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Molecular Characterization and Phylogenetic Analysis of *Rhizoctonia solani* Kuhn. Infecting Tomato

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Abstract

Tomato is one of the important vegetable crops grown across various parts of the world. It suffers from various diseases among them, dry root rot caused by soil-borne pathogen *Rhizoctonia solani* Kuhn. is one of the major threat for tomato production. In this context, tomato plants suffering from dry root rot disease were collected, processed, pure culture of *Rhizoctonia* sp. was isolated on PDA media and designated as *Rhizoctonia* RSK isolate. The pure culture of isolated fungus was studied for its morphological characters through microscopic examination where, right angle branching of mycelium and sclerotial bodies formation typical to genus, *Rhizoctonia* were observed. Mass multiplied *Rhizoctonia* RSK isolate inoculum was inoculated to thirty days old tomato seedlings (Variety: Arka Vikas) which produced symptoms *viz.,* yellowing of older leaves, necrotised lesion and rotting at the collar and root region similar to the symptoms observed for dry root on tomato in the field, confirming its pathogenicity. Further, to confirm the species level, genomic DNA was isolated from the *Rhizoctonia* RSK isolate, internal transcribed spacer (ITS) region was amplified using ITS1 and ITS4 primers and the amplicon was sequenced. ITS sequence and phylogenetic analysis revealed that the *Rhizoctonia* RSK isolate infecting tomato in the current study is *Rhizoctonia solani*, which shared maximum nucleotide identity with *Ceratobasidium* sp.

Keywords : Tomato, *Rhizoctonia solani*, Morphology, Pathogenicity, Molecular characterization and phylogeny

Tomato, *Lycopersicon esculentum* Mill. belongs to the family Solanaceae and is one of the important vegetable crop grown across the world. Cultivated tomato was originated from South America including the Galapagos Islands. However, the centre of its domestication and diversification was Mexico. In world, tomato is the second largest produced vegetable after potato with 182.25 MT of production. India stands 2nd in both the production (19.00 MT) and area (0.78 M ha) of tomato. Among the different states growing tomato during 2018-19, Karnataka has 2.08 MT of production with 0.064 M ha area (Anonymous, 2019).

Tomato is herbaceous annual plant, however biennial and perennial forms exist and mostly grown as an annual crop. It is cultivated in tropical and temperate climates in the open field or under greenhouse. Tomato can be easily and widely cultivated, not limited by day length or any other special condition for its growth and reproduction.

Tomato crop gets affected by various biotic and abiotic stresses leading to reduction in both quality and quantity of the produce. Among the biotic stresses, diseases such as early blight, late blight, powdery mildew, rhizoctonia root rot, fusarium wilt, sclerotium wilt, viral diseases, etc. are known to have a devastating effect on production of tomato. Soil-borne pathogen, *Rhizoctonia solani* was reported to cause root rot disease in tomato crop and is one of the limiting factor in tomato production. It belongs to the family, Ceratobasidiaceae and the division, Basidiomycota. In nature, usually *R. solani* has asexual reproduction and exists primarily as vegetative mycelium and / or sclerotia. The teleomorph of *R. solani*, *Thanatephorus cucumeris*, is classified in the phylum, Basidiomycota. The typical symptoms induced by this pathogen were initial yellowing of older leaves, necrotised sunken area at the collar region and also on the root tissues and finally the whole plant will die.

Studying the morphological features, molecular characterization and phylogenetic relationship of *Rhizoctonia* sp. infecting tomato will provide a better understanding for development of management practices. With this background, current study was undertaken with the objectives of isolation, molecular characterization and phylogenetic analysis of *Rhizoctonia* sp. infecting tomato and results are discussed here.

Material and Methods

Isolation and Morphological Characterization of *Rhizoctonia* sp. Infecting Tomato

Tomato plants showing symptoms typical to dry root rot were collected from the farmer's field at Kodi Ramasandra village, Kolar district, Karnataka State, India. The collected sample was observed for the symptoms and used for isolation of pathogen. Roots and collar regions of the infected plant sample was initially cut into pieces of one cm size separately and washed through running tap water. Further, these small tissue segments were surface sterilized with one per cent sodium hypochlorite for 30-45 seconds, rinsed in sterile distilled water and air dried. The sterilized collar and root tissue segments were placed in the Petri dishes containing potato dextrose agar (PDA) media. Petri dishes were wrapped with saranwrapper and incubated at 27 ± 1 ºC. The pure culture of *Rhizoctonia* sp. obtained was designated as RSK isolate and used for further studies.

