ISSN 1996-0719

International Journal of Plant Pathology

http://knowledgiascientific.com

Knowledgia SCIENTIFIC
A Place to Publish Outstanding Research

International Journal of Plant Pathology 3 (2): 56-65, 2012
ISSN 1996-0719 / DOI: 10.3923/ijpp.2012.56.65

Cultural, Morphological and Biochemical Variability among the Isolates of *Phomopsis azadirachtae* from Karnataka

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

[1]Post Graduate Department of Microbiology, Maharani's Science College for Women, JLB Road, Mysore-570005, Karnataka, India

[2]Department of Studies in Microbiology, Manasagangotri, University of Mysore, Mysore-570006, Karnataka, India

[3]Labland Biodiesel Private Limited, No. 98, 7th Main, Jayalakshmipuram, Mysore-570012, Karnataka, India

Corresponding Author: Syeda Kousar Fathima, Post Graduate Department of Microbiology, Maharani's Science College for Women, JLB Road, Mysore-570 005, Karnataka, India

ABSTRACT

A study on cultural, morphological and biochemical variability among the isolates of *Phomopsis azadirachtae* Sateesh, Bhat and Devaki collected from sixteen districts of Karnataka state, South India was carried out. The isolates were compared for the cultural and biochemical variability. It was observed that the isolates varied considerably for the cultural characteristics such as mycelial growth pattern, colour of the colony, sporulation behaviour, texture of pycnidia, production of alpha and beta conidia and the dimension of conidia produced. Estimation of water soluble mycelial proteins and toxicity of culture filtrate of *P. azadirachtae* isolates revealed a remarkable difference in the mycelial protein content of the isolates and different isolates exhibited various extent of toxicity on the germination of neem seeds.

Key words: *P. azadirachtae,* morphology, biochemical variability, protein, culture filtrate

INTRODUCTION

Neem (*Azadirachta indica* A. Juss.) a member of mahogany family Meliaceae is well-known for its biomedical properties. It is an evergreen, ecofriendly, native tree of Indian sub-continent. Neem is a source of effective bio-pesticides and provides a cure for many ailments right from common cold to cancer and AIDS (Anonymous, 2009; Girish and Bhat, 2008a). The eco-friendly neem tree, in spite of having antimicrobial properties, is infected by various pathogens belonging to bacteria and fungi. The most destructive pathogen of neem at present is *Phomopsis azadirachtae* Sateesh, Bhat and Devaki which causes die-back disease (Sateesh *et al*., 1997). The chief symptoms of the disease are twig blight, inflorescence blight and fruit rot. The disease results in almost 100% loss of fruit production (Bhat *et al*., 1998; Girish and Bhat, 2008b), because of which, neem seeds used as a raw material in the preparation of bio-pesticides, medicines and various industrial products are not obtained.

Plant pathogens are important components of the biodiversity of all natural ecosystems. Remarkable differences in cultural, morphological and biochemical characteristics are observed among pathogen populations from different geographical locations (Thakur, 1999). Differences in cultural, morphological and biochemical characteristics have been reported among populations of

various plant pathogens (Sharma *et al.*, 2002; Basandrai *et al.*, 2005; Khurana *et al.*, 2005; Hosen *et al.*, 2010). Phytopathogenic fungi exhibit intraspecific variability in toxin production (Nandakumar *et al.*, 2007; Asran and Amal, 2011; Singh and Kumar, 2011).

A good number of *Phomopsis* species are reported to exhibit remarkable intraspecific variability (Brayford, 1990; Shivas *et al.*, 1991). Die-back disease of neem caused by *P. azadirachtae* is widespread in different parts of Karnataka State, South India (Sateesh, 1998). Girish and Bhat (2010) reported the presence of morphological, cultural and biochemical variability among the *P. azadirachtae* collected from different regions of Tamil Nadu state, India. Significant differences were found in the protein banding patterns of *P. azadirachtae* isolates from Karnataka (Fathima *et al.*, 2004) and Tamil Nadu (Girish *et al.*, 2009), India. The present investigations were undertaken to study the morphological, cultural and biochemical variability among the isolates of *P. azadirachtae* collected from 16 districts of Karnataka State.

MATERIALS AND METHODS

Sites sampled: The collections of infected neem twig samples were made from Mandya, Gulbarga, Mysore, Bijapur, Hassan, Chikmagalur, Shimoga, Kolar, Raichur, Tumkur, Chamarajnagar, Chitradurga, Belgaum, Davanagere, Bengaluru and Bellary. The twigs were either used immediately after their collection or stored in brown paper bags or polythene covers in a refrigerator at 4°C until used for further study.

