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Research Article

New Anastomosis Group F (AG-F) of binucleate *Rhizoctonia* causing root and stem rot of *Pistacia vera*

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Abstract: A total of ten isolates of fungi with *Rhizoctonia*-like mycelia were obtained from infected roots and stems of Pistachio *Pistacia vera* grown in a commercial nursery in Rafsanjan, Iran, during the autumn of 2011. The infected seedlings showed symptoms of chlorosis that later turned to necrosis. All of the isolates were identified as binucleate *Rhizoctonia* on the basis of hyphal characteristics and nuclear number. They were tested for detection of the anastomosis group, optimum growth temperature, rDNA-ITS region traits and pathogenicity on pistachio seedlings *in vitro* and *in vivo*. The analysis of hyphal anastomosis reaction was carried out with the tester isolates of binucleate *Rhizoctonia* AG-A, AG-Ba, AG-G and AG-F as well as multinucleate *Rhizoctonia* AG4 as already detected on pistachio seedlings. The optimum temperature for growth of binucleate *Rhizoctonia* sp. was 35 °C. In *in vivo* test, the symptoms of root rot were observed 30 days after inoculation and mortality happened two weeks thereafter. According to molecular characteristics and anastomosis test groups, all isolates showed greatest similarity to anastomosis group AG-F. This finding is the first report of anastomosis group F (AG-F) of binucleate *Rhizoctonia*, as causal agent of root and stem rot disease of pistachio in the world and Iran.

Keywords: Pistachio, Pathogenicity, ITS-rDNA, DNA extraction, PCR

Introduction

Pistachio *Pistacia vera* L. is one of the most important commercial products in Iran where about 450000 hectares of land area is under pistachio cultivation (Statistical Center of Iran, 2014). Kerman province is the largest region for pistachio cultivation as well as the center of pistachio seedling production. Soil-borne plant pathogenic fungi and fungus-like agents, including *Pythium*, *Phytophthora*, *Verticillium*, *Armillaria* and *Rosellinia,* are known as serious pathogens of pistachio trees with a worldwide distribution (Teviotdale *et al.*, 2002). Damping off, root rot and stem rot caused by *Rhizoctonia solani* AG-4 are the most serious diseases of this crop in nurseries and are associated with severe economic losses (Ashkan and Abusaidi, 1995; Ilkhan *et al.*, 2011). *Rhizoctonia* spp. have been classified into uninucleate, binucleate and multinucleate groups based on the cell nuclear number (Sharon *et al.*, 2007; Sharon *et al.*, 2006; Sneh *et al.*, 1998). Affinity for hyphal fusion (anastomosis) (Burpee *et al.*, 1980; Ogoshi *et al.*, 1983; Parmeter and Whitney, 1970) has been used to characterize isolates among *Rhizoctonia* spp. and binucleate *Rhizoctonia* (BNR) species with *Ceratobasidium* teleomorphs (Yang and Li, 2012). To date, isolates of *R. solani* have been assigned to 13 anastomosis groups (AG-1 to AG-13) based on hyphal anastomosis reactions

