0= no infection; 1= up to 5%; 2=6-10%; 3=11-20%; 4=21-50% and 5=> 50% leaf area affected by the disease. Based on this numerical rating a Percent Disease Index (PDI) was calculated using the formula:

PDI=[total numerical ratings/(number of leaves examined x max rating scale)] x 100.

3.22. Extraction and assay of defense enzymes activities

3.22.1. β-1,3-glucaanase (E.C.3.2.3.3.9)

Extraction of β-1,3-glucanase (E.C.3.2.3.3.9) was done following the method described by Pan *et al*., (1991). Sorghum leaf samples (1 g) were crushed in liquid nitrogen and extracted using 5 ml of chilled 0.05 M sodium acetate buffer (pH 5.0) by grinding at 4^{O}C using mortar and pestle. The extract was then centrifuged at 10000 rpm for 15 min at 4^{O}C and the supernatant was used as crude enzyme extract. Estimation of the β-1,3-glucanase was done by following the Laminarin dinitrisalicylate method (Pan *et al*., 1991). The crude enzyme extract of 62.5 µl was added to 62.5 µl of laminarin (4%) and then incubated at 40^{O}C for 10 minutes. The reaction was stopped by adding 375 µl of dinitrosalicylic reagent and heating for 5 min in boiling water bath. The resulting colored solution was diluted with 4.5 ml of water, vortexed and absorbance was recorded at 500 nm. The blank was the crude enzyme preparation mixed with laminarin with zero time incubation. The enzyme

activity was expressed as µg glucose released min^{-1} g^{-1} fresh tissue.

3.22.2. Chitinase (E.C.3.2.1.14)

Extraction of chitinase (E.C.3.2.1.14) was done by following the method described by Boller and Mauch (1988) with modifications. 1 gm leaf sample of sorghum plants were crushed in liquid nitrogen and extracted using 5 ml of chilled 0.1 M sodium citrate buffer (pH 5). The homogenate was centrifuged for 10 minutes at 12,000 rpm and the supernatant was used as enzyme source.

Chitinase activity was measured according to the method described by (Boller and Mauch, 1988). The assay mixture consisted of 10 µl Na-acetate buffer (1M) pH4, 0.4 ml of enzyme solution, 0.1 ml of colloidal chitin (1 mg). Colloidal chitin was prepared as per the method of (Roberts and Selitrennikoff, 1988). After 2h of incubation at $37^{O}C$ the reaction was stopped by centrifugation at 10,000 g for 3 minutes. An aliquot of supernatant (0.3 ml) was pipette into a glass reagent tube containing 30 µl of potassium phosphate buffer (1 M) pH 7.1 and incubated with 20 µl of (3% w/ v) desalted snail gut enzyme Helicase (Sigma) for 1 hour. After 1 h, the pH of pH of the reaction mixture was brought to 8.9 by addition of 70 µl of sodium borate buffer (1 M) pH 9.8. The mixture was incubated in a boiling water bath for 3 minutes and then rapidly cooled in an ice water bath. After addition of 2 ml DMAB (ρ-dimethyl amminobenzaldehyde) reagent, the mixture was incubated for 20 minutes at $37^{O}C$. There of absorbance value at 585 nm was measured using a UV-VIS spectrophotometer. N-acetyl glucosamine (GlcNAc) was used as standard. The enzyme activity was expressed as µg GlcNAc min^{-1} mg^{-1} fresh tissues.

3.22.3. Phenylalanine ammonia lyase (PAL) (E.C.4.3.1.5)

Extraction of PAL (E.C.4.3.1.5) was done by following the method described by Chakraborty *et al.* (1993) with modifications. 1 gm of leaf sample was crushed in 0.1 M sodium borate buffer pH 8.8 (5ml/gm) with 2 mM of β mercaptoethanol in ice cold temperature. The slurry was centrifuged in 15,000 rpm for 20 minutes at $4^{O}C$. Supernatant was collected and after recording its volume, was immediately used for assay or stored at $-20^{O}C$.

