Journal of Yeast and Fungal Research Vol. 2(5), pp. 65 - 74, May 2011
Available online http://www.academicjournals.org/JYFR
ISSN 2141-2413 ©2011 Academic Journals

Full Length Research Paper

Physiological variability among isolates of *Phomopsis azadirachtae* from Tamil Nadu

K. Girish[1,2]* and S. Shankara Bhat[1,3]

[1]Department of Studies in Microbiology, Manasagangotri, University of Mysore, Mysore – 570 006, Karnataka, India.
[2]Postgraduate Department of Microbiology, Maharani's Science College for Women, JLB road, Mysore - 570 005, Karnataka, India.
[3]Labland Biodiesel Private Limited, # 98, 7th main, Jayalakshmipuram, Mysore -570 012, Karnataka, India.

Accepted 16 May, 2011

16 isolates of *Phomopsis azadirachtae*, causing die-back of neem were isolated from 16 different geographical regions of Tamil Nadu. Their growth response to physical (temperature and pH) and chemical (carbon and nitrogen sources) factors were investigated. The toxicity of culture filtrate of each isolate on neem seed germination was also studied. Several isolates exhibited maximum growth at 35°C. Maximum growth of all the isolates was observed at pH 6.0. Many isolates preferred starch and a few, sucrose, as carbon sources. Ammonium sulphate formed the good nitrogen source for most of the isolates. However, all the isolates displayed significant differences among themselves in their growth response to each treatment. The culture filtrate of each isolate inhibited the germination of neem seeds but exhibited varying degree of toxicity. The results obtained suggest the existence of physiological variability within *P. azadirachtae*.

Key words: Die-back of neem, carbon source, culture filtrate, isolates, nitrogen source, pH, *Phomopsis azadirachtae,* temperature, variation.

INTRODUCTION

Fungi are known to vary in their morphology, reproduction, phylogeny and occupy varied ecological niches (Manoharachary, 2000). Plant pathogenic fungi are heterogeneous group of organisms and exhibit an enormous diversity in their life-history strategies. The host variety and prevailing environmental conditions influence the development of pathogenic variability (Moore, 1996). Studying the plant pathogen variation is a basic requirement to understand evolution, life-cycles and population dynamics of the pathogen (Thakur, 1999). Pathogen variability affects the effective control of the disease incited by that pathogen. Variability in pathogen populations provides them the capacity to invade a wide variety of host plants, thereby reducing the possibility of evolving disease management methods (Moore, 1996). The primary objective of studies on variability among pathogen populations is to breed resistant host varieties for effective disease management. Variation studies help to know the genetic constitution of host and can be used for deployment of resistant genes (Fathima, 2004; Roustaee et al., 2000). Phytopathogenic fungi exhibit intraspecific variations (Appiah et al., 2003; Mishra and Raj, 1992). Physiological variations among many fungal species were reported (Cairney, 1999; Higley and Tachibana, 1987). Physical, chemical or nutritional factors influence the growth and physiological process of fungi and in inter-related ways (Moore, 1996). Fungi for their heterotrophic life require carbon in large quantities as an essential macro-element and they can utilize many organic compounds. Carbohydrates constitute the major source of carbon (Moore, 1996). Another essential macro-element required for fungal growth, next to carbon in quantity is the nitrogen. Fungi may utilize nitrates and nitrites (inorganic nitrogen) or amino acids (organic nitrogen) for their growth (Deacon, 1997).

Pathogenic fungi exhibit variation in their requirement of carbon and nitrogen, both qualitatively and quantitatively (Gurgel et al., 2002; Mo et al., 2005). Fungi can grow in wide temperature range (Deacon, 1997). Fungi

*Corresponding author. E-mail: girishk77@yahoo.com Tel: +91-9743665772.

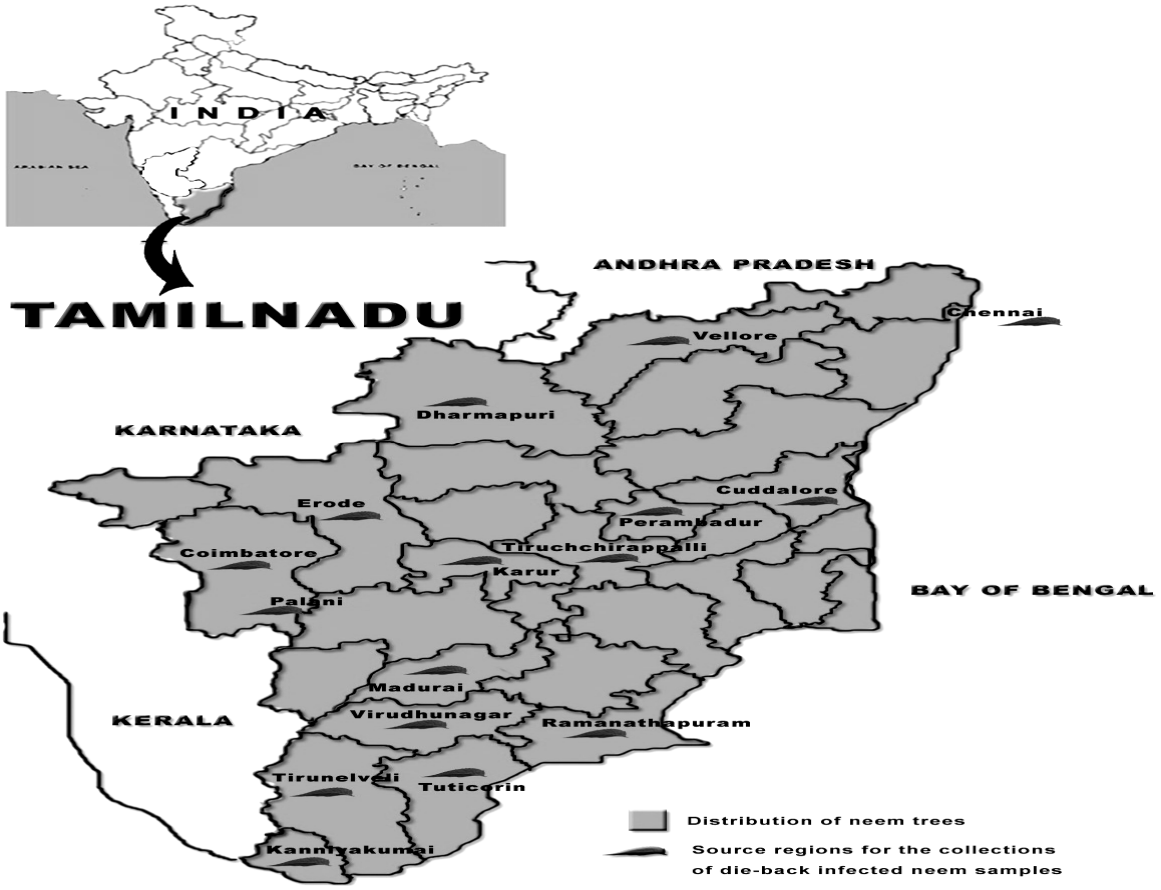

Figure 1. Tamil Nadu map showing source regions for the collection of die-back affected neem twigs.