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(Rinehart *et al.*, 2007; Sneh *et al.*, 1998) and those of *R. zeae* and *R. oryzae* have each been assigned to their own one group (Carling, 1996; Carling *et al.*, 2002; Carling *et al.*, 1999; Sneh *et al.*, 1998). Most of the *R. solani* AGs are recognized as important plant pathogens, while the BNR species do not form a homogenous species and are mostly considered as non-pathogenic mycorrhizal fungi or having a role as biocontrol agents and commonly found in soil organic matter or in plant debris (Adams, 1988; Andersen and Rasmussen, 1996; Anderson, 1982; Burpee and Goulty, 1984). Only a few AGs of the known BNR species are pathogenic. The BNR species as pathogens cause several diseases such as damping-off, root rot, stem rot, sheath blight, fruit decay and foliar blight on a wide range of important agricultural plants (Parmeter and Whitney, 1970; Tanaka *et al.*, 1994). The BNR have been divided into 21 anastomosis groups (AG-A to AG-U), although the tester strains of AG-J and AG-M have been excluded from the BNR species because they have clamp connection (Hyakumachi *et al.*, 2005; Ogoshi *et al.*, 1983; Sneh *et al.*, 1998). AG-B has been further divided into three subgroups AG-Ba, AG-Bb and AG-Bo based on the frequency of hyphal anastomosis and cultural characteristics (Sneh *et al.*, 1998). Differentiation of these three subgroups of AG-B is supported by polymorphism in the ribosomal RNA gene (Cubeta *et al.*, 1991). Also AG-D has been subdivided into two subgroups AG-D (I) and AG-D (II) based on cultural morphology and pathogenicity (Tanaka *et al.*, 1994). Pistachio damping off caused by *Rhizoctonia* solani AG-4 was reported from a nursery in Kerman province, Iran (Ashkan and Abusaidi, 1995) as well as California, USA (Holtz *et al.*, 1996) with a severe economic damage. To our knowledge, to date there is no report on the BNR causing root and stem rot on pistachio in Iran or anywhere else in the world. However, the BNR species have been reported as pathogens causing root rot on strawberry (Martin, 1988; Perez Vicente *et al.*, 2008; Sharon *et al.*, 2007), bean (Xue *et al.*, 1998) and yacon (Fenille *et al.*, 2005). During the summer and fall of 2010 and 2011 root and stem rots symptoms leading to plant death were observed in commercially grown pistachio nursery in Rafsanjan, Iran. Considering the local importance of this *Rhizoctonia* like disease for pistachio production, the objective of this study was to characterize the *Rhizoctonia* spp. associated with root and stem rot of pistachio by determining their nuclear condition, hyphal anastomosis grouping, morphology and mycelia growth rate at different temperatures, virulence and genetic identity with other *Rhizoctonia* spp. based on sequencing analysis of the ITS-5.8S rDNA region and PCR-RFLP.

Materials and Methods

Sampling, fungal isolation and culture maintenance

Infected pistachio seedling samples with chlorotic leaves and/or necrotic lesions and dry brown root and stem rot symptoms were collected from a commercial pistachio nursery in Rafsanjan, Iran, during the summer and autumn of 2010 and 2011. Symptomatic roots were washed thoroughly in running tap water for 30 min to remove adhered soil particles, air dried, then cut into 5 mm pieces. Roots and stems were surface disinfected with 0.5% commercial sodium hypochlorite solution for 2 min and rinsed three times with sterile distilled water. Pieces of root and stem were dried separately on sterilized filter paper, placed on petri dishes containing acidified water agar (AWA, pH 4.5) using10% lactic acid. Cultures were incubated for 2 to 3 days at 25 °C in dark (Carling *et al.*, 2002). Cultures were examined microscopically and fungal colonies with characteristics typical of *Rhizoctonia* like fungi were hyphal tipped and sub-cultured onto potato dextrose agar (Merck, Germany). Pure cultures were stored in PDA slant tubes or sterile barley grain at 4 °C.

Cultural appearance and Induction of teleomorph

To evaluate the cultural appearance of the isolates, a single agar disks (7-mm diameter) containing mycelium from 2- to 3-day-old cultures of *Rhizoctonia* sp. growing on PDA were placed in the center of 9 mm petri dishes

containing PDA and incubated either at 25 °C in dark or in the refrigerator. Cultures were evaluated 25 days after incubation. The sclerotia production of each isolate was investigated according to the method used by Oniki *et al.* (1985). In brief fresh cultures of isolates were grown on a modified Potato Yeast Extract Agar (PYEA) which was acidified to pH 4.5 using 10% lactic acid. Cultures were incubated at 27 °C and when the hyphae reached to the margin of the dish, they were covered to the rim with air dried soil aggregates. The cultures were incubated at room temperature and the petri dish lids removed. Humidity was maintained by watering the soil 1-3 times daily, while excess moisture was drained. Production of hymenia on the surface of soil would be expected within 12-14 days afterwards.

