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MORPHOLOGICAL AND GENOMIC VARIABLITY OF *SCLEROTIUM ROLFSII* SACC. CAUSING STEM ROT OF TUBEROSE

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ABSTRACT

Tuberose is one of the most important ornamental bulbous flower crop cultivated for cut and loose flower trade. The flower has been used for ornaments, bouquets and buttonholes or crown and frequently used during marriages and religious ceremonies. The tuberose was often infected by various numbers of diseases; among that *S. rolfsii* is the one of the major disease which causes stem rot disease. The *S. rolfsii* were collected from various locations of Tamil Nadu were examined for morphological and genomic variability. Fifteen isolates of *S. rolfsii* was assessed and various morphological growth parameters (Mycelia growth, No. of sclerotia/ plate, colour of sclerotia, time taken for sclerotial production (days) and variations among *S. rolfsii* isolates was recorded. ITS region of rDNA amplification with specific ITS1 and ITS4 universal primers produced approximately 600 to 700 bp in all the isolates confirmed that all the isolates obtained are *S. rolfsii*. The sequences of isolates viz., Sr1 and Sr2 were identified as *S. rolfsii* through BLAST search in NCBI website (www.blast.ncbi.nlm.nih.gov/Blast). The sequences were deposited in the Gene Bank with the accession numbers MK880692, MK880693.

Keywords: *S. rolfsii,* variability, GenBank, gene sequence, genomic variability.

INTRODUCTION

Tuberose (*Polianthes tuberosa* L.) is a commercially important ornamental bulbous plant cultivated in the world as well as in India for cut and loose flower trade. Flowers are also used for the extraction of its highly valued natural flower oil which is one of the most expensive raw materials of high-grade perfumes, cosmetic products (Mallavarapu, 1995) and aromatic compounds. Tuberose is a multipurpose ornamental plant and in the past it was considered as pest and disease free crop. Since last few years the crop is found badly infected with diseases like Stem rot, Leaf blight, *Sclerotial wilt* and *Alternaria* leaf spot. The *sclerotium* is often affected by tuberose and causes stem rot of tuberose, the symptoms which include flaccidity and drooping of leaves and later both tubers and roots show rotting symptoms (Anita Mohanthy *et al*., 2016). *Sclerotium* often has extensive host range, profuse growth and ability to produce numerous numbers of sclerotia that may persist in soil for many years. Different molecular approaches have been employed for identification of *S. rolfsii* isolates from other fungal pathogen (McDonald, 1993; Bruns, 1991; Okabe, 2001; Tyson, 2002). DNA has been extracted by using primer base pair of ITS1 and ITS4 produced a gene product of 600-700bp confirming the isolates to be *Sclerotium* spp and amplified by using PCR. The isolates are sequenced and deposited in the GenBank.

MATERIALS AND METHODS

Isolation of *S. rolfsii*

Isolations were made from roots and lower stems of affected tuberose plants. Uprooted plants were rinsed under running tap water and blotted dry with a sterile filter paper. Separate sub samples of roots and stems of each plant were treated by immersing in 1 per cent sodium hypochlorite for 1 min to eliminate secondary invaders, followed by rinsing for 30 sec in sterile distilled water. Tissues excised from dying rootlets, necrotic taproots and stems were cut into 2–3 mm long fragments and they were plated on Petri plates containing Potato Dextrose Agar (PDA) medium amended with 50 µg ml^{-1} of streptomycin sulphate incubated at 28 ± 2°C for 5–8 days (Anita mohanty *et al*., 2016). The fungus identification was based on the symptoms on the host plants, colony colour and morphology and young white *sclerotium*. The culture were examined and purified by using tissue segment method (Rangaswami and Mahadevan, 1999).