generally grow well in acidic conditions (Dix and Webster, 1995), but some species favour neutral to slightly alkaline conditions (Yamanaka, 2003). Isolates of many fungal species differ in their response to pH and temperature (Fargues et al., 1997; Hung and Trappe, 1983). Phytopathogenic fungi produce a wide variety of phytotoxins (Geraldo et al., 2006; Walker et al., 2001). Fungal isolates vary in their toxigenic behaviour (Bamba and Sumbali, 2006; Horn et al., 1996). Intraspecific variations were reported in many *Phomopsis* spp. (Brayford, 1990; Schilder et al., 2005). *Phomopsis azadirachtae* Sateesh, Bhat and Devaki (family: Sphaeropsidaceae), the causal organism of die-back of neem (Sateesh et al., 1997) is wide spread and found in almost all neem growing regions of India (Girish and Shankara Bhat, 2008). Cultural, morphological and biochemical variations were observed among the *P. azadirachtae* isolates from different regions of Karnataka state, India (Fathima, 2004). Mycelial protein profiles of *P. azadirachtae* isolates have also revealed high polymorphism among the isolates (Fathima et al., 2004; Girish et al., 2009). However, physiological variability in *P. azadirachtae* is not well documented.

The purpose of the present study was to understand physiological variability among *P. azadirachtae* isolates collected from 16 different geographical locations of Tamil Nadu, South India.

MATERIALS AND METHODS

Sites sampled

Die-back affected neem twigs were collected from 16 different agroclimatic regions of Tamil Nadu, South India such as Chennai, Coimbatore, Cuddalore, Dharmapuri, Erode, Kanniyakumari, Karur, Madurai, Palani, Perambalur, Ramanathapuram, Tiruchchirappalli, Tirunelveli, Tuticorin, Vellore and Virudhunagar (Figure 1), separately in polythene bags. The twigs were used immediately after bringing to the lab or otherwise kept at 4°C until further study.

Isolation of *P. azadirachtae* from infected neem twigs

The neem twigs (both healthy and infected) were cut into 1 to 1.5 cm segments using sterile blades, with middle transition zone of healthy and infected portion in the case of infected twigs. Healthy twigs were used as control. The bark was removed and the segments were washed thoroughly with running tap water, surface-sterilized using sodium hypochlorite solution (with 5% available chlorine) and washed 5 times with sterile distilled water. The surface-sterilized twig segments were placed aseptically at the rate

of three segments per plate in Petri dishes containing potato dextrose agar (PDA, Himedia, Mumbai, India) amended with 100 ppm of chloramphenicol (20 ml/plate) (Fathima, 2004; Sateesh, 1998). The inoculated plates were incubated at 26 ± 2°C with 12 h photoperiod for 10 days and observed for the growth of the pathogen from the twig segments. The incubation was continued for 15 days to allow sporulation. The spores were identified microscopically and the presence of *P. azadirachtae* was confirmed as per Sateesh et al. (1997). All the isolates were subcultured on to fresh PDA plates by transferring 5 mm mycelial-agar disc drawn from the margin of mycelial mat of *P. azadirachtae* and incubating as mentioned earlier. Three replicates of each isolate were maintained.

Effect of physical and chemical factors on the growth of *P. azadirachtae* isolates

Growth of *P. azadirachtae* was determined by dry weight method (Dhingra and Sinclair, 1995). Liquid basal medium (LBM) was prepared using potassium nitrate (2.0 g), potassium dihydrogen phosphate (1.0 g), magnesium sulphate (0.5 g), ferrous sulphate (0.05 g), glucose (20.0 g) and double distilled water (1 L). The pH of the medium was adjusted to 6.0 using either 0.1 N NaOH or 0.1 N HCl (Deacon, 1997; Sateesh, 1998). The medium was amended with 100 ppm of chloramphenicol. To study the effect of temperature potato dextrose broth (PDB, Himedia, Mumbai, India) amended with 100 ppm of chloramphenicol was used. 75 ml of medium was taken in 250 ml Erlenmeyer flasks and autoclaved for 20 min. After cooling the medium to RT, the flasks were inoculated with 5 mm mycelial-agar disc drawn from the margin of mycelial mat of 7-day-old culture of each *P. azadirachtae* isolate separately and incubated aerobically in a controlled environment incubator shaker at 26°C and 25 rpm for 25 days. Whatman No. 1 filter papers were placed in an oven at 70°C for 24 h and then cooled to RT in a desiccator. The initial weights of filter papers were recorded separately. After incubation period, the mycelial mats were collected on to these pre-weighed filter papers by suction filtration using Buchner funnel. The mycelial mat on filter paper was gently washed twice with distilled water. Then it was air-dried for 12 h and later oven-dried at 70°C till a constant weight was obtained. This was taken as final weight and actual mycelial dry weight was calculated by subtracting the initial paper weight from the final weight. All the experiments contained three replications and the experiment was repeated thrice.

Effect of temperature on the growth of *P. azadirachtae* isolates

PDB was prepared, autoclaved and inoculation was carried out as mentioned earlier. The flasks were incubated at different temperatures namely, 20, 25, 30, 35 and 40 ± 1°C in an incubator for 25 days. Then the mycelial dry weight was calculated as mentioned earlier.

Effect of pH on the growth of *P. azadirachtae* isolates

The pH of liquid basal medium in Erlenmeyer flasks was adjusted to 4.0, 5.0, 6.0, 7.0 and 8.0 separately by using either 0.1 N NaOH or 0.1 N HCl before autoclaving. The pH value in each flask was checked again after autoclaving. All the flasks were inoculated, incubated and the mycelial dry weight was calculated as mentioned earlier.

Effect of carbon sources on the growth of *P. azadirachtae* isolates

The liquid basal medium was amended with 2% of cellobiose, dextrose, sorbitol, starch and sucrose separately. All the media were transferred to different flasks, autoclaved and all the flasks were inoculated, incubated and the mycelial dry weight was calculated as mentioned earlier.

Effect of nitrogen sources on the growth of *P. azadirachtae* isolates

Different nitrogen sources such as ammonium sulphate (2.0 g), L-asparagine (2.0 g), potassium nitrate (3.033 g), sodium nitrite (2.07 g) and urea (0.9 g) were added to a liter of liquid basal medium separately so as to give the same amount of nitrogen (Sateesh, 1998). All the media were transferred to different flasks, autoclaved, and all the flasks were inoculated, incubated and the mycelial dry weight was calculated as mentioned earlier.

