"Unveiling the Hidden Enemies: Morphological and Molecular Characterization of Pod Rot Pathogens in Groundnut (*Arachis hypogea* L.) in India"

Abstract

Pod rot of groundnut is a complex disease caused by multiple pathogens causes significant economic losses. Present study was undertaken to know the pathogens associated with the disease. In Chittoor, Andhra Pradesh, roving survey against pod rot of groundnut revealed association of three fungal species: *Sclerotium rolfsii, Rhizoctonia bataticola* and *Fusarium* spp. Molecular analysis with modified CTAB method and performing PCR using ITS1 and ITS4 primers yielded DNA bands of 650-700 bp, 550-600 bp, and 500-600 bp for *Sclerotium, Rhizoctonia* and *Fusarium* species, respectively. Sequencing of the rDNA ITS region confirmed the identities of the pathogens as *Sclerotium rolfsii, Rhizoctonia bataticola, Fusarium solani, Fusarium oxysporum*, and *Fusarium keratoplasticum*, based on similarity with NCBI reference sequences. This study confirms that *Sclerotium rolfsii*, *Rhizoctonia bataticola* and *Fusarium* spp. are the primary causal agents of pod rot disease in Andhra Pradesh.

Keywords:*Scleroium rolfsii, Rhizoctonia bataticola, Fusarium solani, Fusarium oxysporum* and *Fusarium keratoplasticum*, Charaterization

1. Introduction

Groundnut (*Arachis hypogaea* L.) a cleistogamous allotetraploid legume cultivated widely in tropical, subtropical and warm temperate zones. India is the second largest producer of groundnut in the world after China together which accounts for 51 per cent of the world's production of groundnut. India cultivates about 4.82 M ha area and produces 9.95 MT of groundnut with the yield of 2063 kg/ha per hectare [1]. There is large difference between the realized pod yield in relation to the potential yield in majority of the situations as the crop subjected to many biotic and abiotic constraints [2]. The groundnut crop known to be affected by many fungal, bacterial and viral pathogens during different stages of growth and cause severe yield losses and in some instances impairing the quality of the produce [3,4,5,6,7,8]. Among them pod rot disease of groundnut is fast becoming an economically important disease wide spread across the tropical, subtropical and warm temperate regions causing severe damage to groundnut production in a number of countries like India, Israel, USA and East Africa [9,10,11,12]. The loss in yield due to preharvest pod rot may vary in the range of 5-50 per cent and can reach to 100 per cent depending on the geographical location, soil type and climate [13, 14, 15, 16,17].

Pod rot is usually considered to be of complex etiology. The underground pods are subjected to attack by more than one soil borne pathogens such as *Fusarium* spp., *Sclerotium rolfsii*, *Macrophomina phaseolina, Pythium* spp .*Rhizoctonia solani, Aspergillus* spp. *etc* [9, 10, 11, 12,] which causes different symptoms on affected pod by changing colour and texture of pods to either tan or brown, dry decay or greasy, dark brown/black, wet decay with/without mycelia

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growth depending on the associated pathogens, edaphic factors and prevailing environment conditions [18]. For fungal pathogens, different morphotypes can play different roles during the host-pathogen interaction to optimize overall pathogen fitness. Plant pathologists especially mycologists have traditionally utilized morphological characters such as spore producing structures, colony colour *etc* as a means of species identification within the fungal community. However morphological approaches may not perform well for identifications as these morphological characters can be problematic even for trained mycologists [19]. Morphological characters can often misleading due to hybridization [20], cryptic speciation [21] and convergent evolution [22]. At present molecular markers are being extensively used to characterize populations of plant pathogens [23,24,25,26,27]. The polymerase chain reaction (PCR) and DNA sequencing, among others, have been used to study fungal populations [28,29,30].

Many scientist worked on groundnut pod rot have given the whole range of microorganism associated with pod rot of groundnut but lack to provide the main causal agent of pod rot disease. Hence this paper focus on the main causal agents of pod rot of groundnut instead of focus on whole microbes associated with pod rot of groundnut as secondary pathogens. The study also provides morphological and molecular characterization of pathogens.

