Pak. J. Bot., 50(1): 415-420, 2018.

MOLECULAR IDENTIFICATION AND CHARACTERIZATION OF *RHIZOCTONIA SOLANI* AG-3 ISOLATES CAUSING BLACK SCURF OF POTATO

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Abstract

Twenty-six isolates of *Rhizoctonia solani* AG-3 were collected from four potato growing area of Saudi Arabia. Yield damages due to this infection is reported to range from 7-64% (average of 35%), depending on many factors. Molecular identification of *R. solani* AG-3 isolates by ITS-regions and characterization was done by inter simple sequence repeat (ISSR) markers. Twenty-six isolates of *R. solani* used in the current study were isolated from potato fields in four major potato-producing regions of Saudi Arabia. All isolates were inoculated on potato and observations on the percentage of disease incidence were recorded. Genomic DNA extraction of *R. solani* AG-3 isolates was used by A specific and sensitive PCR and ISSR primers. A single splinter of nearly 500 bp was only amplified once genomic DNA from *R. solani* AG-3 isolates. Amplicon size of three ISSR primers ranged from 0.3 to 2.8 Kb in isolates. Using the three primers, the tested isolates were separated on the basis of genetic similarity coefficients (GSC). The range of the GSC was beginning at 0.62 and ending at 1.00. In unweighted pair-group method arithmetic averages (UPGMA) analysis, the *R. solani* isolates grouped into five clusters. The present method provided rapid and reliable detection of *R. solani* AG-3 isolates. Molecular characterization have great genetic variation in the *R. solani* AG-3 population, without any correlation between aggressiveness, geographical regions and genetic variation based on ISSR markers.

Key words: *Rhizoctonia solani*, Potato, Genetic variation, Pathogenicity, Inter simple sequence repeat (ISSR).

Introduction

Rhizoctonia solani **[teleomorph:** *Thanatephorus cucumeris*], the most recognized species within genus *Rhizoctonia*, is important plant pathogen of economically crop plants and causes 65 diseases Young *et al.* (2010). *R. solani* shows diversity in morphology, geographic location, host specificity and pathogenicity (Ogoshi, 1987), genetically distinct (Sneh *et al*., 1991). *R. solani* causes canker as well as black scurf indications on potato stem, stolon in addition to tubers, and cuts both yields as well as the quality of potato (Jeger *et al*., 1996). Yield damages due to this infection is reported to range from 7-64% (average of 35%), depending on precence of causal pathogen, environmental conditions, crop stages, cultivation practices and resistance of cultivars (Carling *et al*., 1989). Isolates of *Rhizoctonia* species are categorized into group or subgroups grounded on their hyphal anastomosis reactions. Recently, 13 multinucleate AGs besides 16 binucleate *Rhizoctonia* AGs have been defined (Sharon *et al*., 2008). The methods used for identification of fungal plant pathogens generally fall into two groups, (i) genetic, based on PCR technologies have revolutionized research on fungal detection and identification (Tsui *et al*., 2011), and (ii) biochemical, based on electrophoresis of soluble proteins (Reynolds *et al*., 1983) and the examination of Pectic zymograms (Neate *et al*., 1998). Techniques of molecular biology have become reliable and are accurate tools for assessing genetic variation of plant pathogen and populations structure (Sundravadana *et al*., 2011). The inter-simple sequence repeat (ISSR) is a technique, which involves the use of microsatellite sequences as primers in PCR to generate multilocus markers (Young *et al*., 2010). The ISSR markers are greatly polymorphic and are beneficial for the studies on genetic variety, phylogeny, gene tagging, genome mapping, as well as, evolutionary biology (Reddy *et al*., 2002). Till date, no reportable data exists on the genetic variation among the black scarf isolates of *R. solani* in Saudi Arabia (Rauf & Khan, 2002).

Present research was molecular identification for *R. solani* AG-3 isolates and assess the genetic variation of *R. solani* AG-3 isolates using ISSR markers, at two levels, pathogenic and geographic origin of the *R. solani* isolates.

