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Studies on cultural, morphological variability in isolates of *Fusarium solani* (Mart.) Sacc., incitant of dry root-rot of Citrus

M. Ravi Chandran* and *M. Reddi Kumar*

Department of Plant Pathology, S.V. Agricultural College, Tirupati -517502, A. P., India
*Present address: Regional Agricultural Research Station, ANGRAU, Tirupati, A. P., India
*E-mail : reddi_kumar01@yahoo.com

ABSTRACT

Thirteen isolates of *Fusarium solani* (Mart.) Sacc., incitant of dry root-rot in citrus were studied for its cultural and morphological variability. The *F. solani* isolates, AFS_1, AFS_2, AFS_4, CFS_9 and CFS_{13} grew more than 85 mm after 7 days of inoculation and considered as fast growing category. Among all the isolates of *F. solani* CFS_9 isolate showed significant variation in radial growth (90 mm) on PDA medium. The size of macro conidia ranged from 13-15 x 3-4 µm to 27-29 x 4-5 µm and the size of micro conidia ranged from 3-4 x 1-2µm to 9-10 x 1-3µm. The number of septa in macro conidia and in micro conidia is 3-5 and 0-1, respectively and conidia are hyaline. The macro conidia is sickle shaped with blunt end and micro conidia is round to oval shaped. Intercalary and terminal chlamydospores were observed in all the *F. solani* isolates.

KEY WORDS : *Fusarium solani*, variability, cultural, morphological, dry root rot, citrus

INTRODUCTION

Acid lime (*Citrus aurantifolia* Swingle) is one of the important citrus fruits that constitute nearly 20% of the total citrus production in India. Andhra Pradesh is one of the important citrus producing states in the country with a total area of about 1.66 lakh hectares and an annual production of 2.23 lakh tones. Acid lime is affected by several soil borne pathogens like *Fusarium, Rhizoctonia,* and *Diplodia* species causing root-rot disease both in nursery and main field affecting life and production of acid lime. Among these, *Fusarium solani* (Mart.) Sacc. causes dry root-rot in citrus seedlings and also in the developed plants. In nature, plant pathogens exist as different strains that exhibit variation in their morphological and cultural characters, pathogenicity and virulence. To understand the present plant disease situations and to predict the possible future development it is essential to learn as much as possible about the variability in fungi that are pathogenic to plants. Morphological and pathogenic variations are known in many fungal pathogens (Kumar *et al.,* 1995).

Due to variation in the distribution of *F. solani* across environments, it is important to know which isolate is most virulent within a given species especially for germplasm evaluations where the goal is to identify genetic resistance to highly virulent isolates. Keeping in view, the importance of the crop, severity of the disease and advantages of biological control, present investigation was taken up to study the variability in cultural and morphological characters of *F. solani* isolates.

MATERIALS AND METHODS

Survey was conducted in major acid lime growing areas of Ananthapur and Chittoor districts. In each district, three to four mandals were selected and in each mandal four villages were chosen based on prevalence of disease and totally 89 rhizosphere soil samples from healthy plants of diseased orchard were collected from two districts. Serial dilution technique was used to isolate antagonistic mycoflora from rhizosphere soil of acid lime plants. Composite soil samples collected from rhizosphere of healthy plants of dry root-rot infected acid lime trees were shade dried and then used for serial dilution. The general laboratory techniques followed were those described by Dingra and Sinclair (1994) with slight modifications wherever necessary. Acid lime plants infected with dry root-rot pathogen, *Fusarium solani* were collected from Ananthapur and Chittoor districts. The pathogen was isolated from the roots of infected acid lime by tissue segment method (Rangaswami and Mahadevan, 1999) on *Fusarium* specific medium. Axenic culture of the pathogen was obtained by single hyphal tip method and maintained on PDA throughout the present investigation. The pathogen isolates were mainly identified on the basis of cultural and morphological characters as *Fusarium solani* (Mart) Sacc. (Subramanian, 1971). The pure cultures of different isolates of *Fusarium solani* were maintained on PDA slants. Different isolates of *F. solani* were multiplied on Sorghum sand medium and added to soil in pots at 7.5 percent (W/W) by soil infestation following the procedure given by Haware (1980). Soil mixture without inoculum served as control. Each pot was planted with acid lime seedlings and symptoms development was observed. Seedling mortality percent was recorded at 43 DAS. Based on percent mortality of seedlings, virulent isolate was identified. Sand Sorghum medium (SSM) was used for developing mass production of inoculum for carrying out the pathogenicity tests. Different isolates of *F. solani* were multiplied on SSM. Pathogenicity was proved by soil infestation following the procedure given by Haware (1980). Based on the disease incidence, the virulent isolate was identified and used in further studies. SSM was prepared by mixing 100 g sorghum grain flour, 50 g sand, 50 ml distilled water in 250 ml conical flask and sterilized at 15 psi for one hr for three consecutive days. Flasks were subsequently inoculated with 5mm diameter discs of culture of the test fungus and incubated at 28 ± 2^{o}C. For mass multiplication of *Trichoderma* isolates, 50 g sorghum grain flour with 25 ml tap water (adjusted level to the 50 per cent (w/v)) was taken in 500ml conical flasks and autoclaved at 15 psi for 60 minutes for three successive days. Then seven day-old *Trichoderma* culture was added under aseptic conditions and incubated at room temperature at 28 ± 2^{o}C.