2. Material and methods

2.1. Isolation of pod rot fungal pathogens of groundnut

A total of 48 pod rot samples were collected from 19 major groundnut growing villages spread across Chittoor district of Andhra Pradesh during 2020. The pods were originated from the most popular groundnut varieties grown in Chittoor such as 'Kadiri-6', 'TAG-24', 'Dharani' and 'Narayani' which were cultivated both under rainfedand irrigated conditions with the occurrence of pod rot disease in almost all the cultivars irrespective of the season. The survey ascertained the occurrence of the pod rot disease with the mean pod rot disease incidence varied from 5.0 per cent to 35.4 per cent. A total of 29 isolates were used for morphological and molecular characterization. To isolate pathogens species, groundnut pods with typical symptoms of pod rot disease were cleaned with sterilized distilled water to remove surface debris and infectedpodportions made into small bits of 1×1 cm^2 using sterile knife. These bits were surface sterilized with 1 % sodium hypochlorite (NaOCl) solution followed by rinsing with three changes of sterile distilled water and plated on the potato dextrose agar medium (PDA) amended with streptomycinsulphate (0.05 g/L). The Petri plates were incubated at 28 ± 2 ºC with 12/12h photoperiod for 7 days and observed for the growth of fungal colonies. The pure culture of pathogens was obtained by single hyphal tip method [31].

2.2. Morphological and cultural variability

Morphological and cultural variability for all 28 isolates was studied on PDA medium under *in vitro* condition. Mycelial disks of 5 mm diameter made from the margins of actively growing culture were inoculated in the centre of 90

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mm Petri plates containing 20 ml of PDA in three replications. Inoculated plates were incubated at 28±2ºC. After seven days of incubation, the cultures was used to assess the colony characteristics such as colony colour, radial growth rate, conidial morphology and pigmentation. Confirmation of the genera was done by observing the fungal spores, their shape, size and colour under light microscope at 10X and 40X magnification [32, 33, 34].

2.3. Molecular characterization

2.3.1. DNA extraction

To verify the morphological identification of isolated pathogen species 29 selected isolates were further investigated by molecular techniques using ITS-PCR assays.

The total genomic DNA of all the isolates was extracted using Cetyl Tri Methyl Ammonium Bromide (CTAB) method [35]. Mycelial mat of all the isolates were grown on 100ml Potato Dextrose Broth in 250 ml conical flask and incubated at 28 ± 2 ºC for 3 days. Powdered mycelium was transferred into a 2 ml Eppendorf tube, to this 500 µl of CTAB extraction buffer (0.13MTris-HCl, pH 8.9; 0.017M EDTA pH 7.0; 0.83 % SDS, 5 % PVP and 1 M LiCl) was added. Subsequently, the tubes were incubated in the water bath at 65 ºC for 45 min with occasional shaking at every 10 min interval to ensure even spread of the heat and extraction buffer. After incubation, tubes were then centrifuged at 10,000 rpm for 10 min at 15 ºC. Then an equal amount of Phenol: Chloroform: Isoamyl alcohol (25: 24: 1 v/v) was added and mixed thoroughly by gentle inversions and centrifuged at 12000 rpm for 10 min at 25 ºC. The supernatant was collected in separate 2 ml Eppendorf tube and Chloroform: Isoamyl (24:1) alcohol was added and mixed thoroughly by gentle inversions and centrifuged at 12000 rpm for 10 min at 25 ºC. Again supernatant was collected in fresh tubes and isopropyl alcohol of 2/3rd volume of supernatant and 50 µl of 3M sodium acetate was added and were kept undisturbed overnight at -20 ºC. Later the samples were centrifuged at 13000 rpm for 20 min at 4 ºC to pellet out the nuclear DNA. The supernatant was discarded and the DNA pellet was washed with 100 µl of 70 per cent ethanol and centrifuged at 12000 rpm for 10 min. The supernatant was removed. The ethanol was left to evaporate, and the pellet was dissolved in 50 µl of Tris-EDTA (TE) buffer (pH 8.0) and stored at -20 ºC.

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2.3.2. Qualitative and quantative verification of DNA

The quality and quantity of DNA was analyzed by running 2 µlof each sample mixed with 2 µlof 10x loading dye in 1% agarose gel. The DNA from all isolates produced clear sharp bands in one per cent agarose gel indicating the good quality of DNA. The DNA has been quantified by comparing with the 1 kb size marker (Thermo Scientific, Tirupati) and by spectrophotometer (Nanodrop ND1000).

