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RESEARCH ARTICLE

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Identification and pathogenicity of *Rhizoctonia solani* AG-4 causing root rot on chickpea in Turkey

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Abstract

In the 2016-17 growing seasons, surveys were conducted in the Isparta, Uşak, Kütahya and Denizli provinces of Turkey to identify the *Rhizoctonia solani* AG-4 associated with root and crown rot of chickpea. A total of 75 isolates of *Rhizoctonia* were obtained from surveyed areas. Visual diagnostic, isolation and microscopic observation identified the causal organism as *R. solani*. Sequence data of the ITS rDNA region confirmed the species identity and revealed that the anastomosis group of the 23 isolates were AG-4 HGII. The isolates were variable in their morphological characters. The sequences generated during this study were clustered in the same branch with the reference isolates of *R. solani* AG-4 HGII based on their ITS sequencing on chickpea and the isolate grouping was not related to their geographic origins or virulence pattern. Pathogenicity tests revealed that all AG-4 isolates were pathogenic on chickpea and the disease severity values of 23 isolates varied between 42.8% and 100%. Based on the virulence, the isolates were grouped into two categories: 5 of them exhibited moderately virulence and 18 of them exhibited highly virulence reaction on chickpea. The high virulent isolate level (>50% disease severity) was determined as 78.2% of all 23 isolates. This is the first report of *R. solani* AG-4 as a pathogen of chickpea in Turkey.

Additional keywords: anastomosis group; ITS sequence; morphological characters; phylogenetic tree; radical assay.

Abbreviations used: AG (anastomosis group); DS% (percentage of disease severity); HV (highly virulent); ITS (internal transcribed spacer); LSD (least significance difference); LV (less virulent); MV (moderately virulent); PCR (polymerase chain reaction); PDA (potato dextrose agar).

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Introduction

Chickpea (*Cicer arietinum* L.) is one of the most extensively grown legume crops in Turkey. However, chickpea production and yield level are limited because of biotic and abiotic stress factors in Turkey. Root rot pathogens are very important within biotic factors including plant diseases. To date, more than 50 pathogens have been reported on chickpea from different parts of the world. However, only a few of them cause serious economic losses, such as Ascochyta blight caused by *Ascochyta rabiei* (Pass.) Labr., Fusarium wilt caused by *Fusarium oxysporum* f. sp. *ciceris* (Pad.), and root rot caused by a number of fungi, including *Rhizoctonia bataticola* (Taub.) Butler [*Macrophomina phaseolina* (Tassi) Goid] and *Rhizoctonia solani* Kuhn [teleomorph: *Thanatephorus cucumeris* (Frank)] (Nene & Reddy, 1987; Dolar, 1996; Bayraktar & Dolar, 2009). For management of the root rot pathogens firstly it is necessary to determine the disease agents which dominate and destroy the plant. *Rhizoctonia* species, causal agent of root rot of chickpea are wide spread on chickpea crops in the world where it is reported to cause considerable damage (Hwang *et al.*, 2003; Gonzalez *et al.*, 2006). The dry root rot of chickpea caused by necrotrophic fungus *R. bataticola* has emerged as a serious threat to the chickpea production worldwide. If the plant is exposed to moisture stress conditions, the disease severity is higher (Sharma *et al.*, 2010). The disease generally

appears during late flowering and podding stages and the infected plants appear completely dried (Pande *et al.*, 2004). *R. solani* is a soil and seed borne pathogen which has a wide host range and causes various diseases in important agricultural and horticultural crops due to its polyphagic nature and high saprophytic ability (Anderson, 1982; Nelson *et al.*, 1996). *R. solani*, which causes wet root rot, can be seen in early periods when soil moisture is high, and it can cause infection in every growth stage of a plant. It usually causes root rot which starts on the tip of young roots, and gradual yellowing and wiltings of the leaves (Dubey & Dwivedi, 2000). *Rhizoctonia* is typically a sterile fungal genus and has been characterized by division into binucleate and multinucleate groups. Hyphal anastomosis concept was introduced by Parmeter & Whitney (1970) for identification and characterization of *Rhizoctonia* isolates. Ogoshi (1987) classified *R. solani* primarily based on anastomosis behaviour; at present, 14 anastomosis groups (AGs) are recognised: AG-1-13 and AG-BI, and some that include several subgroups and isolates of *R. zeae* and *R. oryzae* have been assigned to WAG-Z and WAG-O, respectively (Sneh *et al.*, 1991, 1996; Carling *et al.*, 1999, 2002; Yang & Li, 2012). Various molecular markers have been used for characterization and grouping of *Rhizoctonia* species. The genetic diversity of *Rhizoctonia* isolates has been studied using RAPD-PCR, SSR-PCR, rDNA-RFLP, rDNA-ITS sequence analysis, universally primed-PCR and rep-PCR (Sharon *et al.*, 2008). Currently, the rDNA-ITS sequence analysis is the most appropriate method for classification of *Rhizoctonia* spp. and sequence analysis of the ITS-5.8S rDNA region has been used as a suitable molecular tool for identification of *R. solani* subgroups (Hyakumachi *et al.*, 1998; Salazar *et al.*, 2000; Priyatmojo *et al.*, 2001; Carling *et al.*, 2002). Genetic heterogenicity between, and within, anastomosis groups was evaluated by Fenille *et al.* (2003), using sequence analysis of the internal transcribed spacer (ITS) region of the ribosomal DNA. Comparison of the ITS region is significant in the determination of anastomosis groups, and also these sequences are useful for verifying subsets. Ganeshamoorthi & Dubey (2013) firstly used ITS region to determine anastomosis grouping of *R. solani* isolates from chickpea by using ITS1 and ITS4 primers. Different anastomosis groups such as AG-1, AG-2-2, AG-2-2LP, AG-2-3, AG-3, AG-4 and AG-5 were reported on chickpea in the world (Dubey *et al.*, 2011). Chickpea is sensitive to infection by several anastomosis groups (AG-4 and AG-5) (Hwang *et al.*, 2003; Dubey *et al.*, 2011; Ganeshamoorthi & Dubey, 2015). The presence of pathogenic isolates of AG-4 of *R. solani* in chickpea has been reported by many researchers in different country (Hwang *et al.*, 2003; Dubey *et al.*, 2011, 2014; Ganeshamoorthi & Dubey, 2013, 2015). In Turkey, isolates of *R. solani* AG-5 have already been determined to be pathogenic on chickpea (Tuncer & Erdiller, 1990; Demirci *et al.*, 1998), but so far the presence and pathogenicity of *R. solani* AG-4 have been not reported.

