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Serological and molecular detection of *Macrophomina phaseolina* causing root rot of *Citrus reticulata*

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Abstract
Polyclonal antibodies (PAbs) were raised against mycelial antigens of *Macrophomina phaseolina* a causal organism of root rot disease of mandarin plants. IgG was purified and further packaged into immunological formats such as immuno diffusion, Plate trapped antigen (PTA)-ELISA, dot immunobinding assay, Western blot analysis and indirect immunofluorescence for quick and accurate detection of pathogen from soil. Indirect staining of mycelia and sclerotia of *M. phaseolina* with homologous PAb and labeling with goat antirabbit IgG conjugated with FITC developed strong fluorescence in young hyphal tips and sclerotia of *M. phaseolina*. Genomic DNA prepared from mycelia of *M. phaseolina* was purified and PCR amplification of 18S rDNA was done using ITS region specific primer pair. The amplified DNA was sequenced and aligned against ex-type strain sequences from NCBI GenBank using BLAST and phylogenetic analysis was obtained using MEGA4 software. Amplification of ITS1 region of the rDNA can be considered as a rapid technique for identifying pathogens successfully in all cases.

Keywords: *Citrus reticulata, Macrophomina phaseolina*, PTA-ELISA, DIBA, immunofluorescence, rDNA, ITS region

The most widely cultivated cultivar in India is the mandarin (*Citrus reticulata* Blanco) followed by sweet orange (*Citrus sinensis* Osbeck) and acid lime (*Citrus aurantifolia* Swingle), sharing 65, 25 and 10 percent of total production respectively but still remains unexplored for systemic collection, evaluation and characterization to shortlist them in order to identify active germplasm. There are four different strains of mandarin cultivated in India viz. Khasi mandarin grown in north-eastern states, Darjeeling mandarin grown in the hills of Darjeeling (Figure.1A) and Sikkim, Nagpur mandarin grown in Maharashtra, Coorg mandarin grown in south India (Allay and Chakraborty, 2010).

The mandarin cultivation in Darjeeling has a massive decline due to various pathological, entomological and nutritional stresses. *Macrophomina phaseolina, Fusarium solani* and *Fusarium oxysporum* infects roots of both nursery grown and field grown mandarin plants. The use of immunological assays for both detection and diagnosis of plant diseases have increased rapidly (Chakraborty and Chakraborty, 2003). It has long been known that most plant pathogens possess as part of their structures, specific antigenic determinants or recognition factors in the form of proteins, glycoproteins, complex carbohydrate polymers or other complex molecules (Chakraborty, 1988).

Since it is difficult to detect *M. phaseolina* in root tissue or in soil until it is too late, the present study was undertaken for early and accurate detection of the pathogen by serological techniques. It is expected that such an early detection would prove useful for better management practices

The development of serological techniques has produced a number of highly sensitive methods for identifying microorganisms in diseased plant tissue (Chakraborty *et al.* 1995). These rely on the recognition of the solid or solid antigenic materials by antibodies raised against the organisms and the subsequent use of an enzyme labeling system. The purpose of the present study was rapid identification of *Macrophomina phaseolina*, a root rot pathogen of *Citrus reticulata* (Blanco) in Darjeeling hills based on the sequence analysis of ITS regions of the rDNA gene and development of rDNA markers for analysis of genetic variability.

Materials and Methods

Fungal culture

Fungal pathogen (*Macrophomina phaseolina*) was isolated from samples of diseased roots of mandarin (*Citrus reticulata*) plants grown in Mirik busty by culturing pieces of internal tissues. Infected root tissues were thoroughly washed in sterile water, treated with 0.1% $HgCl_2$ for 2-3 minutes, rewashed with sterile distilled water, transferred to potato dextrose agar (PDA) slants and incubated at 28°C for two weeks. The isolated organism was examined under microscope. Healthy seedlings of mandarin (*Citrus reticulata*) plants (1-year-old) were further inoculated with this isolated organism and incubated for a period of 4 weeks for completion of Koch's postulate. Subsequently, the infected roots were collected, washed, cut into small pieces, treated with 0.1% $HgCl_2$ for 2-3 minutes,

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rewashed with sterile distilled water, transferred to PDA slants and incubated at 28°C. At the end of two weeks, the reisolated organism was examined, compared with the original stock culture and its identity was confirmed following microscopic observations as *Macrophomina phaseolina*. Mycelia – septate, branched, hyaline when young becoming brown with age. Advancing zone of mycelia mat even and appressed. Sclerotia – black, moderate size (34-78 μ in diameter), round or irregular uniformly reticulate with no difference in internal structure. The culture was maintained on PDA slants and stored under three different conditions (5°C, 20°C and 30°C (room temperature)) in sterile liquid paraffin. The culture was examined at a regular interval to test its viability and pathogenicity of the fungus.

Inoculation techniques and disease assessment

M. phaseolina was grown in sand maize meal medium supplemented with citrus root pieces, for inoculation of healthy mandarin seedlings as it increases the survival capacity and viability of the pathogen in the soil. Initially, *M. phaseolina* was grown on PDA in Erlenmeyer flasks (250ml) for 2 days. Subsequently 30 sterilized mandarin root pieces (one inch long) were transferred to each flask and incubated for 15 days. Sand maize meal medium (50g) containing five such pieces covered with the mycelia and sclerotia were inserted in the rhizosphere of each plant. The inoculated plants were examined at an interval of 7 days up to a period of 28 days. Each time, the plants were uprooted, washed and symptoms noted. Finally roots were dried at 60°C for 96h and weighed. Root rot index was calculated on the basis of percentage root area affected and they were graded into 6 groups and a value was assigned to each group (viz. no. root rot = 0; up to 10% root area affected = 0.10; 11-25% = 0.25; 26-50% = 0.50; 51-75% = 0.75; 76-100% = 1.0). The root rot index in each case was the quotient of the total values of the replicate roots and the number of roots (i.e. number of plants).