Nuclear conditions and AG determination of isolates

The number of nuclei per hyphal cell was determined by the procedure of Bandoni (1979). A single agar disk (7-mm diameter) containing mycelium from 2- to 3-day-old cultures of *Rhizoctonia* sp. growing on PDA was placed on a clean, sterile, microscopic glass slides and incubated for 1-2 days in a moist chamber at 25 °C in dark. Nuclei were stained using a drop of safranin O and 3% KOH (1:1v/v). Twenty five cells of each isolate were examined at × 400 magnifications using bright field microscopy for the number of nuclei. To determine anastomosis group of isolates, the glass-slide technique was used as described by Kronland and Stanghellini (1988). A single agar disk (7 mm- diameter) was cut from the edge of a 2- to 3-day-old colony on PDA and placed on a clean glass slide. Tester isolates AG-A, AG-Ba, AG-G and AG-F as well as multinucleate *Rhizoctonia* AG4 were placed 3 to 4 cm away from each tested isolates. Slides were put in a moist chamber and incubated at 25 °C for 24 to 48 h in the dark. Excess moisture was wiped from the bottom of the slide. When the hyphae from the two disks were overlapping, they were stained using safranin O and 3% KOH (1: 1 v/v) and examined microscopically to determine anastomosis reaction (Carling, 1996; Kronland and Stanghellini, 1988).

Hyphal growth rates and diameter determination

A single agar disk (7-mm diameter) containing mycelium from 2- to 3-day-old cultures of *Rhizoctonia* sp. growing on PDA was placed on a clean, sterile, microscopic glass slide that was covered by 2% WA and incubated for 1-2 days in a moist chamber at 25 °C in dark. After 24-48h of incubation, the hyphae were stained with Fushin acid and evaluated using the optical microscope with × 400 magnifications. In order to measure the hyphal diameter at least 50 samples were measured and their mean was takenas the hyphae diameter (Sneh *et al.*, 1998). Radial growth rates of isolates were determined at 5, 15, 20, 25, 30 and 40 °C. A single agar disk (7 mm diameter) containing mycelium from the margin of a 2- to 3-days colony was transferred to PDA in the center of 9 cm petri dish. Measurements were taken 12h after incubating petri dishes to allow temperature diffusion to agar. Colony radius was measured at 24h intervals until the colony reached the edge of the petri dish. Treatments were replicated three times and experiment was repeated twice.

***In vitro* and *in vivo* pathogenicity test**

In vitro pathogenicity test of the isolates on seedlings in petri-dish were based on the method described by Ichielvich-Auster (1985), using pistachio seeds and other host plants including radish, melon, watermelon, lentil, sugar-beet, eggplant, tomato, sunflower, tare and wheat. Isolates were grown on PDA and incubated at 25 ºC for 2-3 days, then a mycelial agar disc (6 mm diameter) was excised and inoculated centrally on agar (2% Merck) plate and incubated at 25 ºC for 24h. Three replicate plates were inoculated per each isolate. Five germinated pistachio seeds or other tested host plants were placed around the periphery of each colony 3–4 cm apart. The rootlets of the plants were examined after 7 days incubation at 25 ºC. Disease severity was evaluated based on the relative size of necrotic area on the roots using the scale of 0-5 (Ichielvich-Auster, 1985) with some modification as follow: 0 = no disease; 1 = 1-10%; 2 = 11-30%; 3 = 31-50%; 4 = 51-80%; and 5 = 100%.

Isolates causing a mean disease severity between 0 and 1 were considered non-pathogenic.