Effect of culture filtrates of *P. azadirachtae* isolates on germination of neem seeds

100 ml of PDB in 250 ml Erlenmeyer flask was inoculated with 5 mm diam. mycelial agar disc of each *P. azadirachtae* isolate separately. The inoculated flasks were incubated for 25 days at 26 ± 2°C with 12 h photoperiod. Then the culture broth was filtered using Whatman No. 1 filter paper and the culture filtrate of each isolate was collected separately. The culture filtrates of each isolate thus obtained were filter-sterilized using 0.45 µm membrane filter discs (Sartorius, Goettingen, Germany) and used immediately or maintained at 4°C. Freshly harvested healthy neem fruits were washed and the pulp was removed. The seeds were then washed with running tap water and air-dried at RT for 24 h. Later, the seeds were soaked in water for 10 to 15 min. The hard endocarp was dissected out and the seeds were collected, washed thoroughly with water and surface-sterilized using sodium hypochlorite solution (with 5% available chlorine) for 15 min. Then the seeds were rinsed well in sterile distilled water for 5 times. Healthy surface-sterilized neem seeds were treated separately by incubating 100 seeds for 24 h in 25 ml of culture filtrate of each isolate taken in 100 ml beakers. The control treatment included medium alone and sterilized distilled water. The seeds were plated on sterile moist blotter in Petri plates and on standard paper towels (ISTA, 1993) and incubated at RT with natural alternate day and night photoperiod for germination. Each treatment had 4 replications.

After 15 days of incubation shoot length, root length and percentage germination were recorded. Vigour index was calculated using the formula given by Abdul-Baki and Anderson (1973).

RESULTS

Isolation of *P. azadirachtae* from infected neem twigs

The pathogen was isolated from all the infected neem twig samples collected from different places of Tamil Nadu and the isolates were designated as mentioned in Table 1. All the isolates produced characteristic alpha (α)

Table 1. Designation of *P. azadirachtae* isolates collected from different regions of Tamil Nadu, India.

Place of isolation	Designation of isolate
Chennai	TN 01
Coimbatore	TN 02
Cuddalore	TN 03
Dharmapuri	TN 04
Erode	TN 05
Kanniyakumari	TN 06
Karur	TN 07
Madurai	TN 08
Palani	TN 09
Perambalur	TN 10
Ramanathapuram	TN 11
Tiruchchirappalli	TN 12
Tirunelveli	TN 13
Tuticorin	TN 14
Vellore	TN 15
Virudhunagar	TN 16

and beta (β) – conidia.

Effect of temperature on the growth of *P. azadirachtae* isolates

Significant differences among the isolates were found for each temperature and between temperatures for each isolate. The data showed significant effects of temperature ($F = 88733.724$; $P \leq 0.000$) and isolate ($F = 3261.164$; $P \leq 0.000$) on growth, as well as significant isolate-temperature interaction ($F = 875.601$; $P \leq 0.000$). Several isolates exhibited maximum growth at 30°C (TN 01, TN 02, TN 07, TN 10, TN 11, TN 13, TN 14, TN 16) while the others exhibited maximum growth at 35°C (TN 03, TN 04, TN 05, TN 06, TN 08, TN 09, TN 12, TN 15). Temperature 25°C also supported good growth. The growth of majority of the isolates increased from 20 to 35°C and reduced drastically at 40°C. Mean maximum growth with respect to temperatures was displayed by isolate TN 13 (492.05 mg) and least growth was displayed by TN 14 (304.41 mg). Isolate TN 13 exhibited maximum growth at 30°C (682.0 mg) and isolate TN 05 displayed maximum growth at 35°C (641.87 mg). Isolate TN 07 showed minimum growth at 30°C (417.90 mg) while isolate TN 14 displayed minimum growth at 35°C (383.07 mg). Maximum mean growth was found at temperature 30°C (517.91 mg) followed by (508.46 mg) and 25°C (269.09 mg). Minimum mean growth was observed at 40°C (249.27 mg). Thus the temperature range between 30 to 35°C was found to be excellent for the growth of all the isolates (Table 2).

Effect of pH on the growth of *P. azadirachtae* isolates

There were significant effect of pH ($F = 18866.655$; $P \leq 0.000$) and isolate ($F = 4061.284$; $P \leq 0.000$) on growth along with isolates-pH interactions ($F = 249.442$; $P \leq 0.000$). Significant differences between isolates were found for each pH and between pH for each isolate. pH 6.0 was preferred by all the isolates with occurrence of maximum mean growth of 536.69 mg and least preferred pH was 4.0 wherein a minimum mean growth of 373.57 mg was observed. The mean growth was similar at pH 5.0 and 7.0 with values of 477.80 mg and 473.44 mg respectively. At pH 6.0 maximum growth was evident with isolate TN 12 (613.73 mg) and least growth was seen with the isolate TN 03 (432.5 mg). Isolate TN 10 exhibited maximum mean growth (539.23 mg) and minimum mean growth was showed by the isolate TN 13 (357.46 mg). Clear differences in the growth among the isolates within the same pH were evident (Table 3).

Effect of carbon sources on the growth of *P. azadirachtae* isolates

There were significant differences between isolates ($F = 1027.343$; $P \leq 0.000$) carbon sources ($F = 38581.252$; $P \leq 0.000$) and their interactions ($F = 337.186$; $P \leq 0.000$). The mean mycelial weight as influenced by different carbon sources revealed maximum weight in starch amended LBM medium (506.43 mg) followed by sucrose (485.29 mg), dextrose (330.84 mg), sorbitol (319.66 mg) and cellobiose (159.33 mg) amended media. Cellobiose

Table 2. Effect of temperature on the growth of *P. azadirachtae* isolates in potato dextrose broth.