Preparation of antigen

Antigens were prepared from mycelia of *M. phaseolina*, healthy and artificially inoculated root tissue of mandarin plants following the methods as described by Chakraborty and Purkayastha (1983). They were stored at –20°C and used as mycelial and root antigens.

Production and purification of polyclonal antibody

New Zealand white male rabbits were used to raise polyclonal antibodies against mycelial antigens of *M. phaseolina* following the method of Chakraborty and Purkayastha (1983). Normal sera were collected from the rabbit by ears vein puncture before immunization. The antigen emulsified with an equal volume of Freund's complete/incomplete adjuvant was injected subcutaneously at weekly interval for six consecutive weeks. The blood samples were collected after six weeks following injection and kept for 1h at 30°C. The clots were loosened and stored at 4°C. The antisera were then clarified by centrifugation and stored at –20°C until required. IgGs were purified by DEAE-Sephadex column chromatography following the protocol of Clausen (1988).

Immunodiffusion

Agar gel double diffusion tests were performed using PAb raised against *M. phaseolina* following the method of Ouchterlony (1967).

PTA-ELISA

Optimization of ELISA was done using purified IgGs of known concentration which was predetermined using the referred formula. Goat antirabbit IgG (whole molecule) alkaline phosphatase (Sigma) conjugate (1:10000) and p-nitrophenyl phosphate (100 mg ml^{-1}) were used for PTA-ELISA as enzyme substrate (pNPP), reaction was terminated after 60 min and the absorbance values were recorded as mean of five adjacent wells measured at 405 nm essentially as described by Chakraborty and Sharma (2007). Antigens from fungal pathogen was diluted with coating buffer and IgGs were diluted to 1:125 with PBS-Tween containing 0.5% BSA. Goat antirabbit IgG (whole molecule) alkaline phosphatase (Sigma) conjugate and 4-nitrophenyl phosphate (pNPP) as enzyme-substrate, were used for ELISA tests. Absorbance values were measured at 405 nm in an ELISA reader (Multiskan EX, Labsystems). Absorbance values in wells not coated with antigens were considered as blanks.

Dot immunobinding assay

Mycelial antigens prepared *M. phaseolina* were loaded on nitrocellulose membrane filters using Bio-Dot apparatus (Bio-Rad). Dot immunobinding assay was performed using PAb of *M. phaseolina* as outlined by Lange *et al.* (1989).

Western Blotting

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

Immunofluorescence

PAbs of *M. phaseolina* and goat antisera specific to rabbit globulins conjugated with Fluorescein isothiocyanate (FITC) were used for indirect immunofluorescence study to detect the pathogen. Observations were made using a Biomed microscope (Leitz) equipped with an I3 filter block ideal for FITC fluorescence under UV light in the dark. Photographs were taken by Leica Wild MPS 48 camera on Kodak 800 ASA film.

Genomic DNA extraction

Genomic DNA was isolated from 4 day old fungal mycelia *M. phaseolina* by a modified method of Raeder and Broda 1985. Fungal mycelia from 3-4 days old cultures grown on potato dextrose broths was crushed with liquid nitrogen and incubated with lysis buffer containing 250 mM Tris-HCl (pH 8.0), 50 mM EDTA

(pH8.0), 100 mM NaCl and 2% SDS, for 4 h at 65°C followed by centrifugation at 12,000 rpm for 15 min. The supernatant was extracted with equal volume of water saturated phenol, centrifuged at 12,000 rpm for 15 min, and further extracted with equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) by centrifugation at 12000 rpm for 15 min; the aqueous phase was transferred in a fresh tube and chloroform (in the ratio of 1:4 v/v) was added followed by 0.5M Na-acetate (in the ratio of 1:10 v/v). Next, isopropanol was added to the above mixture (0.7 times the final volume) and centrifuged. DNA was precipitated from the aqueous phase with chilled ethanol (100%) and pelleted by centrifuging at 12000 rpm for 15 min followed by washing in 70% ethanol and centrifugation. The pellets were air dried and suspended in TE buffer pH 8.

Qualitative and quantitative estimation of DNA

The extraction of total genomic DNA as per the above procedure was followed by RNAase treatment. Genomic DNA was re-suspended in 100 µl 1 X TE buffer and incubated at 37°C for 30 min with RNAse (60µg). 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.

PCR amplification of ITS region and sequencing

Genomic DNA was amplified by mixing the template DNA (50 ng), with the polymerase reaction buffer, dNTP mix, primers and Taq polymerase. Polymerase Chain Reaction was performed in a total volume of 100 µl, containing 78 µl deionized water, 10 µl 10 X Taq polymerase buffer, 1 µl of 1U Taq polymerase enzyme, 6 µl 2 mM dNTPs, 1.5 µl of 100 mM reverse and forward primers. For amplification of the ITS regions of the ribosomal DNA primer pairs, ITS1 and ITS4 were used. PCR was programmed with an initial denaturing at 94°C for 5 min. followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 61°C for 30 sec and extension at 70°C for 2 min and the final extension at 72°C for 7 min in a Primus 96 advanced gradient Thermocycler.

PCR product (10 µl) was mixed with loading buffer (5ml) containing 0.25% bromophenol blue, 40 % w/v sucrose in water, and then loaded in 1.5% Agarose gel with 0.1 % ethidium bromide for examination with horizontal electrophoresis. Nucleotide base pairs of the amplicons were determined on the basis of its migration and conformation relative to the molecular size marker (1000 base pair, wide range DNA ladder, Genie,Bangalore) PCR products were sent for sequencing to Chromous biotech, Bangalore, India.

