Egyptian Journal of Phytopathology,Vol. 52, No.1, pp 83- 90 (2024)
DOI 10.21608/ejp.2024.284697.1117

ORIGINAL PAPER

Genetic Analysis of *Rhizoctonia solani* Isolates from Potato Stem Cankers and Black Scurf Diseases using rDNA ITS1-5.8S-ITS2 Region Sequencing and PCR-RFLP Techniques

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Received: 23 April 2024 / **Accepted**: 15 June 2024 / **Published online**: 27 June 2024

ABSTRACT

Rhizoctonia solani, a common pathogenic fungus of potato plants, causes stem cankers and black scurf. This study aims to investigate the genetic diversity of *Rhizoctonia solani* isolates; causes stem cankers and black scurf diseases from various potato fields. This study utilized sequence analysis and restriction digestion of PCR-amplified internal transcribed spacers (ITS1, ITS2) and the 5.8S region of ribosomal DNA (rDNA) genes. The amplification of the ITS1-5.8S-ITS2 region using universal primers (ITS1 and ITS4) yielded an approximately 700 bp amplicon, revealing notable sequence heterogeneity among the isolates examined. Subsequent digestion of the amplified products with *HaeIII*, *HinfI*, and *XbaI* restriction enzymes unveiled distinct banding patterns across the tested isolates, distinguished by varying numbers of bands and fragment sizes. This study underscores the efficacy of DNA sequencing and ITS restriction fragment length polymorphism (RFLP) as valuable molecular techniques for exploring nucleotide polymorphisms within genetically diverse *R. solani* isolates.

Keywords: *Rhizoctonia solani*, ITS1, ITS2, Restriction enzyme

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INTRODUCTION

Rhizoctonia solani Kühn (teleomorph *Thanatephorus cucumeris* [Frank Donk]) is a necrotrophic fungal pathogen with a wide host range (Ogoshi 1987; El-bebany et al. 2017; Abdelsalam et al. 2020). Black scurf and stem canker, caused by *R. solani*, are important constraints to potato production not only in Egypt but also in other regions (Balali-dehkordi 1996; El-bebany et al. 2017; Kankam et al. 2021).The disease appears as a hard black mass of hyphae (sclerotia) on potato tubers, along with cankers on stems and stolons (Ferrucho et al. 2012; Kumar et al. 2017) resulting in significant quantitative and qualitative yield reductions worldwide (Bounou et al. 1999; Kankam et al. 2021; Muzhinji et al. 2018). Primarily, chemical fungicides are the effective control measures used in the fields to combat plant diseases (Deresa and Diriba 2023). Therefore, studying the genetic structure of the phytopathogens helps in tracking phytopathogens, detecting the connections between isolates assuming that may reduce the disease incidence without the extensive use of harmful agrochemicals (Das et al. 2014; Hansen et al. 2016).

Plant pathogens are known to rapidly evolve to overcome plant resistance and environmental stressors (Dolatabadian and Fernando 2022). *R. solani* is known to have a great variability in terms of morphology,

pathogenicity, and genetics and these criteria are used to differentiate *R. solani* isolates (Ogoshi 1987; Matsumoto 1996;K et al. 2022; Moni et al. 2016) . Genetic variations among *R. solani* isolates have been investigated based on several criteria, including pathological, morphological, physiological characteristics (Matsumoto 1996). *R. solani* is a complex pathogen categorised into 14 different anastomosis groups (AGs), which is a useful tool for measuring genetic relatedness between *R. solani* isolates from different AGs. (Elbakali et al. 2003). Genetic variability can be examined *via* more robust nucleic acid-based techniques such as sequencing and restriction fragment length polymorphism (RFLP) (Elbakali et al. 2003). The sequence of the ribosomal DNA (rDNA) is of great importance to distinguish the minor DNA sequence changes among the pathogen isolates. Considerable genetic variation has been reported in *R. solani* internal transcribed region (ITS) sequences (Kuninaga et al. 1997). This variability can be readily assessed by examining changes in restriction sites within ITS regions among *R. solani* isolates. This provides insight into the evolutionary dynamics of the entire genome. (Pannecoucque and Höfte 2009).