Phenylalanine ammonia lyase activity in the supernatant was determined by measuring the production of cinnamic acid from L-phenylalanine spectrophotometrically. The reaction mixture contained 0.3 ml of 300 µM sodium borate (pH 8.8), 0.3 ml of 3 µM L-phenylalanine and 0.5 ml of supernatant in a total volume of 3 ml. Following incubation for 1 h at $40^{O}C$ 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 1 min g^{-1} fresh weight of tissues.

3.22.4. Peroxidase (E.C.1.11.1.7)

For the extraction of peroxidase (E.C.1.11.1.7) the plant tissues were macerated to powder in liquid nitrogen and extracted in 0.1 M sodium borate buffer (pH 8.8) containing 2mM β mercaptoethanol under ice cold conditions, the homogenate was centrifuged immediately at 15,000 rpm for 20 minutes at $4^{O}C$. After centrifugation the supernatant was collected and after recording its volume was immediately used for assay or stored at $-20^{O}C$ (Chakraborty *et al.,* 1993).

For determination of peroxidase activity, 100 µl of freshly prepared crude enzyme extract was added to the reaction mixture containing 1 ml of 0.2 M sodium phosphate buffer (pH 5.4), 100 µl of 4 mM H_2O_2, 100 µl O-dianisidine (5 mg ml^{-1} methanol) and 1.7 ml of distilled water. Peroxidase activity was assayed spectrophotometrically at 460 nm by monitoring the oxidation of O-dianisidine in presence of H2O2 (Chakraborty *et al.,* 1993). Specific activity was expressed as the increase in absorbance at 460 nm g^{-1} tissue/min^{-1}.

3.22.5. Isozyme analysis of peroxidase

Polyacrylamide gel electrophoresis (PAGE) was performed for isozyme analysis of peroxidase. Extract for isozyme analysis was prepared by crushing 1 gm of leaf tissue in a mortar and pestle in 2 M sodium phosphate buffer (pH 7.0) in ice cold condition as described by Davis (1964) and used immediately for the isozyme analysis.

3.22.5.1. Preparation of the stock solution

Solution A: Acrylamide stock solution (Resolving gel)

For the preparation of acrylamide stock

solution for resolving gel 28 gm of acrylamide and 0.74 gm of N' N' methylene bisacrylamide was dissolved in 100 ml of distilled water. The stock solution was filtered with Whatman No. 1 filter paper and stored at $4^{O}C$ in dark bottle.

Solution B: Acrylamide stock solution (stacking gel)

For the preparation of acrylamide stock solution for stacking gel 10 gm of acrylamide and 2.5 gm of N' N' methylene bisacrylamide was dissolved in 100 ml of distilled water. The stock solution was filtered and stored at $4^{O}C$ in dark bolttle.

Solution C: Tris-HCL (Resolving gel)

36.6 gm of Tris base was mixed with distilled water and 0.25 of N,N,N',N'-tetramethyl ethylene diamine (TEMED) was added. The pH was adjusted to 8.9 with concentrated HCL. The volume of the solution was then stored at $4^{O}C$ for further use.

Solution D: Tris-HCL (Stacking gel)

5.98 gm of Tris base was mixed with distilled water and 0.46 ml of TEMED and the pH was adjusted to 6.7 with concentrated HCL. The volume of the solution was made up to 100 ml with distilled water. The solution was stored at $4^{O}C$ for further use.

Solution E: Ammonium persulphate solution (APS)

Fresh solution of APS was prepared by dissolving 0.15 gm of APS in 10 ml of distilled water.

Solution F:Riboflavin solution

Fresh solution of Riboflavin was prepared by dissolving 0.4 mg of Riboflavin in 10 ml distilled water. The solution was kept in dark bottle to protect from light.

Solution G: Electrode buffer

Electrode buffer was prepared freshly by dissolving 0.6 gm of Tris base and 2.9 gm of Glycine in 1 L of distilled water.