The objectives of the present study were to (i) identify and characterize the *R. solani* AG-4 isolates associated with root and crown rot of chickpea in Turkey, and (ii) determine the pathogenicity of the isolates.

Material and methods

Survey and fungal isolation

Survey studies were conducted in 2016 and 2017 during the chickpea production seasons in the Isparta, Uşak, Kütahya and Denizli provinces in Turkey (Table 1, Fig. 1).

During these surveys, the infected plants, which had dark-brown lesions on the roots and crown, were collected. Depending on the size of the fields, at least 5 plants were collected from each field. To isolate the pathogen, root parts of diseased plants were washed in tap water and dried on sterile blotter paper. Symptomatic root and crown tissues were cut into 0.2-0.5 cm pieces, then surface-sterilized with 1% sodium hypochloride (NaOCl) for 2-3 min. After rinsing in sterile distilled water five times and subsequently drying on blotter paper, 5 or 6 tissue pieces were placed in Petri dishes containing potato dextrose agar (PDA, Merck) and amended with streptomycin sulphate (50 mg/L). Petri dishes were incubated at 23±1°C with a 12-h photoperiod for 7-10 days. For the purification of the isolated fungus, single hyphal tips were transferred onto new PDA plates from the fungal cultures on 1.5% water agar. Purified isolates were stored on PDA, filter papers in eppendorf tubes at +4°C and colonized wheat seed at -20°C.

Morphological characterization and AG typing of isolates

All isolates were characterized based on cultural morphology, cellular nuclei number in young vegetative hyphae, and anastomosis reactions with tester isolates. Cultural characteristics were determined by growing each isolate on PDA and incubating at 25°C for 14 days (Sneh *et al.*, 1991). Cultures were examined for colony color, sclerotia formation and color, and aerial mycelia formation. Observations were recorded visually by the method described by Burpee *et al.* (1980). Nuclear

Table 1. Location of survey area in Turkey and number of samples (2016-17 growing seasons).

Province	District	Growth stages of chickpea	Number of fields	Number of collected plants
Denizli	Serinhisar	Pre-flowering	1	6
	Tavas	Seedling	2	42
	Çal	Pre-flowering	1	17
Uşak	Banaz	Pre-flowering	1	11
	Ulubey	Seedling	1	14
	Bölme	Pre-flowering	1	27
	Güney	Flowering	1	18
Kütahya	Gediz	Pre-flowering	1	24
	Dumlupınar	Seedling	1	15
Isparta	Senirkent	Pre-flowering	2	47
	Yalvaç	Flowering	1	47
Total			**13**	**268**

condition of isolates was determined using the Safranin O staining technique (Bandoni, 1979). For anastomosis groups determination, the slide technique coupled with Safranin O staining was used for observing hyphal fusion reactions between testers and unknown isolates (Kronland & Stanghellini, 1988). Briefly, *Rhizoctonia* isolates and testers initially were grown on PDA at 25 °C in the dark. A sterilized coverslip was coated with a thin layer of 0.5% PDA and placed on Petri dishes containing 1.5% water agar medium. Agar disks of *Rhizoctonia* isolates and the tester isolates were cut from the growing edge of the plate and transferred to opposite ends of the coverslip on the water agar plates. After incubation at 25°C for 24-72 h in the dark, when overlapping mycelia of two isolates were observed, the coverslip was removed from the plate and placed on a microscope glass slide, and stained with safranin O and 3% KOH. Sites of hyphal interaction were examined under a light microscope and occurrence of anastomosis was determined when the hyphae of paired isolates were fused and exchanged cytoplasms. Reaction types observed between interacting hyphae were assigned to one of the four categories (C0, C1, C2 or C3) described by Carling *et al.* (1988). For the anastomosis testing, pairing for each unknown isolate was replicated three times.