Isolates	Mycelial dry weight (mg)					
	Temperature					
	20°C	25°C	30°C	35°C	40°C	Mean
TN 01	$^{q}297.30^{h}$	$^{r}463.63^{i}$	$^{t}570.77^{j}$	$^{s}491.77^{e}$	$^{p}289.17^{k}$	422.53
TN 02	$^{q}362.53^{jk}$	$^{r}428.43^{fg}$	$^{t}598.23^{k}$	$^{s}502.93^{fg}$	$^{p}227.37^{f}$	423.90
TN 03	$^{q}249.53^{de}$	$^{r}417.63^{e}$	$^{s}478.53^{d}$	$^{t}495.33^{ef}$	$^{p}237.90^{g}$	375.79
TN 04	$^{q}268.83^{f}$	$^{r}523.77^{k}$	$^{r}528.97^{h}$	$^{s}584.07^{k}$	$^{p}256.43^{i}$	432.41
TN 05	$^{p}157.93^{b}$	$^{r}501.73^{j}$	$^{s}520.97^{h}$	$^{t}641.87^{m}$	$^{q}298.10^{l}$	424.12
TN 06	$^{q}257.57^{e}$	$^{r}395.70^{cd}$	$^{s}478.83^{d}$	$^{t}527.90^{j}$	$^{p}204.17^{bc}$	372.83
TN 07	$^{p}152.23^{b}$	$^{r}389.90^{c}$	$^{s}417.90^{a}$	$^{r}397.73^{b}$	$^{q}286.67^{k}$	328.89
TN 08	$^{p}286.47^{g}$	$^{r}437.30^{g}$	$^{s}448.73^{b}$	$^{t}509.87^{gh}$	$^{q}306.60^{m}$	397.79
TN 09	$^{q}375.63^{l}$	$^{r}471.57^{i}$	$^{s}492.77^{ef}$	$^{t}521.13^{ij}$	$^{p}247.53^{h}$	421.73
TN 10	$^{p}217.73^{c}$	$^{q}403.90^{d}$	$^{s}559.83^{i}$	$^{r}471.97^{d}$	$^{p}213.87^{de}$	373.46
TN 11	$^{p}241.93^{d}$	$^{r}518.27^{k}$	$^{t}626.97^{l}$	$^{s}574.80^{k}$	$^{q}276.40^{j}$	447.67
TN 12	$^{q}246.50^{d}$	$^{r}425.40^{ef}$	$^{s}463.70^{c}$	$^{s}470.33^{cd}$	$^{p}209.03^{cd}$	362.99
TN 13	$^{q}368.63^{kl}$	$^{r}580.50^{l}$	$^{t}682.23^{m}$	$^{s}631.07^{l}$	$^{p}197.80^{ab}$	492.05
TN 14	$^{p}124.87^{a}$	$^{r}313.87^{a}$	$^{t}439.87^{b}$	$^{s}383.07^{a}$	$^{q}260.37^{i}$	304.41
TN 15	$^{q}358.53^{ij}$	$^{r}448.93^{h}$	$^{s}508.17^{g}$	$^{t}517.20^{hi}$	$^{p}315.90^{n}$	429.75
TN 16	$^{q}256.43^{e}$	$^{r}369.83^{b}$	$^{t}502.17^{fg}$	$^{s}459.97^{c}$	$^{p}220.33^{ef}$	361.75
Mean	269.09	446.54	517.91	508.46	249.27	

Source	F value	Significance
Isolates	3261.164	0.000
Temperatures	88733.724	0.000
Isolates* Temperatures	875.601	0.000

Values are means of three experiments and each with three replications. Figures followed by different superscript letters differ significantly when subjected to Tukey's HSD (honestly significant differences) (α = 0.05). Letters to the right of each value refer to differences between isolates (read vertically); letters to the left of each value refer to differences between temperatures (read horizontally).

was the least preferred carbon source by all the isolates. Most of the isolates preferred starch (TN 01, TN 05, TN 06, TN 07, TN 08, TN 10, TN 13, TN 14, TN 15 and TN 16) while several isolates preferred sucrose (TN 02, TN 03, TN 04, TN 09, TN 11, TN 12). Isolate TN 04 exhibited maximum growth with sucrose (645.0 mg) and isolate TN 08 displayed maximum growth with starch (637.67 mg). Isolate TN 13 showed minimum growth with sucrose (357.93 mg) while isolate TN 11 displayed minimum growth with starch (390.83 mg). Maximum mean growth was showed by isolate TN 04 (442.66 mg) and minimum mean growth was observed with isolate TN 16 (304.97 mg). The isolates also significantly varied in their growth with respect to utilization of each carbon source (Table 4).

Effect of nitrogen sources on the vegetative growth of *P. azadirachtae* isolates

The data revealed significant effects of isolate (F = 945.365; P ≤ 0.000) and nitrogen source (F =106418.634; P ≤ 0.000) on growth with the occurrence of isolates - nitrogen sources interactions (F = 696.865; P ≤ 0.000). Maximum mean weight was observed with the ammonium sulphate amended medium (510.57 mg) revealing its preference against other nitrogen sources by most of the isolates. This was followed by potassium nitrate (486.35 mg), asparagine (426.69 mg). Mean growth decreased drastically with sodium nitrite (255.61 mg) and urea (179.87 mg), and urea was the least preferred nitrogen source by all the isolates. Isolates TN 01, TN 02, TN 03, TN 12, TN 13, TN 14 and TN 16 exhibited best growth with ammonium sulphate. Isolates TN 04, TN 09 and TN 15 showed maximum growth with potassium nitrate. Isolates TN 06, TN 08 and TN 10 produced maximum growth using asparagine. Isolates TN 05, TN 06, TN 07 and TN 11 exhibited similar amount of growth with both ammonium sulphate and potassium nitrate (468.27 and 472.37 mg; 418.70 and 412. 87 mg; 543.47 and 543.30 mg; 576.47 and 575.20 mg respectively). Isolate TN 03 exhibited maximum growth with ammonium sulphate (597.80 mg) and isolate TN 11 displayed maximum growth with potassium nitrate (575.20 mg).

Table 3. Effect of pH on the growth of *P. azadirachtae* isolates in liquid basal medium.