Cultural and morphological variability of *S. rolfsii*

Fifteen ml sterilized PDA medium was poured into sterile Petri dishes and allowed to solidify. A nine mm culture disc of *S. rolfsii* obtained from actively growing region was aseptically placed at the centre of the dish and incubated at room temperature (28 ± 2°C). The radial growth of the isolate (in mm) was measured at 5 days after inoculation (Vincent 1927). Radial growth of each colony in two directions at right angle was measured. Visual observation on the sclerotial formation was recorded. The pathogen was identified based on standard mycological notes (Barnett and Hunter, 1972) like morphological characters based on mycelia (colony morphology, mycelial growth) and sclerotial characters (sclerotial colour, number of sclerotic/ plate), time taken for sclerotial production were

Profile	Step	Temperature	Duration	No. of cycles
1	Initial denaturation	95°C	5 min	1
2	Denaturation	94°C	30 sec	35
3	Annealing	52°C	45 sec	
4	Extension	72°C	45 sec	
5	Final extension	72°C	7 min	1

Table 1. Cultural characteristics of various isolates of *Sclerotium rolfsii*

S.No	Isolate	Colony morphology	Mycelia growth (mm)	No. of sclerotia/ plate	Colour of sclerotia	Time taken for sclerotial production (days)
1	Sr1	Fluffy growth of mycelium	90.00	187	Brown	8
2	Sr2	White cottony growth of mycelium	87.34	132	Dark brown	12
3	Sr3	Cottony profuse growth of mycelium	87.17	115	Reddish brown	12
4	Sr4	Cottony white growth of mycelium	87.67	154	Dark brown	11
5	Sr5	Fluffy growth of mycelium	90.00	174	Pale brown	8
6	Sr6	Profuse cottony growth of mycelium	88.03	165	Pale brown	9
7	Sr7	Cotton white mycelial growth	85.08	83	Brown	15
8	Sr8	Cotton white mycelial growth	85.41	97	Brown	14
9	Sr9	Dull white profuse mycelium	86.31	103	Dark brown	14
10	Sr10	Cotton white mycelium	85.34	89	Dark brown	14
11	Sr11	Dull cottony white mycelial growth	79.85	32	Pale brown	24
12	Sr12	Profused white cottony mycelial growth	82.63	54	Dark brown	21
13	Sr13	Profused white cottony mycelial growth	84.68	80	Dark brown	17
14	Sr14	Pluffy cottony growth of mycelium	83.83	63	Dark brown	18
15	Sr15	White cottony mycelial growth	82.13	41	Pale brown	21

Plate 1 (a) : Gel image of *S. rolfsii*

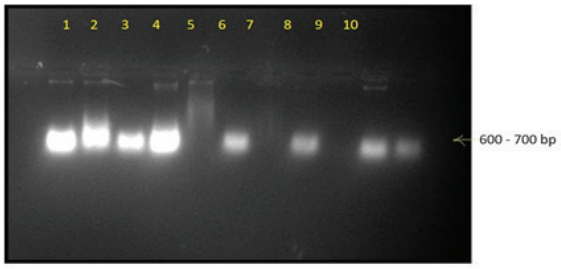

Plate 1 (b) : PCR image of *S. rolfsii*

recorded after 7 days of incubation.

DNA extraction

100 mg of mycelia was ground to a fine powder using liquid nitrogen. Pre warmed extraction buffer (1 ml) was added to the samples and it was ground once more in the buffer. All the samples were transferred to 2.0 ml Eppendorf tubes, 5µL Proteinase K (10 mg/ ml) was added. The tube was incubated in 37°C for 30 min and then at 65°C for another 30 min with frequent swirling. Samples were centrifuged at 10,000 rpm for 10 min and supernatant was transferred to a fresh Eppendorf tube. To the supernatant, 100 µl of 7.5 M potassium acetate was added and incubated at 4°C for 30 min. It was observed that addition of this solution and incubation of the samples for at least 15 min at 4°C increased the recovery of DNA yield with high quality.