In vivo pathogenicity test was determined on six months and one-year old pistachio seedlings using the methods described by Yang and Verma (1992). Inoculum of *Rhizoctonia* isolates was prepared by growing each isolate in a 500 mL erlenmeyer flask containing 100g of barley grain and 100 mL of distilled water. Flasks were sterilized at 121 °C for 20 min and inoculated with three 7 mm- diameter mycelial disks of the isolates cut from the edges of 3-day-old *Rhizoctonia* sp. growing on PDA. Flasks were incubated at 25 °C for 10 days in the dark and shaken regularly to aid uniform colonization. Infested barley grain was air dried for 1 week and stored at 4 °C until use. Soil containing gravel was partially sterilized on two consecutive days at 121 °C for 30 min, and then infested with 2% (w/w) barley grain colonized with *Rhizoctonia*. Healthy, uniform seedlings were carefully transferred into 20g of *Rhizoctonia*-infested soil in 15 cm-diameter plastic pots. Soils inoculated with sterile barley grain served as negative controls. Pots were covered with black vinyl sheet, incubated at room temperature for 24 h to stimulate the growth of *Rhizoctonia* sp., and then transferred to the greenhouse. Disease severity was determined five weeks after inoculation based on rating disease symptoms on roots as described by Ruppel's scale (Ruppel *et al.*, 1979): (0) No visible lesions, plants apparently healthy, (1): < 1% of the root surface with visible *Rhizoctonia* lesions, (2): > 1–5% of the root surface with visible lesions, (3): > 5–10% of the root surface with dry-rot canker (4): > 10–25% of the root surface with dry-rot canker (5): > 25–50% of the root surface with dry-rot canker, (6): > 50–75% of the root surface with dry-rot canker (7): > 75% of the root surface with dry-rot canker (8): seedling dead with root rot.

Molecular characterization of rDNA-ITS

DNA Extraction

Each isolate was grown on 100 ml of liquid potato dextrose medium. Cultures were maintained at 25 ± 1 °C with shaking (150 rpm) for one week. Mycelia mats were harvested by filtration, washed three times with sterile distilled water and powdered with liquid nitrogen using a mortar and pestle, kept at -80 °C. Genomic DNA was extracted from mycelium using a modification of the cetytrimethylammonium bromide (CTAB) extraction procedure described by Murray and Thompson (1980). In brief, 450µl extraction buffer (0.7M NaCl (Merck, Germany), 50mM Tris-HCl (pH 8.0) (sigma, St. Louis, USA), 0.01M Na_2-EDTA (Merck), 1% (v/v) β-mercapto ethanol (Sigma) and 1% CTAB (Sigma)) were added to the pellet of mycelium. The mixture was briefly mixed and incubated at 65 ºC in a water bath for 30 min. Then an equal volume of chloroform: isoamylalcohol (24:1 (v/v)) was added to the sample. The mixture was emulsified using a vortex and subsequently centrifuged at 16,000 g for 15 min. The clear supernatant was transferred to a new tube and the nucleic acids were precipitated with isopropyl alcohol and centrifuged at 16,000 g for 5 min. The pellet was washed in 70% EtOH and re-centrifuged. Finally the pellet was dried at room temperature re-suspended in 50µl of sterilized milliQ water and stored at -20 ºC. DNA concentrations were determined using the Nanodrop (Thermo Scientific, USA).

Polymerase chain reaction and PCR-RFLP

PCR amplification of the ITS1-5.8S-ITS2 region of the rDNA was achieved with a GeneAmp PCR System C-1000 (BioRad, USA) thermocycler. The primer combinations were ITS1-F (5'-CTT-GGT-CAT-TTA-GAG-GAA-GTA-A-3') (Gardes and Bruns, 1993) and ITS4 (5'-TCC-TCC-GCT-TAT-TGA-TAT-GC-3') (White *et al.*, 1990). The PCR reaction mixture (25 µL) contained PCR Buffer (10 mM Tris-HCl, 50 mM KCl; pH 8.8) (Sinaclon, Iran), 2.5 mM $MgCl_2$ (Sinaclon), 0.2 mM of dNTP (Roche, Mannheim Germany), 0.2 µM of each primer, 1.5 units Ampli *Taq* polymerase (Sinaclon), and 5 µL of DNA template. The PCR profile was: an initial preheat at 94 °C (5 min), 40 cycles of denaturation at 94 °C (1 min), annealing at 45 °C (1min) and extension at 72 °C (1 min), followed by a final extension