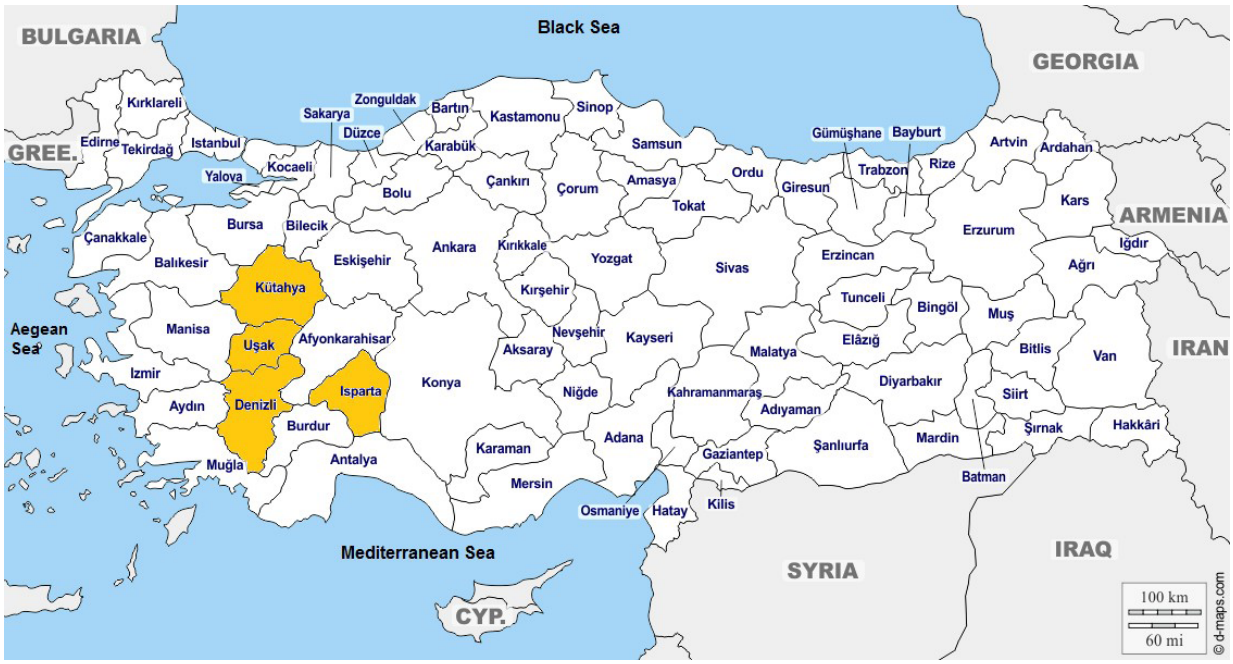

Figure 1. Location of survey area (orange) in Turkey.

ITS-rDNA gene sequencing

For molecular characterization, each isolate was grown on PDA (Merck) at 25±1°C. After 5-7 days, about 300 mg mycelium was harvested by removing excess of the solid media using a sterile scalpel, and stored at -20°C until used. Genomic DNA was extracted using the Plant/Fungi DNA Isolation Kit (Norgen, Biotek) following the manufacturer's recommendations for fungal DNA isolation. The ITS region of the rDNA was amplified using ITS 1 (5' TCC GTA GGT GAA CCT GCGG 3') and ITS 4 (5' TCC TCC GCT TAT TGA TATGC 3'), primers described by White *et al.* (1990). The PCR reactions were carried out in a 50 µL final volume containing 25 µL of PCR Master Mix (Norgen Biotek Corporation, Canada), 2 µL of each primer (concentration 10 pmol/µL), 5 µL of DNA template and 16 µL of PCR-grade water. The DNA amplifications were performed in a thermocycler (Eppendorf AG, Hamburg, Germany) using the following cycle parameters: initial denaturation at 95°C for 2 min; 35 cycles of denaturation at 95°C for 20 s, annealing at 55°C for 30 s, and extension at 72°C for 45 s; and a final extension step at 72°C for 5 min. Following the PCR reaction, the amplified products were loaded in a 1.5% agarose gel stained with GelRed, together with 100 bp DNA marker (Norgen Biotek Corporation, Canada). Before loading, both samples and marker were stained with Blue/Orange 6X Loading Dye (Norgen Biotek Corporation, Canada) used for tracking migration during electrophoresis. Electrophoresis was run at 100 V for 1 hour. The DNA bands were visualised using a Quantum Capt. ST4 Imaging System (Quantum Corporation, France). They were sequenced by ALTIGENBIO Life Science (International Biotechnology Company, Izmir, Turkey). ITS sequence analysis was performed using BLAST via http://www.ncbi.nlm.nih.gov. Sequences of each isolate were deposited in GenBank (see accession numbers in Table 3).

Pathogenicity tests

According to the results of AG typing and ITS-rDNA gene sequencing, isolates identified as AG-4 of *R. solani* were tested for pathogenicity by radicle assay in petri dishes under in vitro conditions, then virulence scales were determined based on the percentage of disease severity. The isolates were allowed to incubate at 25±2°C for 10-15 days, then mycelial discs were taken with a 4 mm diameter cork borer from an actively growing edge of the fungal culture. Each piece was placed in the center of a petri plate containing 2% water agar and incubated at 25±2°C for 48 h. Susceptible chickpea cultivar seeds (cv. ILC 482) were immersed in 1% NaOCl for 5 min for surface disinfection and washed with sterile distilled water 3 times. Then 7 seeds were placed at equal distances around the fungus piece. As a control treatment, seeds were placed around the sterile PDA discs. All petri plates were wrapped with parafilm and then incubated at 25±2°C with 12 h photoperiod for 10-12 days. Subsequently, by examining the hypocotyls of germinated seeds, the disease development was assessed according to the scale of 0 to 5 (Fig. 2) based on the size of the necrotic area in the hypocotyl (Ichielevich-Auster *et al.*, 1985). Three plates were used for each isolate.