The pure culture of RSK isolate from dry root rot exhibiting tomato sample was morphologically studied to confirm the initial identity. The pure culture of fungus isolated from tomato on PDA media was assessed for its colony features, microscopically observed for its mycelial characters and resting structures based on the available taxonomic guidelines (Sharma *et al*., 2005).

Pathogenicity Assay

To confirm the pathogenicity of *Rhizoctonia* RSK isolate, pathogenicity assay was conducted. Initially, the sorghum seeds were boiled and autoclaved for 15 minutes at 121 ºC temperature, 15 kg / cm^2 pressure for 15 min in autoclavable polythene covers. Eight mycelial discs (10 mm) of actively growing five days old *Rhizoctonia* RSK isolate on PDA media were taken and inoculated into the polythene covers containing 100 grams of autoclaved sorghum seeds under aseptic conditions and incubated at 27 ± 1 ºC (Upadhyay and Mukhopadhyay, 1986). After seven days of incubation the mass multiplied pathogenic inoculum of *Rhizoctonia* RSK isolate at the rate of three per cent (w/w) (Jinantana and Sariah, 1998) was applied to the pots containing 30 days old tomato seedlings (Variety - Arka Vikas from Indian Institute of Horticultural Research, Hessaraghatta, Bengaluru) and pots mock was inoculated with the autoclaved sorghum seeds without pathogen and transplanted with tomato plants that served as control. Finally, observations were recorded for symptom expression at regular interval.

Isolation of Fungal DNA

Actively growing mycelium of *Rhizoctonia* RSK isolate on PDA media was inoculated into conical flask containing potato dextrose broth (PDB) under aseptic condition and incubated for four days at 27 °C. After four days, the mycelial mat was harvested, air-dried and the genomic DNA was isolated by following the Cetyl (hexadecyl) trimethyl ammonium bromide (CTAB) method as described by Csaikl *et al*., 1998. The presence of DNA was confirmed by agarose gel electrophoresis (0.8 %) of the sample and quantified using spectrophotometer using A260 / 280 readings.

PCR Amplification of the Internal Transcribed Spacer (ITS) Region

The sequence of ITS region is extensively used for taxonomic profiling of fungi at present due to its conserved nature. The DNA isolated from *Rhizoctonia* RSK isolate in the current study was used for amplification of ITS region by PCR using ITS1 and ITS4 primer pair (White *et al.,* 1990).

PCR amplification was carried out in 25 ìL reaction volume. The reaction mixture composed of template (100 ng/ìL), Taq buffer (2.5 ìL of 10X), $MgCl_2$(1 ìL of 2 mM), 1 mM dNTPs mixture (2.5 ìL), 5 pM of 1.5 ìL

each primer, 1 unit of Taq polymerase and sterile distilled water. PCR was performed in a ProFlex PCR system. The PCR conditions imposed are 94 °C for 4 min followed by 30 cycles of 94 °C for 60 s, 55 °C for 45 s, 72 °C for 90 s with a final extension step at 72 °C for 10 min. The PCR products were resolved on 1 per cent agarose gel. The amplified products were eluted from the gel (Qiagen gel elution kit; *Cat No.*/ ID: 28706) and were sent for sequencing at Medauxin, Bangalore.

Sequence and Phylogenetic Analysis

Sequencing was done in both directions and the aligned sequence was obtained. Sequence similarity searches was performed for the aligned sequence to all available sequences in GenBank using BLASTn. Sequences showing the maximum identity scores with the present *Rhizoctonia* RSK isolate were retrieved. Sequence identity matrices for ITS region sequence of *Rhizoctonia* RSK isolate with sequences retrieved from GenBank for comparison were generated using the Bioedit Sequence Alignment Editor (Version 7.2.5). Phylogenetic tree was generated using MEGA X software (Kumar *et al*., 2018) by Neighbour-Joining method (Saitou and Nei, 1987) with 1000 bootstrap replications to estimate evolutionary distances between all pairs of sequences.

Results and Discussion

Isolation of Pathogen

The collar and root segments from the tomato plants showing typical dry root rot symptom was collected and processed for isolation of *Rhizoctonia* sp. by inoculating into Petri dish containing PDA media. The mycelial growth from the tip of inoculated collar and root segments was sub-cultured and purified. The pure culture of the *Rhizoctonia* sp was isolated from the tomato dry root rot disease sample cultured on PDA slants and stored in the refrigerator at 4 °C for further studies.