Isolates	Mycelial dry weight (mg)					
	pH					
	4.0	5.0	6.0	7.0	8.0	Mean
TN 01	$^{p}379.37^{fg}$	$^{s}484.93^{g}$	$^{t}528.37^{f}$	$^{r}432.33^{b}$	$^{q}404.93^{f}$	445.99
TN 02	$^{p}385.20^{gh}$	$^{r}491.80^{gh}$	$^{t}577.03^{hi}$	$^{s}550.17^{h}$	$^{q}461.07^{j}$	493.05
TN 03	$^{p}275.30^{a}$	$^{q}352.67^{a}$	$^{t}432.50^{a}$	$^{s}386.03^{a}$	$^{r}367.77^{cd}$	362.85
TN 04	$^{p}373.43^{f}$	$^{r}465.90^{f}$	$^{t}515.40^{d}$	$^{s}493.93^{fg}$	$^{q}450.80^{i}$	459.89
TN 05	$^{p}315.80^{c}$	$^{r}422.30^{d}$	$^{t}465.80^{b}$	$^{s}435.80^{b}$	$^{q}412.47^{f}$	410.43
TN 06	$^{p}402.27^{i}$	$^{s}573.43^{j}$	$^{t}604.43^{jk}$	$^{r}454.60^{c}$	$^{q}445.23^{hi}$	495.99
TN 07	$^{p}414.03^{j}$	$^{r}496.10^{h}$	$^{s}525.03^{ef}$	$^{r}498.93^{g}$	$^{q}476.03^{k}$	482.03
TN 08	$^{p}334.83^{d}$	$^{r}408.07^{c}$	$^{t}501.20^{c}$	$^{s}435.90^{b}$	$^{q}373.33^{d}$	410.67
TN 09	$^{p}451.37^{l}$	$^{r}558.80^{i}$	$^{s}584.83^{i}$	$^{q}466.23^{d}$	$^{q}460.40^{j}$	504.33
TN 10	$^{p}440.13^{k}$	$^{r}570.17^{j}$	$^{s}599.87^{j}$	$^{r}574.80^{i}$	$^{q}511.20^{l}$	539.23
TN 11	$^{p}340.67^{de}$	$^{r}408.67^{c}$	$^{t}507.37^{cd}$	$^{s}481.03^{e}$	$^{q}385.77^{e}$	424.70
TN 12	$^{q}392.63^{hi}$	$^{s}553.90^{i}$	$^{t}613.73^{k}$	$^{r}487.70^{ef}$	$^{p}356.27^{b}$	480.85
TN 13	$^{p}287.97^{b}$	$^{r}369.10^{b}$	$^{t}434.57^{a}$	$^{s}379.87^{a}$	$^{q}315.80^{a}$	357.46
TN 14	$^{p}396.33^{i}$	$^{r}487.47^{gh}$	$^{s}516.53^{de}$	$^{r}491.93^{fg}$	$^{q}427.07^{g}$	463.87
TN 15	$^{p}350.13^{e}$	$^{r}448.13^{e}$	$^{t}551.93^{g}$	$^{s}479.23^{e}$	$^{q}360.87^{bc}$	438.06
TN 16	$^{p}369.90^{f}$	$^{qr}452.07^{e}$	$^{s}568.33^{h}$	$^{r}453.07^{c}$	$^{q}441.43^{h}$	456.96
Mean	373.57	477.80	536.69	473.44	422.58	

Source	F value	Significance
Isolates	4061.284	0.000
pH values	18866.655	0.000
Isolates * pH values	249.442	0.000

Values are means of three experiments and each with three replications. Figures followed by different superscript letters differ significantly when subjected to Tukey's HSD (honestly significant differences) (α = 0.05). Letters to the right of each value refer to differences between isolates (read vertically); letters to the left of each value refer to differences between pH (read horizontally).

Isolate TN 06 showed minimum growth with ammonium sulphate (418.70 mg) while isolate TN 13 displayed minimum growth with potassium nitrate (401.73 mg). Maximum mean growth was evident with isolate TN 11 (415.44 mg) and minimum mean growth with isolate TN 13 (303.07 mg).

All the isolates displayed significant difference among themselves in their growth in each nitrogen source (Table 5).

Effect of culture filtrates of *P. azadirachtae* isolates on germination of neem seeds

Significant differences were observed between isolates in the toxic effect of their culture filtrate on shoot length (F = 446.29; P ≤ 0.000) root length (F = 925.833; P ≤ 0.000), percentage germination (F = 11.108; P ≤ 0.000) and vigour index (F = 137.690; P ≤ 0.000) of neem seeds. The culture filtrates of *P. azadirachtae* isolates considerably reduced the germination and seed vigour of neem seeds in comparison with the control seeds treated with sterile distilled water. Culture filtrate of isolate TN 02 (Coimbatore) was most toxic and of TN 11 (Ramanathapuram) was least toxic against germination of neem seeds in comparison with all other isolates. The toxicity of culture filtrate of each isolate against neem seed germination is mentioned in Table 6.

DISCUSSION

The results of the present study reveal wide variation in the physiological characteristics among Tamil Nadu isolates of *P. azadirachtae*. The 16 *P. azadirachtae* isolates of Tamil Nadu varied among themselves with respect to temperature, pH, carbon and nitrogen sources requirement, and toxigenic behaviour. Plant pathogen populations exhibit geographical variability (Okabe et al., 1998). The initial study of differences among fungal isolates usually includes the morphological characteristics. However, the possibility that the strains no longer produce typical morphological structures makes it necessary to examine non-morphological characteristics

Table 4. Effect of different carbon sources on the growth of *P. azadirachtae* isolates in liquid basal medium.

Isolates	Mycelial dry weight (mg)					
	Carbon sources					
	Cellobiose	Dextrose	Sorbitol	Starch	Sucrose	Mean
TN 01	$^{p}120.90^{c}$	$^{r}388.57^{j}$	$^{q}285.13^{de}$	$^{t}593.90^{j}$	$^{s}509.80^{g}$	379.66
TN 02	$^{p}188.83^{i}$	$^{q}287.07^{cde}$	$^{r}387.77^{l}$	$^{s}498.00^{f}$	$^{t}529.40^{hi}$	378.21
TN 03	$^{p}203.43^{k}$	$^{q}430.50^{k}$	$^{r}455.10^{m}$	$^{s}500.10^{f}$	$^{t}552.00^{j}$	428.23
TN 04	$^{p}240.30^{l}$	$^{r}427.90^{k}$	$^{q}372.90^{k}$	$^{s}527.20^{g}$	$^{t}645.00^{l}$	442.66
TN 05	$^{p}161.23^{g}$	$^{r}393.13^{j}$	$^{q}300.47^{f}$	$^{t}573.40^{i}$	$^{s}562.33^{k}$	398.11
TN 06	$^{p}238.77^{l}$	$^{q}319.40^{fg}$	$^{q}290.30^{e}$	$^{s}404.83^{b}$	$^{r}382.67^{b}$	327.19
TN 07	$^{p}173.73^{h}$	$^{q}250.73^{a}$	$^{r}320.30^{gh}$	$^{t}633.33^{kl}$	$^{s}501.27^{g}$	375.87
TN 08	$^{p}113.23^{b}$	$^{q}315.37^{ef}$	$^{r}345.17^{i}$	$^{t}637.67^{l}$	$^{s}456.87^{e}$	373.66
TN 09	$^{p}138.83^{e}$	$^{r}374.23^{ij}$	$^{q}277.27^{cd}$	$^{s}400.90^{b}$	$^{t}533.63^{i}$	344.97
TN 10	$^{p}196.27^{j}$	$^{q}340.23^{fgh}$	$^{r}360.67^{j}$	$^{t}627.30^{k}$	$^{s}547.90^{j}$	414.47
TN 11	$^{p}147.43^{f}$	$^{q}275.80^{abc}$	$^{q}313.00^{g}$	$^{r}390.83^{a}$	$^{r}418.97^{c}$	309.21
TN 12	$^{p}136.10^{e}$	$^{q}275.13^{abc}$	$^{r}324.10^{h}$	$^{s}432.17^{c}$	$^{t}520.17^{h}$	337.53
TN 13	$^{p}140.40^{e}$	$^{q}351.27^{hi}$	$^{q}341.90^{i}$	$^{r}425.10^{c}$	$^{q}357.93^{a}$	323.32
TN 14	$^{p}101.87^{a}$	$^{r}283.13^{bcd}$	$^{q}237.20^{a}$	$^{t}549.47^{h}$	$^{s}431.47^{d}$	320.63
TN 15	$^{p}115.27^{b}$	$^{r}312.23^{def}$	$^{q}262.80^{b}$	$^{t}501.27^{f}$	$^{s}434.80^{d}$	325.27
TN 16	$^{p}128.10^{d}$	$^{q}253.27^{ab}$	$^{r}288.33^{e}$	$^{t}464.50^{e}$	$^{s}390.63^{b}$	304.97
Mean	159.33	330.84	319.66	506.43	485.29	

