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Variability of rice sheath blight pathogen, *Rhizoctonia solani* fromTamil Nadu

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Abstract

A survey was conducted in five different major rice growing areas of Tamil Nadu *viz*., Mayiladuthurai, Aduthurai, Madurai, Vellore and Thiruvarur for the occurrence of Sheath Blight (ShB) disease, which revealed that Aduthurai in Thanjavur district is the hot spot area for ShB with highest disease incidence of 64%. Among the five isolates, *Rhizoctonia solani* (Rs_2) collected from Aduthurai recorded the higher severity of ShB (53.80%) followed by Thiruvarur isolate (Rs_5) (40.23%). *R. solani* isolates (Rs_2) were successfully grown on different solid media *viz.*, potato dextrose agar, beetroot dextrose agar, carrot dextrose agar, Czapek's dox agar and Richard's agar. Among the nitrogen sources tested for supporting the growth of *R. solani*, peptone recorded the maximum mycelial growth of 8.57 cm with sclerotial production of 86.67 per plate. Of the six carbon sources tested for supporting the growth of *R. solani*, glucose recorded the maximum mycelial growth of 7.92. All the five isolates of *R. solani* were molecularly identified using ITS 1F and ITS 4 R primer pairs. The results indicated an amplicon of 600 bp in all the isolates of *R. solani*.

Key words: *R. solani*- nitrogen sources-carbon source- pH-Internal transcribed spacer

Introduction

Rice (*Oryza sativa* L.) is one of the widely cultivated important food crops in the world and over half of the world population relies on it. It is a chief food for more than 65% of the world's population and India's pre-eminent crop. Rice provides 20 per cent of the world's dietary supply and good source of thiamine, riboflavin, niacin and dietary fiber. In Asia, where 95 per cent of the world's rice is produced and consumed, it contributes 40 – 80 per cent of the calories of Asian diet (Kanchana *et al.*, 2012). Globally, more than three million people have rice as staple food, and it accounts for 50 to 80 per cent of their daily calorie intake (Delseny *et al.*, 2001). Over the next 20 years it is expected that demand for rice will grow by 2.5 per cent per year (Hobbs, 2001). In India, rice is grown in 43.86 million ha with the production of 104.80 million tonnes and the productivity of about 2390 kg/ha (Statistics, 2005). In Tamil Nadu, rice crop was cultivated in about 20 lakh hectares with an annual production of about 75.17 lakh tonnes and productivity of 3758 kg/hectare during the year 2015-16 (INDIASTAT).

Diseases are serious constraints to rice cultivation in India. More than 70 diseases caused by fungi, bacteria and viruses have been recorded on rice (Ou, 1985). Among them, sheath blight caused by *Rhizoctonia solani* (Teleomorph: *Thanatephorus cucumeris* (Frank) Donk) anastomosis group 1 IA (AG-1 IA) is a serious disease worldwide (Rush and Lee, 1983). The sheath blight disease of rice is prevalent in almost all the rice growing countries of the world. Sheath blight is an important soil borne fungal disease causing annually up to 40 per cent of yield losses. The

symptoms of the disease include greenish grey water-soaked spots development on the outer most sheaths of rice. As the disease progresses, spots enlarge and coalesce forming larger lesions with grayish-white center surrounded by tan to brown irregular borders or outlines. Sclerotia surviving in the soil are the major source of infection (Damicone and Jackson, 1996). When sclerotia come into contact with a rice plant, they germinate and the hyphae produce infection cushions on the exposed leaf sheath. Haustoria grow from the infection cushions, penetrate the host tissue, and develop typical green gray, water-soaked lesions (Dodman and Flentje, 1970).

Among the cultivated rice varieties, the level of resistance to rice sheath blight is low. The conventional method for the control of fungal diseases has been mainly the use of synthetic fungicides. However, chemical control is less acceptable due to the increase in the development of resistance, lack of specificity towards the target pathogen and adverse effect on environment and beneficial microbes (Mcneil *et al.*, 2010). Fungicide application may not be economically feasible and the time of application appears to be important.

Keeping this background, the following objectives were formulated to manage the sheath blight disease of rice, viz., 1) Isolation of the causal organism of rice sheath blight (*Rhizoctonia solani*) and proving the pathogenicity 2) Physiological and nutritional requirement by the pathogen for its growth and 3) Morphology and molecular characterization of *R. solani* by using ITS primers.