The sequenced PCR product was aligned with ex-type strain sequences from NCBI Gene Bank and established fungal taxonomy for identification. Sequences were aligned following the Clustal W algorithm included in the Megalign module (DNASTAR Inc.) Multiple alignment parameters used were gap penalty = 10 and gap length penalty = 10. Both of these values are aimed to prevent lengthy or excessive numbers of gaps. The default parameters were used for the pair wise 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 made manually in order to remove artificial gaps. Phylogenetic analyses were completed using the MEGA package (version 4.01; Institute of Molecular Evolutionary Genetics, University Park, PA). 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 inference was performed by the UPGMA method. Bootstrap tests with 1,000 replications were conducted to examine the reliability of the interior branches and the validity of the trees obtained (Felsenstein 1985). An additional standard error test was performed with the data set using the same characters in order to evaluate the statistical confidence of the inferred phylogeny. There were a total of 138 positions in the final dataset. Phylogenetic analyses were conducted in MEGA 4 as described by Tamura *et al.* (2007).

Results and Discussion

Charcoal root rot incidence of Citrus reticulata

Symptoms of Charcoal root rot caused by *M. phaseolina* were characterized by a gradual decay of the root tips, lateral roots and root crown. This gradual destruction of the root system causes the seedlings to become stunted and chlorotic, and finally to die. In some cases, the margin of the infected area breaks away from the healthy area and may curl back. Lesions can eventually girdle the entire tree trunk leading to the death of the tree. Mandarin seedlings were collected from eight different locations, viz. Rangali Rangliot, Bijanbari, Sukhia Pokhari, Kurseong, Mirik, Kalimpong Block I, Kalimpong Block II and Gorubahan and maintained in

the Glass house conditions. Charcoal root rot pathogen (*M. phaseolina*) isolated from mandarin orchards of Darjeeling hill was used for present study. after completion of Koch's postulate. Mycelia – septate, branched, hyaline when young becoming brown with age. Advancing zone of mycelia mat was even and appressed. Sclerotia – black, moderate size (34-78 u in diameter), round or irregular (Figure 1 G) uniformly reticulate with no difference in internal structure.

Healthy seedlings of mandarin (*Citrus reticulata*) plants (1-year-old) grown in earthenware pots were inoculated with this isolated organism and incubated for a period of 4 weeks. Pathogenicity of *M. phaseolina* was tested on twenty mandarin plants each of eight different locations. The inoculated plants were examined after 4 weeks. Colour of root, root rot index and percentage loss in dry weight of roots were noted. Young seedlings showed light brown discolouration of the root at the soil line initially which gradually turned dark brown to blackish brown and finally to black. In advanced stage of disease symptoms also appeared at ground level. Lower leaves turned yellow and remained attached, sometimes

showed wilting symptoms. In advanced stage defoliation of lower leaves were evident (Figure 1 H). When epidermis was removed, small, black bodies (sclerotia) were discerned. These propagating bodies were abundant enough to impart a grayish black colour like charcoal to the tissues. The root rot index as well as percentage loss in dry weight of roots were very low at the initial stage of infection which increased significantly with time in compatible interaction. Mandarin seedlings of three locations (Mirik, Kalimpong Block-I and Sukia Pokhri) were found to be highly susceptible (Table 1).

Culture filtrate of the pathogen (*M. phaseolina*) following two weeks growth in Richards' media at 28°C, was collected and the young seedlings of mandarin of three different locations ((Mirik, Kalimpong Block-I and Sukia Pokhri) which showed susceptible reaction, were further tested in *in vitro* conditions in comparison with sterile distilled water control (Figure 1 B-E). It is interesting to note that seedlings showed same symptoms in this case also. Wilting followed by chlorosis and browning reaction of green leaves were evident in seedlings grown in culture filtrate of the pathogen. Wilting symptoms first appeared 7 days after treatment. However within two weeks leaves of all the seedlings turned into brown colour.

Cultural conditions affecting growth of the pathogen

Macrophomina phaseolina infect mandarin plant roots and their interactions affect the development of root rot disease. Initially it was considered worthwhile to study the effects of some major factors such as incubation time, temperature and pH of substrate on growth of the pathogen *in vitro*.

Effect of incubation time.

The effect of incubation time on the growth of *M. phaseolina* was studied *in vitro*. *M. phaseolina* was grown in Richard's media for a period of 24 days at 28°C. Mycelial growth of the fungus was recorded after 2,4,8,12,16,20, and 24 days. The results are embodied in Table 2. Maximum growth of *M. phaseolina* (755 mg) was observed after 12 days of incubation and then the rate of growth declined. Mycelial growth increased by 24% from 8 to 12 days of incubation and decreased by 5% from 12 to 16 days (Table 2).

Effect of pH on growth.

It is well known that the pH of the medium usually plays an important role in the growth of microorganisms. The utilization of nutrients depends partially upon the pH of the culture medium. Therefore, it was considered imperative to use a buffer system to stabilize the pH of the culture medium during incubation. In the present study, buffer solutions with pH values ranging from 4-8 (4.0, 5.0, 6.0, 7.0 and 8.0) were prepared by mixing KH_2PO_4 and K_2HPO_4 each at a concentration of M/30. The pH of the medium was adjusted using N/10 NaOH or N/10 HCl to obtain the corresponding range of pH values (4.0-8.0). Both the medium and the phosphate buffer were sterilized. Equal parts of the buffer solution and medium were mixed before use. Each flask containing 50ml of the medium was inoculated with fungus and incubated for 12 days at 28°C. The results are given in Table 3. It appears that *M. phaseolina* grew well over a range of pH (4.0-8.0) and optimum growth was recorded at pH 5.5. It is necessary to mention that mycelia growth increased up to pH 5.5 and then gradually declined.