3.22.5.2. Preparation of gel

For the polyacrylamide gel electrophoresis of peroxidase isozymes mini slab gel was prepared. For slab gel preparation, two glass plates were thoroughly cleaned with dehydrated alcohol to remove any trace of grease and then dried. 1.5 mm thick spacers were placed between the glass plates on three sides and these were sealed with high vacuum grease and clipped thoroughly to prevent any leakage of

the gel solution during pouring. 7.5 % resolving gel was prepared by mixing solution A:C:E: distilled water in the ratio of 1:1:4:1 by pipette leaving sufficient space for (comb + 1 cm) the stacking gel. This resolving gel was immediately over layered with water and kept for polymerization for 2 hours. After polymerization of the resolving gel was complete, over layer was poured off and washed with water to remove any unpolymerized acrylamide. Stacking gel solution was poured over the resolving gel and comb was inserted immediately and over layered with water. Finally the gel was kept for polymerization for 30-45 minutes in strong sunlight. After polymerization of the stacking gel the comb was removed and washed thoroughly. The gel was now finally mounted in the electrophoretic apparatus. Tris-Glycine running buffer was added sufficiently in both upper and lower reservoir. Any bubble, trapped at the bottom of the gel, was removed carefully with a bent syringe.

3.22.5.3 Sample preparation

Sample (32 µl) was prepared by mixing the sample enzyme (20 µl) with gel loading dye (40% sucrose and 1% bromophenol blue in distilled water) in cyclomixture in ice cold condition. All the solutions for electrophoresis were cooled. The samples were immediately loaded in a predetermined order into the bottom of the wells with a microlitre syringe.

3.22.5.4. Electrophoresis

Electrophoresis was performed at constant 15 mA current for a period of 3-4 h at 4^{O}C until the dye front reached the bottom of the gel.

3.22.5.5. Fixing and staining

After electrophoresis the gel was removed carefully from the glass plates and then the stacking gel was cut off from the resolving gel and finally stained. Staining of the gel was performed following the method Reddy and Gasber (1973). The gel was incubated in the aqueous (80 ml) solution of Benzidine (2.08 gm), Acetic acid (18 ml), 3% H_2O_2 (100 ml) for 15 minutes. The reaction was stopped with 7% Acetic acid. After the appearance of clear blue coloured bands, analysis of isozyme was done immediately.

3.22.6. Extraction and estimation of total sugar

Estimation of total sugar was done by Anthrone reagent following the method

of Plummer (1978). To 1 ml. of test solution, 4 ml. of Anthrone reagent (0.2% Anthrone in conc. H_2SO_4) was added. The reaction mixture was mixed thoroughly and was incubated in boiling water bath for 10 mins. Then the reaction mixture was cooled under running tap water and absorbance was measured in a colorimeter at a wavelength of 620 nm and sugar content was quantified using a standard curve of D-glucose.

3.22.7. Extraction and estimation of total chlorophyll

For the extraction of chlorophyll from leaves of plants the method of Harborne (1973) with modifications was used. 1gm leaf tissues were crushed in a mortar and pestles using 80% acetone in the dark to prevent the photo oxidation of chlorophyll. The crushed samples were filtered through Whatman No. 1 filter paper. Final volume was made up 25 ml. with adding sufficient amount of acetone. A tube containing 80% acetone was used as blank.

Estimation of chlorophyll was done by measuring the absorbance at 645 nm and 663 nm respectively in a UV-VIS spectrophotometer against blank of 80% acetone and calculated using the formula as given by Arnon (1949).

Total chlorophyll = (20.2 A_{645} + 8.02 A_{663}) mg g^{-1} fr. Wt.

3.22.8. Extraction and estimation of phenol content

Phenol was extracted from the fresh young leaves following the method of Mahadevan and Sridhar (1982). One gm of sample were cut into pieces and immediately immersed in 10 ml of boiling water. After 15 minutes of boiling it was cooled and crushed in mortar using pestle thoroughly at room temperature. The extract was filtered thorough Whatmann No.1 filter paper. Final volume was adjusted was 80% ethanol. The whole extraction of phenol was done in dark to prevent light induced degradation of phenol.