Materials and Methods

Collection of *R. solani* isolates: Twenty-six isolates of *R. solani* used in the current study were isolated from potato tuber showing typical symptoms of black scurf. The isolates collected from potato fields in four major potato-producing regions of Saudi Arabia. All isolates were isolated following the method described by (Papavizas & Davey, 1959). Each isolate was purified by hyphal tip method (Mundkur, 1959) and maintained in pure culture and stored at 4°C for further study. The isolates designated as RS1-RS26.

Genomic DNA extraction of *R. solani* isolates: Twenty-six isolates of *R. solani* cultured on double-layer (one solid and one liquid) media in 50-mm Petri dishes. The base solid medium was potato dextrose agar as a film, and the top liquid medium was peptone yeast glucose (1200 µl) continuous method according to (Mahmoud, 2015).

Specific PCR amplification of *R. solani* AG-3: Specific PCR assays used two primers Rs1F2 (5′-TTGGTTGT AGCTGGTCTATTT-3′) and Rs2R1 (5′-TATCACG CTGAGTGGAACCA-3′). PCR was performed in a Techne TC-312 (Techne, United Kingdom). PCR protocol was completed according to a protocol described by (16). A 100-bp DNA ladder was used as the molecular marker, (Intron Biotechnology, South Korea).

Pathogenicity investigation: The growing substrate for the *R. solani* AG-3 isolates were primed in 500 ml glass bottles; every bottle contained 50 g sand, 100 g sorghum grains, in addition to 90 ml tap water. The substances from each bottle were autoclaved for 30 min. The isolate inoculum at 20°C, obtained from a one-week-old culture on PDA, Pathogenicity test was applied according to (Abd El-Aziz *et al*., 2013). The incidence of black scurf and stem canker was recorded at 90 days after planting. After incubation, the tubers of every plant were assessed for infection indicators according to (Lees, 2002).

$$\text{Disease incidence} = \frac{\text{No. of tubers infected}}{\text{Total tubers observed}} \times 100$$

ISSR by PCR: DNA extraction was achieved by means of the method defined in the preceding section. Three ISSR primers that were used for amplification of genomic DNA isolates. These primers were: PCMS: $(GTC)_7$, ISSR-P1: $(TC)_8C$, and ISSR P4: $(ATG)_6$. PCR reaction was carrying out according to (18).

DNA electrophoresis: For all samples, the amplified DNA (15 µL) was electrophoresed (Wide Mini-Sub-Cell GT System, Bio-RAD) on 1.5% agarose gels containing 0.5 µg/mL ethidium bromide, at a constant 75 V and 60 mA, and visualized with UV Transilluminator with DNA standard DNA marker 100-bp (Intron Biotechnology, South Korea).

Results

Specific PCR of *R. solani* AG-3: Two primers Rs1F2 and Rs2R1 were used to amplify genomic DNA extracted from 26 isolates of *R. solani* AG-3 from various regions (Fig. 1). A single splinter of nearly 500 bp was only amplified once genomic DNA from *R. solani* AG-3 isolates was used.

Pathogenicity test: All isolates were inoculated on potato and observations on the percentage of disease incidence were recorded (Table 1). The disease incidence varied from 8.2 to 17.4%. One *R. solani* isolate (RS20) collected from Hail was the high disease incidence (17.4%). While, an isolate (RS5) isolated from Dammam was the least disease incidence (8.2%). Clustering the results of disease severity indicated that 26 isolates were divided into four groups (Fig. 2): Five *R. solani* isolates (RS20, RS17, RS11, RS10, and RS4) were categorized as high virulence (HV) group. Six *R. solani* isolates (RS1, RS6, RS15, RS22, RS8, and RS13) were grouped as virulence (V). The MV groups with moderate virulence comprise eight *R. solani* isolates (RS25, RS24, RS14, RS26, RS7, RS12, RS19, and RS3). The group with weak virulence (W) comprises nine *R. solani* isolates (RS23, RS2, RS18, RS21, RS9, RS16, and RS5).

Table 1. Pathogenicity test of 26 isolates of *R. solani* on potato under greenhouse conditions.