Table 1: Pathogenicity test of *Macrophomina phaseolina* on different root samples of *Citrus reticulata*

Locality of saplings	* Root rot index	**Colour intensity
Rangli Rangliot	0.10	+
Bijanbari	0.25	++
Sukhia Pokhari	0.75	++++
Kurseong	0.25	++
Mirik	0.75	++++
Kalimpong Block I	0.75	++++
Kalimpong Block II	0.50	+++
Gorubathan	0.50	+++

* On the basis of root area affected; 0-10% (0.10); 11-25% (0.25); 26-50% (0.50); 51-75% (0.75); 76-100% (1.0). ** + Light brown, ++ Deep brown , +++ Blackish brown, ++++ Black

Table 2: Effect of incubation time on growth of *M. phaseolina*

Incubation Time (days)	Average dry weight of mycelia (mg)
2	97.20 ± 2.24
4	214.35 ± 4.44
8	618.00 ± 3.72
12	755.00 ± 1.81
16	717.52 ± 1.58
20	689.32± 2.86
24	653.62± 2.43

Average of 3 replicates/treatments; Temperature 28°C; pH of medium – 5.4

Effect of temperature on growth

Temperature is also a major factor affecting growth of a pathogen. Therefore, the effects of different temperatures (15, 20, 25, 28, 30, 35, 40°C) on growth of *M. phaseolina* was studied *in vitro*. Maximum mycelial growth was noted at 30°C with a decline at 40°C (Table 4).

Immunological assays

Serological assays were performed using Polyclonal antibodies (PAb) raised against mycelial protein of *M. phaseolina*. Effectiveness of antigen in raising antibodies were checked initially using agar gel double diffusion technique. Strong precipitin reactions were noticed in homologous reaction with antigen and antibody raised against *M. phaseolina* (Figure 1). Dot immunobinding assay using mycelia antigen and PAb of *M. phaseolina* was also carried out. For this, soluble protein obtained from seven-day-old mycelia of *M. phaseolina* were reacted on nitrocellulose paper with

PAb of the pathogen (*M. phaseolina*). Results shows development of deep violet colour indicating a positive reactions suggestive of effectiveness of mycelial antigen in raising PAb against the pathogen (Figure 1 J). Optimization of ELISA was done by considering two variables, dilution of the antigen extract and dilution of the antiserum to obtain maximum sensitivity (Figure 2).

Root antigens were prepared from healthy as well as artificially inoculated plants of *C. reticulata*. Three days and seven days following inoculation with *M. phaseolina*, root antigens were prepared along with healthy root antigens and reacted with PAbs of *M. phaseolina* for comparison. Absorbance values were higher in those root samples which showed susceptible reaction when tested against root pathogens. Following inoculation with the pathogens absorbance values were always higher in artificially inoculated plant roots in comparison with healthy root antigens when tested against PAbs of the respective pathogens (Table 5).

Western blot analysis using PAb of *M. phaseolina* was also performed to develop strategies for rapid detection of the pathogen. For this total soluble protein of young mycelia was used as antigen source and SDS-PAGE was performed as described previously followed by probing of the localized antigen with alkaline phosphatase conjugate. The bands on nitrocellulose membrane was compared with corresponding protein bands on the SDS-PAGE. Bands of varying intensities was observed ranging from 14 KDa to 95 KDa (Figure 1. K&L). Bands of lower molecular weight were more in number. Hence the result suggests that Western blot formats could be used as one of a refined tool for detection of pathogen. Indirect immunoflorescence of hyphae and young sclerotia of *M. phaseolina* were conducted with homologus antibody (PAb of *M. phaseolina*) and reacted with fluorescein isothiocyanate (FITC) labeled antibodies of goat specific for rabbit globulin. Strong apple green fluorescence were evident in both mycelia and scletoria which confirmed the detection of the pathogen (Figure 1. M&N).

Identification of M. Phaseolina using rDNA sequence

Genomic DNA of *M. phaseolina*, the isolate obtained from mandarin root tissue collected from Mirik orchard, the causal organism of root rot of *Citrus reticulata* was amplified. Main focus was on the ITS regions of ribosomal genes for the construction of primers that can be used to identify *M. phaseolina*. ITS region of rDNA was amplified using genus specific ITS-1 and ITS4 for sequencing of its 18S rDNA region. PCR products produced sequences and chromatogram and 18S rDNA sequence of *M. phaseolina* that could be aligned and showed satisfactory homology with ex-type strain of *M. phaseolina* sequences from the NCBI Genbank data base. The priming site of the ITS1 and ITS4 primers were determined in order to confirm that the sequences obtained corresponded to the actual ITS 1 region. ITS1 showed the highest number of nucleotide substitutions, and it was used for the phylogenetic study.

Phylogenetic analysis

Studies involving isolates of *M. phaseolina* revealed that the partial sequence of ITS1- 5.8S-ITS4 rRNA gene is as variable as rDNA regions. The sequence information was then analysed through BLASTn program which indicated that the sequences contain the genetic information of internal transcribed spacer region of rDNA gene of *M. phaseolina* with 100% similarity. This sequence has been deposited to NCBI genebank to get accession number.

Identified *M. phaseolina* rDNA gene sequences obtained from NCBI genebank of various host plants were selected for comparison with the rDNA gene sequence of *M. phaseolina* isolate of mandarin plant. The sequence alignment of the isolate of *M. phaseolina* shows variation in this gene. These available sequences of *M. phaseolina* from NCBI were used in the pair wise and multiple sequence alignment using Bioedit software (Figure 3) for determining the conserved regions of rDNA gene. This partial sequence was deposited to NCBI database (Acc. No.JN241996) (Table 6).