2.3.3. PCR amplification of ITS region

The PCR was performed using Eppendorf Master-cycler X50s (model AG22331, Made in Germany). The primers ITS1 (5'-TCCGTAGGTGGACCTGCGG-3') and ITS4 ('5-CCTCCGCTTATTGATATGC - 3') were synthesized and obtained from Thermo Scientific, Tirupati, India [36]. The PCR amplification was carried out in 25 µl reaction mixture containing 1 µl of DNA sample with 2.5 µl of 10X PCR buffer, 2.5 mM MgCl2, 1.0 µl of 2 mM dNTPs, 20 pmol of each primer (1.0 µl) and 0.2 µl of Taq DNA Polymerase and made up to 25 µl with 14.8 µl of nuclease free water.

The PCR conditions included an initial denaturation at 94 °C for 2 min, 35 cycles of denaturation at 94 °C for 1 min, primer extension for 30 s at 72 °C and final extension at 72 °C for 10 min with only change in annealing temperature of 56 °C**,** 54°Cand 58 °C for 1 min respectively for *Sclerotium* spp., *Rhizoctonia* spp and *Fusarium* spp. After completion of the PCR reaction, the products were loaded into the wells of 1.5 per cent (w/v) agarose gel prepared in 1X TBE containing ethidium bromide as described earlier by mixing with 2 µl of 6X loading dye (Thermo Scientific, Tirupati). A GeneRuler 100 bp DNA ladder (Thermo Scientific, Tirupati) was loaded as a standard reference. The gel was run at constant voltage of 60 V for about 1-2 hours. The banding profiles of ITS-PCR products were documented in gel documentation system (Biorad Gel Doc XR+ Imaging System) and saved the image for later use.

2.3.4. ITS data analysis

The ITS nucleotide sequences for each isolate were then compared to those in the public domain databases NCBI (National Center for Biotechnology information; www.ncbi.nih.gov) using Basic Local Alignment Search Tool for Nucleotide Sequences (BLASTN). Alignment of ITS DNA sequences was done using Clustal_W program [37]. Phylogenetic tree was created using CLC Sequence Viewer Version 6.3 based on UPGMA (unweighted pair group method for arithmetic analysis). The confidence of the branching was estimated by bootstrap analysis.

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3. Result and discussion

3.1. Isolation of pathogens

The study provides an abstract into the predominance and composition of the fungal populations associated with the pods causing damage to the crop in the Chittoor district.During the survey a total of 41 pod rot affected samples were collected in farmer fields spread across twenty villages in twelve mandals of Chittoor district. Twenty-nine isolates belonging to three pathogenic fungi *viz*.,

Fusarium spp., *Rhizoctonia* spp. and *Sclerotium* spp. were isolated and such diversity was reported in several of the survey studies by the researchers [17, 38].

It is reported that pod rot disease of groundnut can be caused by a number of different fungi including *R. solani*, *Pythium* spp., *Sclerotium rolfsii*, and *Fusarium* spp. *F. oxysporum, F. semitectum* and *F. moniliforme* , *Aspergillus fumigatus, A. flavus, A. niger, P. chrysogenum* and *F. oxysporum*[18]. It is observed that the fungal pathogens, *Sclerotium rolfsii*, *Rhizoctoniabataticola*, and *Fusarium* spp. which are typically reported as pathogens of the pod rot were associated with the pod samples collected in the 12 major groundnut growing mandals of Chittoor district [13, 39]

3.2. Morphological and cultural variability

3.2.1. *Sclerotium rolfsii*

All the ten isolates of *Sclerotium* spp. isolated from the pod rot samples collected in Chittoor district were studied morphologically on the basis of mycelial growth, colony colour, mycelial dispersion and appearance (Table 1). The sclerotial morphology was characterized based on sclerotial colour, shape, number, sclerotial intiation and their arrangement on PDA surface as mentioned in Table 2