Isolation of *P. azadirachtae* from infected neem shoot: The infected twigs collected from each district were cut into 2-3 cm pieces with middle transition region of healthy and infected portion. Healthy twig explants served as control. Both healthy and infected twig pieces were washed separately with running tap water for one hour. Later they were cut into 1-1.5 cm segments with transition zone at the middle portion. The bark was removed and the segments were washed thoroughly and were surface-sterilized using sodium hypochlorite solution (with 5% available chlorine) (Sauer and Burroughs, 1986). Then they were rinsed six times with sterile distilled water. The explants were plated on Potato Dextrose Agar (PDA) medium amended with 200 ppm of chloramphenicol in Petri plates at the rate of four segments per plate (Sateesh, 1998). The inoculated Petri plates were incubated for 3-7 days in dark and observed for the growth of the pathogen from the twig segments (Alexopoulos *et al.*, 1996). Later the plates were kept near the window on the laboratory bench for natural photoperiod and the pathogen was allowed to sporulate.

Mycelial plugs (5 mm diam.) were removed from the advancing margins of 7 day old cultures of each isolate and transferred onto fresh PDA in Petri plates (90 mm diam.). Three replicates of each isolate were maintained and the plates were incubated for 10 days at 26±2°C for 12 h photoperiod. The isolates were identified as per Sateesh *et al.* (1997). The isolates were designated as follows: Mandya-Pa 01, Gulbarga-Pa 02, Mysore-Pa 03, Bijapur-Pa 04, Hassan-Pa 05, Chikmagalur-Pa 06, Shimoga-Pa 07, Kolar-Pa 08, Raichur-Pa 09, Tumkur-Pa 10, Chamarajnagar-Pa 11, Chitradurga-Pa 12, Belgaum-Pa 13, Davanagere-Pa 14, Bengaluru-Pa 15 and Bellary-Pa 16.

Cultural characteristics: Mycelial plugs were removed from the advancing margins of 7 day old cultures of each isolate and transferred onto fresh PDA plates. Three replicates of each isolate were

maintained and incubated for 10 days at room temperature (26±2°C) with 12 h photoperiod. The single spore culture (pure culture) was obtained on PDA medium by inoculating a single spore (Tuite, 1969). The cultures thus obtained were compared for their cultural characteristics. The colony morphology was assessed for growth characteristics, mycelial type, colour of the colony, colony diameter and texture and growth pattern of pycnidia. To measure the conidial dimension, the spores were collected onto the microscope slides, by touching the spore ooze. They were spread by adding a drop of cotton blue in lactophenol. About 100 alpha (α) and 100 beta (β)-conidia were measured in case of each isolate (Uecker and Caruso, 1988).

Estimation of water soluble mycelial proteins of *P. azadirachtae* isolates: All the 16 isolates of *P. azadirachtae* were grown separately on Czapek Dox broth for seven days. Mycelial mat was taken out, washed thoroughly with distilled water and dried using filter paper. One gram of the dried mycelium of each isolate was homogenized in 10 mL of TCA and centrifuged at 3000 xg for 20 min. The residue was re-dissolved in 10 mL of phosphate buffer and centrifuged. The supernatant was used for protein estimation as per Lowry *et al.* (1951).

Effects of culture filtrate of *P. azadirachtae* isolates on germination of neem seeds: To determine the variability in toxicity of culture filtrate among the isolates of *P. azadirachtae*, the filtrate of each isolate was obtained from 25 day old culture grown on potato dextrose broth. About 100 healthy surface-sterilized neem seeds were treated with the culture filtrate of each isolate separately by incubating in a beaker for 24 h. The control treatment included medium alone and sterile distilled water. The seeds were plated on sterile moist blotter in petri plates or on standard paper towels (ISTA, 1993) and incubated at room temperature (26±2°C). Each treatment had four replications. After 10 days shoot length, root length and percentage germination were recorded. Vigour index was calculated using the formula given by Abdul-Baki and Anderson (1973).

RESULTS

Sites sampled and isolation of *P. azadirachtae* from infected neem shoots: The field survey of various districts of Karnataka state, South India revealed the occurrence of die-back disease in all the 16 districts of Karnataka state, South India. The infected neem segments collected from all the locations developed fungal colonies whose identification was confirmed as *P. azadirachtae* as per Sateesh *et al.* (1997). All the isolates produced characteristic α and β-conidia.