Materials and Methods

Plant materials and experimental site

Paddy variety ADT 49 was used throughout the experiment. Seeds were obtained from Tamil Nadu Rice Research Institute (TRRI), Aduthurai. The experiments on proving pathogenicity of rice sheath blight (ShB) pathogen *R. solani*, isolation of bacterial antagonists from rice rhizosphere, *in vitro* studies and induced systemic resistance were conducted at the TRRI, Aduturai, Thanjavur district.

ShB disease assessment - *Symptomatology*

ShB disease of rice caused by *R. solani* inflicts damage to the outer most sheath covering the stem portion at maximum tillering or panicle initiation stage. Symptoms initially occur on leaf sheaths near the water line as water-soaked lesions which are greenish gray in colour. As the disease progresses, they enlarge and tend to coalesce. Secondary infections are caused by hyphae growing upward towards uninfected plant parts, producing additional lesions and sclerotia to complete the 1st cycle. Such sclerotia fall down and spread to adjacent plants through irrigation water. These sclerotia can survive in soil for longer period and the inoculum can survive in the infected plant debris. Large lesions formed on infected sheaths of lower rice leaves may lead to softness of the stem and lodging thereby causing considerable yield loss. Visible disease symptoms include formation of lesions, plant lodging and presence of ill-filled grains.

Survey

A survey was conducted during the year 2017 to assess the severity of sheath blight disease in different rice growing areas of Tamil Nadu *viz*., Mayiladuthurai, Aduthurai, Madurai, Vellore and Thiruvarur districts during August, 2017. Per cent disease index (PDI) and severity was calculated by the following formula using the Relative Lesion Height (Singh *et al.*, 2015) and ShB grade chart (IRRI SES, 2014).

$$PDI = \frac{\text{Sum of individual ratings}}{\text{Total number of plants observed}} \times \frac{100}{\text{Maximum grade}}$$

Rice sheath blight grade chart

Disease grade	Lesion height
0	No infection observed
1	Lesion limited to lower 20 per cent of the height of the plant
3	Lesion limited to 21-30 per cent of the height of the plant
5	Lesion limited to 31-45 per cent of the height of the plant
7	Lesion limited to 46-65 per cent of the height of the plant
9	Lesion more than 65 per cent of the height of the plant

$$\text{Relative Lesion Height (RLH)} = \frac{\text{Lesion height}}{\text{Plant height}} \times 100$$

The severity of the disease was calculated by using the following formula (Knaus *et al.,* 1985).

$$\text{Severity} = \frac{0(N0) + 5(N1) + 10(N3) + 30(N5) + 50(N7) + 100(N9)}{\text{Total number of tillers observed}} \times 100$$

Where, N0-N9 = Number of tillers classified as grade 0-9

Isolation and purification of rice ShB pathogen

Preparation of Potato Dextrose Agar (PDA) **-** PDA was prepared as described by Islam *et al., (*2009). Two hundred grams of peeled and sliced potato was boiled in 500 ml water in a bowl for about half an hour. The extract of potato was filtered through a filter. The other ingredients *viz*., 20g each of dextrose and agar were added in the extract and the volume was made up to 1 liter. The prepared PDA was poured in 1000ml conical flask and sterilized in autoclave 121ºC with 1.1 kg/cm^2 pressure for 15 minutes.

Isolation of R. solani from infected samples **-** The infected rice sheaths showing the typical symptoms of rice sheath blight collected from different rice growing areas were used for the isolation of the pathogen. The diseased sheath tissues were washed in running tap water, cut into small bits of approximately 5mm long by means of a surface sterilized scalpel. The bits were further surface sterilized in 0.1 per cent mercuric chloride for 30 seconds and subsequently, washed three times in repeated changes of sterile distilled water. They were then placed on sterilized Petri dish containing PDA medium amended with streptomycin sulphate (16mg/l) by means of forceps. All these steps were carried out under aseptic conditions in the culture room. The plates were incubated at room temperature (28 ±2°C) for 4 days and observed for growth of the pathogen. The pure cultures of the pathogen were obtained by single hyphal tip method (Rangaswami and Mahadevan, 1998). The purified cultures of five isolates from five districts were maintained on PDA slants for further studies. They were designated as Rs_1, Rs_2, Rs_3, Rs_4 and Rs_5. Based on the morphological and microscopic observations, the isolates were confirmed as *R. solani.*