Total phenol content was estimated by Folin Ciocalteau's reagent, following the method of Mahadevan and Sridhar (1982). To 1 ml of the alcoholic extract, 1 ml of 1 N Folin Ciocalteau's reagent followed by 2 ml of 20% sodium carbonate solution was added in a test tube. The test tube was shaken and heated on a boiling water bath for 1 minute. After cooling, the volume of the reaction mixture was raised to 25 ml. absorbance of the blue colored solution was measured at 560 nm.

Quantity of total phenol was estimated using caffeic acid as standard.

O-dihydroxy phenol was also estimated following the method of Mahadevan and Sridhar (1982). 1 ml of alcoholic extract was mixed with 2 ml of 0.05 N HCl, 1 ml of Arnow's reagent ($NaNO_2$-10 gm, Na_2MoO_4-10 gm, distilled water-100 ml) and 2 ml of 1 N NaOH and mixed thoroughly at room temperature following which the volume of the reaction mixture was raised to 10 ml. absorbance of the colored solution was recorded at 515 nm. Quantity of the O-dihydroxy phenol was estimated using caffeic acid as standard.

3.22.9. Extraction and estimation of proline

Extraction of proline from the leaves and roots was done by the method of Bates *et al.* (1973). 1 gm of plant tissue was homogenized in 10 ml of 3% Sulfosalicylic acid and filtered through Whatman No. 1 filter paper. The supernatant was collected for estimation. To 1 ml of extract, 3 ml of distilled water and 1 ml pf Ninhydrin solution (1 g Ninhydrin in 10 ml acetone and 15 ml distilled water) was added. The reaction mixture was kept on a boiling water bath for 30 mins and then cooled at room temperature. The reaction mixture was transferred in separating funnel and 5 ml of Toluene was added and mixed vigorously. The lower coloured layer was taken and the OD values were measured at 520 nm in a colorimeter against a blank and quantified from a standard curve of proline.

3.22.10. Extraction and estimation of malonaldehyde

Malondialdehyde (MDA) was measured following the method of Heath and Packer (1968). 0.5 gm leaf sample was homogenized in 0.1 % (w/v) TCA and centrifuged for 10 min at 10,000 rpm. For estimation, 0.5 ml of the supernatant was mixed with 2 ml of 0.5% (w/v) TBA in 20% TCA, followed by heating for 30 min at $95^{O}C$ and cooling it on ice. The absorbance of the reaction mixture was determined at 352 and 600 nm. MDA content was then calculated using an extinction coefficient of 155 mM^{-1} cm^{-1}.

3.22.11. Extraction and estimation of hydrogen peroxide

The extraction of hydrogen peroxide was done by the method given by Jena and Choudhuri (1981) with slight modifications. 0.5 gm leaf extract was homogenized in 10 ml of phosphate

buffer (50 mM, pH 6.5) in a dark chamber and centrifuged at 6000 rpm for 25 mins. The estimation of hydrogen peroxide was done by the method given by Jena and Chaudhuri (1981). The supernatant was used for estimation, where 3 ml of extract was mixed with 1 ml. of 0.1% titanium sulphate in 20% (v/v) H_2SO_4 along with. Both the blank and the reaction mixture was centrifuged at 6000 rpm for 15 mins and the intensity of yellow colour obtained was measured at 410 nm in a UV-VIS spectrophotometer. H2O2 content was quantified using the extinction coefficient value of € = 0.28 μmol^{-1} cm^{-1}.

3.22.11.1. Localization of hydrogen peroxide

The detection of hydrogen peroxide in the leaf tissue was done according to the method given by Thordal-Christensen *et al*., (1997) with minor modifications. The leaf tissues were washed thoroughly with doubled distilled water and cut into leaf discs. Then these discs were incubated in dark for 24 h in 1 mg/ml of 3,3'-Diaminobenzidine (DAB) having pH 3.8. after 24 h the leaf disc was transferred to a beaker containing ethanol/lactic acid/glycerol in the ratio 4:1:1 and was heated at a temperature of $70^{O}C$ until all the chlorophyll was removed. Hydrogen peroxide was visualized as reddish-brown colour at the site of Diaminobenzidine polymerization.