Multiple sequence alignment revealed that there were quite a number of gaps introduced in the alignment within the ITS region which were closely related. Similar sequence indicated that the isolates were closely related. From the sequence alignment, variations were observed between other *M. phaseolina* isolates in species level. Multiple and pair wise sequence alignment were generated and used to calculate evolutionary distances and percent of sequence similarity values (Table 7) and to construct a phylogenetic tree.

The evolutionary history was inferred using the UPGMA method. The optimal tree with the sum of branch length = 0.56368608 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. Codon positions included were 1st+2nd+3rd+ Noncoding. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There were a total of 294 positions in the final dataset. Phylogenetic analyses were conducted in MEGA4 (Figure 4).

PAbs raised against *M. phaseolina* was tested with homologous and heterologous antigens of mandarin roots. Strong precipitation reactions occurred in homologous reactions in immunodiffusion test. Among the root antigens of mandarin plants of eight different locations tested against PAbs of *M. phaseolina*, strong and positive reactions were noticed in root antigens of four specific locations. Previous studies have also suggested that common antigens may be indicators of plant host-parasite compatibility (Chakraborty, 1988). Optimization of ELISA was done by considering two variables, dilution of the antigen extract and dilution of the antiserum to obtain maximum sensitivity. ELISA values increased with a concomitant increase of antigens concentrations. Concentration as low as 25 µg/L, could

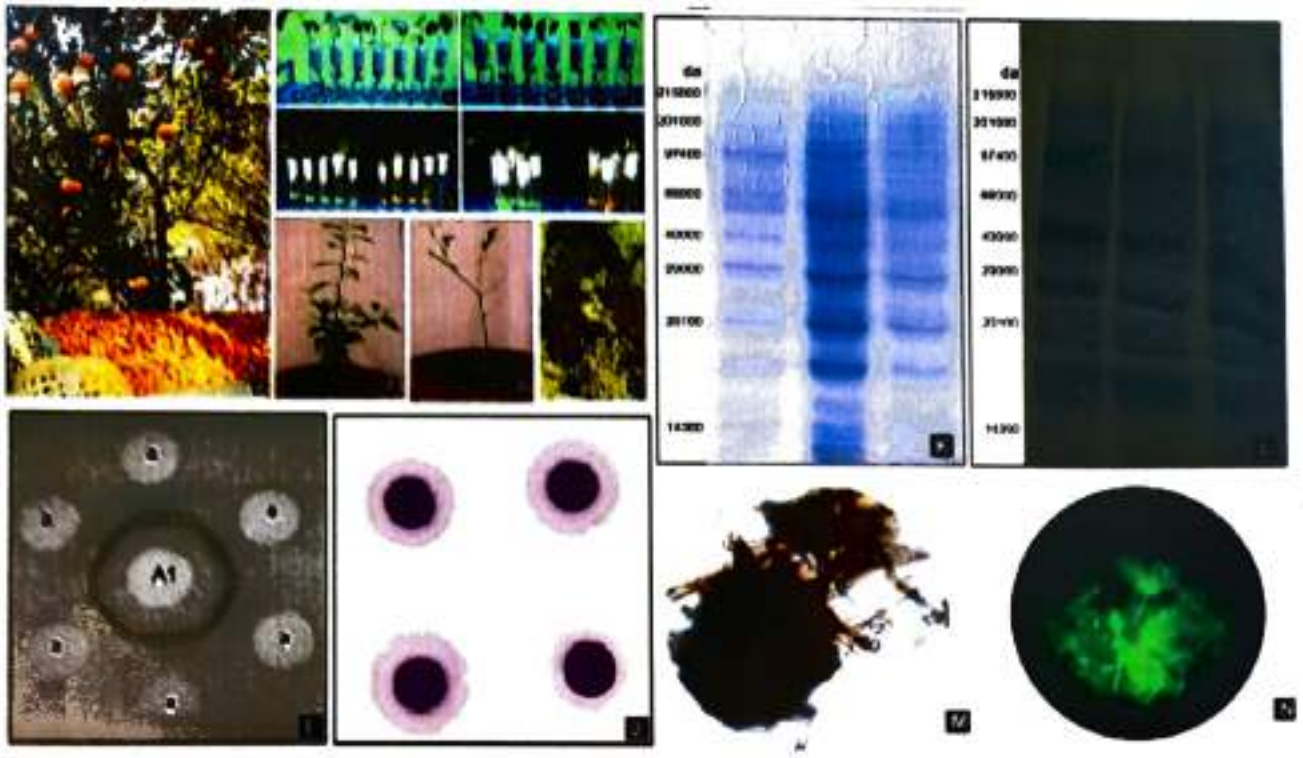

Figure 1: *C. reticulata* in Mirik orchard and harvested mandarin fruits in market place (A). Mandarin seedlings grown in distilled water (B, D & E - left) and culture filtrate of *M. phaseolina* (C, D & E - right); Healthy mandarin plants in pots (F), Infected mandarin plants showing symptoms 30 days following artificial inoculation with *M. phaseolina* (G); *M. phaseolina* (root rot pathogen) (H). Immunodiffusion (I, Dot immunobinding assay (J), SDS PAGE analysis (K) and Western blot analysis(L) of *M. phaseolina* using mycelial antigen (a) and PAb of M. phaseolina (A1); Microscopic observation of *M. phaseolina* under bright field (M). Mycelia of *M. phaseolina* treated with PAb of the pathogen and labeled with FITC (N)

Table 3: Effect of different pH on the growth of *M. phaseolina*

pH	Average dry weight of mycelia (mg)
4.0	310.50 ± 2.33
4.5	581.25 ± 3.40
5.0	616.00 ± 3.12
5.5	695.00 ± 2.61
6.0	437.55 ± 2.53
6.5	409.32± 3.86
7.0	325.12± 2.63
8.0	310.33± 1.95

