# "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:***Scleroiumrolfsii, 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 5.8 M ha area and produces 6.7 MMT of groundnut with the yield of 1.2 MT 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*, *Macrophominaphaseolina*, *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

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 rainfed and 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 were obtained by single hyphal tip method [31].

### 2.2. Morphological and cultural variability

Morphological and cultural variability for all 28 isolateswas 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 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 $2/3^{rd}$ 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.

#### 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 isolatesproduced clear sharp bands in one per centagarose 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 ℃ for 2 min, 35 cycles of denaturation at 94 ℃ for 1 min, primer extension for 30 s at 72 ℃ and final extension at 72 ℃ for 10 min with only change in annealing temperature of 56 ℃**,** 54℃and 58 ℃ 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 bybootstrap analysis.



## 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 isolatedand 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, sclerotialintiation 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 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 *et al.* (2011) 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]. 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].

**Table 1Colony 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 2Sclerotial 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 |

### 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].

**Table 3Colony 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 4Colony 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 |

### 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].

**Table 5Morphological 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 |

### 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 BioSciencePvt.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 Adandononet al. (2005) 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 et al. (1995) 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 et al. (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 et al. (2019) 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

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 et al. (2020) amplified rDNA gene cluster consisting of ITS 1, ITS 2 and 5.8S rDNA using ITS 1 and ITS 2 to characterize the *M. phaseolina* isolates [70].

**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 |

**Table7Molecular 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 | *Macrophominaphaseolina* | 1088 | 1757 | 76% | 0 | 94.02 | 1331 | MG372013.1 |
| 2 | CRb2 | *Macrophominaphaseolina* | 1090 | 1536 | 70% | 0 | 94.14 | 1331 | MG372013.1 |
| 3 | CRb3 | *Macrophominaphaseolina* | 1053 | 1185 | 55% | 0 | 95.87 | 785 | MN096210.1 |
| 4 | CRb4 | *Macrophominaphaseolina* | 1101 | 1531 | 72% | 0 | 93.54 | 1331 | MG372013.1 |
| 5 | CRb5 | *Macrophominaphaseolina* | 1062 | 1503 | 68% | 0 | 93.47 | 1331 | MG372013.1 |
| 6 | CRb6 | *Macrophominaphaseolina* | 1072 | 1512 | 70% | 0 | 93.17 | 1331 | MG372013.1 |
| 7 | CRb7 | *Macrophominaphaseolina* | 1059 | 1766 | 98% | 0 | 92.49 | 1331 | MG372013.1 |
| 8 | CRb8 | *Macrophominaphaseolina* | 1066 | 1204 | 61% | 0 | 96.18 | 785 | MN096210.1 |
| 9 | CRb9 | *Macrophominaphaseolina* | 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 |

## 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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