Given the significant reliance on potato production for both domestic consumption and export, minimizing crop losses is imperative for a country. Understanding the genetic diversity and population structure of *R. solani* could greatly aid in disease management, tracking the spread of the pathogen, and targeting disease control strategies (Chen et al. 2007). Furthermore, this research could potentially enhance breeding programs by facilitating the development of resistant cultivars (Narayanasamy 2011). Therefore, the current study aimed to investigate sequence heterogeneity among amplified ITS1-5.8S-ITS2 regions of various *R. solani* isolates through DNA sequencing and RFLP analyses.

MATERIALS AND METHODS

Fungal Isolates

Infected potato samples were obtained from different geographic locations in Egypt table 1. Twenty isolates of *R. solani* were obtained from potato plants with a potential symptoms of stem canker and black scurf. The *R. solani* isolates identified by El-bebany and his colleagues (El-bebany et al. 2017) and used in this study.

Genomic DNA extraction

A pure culture of each isolate was grown in potato dextrose broth (PDB) for 4 days in an incubator at 25°C, and then mycelia were harvested and blotted by filter paper. DNA was extracted from *R. solani* isolates using CTAB buffer procedure (Clapp 1996)**.**

Polymerase Chain Reaction (PCR)

Primer pairs [ITS-1(5' TCCGTA GGTGAACCTGCGG 3')] and [ITS-4 (5' TCCTCCGCTTATTGATATGC 3')] were used for the amplification of rDNA ITS as described by (White et al. 1990). PCR amplification was performed using Dream Taq Green PCR master mix (Thermo Scientific) according to the manufacturer recommendations. The PCR program consisted of an initial denaturation step of 94.0 °C for 1 min followed by 35 cycles, each with 1 min at 94°C for denaturation, 2 min at 55°C for annealing and 1 min at 72°C for extension. Reaction mixtures were then incubated at 72°C for 10 min for final extension. Amplified PCR products were separated on a 1.5% agarose gel, stained with RedSafe™ (nucleic acid stain), and visualized using UV light.

Sequencing of the amplified ITS1-5.8s-ITS2 region

The amplified fragments of ITS1-5.8s-ITS2 region (≈700bp) of the tested isolates named (RS 13-82, RS 13-88, RS 13-92, RS 13-99, RS 13-32, RS 13-71, RS 13-56, RS 13-49, RS 13-19, RS 13-76, RS 13-77, RS 13-78, RS 13-79, RS 13-39,RS 14-125, RS 14-126, RS 14-127, RS 14-128, and RS 14-129) were sent abroad for sequencing. Pairwise alignments were made using Molecular Evolutionary Genetics Analysis

(MEGA 11) software Version 11, and then phylogenetic tree was constructed using neighbour joining method from CLUSTALW alignment (Tamura et al. 2021). Obtained sequences were compared with eleven accessions (U57744, U57878, U57731, U57723, JQ946296.1, DQ452128.1, EU513130, MIAE00227, FJ588594, FJ588598, FJ588590) obtained from GenBank.

Restriction Fragment Length Polymorphism (RFLP) analysis

The PCR products of ITS1-5.8s-ITS2 region of the 20 *R. solani* isolates was digested using three restriction enzymes (*HaeIII, Hinf1*,and *Xba1*). Restriction fragments were separated by electrophoresis in 2.5% agarose gel, stained with RedSafe™ (nucleic acid stain). Restriction profiles were visually analysed.