For evaluation of each isolate, scale values were given to all the seeds in each replicate. No scale value was given as no infections were seen in the control treatment. Percentage of disease severity (DS%) was calculated from the Townsend-Heuberger´s (1943) formula based on scale values obtained by pathogenicity tests.

According to the results of the in vitro tests, pathogenicity was also tested on seed in pots with the most virulent isolate on susceptible chickpea cultivar (cv. ILC482). Inoculum was prepared on the sterile wheat grains in tests tubes. The mixture of perlite: silt loam+fine sand (2:1) was sterilized and filled in each plastic pot (13 cm in diameter). Susceptible chickpea cultivar seeds were immersed in 1% NaOCl for 5 min for surface disinfection and washed with sterile distilled water three times. Five chickpea seeds were placed in each pot and five pathogen-colonized wheat grains were placed near the seeds to serve as inoculum. The control consisted of pots without inoculum. There were five replicate pots per treatment. The pots were incubated at 25±2°C with 12 h photoperiod for 10-12

Figure 2. The 0-5 scale used in disease development assessment: 0=healthy, 1=1-10% infection of hypocotyls, 2=11-30% infection of hypocotyls, 3=31-50% infection of hypocotyls, 4=51-80% infection of hypocotyls and 5=plant dead.

days in a growth chamber. Re-isolations were made from the roots of diseased plants to confirm the identity of the causal agent.

Data analyses

Radicle assay test for pathogenicity was carried out in a completely randomized design of three replicates. For all pathogenic isolates, the mean DS% was used to categorize their relative virulence, where isolates were categorized as highly virulent (HV) if they showed a mean DS% between 50.1 and 100%, moderately virulent (MV) between 20.1 and 50%, and less virulent (LV) between 0 and 20%.

Percentage data was transformed into angular values to produce an approximately constant variance before carrying out the analysis of variance (ANOVA). The statistical significance was assessed at $p < 0.05$ and Fisher's least significance difference (LSD) test was used to separate means. Statistical analyses were performed using the JMP 14.0 (SAS Institute, Cary, NC, USA) software package. A phylogenetic tree of the *R. solani* isolates was constructed based on ITS sequencing using MEGA 10.0.5 via the neighbor-joining bootstsap method. The tester isolate of *R. solani* AG-4 which was obtained from Japan and the reference isolates of *R. solani* AG-4 HGI (AY152704.1, AB000007.1), HGII (AY154308.1, AB000006.1) (Kuninaga *et al.*, 1997; Kuramae *et al.*, 2003) and HGIII (DQ102449.1, AY154659.1) (Kuramae *et al.*, 2003; Sharon *et al.*, 2007) were also used on the tree for comparison with the isolates generated in this study.

Results

Morphological and molecular characterization of isolates

Two-hundred-sixty-eight plant samples were collected from chickpea growing areas in the Isparta, Uşak, Kütahya and Denizli provinces in Turkey (Table 1). A total of 75 isolates of *Rhizoctonia* were obtained from damaged tissues and twenty-three of them were identified as *R. solani* AG-4 according to cultural morphology and anastomosis reactions with tester isolates. Microscopic inspection of twenty-three *R. solani* AG-4 isolates revealed a buff-colored to dark-brown, regularly septate mycelium with a slight constriction at the septum. Each hypha cell contained more than two nuclei (Fig. 3a).

The color of the colony varied from whitish brown (Fig. 3b) to dark brown. Among the 23 isolates studied, 4 isolates were whitish brown, 16 isolates were light brown and 3 isolates were dark brown. On the basis of growth pattern, 5 isolates produced abundant mycelium, while 11 isolates had moderate mycelium