Morphological studies were conducted by taking a small amount of pure culture using a sterile needle and transferred onto a clean glass slide. The culture was taken from four positions of the culture plate, two at right angle to each other, one from very close to the inoculation point and another mid point of radius. Total three culture plates of each isolate were used for the morphological studies after 7, 15, 25 days after incubation at 25 ± 1^{o}C. The culture was stained with 0.1% lacto phenol cotton blue and observed for the micro conidia, macro conidia and chlamydospores using a compound microscope. Micro Conidial size was measured with the help of an ocular micrometer after calibrating the microscope using seven day old culture of each isolate which was replicated thrice with 100 conidia in each replication. Length and breadth of 100 macro conidia for each of three

replications of all the isolates were measured using 15 days old culture. The mode of chlamydospore production viz., solitary, pairs, chains and location were observed using 25 day old culture. Cultural characteristics viz; the substrate, nature of mycelium and pigmentation of the different isolates were observed in PD broth cultures after 7-8 days of inoculation. The radial growth of pathogen was recorded up to 8 days after inoculation.

RESULTS AND DISCUSSION

A preliminary survey was carried out on the occurrence of dry root-rot disease of acid lime in major acid lime growing areas of Ananthapur and Chittoor districts of Andhra Pradesh. In the present study, survey was conducted in four major acid lime growing mandals of Ananthapur *viz.*, Narpala, Singanamala, Putlur and Thadipatri and the average percentage of disease incidence was 22.14, 16.07, 15.4 and 13.91 per cent respectively. The highest average per cent disease incidence was recorded in Narpala mandal (22.14%) of Ananthapur district. In Chittoor district, survey was conducted in Punganur, B.N. Kandriga and Tirupati rural mandals and the average per cent incidence recorded were 18.04, 14.38 and 14.7 per cent, respectively. Among the two districts, the highest mean per cent disease incidence was recorded in Narpala mandal of Ananthapur district (22.4 per cent) which might be due to the presence of calcareous soils, high soil pH that predisposes the acid lime trees to dry root-rot infection. The pathogen was isolated from the root-rot affected acid lime plants by tissue segment method on *Fusarium* selective medium (Rangaswami and Mahadevan, 1999).White, cottony and fluffy growth with smooth margin colonies were observed on fourth day after inoculation. A total of 96 root and soil samples of dry root-rot infected acid lime plants were collected from Ananthapur and Chittoor districts and subjected to isolation of test pathogen. A sum of 43 *Fusarium solani* isolates was isolated using *Fusarium* selective medium. Based on colony characters, pigmentation, production of macro and micro conidia and chlamydospore similarities, all 43 isolates were pooled together into 13 pure cultures of *Fusarium solani* isolates. Ghasolia and Shivpuri (2007) studied variability in 38 isolates of *Sclerotinia sclerotiorum* and according to similarities, 9 groups (G_1 to G_9) of 38 isolates were established. Among these, 3 isolates were grouped in G1 based on similar growth characters like profuse and white mycelial growth, small size sclerotia, less number of sclerotia, cottony in appearance and randomly scattered over colony surface; 5 isolates grouped in G2 on the basis of raised, smooth, dull white mycelial growth and remaining all isolates were established under different groups based on cultural and morphological similarities of the pathogen.