RESULTS AND DISCUSSION

The aim of this study was to investigate the sequence heterogeneity of the ITS1-5.8s-ITS2 region. The amplification of ITS1–5.8s–ITS2 rDNA region of all isolates was carried out using the primers ITS1 and ITS4. A unique single specific band of approximately 700 bp was obtained for all isolates (Fig. 2A) then subjected to DNA sequencing. The obtained sequences displayed length heterogeneity at the sequence level ranged from 539 to 700 bp. That kind of variation potentially due to limitations of sequencing method used or the ITS multicopy nature of *R. Solani* (Pannecoucque and Höfte 2009). Alignment of the obtained sequences of the amplified region (ITS1-5.8s-ITS2) displayed sequence variations. This is in agreement with (Kuninaga et al. 1997) who reported that *R. solani* isolates are known to display sequence variation in rDNA. The phylogenetic analysis based on nucleotide sequence of ITS1-5.8s-ITS2 region (Fig. 1) grouped the tested isolates into two main clusters, I and II. Cluster I includes isolate RS14-126 and RS14-129 from one origin (EL Khatatba) and cluster II is divided into two sub-clusters. Sub-cluster I includes isolate RS13-91(El-Nahda), YEG5 (AL-Gharbia), YEG8 (Damitta) and YEG3 (Kafr Al-Sheikh). The remaining isolates grouped in sub-cluster II including three isolates from Kafr Al-Sheikh (RS 13-77, RS13-79 and RS13-76) , six isolates from El-Beheira (RS13-19, RS13-49, RS13-32, RS13-39, RS13-71, RS13-56) and four isolates from Alexandria RS13-99, RS13-92, RS13-88, RS13-82. Despite that grouping, no clear correlation was found between the geographic origin and sequences of the ITS1-5.8s-ITS2 region of the *R. solani* isolates.

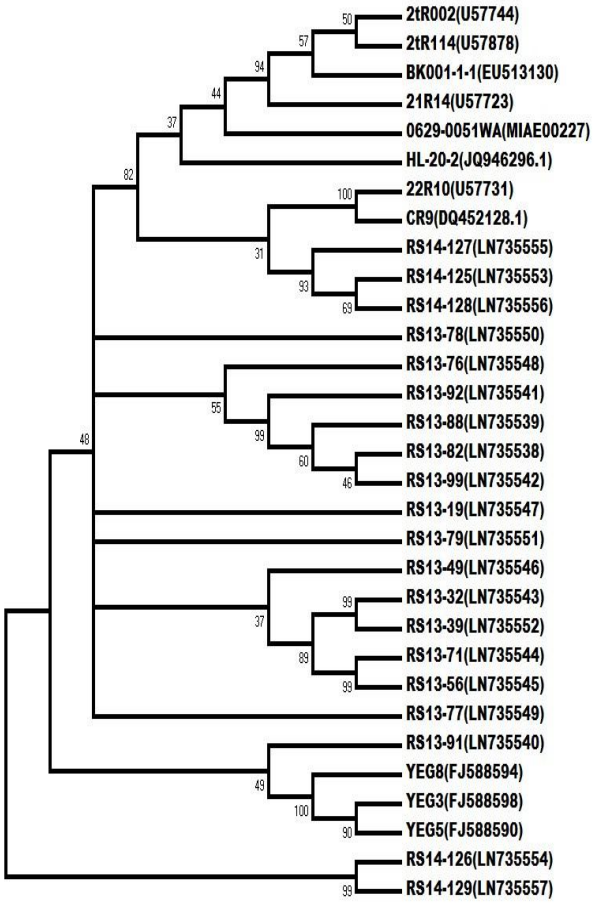

Fig. 1: Condensed neighbor-joining tree with cutoff value (25%) of the nucleotide sequence of ITS1-5.8s-ITS2 region of rDNA of *R. solani* isolates based on sequence alignment of PCR product (≈700 bp) amplified using specific primers (ITS1/ITS4) compared with other selected isolates from GenBank, accession numbers between parentheses, bootstrap support values (%) are indicated at the node. The isolates obtained in this study are identified by accession numbers which range from LN735538 to LN735557 and are given in brackets.

RFLP is a simple technique requires small amount of DNA and could discriminate and estimate the genetic distance between *R. solani* isolates (Vilgalys and Hester 1990; Çebi Kiliçoğlu and Özkoç 2010). Therefore, the genetic variability among the tested isolates was investigated based on variation in the banding patterns of the digested ITS1–5.8s–ITS2 rDNA region using RFLP.