Source	F value	Significance
Isolates	1027.343	0.000
Carbon sources	38581.252	0.000
Isolates * Carbon sources	337.186	0.000

Values are means of three experiments and each with three replications. Figures followed by different superscript letters differ significantly when subjected to Tukey's HSD (honestly significant differences) (α = 0.05). Letters to the right of each value refer to differences between isolates (read vertically); letters to the left of each value refer to differences between carbon sources (read horizontally).

such as hydrocarbon and inorganic elements metabolism (Jernejc and Cimerman, 2001). Temperature and hydrogen ion concentration are the major physical factors (Madigan and Martinko, 2006) and the sources of carbon and nitrogen are the major chemical or nutritional factors that affect considerably growth, physiology and sporulation in fungi (Garraway and Evans, 1984). The studies on the effect of temperature, pH, carbon and nitrogen sources revealed the presence of physiological variations among the Tamil Nadu isolates of *P. azadirachtae.* The isolates preferred starch and sucrose as carbon source, ammonium nitrate and potassium nitrate as nitrogen source for their growth. Maximum growth of all the isolates was observed at pH 6.0. These results are in accordance with the investigations of Sateesh (1998). using Mysore isolate of *P. azadirachtae*. However, each isolate differed in its rate of growth in every treatment. Sateesh (1998) reported that *P. azadirachtae,* the Mysore isolate, showed maximum growth at 30°C and with increase in temperature to 35°C there was drastic decrease in the growth. But several Tamil Nadu isolates exhibited maximum growth at 35°C. This may be due to the fact that the isolates included in both of these studies originated from different regions. Environment is one of the components of the disease triangle and the knowledge of the effect of environmental factors is very important in disease control (Maloy, 1993). Similar results in other fungi were reported by many workers (Roustaee et al., 2000; Schilder et al., 2005; Yamanaka, 2003). The ability of the pathogen to utilize various carbon and nitrogenous sources and to grow at various temperatures and pH conditions may be the reason for its occurrence at different climatic conditions and widespread nature. Phytopathogenic fungi differ in their toxin producing ability (Bamba and Sumbali, 2006; Horn et al., 1996; Shivas et al., 1991; Walker et al., 2001). Many phytopathogenic fungi release toxic secondary metabolites into the media (Agrios, 2004; Maude, 1996). *P. azadirachtae* released toxic metabolite into media. The culture filtrates of all the Tamil Nadu isolates of *P. azadirachtae* were capable of inhibiting germination of neem seeds but at different rates exhibiting significant variability in the toxicity of culture filtrates among themselves. This indicates differences in virulence.

Table 5. Effect of different nitrogen sources on the growth of *Phomopsis azadirachtae* isolates in liquid basal medium.

Isolates	Mycelial dry weight (mg)					
	Nitrogen sources					
	Ammonium sulphate	Asparagine	Potassium nitrate	Sodium nitrite	Urea	Mean
TN 01	$^{t}588.70^{k}$	$^{r}348.33^{c}$	$^{s}537.10^{i}$	$^{q}289.73^{k}$	$^{p}226.53^{g}$	398.08
TN 02	$^{t}536.10^{hi}$	$^{r}408.43^{e}$	$^{s}474.13^{f}$	$^{q}261.30^{hi}$	$^{p}200.20^{hi}$	376.03
TN 03	$^{t}597.80^{k}$	$^{r}391.33^{d}$	$^{s}535.23^{i}$	$^{q}246.26^{fg}$	$^{p}190.83^{j}$	392.29
TN 04	$^{s}460.40^{cd}$	$^{r}336.77^{b}$	$^{t}519.30^{h}$	$^{q}241.23^{ef}$	$^{p}205.40^{l}$	352.62
TN 05	$^{s}468.27^{de}$	$^{r}426.40^{f}$	$^{s}472.37^{f}$	$^{q}294.17^{k}$	$^{p}178.90^{k}$	368.02
TN 06	$^{r}418.70^{a}$	$^{s}501.30^{j}$	$^{r}412.87^{b}$	$^{q}210.53^{ab}$	$^{p}183.13^{b}$	345.31
TN 07	$^{s}543.47^{i}$	$^{r}438.33^{g}$	$^{s}543.30^{i}$	$^{q}218.77^{bc}$	$^{p}148.80^{g}$	378.51
TN 08	$^{s}505.17^{f}$	$^{s}511.80^{k}$	$^{r}419.37^{bc}$	$^{q}268.83^{ij}$	$^{p}196.93^{e}$	380.42
TN 09	$^{s}426.93^{ab}$	$^{r}404.67^{e}$	$^{t}538.50^{i}$	$^{q}244.83^{f}$	$^{p}172.17^{i}$	357.42
TN 10	$^{s}477.87^{e}$	$^{t}570.83^{l}$	$^{r}447.50^{d}$	$^{q}274.77^{j}$	$^{p}191.23^{j}$	392.44
TN 11	$^{s}576.47^{j}$	$^{r}473.67^{i}$	$^{s}575.20^{j}$	$^{q}233.90^{de}$	$^{p}217.97^{c}$	415.44
TN 12	$^{t}525.20^{g}$	$^{s}478.43^{i}$	$^{r}426.93^{c}$	$^{q}295.23^{k}$	$^{p}132.07^{h}$	371.57
TN 13	$^{t}436.43^{b}$	$^{r}325.07^{a}$	$^{s}401.73^{a}$	$^{q}225.07^{cd}$	$^{p}127.07^{a}$	303.07
TN 14	$^{t}530.87^{gh}$	$^{r}452.37^{h}$	$^{s}476.67^{f}$	$^{q}205.80^{a}$	$^{p}160.10^{d}$	365.16
TN 15	$^{s}450.17^{c}$	$^{r}344.37^{bc}$	$^{t}507.60^{g}$	$^{q}255.43^{gh}$	$^{p}209.20^{d}$	353.35
TN 16	$^{t}570.53^{j}$	$^{r}427.23^{f}$	$^{s}458.17^{e}$	$^{q}314.80^{l}$	$^{p}160.77^{b}$	386.30
Mean	510.57	426.69	486.35	255.61	179.87	

Source	F value	Significance
Isolates	945.365	0.000
Nitrogen sources	106418.634	0.000
Isolates * Nitrogen sources	696.865	0.000

Values are means of three experiments and each with three replications. Figures followed by different superscript letters differ significantly when subjected to Tukey's HSD (honestly significant differences) (α = 0.05). Letters to the right of each value refer to differences between isolates (read vertically); letters to the left of each value refer to differences between nitrogen sources (read horizontally).