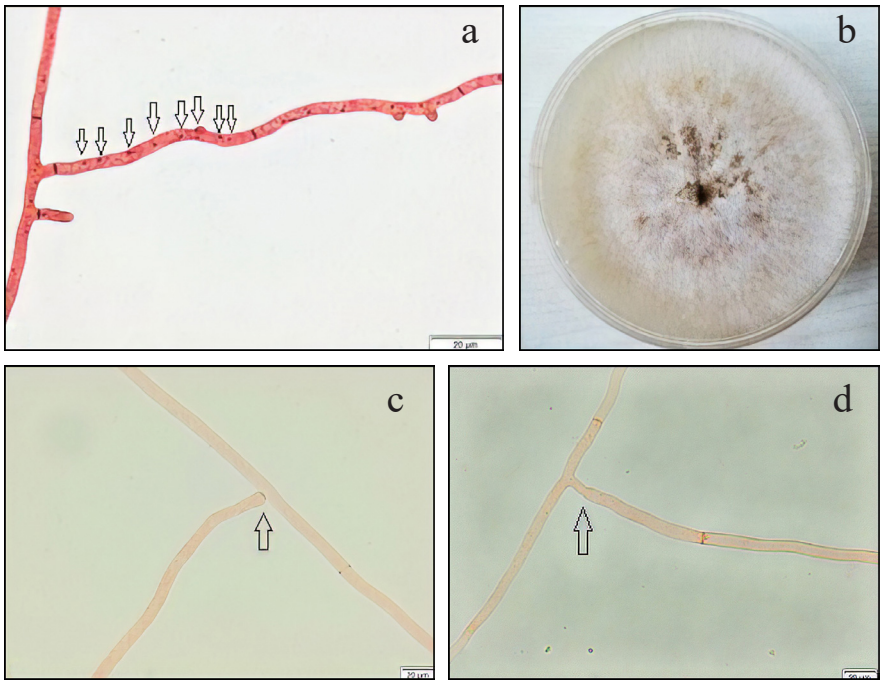

Figure 3. Multinucleate hyphal cell (a) and colony growth of *R. solani* AG-4 on PDA (b), C1 anastomosis reaction (contact) between hyphae (c), and C3 anastomosis (hyphal fusion) reaction between hyphae (d).

and the remaining 7 isolates recorded slight mycelium. Although 13 isolates produced sclerotia, 10 isolates did not. Among the 13 isolates that produced sclerotia, 9 of them showed light brown sclerotia color while the rest of them were dark brown. Based on the formation of sclerotia, 8 of them had scattered form, 4 of them had central form and only 1 of them had peripheral form (Table 2). On the other hand, C1 and C3 anastomosis reactions were observed between AG-4 isolates and test isolates diagnosed in anastomosis reaction studies (Fig. 3c, d).

After the pathogenicity tests, re-isolated fungi were characterized based on cultural morphology and anastomosis reactions with tester isolate of AG-4 of *R. solani.*

The ITS sequences of 23 isolates of *R. solani*, which belonged to AG-4 were recovered from the GenBank (Acc. no. MH231493 to MH231515). The sequences were compared to reference sequences of *R. solani* anastomosis groups in GenBank, and were found to be most similar to group AG-4, the intraspecific group HG-II at 99 to 100% identity (Table 3). DNA bands of 23 *R. solani* AG-4 isolates varied from 615 to 667 bp long.

The phylogenetic tree was constructed from the nucleotide sequence similarity of 23 isolates along with the tester isolate of *R. solani* AG-4 which was obtained from Japan and the reference isolates of *R. solani* AG-4 HGI, HGII and HGIII (Fig. 4). The sequences generated during this study were clustered in the same branch with the tester isolate of *R. solani* AG-4 (MK280743.1) and the reference isolates of *R. solani* AG-4 HGII. The reference isolates of *R. solani* AG-4 HGI and the reference isolates of *R. solani* AG-4 HGIII clustered seperately. Grouping the isolates based on their ITS sequencing was not related to their geographic origins or virulence patterns.

Pathogenicity of the isolates

According to pathogenicity test under in vitro conditions, all AG-4 isolates were found to be

Table 2. Cultural and sclerotial characteristics and pathogenic behaviour of AG-4 isolates of *R. solani.*

Province	District	Isolate No.	Colony characters		Sclerotia characters		Pathogenic behaviour	
			Colony color	Growth pattern	Sclerotia color	Formation of sclerotia	Disease severity (%)[1,2]	Virulence[3]
Denizli	Serinhisar	D1	Whitish brown	Moderate	Light brown	Central	58.8 (50.14)^{cde}	HV
	Serinhisar	D2	Light brown	Abundant	Light brown	Scattered	65.7 (54.35)^{bcde}	HV
	Tavas	D4	Light brown	Slight	Dark brown	Peripheral	72.3 (58.86)^{bcde}	HV
	Tavas	D5	Light brown	Slight	Dark brown	Scattered	47.6 (43.62)^{de}	MV
	Tavas	D6	Light brown	Slight	Not observed		73.3 (59.60)^{bcde}	HV
	Çal	D8	Light brown	Moderate	Not observed		79.0 (62.89)^{bcd}	HV
	Tavas	D10	Light brown	Moderate	Not observed		60.0 (50.83)^{bcde}	HV
Uşak	Ulubey	U1	Whitish brown	Slight	Light brown	Scattered	75.2 (60.21)^{bcde}	HV
	Ulubey	U2	Dark brown	Moderate	Not observed		51.4 (45.53)^{cde}	HV
	Bölme	U6	Light brown	Moderate	Light brown	Scattered	72.0 (58.15)^{bcde}	HV
	Güney	U11	Light brown	Slight	Not observed		68.5 (56.12)^{bcde}	HV
	Banaz	U12	Light brown	Moderate	Not observed		100 (99.33)^{a}	HV
	Banaz	U13	Light brown	Moderate	Light brown	Central	73.3 (59.99)^{bcde}	HV
	Banaz	U14	Light brown	Abundant	Not observed		74.2 (59.49)^{bcde}	HV
Kütahya	Dumlupınar	K7	Light brown	Slight	Not observed		47.1 (43.33)^{de}	MV
	Dumlupınar	K8	Dark brown	Moderate	Light brown	Central	72.3 (59.00)^{bcde}	HV
	Gediz	K9	Light brown	Abundant	Light brown	Scattered	88.5 (71.24)^{b}	HV
	Gediz	K10	Whitish brown	Slight	Not observed		57.1 (50.92)^{bcde}	MV
Isparta	Senirkent	IS7	Whitish brown	Moderate	Light brown	Scattered	69.5 (64.97)^{bc}	MV
	Senirkent	IS9	Light brown	Abundant	Dark brown	Scattered	71.4 (58.33)^{bcde}	HV
	Senirkent	IS10	Dark brown	Moderate	Dark brown	Scattered	82.8 (65.54)^{bc}	HV
	Senirkent	IS15	Light brown	Abundant	Not observed		72.3 (58.54)^{bcde}	HV
	Yalvaç	IS29	Light brown	Moderate	Light brown	Central	42.8 (40.52)^{e}	MV

[1]Percentage data were transformed into angular values. [2]The values within a column with different letters are significantly different ($p \leq 0.05$). [3]HV: Highly virulent, MV: Moderately virulent.