Morphological Characterization

Morphological characters of the *Rhizoctonia* RSK isolate grown on PDA were studied through visual and microscopic observations. The *Rhizoctonia* fungus has fast growth on the PDA media which covers entire Petri dish area (9 cm diameter) within four days of inoculation and sclerotial bodies were formed at the periphery of the Petri dish (Fig. 1a) after seven to eight days of inoculation. Microscopic examination of four days old fungus culture showed the right angle branching of mycelium with constricted region at the base which is the typical morphological feature of the genus *Rhizoctonia* (Fig. 1b). Initial identity of the *Rhizoctonia* sp. in the current study was confirmed based on the colony characters and morphological features in comparison with earlier reports (Sharma *et al*., 2005).

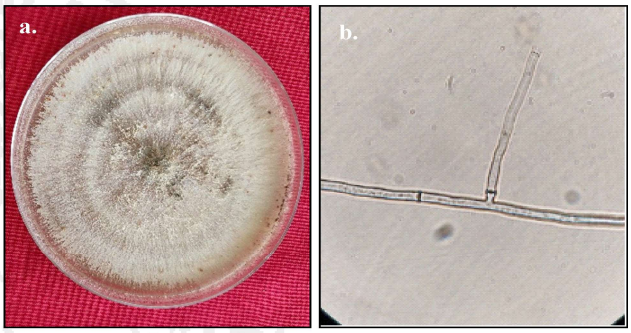

Fig. 1: a) *Rhizoctonia* RSK isolate grown on PDA media
b) Right angle branching of *Rhizoctonia* mycelium observed through microscope

Pathogenicity Assay

To confirm the virulence of isolated *Rhizoctonia* RSK isolate from tomato plants, pathogenicity assay was conducted by inoculating the tomato plants with RSK isolate. After four days of inoculation, symptom expression appeared in pathogen treated plants, where, older leaves of infected plants became yellow. Further, rotting of tissues at the collar region and on root tissues was observed, which is typical symptom of *Rhizoctonia* sp. infection (Fig. 2). No symptoms were observed in the mock inoculated control plants. The observed symptoms were similar to the symptoms recorded in the diseased plant sample collected from a farmer's field for pathogen isolation and also with the earlier reports of symptoms produced by *R. solani* (Taheri and Tarighi, 2012).The fungal pathogen was reisolated from diseased plant tissues where it was

identical with the original culture of RSK isolate with respect to the morphological features confirming the pathogenicity of the isolated RSK isolate from tomato.

Fig. 2: a) Pathogenicity assay showing symptomatic expression of *Rhizoctonia* RSK isolate infected plants b) *Rhizoctonia* RSK isolate infected tomato seedlings showing rotting at the collar region

Molecular Characterization and Phylogenetic Analysis

The ITS region in fungi are highly conserved and can be used for distinguishing the fungi upto species level. The genomic DNA isolated from the RSK isolate was subjected to PCR amplification using ITS-1 forward and ITS-4 reverse primers. This resulted in the expected amplification product of approximately 650 bp (Fig. 3). The amplified product was eluted and sequenced. The obtained sequence was queried in BLASTn available in NCBI GenBank. The GenBank accessions showing maximum identity were retrieved, multiple aligned and identity matrices was obtained.

The pathogen isolate in the current study shared maximum nucleotide identity of 82 per cent with *Ceratobasidium* sp. (Ac.No.:MT522873), which is followed by several sequences derived from *Ceratobasidium* sp. and *Rhizoctonia solani* reported from different parts of the world (Table 1). This was further confirmed with the phylogenetic analysis

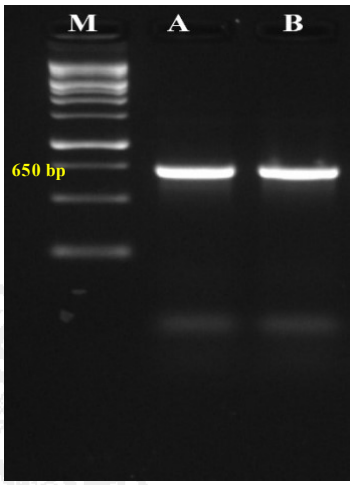

Fig. 3: Ethidium bromide stained agarose gel showing amplification product of approximately 650 bp PCR amplicon specific to Internal Transcribed Spacer (ITS) region, A and B *Rhizoctonia solani* isolate (M: Ladder 1kb).

showing close clustering of RSK isolate with already reported isolates of *R. solani* indicating it as *Rhizoctonia solani* (Fig. 4). *Ceratobasidium* and