In the present study, the isolates were designated as AFS_1 to AFS_7 for the isolates of *F. solani* collected from Ananthapur and CFS_8 to CFS_{13} for Chittoor *F. solani* isolates. The pathogen was identified as *F. solani* based on mycological characters of the pathogen reported by Subramanian (1971. Abundant micro conidia were noticed on 3rd day and macro conidia were observed on 12th day after inoculation. Booth (1971) reported that abundant micro and macro conidia production takes place by the virulent isolate on 11-12th day after inoculation of pathogen in culture plates.

Serial dilution technique was used to isolate antagonistic mycoflora from rhizosphere soil. Colonies of fungal mycoflora such as *Trichoderma* sp.,

Aspergillus flavus, A. niger, Penicillium sp., and *Rhizopus stolonifer* were obtained on Rose Bengal Agar medium on 7th day after inoculation. Bacterial colonies (*Pseudomonas fluorescens*) were observed after two days of inoculation on Kings' B Medium. *Trichoderma* sp. i.e. *T. hamatum, T.harzianum, T.viride, T.longibrachiatum and Aspergillus flavus, A.niger, Penicillium* spp., *Rhizoctonia solani* were identified based on mycological keys described by Subramanian (1971). In a similar study, Upadhyay and Raj (1989) isolated *T. viride* and *T. harzianum* from rhizosphere of pigeon pea for the control of *Fusarium* wilt caused by *F.udum.* Muthukumar (2005) isolated *T.viride, Trichoderma* sp. and *Bacillus subtilis* for the management of tuber rot of tuberose caused by *Fusarium oxysporum*. The colony characters include white, cottony and fluffy growth with smooth margin, produced pale yellow to dusky red colour pigmentation in culture plates and in PD broth. The three days young culture showed sparse, slimy growth of mycelium with huge number of micro conidia when compared to seven days old cultures. Soil inoculation method was followed to establish the association of pathogen with disease and the most virulent race. The studies on pathogenicity test in pot culture revealed that per cent disease incidence was more in CFS_9 isolate collected from Tirupati rural mandal of Chittoor district followed by AFS_4 isolate of Ananthapur district. There is direct relationship between virulence of the pathogen and disease development in acid lime seedlings. The acid lime seedlings showed symptoms of wilting, drooping and yellowing of leaves in pots inoculated with CFS_9 isolate on 43rd day after inoculation. Consequently, the acid lime seedlings became dried due to disintegration of roots, the roots of such infected plants showed dark brown to black discoloration of vascular tissues when split open. In a similar study, Graham *et al.* (1985) when inoculated young potted citrus plants of rough melon with *F.solani* observed typical symptoms of the disease one month after inoculation. Nemec *et al.* (1980) described symptoms due to *Fusarium solani* causing dry root-rot of citrus seedlings. They observed dull green, dehydrating leaves leading to severe leaf wilt followed by desiccation and leaf drop. Roots of infected seedlings showed vessel plugging and rotting of secondary fibrous roots. The pathogen was reisolated from the infected plants and the characters of the pathogen were similar to that of with original strain (CFS_9) isolated from the field and thus proved Koch's postulates. Thus CFS_9 isolate was identified as virulent pathogen, used in the pot culture studies and maintained in PDA slants.

Cultural variability among *F. solani* isolates

Based on radial growth, the *F. solani* isolates were characterized as fast growing, moderately growing and slow growing. The growth of 13 isolates of *F. solani* was observed up to 8 days. The radial growth of isolates AFS_1, AFS_2, AFS_4, CFS_9 and CFS_{13} was more than 85 mm after 7 days of inoculation and considered as fast growing category (Table 3). Among all the isolates of *Fusarium solani*, CFS_9 isolate showed significant variation in radial growth (90 mm) (Plate 1). The radial growth of isolates AFS_3, AFS_6, CFS_8 and CFS_{11} was between 70 to 87 mm and considered as moderate growth category. The remaining all isolates of pathogen grown less than 70 mm even after 7 days of inoculation were grouped under slow growth category (Table 1). White dense, fluffy mycelium with concentric rings and raised mycelium with smooth margins was observed in AFS_1, AFS_3, AFS_4, AFS_5, CFS_8 and CFS_{11} isolates. Moderate to profuse sporulation

was observed in most of the *F. solani* isolates. CFS_9 isolate of *F. solani* showed white sparse mycelium in concentric rings and profuse sporulation.