at 72 C (10 min). Five µl of PCR product were analyzed by electrophoresis on 1.5% agarose gel followed by staining with ethidium bromide and photographing under a UV trans-illuminator. The obtained PCR products were also digested with two restriction endonuclease enzymes *Bstf5I* and *Tru9I* (MBI Fermentas, Lithuania) and the RFLP patterns were obtained (Falahati-Rastegar *et al.*, 2010; Kilicoglu and Ozkoc, 2010). Typical restriction enzyme reactions consisted of 8 µl of PCR product, 10 units of restriction enzyme, 2.5 µl of reaction buffer and MiliQ-water to a total volume of 20 µl. Reactions were incubated for 4 h at 65 °C according to manufacturer's recommendations.

Sequencing and phylogenetic analysis

PCR products were cleaned using the AxyPrep® PCR Clean-up Kit (Roche Molecular Biochemicals) according to the manufacturer's instructions. Direct sequencing of the PCR products was done by the DNA sequencing laboratory of MilleGen (Labege, France). The complete rDNA ITS regions were sequenced in both directions using the primers ITS1F and ITS4. Sequence data were analysed using Chromas 1.45 (copyright ©1996-1998, Conor McCarty) and verified manually. DNA sequence data obtained in this study has been deposited in GenBank. DNA sequences were compared to homologous sequences registered in GenBank using the standard nucleotide-nucleotide BLAST protocol (http://www.ncbi.nlm.nih.gov/BLAST/). In order to determine the extent and location of ITS sequence variation between different isolates, the sequences were aligned using ClustalX 1.81 (Thompson *et al.*, 1997), followed by minor manual editing to uniformly present the location of nucleotides in repeat sequences. For phylogenetic analysis, the alignment of sequence data was performed comprising complete ITS1, 5.8S and ITS2 sequences of representative isolate along with the sequences of the identified species obtained from Genebank, including *Ceratobasidium* sp. AG-F (DQ102440.1, DQ102439.1, DQ102436.1, DQ102437.1, DQ102438.1, DQ102441.1, DQ102435.1, JF705215.1, DQ102433.1, DQ102432.1, AF354085.1), *R. solani* AG-4 (LC017861.1, JQ669932.1), *Rhizoctonia* sp. AG-F (JF519832.1), *Rhizoctonia* sp. AG-Fb (FR734297.1) and *Rhizoctonia* sp. AG-G (KC825348.1). Single gaps were treated as missing data. *R. oryzae (*FJ766520.1, KT362135) was used as outgroup. Sequences were aligned with the help of MAFFT v7 (Katoh and Standley, 2013) under the default settings using the server available at https://toolkit.tuebingen.mpg.de/mafft (Alva *et al.*, 2016). The model of sequence evolution was optimized with the help of ModelTest using Mega 7.0 software (Kumar *et al.*, 2016). The model indicated as the best fit model was T92 + G. The clustering methods, Neighbor-joining (NJ) (Saitou and Nei, 1987) as well as Maximum Parsimony (MP) were used to estimate the phylogeny with the aid of Mega 7.0 software (Kumar *et al.*, 2016) and Maximum Likelihood (ML) distances. 1000 bootstrap replicates were performed.

Results

Sampling and morphological identification of isolates

A total of twelve infected pistachio samples were collected. Infected root and stems turned dark brown or black (Fig. 1A). The fresh shoots and leaves of the infected plants showed chlorosis, later turned necrotic. Because roots and stems suffered from severe rot, plants died a few days later due to disruption of water and nutrients translocation. Mycelia were often found on infected roots and stems near the soil surface. Disease was frequently observed on young (six months and one-year old) plants. Twelve isolates were obtained from infected root and stem rot including two isolates of *Fusarium* sp. and ten isolates showed typical *Rhizoctonia* like hyphal characteristics. The vegetative myceliums of *Rhizoctonia* isolates on PDA were in shades of white to light color when young and turned into production of aggregated mycelia at later age. Aerial mycelia were produced in all isolates after 7 days but sclerotia were neither produced on PDA at room temperature nor in the refrigerator temperature (Fig. 1B).