TABLE 1

Comparison of Internal Transcribed Spacer (ITS) region nucleotide sequences *Rhizoctonia solani* RSK isolate with other isolates available in NCBI, GenBank

Accession No.	Organism	Country	Host	Per cent nucleotide identity of ITS region sequence of *R. solani* isolate with others
MT522873	*Ceratobasidium* sp.	India	-	82.00
JX913821	*Ceratobasidium* sp.	South Korea	Chinese yam	77.30
KC193238	*Ceratobasidium* sp.	India	Marigold	77.30
MG920780	*Ceratobasidium* sp.	South Korea	Sweet potato	77.90
MH517362	*Rhizoctonia solani*	India	Elephant foot yam	76.20
KX118357	*Ceratobasidium* sp.	USA	Soybean	78.90
KF372662	*Rhizoctonia solani*	Iraq	Pepper	76.00
KX674533	*Rhizoctonia solani*	Malasiya	-	75.70
JX913818	*Ceratobasidium* sp.	South Korea	Chinese yam	77.10

Table 1 (Contd.)

Accession No.	Organism	Country	Host	Per cent nucleotide identity of ITS region sequence of *R. solani* isolate with others
KJ834072	*Ceratobasidium* sp.	China	Ginger	77.00
JX913826	*Ceratobasidium* sp.	South Korea	Chinese yam	77.00
JX913819	*Ceratobasidium* sp.	South Korea	Chinese yam	77.10
MK621284	*Rhizoctonia solani*	India	-	77.10
MK027051	*Rhizoctonia solani*	Mexico	Chilli	77.30
MF716663	*Rhizoctonia solani*	Pakistan	Chilli	75.20
KF372653	*Rhizoctonia solani*	Iraq	Tomato	76.60
KF372646	*Rhizoctonia solani*	Iraq	Tomato	76.00
MW369735	*Rhizoctonia solani*	Egypt	-	76.90
MH520072	*Rhizoctonia solani*	Iraq	Potato	75.30
KF372645	*Rhizoctonia solani*	Iraq	Tomato	76.00

Thanatephorus were reported as teleomorphic stage of *Rhizoctonia* sp. isolated from different crops (Gonzalez *et al.*, 2001). This variation could be due the anastomosis of *Rhizoctonia* sp. quite often leading to variability. However, *Rhizoctonia* RSK isolate in the study shared maximum nucleotide identity of 82 per cent in the ITS region with other reported related species, indicating that it may be a novel isolate compared to the already reported ones. More sampling of *Rhizoctonia isolates* infecting Solanaceae crops will reveal deeper information in this regard.

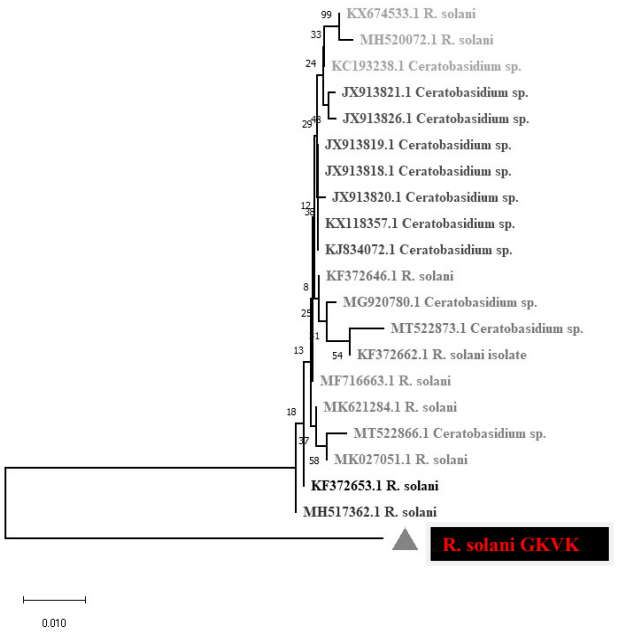

Fig. 4 : Phylogenetic tree constructed from sequences of Internal Transcribed Spacer (ITS) region of *Rhizoctonia solani* RSK isolate infecting tomato with sequences of *Rhizoctonia* spp. retrieved from NCBI GenBank using Neighbor-joining method with 1000 bootstrap replication integrated in MEGA X software.

In conclusion, *Rhizoctonia* RSK isolate infecting tomato was confirmed as *R. solani* based on its morphological features, ITS region nucleotide sequence comparisons and phylogenetic analysis.

Acknowledgement

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