Governorate	Isolate code No.	Percentages disease incidence (Mean ± SE)
Dammam	RS1	14.3 ±^{b}
	RS2	9.7 ±^{e}
	RS3	10.3 ±^{de}
	RS4	16.1 ±^{a}
	RS5	8.2 ±^{f}
	RS6	14.1 ±^{b}
	RS7	11.1 ±^{cd}
Tabuk	RS8	13.3 ±^{b}
	RS9	9.1 ±^{e}
	RS10	16.4 ±^{a}
	RS11	17.6 ±^{a}
	RS12	10.9 ±^{cd}
	RS13	13.1 ±^{d}
	RS14	12.1 ±^{c}
Hail	RS15	13.9 ±^{b}
	RS16	8.7 ±^{f}
	RS17	16.9 ±^{a}
	RS18	9.7 ±
	RS19	10.5 ±^{de}
	RS20	17.4 ±^{a}
	RS21	9.2 ±e
Buraydah	RS22	13.7 ±^{b}
	RS23	9.9 ±^{e}
	RS24	12.5 ±^{c}
	RS25	12.8 ±^{c}
	RS26	11.5 ±^{cd}
L.S.D. $_{0.05}$		0.79

- Each value represents the mean ± S.E (Standard Error) and mean of 4 replicates
- Values in the same column with the same letter are not significantly different at ($p \leq 0.05$)

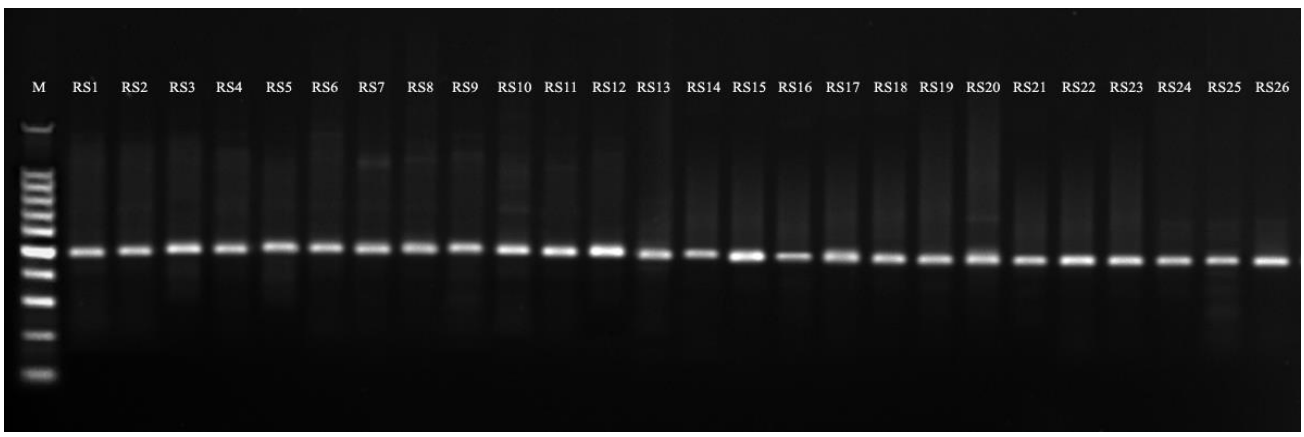

Fig. 1. Amplification of DNA from 26 isolates of various *R. solani* AG-3 (lanes 1–22) using primer pair Rs1F2 and Rs2R1. Lanes 1–22, RS1, RS2, RS3, RS4, RS5, RS6, RS7, RS8, RS9, RS10, RS11, RS12, RS13, RS14, RS15, RS16, RS17, RS18, RS19, RS20, RS21, RS22, RS23, RS24, RS25, RS26. M indicates a 100-bp size marker.