Pathogenicity and Virulence study

Preparation of inoculums **-** Fresh stem bits of rice plants were collected from the field. Stem bits were washed with running tap water to remove soil particles. They were cut into small pieces of 2cm length by using sterilized scissor, washed with sterile water and spreaded on blotter paper. Then the bits were filled in conical flask and plugged with nonabsorbent cotton and sterilized in autoclave at 1.4 kg cm^2 pressure for 30 minutes. The flasks containing stem bits were transferred to laminar air flow chamber. Three numbers of 8 mm mycelial discs taken from 5 days old *R. solani* culture were transferred to each conical flask. They were incubated at room temperature for 15 days. Similar inoculation was done for all the isolates of *R. solani*. The mycelial growth covered all the bits in flask and produced brown sclerotia after fifteen days of inoculation. These stem bits without sclerotia were used as source of *R. solani* inoculum for artificial inoculation.

Inoculation for proving pathogenicity **-** The pathogenicity of *R. solani* isolates was proved by Koch's postulates on the paddy variety ADT 49 under pot culture conditions. The plants were raised in earthen pots of 30cm diameter containing 3kg of pot mixture soil. Three seedlings per pot were maintained. At panicle initiation stage, the rice stem bit inoculum of *R. solani* was placed between the leaf sheath and culm just above the water level. Three sheaths of inner tillers per hill and three hills per pot were inoculated and replicated thrice. Similar inoculation was done for all the isolates separately. Inoculated plants were observed for the ShB symptoms regularly. After six days, the lesions produced by different isolates were measured by following the Standard Evaluation System (SES) developed by IRRI (1980). The plants inoculated with uninoculated autoclaved stem bits served as control. The pathogen was re-isolated from the typical ShB lesions and the characters were compared with those of original. The ShB severity

and Relative Lesion Height (RLH) was measured for each isolate. The isolate which showed maximum ShB severity and RLH was considered as most virulent one and taken for further studies.

Growth and cultural characters of *R. solani*

Morphological characters - Nine mm mycelial disc was cut from 5 days-old culture using sterilized cork borer. Then it was placed at the center of petri plates containing 20 ml of solidified PDA, replicated thrice and incubated at room temperature (28 ±2°C). The growth and morphological characters *viz.*, colour of mycelium, colony diameter and morphology, number of sclerotia per Petri plate, colour of sclerotia and arrangement pattern on PDA were recorded. The colony diameter was measured using a meter scale.

Stem bit culture **Inoculation of stem bits at Centre of the sheath portion**

Expression of symptoms on outer leaf sheath

Figure 1: Artificial inoculation of Rhizoctonia solani in rice plants by stem bit culture method

Cultural characters

Growth on different media - Seven different growth media *viz.*, PDA, Czapek's Dox agar, carrot dextrose agar, Richards's agar and beetroot dextrose agar were prepared separately and autoclaved at 1.4 kg/cm^2 pressure for twenty minutes. Nine mm mycelial disc of *R. solani* was cut from 5 days-old culture using cork borer and placed at the center of each Petri plate containing 20 ml of different growth media. The plates were incubated for five days at room temperature (28±2ºC). Three replications were maintained for each treatment. The mycelial growth on different growth media was observed regularly.

Growth in different carbon, nitrogen sources, pH of solid media - The Czapek's Dox medium with carbon sources *viz.*, sucrose or glucose, or dextrose or starch or maltose and of nitrogen sources *viz.*, ammonium nitrate or peptone or sodium nitrate or potassium

nitrate or ammonium sulphate along with different pH *viz*., 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0 using 0.1 N HCl or NaOH was prepared and autoclaved at 1.4 kg/ cm^2 pressure for 20 minutes. Czapek's Dox medium without any carbon and nitrogen sources was kept as control. Three replications were maintained for each treatment. Nine mm mycelial disc of *R. solani* was cut from 5 days old culture using cork borer and placed at the center of each Petri plate containing 20 ml of medium. The plates were incubated for five days at room temperature (28±2ºC) and growth was observed regularly.