The isolates AFS_3, AFS_6, AFS_7, CFS_9 and CFS_{11} produced pale pink to dusky red colour pigmentation in PDA medium and potato dextrose broth culture. The remaining all isolates produced pale yellow to dark yellow pigmentation. Most of the isolates produced profuse sporulation, whereas others produced moderate sporulation. In a similar study, Madhukeshwara (2000) studied cultural variability among six isolates of *F. udum* causing wilt of pigeon pea. All the isolates varied with each other in terms of growth, mycelium, pigmentation and sporulation. Most of the isolates produced cottony white raised mycelium, pale yellow to dusky red colour pigmentation and moderate to profuse sporulation on PDA medium. Champawat and Pathak (1989) studied cultural variability among nine isolates (I_1 to I_9) of *F. oxysporum* f.sp. *cumini* causing wilt of cumin. The isolates I_2, I_3 and I_8, I_9 showed white mycelial and different colours of substrate pigmentation on PDA media. Isolate I_3 produced light yellow coloured substrate pigmentation on PDA medium.

Morphological variability among *F. solani* isolates

Morphological characters such as size, shape, septation and colour of conidia were studied using PDA medium. Conidiophores were elongated and sparsely branched, each branch usually terminated with a spore bearing phialide. The pathogen produced two types of asexual spores *viz.*, micro conidia and macro conidia. The resting spores namely chlamydospores also were observed in age old cultures. The size of macro conidia was ranged from 13-15 x 3-4 µm to 27-29 x 4-5 µm, size of micro conidia was ranged from 3-4 x 1-2 µm to 9-10 x 1-3 µm, the number of septa in macro conidia and in micro conidia are 3-5 and 0-1 respectively and the colour is hyaline (Table 3). The shape of macro conidia is sickle shaped with blunt ends and micro conidia is round to oval shaped. The chlamydospores located in middle of hyphae (intercalary), on tip of the hyphae (terminal) and some chlamydospores were seen in middle of macro conidia. The isolates AFS_2, AFS_4, AFS_6, CFS_{10}, CFS_{12} and CFS_{11} were produced large sized macro conidia (21-23 x 3-4 µm to 27-29 x 4-5 µm) and these macro conidia are elongated, sickle shaped cells with blunt ends and hyaline cells (plate 2 & 3). The remaining isolates produced medium sized macro conidia with 2-3 septa and sickle shaped with blunt end. The *F. solani* isolates AFS_5, CFS_8 and CFS_{11} produced abundant, profuse micro conidia rather than macro conidia. The micro conidia are mostly single celled, round to oval shaped and hyaline in nature. Chlamydospores were produced in the culture plates on 12^{th} day after plating. AFS_6, AFS_7, CFS_{13} isolates of *F. solani* produced abundant chlamydospores, both intercalary and terminal chlamydospores were observed. The chlamydospores are thick walled, rough, globose to oval shaped and measured 8.95-12.65 x 6.10-9.95 µm. Intercalary chlamydospores were observed in macro conidia of *F. solani* isolate CFS_{11}. Madhukeshwara (2000) reported the size of micro and macro conidia were 6 – 8 x 2 – 3µm and 19 – 26 x 3.5µm respectively, in most of the isolates. Septation ranged from 2 to 5 in macro conidia and 0 – 1 in number in case of microconidia. Macroconidia is sickle shaped with pointed ends and hyaline. The micro conidia are oval shaped and hyaline. Desai *et al.* (2003) observed that the size of micro and macro conidia of *F.oxysporum* f.sp. *ricini* ranged from 5.25 – 14.00 x 3.50 – 7.00 µm and 17.5 – 70.00 x

3.50 – 5.25 µm, respectively. The isolates which were highly virulent produced abundant sporulation, while moderately virulent isolates were having poor sporulation.