Toxins are implicated as virulence factors on vegetative tissues (Schilder et al., 2005). The results clearly state that all the isolates of *P. azadirachtae* were pathogenic but the degree of pathogenecity was statistically different among the isolates. Fathima (2004) reported similar observation. The *P. azadirachtae* isolated from various regions of Karnataka, South India, exhibited remarkable differences in the toxicity of their culture filtrates against the germination of neem seeds.

The data obtained from this study indicate the diversity of *P. azadirachtae* population in Tamil Nadu, South India. The phenotypic variations exhibited by plant pathogenic fungi finally depend on the genetically based variation which usually occurs due to mutation, recombination, and gene flow between populations of the pathogen (Burdon and Silk, 1997; Roustaee et al., 2000). Presence of considerable intraspecific differences among the isolates of *P. azadirachtae* revealed its heterogeneous nature and genetic diversity. The study of differences among fungal isolates is required for clear taxonomic schemes (Jernejc and Cimerman, 2001). Many fungi were characterized into sub-specific taxa utilizing the data of differences among their isolates (Pioli et al., 2003; Shivas et al., 1991). The differences observed among the *P. azadirachtae* isolates in the present study could be utilized for the characterization of the pathogen into sub-specific taxa. The data obtained clearly suggest the existence of physiological races within the fungus. Until more technical classification methods such as molecular characterization becomes more routine, morphological and physiological data must be relied upon to distinguish phytopathogenic isolates (Kellam and Zentmyer, 1986). To understand the mechanism of resistance and to breed resistant varieties it is very important to have precise knowledge of the spectrum of diversity in pathogen population (Fathima, 2004; Roustaee et al., 2000). The intraspecific variations among *P. azadirachtae* isolates in physiological traits identified by present work may be helpful for this purpose. However, it is necessary to increase the isolate pool and to include biochemical and/

Table 6. Effect of culture filtrates of *P. azadirachtae* isolates on the germination of neem seeds.

Isolates	Mean shoot length (cm)	Mean root length (cm)	Percentage germination	Vigour index
Control	3.85 ± 0.042^{k}	8.24 ± 0.046^{k}	88.75 ± 0.30^{c}	1073.13 ± 37.93^{k}
TN 01	1.15 ± 0.042^{f}	4.00 ± 0.060^{ef}	68.75 ± 0.30^{b}	353. 88 ± 9.47^{efg}
TN 02	0.65 ± 0.042^{ab}	2.91 ± 0.30^{a}	51.25 ± 0.30^{a}	182.50 ± 10.75^{ab}
TN 03	1.54 ± 0.042^{hij}	4.74 ± 0.042^{h}	72.50 ± 0.31^{b}	455.50 ± 21.90^{hij}
TN 04	0.89 ± 0.035^{de}	3.44 ± 0.038^{d}	68.75 ± 0.30^{b}	296.25 ± 12.26^{cde}
TN 05	0.76 ± 0.026^{bcd}	3.15 ± 0.042^{b}	58.75 ± 0.30^{ab}	229.13 ± 9.67^{abc}
TN 06	1.28 ± 0.025^{fg}	4.18 ± 0.037^{fg}	71.25 ± 0.35^{b}	388.50 ± 19.82^{fgh}
TN 07	0.92 ± 0.027^{de}	3.80 ± 0.057^{e}	62.50 ± 0.31^{ab}	295.13 ± 13.27^{cde}
TN 08	0.71 ± 0.030^{bc}	3.16 ± 0.057^{b}	58.75 ± 0.30^{ab}	227.38 ± 13.33^{abc}
TN 09	0.81 ± 0.030^{bcde}	3.33 ± 0.041^{bcd}	62.50 ± 0.31^{ab}	258.00 ± 11. 68^{bcd}
TN 10	0.85 ± 0.033^{cde}	3.39 ± 0.030^{cd}	58.75 ± 0.30^{ab}	248.75 ± 12.21^{bcd}
TN 11	1.63 ± 0.031^{j}	5.65 ± 0.042^{j}	73.75 ± 0.32^{bc}	535.50 ± 20.92^{j}
TN 12	0.78 ± 0.018^{bcde}	3.20 ± 0.046^{bc}	60.00 ± 0.27^{ab}	238.50 ± 13.02^{bc}
TN 13	1.38 ± 0.045^{gh}	4.23 ± 0.037^{g}	68.75 ± 0.30^{b}	385.00 ± 16.72^{fgh}
TN 14	1.44 ± 0.042^{ghi}	4.33 ± 0.031^{g}	71.25 ± 0.35^{b}	421.13 ± 24.06^{ghi}
TN 15	1.58 ± 0.031^{ij}	5.38 ± 0.045^{i}	72.50 ± 0.31^{b}	503.25 ± 20.07^{ij}
TN 16	0.95 ± 0.042^{e}	3.93 ± 0.056^{e}	67.50 ± 0.25^{b}	329.88 ± 15.92^{def}
F value	446.29	925.833	11.108	137.690
Significance	0.000	0.000	0.000	0.000

Values given are means of four replicates ± S.E. Figures followed by different superscript letters differ significantly when subjected to Tukey's HSD (honestly significant difference) (α = 0.05).

or molecular characterization to have precise knowledge.

ACKNOWLEDGMENTS

The authors thank University of Mysore for facilities. K. Girish acknowledges DST, New Delhi for financial support (Grant Number: SP/ SO/ A-26/ 99).

REFERENCES

Abdul-Baki AA, Anderson JP (1973). Vigour determination in soybean seed by multiple criteria. Crop Sci., 13: 630-633.

Agrios GN (2004). Plant Pathology, 4th ed. Academic Press, New York, p. 635.

Appiah AA, Flood J, Bridgeand PD, Archer SA (2003). Inter- and intraspecific morphometric variation and characterization of *Phytophthora* isolates from cocoa. Plant Pathol., 52: 168-180.