Molecular characterization of *R. solani* isolates

Genomic DNA extraction from R. solani - Genomic DNA was extracted from the pure cultures of *R. solani* isolates using CTAB (Zolan and Pukkila, 1986). Fresh mycelium of approximately 100 - 200g was ground by using liquid nitrogen until it gets powdered. The powdered mycelium was transferred into Eppendorf tube and added with 700 µl of CTAB buffer (700 mM of NaCl, 50 mM of Tris HCl with pH 8.0, 10 mM of EDTA, 2% CTAB, and 1% mercapto-ethanol). The samples were then incubated at 65°C for 15 minutes and added with 750µl of Phenol: Chloroform: Isoamyl alcohol (25:24: 1, v/v). The tube was inverted to form an emulsion and centrifuged at 10000 rpm for 10 min at 4°C. The upper phase was transferred into new tube, added with equal volume of Phenol: Chloroform: Isoamyl alcohol, (25:24: 1, v/v) and centrifuged at 4°C for 10 minutes. The upper phase was transferred into new Eppendorf tube and added with 0.5 vol (150 µl) of 3M sodium acetate or 5M NaCl and 600 µl of ice cold isopropanol. The content was mixed well and incubated at -20°C for overnight. Finally, the supernatant was precipitated by centrifugation at 10,000 rpm and the pellet was washed twice with 70 per cent cold ethanol, dried and dissolved in 50 µl TE buffer. DNA was stored at -20°C for further studies.

PCR amplification of ITS region of R. solani isolates - The Internal Transcribed Spacer (ITS) gene region of *R. solani* isolates was amplified using following primer pairs as per the protocol (White *et al.*, 1990).

Forward primer: ITS1 (5'-TCCTGTAGGTGAACCTGCGG–3')

Reverse primer: ITS4 (5'-TCCTCCGCTTATTGATATGC - 3')

The PCR mixture consisted of 5-10 ng of genomic DNA, 1µM each of ITS1 and ITS4 primers, 3 mM MgCl2, 200µM each of dNTP and 2.5 U of Taq DNA polymerase in a total volume of 50 µl. The PCR was carried out with a Master cycler gradient (Eppendorf, Germany) programmed for preheating at 95°C for 5 minutes followed by 40 cycles consisting of denaturation at 94°C for 45 seconds, annealing at 58°C for 1 min and extension at 72°C for 2 min with a final extension at 72°C for 8 min and final hold at 4°C. The PCR products were separated by electrophoresis in a 1.2 per cent agarose gel stained with Ethidium bromide and visualized under UV light and documented using Alpha imager (Alpha Innotech, California, USA). The size of PCR products was measured using 100 bp DNA ladder (Bangalore Genei, Pvt. Ltd., Bangalore).

Results and Discussion

Survey on rice ShB - The rice ShB disease severity ranged from 16 to 64 per cent in different rice growing areas of Tamil Nadu. The maximum severity index of 64 per cent was recorded at TRRI, Aduthurai in Thanjavur district of Tamil Nadu followed by Tiruvayur district with 56% PDI.. This might to be due to the high population and virulence of the pathogens, sclerotia present in the soil, continuous cropping of rice, intensive cultivation and the susceptibility of host to pathogen. Neha *et al.,* (2016) reported higher incidence of sheath blight in Naduthittu followed by Vadakkumangudi in Cuddalore district of Tamil Nadu. Savary *et al.,* (2000) estimated a yield loss up to 69% by rice sheath blight in Tamil Nadu. Losses due to rice sheath blight generally varies from 30 to 40 per cent (Srinivas *et al.,* 2013).

Table 1: Survey on assessment of sheath blight disease in rice in Tamil Nadu

S. No	Isolate	Places	District	Stage of the crop	Percent Disease Index (PDI)	Sclerotial colour	Sclerotial pattern	No of sclerotia / plate	Disease severity (Artificial condition)
1.	Rs_1	Mayiladuthurai	Nagapattinam	Grain filling	30	Dark brown	Scattered	53	25.83
2.	Rs_2	Aduthurai	Thanjavur	Tillering	64	Light brown	Periphery	86	53.80
3.	Rs_3	Madurai	Madurai	Tillering	24	Light brown	Scattered	38	23.45
4.	Rs_4	Vellore	Vellore	Grain filling	16	Light brown	Periphery	17	16.18
5.	Rs_5	Thiruvarur	Thiruvarur	Tillering	56	Dark brown	Centre	82	40.23