The mycelial growth in all the isolates was first pure white in colour and later some isolates turned into dull white with radial spreading having fan like appearance. Isolates CSr1, CSr2, CSr3, CSr4, CSr5, CSr6, CSr9, CSr10 had pure white mycelium; while isolates CSr7, CSr8 had dull white mycelium. Further the isolates were categorized based on their growth rate which varied from 72 h to 132 h. Isolate CSr1 categorized as fast growing (72 h), CSr4, CSr6, CSr8 as medium growing (96-108 h) and CSr2, CSr3, CSr5, CSr7, CSr9 as slow growing (120-132 h) isolates on the basis of time taken to complete full growth on PDA plates. Microscopic examination of the fungal culture revealed the aerial hyaline, thin walled, septate hyphae with profusely branched mycelium. The mycelium after approaching the edges of the Petri plate between 9 to 15 days of inoculation, small white mycelial knots were formed which later turned into mustard seed like sclerotial bodies with light brown to deep dark brown or brownish black in colour and round to irregular in shape (Table 2). Among the ten isolates of *Sclerotium*, CSr6 initiated sclerotial bodies production at 9 days after inoculation (DAI); while isolates CSr1 and CSr7 produced sclerotial bodies at 10 to 11 DAI; majority of the isolates like CSr3, CSr4, CSr5, CSr8, CSr9, CSr10 initiated sclerotial bodies formation between 12 to 13 DAI; whereas isolate CSr2 took 15 DAI to initiate sclerotial bodies formation. Twenty-five days after inoculation, the colour of sclerotial bodies were recorded by comparing with the standard colour chart. Sclerotia of isolates CSr4, CSr6, CSr9, CSr10 were light brown; while CSr2, CSr3, CSr5, CSr8 were dark brown; CSr1, CSr7 were deep dark brown. On the basis of size and shape sclerotia were characterized into minute, small, medium and large in size; round, oval, irregular in shape. Isolates CSr1, CSr7 were minute round; CSr3, CSr4 were small round; CSr10 was small, round to irregular; CSr6, CSr9 were medium round; CSr8 was medium irregular and CSr2, CSr5 were large

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irregular. The sclerotial arrangement on PDA media was observed with sclerotia scattered all over the plate (CSr4, CSr10); sclerotia placed on the surface of lid (CSr2, CSr5,); on the surface of lid and scattered (CSr6); peripheral (CSr3, CSr7); sclerotia placed both at peripheral and central (CSr1); sclerotia placed at as centre large cluster and scattered (CSr8). The sclerotial number varied from 10 to 167 among the isolates and the highest sclerotial production was observed in isolate CSr9. while the test weight of 10 sclerotial bodies among the isolates varied from 4 mg to 49 mg with isolate CSr2 recording highest test weight of 49 mg (Table 2).

The results of the present study revealed presence of wide variation among isolates of *S. rolfsii* from Chittoor district associated with pod rot disease of groundnut. Rakholiya and Jadeja and coworkers observed considerable variability in mycelial characteristics and sclerotial dimensions in 30 isolates of *S. rolfsii* in groundnut [40]. Variations in mycelial growth rates, colony morphology, sclerotial production, arrangement, number, colour, shape and size have been reported by different scientists on various hosts and media. The pattern of sclerotial distribution varied among the isolates as aerial, scattered, peripheral and aerial-scattered [39,41,42,43,44,45,46,71]. The sclerotial weight in most of the isolates varied from 3.3 to 12.0 mg/10 sclerotia with maximum weight of 26.0 mg/10 sclerotia in one of the isolate was reported [39,40].

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Table 1.Colony characteristics among isolates of *Sclerotium* spp.

S.No	Isolate	Mycelial colour	Type of growth	Hours to grow full plate (h)	Mycelial appearance and dispersion
1	CSr1	White	Fluffy	72	Loose cottony, sparse, peripheral upright growth
2	CSr2	White	Fluffy	120	Loose cottony, sparse, irregular upright growth
3	CSr3	White	Fluffy	120	Dense cottony, aggregated upright fluffy growth
4	CSr4	White	Fluffy	96	Dense cottony, aggregated peripheral upright growth
5	CSr5	White	Flat	120	Loose cottony, sparse growth
6	CSr6	Dull white	Fluffy	96	Dense cottony, aggregated circular upright growth
7	CSr7	Dull white	Flat	132	Loose cottony, sparse, growth
8	CSr8	White	Fluffy	108	Dense cottony, aggregated circular upright growth
9	CSr9	White	Fluffy	120	Loose cottony, sparse, concentric upright growth
10	CSr10	White	Fluffy	96	Dense cottony, aggregated growth

Table 2.Sclerotial characteristics among isolates of *Sclerotium* spp.