Cultural characteristics: The isolates of *P. azadirachtae* derived from naturally infected neem shoots exhibited variable colony characteristics on PDA plates at 26±2°C. The isolates varied considerably for mycelial growth, colour of the colony, sporulation behaviour, growth pattern, texture and formation of pycnidia, production of alpha and beta conidia and their length and breadth (Fig. 1). The cultural characteristics of ten of the isolates are presented in the Table 1. Isolate Pa 14 had the longest alpha conidia (11.2×3.0 μm) while Pa 09 had the shortest alpha conidia (4.2×1.0 μm). Isolate Pa 04 had the longest beta conidia (24.2×1.8 μm) while shortest beta conidia (15.3×1.4 μm) were observed in isolate Pa 13. The isolates also varied significantly for colony growth and sporulation rate. Isolate Pa 07 had the largest colony (74 mm diam.) while Pa 06 had the smallest (32 mm diam) (Fig. 2). Maximum sporulation was observed in Pa 08 (280 to

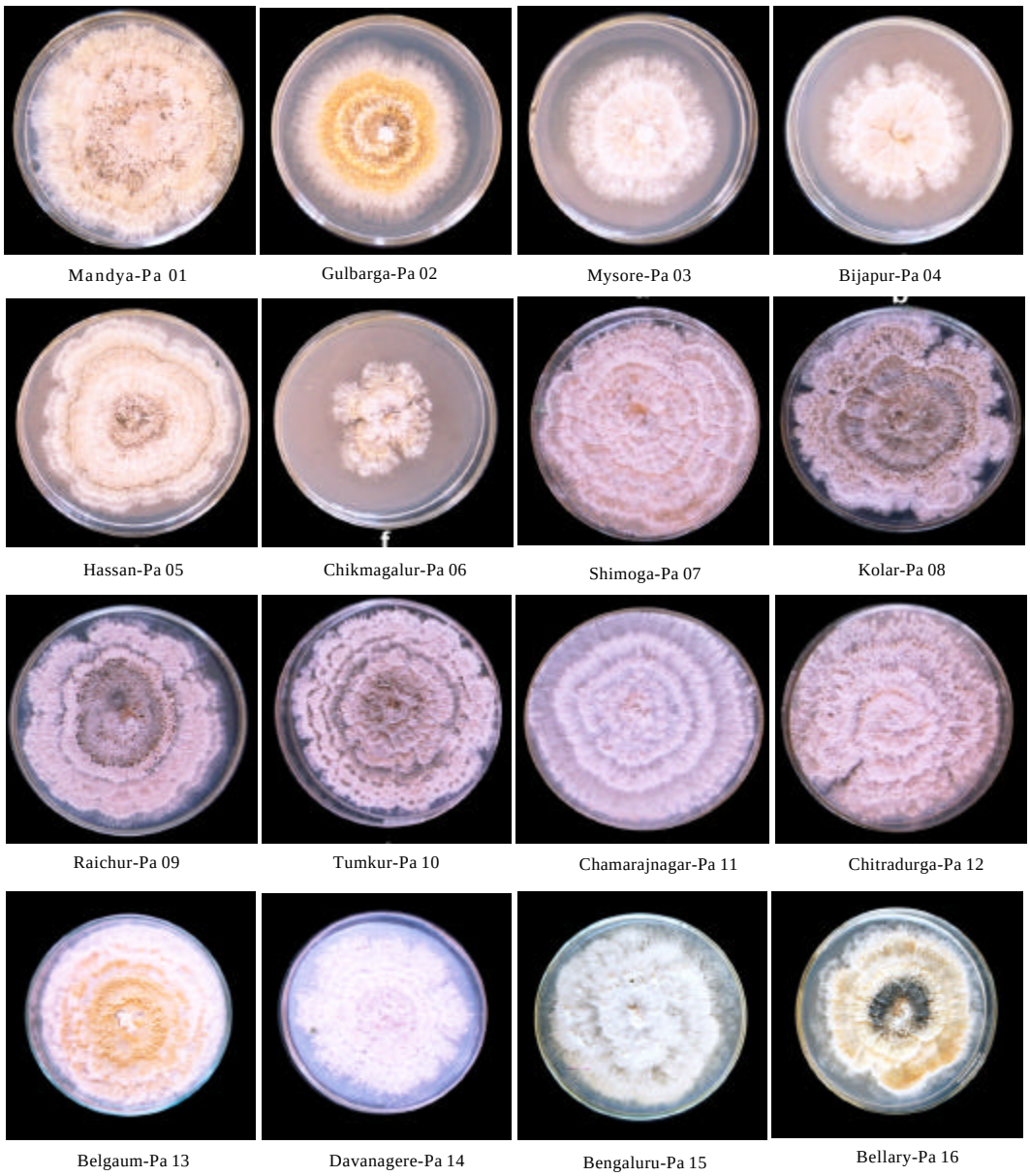

Fig. 1: Ten day old colonies of *P. azadirachtae* collected from different regions of Karnataka on potato dextrose agar