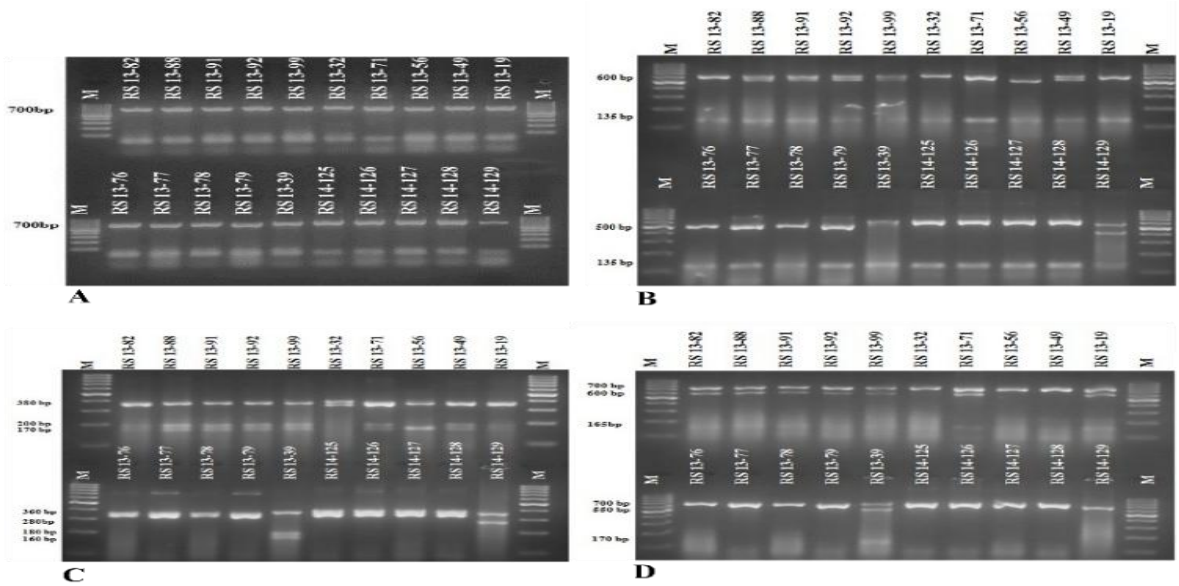

Fig. 2: PCR amplification and RFLP analysis of ITS1 -5.8s-ITS2 region of *R. solani* isolates. A) PCR amplification showing characteristic amplicons of nearly 700 bp (in 1.5% agarose gel) of ITS1 -5.8s-ITS2 region of *R. solani* using ITS1 and ITS4 primers. B, C, and D) Restriction fragment patterns of ITS1-5.8s-ITS2 region for 20 *Rhizoctonia solani* isolates digested with restriction enzyme *HaeIII, Hinf1, and Xba1* respectively. Electrophoresis was caried out using 2.5% and 1.5% agarose gels for PCR and RFLP analysis respectively. M, a 100 bp DNA marker. Gels were stained with RedSafe™ and were viewed using UV-transilluminator.

Restriction digestion of ITS1-5.8s-ITS2 region in the tested isolates using three restriction enzymes (*HaeIII, Hinf1, and Xba1*) revealed variations in banding patterns among tested isolates (Tables 1). Different restriction patterns were observed among tested isolates based on the number of restriction sites (Fig.2 B, C, and D) which indicates that the tested isolates of *R. solani* varied in the sequence of ITS1-5.8s-ITS2 region.

This study showed that the target ITS1-5.8s-ITS2 region may has one restriction site for *HaeIII* and *Xba1* but have two restriction sites for *Hinf1*. The number of bands produced from some isolates indicated that *R. solani* probably has a multicopy of ITS1-5.8s-ITS2 region due to its multinucleate nature or due to the existence of base substitution and insertion/deletion (Pannecoucque and Höfte 2009; Sanders et al. 1995). Digested ITS region of *R. solani* by *HaeIII* produced a restriction pattern of two fragments (600 bp and 140 bp) (Hyakumachi et al. 1998), but our data showed that the restriction patterns produced by *HaeIII* in addition to *Xba1* were of three fragments including almost non-digested band (Table 1).

Fungal rDNA is widely used to discriminate between genera (Bowman et al. 1992; O'Brien 1994). ITS1, ITS2, and 5.8s could reveals the variability within the species level (Chen et al. 2007). *R. solani* isolates could be separated into groups according to their origin using RFLP procedures (Boysen et al. 1996; Redecker et al. 1997; Abbas et al. 2022; Çebi Kiliçoğlu and Özkoç 2010). In this study, the tested isolates were found to be assigned into two groups based on restriction sites that found with *HaeIII*, group I encompasses 6 isolates (RS 13-88, RS 13-91, RS 13-92 and RS 13-99 from Alexandria, RS13-56 from EL-Beheira and RS 14-129 from Minufiya) which have two restriction sites and group number II includes 14 isolates that have one restriction site. Differences in restriction

patterns were observed between and within these groups. Deletion/insertion could trigger changes in sequence of restriction sites in the ITS region of *R. solani* (Kuninaga et al. 1997).