The samples were centrifuged at 13,000 rpm for 10 min; the supernatant was transferred to a fresh tube, an equal volume of chloroform: isoamyl alcohol (24:1) was added and mixed by gentle inversion 30 - 40 times. The samples were centrifuged at 10,000 rpm for 10 min. The supernatant was transferred to a fresh tube and precipitated with 2/3 volume of isopropanol. The precipitated nucleic acids were collected and washed twice with wash solution. The obtained nucleic acid pellet was air-dried until the ethanol was removed and dissolved in an appropriate amount of TE buffer (50-70µL). The nucleic acid dissolved in TE buffer were treated with ribonuclease (RNase, 10 mg/ml), incubated at 37°C for 30 min and stored at –20°C until use. The experiment was repeated thrice and result described as the mean of three independent experiments.

Nanodrop 100 spectrophotometer (Biotech, Faculty of Agriculture, Annamalai University) was used for measuring the absorbance of each sample at 280 nm for examining the quality and quantity of extracted DNA. DNA isolated was stored at -70°C for further use.

PCR Amplification

PCR amplification was carried out to amplify the internal transcribed spacer (ITS) region in the DNA of the *Sclerotium* isolates.

Forward primer: ITS 1 5' TCCGTAGGTGAACCTGCGG 3'
Reverse primer: ITS 4 3' TCCTCCGCTTATTGATATGC 5'

RESULTS AND DISCUSSION

Cultural characteristics of *S. rolfsii* isolates

Mycelial growth

All the fifteen isolates of *S. rolfsii* produced cottony profuse growth mycelium with radial spread giving fan like appearance on Potato Dextrose Agar (PDA) medium which was first silky white in color later turned to dull white. Among the isolates Sr1 and recorded the maximum (90 mm) mycelial growth which was followed by Sr5 (90 mm), Sr6 (88.03 mm), Sr4 (87.67), Sr2 (87.34 mm), Sr3 (87.17 mm) in the decreasing order of merit while it was the minimum (79.85 mm) in the case of Sr_{11}. (Table 1).

Sclerotial number

All the isolates of *S. rolfsii* varied in their ability to produce sclerotia on PDA medium. The maximum sclerotial number of 187 per nine mm culture disc was obtained from Sr_1 which was also the most virulent isolate. This was followed by the isolates Sr_5, Sr_6, Sr_4 and Sr_2, which produced 174, 165, 154 and 132 numbers of sclerotia, respectively. The minimum number of sclerotia of 32 was recorded by Sr_{11} the least virulent isolate (Table 1).

Sclerotial color

The isolates of *S. rolfsii* produced different colour of sclerotia on PDA. The isolate Sr_1produced brown colour, Sr_3 produced Reddish brown colour, Sr_2, Sr_4, Sr_9, Sr_{10}, Sr_{12} and Sr_{13}, Sr_{14} produced dark brown colour and Sr_5, Sr_6, Sr_{11} and Sr_{15} produce pale brown colour.

Molecular analysis by ITS and PCR amplification

In the present study PCR amplification of ITS region with primer pair of ITS1 and ITS4 produced a gene product of 550-650 bp confirming the isolates to be *Sclerotium* spp. Among them, the PCR products of three most virulent isolates were sequenced. This sequence consisted of rDNA in the order of partial 18S sequence; ITS 1 and ITS 4 sequence. The sequences of isolates *viz.,* Sr_1 and Sr_2 were identified as *S. rolfsii* through BLAST search in NCBI website (www.blast.ncbi.nlm.nih.gov/ Blast). The sequences were deposited in the GenBank with the accession numbers MK880692, MK880693 (Plate a and b).

Mycelial growth

All the 15 isolates of *S. rolfsii* produced cottony profuse growth mycelium with radial spread giving fan like appearance on Potato Dextrose Agar (PDA) medium which was first silky white in color later turned to dull white. Among the isolates Sr1 recorded the maximum (90 mm) mycelial growth which was followed by Sr5, Sr6, Sr4, Sr2, Sr3 in the decreasing order of merit while it was the minimum (79.85 mm) in the case of Sr_{11}. (Table 1).