300 pycnidia/colony), while it was minimum in Pa 11 (25 to 40 pycnidia/colony) (Fig. 3). Further almost all the isolates produced more number of α conidia than β-conidia except the isolate Pa 04 which produced large number of beta conidia and very few alpha conidia with an alpha to beta conidia ratio of 8:42 unlike rest of the 15 isolates in which the ratio ranged between 35:15 to 44:6.

Estimation of water soluble mycelial proteins of *P. azadirachtae* isolates: The isolates of *P. azadirachtae* showed significant difference in their mycelial protein content. The protein content

Table 1: Colony characteristics of the 10 isolates of *P. azadirachtae* on potato dextrose agar plates after 10 days of incubation at 26±2°C

			Conidia	
Isolate	Colony characteristics	Pycnidia formation	Alpha	Beta
Pa 01	Raised felty creamish white mycelium with concentric rings at periphery, margin was irregular	Submerged and scattered	Abundant 7.4×2.0 µm	Sparse 17.8×1.8 µm
Pa 05	Thin adpressed pale brown mycelium with concentric rings at the center, sparse mycelium at periphery, margin was wavy	Distinct in concentric rings	Abundant 5.9×1.6 µm	Sparse 19.5×1.7 µm
Pa 06	Raised yellowish white mycelium, margin was wavy	Distinct in concentric rings	Abundant 9.2×2.4 µm	Sparse 17.2×1.6 µm
Pa 07	Raised wooly pale yellow mycelium with concentric rings towards periphery, margin was even	Submerged in concentric rings	Abundant 8.5×2.1 µm	Sparse 18.6×1.6 µm
Pa 08	Raised felty brownish mycelium at the center and whitish mycelium towards periphery, margin was wavy	Distinct and scattered	Abundant 9.8×2.6 µm	Sparse 21.3×1.9 µm
Pa 10	Thick adpressed pale brownish mycelium with dark brown concentric rings, margin was wavy	Submerged in concentric rings	Abundant 6.9×1.7 µm	Sparse 19.9×1.8 µm
Pa 11	Thin filmy whitish adpressed mycelium with concentric rings of dense and sparse hyphae, margin was circular	Submerged and scattered	Abundant 9.9×2.3 µm	Sparse 18.2×1.8 µm
Pa 12	Raised and wooly grayish white mycelium with scattered, dense and sparse mycelium, margin was irregular	Distinct and scattered	Abundant 9.6×2.3 µm	Sparse 19.3×1.9 µm
Pa 15	Adpressed cottony grayish white mycelium, sparse towards periphery, margin was wavy	Submerged and scattered	Abundant 8.3×2.3 µm	Sparse 18×1.8 µm
Pa 16	Raised felty pale orange mycelium with a dark gray center mycelium sparse towards periphery, margin was wavy	Distinct in concentric rings	Abundant 6.0×1.7 µm	Sparse 17.0×1.6 µm

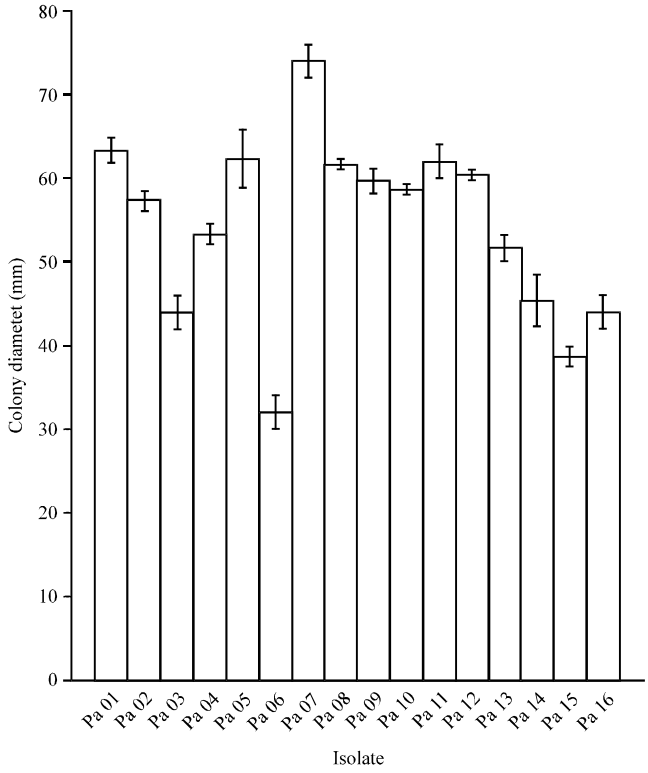

Fig. 2: Colony diameter of 16 isolates of *P. azadirachtae* on potato dextrose agar medium after 10 days of incubation at 26°C, Bars indicate standard deviation