S. No.	Isolate	Sclerotial initiation (Days after inoculation)	Sclerotial distribution pattern	Sclerotial No./plate	Shape	Colour	Test weight (mg)
1	CSr1	11	Peripheral and central	58	Minute, round	Deep dark brown	10
2	CSr2	15	Surface of lid	78	Large, irregular	Dark brown	49
3	CSr3	12	Peripheral	49	Small, round	Dark brown	15
4	CSr4	12	Scattered all over plate	10	Small, round	Light brown	12
5	CSr5	13	Surface of lid	55	Large, irregular	Dark brown	37
6	CSr6	9	Surface of lid and scattered	74	Medium, round	Light brown	20
7	CSr7	10	Peripheral	113	Minute, round	Deep dark brown	4
8	CSr8	13	Central large cluster and scattered	91	Medium, irregular	Dark brown	18
9	CSr9	12	Surface of lid	167	Medium, round	Light brown	22
10	CSr10	13	Scattered all over plate	62	Small, round to irregular	Light brown	13

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3.2.2. *Rhizoctonia bataticola*

The obtained nine isolates of *Rhizoctonia* spp. from the collected samples were studied morphologically on the basis of mycelial colour, presence of aerial mycelium and colony appearance as presented in the table 3. The colour of mycelium varied from creamy white to dark grey. Isolate CRb4 showed creamy white mycelium, while isolates CRb3, CRb7 were with light grey mycelium; isolates CRb1, CRb2 with grey mycelium and isolates CRb5, CRb6, CRb8 and CRb9 with dark grey mycelium.

The isolates showed difference in colony morphology as regular (CRb1, CRb8); regular with fluffy white mycelial growth (CRb9); regular with white mycelial mosaic (CRb5); reticulation (CRb2, CRb6); appressed (CRb3, CRb4); concentric zonation (CRb7). Isolates also showed variation in number of days taken to complete full plate growth. Isolates CRb7 and CRb8 covered entire Petri plate within four days; while isolates CRb2, CRb4, CRb6 and CRb9 covered plate in seven days; isolates CRb1, CRb3 and CRb5 entire plate in eight days to grow full plate (Table 3).

The morphological variability observed in the present study is in accordance with the earlier reports of colony colour among isolates of *R. bataticola* varying from light black to black and light grey to grey with presence or absence of aerial mycelium on PDA medium [47,48]. The isolates of *R. bataticola* showed difference in the colony morphology as regular, regular zonation, concentric zonation, regular fluffy and irregular appressed or completely or partially suppressed. The growth rate of the pathogen varied from 102 h to 216 h [39,47].

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Table 3. Colony characteristics among isolates of *Rhizoctonia* spp.

S. No.	Isolate	Colony colour	Aerial mycelium	Colony morphology	Days taken to grow full plate
1	CRb1	Grey	Present	Regular	8
2	CRb2	Grey	Present	Reticulation	7
3	CRb3	Light grey	Absent	Appressed	8
4	CRb4	Creamy white	Absent	Appressed	7
5	CRb5	Dark grey	Absent	Regular with white mycelia mosaic	8
6	CRb6	Dark grey	Absent	Reticulation	7
7	CRb7	Light grey	Present	Concentric zonation	4
8	CRb8	Dark grey	Present	Regular	4
9	CRb9	Dark grey	Present	Regular with fluffy white mycelia growth	7

Table 4.Colony characteristics among isolates of *Fusarium* spp.

S.No.	Isolate name	Colony morphology	Mycelium colour		Days taken to grow full plate
			Top side of Petri plate	Reverse side of Petri plate	
1	CF1	Aerial mycelium with irregular surface	White	Light brown	15 d
2	CF2	Aerial mycelium with septation	White	Dark red	15 d
3	CF3	Aerial mycelium with septation	Purple	Dark red	14d
4	CF4	Aerial mycelium with irregular surface	White	Light brown	15 d
5	CF5	Aerial mycelium with smooth surface	White	Orange	13d
6	CF6	Aerial mycelium with septation	Purple	Dark red	15 d
7	CF7	Aerial mycelium with smooth surface	White	Creamy white	14 d
8	CF8	Appressed mycelium with zonation	Orange	Creamy white	18 d
9	CF9	Aerial mycelium with smooth surface	White	Orange	12 d 6 hr
10	CF10	Aerial mycelium with irregular surface	White	Brown	13 d

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3.2.3. *Fusarium* spp.

All ten isolates showed a wide variation with respect to the colony colour; pigmentation of the top view of the colony varied from white (CF1, CF2, CF4, CF5, CF7, CF9, CF10), orange (CF8) and purple (CF3, CF6), while the colony on the reverse side of plate showed colours from creamy white (CF7, CF8), orange (CF5, CF9), light brown (CF1, CF4), brown (CF10) and dark red (CF2, CF3, CF6). The colony morphology of the isolates varied as aerial mycelium with smooth surface (CF5, CF7, CF9), aerial mycelium with irregular surface (CF1, CF4, CF10), aerial mycelium with septation (CF2, CF3, CF6), appressed mycelium with zonation (CF8) (Tabel 4).