Average of 3 replicates/treatment; Temperature 28°C; Incubation time – 12 days

Table 4: Effect of different temperature on the growth of *M. phaseolina*

Temperature (°C)	Average dry weight of mycelia (mg)
15	95.00 ± 2.43
20	181. 05 ± 2.40
25	246.00 ± 3.22
28	595.00 ± 2.82
30	737.55 ± 3.53
35	665.42± 2.85
40	125.25± 3.63

Average of 3 replicates/treatment; pH adjusted to 5.5 ; Incubation time – 12 days

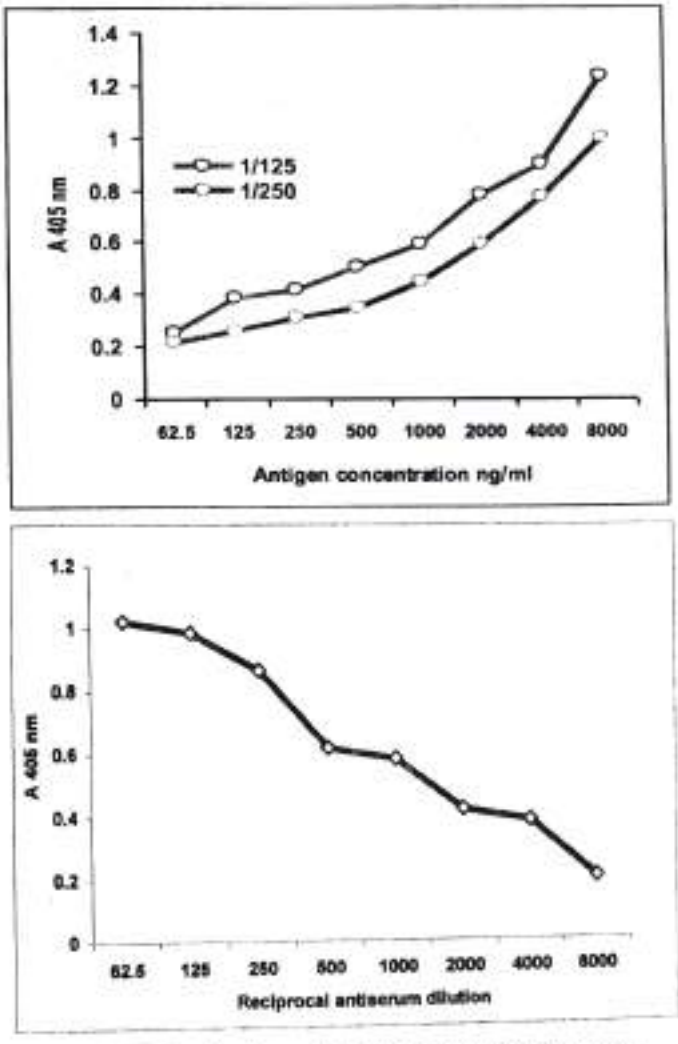

Figure 2: Optimization of ELISA by considering two variables, dilution of the antigen extract (A) and dilution of the antiserum (B)

Table 5: PTA-ELISA values showing reaction of PAbs of *M. phaseolina* with antigens of healthy and inoculated roots of *C. reticulata*

Citrus saplings	Antigen concentration (40 mg/L)	
Locality	Healthy	Inoculated
		M.phaseolina
Rangli Rangliot	0.812	1.264
Bijanbari	0.890	1.149
Sukhia Pokhari	1.115	1.774
Kurseong	0.972	1.880
Mirik	1.064	1.993
Kalimpong Block I	1.007	1.887
Kalimpong Block II	1.187	1.932
Gorubathan	0.938	1.872

PAb of *M. phaseolina* was used at 1:125 dilution; 7 days after inoculation; absorbance at 405 nm

Table 6: NCBI GenBank sequences of *M. phaseolina*

Acc. No.	Sequences	Country
JN241996	**310bp**	**India**
DQ314733	527 bp	India
DQ233666	495 bp	India
DQ233664	441 bp	India
DQ233663	519 bp	India
DQ233662	432 bp	India
EU250575	582 bp	China
HQ713771	511 bp	Switzerland
HQ380051	685 bp	Turkey
HM990163	534 bp	India
EF446288	562 bp	India
HQ660591	583 bp	China
HQ660590	584 bp	China
HQ660589	583 bp	China
JF710587	583 bp	China
FJ960442	582 bp	China
EF570501	642 bp	Canada

be easily detected by ELISA, in both antisera dilutions. In time course experiment involving artificial inoculation of roots with the fungal pathogen (s).infection could be detected from 20h onwards in ELISA on the basis of significantly higher ($p = 0.01$) absorbance values of infected root extracts in comparison with healthy root extracts. Absorbance values in PTA-ELISA were also significantly higher for infected root extracts than for healthy controls up to a concentration of 2mg/L. Kitagawa *et al.* (1989) successfully used a competitive ELISA technique to develop an assay to identify *F. oxysporum* f. sp. *cucumerinum* among other *Fusaria*. Mycelial antigen of the pathogen was analysed on SDS-PAGE and then western blot analysis was done using homologous PAb.