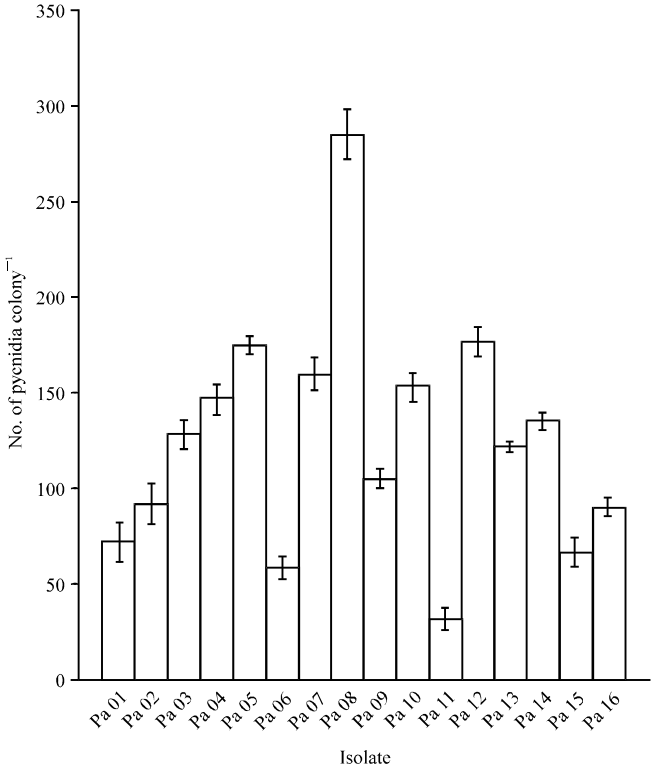

Fig. 3: Sporulation rate of 16 isolates of *P. azadirachtae* on potato dextrose agar medium after 10 days of incubation at 26°C, Bars indicate standard deviation

Table 2: Water soluble mycelial protein of 16 isolates of *P. azadirachtae*

Isolate	Water soluble protein (μg g^{-1})
Pa 01	102.6±0.40^{de}
Pa 02	99.8±0.37^{d}
Pa 03	117.8±1.39^{g}
Pa 04	149.3±0.30^{l}
Pa 05	129.4±0.40^{i}
Pa 06	143.2±0.80^{k}
Pa 07	91.0±0.44^{bc}
Pa 08	139.4±0.40^{j}
Pa 09	123.8±0.58^{h}
Pa 10	132.6±0.74^{j}
Pa 11	83.8±0.58^{a}
Pa 12	114.0±0.54^{f}
Pa 13	104.2±0.37^{e}
Pa 14	119.6±0.50^{g}
Pa 15	93.2±0.96^{c}
Pa 16	89.7±0.30^{b}

Values given are mean of three replicates ±SE, Values followed by different superscript letters differ significantly when subjected to Tukey's HSD (honestly significant difference)

of different *P. azadirachtae* isolates is presented in Table 2. Isolate Pa 04 had the highest mycelial protein content 150 μg g^{-1} whereas the isolate Pa 11 had the lowest mycelial protein content 85 μg g^{-1}.

Table 3: Effect of culture filtrate of 16 isolates of *P. azadirachtae* on germination of neem seeds