Nuclear condition and AG determinations

Rhizoctonia isolates recovered from infected roots and stems of pistachio seedlings had binucleate vegetative hyphal cells. The BNR isolates failed to anastomose with tester isolates of AG-A, AG-Ba, AG-G as well as multinucleate *Rhizoctonia* AG4 but anastomosed with AG-F. This anastomosis reaction was in C2 category.

***In vitro* and *in vivo* pathogenicity test**

The results of *in vitro* pathogenicity test showed that all isolates of *Rhizoctonia* sp. could infect germinated seeds of pistachio with disease severity of 100% as well as melon (100%), watermelon (90%), radish and lentil (90%), sugar-beet (80%), eggplant and tomato (60%), sunflower and tare (40%) and wheat (20%). High disease severity on germinated seeds was observed 7 days after inoculation (Fig. 1C). All isolates of *Rhizoctonia* that were originally isolated from pistachio caused disease symptoms after five weeks on six-month old seedlings. No symptoms were observed on one-year old seedlings. Disease symptoms began to appear about 30 to 34 days after inoculation. The first symptoms were chlorosis and necrosis on the upper part of the plants and later turned to defoliation. The infected plants had rotted and dried roots. All lateral roots were destroyed and the main root showed dry rot symptoms. Plants showing severe damage declined and finally mortality occurred. Based on Koch's postulates, the binucleate *Rhizoctonia* sp. was also re-isolated from inoculated tissue. No symptoms were observed on control treatment.

Hyphal growth rates and diameter determination

Hyphal growth rates of all isolates of the BNR were similar at all temperatures. All BNR isolates studied in this research could not grow at 5 °C but they were able to grow at 10 to 40 °C (Fig. 2). The optimum growth temperature for all isolates was 35 °C. The diameter of hyphal cell wall ranged from 3−5µm, which were thinner than those of *R. solani*.

Characterization of the rDNA ITS sequences and PCR-RFLP

PCR amplification of the rDNA ITS region of *Rhizoctonia* isolate using the ITS1F-ITS4 primer pairs was successful and gave PCR products of 718 base pair (bp) (results not shown). The complete genomic DNA sequences between the primers ITS1F-ITS4 was obtained for the BNR isolate VRU-R1. The rDNA ITS nucleotide sequences of the isolates showed high sequence homology (100% identity). No significant intra-specific variation was observed. The sequence of representative isolate was submitted to GenBank with accession number of KF841441. The GenBank-BLAST homology search using these ITS sequences revealed binucleate *Rhizoctonia* sp. AG-F as the most similar sequence (> 99% identity) with GenBank entries FR734294 and JF519832, which is a partial and complete sequence of ribosomal RNA genes derived from *Nicotiana tabacum* and apple root stock M9 respectively (Gurkanli and Ozkoc, 2011; Kelderer *et al.*, 2012).