Table 3. ITS sequencing data of AG-4 isolates of *R. solani*.

Isolate No.	ITS sequencing		Match in GenBank	
	GenBank Acc. No.	Query length (bp)	Anastomosis grouping and similarity rate	Acc. No.
D1	MH231493.1	628	AG-4 HGII 100%	KX631315
D2	MH231494.1	653	AG-4 HGII 99%	KC590607
D4	MH231495.1	649	AG-4 HGII 100%	KC590589
D5	MH231496.1	625	AG-4 HGII 100%	KC590605
D6	MH231497.1	663	AG-4 HGII 99%	JX843818
D8	MH231498.1	636	AG-4 HGII 99%	KJ170352
D10	MH231499.1	628	AG-4 HGII 99%	KJ170349
U1	MH231505.1	626	AG-4 HGII 100%	KX118330
U2	MH231506.1	622	AG-4 HGII 100%	KX468074
U6	MH231507.1	655	AG-4 HGII 100%	KC590563
U11	MH231508.1	645	AG-4 HGII 99%	KC590535
U12	MH231509.1	649	AG-4 HGII 100%	KC590571
U13	MH231510.1	641	AG-4 HGII 100%	KC590533
U14	MH231511.1	641	AG-4 HGII 99%	KC590602
K7	MH231512.1	615	AG-4 HGII 100%	KX631299
K8	MH231513.1	640	AG-4 HGII 100%	HQ629866
K9	MH231514.1	631	AG-4 HGII 100%	KX631298
K10	MH231515.1	667	AG-4 HGII 100%	KX468071
IS7	MH231500.1	636	AG-4 HGII 100%	HQ629864
IS9	MH231501.1	636	AG-4 HGII 100%	HQ629864
IS10	MH231502.1	623	AG-4 HGII 100%	KX631250
IS15	MH231503.1	638	AG-4 HGII 100%	KX468070
IS29	MH231504.1	641	AG-4 HGII 100%	HQ629868

pathogenic on chickpea. The control seeds did not develop symptoms (Fig. 5). The results showed that the disease severity values of 23 isolates varied between 42.8% and 100% (Table 2).

According to the virulence of isolates, 23 isolates of *R. solani* AG-4 were grouped into two categories: MV and HV. Five isolates were found to be MV and 18 isolates were HV on chickpea (Table 2). No isolate was in the 0-20% (LV) range. According to the values, HV isolate level (>50% disease severity) was determined as 78.2% of all 23 isolates.

The pathogenicity of U12, K9 and IS29 differed significantly ($p < 0.05$) from the other isolates, while there were no statistically significant differences ($p > 0.05$) among other isolates according to Fisher's LSD test (Table 2). Isolate U12 was the most virulent whereas isolate IS29 was the least virulent (100% and 42.8%, respectively). According to the pot trial results, U12 caused 100% pre-emergence damping-off in chickpea (Fig. 6).

Discussion

A total of 75 isolates of *Rhizoctonia* were obtained from surveyed areas in Turkey. Twenty-three of them were multinucleate and belonged to anastomosis group 4 (AG-4 HGII). The isolates were variable in their morphological characters. Based on the colony color, 23 isolates of *R. solani* were assigned into three categories and most of them had a light brown colony color. Ganeshamoorthi & Dubey (2015) characterized 50 isolates in terms of cultural variability obtained from chickpea and among four isolates of AG-4 of *R. solani* two isolates showed light brown, while two isolates had dark brown colony color. In the present study, moderate growth pattern was the dominant character among the isolates. Sclerotia were central, peripheral or scattered, and light brown to dark brown. According to the study reported by Ganeshamoorthi & Dubey (2015), three isolates of AG-4 showed dark and scattered, one isolate showed dark and peripheral