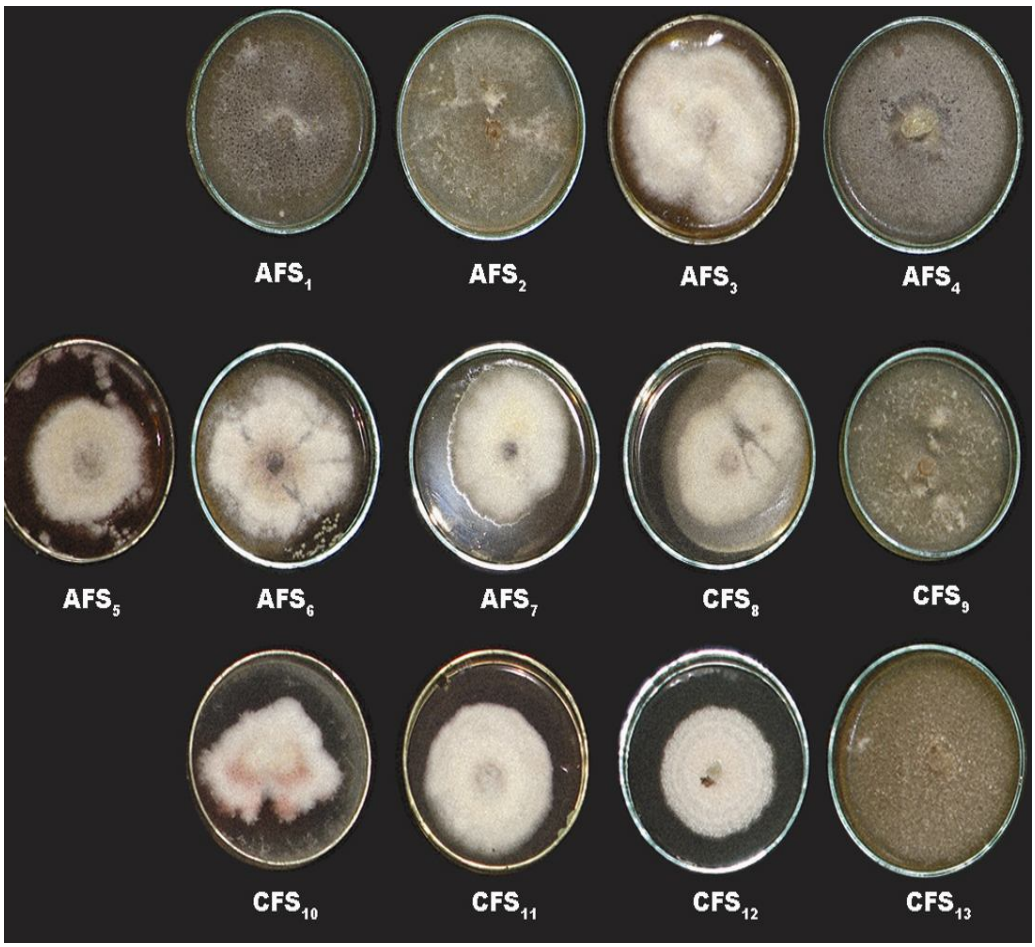

Plate 1: Cultural variability among isolates of *Fusarium solani*

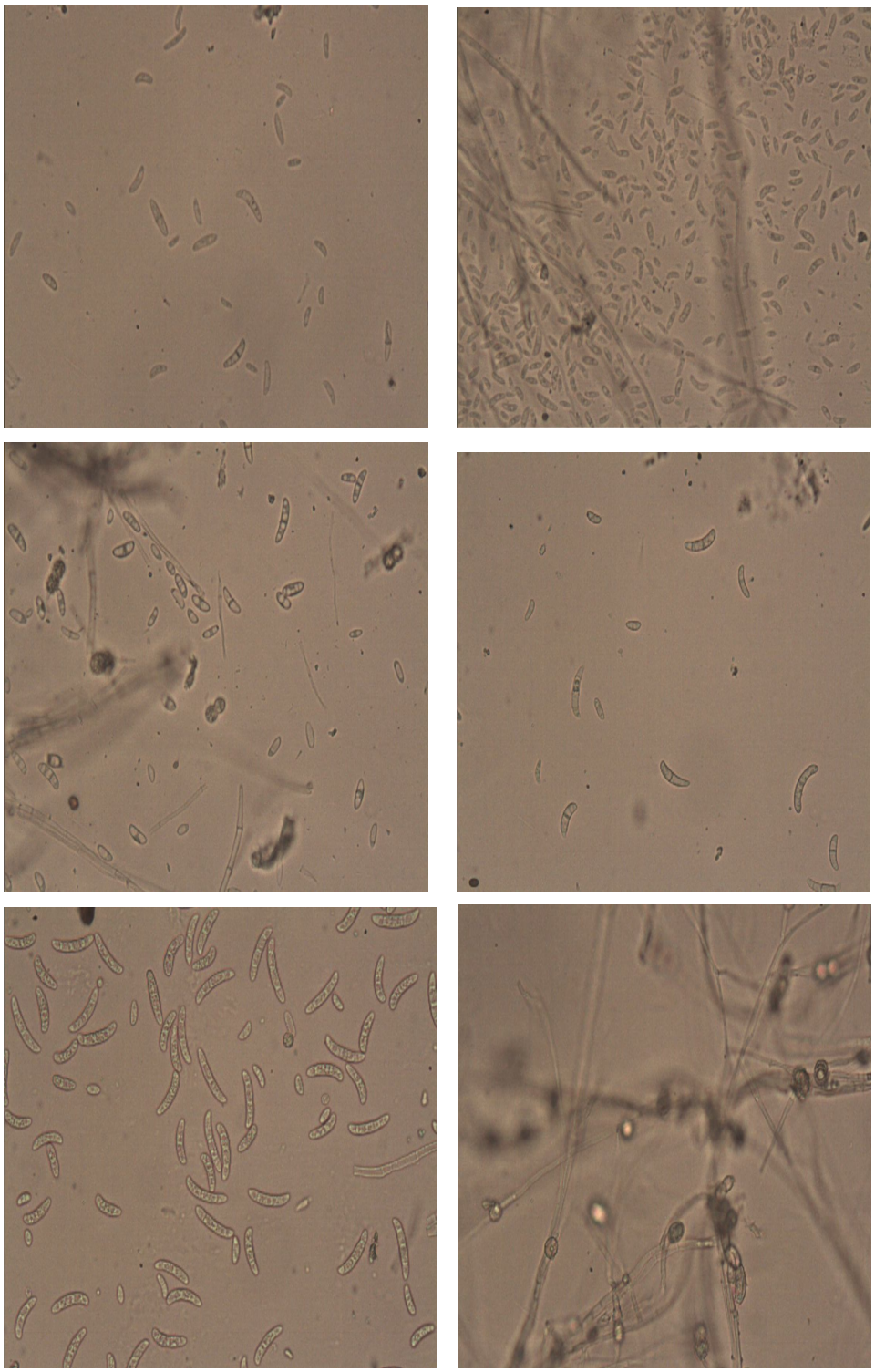

Plate 2: Microphotograph with macro conidia and micro conidia of *Fusarium solani* isolates AFS1, AFS2, AFS3, AFS4, AFS5 and AFS6 (Ananthapur *F.solani*)