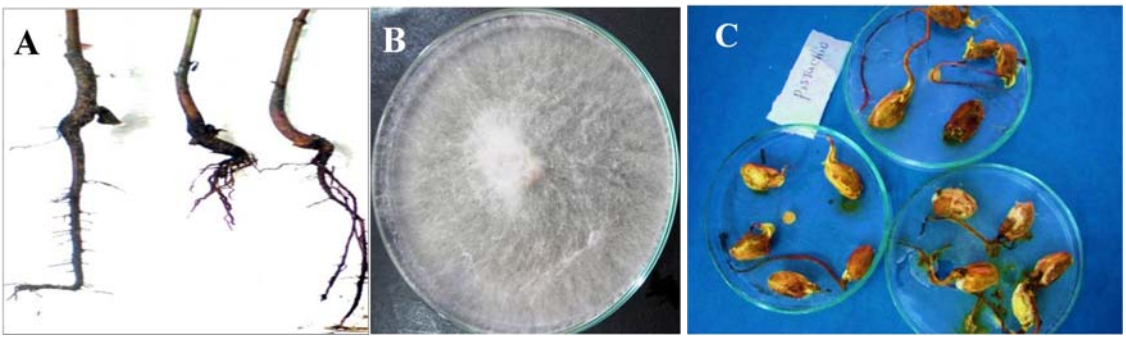

Figure 1 *Rhizoctonia* sp. AG-F. (A) Symptom of root and stem rot on pistachio seedlings, (B) Morphology of 7-day-old colony on PDA at 28 °C, (C) *in vitro* pathogenicity test on pistachio germinated seeds.

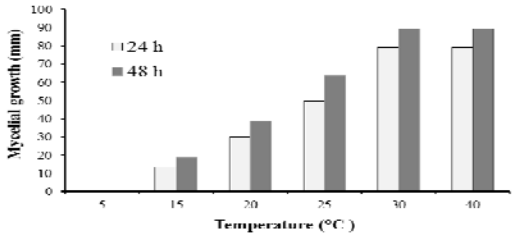

Figure 2 Mycelial growth of binucleate *Rhizoctonia* sp. AG-F on PDA at different temperatures after 24 and 48 h.

The length of the amplified fragments in our study was 717 bp, which consisted of 71 bp of the 3' end of 18S rDNA, 201 bp of ITS1 region, 162 bp of 5.8S rDNA, 254 bp of the ITS2 region, and 29 bp of the 5' end of the 28S rDNA (Fig. 3). The nucleotide composition of the ITS1-5.8S-ITS2 sequences averaged 18.38% C, 32.17% T, 28.69% A and 20.75% G. The rDNA-ITS region, including ITS1, 5.8S, and ITS2 for the isolates of binucleate *Rhizoctonia* sp. AG-F and the tester isolates of *R. solani* anastomosis group of AG-4, AG-3 and AG2-2 were amplified and digested using two discriminating restriction enzymes (*Bstf5I* and *Tru9I*) (Fig. 4, Table 1). Phylogenetic analyses aimed to determine the phylogenetic position and relationship of the obtained isolates among the identified species of *Rhizoctonia*. The topology of trees obtained from different analysis methods were the same (data not shown) and binucleate *Rhizoctonia* sp. AG-F was monophyletic group and well supported with bootstrap of 98% in all analysis (Fig. 5).