3.23. Analysis of antifungal compounds from plants

3.23.1. Sample preparation

An attempt was made to characterize the various antifungal and chemical compounds present in methanolic extracts of healthy and treated infected plant samples through Gas Chromatography- Mass Spectrometry (GC-MS analysis).

50 gm of leaf samples were cut into small pieces and emerged in 100 ml of HPLC grade methanol for 48 hr under darkness and normal room temperature. Then the extracted solvent was filtered through Buckner's funnel with Whatman no. 1 filter paper. The extracted solvent was then used for further analysis.

3.23.2. Spore germination and Radial growth bioassay

Germination of spores was observed at 24 hr, 48 hr interval in *in vitro* condition in glass slides supplemented with antifungal compound extracted

from treated and inoculated plants and control set was supplemented with sterile distilled water.

Radial growth inhibition bioassay was performed for determining antifungal activity of plants extracts. The sample (50 µl) was taken in sterile Petri dishes and allowed to evaporate. Subsequently 10 ml sterilized PDA medium was poured in each Petri dish, thoroughly mixed and allowed to solidify. Agar block (5 mm dia) containing mycelia of *Bipolaris sorokiniana* (7 days old culture) were taken from the advancing zone and transferred to each Petri dish these were incubated at 28±2^{o}C, until inhibition of mycelia growth was observed. Percentage of mycelia inhibition was calculated using the following formula:

Percentage of mycelia inhibition = (C -T)/C X 100

Where, C and T are the growth diameter (mm) in control and treated samples respectively.

3.23.3. GCMS analysis

In the present study methanolic fractions of different treatments of sorghum plants were used separately for carrying out the GC-MS analysis for various phytochemical compounds present in the plant samples. GC-MS analysis of the extract was carried out with GCMS-QP-2010 Ultra, Shimadzu, Japan with Thermal Desorption System TD 20. The instrument was equipped with programmable head space auto sampler and auto injector. The capillary column used was DB-1/ RTXMS (30 m) with pure helium (99.999%) as a carrier gas, at a constant flow rate of 3 ml min^{-1} and 1 µl injection volume. Column oven temperature and injection temperature were set at 60^{o}C and 260^{o}C respectively. Samples were analyzed with the column held at different temperature. Pressure was established as 72.3 KPa and the sample was run for 60 min. Temperature and column flow for flame ionization detectors were set as 230^{o}C and 1.20 ml min^{-1}, correspondingly. MS parameters were as follows: scan range: 40 to 650 m/z with a scan speed of 3333. The relative percentage of the chemical constituents present in the was expressed as percentage by peak area normalization.

The chemical compounds were identified on the basis of comparison of their retention time and mass spectra and computer matching with WILEY 8.0 libraries and National Institute of

Standards and Technology (NIST 14.0) database provided with computer controlling the GC-MS system. The spectrum of the unknown component was compared with the spectrum of the known compounds stored in the library. The name, molecular weight and structure of the compounds of the test plants were ascertained.

3.23.4. TLC analysis of phenolics

Thin- layer chromatography was performed on plates of 15 cm-6cm silica gel Polygram Sil G (Marck). In each case 1.5 cm was measured from the base of the TLC plate, marked with a pencil and labeled. Before applying the sample the TLC plate was charged by heating at 80^{o}C for 1 hours. Thin layer chromatography was performed on the methanolic crude extracts of sample. Capillary tube was used to spot the plates with the sample extract. The spot plates were then placed in a vertical chamber saturated with butanol: acetic acid: water (80:20:20) and covered and ensuring that the solvent was just below the spot. The plate was removed after about two hours when the solvent had risen close to the top edge, marking the distance travelled by solvent with a pencil. It was then dried at room temperature. The dried plate was then placed in a container and folin ciocalteau solution was sprayed over the plate. Blue colour spots developed according the separation of phenolics. The samples were run along with standard phenolics to identify the presence of phenolics in the samples.