Isolate	Mean shoot length (cm)	Mean root length (cm)	Germination (%)	Vigour index
Control	3.760±0.38^{g}	7.13±0.120^{k}	86.6±3.33^{e}	947.00±65.96^{h}
Pa 01	0.603±0.33^{abc}	3.43±0.12^{fgh}	56.6±3.33^{abc}	228.70±15.3^{bc}
Pa 02	0.633±0.03^{abc}	2.80±0.15^{bcde}	66.6±3.33^{bcd}	228.30±8.64^{bc}
Pa 03	0.333±0.03^{a}	1.73±0.12^{a}	43.3±3.33^{a}	89.60±9.59^{a}
Pa 04	0.730±0.03^{abcd}	2.66±0.08^{bcd}	70.0±0.00^{cd}	238.00±4.04^{bcd}
Pa 05	0.530±0.03^{ab}	2.50±0.05^{b}	56.6±3.33^{abc}	172.00±11.13^{ab}
Pa 06	0.730±0.03^{abcd}	2.66±0.08^{bcd}	66.6±3.33^{bcd}	226.60±13.92^{bc}
Pa 07	0.760±0.03^{abcd}	3.06±0.06^{cdef}	66.6±3.33^{bcd}	255.00±7.76^{bcde}
Pa 08	0.560±0.03^{abc}	2.60±0.05^{bc}	53.3±3.33^{ab}	169.30±14.62^{ab}
Pa 09	1.900±0.057^{f}	6.06±0.06^{j}	76.6±3.33^{de}	610.60±25.43^{g}
Pa 10	0.860±0.03^{abcd}	3.20±0.05^{efg}	63.3±3.33^{bcd}	258.00±18.3^{bcde}
Pa 11	0.660±0.03^{abc}	2.76±0.08^{bcde}	56.6±3.33^{abc}	194.00±8.71^{abc}
Pa 12	1.000±0.03^{abcde}	3.70±0.01^{ghi}	63.3±3.33^{bcd}	296.60±7.68^{cdef}
Pa 13	1.230±0.03^{de}	3.83±0.03^{hi}	66.6±3.33^{bcd}	338.00±19.42^{def}
Pa 14	1.460±0.06^{ef}	3.96±0.03^{i}	70.0±0.00^{cd}	380.00±2.33^{f}
Pa 15	0.860±0.03^{abcd}	3.13±0.18^{def}	56.6±3.33^{abc}	225.60±5.60^{bc}
Pa 16	1.100±0.010^{bcde}	3.96±0.03^{i}	70.0±0.00^{cd}	354.60±6.17^{ef}
F value	57.388	190.815	10.705	98.553
Significance	0.000	0.000	0.000	0.000

Values given are means of three replicates ±SE, Values followed by different superscript letters differ significantly when subjected to Tukey's HSD (honestly significant difference)

Effects of culture filtrate of *P. azadirachtae* isolates on germination of neem seeds: The neem seeds treated with culture filtrate of isolates of *P. azadirachtae* collected from various districts showed reduction in their germination and seed vigour when compared to control seeds in both Petri plates and paper towels. Further it was observed that the culture filtrate of different isolates exhibited different extent of toxicity on the germination of neem seeds. The difference in the level of toxicity is presented in the Table 3. The seeds treated with isolate Pa 03 culture filtrate showed lowest percentage of germination and the seedling quality was also significantly affected. Isolate Pa 09 culture filtrate was least toxic compared to all other isolates.

DISCUSSION

Fungi are a unique group of microorganisms with their wide range of adaptability in different ecological situations (Moore, 1996). The genus *Phomopsis* is important plant pathogen (Raeisi *et al*., 2011) and reported to be a highly variable pathogen (Brayford, 1990; Shivas *et al*., 1991; Akhtar and Chaube, 2002). The present study revealed remarkable differences in cultural, morphological and biochemical characteristics of various *P. azadirachtae* isolates collected from different agroclimatic regions of Karnataka State, South India. Similar differences were observed among the *P. azadirachtae* isolates of Tamil Nadu, in radial growth, colony morphology, colour, texture, pycnidial density and arrangement, conidial number and size (Girish and Bhat, 2010). Similar variation was reported in *Phomopsis oblonga* isolated from *Ulmus* species in the British Isles and Italy (Brayford, 1990). Intraspecific variation was demonstrated among the isolates of *Phomopsis leptostromiformis* the causal agent of stem canker on *Lupinus angustifolius* using cultural and biochemical techniques (Shivas *et al*., 1991). Similar variability among the isolates of other phytopathogenic fungi was also reported. *Rhynchosporium secalis* isolates from

barley in different agroecological zones of Ethiopia differed markedly in several cultural characteristics like colony and conidial morphology, colony growth rate and sporulation (Meles *et al*., 2004). Isolates of *Sclerotinia sclerotiorum* collected from infected lentil plants from two agroecological zones of Syria showed considerable variation in cultural characteristics such as mycelia growth, mycelia pigmentation and sclerotial production (Akem *et al*., 2006). Two isolates of *Pythium ultimum* var. *ultimum* isolated from Egypt and Germany varied in the size of sexual organs, oospore production, growth rate and other morphological and physiological characteristics (Al-Sheikh and Abdelzaher, 2010).