Table 1. The Apparent lengths (bp) of the restriction fragments of PCR-amplified ITS1-5.8S-ITS2 region of *R. solani* isolates collected from different geographic locations in Egypt.

Isolate	Origin	District	Fragment length(s) (bp)								
			HaeIII			*HinfI*			*XbaI*		
RS 13-19	El-Beheira	Badr	135		600	160	180	360	170	600	700
RS 13-32	"	Abou Elmatamir	"		"		350	390			"
RS 13-39	"	Hosh Eissa	"		"	160	180	360	177	555	"
RS 13-49	"	Kafr El-Dawar	"		"	160	180	360			"
RS 13-56	"	Itay Albaroud	"	500	550	"	"	"			"
RS 13-71	"	Wadi Alnetroun	"		600	"	"	"	150	600	"
RS 13-82	Alexandria	El- Nahda	"	570		175	200	360	165	"	"
RS 13-88	"	"	"	"	640	"	"	"	"	"	"
RS 13-91	"	"	"	"	"	"	"	"	"	"	"
RS 13-92	"	"	"	"	"	"	"	"	"	"	"
RS 13-99	"	"	"	"	"	"	"	"	"	"	"
RS 13-76	Kafr El-Sheikh	Sakha	"	525			340	"			"
RS 13-77	"	"	"	500			"	"			"
RS 13-78	"	"	"	525			"	"			"
RS 13-79	"	"	"	500			"	"			"
RS 14-125	Minufiya	El Khatatba	"		600		320	380			"
RS 14-128	"	"	"		"		"	"			"
RS 14-126	"	"	"		"		"	"			"
RS 14-127	"	"	"		"		"	"			"
RS 14-129	"	"	"	420	570	280	"	"			660

Different restriction sites were observed among tested isolates using *Hinf1*, whereas 5 isolates from Alexandria (RS 13-82, RS 13-88, RS 13-91, RS 13-92, and RS 13-99) have two restriction sites and shears the same restriction pattern. Other 5 isolates obtained from EL-Beheira (RS 13-71, RS 13-56, RS 13-49, RS 13-19, and RS 13-39) have two restriction sites with identical banding patterns. Four isolates RS 13- RS 13-76, RS 13-77, RS 13-78 and RS 13-79 from EL-Beheirathey have the same restriction patterns. Tested isolates subjected to *Xba1*shows differences among restriction patterns produced, whereas 12 isolate have no restriction site, the other 8 isolates (RS 13-82, RS 13-88, RS 13-91, RS 13-92 and RS 13-99 (from Alexandria), RS 13-71, RS 13-19, RS 13-39 (obtained from EL-Beheira)) have one restriction site.

Sequence analysis of *R. solani* isolates with a monomorphic ITS region has been found in different geographical areas, suggesting that the spread of these common clonal isolates is likely due to the movement of infected potato tubers both nationally and internationally (Das et al. 2014). Moreover, due to its multinucleate nature, different types of *R. solani* ITS regions can be found within a single isolate from different locations, each showing different degrees of pathogenicity to different parts of the potato plant. Therefore, the detection of genetic variability within specific populations of a plant pathogen can help to identify their potential origin (Das et al. 2014; Hansen et al. 2016). In summary, this study underscores the advantages of employing DNA sequencing and RFLP as molecular tools for elucidating sequence heterogeneity in *R. solani*. Therefore, exploring the genetic variability within *R. solani* populations in some potato production regions of Egypt could

facilitate the tracking of Rhizoctonia disease. This, in turn, could enable the implementation of effective management strategies to safeguard potato plants in the future, ultimately enhancing potato production in a cost-effective manner.

Acknowledgement

This research was financially supported by Alexandria University research enhancement program (Alex-Rep) project number AGRV-11-03

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