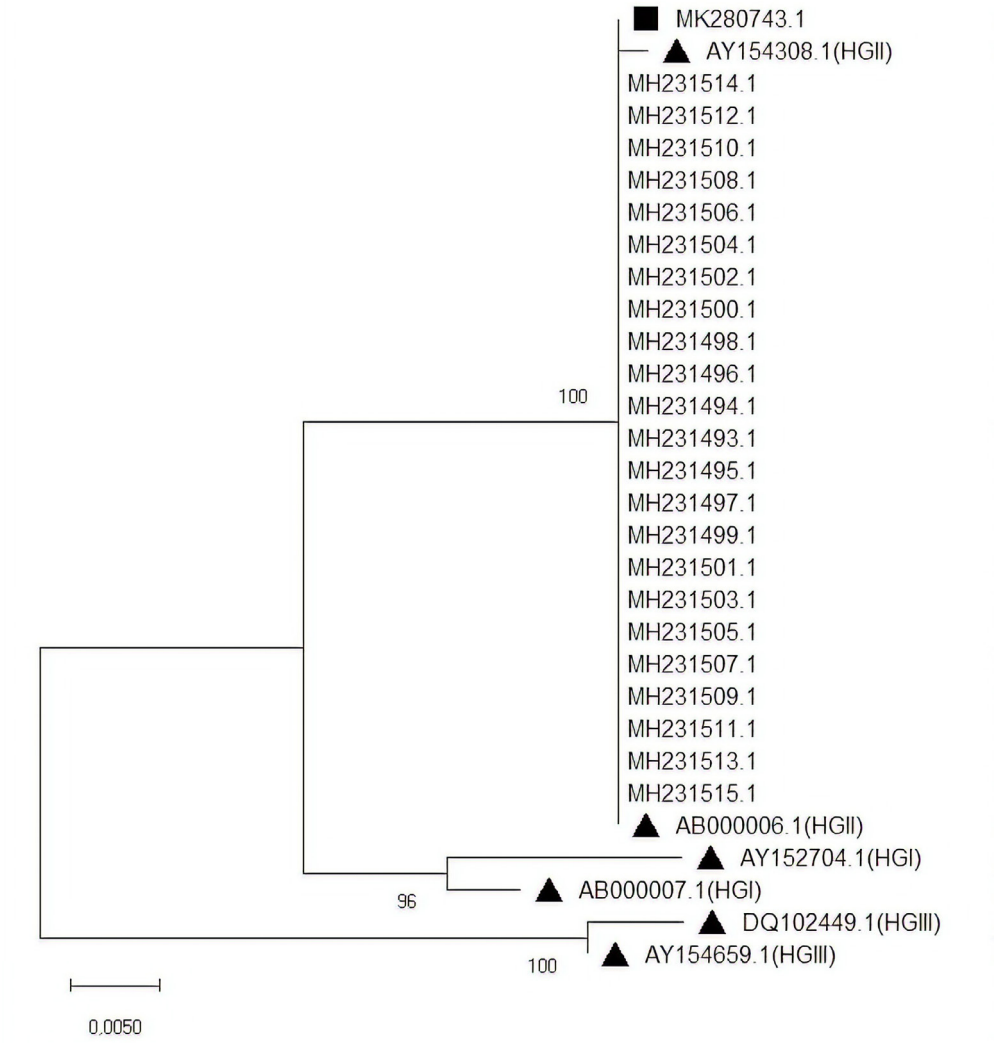

Figure 4. Neighbor-joining tree showing the phylogenetic relationship among isolates of *R. solani* AG-4 based on their ITS sequences. The tester isolate of *R. solani* AG-4 obtained from Japan were labelled square "■"; the reference isolates of *R. solani* AG-4 HGI, HGII and HGIII were labelled triangle "▲".

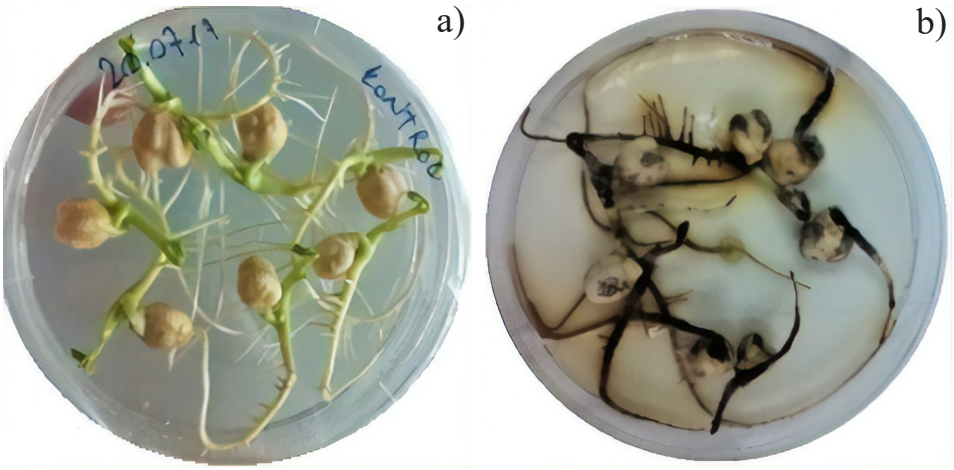

Figure 5. Pathogenicity test: healthy hypocotyls in control plate (a), hypocotyl infection at 11 days after inoculation (b).

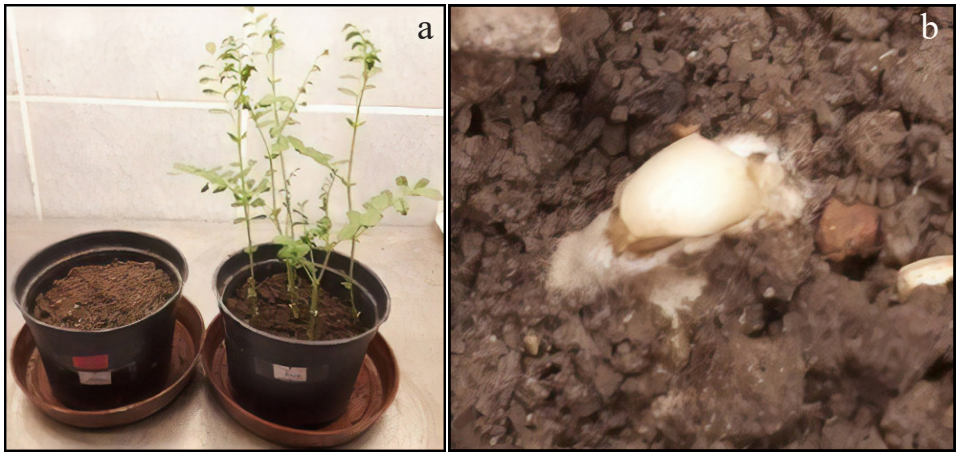

Figure 6. Inoculated pot with isolate U12 (left) and control pot (right) (a); pre-emerging damping-off in chickpea (b).