The present investigations revealed that in one of the isolates of *P. azadirachtae*, designated as Pa 04 collected from Bijapur district, there was abundant production of beta conidia. Generally beta conidia production is influenced markedly by the substrate (Parmeter, 1958). The remarkable difference in alpha to beta conidia ratio in the isolate Pa 04 in the present study could be possibly due to the difference in the host growing in different geographical locations.

Studies on non-morphological characteristics such as wall composition, proteins and other hydrocarbons, of fungal isolates becomes important to differentiate among isolates when the strains no longer produce typical morphological structure (Jernejc and Cimerman, 2001). A remarkable variation in the mycelial protein content of various isolates of *P. azadirachtae* was observed in the present study. Similar observations were made by Girish and Bhat (2010) among the Tamil Nadu isolates of *P. azadirachtae* and Khurana *et al*. (2005) among the *Alternaria brassicae* isolates. The variations could be possibly due to the difference in the geographical locations from where the isolates are collected.

A good majority of deuteromycetes are reported to release toxic secondary metabolites into the media (Maude, 1996; Agrios, 2004). Sateesh, (1998) indicated the release of a toxic metabolite by *P. azadirachtae* into the medium which reduces the seed vigour and seed quality, based on the study of effects of culture filtrate on germination of neem seeds. In the present investigations, a remarkable difference in toxicity of culture filtrate was observed among various isolates of *P. azadirachtae* on the germination of neem seeds. Similar observation was reported by Shivas *et al*. (1991) among *Phomopsis leptostromiformis* isolates. Variation in toxin production among the isolates of *Sarocladium oryzae* causing rice sheath blight was reported by Nandakumar *et al*. (2007). Isolates of *Fusarium moniliforme* from pepper plants produced different quantities of each of fumonisin, zearelenone *in vitro* (Abo-Elnaga and Ahmed, 2007). A remarkable difference in toxin production was observed among the isolates of *Fusarium oxysporum* f. sp. *chrysanthemi* pathogenic to *Chrysanthemum* (Singh and Kumar, 2011).

The results of present study suggest that *P. azadirachtae* is a highly variable pathogen. These variations can be attributed to the influence of host variety and prevailing environment. This variability in pathogen populations makes them capable of invading a wide variety of host plants, thereby reducing the possibility of evolving disease management methods (Moore, 1996). The primary objective of studies on variation in pathogenicity or virulence is breeding and exploitation of resistance for disease management. The study of variations among the fungal isolates will also be helpful in characterizing sub-specific taxa and thereby in fungal taxonomy (Jernejc and Cimerman, 2001).

ACKNOWLEDGMENTS

The authors thank University of Mysore for facilities. Syeda Kousar Fathima acknowledges UGC, New Delhi for granting FIP fellowship.

REFERENCES

Abdul-Baki, A.A. and J.D. Anderson, 1973. Vigour determination in soybean seed by multiple criteria. Crop Sci., 13: 630-633.

Abo-Elnaga, H.I.G. and N.G. Ahmed, 2007. Pathogenicity, toxicity and gibberellic acid content of *Fusarium moniliformae* causing root rot and damping off of pepper. Plant Pathol. J., 6: 318-323.

Agrios, G.N., 2004. Plant Pathology 4th Edn., Academic Press, New York, USA.

Akem, C., M. Bellar and B. Bayaa, 2006. Comparative growth and pathogenecity of geographical isolates of *Sclerotinia sclerotiorum* on lentil genotypes. Plant Pathol. J., 5: 67-71.

Akhtar, J. and H.S. Chaube, 2002. Morphological and pathogenic variability in *Phomopsis vexans*, the incitant of fruit rot of brinjal. Proceedings of Asian Congress of Mycology and Plant Pathology, October 1-4, 2002, Mysore, India, pp: 130-131.

Al-Sheikh, H. and H.M.A. Abdelzaher, 2010. Differentiation between two isolates of *Pythium ultimum* var. *ultimum* isolated from diseased plants in two different continents. J. Biol. Sci., 10: 306-315.

Alexopoulos, C.J., C.W. Mims and M. Blackwell, 1996. Introductory Mycology. 4th Edn., John Wiley and Sons Inc., USA., ISBN: 0-471-52229-5.

Anonymous, 2009.. Neem-growing neem, organic farming, health, animal health, environmental use, home uses, economic potential, patents, new bazaars, research papers, world neem conference. Neem foundation (Internet). Mumbai, India. http://www.neemfoundation.org/

Asran, M.R. and M.I.E. Amal, 2011. Aggressiveness of certain *Fusarium graminearum* isolates on wheat seedlings and relation with their trichothecene production. Plant Pathol. J., 10: 36-41.