The growth rate of isolates were recorded in terms of number of days taken for spreading to full Petri plate which varied from 12 days to 18 days as mentioned in table 4. Isolate CF9 was fast growing isolate which covered an entire plate (9 cm) within 12 days, while isolates CF5 and CF10 took 13 days ; isolates CF3, CF7 covered in 14 days; isolates CF1, CF2, CF4, CF6 covered the Petri plates in15 days and isolate CF8 covered in 18 days to grow full Petri plate.

All the 14 isolates produced two types of asexual spores *viz.*, micro and macro conidia. The resting spores, chlamydospores also were observed in 10-15 days age old cultures. The number of septa in the micro and macro conidia was 0-1 and 1-4 respectively which were hyaline. Microconidia of all the isolates (CF1, CF2, CF4, CF6, CF7, CF8, CF10) were 0-1 septate except isolates CF3, CF5, CF9 were aseptate. Whereas macroconidia of most of the isolates (CF1, CF4,CF5, CF7, CF8, CF10) were 2-3 septate; while isolates CF2, CF3, CF6 were having 1-2 sepatate; isolate CF9 was 3-4 sepatate. The shape of macroconidia was sickle shaped (CF4, CF6, CF7, CF9); sickle shaped with blunt ends (CF1, CF10); elongated with blunt ends (CF2, CF3, CF5) and elongated with sharp ends (CF10); microconidia of all the isolates were oval shaped. The chlamydospores were globose, intercalary and terminal among the isolates (Table 5).

The variability for the cultural and morphological traits was observed in several of the previous studies of *Fusarium* spp. [49,50,51]. The results indicate that *Fusarium* isolates were highly variable in their colony growth pattern, size of colony and pigmentations. Researchers have found variation in isolates with respect to their mycelium type, colony colour, toxin production, pathogenicity [52] and also various types of pigmentations (yellow, brown, crimson) in culture [53, 54, 55, 56]. The variation in number of septa of macro and microconidia of *Fusarium solani* was earlier reported among the isolates [57]. The vaiability in chlamydospore formation and position among the isolates was also observed as reported by earlier researchers [39,58].

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Table 5.Morphological characteristics among isolates of *Fusarium* spp.

S.No.	Isolate	Conidial Shape		Septation		Chlamydospore position	Colour
		Micro conidia	Macroconidia	Micro conidia	Macro conidia		
1	CF1	Oval	Sickle shaped with blunt end	0-1	2-3	Both terminal and intercalary	Hyaline
2	CF2	Oval	Elongated with blunt end	0-1	1-2	Both terminal and intercalary	Hyaline
3	CF3	Oval	Elongated with blunt end	0	1-2	Only terminal	Hyaline
4	CF4	Oval	Sickle shaped	0-1	2-3	Only terminal	Hyaline
5	CF5	Oval	Elongated with blunt end	0	2-3	Both terminal and intercalary	Hyaline
6	CF6	Oval	Sickle shaped	0-1	1-2	Both terminal and intercalary	Hyaline
7	CF7	Oval	Sickle shaped	0-1	2-3	Both terminal and intercalary	Hyaline
8	CF8	Oval	Elongated with sharp end	0-1	2-3	Both terminal and intercalary	Hyaline
9	CF9	Oval	Sickle shaped	0	3-4	Both terminal and intercalary	Hyaline
10	CF10	Oval	Sickle shaped with blunt end	0-1	2-3	Both terminal and intercalary	Hyaline

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3.3. Molecular characterization of isolates using ITS-PCR