The isolated pathogen was identified as *S. rolfsii* based on mycological characters; the fungal mycelium was first silky white in colour later turned to dull white with radial spreading given fan like appearance. Microscopic examination of the fungal culture revealed the aerial hyaline, thin walled, septate hyphae with profusely branched mycelium when fungus attained maturity small mycelial knots were formed which later turned to mustard seed like sclerotia which were deep brown or brownish black, shiny, hard and spherical to irregular in shape. Similar, reports were given by Barnett and Hunter (1972), Mahmood *et al.,* (1976), Singh (1987), Mirza and Aslam (1993), Mohan *et al.,* (2000), Savita Ekka *et al.,* (2016).

Variability in cultural morphology mycelia rate, *sclerotium* formation, sclerotia size and colour among *S. rolfsii* isolates were observed by many researchers (Almeida *et al.*, 2001; okereke and wokocha, 2007; Kokub, 2007). Thilagavathirasu *et al*., (2013) observed that out of the 17 isolates, colonies of 5 isolates were fluffy and 12 isolates were compact. Also similar such variation in the cultural characteristics of *S. rolfsii* on PDA was reported by Sivakumar *et al.,* (2016). Similar result was observed by Rakholiya *et al.,* (2011) who studied variability of 30 isolates of *S. rolfsii* and reported considerable variability in mycelial and sclerotial dimensions. Also, similar such variation in the cultural characteristics of *S. rolfsii* on PDA was reported by Madiya Waskale (2016). These earlier reports lend support to the present investigations.

Sclerotial color

The isolates of *S. rolfsii* produced different colour of sclerotia on PDA. The isolate Sr_1produced brown color, Sr_3 produced Reddish brown color, Sr_2, Sr_4, Sr_9, Sr_{10}, Sr_{12} and Sr_{13}, Sr_{14} produced Dark brown color and Sr_5, Sr_6, Sr_{11} and Sr_{15} produced pale brown color. Initially white color sclerotia were formed, then the colour changed from white to light brown or chocolate, dark brown or brown as they attained maturity after utilization of nutrients, the plates become dry. However, dark brown coloured sclerotia survived for long period. The change in colour of sclerotia might also be due to utilization or exhaustion of nutrients. Similar such colour change was reported earlier (Venkatesh *et al.,* 2014; Reddi Kumar *et al*., 2014)

Sclerotial number

All the isolates of *S. rolfsii* varied in their ability to produce sclerotia on PDA medium. The maximum sclerotial

number of 187 per nine mm culture disc was obtained from Sr_1 which was also the most virulent isolate. It was further observed in our studies that isolates with heavy mycelial growth produced more number of sclerotia. These finding were consistent with the earlier reports (Abida Akrams *et al.,* 2008; Asish Mahato and Bholanath Mondal, 2014, Mohd Akram *et al*., 2015; Chanandra Sekhar *et al.*, 2017; Manu *et al*., 2018).

Molecular variability of *S. rolfsii* isolates by PCR

In the present study, the genomic DNA amplification of ITS region of different isolates yielded a fragment of approximately 600-700 bp. Among them, the two isolates viz., Sr_1 and Sr_2 were sequenced and identified as *S. rolfsii* through BLAST search in NCBI website (ww.blast.ncbi.nlm.nih.gov/Blast). Our results are in agreement with those of Adandonon *et 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 600-700 bp which suggests that these isolates belong to *S. rolfsii*.

Similarly, Jeeva *et al*., (2008) isolated rDNA-ITS region of *S. rolfsii* using ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) and ITS 6, (5′ GAAGGTGAAGTCGT AACAAGG -3′) primers amplified and extracted genomic DNA of isolates yielded a single 700-bp product of *Sclerotium* isolates.

Poornima *et al.,* (2018) analysed genetic variability among the 24 isolates of *S. rolfsii* was studied by using molecular markers like ITS-PCR and RAPD primers. Amplification of ITS region of rDNA with specific ITS1 and ITS4 universal primers produced approximately 650 to 700 bp in all the isolates of the fungus confirmed that all the isolates obtained are *S. rolfsii* and were sequenced. Identity of the isolates was confirmed with sequences of NCBI data base of *S. rolfsii*. Among the twenty-four isolates, four random primers viz., UBC-467, UBC482, UBC-485 and UBC-489 generated reproducible polymorphism.

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