Amplification of target DNA through PCR with sequence specific primers is potentially more sensitive and rapid than microbiological techniques, as a number of constraints are removed. Unlike culture, PCR does not require the presence of viable organisms for success and may be performed even when sample volumes are small. Differences in the nucleotide composition of the variable ITS region have been successfully employed to

Table 7: Similarity of rDNA sequences within groups of *M. phaseolina*

	JN241996	DQ233663	HM990163	HQ660594	HQ660593	HQ660592	HQ660591	HQ660590	HQ660589	JF710587	DQ233662	EF446288	HQ713771	J960442	EU250575	EF570501	DQ314733	DQ233666	DQ233664	HQ380051
JN241996																				
DQ233663	0.26																			
HM990163	0.26	0.55																		
HQ660594	0.26	0.55	0.00																	
HQ660593	0.22	0.55	0.00	0.00																
HQ660592	0.25	0.60	0.00	0.00	0.00															
HQ660591	0.23	0.47	0.00	0.00	0.01	0.01														
HQ660590	0.34	0.27	0.10	0.10	0.11	0.13	0.07													
HQ660589	0.26	0.52	0.00	0.00	0.01	0.01	0.00	0.09												
JF710587	0.26	0.55	0.00	0.00	0.00	0.00	0.00	0.10	0.00											
DQ233662	0.26	0.55	0.00	0.00	0.00	0.00	0.00	0.10	0.00	0.00										
EF446288	0.25	0.42	0.01	0.01	0.02	0.02	0.00	0.04	0.01	0.01	0.01									
HQ713771	0.32	0.67	0.01	0.01	0.01	0.01	0.02	0.14	0.02	0.01	0.01	0.03								
J960442	0.33	0.43	0.03	0.03	0.04	0.05	0.02	0.02	0.03	0.03	0.03	0.01	0.05							
EU250575	0.26	0.55	0.00	0.00	0.00	0.00	0.00	0.10	0.00	0.00	0.00	0.01	0.01	0.03						
EF570501	0.26	0.55	0.00	0.00	0.00	0.00	0.00	0.10	0.00	0.00	0.00	0.01	0.01	0.03	0.00					
DQ314733	0.99	0.15	0.94	0.94	0.98	1.02	0.93	0.49	0.99	0.94	0.94	0.76	1.11	0.72	0.94	0.94				
DQ233666	0.26	0.55	0.00	0.00	0.00	0.00	0.00	0.10	0.00	0.00	0.00	0.01	0.01	0.03	0.00	0.00	0.94			
DQ233664	0.26	0.55	0.00	0.00	0.00	0.00	0.00	0.10	0.00	0.00	0.00	0.01	0.01	0.03	0.00	0.00	0.94	0.00		
HQ380051	0.26	0.55	0.00	0.00	0.00	0.00	0.00	0.10	0.00	0.00	0.00	0.01	0.01	0.03	0.00	0.00	0.94	0.00	0.00	0.00

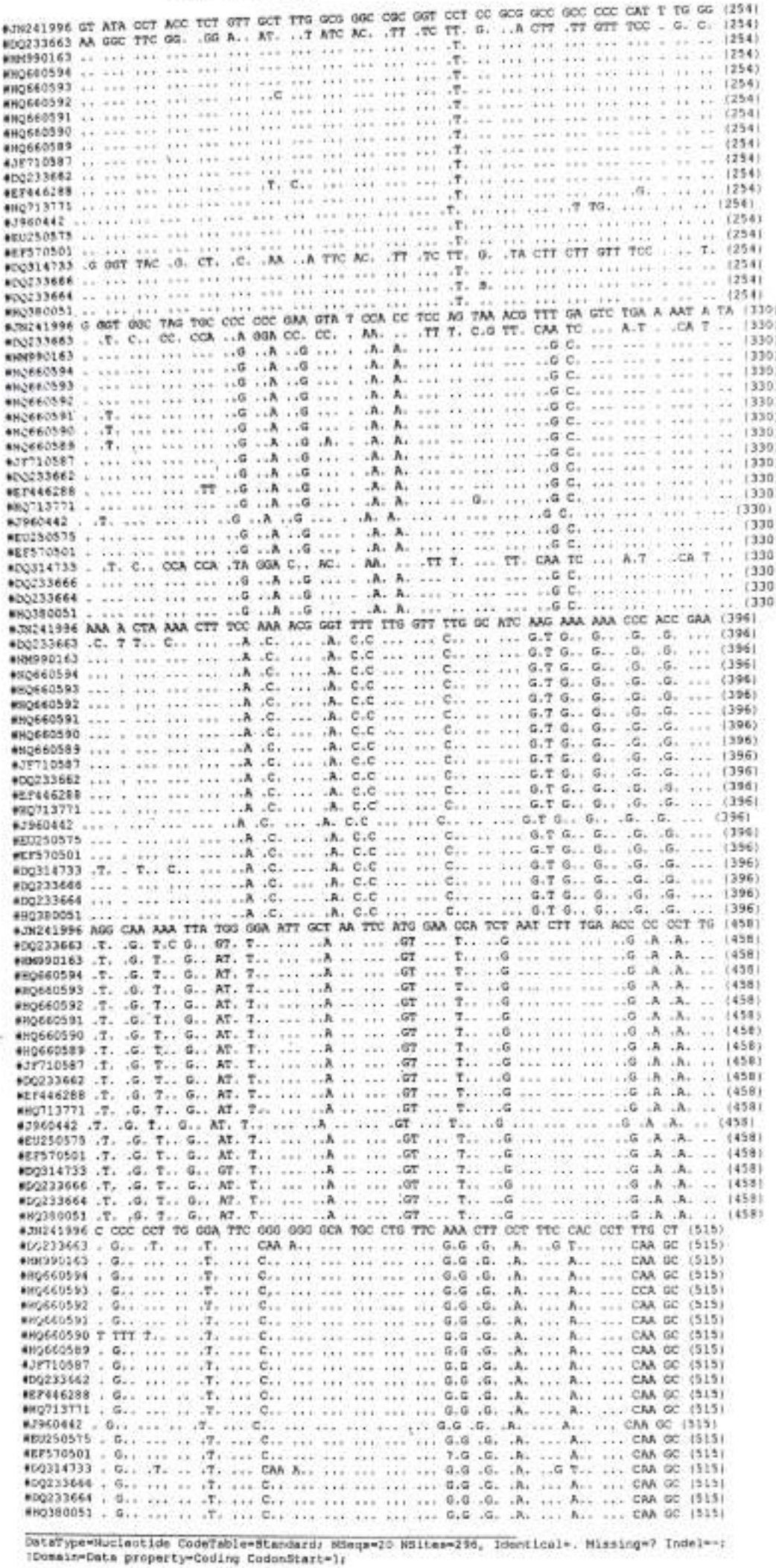

Figure 3: Nucleotide sequence alignments of the parts of the rDNA repeats encoding ITS region of different isolates of *M. phaseolina* used for analysis