Amplification of the ITS region of 18S rRNA gene and 5.8s rDNA using ITS 1 and ITS 4 primers of all ten isolates of *Sclerotium* spp. varied from 650–700 bp fragment which is specific to *S. rolfsii.* Similarly genomic DNA of *Rhizoctonia* spp. was amplified into a region of 550 bp - 600bp fragment which is specific to *Rhizoctonia* spp. and 500 bp to 600 bp fragment specific to *Fusarium* spp. The structure of rDNA cluster and the expected amplified products with ITS-1 and ITS-4 primers are shown in Fig. 1.These PCR products were sequenced using forward and reverse primers at Barcode BioScience Pvt.Ltd., Bengaluru. Homology search was done using BLAST algorithm available at the http://www.ncbi.nlm.nih.gov. Multiple alignments for homology search were performed using the Cluster W algorithm software and the phylogenetic tree was constructed.nBlast was used to perform similarity search. The BLAST data results revealed that the *Sclerotium* species matched with the reference strains of NCBI results and identified as *Sclerotium rolfsii* (Table 6). Similarly *Rhizoctonia* species matched with reference strains of NCBI and identified as *Rhizoctonia bataticola* (Table 7). Whereas among 10 isolates of *Fusarium,* eight isolates were identified as *Fusarium solani* while isolate CF2 identified as *Fusarium keratoplasticum* (member of *F. solani* complex), while isolates CF8 and CF9 were identified as *Fusarium oxysporum* (Table 8).

The present results are in agreement with Adandonon and coworkers, who studied genetic variation among *S. rolfsii* isolates of cowpea by using mycelial compatibility and ITS rDNA sequence data and obtained an amplification fragment of about 700 bp which is specific for *S. rolfsii*. In the present study, all isolates gave the same size of the fragment that is 650–700 bp, which suggests that these isolates are the same species [59]. Harlton and coworkers screened a worldwide collection of *S. rolfsii*, using universal primer pairs ITS 1- ITS 4, ITS 1 – ITS 2 and ITS 3 – ITS 4 and revealed variation in ITS regions with 12 sub-groups with *S. rolfsii* and *S. delphinii* yielded a common unique band of about 720 bp [60]. Prasad *et al.* (2010), Kwon *et al.* (2011), Mahadevkumar *et al.* (2015), Gururaj et al. (2016), Poornima *fig.* (2018) and Swain *et al.* (2018) performed rDNA amplification with specific ITS 1 and ITS 4 that produced approximately 650 to 700 bp in all isolates confirming the isolates obtained were *Sclerotium rolfsii* [61, 62, 63, 64, 65, 66]. The total size of the ITS 1 and ITS 4 regions including 5.8S rDNA gene of different *Fusarium* spp. studied by Singh and kumar (2011) varied from 380 to 620 bp. These were then sequenced and compared with NCBI database using BLAST and identified as *Fusarium oxysporum*, *F. equiseti*, *F. proliferatum* and *Fusarium* sp [56]. Whereas Aydin and coworkers obtained PCR amplified product of *Fusarium solani*, *F.oxysporum*, *F. sambucinum* and *F. equisetti* was amplified into region of about 500-600 bp length using ITS 4 and ITS 5 [67]. Similarly analysis of nucleotide sequences of amplified products using ITS 1 and ITS 4 allowed the identification of *Fusarium oxysporum* and *Fusarium solani* with more than 90 % similarity with the reference sequences in NCBI-BLAST program database search system [68]. The ITS regions and the 5.8S rDNA

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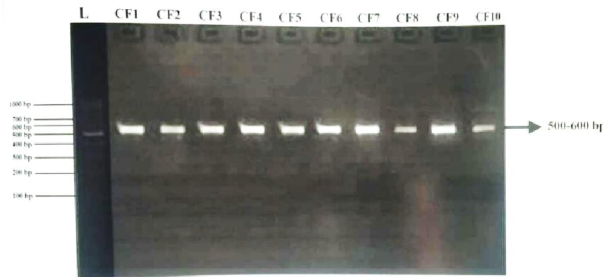
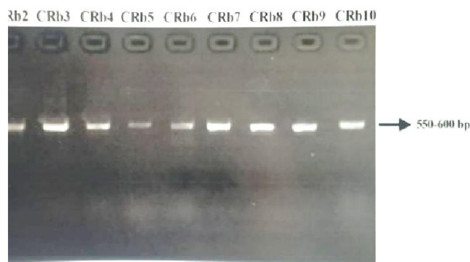
of 20 *M. phaseolina* isolates were amplified with ITS 1 and ITS 4 primers produced bands of ~500-600 bp with 65.28 to 100 per cent nucleotide similarity with already deposited *M. phaseolina* sequences in NCBI database [69]. Whereas Pandey and coworkers amplified rDNA gene cluster consisting of ITS 1, ITS 2 and 5.8S rDNA 2 to characterize the *M. phaseolina*





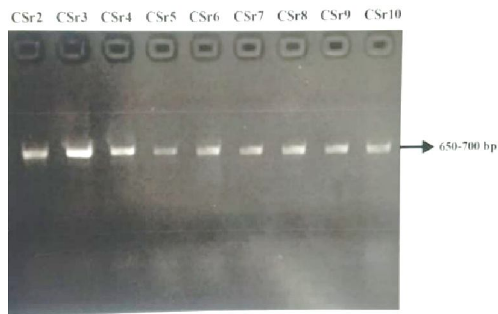


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Fig.1. PCR amplification of ITS region of pod rot associated patogens with ITS1 and ITS 4 primers. A. *Fusarium* spp. B. *Rhizactonia* spp. C. *Sclerotium* spp.