3.23.5. HPLC analysis of phenolic compounds

Fresh leaves of sorghum plant were chopped into pieces and soaked overnight in methanol in the ratio of 1:3 (w/v), filtered through Buckner's funnel and the solvent was evaporated using lyophilizer as described by Pari and Latha (2004). The dried powder was finally mixed in HPLC grade methanol and stored at 4^{O}C for further analysis. HPLC analysis of phenolic compounds present in the extracts was done using SPD-10A VP Shimadzu UV-VIS Detector. A flow rate of 1 ml/ min, and gradient elution of acetonitrile -water-acetic acid (5:93:2, v/v/v) [solvent A] and of acetonitrile-water-acetic acid (40:58:2, v/v/v) [solvent B], 0-50 min solvent B from 0 to 100%; and injection volume of 20 µl were applied; whereas the separation of compounds was monitored at 280 nm (Pari *et al.,* 2007).

3.24. Scanning Electron Microscopy

Spores of fungal pathogens were examined under scanning electron microscopy (SEM). Selected fungal spores were sonicated under 35 MHz to followed by washing five times in sterile distilled water, surface disinfected with 4% (w/v) chloramines-T and 300 ppm of streptomycin for 1 h, and then rinsed further five times in sterile distilled water and were stored in eppendorf's tube in room temperature. Each sample was placed within separate aluminium "disc cup" (20 mm diam x 5 mm deep). Each sample was lifted from the bottom of the specimen dish with fine forceps and was positioned upright in a disc cup. The samples were then dried. All dried samples were mounted on double-sided tape affixed to SEM specimen mounts and were subsequently sputter-coated with gold. Gold coated samples were examined with Philips 505 scanning electron microscope operating at 9.5-r5 Kev.

3.25. Transmission Electron Microscopy

3.25.1. Specimen preparation

3.25.1.1. Fixation

Control and inoculated leaf samples (1-2 mm) were excised in 0.1 M sodium phosphate buffer pH 7.4. They were immediately transferred to 2.5% Glutaraldehyde in eppendorf tubes for 2-12 hours at room temperature.

3.25.1.2. Dehydration

Dehydration was done in ascending grades of alcohol at intervals of 30 minutes in $4^{O}C$ (30%, 50%, 70%, 80%, 90%) and two changes in absolute alcohol at 1 hr interval each at $4^{O}C$ in PLT-272 (M) Fume Hood (Tanco).

3.25.1.3. Infiltration

Infiltration was done twice in LR White resin (London Redin Co. Ltd) in absolute alcohol (1:1) for 1 hr each at $4^{O}C$.

3.25.1.4. Embedding

The samples were dipped in LR white and kept overnight at $4^{O}C$. They were kept at room temperature for 3 hrs. a fresh change of LR white was done and kept at $56^{O}C$ for 36 hrs.

3.25.2. Viewing preparation

3.25.2.1. Trimming

Moulds containing the samples were roughly trimmed with a block trimmer (Reichert TM60) fitted with a rotating milling cutter.

3.25.2.2. Sectioning

A series of thick sections of the selected blocks were cut with Belgium glass strips in microtome (Leica EM UC7) to observe under an optical microscope.

3.25.3. Immunogold labeling

Ultrathin sections (60 nm) were cut with fresh Belgium glass strips and picked up in nickel grids (100 mesh) for immunogold labeling.

3.25.3.1. Primary antibody

The grids containing ultrathin sections were floated in blocking solution containing 2% skimmed milk agar for 30 minutes. Primary antibody was diluted in 1% fish gelatin in the ratio of 1:20. Grids were incubated in the PAbs for 24 hrs at $4^{O}C$. Grids were washed on drops (100 µl) of fish gelatin pipette on to parafilm 10X2 minutes.

3.25.3.2. Secondary antibody

Grids were incubated with anti-rabbit IgG (Whole Molecule) gold antibody produced in goat affinity isolated antibody (Sigma-G7402) diluted in 1:5.