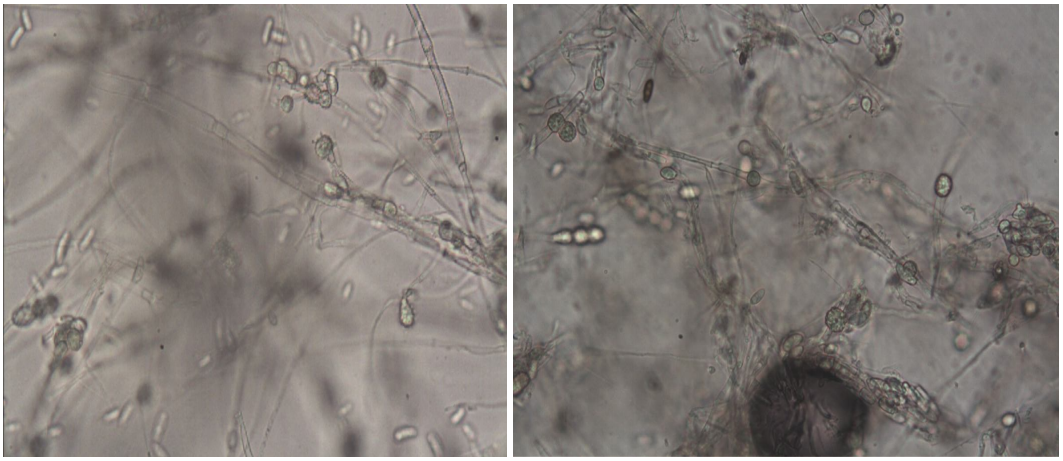

Plate 3: Microphotograph with macro conidia and chlamydospores of *F .solani* isolates AFS7 and CFS13

Table 1 : Cultural characters of *Fusarium solani* isolates in PD broth and PDA medium