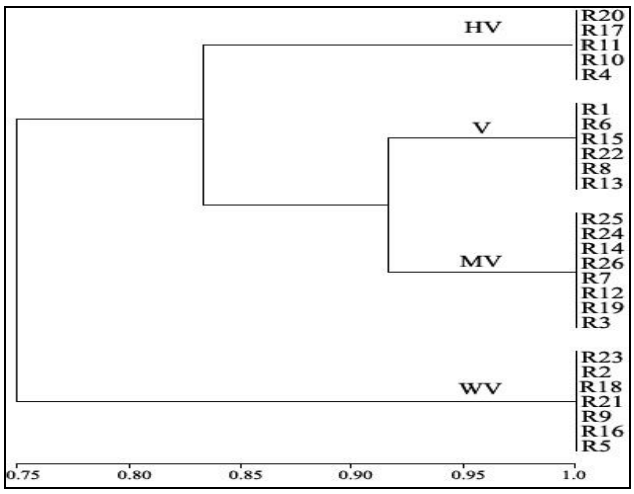

Fig. 2. Cluster of 26 *R. solani* AG-3 isolates based on pathogenic characters.

Genetic variation of the 26 *R. solani* AG-3 isolates based on PCMS: A cluster analysis was conducted based on the GSC, which ranged from 0.63 to 1.0 for the 26 isolates. A cluster analysis appeared using the UPGMA 26 isolates divided into five main clusters (labeled Groups A, B, C, D, and E) (Fig. 3). Group A included eleven isolates (RS1, RS6, RS5, RS10, RS4, RS9, RS25, RS23, RS24, RS13, and RS15). Group B included five isolates (RS20, RS22, RS11, RS2, and RS3). Group C included two isolates (RS8 and RS19). Group D included four isolates (RS12, RS7, RS14, and RS21). Group E included four isolates (RS16, RS17, RS26, and RS18).

The distinction and grouping of different isolates of *R. solani* based on genetic similarity and pathogenicity were studied. Group A included two isolates (RS4 and RS10) were categorized as high virulence (HV), four isolates (RS1, RS6 RS13, and RS15) were categorized as virulence (V). Two isolates (RS24 and RS25) were categorized as moderate virulence (MV), and three isolates (RS5, RS9, and RS23) were categorized as weak virulence (WV). Group B included two isolates (RS20 and RS11) were categorized as high virulence (HV). One isolate (RS22) was categorized as virulence (V), one isolate (RS3) was categorized as moderate virulence (MV), and one isolate (RS2) was categorized as weak virulence (WV). Group C included one isolate (RS8) was categorized as virulence (V), one isolate (RS19) was categorized as moderate virulence (MV). Group E included one isolate (RS17) was categorized as high virulence (HV), one isolate (RS26) was categorized as moderate virulence (MV), and two isolates (RS16 and RS18) were categorized as weak virulence (WV). The correlation between genetic similarity and geographical origin was studied. Group A included four isolates (RS1, RS6 RS5, and RS4) from Dammam region, three isolates (RS9, RS10, and RS13) from Tabuk, one isolate RS 15 from Hail, and three isolates (R23, RS9, and RS23) from Buraydah. Group B included two isolates (RS2 and RS3) from Dammam region, one isolate (RS11) from Tabuk, one isolate (RS20) from Hail, and one isolate (RS22) from Buraydah. Group C included one isolate (RS8) from Tabuk, one isolate (RS19) from Hail. Group D included one isolate (RS7) from Dammam region, two isolates (RS12 and RS14) from Tabuk, one isolate (RS21) from Hail. Group E included three isolates (RS16, RS17, and RS18) from Hail, and one isolate (RS26) from Buraydah. No correlation was detected between the genetic similarity, pathogenicity and geographical origin of tested isolates.

Genetic variation of the 26 *R. solani* AG-3 isolates based on ISSR P1: A dendogam analysis was completed based on the GSC, which ranged from 0.66 to 0.90 for the 26 isolates. A dendrogram grouped 26 isolates into five main clusters (labeled Groups A, B, C, D, and E) (Fig. 4). Group A included thirteen isolates (RS1, RS5, RS3, RS4, RS26, RS2, RS25, RS8, RS24, RS21, RS23, RS7, and RS15). Group B included five isolates (RS22, RS10, RS17, RS20, and RS9). Group C included two isolates (RS16 and RS13). Group D included two isolates (RS19 and RS11). Group E included four isolates (RS14, RS12, RS6, and RS18). These data indicated that this primer was not able to differentiate between isolates according to pathogenicity and geographic regions. No correlation was apreared between the DNA profiling obtained, pathogenicity and geographic regions.