Bamba R, Sumbali G (2006). Differential toxigenic behaviour of *Alternaria alternata* isolates from citrus fruits. Indian Phytopath., 59: 194-198.

Brayford D (1990). Variation in *Phomopsis* isolates from *Ulmus* species in the British Isles and Italy. Mycol. Res., 94: 691-697.

Burdon JJ, Silk J (1997). Sources and patterns of diversity in plant pathogenic fungi. Phytopathology, 87: 664-669.

Cairney JWG (1999). Intraspecific physiological variation: implications for understanding functional diversity in ectomycorrhizal fungi. Mycorrhiza, 9: 125-135.

Deacon JW (1997). Modern mycology, 3rd ed. Blackwell Science Publications, Oxford, p. 303.

Dhingra OD, Sinclair JB (1995). Basic plant pathology methods, 2nd ed. CRC Press, Boca Raton, Florida, p. 448.

Dix NJ, Webster J (1995). Fungal ecology. Chapman and Hall, London, p. 560.

Fargues J, Goettel MS, Smits N, Ouedraogo A, Rougier M (1997). Effect of temperature on vegetative growth of *Beauveria bassiana* isolates from different origins. Mycologia, 89: (Abstr).

Fathima SK (2004). Investigations on the biology and management of *Phomopsis azadirachtae* on neem. PhD dissertation, University of Mysore, Mysore, India.

Fathima SK, Shankara Bhat S, Girish K (2004). Variation in *Phomopsis azadirachtae* the incitant of die-back of neem. Indian Phytopath., 51: 30-33.

Garraway MO, Evans RC (1984). Fungal nutrition and physiology. Wiley publications, New York, p. 412.

Geraldo MRF, Tessmann DJ, Kemmelmeier C (2006). Production of mycotoxins by *Fusarium graminearum* isolated from small cereals (Wheat, Triticale and Barley) affected with scab disease in Southern Brazil. Braz. J. Microbiol., 37: 58-63.

Girish K, Shankara Bhat S (2008). *Phomopsis azadirachtae* – the die-back of neem pathogen. eJBio., 4: 112-119.

Girish K, Shankara Bhat S, Raveesha KA (2009). Intraspecific variability in *Phomopsis azadirachtae* infecting neem. Arch. Phytopathol. Plant Protect., 42: 489-498.

Gurgel LMS, Menezes M, Coelho RSB (2002). A comparative study of *Phomopsis anacardii* and *Phomopsis mangiferae* isolates by pathogenicity and nutrition of C and N, under three light systems. Summa Phytopathol., 28: 160-166.

Higley PM, Tachibana H (1987). Physiologic specialization of *Diaporthe phaseolorum* var. *caulivora* in soybean. Plant Dis., 71: 815-817.

Horn WS, Simmonds MSJ, Schwartz RE, Blaney WM (1996). Variation in production of phomodiol and phomopsolide B by *Phomopsis* spp. Mycologia, 88: 588-595.

Hung LL, Trappe JM (1983). Growth variation between and within species of ectomycorrhizal fungi in response to pH *in vitro*. Mycologia, 75: 234–241.

International seed Testing Association (ISTA) (1993). International rules for seed testing. Seed Sci. Technol., 21: supplement.

Jernejc K, Cimerman A (2001). Morphological characteristics, extracellular and intracellular protein and enzyme patterns of five *Aspergillus* species. Food Technol. Biotechnol., 39: 333-340.

Kellam MK, Zentmyer GA (1986). Morphological, physiological, ecological, and pathological comparisons of *Phytophthora* species isolated from *Theobroma cacao*. Phytopathology, 76: 159-164.

Madigan MT, Martinko JM (2006). Brock Biology of Microorganisms, 11th ed. Pearson Prentice Hall, New Jersey, pp 150-158.

Maloy OC (1993). Plant disease control: Principles and practice. John Wiley and Sons Inc., New York, p 360.

Manoharachary C (2000). Biodiversity, taxonomy and characterization of aquatic fungi. In: Proceedings of an international conference on integrated plant disease management for sustainable agriculture held at New Delhi, India: Indian Phytopathological Society, IARI, pp. 118-122.

Maude RB (1996). Seed-borne diseases and their control: Principles and practice. CAB International, Cambridge, UK, p 304.

Mishra DK, Raj SK (1992). Comparative study of four isolates of *Aspergillus niger* causing collar rot of groundnut. J. Mycopathol. Res., 30: 103-111.

Mo M, Xu C, Zhang K (2005). Effects of carbon and nitrogen sources, carbon-to-nitrogen ratio, and initial pH on the growth of nematophagous fungus *Pochonia chlamydosporia* in liquid culture. Mycopathologia, 159: 381-387.

Moore LE (1996). Fundamentals of the fungi, 4th ed. Prentice-Hall International Inc., New Jersey, p 574.

Okabe I, Morikawa C, Matsumoto N, Yokoyama K (1998). Variation in *Sclerotium rolfsii* isolates in Japan. Mycoscience**,** 39: 399-407.

Pioli RN, Morandi EN, Martinez MC, Lucca F, Tozzini A, Bisaro V, Hopp HE (2003). Morphologic, molecular, and pathogenic characterization of *Diaporthe phaseolorum* variability in the core soybean producing area of Argentina. Phytopathology, 93: 136-146.

Roustaee A, Costes S, Dechamp-Guillaume G, Barrault G (2000). Phenotypic variability of *Leptosphaeria lindquistii* (anamorph: *Phoma macdonaldii*), a fungal pathogen of sunflower. Plant Pathol., 49: 227-234.

Sateesh MK (1998). Microbiological investigations on die-back disease of neem (*Azadirachta indica* A. Juss.). PhD dissertation, University of Mysore, Mysore, India.

Sateesh MK, Shankara Bhat S, Devaki NS (1997). *Phomopsis azadirachtae sp. nov*. from India. Mycotaxon, 65: 517-520.

Schilder AMC, Erincik O, Castlebury L, Rossman A, Ellis MA (2005). Characterization of *Phomopsis* spp. infecting grapevines in the Great Lakes region of North America. Plant Dis., 89: 755-762.

Shivas RG, Allen JG, Williamson PM (1991). Intraspecific variation demonstrated in *Phomopsis leptostromiformis* using cultural and biochemical techniques. Mycol. Res., 95: 320-323.

Thakur RP (1999). Pathogen diversity and plant disease management. Indian Phytopath., 52: 1-9.

Walker SL, Leath S, Hagler WM Jr., Murphy JP (2001). Variation among isolates of *Fusarium graminearum* associated with *Fusarium* head blight in North Carolina. Plant Dis., 85: 404-410.

Yamanaka T (2003). The effect of pH on the growth of saprotrophic and ectomycorrhizal ammonia fungi *in vitro*. Mycologia, 95: 584-589.