Symptomatology **-** Under artificial inoculation, the symptoms of rice ShB appeared on the outer most sheath at maximum tillering and flowering stage of the plants near the water level. Initially the pathogen produces pale green soaked lesions seven days after inoculation

Pathogenicity- In the present study, rice ShB pathogen was inoculated by rice stem bit method. All the isolates were pathogenic and produced water soaked lesions on the sheath portion of plants under pot culture conditions. Among the five isolates of rice sheath blight, Rs_2 was identified as the most virulent one which recorded 53.80 per cent severity followed by Rs_5 with disease severity of 40.23 per cent (Table 1). The most virulent isolate Rs_2 was collected from TRRI, Aduthurai in Thanjavur district while the isolate Rs_4 collected from Vellore district, was less virulent. The results indicated variations in virulence depending on the continuous availability of host. Prasad *et al.,* (2010) inoculated *R. solani* in rice plants by straw bit method and the pathogen produced typical water soaked lesions seven days after inoculation.

Morphological characters **-** Morphological characters are important tool for identification and classification of fungus. The colour of the mycelium initially white later turned to light brown in all the five isolates and the angle of branching of mycelium was right angle. Among five isolates, the mycelium of Rs_1, Rs_2, Rs_3 and Rs_4 isolates were flat in nature. Rs_5 isolate showed fluffy mycelial growth (Table 2). The mycelia of all the isolates were septate and showed right angle branching. The above characters were in agreement with those of the original descriptions given by Guleria *et al.,* (2007) and Lal *et al.,* (2014).

Cultural characters of *R. solani*

Growth of R. solani on different solid media **-** Rs_2 isolate exhibited variation in their growth in different solid media. The maximum mycelial growth of 8.85 cm and sclerotial production of 89 per plate was observed in PDA medium followed by Beetroot Agar (8.62 cm) with sclerotial production of 85 per plate, while Richards's agar medium recorded the minimum mycelial growth of 7 cm with sclerotial production of about 73 per plate (Table 2). Similar findings were reported by several workers (Anilkumar and Sastry, 1982 ; Wasser *et al.*, 1988).

Growth of *Rhizoctonia solani* on different Nitrogen sources **-** The virulent isolate of *R. solani* (Rs_2) obtained from Aduthurai was tested in different nitrogen sources. Among the different nitrogen sources, peptone recorded the maximum mycelial growth of 8.57 cm with number of sclerotia of 86.67 per plate followed by Ammonium sulphate with 7.5 cm growth and 73.67 sclerotia per plate. Potassium

Table 2: Morphological characters and nutrient requirement of *Rhizoctonia solani* isolated from different places

S. No	Isolate	Colony type	Colony colour	Growth rate of mycelium at 4 DAI	Media	Diameter of mycelial growth at 4 DAI (cm)*	Number of sclerotia / plate**	Nitrogen sources	Mycelial growth (cm)*at 4 DAI	Number of sclerotia per plate at 7 DAI*	Carbon sources	Mycelial growth (cm)*at 4 DAI	Number of Sclerotia per plate *
1.	Rs_1	Flat	Dark brown	7.53	Potato dextrose agar	8.85	86	Ammonium nitrate	7.25	73.00	Maltose	6.00	4.00
2.	Rs_2	Flat	Light brown	8.85	Richard's agar	7.20	70	Ammonium sulphate	7.50	73.67	Glucose	7.92	78.67
3.	Rs_3	Flat	Light brown	7.20	Czapek's dox agar	7.60	73	Peptone	8.57	86.67	Sucrose	7.40	72.67
4.	Rs_4	Flat	Light brown	6.76	Beetroot dextrose agar	8.62	83	Potassium nitrate	7.13	70.00	Dextrose	7.59	76.67
5.	Rs_5	Light fluffy	Dark brown	8.27	Carrot dextrose agar	8.30	79	Sodium nitrate	7.25	72.00	Starch	6.96	70.00
								Control	7.00	4.00	Control	5.40	2
					SEd	**0.14**			**0.15**	**1.76**		**0.17**	**1.50**
					CD	**0.26**			**0.33**	**3.84**		**0.36**	**3.95**

** 7 Days after Inoculation; *Mean of three replications; DAI – Days After Inoculation

nitrate showed the minimum mycelial growth (7.13 cm) and sclerotia (70 per plate). Pal and Kaushik (2012) and Saha *et al.,* (2008) also reported the maximum growth of *R. solani* in peptone nitrogen source. (Table 2).