S. No	Isolates	Colony character	Pigmentation	Sporulation
1	AFS_1	White sparse and fluffy mycelial growth	dark yellow	Profuse
2	AFS_2	White sparse mycelium in concentric rings	Pale yellow	Moderate
3	AFS_3	White cottony and fluffy mycelium	Pale pink	Profuse
4	AFS_4	White dense, fluffy mycelium with concentric rings	Straw yellow	Moderate
5	AFS_5	White fluffy and raised colony	Pale yellow	Profuse
6	AFS_6	White sparse growth with smooth margin	Dusky red	Profuse
7	AFS_7	White cottony and fluffy growth with smooth margin	Pink	Profuse
8	CFS_8	White dense growth in concentric rings with smooth margin	Dusky yellow	Moderate
9	CFS_9	White sparse mycelium in concentric rings	Dusky red	Profuse
10	CFS_{10}	White cottony and raised mycelium	Pink	Moderate
11	CFS_{11}	White fluffy growth in concentric rings	Dull red	Profuse
12	CFS_{12}	White dense growth in concentric rings	Pale yellow	Profuse
13	CFS_{13}	White sparse growth	Black to brownish	Profuse

Table 2: Size, shape, colour, septation of the macro conidia and micro conidia of different *Fusarium solani* isolates

S. No	Isolates	Macro conidia (µm) L X B	Micro conidia (µm) L X B	Septation		Shape		Colour
				Micro	Macro	Macro conidia	Micro conidia	
1	AFS_1	14-16 X 3-4	4-5 X 1-2	0-1	3-5	Sickle shaped with blunt ends	Round to oval	Hyaline
2	AFS_2	23-26 X 4-5	6-7 X 1-2	0-1	3-4	Elongated with blunt ends	Round to oval	Hyaline
3	AFS_3	19-20 X 3-4	4-8 X 1-3	0-1	3-4	Sickle shaped	Round to oval	Hyaline
4	AFS_4	21-23 X 3-4	5-7 X 1-3	0-1	2-3	Sickle shaped	Round to oval	Hyaline
5	AFS_5	17-18 X 3-4	9-10 X 1-2	0-1	3-4	Sickle shaped with blunt ends	Round to oval	Hyaline
6	AFS_6	24-26 X 4-5	3-4 X 1-2	0-1	3-4	Sickle shaped	Round to oval	Hyaline
7	AFS_7	13-15 X 3-4	3-5 X 1-2	0-1	2-3	Sickle shaped	Round to oval	Hyaline
8	CFS_8	16-18 X 4-5	8-9 X 1-3	0-1	2-3	Sickle shaped with slight blunts	Round to oval	Hyaline
9	CFS_9	15-17 X 3-4	3-4 X 1-2	0-1	3-5	Sickle shaped	Round to oval	Hyaline
10	CFS_{10}	27-29 X 4-5	5-7 X 1-3	0-1	3-4	Sickle shaped	Round to oval	Hyaline
11	CFS_{11}	25-27 X 4-5	9-10 X 1-3	0-1	3-4	Sickle shaped with blunt ends	Round to oval	Hyaline
12	CFS_{12}	23-28 X 4-5	7-9 X 1-2	0-1	2-3	Sickle shaped	Round to oval	Hyaline
13	CFS_{13}	18-20 X 3-4	3-5 X 1-2	0-1	3-4	Sickle shaped with blunt ends	Round to oval	Hyaline

Table 3: Radial growth (in mm) of *Fusarium solani* isolates up to 8 days after inoculation

S.No	Isolates	24 hrs.	48 hrs.	72 hrs.	96 hrs.	120hrs.	144 hrs.	168 hrs.	192 hrs.	Score
1	AFS_1	14	28	37	47	62	75	89	-	+
2	AFS_2	13	23	32	44	63	74	88	-	+
3	AFS_3	17	24	33	53	68	72	76	79	++
4	AFS_4	13	22	35	46	65	73	83	-	+
5	AFS_5	12	20	31	45	58	53	55	62	+++
6	AFS_6	15	23	34	49	61	68	70	73	++
7	AFS_7	12	19	26	37	42	54	68	70	+++
8	CFS_8	15	21	28	34	48	62	72	75	++
9	CFS_9	18	25	27	51	69	78	90	-	+
10	CFS_{10}	14	24	38	43	57	60	73	78	++
11	CFS_{11}	19	27	30	42	49	58	64	72	+++
12	CFS_{12}	16	20	37	41	49	52	58	69	+++
13	CFS_{13}	14	26	39	47	69	76	89	-	+

+: Fast growing ++: Moderately growing +++: Slow growing

AFS – Ananthapur *F. solani* CFS – Chittoor *F. solani*

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