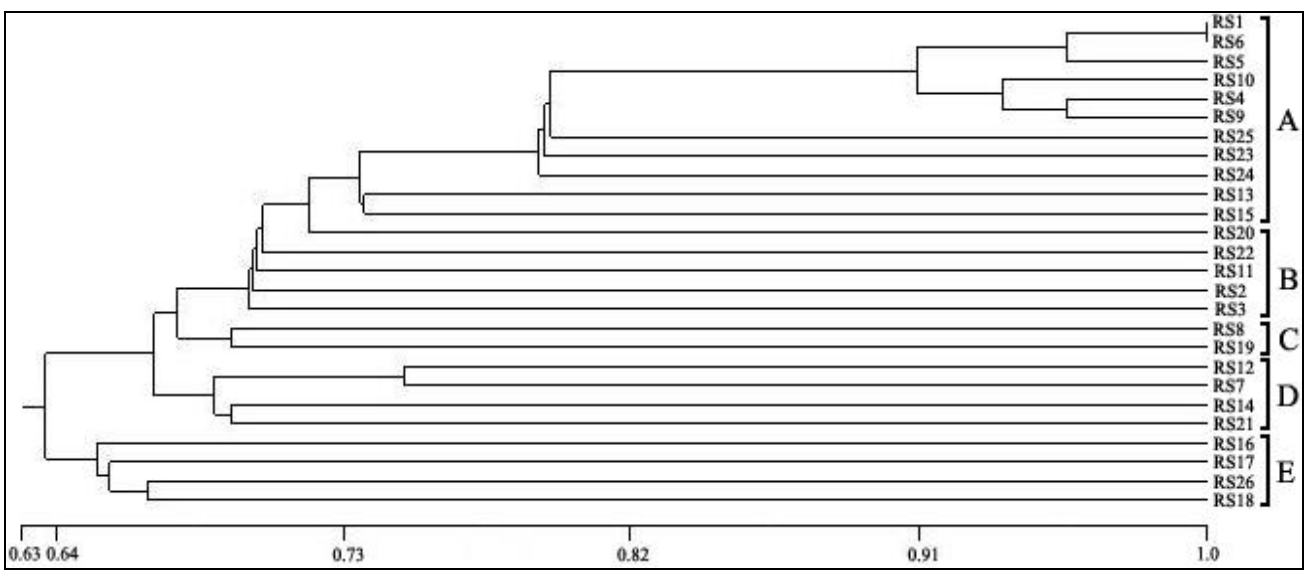

Fig. 3. Dendrogram obtained by UPGMA method derived from PCR-amplification banding of ISSR with primer PCMS for 26 *R. solani* isolates.