Growth of R. solani on different carbon sources **-** With respect to carbon sources, the results revealed that glucose was the best carbon source recording the maximum mycelial growth of 7.92 cm with sclerotial production of 78.67 per plate, followed by dextrose which showed a mycelial growth and sclerotial production of 7.59 cm and 76.67 per plate, respectively (Table 2). Maltose recorded the minimum mycelial growth of 6.8 cm and sclerotia of 64 per plate. Pal and Kaushik (2012) observed the maximum growth of *R. solani* in glucose source.

Growth of R. solani on different pH levels **-** In the present study, maximum mycelial growth was observed at pH 7.0 (8.7 cm) with 84.5 sclerotia per plate followed by pH 6 with a mycelial growth and sclerotia of 8.27 cm and 82 per plate, respectively (Table 3). The least mycelial growth (6.31 cm) and sclerotial production (66 per plate) was observed in pH 8.0. Goswami *et al.,* (2011) also reported that pH 7 supports the maximum mycelial growth followed by pH 6.

Table 3: Effect of pH on the mycelial growth of *Rhizoctonia solani* (Rs_2) under *in vitro* conditions

S. No	pH	Mycelial growth (cm)*at 4 DAI	Number of sclerotia per plate*
1.	5	6.48	72.00
2.	5.5	6.33	68.3
3.	6	8.27	82.00
4.	6.5	8.13	79.67
5.	7	8.7	84.50
6	7.5	6.45	71.60
7	8	6.31	66.00
SEd		**0.09**	**1.52**
CD		**0.22**	**3.27**

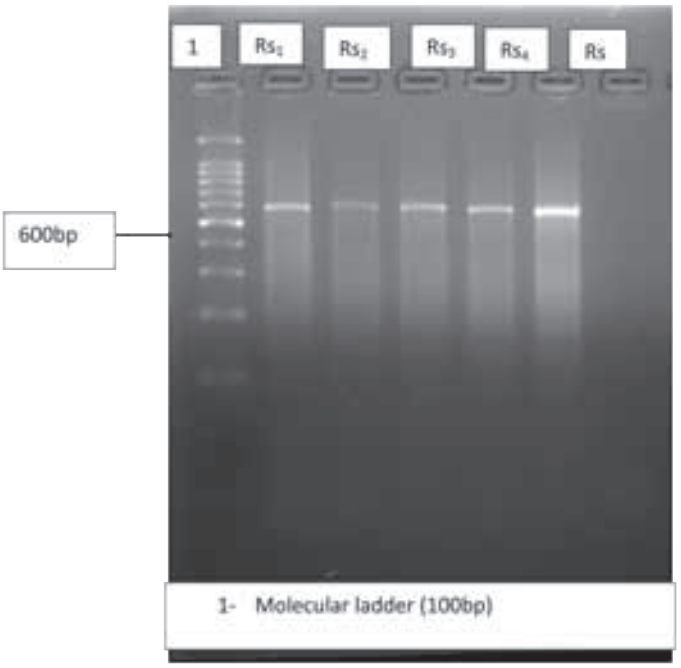

Figure 2: Molecular identification of *Rhizoctonia solani*

*Mean of three replications ; DAI – Days After Inoculation

Molecular identification of *R. solani* isolates - All the isolates were molecularly characterized using ITS 1 and ITS 2 primer pairs. The result stated an amplicon of 600 bp and confirmed that all the five isolates belong to the genus *Rhizoctonia* (Figure 2). Molecular techniques such as using specific primers for species identification has quicker result compared to conventional methods (Moni *et al.,* 2016). Molecular characterization also gives a real time result and can distinguish isolates from each host and even from different geographic areas.

Salazar *et al.,* (2000) designed specific ITS 1F and ITS 4R based on specific sequences of the ITS regions in the *R. solani* species complex. They also showed that PCR detection has time saving advantages over traditional tool for identification. ITS region of the *R. solani* can be amplified using universal primers ITS1 and ITS 4 and it can yield an amplicon of 700 bp size in all the isolates of *R. solani.* Rani *et al.,* (2013) amplified ITS region of *R. solani* from cotton using ITS1 and ITS 4 primers. Nadarajah *et al.,* (2014) characterized the Malaysian isolates of *R. solani* at molecular level.

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