sclerotia formation. These results were parallel with our findings.

The frequency of AG-4 in all isolates collected was found to be quite high with a value of 30.6% whereas Mikhail *et al.* (2010), Ganeshamoorthi & Dubey (2013), and Dubey *et al.* (2014) revealed that the frequency of AG-4 obtained from chickpea was 13.7%, 8% and 7% respectively. On the other hand, Hwang *et al.* (2003) determined that all the isolates obtained from chickpea belonged to AG-4 with a value of 100% frequency, which indicates that the frequency of *R. solani* AG-4 isolates obtained from chickpea varies in different countries.

The present study shows that ITS sequencing is a powerful tool in understanding and determinating the relationship between anastomosis groups and subgroups of *R. solani*. All isolates belong to AG-4 HGII at 99 to 100% identity. DNA bands of 23 *R. solani* AG-4 isolates varied from 615 to 667 bp long in this study. Dubey *et al.* (2014) reported that *R. solani* isolates were variable respect to their nucleotide sequences of ITS region (650–750 bp). According to Ganeshamoorthi & Dubey (2013), ITS region is very useful to study intra-specific diversity of the pathogen and helps in the development of species level diagnostic molecular markers. They found that, using ITS1 and ITS4 primers, 50 isolates of *R. solani* produced between 572 and 715 bp long bands.

In this study, the generated sequences were clustered into the same branch of the reference isolates of *R. solani* AG-4 HGII. However, grouping of the isolates was not related to their geographic origins or virulence pattern. Boysen *et al.* (1996), also observed sequence variations in ITS region of nine *R. solani* isolates of AG-4. The method was also used to express the genetic similarity among 52 isolates of *R. solani* (El-Samawaty *et al.*, 2008). Also, Ganeshamoorthi & Dubey (2013) analysed 50 *R. solani* isolates obtained from chickpea by the phylogenetic tree method and they grouped into two categories. In the present study, the findings clearly indicate that the chickpea populations of *R. solani* AG-4 are highly variable in their ITS region. The results are also in agreement with Kuninaga *et al.* (1997), who found a highly variable ITS 5.8s rDNA sequence in 45 isolates of *R. solani*.

The results of the pathogenicity studies clearly show that all the AG-4 HGII isolates tested are pathogenic on chickpea. AG-4 of *R. solani* has also been reported many times to be pathogenic to chickpea worldwide (Hwang *et al.*, 2003; Dubey *et al.*, 2011, 2014; Ganeshamoorthi & Dubey, 2013, 2015). According to the virulence of isolates, 23 isolates of *R. solani* AG-4 have been categorized into two groups. Five of them were MV and 18 of them were HV on chickpea. In this study, the isolates had high pathogenic behaviour on chickpea, with a value of 78.2% which was the HV category. This also supports the findings of Dubey *et al.* (2011) who found that the disease incidence caused by isolates, including AG-4, varied from 11 to 100% and the HV isolate level was 75.6% on chickpea. However, Ganeshamoorthi & Dubey (2015) found 4 out of 50 *R. solani* isolates which belonged to AG-4 and all of them were MV on chickpea. In the present study, as a result of the pathogenicity test in pots, isolate U12 showed pre-emergence damping-off with 100% disease severity value. Hwang *et al.* (2003) revealed that five isolates of *R. solani* collected from Saskatchewan (Canada) belonged to AG-4 and caused 100% pre-emergence damping-off in chickpea in the pathogenicity test. Results of these studies are in parallel with our findings.

So far, five AG groups (AG-1, AG-2, AG-3, AG-4 and AG-5) of *R. solani* have been detected in chickpea in the world (Hwang *et al.*, 2003; Mikhail *et al.*, 2010; Youssef *et al.*, 2010; Dubey *et al.*, 2011, 2014;

Ganeshamoorthi & Dubey, 2013, 2015). In Turkey, AG-4 of *R. solani* has been reported to be pathogenic to barley (Demirci, 1998; Ünal & Kara, 2017), common bean (Eken & Demirci, 2004; Kılıçoğlu & Özkoç, 2013), pepper (Tuncer & Eken, 2013), tomato (Yıldız & Döken, 2002), soybean (Erper *et al.*, 2011), cotton (Kural *et al.*, 1994), Johnsongrass (Demirci *et al.*, 2002) and wheat (Demirci, 1998; Ünal *et al.*, 2015) but this group had not yet been reported in chickpea in Turkey. The present study shows then that both the morphological and molecular analyses of *R. solani* isolates indicate that this is the first identification of AG-4 on chickpea in Turkey.

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