Basandrai, A.K., S. Pande, G. Krishna Kishore, J.H. Crouch and D. Basandrai, 2005. Cultural, morphological and pathological variation in Indian isolates of *Ascochyta rabiei*, the chickpea blight pathogen. Plant Pathol. J., 21: 207-213.

Bhat, S.S., M.K. Sateesh and N.S. Devaki, 1998. A new destructive disease of neem trees incited by *Phomopsis azadirachtae*. Curr. Sci., 74: 17-19.

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

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

Girish, K. and S.S. Bhat, 2008a. *Phomopsis azadirachtae*-The die-back of neem pathogen. eJBio., 4: 112-119.

Girish, K. and S.S. Bhat, 2008b. Neem-a green treasure. eJBio., 4: 102-111.

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

Girish, K. and S.S. Bhat, 2010. Cultural, morphological and biochemical variability among the isolates of *Phomopsis azadirachtae* from Tamil Nadu. J. Mycol. Plant Pathol., 40: 395-401.

Hosen, M.I., A.U. Ahmed and M.R. Islam, 2010. Variability and pathogenecity in Bangladeshi isolates of *Botrytis cinerea* causing botrytis gray mold in chickpea (*Cicer arietinum*). Plant Pathol. J., 3: 129-134.

ISTA, 1993. Proceedings of the international seed testing association, international rules for seed testing. Seed Sci. Technol., 21: 25-30.

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

Khurana, A., N. Mehta and M.S. Sangwan, 2005. Variation in biochemical contents of *Alternaria brassicae* isolates. J. Mycol. Plant Pathol., 35: 343-345.

Lowry, O.H., N.J. Rosebrough, A.L. Farr and R.J. Randall, 1951. Protein measurement with Folin phenol reagent. J. Biol. Chem., 193: 265-275.

Maude, R.B., 1996. Seed-borne diseases and their control: Principles and practice, CAB international. Cambridge, UK, pp: 304.

Meles, K., M. Hulluka and M.M. Abang, 2004. Phenotypic diversity in *Rhynchosporium secalis* from Ethiopia and host response to barley scald. Plant Pathol. J., 3: 26-34.

Moore, L.E., 1996. Fundamentals of the fungi. 4th Edn., Prentice-Hall International Inc., New Jersey, USA, pp: 574.

Nandakumar, R., S. Babu, G. Amutha, T. Raguchander and R. Samiyappan, 2007. Variation in toxin production among isolates of *Sarocladium oryzae* the rice sheath rot pathogen. Plant Pathol. J., 6: 120-126.

Parmeter, J.R., 1958. An effect of substrate on spore form in *Phomopsis*. Phytopathology, 48: 396-397.

Raeisi, S., A. Puteh and K. Sijam, 2011. First report of *Diaporthe/Phomopsis* complex isolates in soybean from Malaysia and their longevity in stored seeds. Asian J. Plant Pathol., 5: 146-154.

Sateesh, M.K., 1998. Microbiological investigations on die-back disease of neem (*Azadirachta indica* A. Juss.). Ph.D. Thesis, University of Mysore. Mysore, India.

Sateesh, M.K., S.S. Bhat and N.S. Devaki, 1997. *Phomopsis azadirachtae* sp. nov. from India. Mycotaxon, 65: 517-520.

Sauer, D.B. and R. Burroughs, 1986. Disinfection of seed surfaces with sodium hypochlorite. Phytopathology, 76: 745-749.

Sharma, S.K., D.V. Singh, R. Aggarwal and K.D. Srivastava, 2002. Pathogenic and biochemical variations in *Neovossia indica*. Indian Phytopath., 56: 133-139.

Shivas, R.G., J.G. Allen and P.M. Williamson, 1991. Intraspecific variation demonstrated in *Phomopsis leptostromiformis* using cultural and biochemical techniques. Mycol. Res., 95: 320-323.

Singh, P.K. and V. Kumar, 2011. Variability among isolates of *Fusarium oxysporum* f. sp. *chrysanthemi* pathogenicto *chrysanthemum*. Int. J. Plant Pathol., 2: 136-143.

Thakur, R.P., 1999. Pathogen diversity and plant disease management. Ind. Phytopathol., 52: 1-9.

Tuite, J., 1969. Plant Pathological Methods-Fungi and Bacteria. Burgress Publ. Co., Menneapolis, Minnesota.

Uecker, F.A. and F.L. Caruso, 1988. *Synchronoblastia crypta*, a new *Coelomycetous* pathogen of upright stems and fruits of cranberry. Mycologia, 80: 344-347.