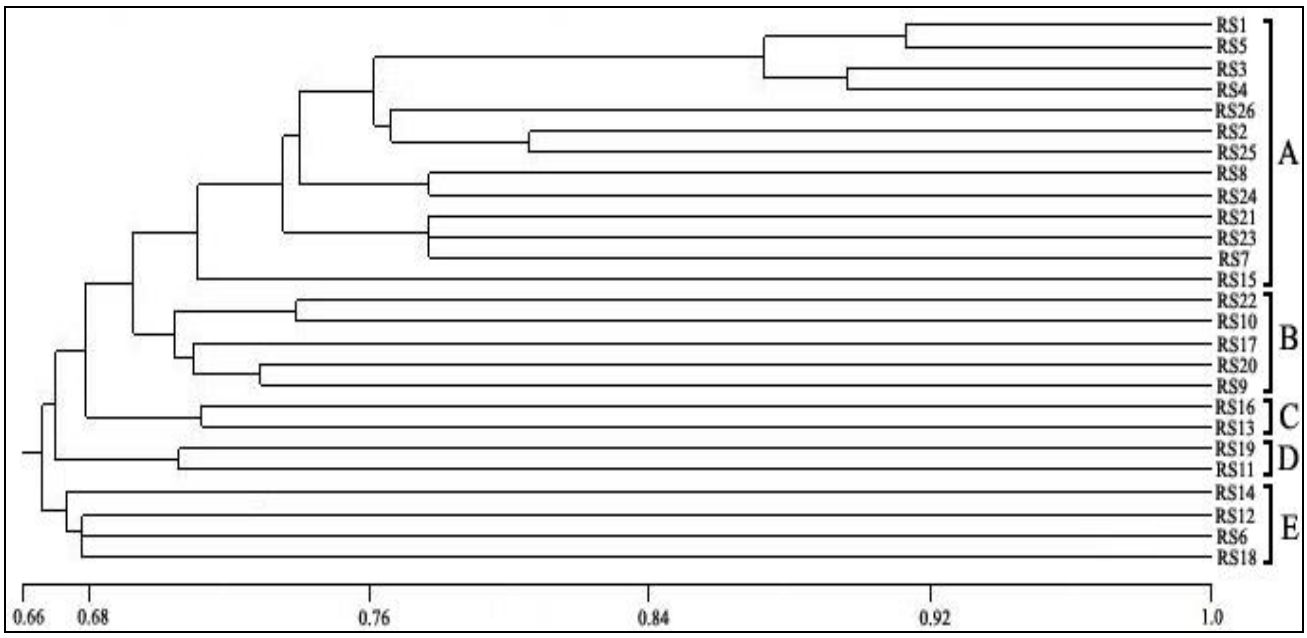

Fig. 4. Dendrogram obtained by UPGMA method derived from PCR-amplification banding of ISSR with primer ISSR P1 for 26 *R. solani* isolates.

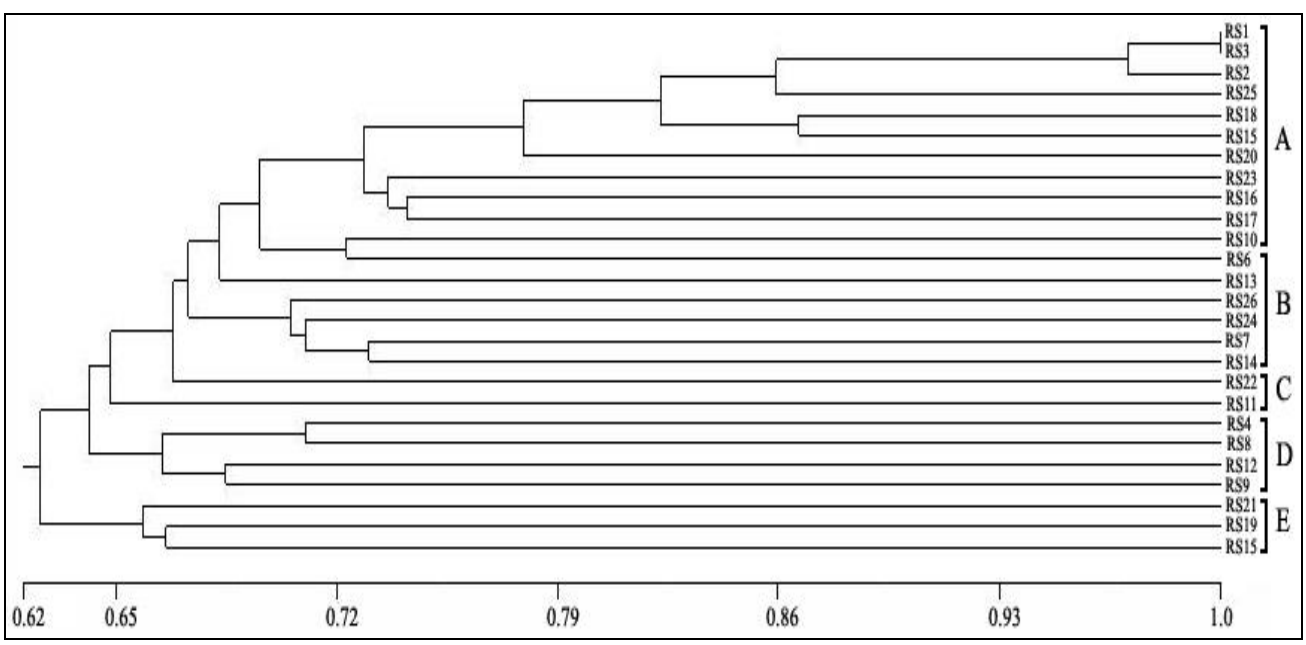

Fig. 5. Dendrogram obtained by UPGMA method derived from PCR-amplification banding of ISSR with primer ISSR P4 for 26 *R. solani* isolates.