Table 6. Molecular identification of *Sclerotium* spp.

S. No	Isolate Name	Molecular Identification	Max Score	Total Score	Query Cover	E value	Per. ident	Acc. Len	Accession
1	CSr1	*Athelia rolfsii*	809	809	77%	0	90.42	615	MK418758.1
2	CSr2	*Athelia rolfsii*	944	944	55%	0	93.42	707	MT012545.1
3	CSr3	*Athelia rolfsii*	1116	1116	99%	0	97.41	687	MN380242.1
4	CSr4	*Athelia rolfsii*	957	957	97%	0	93.26	707	MT017581.1
5	CSr5	*Athelia rolfsii*	632	632	63%	2.00E-176	85.53	642	MW288292.1
6	CSr6	*Athelia rolfsii*	508	508	58%	3.00E-139	81.92	642	MK880693.1
7	CSr7	*Athelia rolfsii*	1131	1131	92%	0	99.36	704	MH514001.1
8	CSr8	*Athelia rolfsii*	965	965	78%	0	93.28	677	KT750883.1
9	CSr9	*Athelia rolfsii*	752	752	61%	0	88.46	637	MN861081.1
10	CSr10	*Athelia rolfsii*	1171	1171	99%	0	98.93	687	MN872304.1

Table 7.Molecular Identification of *Rhizoctonia* spp.

S. No	Isolate Name	Molecular Identification	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
1	CRb1	*Macrophomina phaseolina*	1088	1757	76%	0	94.02	1331	MG372013.1
2	CRb2	*Macrophomina phaseolina*	1090	1536	70%	0	94.14	1331	MG372013.1
3	CRb3	*Macrophomina phaseolina*	1053	1185	55%	0	95.87	785	MN096210.1
4	CRb4	*Macrophomina phaseolina*	1101	1531	72%	0	93.54	1331	MG372013.1
5	CRb5	*Macrophomina phaseolina*	1062	1503	68%	0	93.47	1331	MG372013.1
6	CRb6	*Macrophomina phaseolina*	1072	1512	70%	0	93.17	1331	MG372013.1
7	CRb7	*Macrophomina phaseolina*	1059	1766	98%	0	92.49	1331	MG372013.1
8	CRb8	*Macrophomina phaseolina*	1066	1204	61%	0	96.18	785	MN096210.1
9	CRb9	*Macrophomina phaseolina*	1085	1525	72%	0	93.27	1331	MG372013.1

Table 8. Molecular identification of *Fusarium* spp.

S. No	Isolate Name	Molecular Identification	Max Score	Total Score	Query Cover	E value	Per. ident	Acc. Len	Accession
1	CF1	*Fusarium solani*	974	1128	98%	0	99.63	613	MH890688.1
2	CF2	*Fusarium keratoplasticum*	298	298	82%	2.00E-76	92.09	521	MN559628.1
3	CF3	*Fusarium solani*	926	926	89%	0	97.79	576	MT658112.1
4	CF4	*Fusarium solani*	867	867	98%	0	95.63	559	LC633899.1
5	CF5	*Fusarium solani*	942	942	93%	0	99.81	564	KY785016.1
6	CF6	*Fusarium solani*	1011	1011	99%	0	98.42	571	KX583231.1
7	CF7	*Fusarium solani*	941	941	98%	0	98.33	564	KY785016.1
8	CF8	*Fusarium oxysporum*	806	975	65%	0	82.47	725	MZ661217.1
9	CF9	*Fusarium oxysporum*	806	975	65%	0	82.47	725	MZ661217.1
10	CF10	*Fusarium solani*	1079	1581	89%	0	93.21	808	JQ364977.1

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4. Conclusion

The present study revealed the main casual agents of pod rot of groundnut in Andhra Pradesh suggesting the focus of management of disease should be on *Sclerotium*, *Rhizoctonia* and *Fusarium* which is further supported by morphological and 5.8s RNA based identification.

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