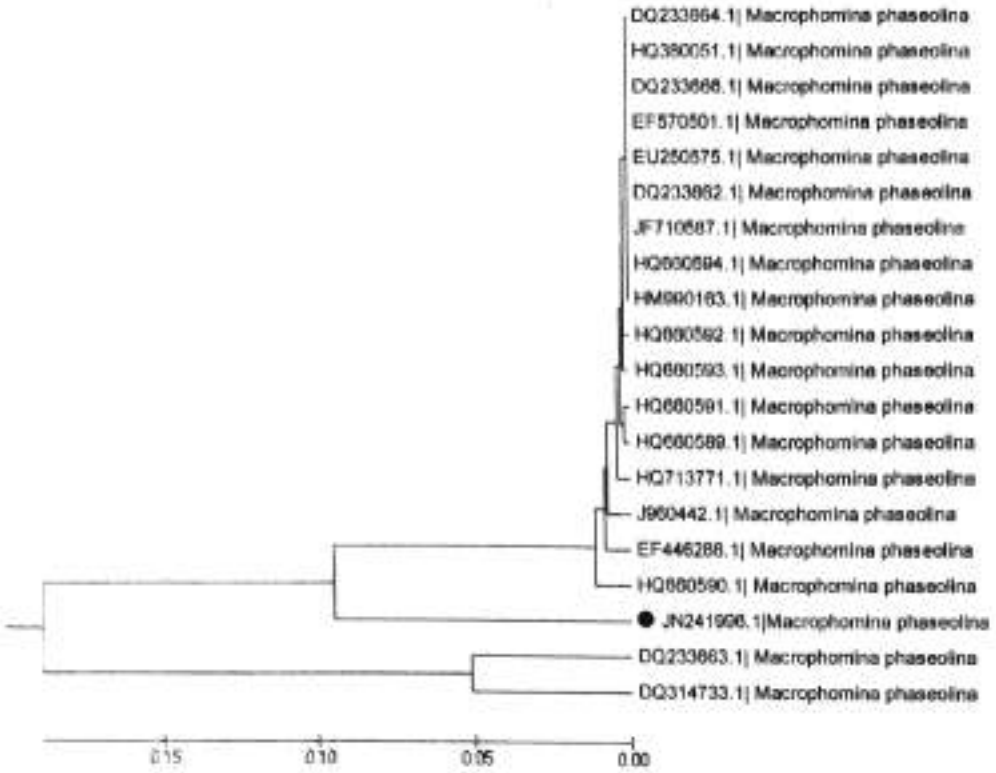

Figure 4: Phylogenetic placement of *Macrophomina phaseolina* (RHS/S565) with extype strains from NCBI genebank

design specific primer sets that amplify DNA selectively among and within species of plant pathogens (Nazar *et al.* 1991, Moukahmedov *et al.* 1994, Schilling *et al.* 1996, Moricca *et al.* 1998). In the broader context, taxon -selective amplification of ITS regions is likely to become a common approach in molecular identification strategies. ITS regions have been used successfully to generate specific primers capable of differentiating closely related fungal species (Bryan *et al.* 1995). These rDNA are highly stable and exhibit a mosaic of conserved and diverse regions within the genome (Hibbett 1992). They also occur in multiple copies with up to 200 copies per haploid genome (Bruns *et al.* 1991) arranged in tandem repeats with each repeat consisting of the 18S small subunit (SSU), the 5.8S, and the 28S large subunit (LSU) genes.

In the present study, ITS regions of ribosomal genes for the construction of primers were used to identify *M. phaseolina*. ITS region of rDNA was amplified using genus specific ITS-1 and ITS4 primers for *M. phaseolina*. Amplified products of size in the range of 550bp were produced by the primer pairs.

Amplification of DNA fragments of *M. phaseolina* with specific primers indicate the usefulness of molecular technique for their detection and identification. Using the specific primers ITS 1 and ITS 4, only a single band of 550 bp was generated in the amplification pattern of all the isolates. *M. phaseolina* as first described by (Pearson *et al.* 1986) suggested that isolates from one specific host are more suited to colonize it. Later, differences in pathogenicity among the isolates of soybean and sorghum have been observed (Cloud and Rupe 1991). This has been further confirmed with isolates from soybean, sorghum and cotton (Su *et al.* 2001). Isolates were clearly grouped according to the host origin. Additionally, no molecular variation could be observed among the isolates tested in PCR of the ITS region. The overall study was established the rDNA gene sequence of *M. phaseolina* a root rot pathogen of *C. reticulata* based on the bioinformatics tools with sequence analysis of ITS regions of the rDNA gene for rapid identification and development of rDNA markers for analysis of genetic variability within the outgrops. We have demonstrated that the analysis of aligned rDNA sequences is a reliable clustering strategy for identification purposes in a variety of taxonomic groups and systemic levels. While this approach was previously applied in analyzing complete genome data, the present study shows that it is also applicable in analyzing much shorter DNA sequences from a single gene, which is going to be the fundamental block in the massive rDNA database. Cloud and Rupe (1991) working with isolates of soybean and sorghum, also observed differences in pathogenicity. This has been further confirmed with isolates from soybean, sorghum and cotton (Su *et al.* 2001). Isolates were clearly grouped according to the host origin. Additionally, no molecular variation could be observed among the isolates tested in PCR of the ITS region.

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