Genetic variation of the 26 *R. solani* AG-3 isolates based on ISSR P4: A clustering tree analysis was executed based on the GSC, which ranged from 0.62 to 0.97 for the 26 isolates. A clustering tree gathered 26 isolates into five main clusters (labeled Groups A, B, C, D, and E) (Fig. 5). Group A included eleven isolates (RS1, RS3, RS2, RS25, RS18, RS215, RS20, RS23, RS16, RS17, and RS10). Group B included six isolates (RS6, RS13, RS26, RS24, RS7, and RS14). Group C comprised of two isolates (RS22 and RS11). Group D included four isolates (RS4, RS8, RS12, and RS9). Group E had three isolates (RS19, RS21, and RS5). With reference to this primer, there was no clear relationship between the ISSR clustering tree, pathogenicity and geographic regions.

Discussion

R. solani AG-3 is an important soil-borne (Khan *et al*., 2017) and causal pathogen of stem canker and black scurf of potato (Young *et al*., 2010). Previously described methods electrophoresis of soluble proteins (Reynolds *et al*., 1983) Pectic zymograms (Neate *et al*.,1988, Hussain *et al*., 2017), restriction fragment length polymorphism (RFLP) (Vilgalys and Gonzalez, 1990) are beneficial for the differentiation of AGs but are unable to detect specific only one groups in the environment. The use of the ITS regions (1 and 2) for the designing of delicate primers has been established good strategy for improving diagnostic assays for many soil-borne fungal pathogens of potato (Cullen *et al*., 2002). In the current study, PCR primers

(Rs1F2 and Rs2R1) generated a sensitive, specific and simple PCR assay to *R. solani* AG-3 (Lees *et al*., 2002). The evaluation of genetic diversity of *R. solani* AG-4 by ISSR profile showed high genetic diversity between various isolates and different biogeographical regions (Mirmajlessi *et al*., 2012). The sugar beet isolates collected from various geographical areas of Iran into two AGs, were divided into two groups, AG-1 and AG-4. Population biology and genetics studies in *R. solani* has been confused, as *R. solani* is actually a large species complex comprised of many genetically featured groups that have very assorted life histories (Young *et al*., 2010). The current classification within the *R. solani* complex is largely based on the grouping of isolates into AGs. At least 12 AGs have been described within the *R. solani* complex. The considerations of mating compatibility systems (homothallic versus heterothallic) and how they may influence the population structures are also presented (Sneh *et al*., 1996). One possible explanation for intra-group variation found in the present studies could be the evolutionary divergence of biological species (Justesen *et al*., 2003). It may be related to the frequency, gene transfer and heterokaryon formation. Further, given the apparent rarity of the perfect state for *R. solani*, it is possible that the variation observed for isolate is a result of parasexual combination (Ogoshi, 1987). Twenty-three isolates of *R. solani* were obtained from cucurbits in Iran, investigation of the AGs indicated that all of the isolates belonged to AG-4. Genetic diversity was performed among isolates of *R. solani* AG-4 by ISSR primers. The results revealed a high genetic variability in the *R. solani* AG-4 population, without any correlation amongst genetic diversity, the host plants and geographical regions (Mirmajlessi *et al*., 2012). In case of Australian *R. solani* (Duncan *et al*., 1993) and Philippine (Pascual *et al*., 2002) isolates, origin related genetic relatedness was reported. However, no such correlation between the geographic origin and genetic relatedness was observed among the Indian isolates (Susheela & Reddy, 2013).

Acknowledgment

This project was supported by the King Saud University, Deanship of Scientific Research, College of Science Research Center.

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(Received for publication 15 March 2017)