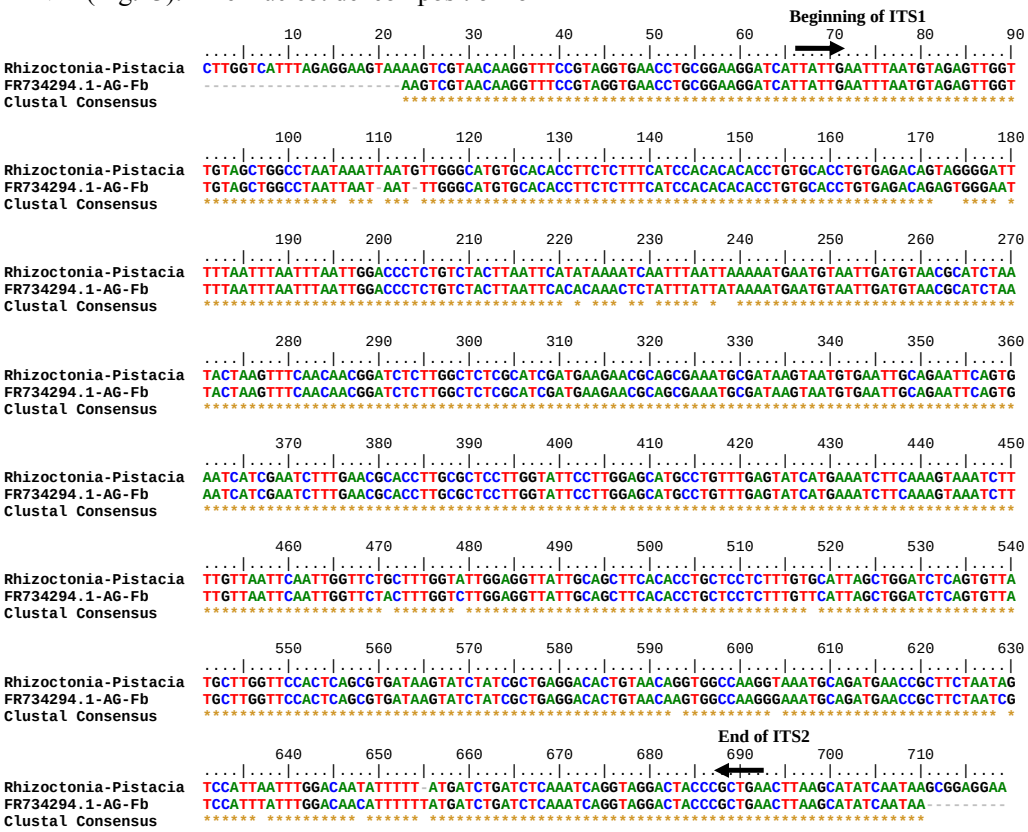

Figure 3 Alignment of the complete nucleotide sequence of the internal transcribed spacer (ITS1 and ITS2) region and the 5.8S subunit of the nuclear ribosomal RNA genes of *Rhizoctonia* sp. AG-F isolate VRU-R1 isolated from pistachio seedlings with accession number FR734294.1(Gurkanli and Ozkoc, 2011). The sequences are written 5′ to 3′. Identical nucleotides are indicated by dots. The ITS1 and ITS2 regions are marked with arrows.

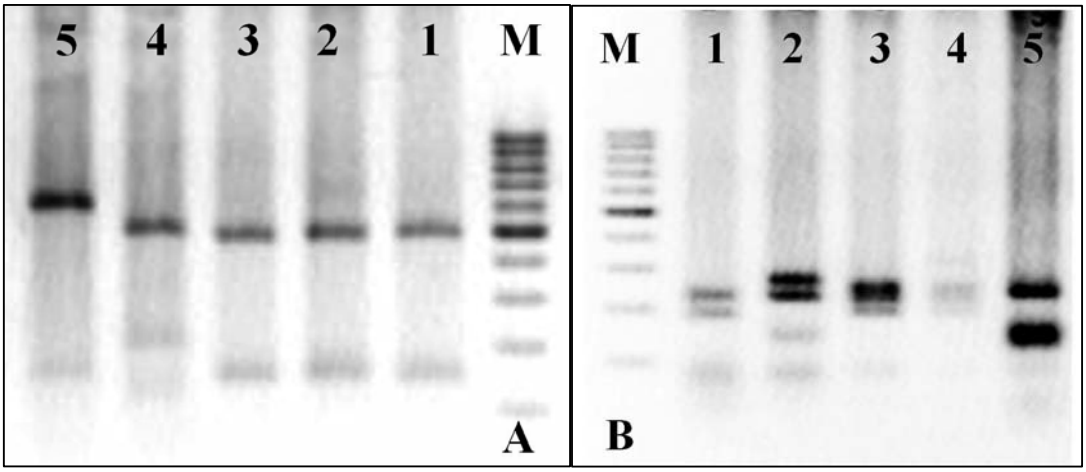

Figure 4 PCR-RFLP pattern of ITS1-5.8S-ITS2 amplified using ITS1F-ITS4 primer with two restriction endonuclease enzymes *Bstf5I* (A) and *Tru9I* (B) in Binucleate *Rhizoctonia* sp. AG-F compared to other Anastomosis Groups on 2% agarose gel. A: (M) 100 bp DNA Ladder, lanes 1-3: Potato, AG-3, lane 4: Pepper, AG-4, lane 5: Pistachio, AG-F. B: (M) 100 bp DNA Ladder, lane 1: Pistachio, AG-F, lane 2: Sugar beet, AG2-2, lane 3, 4: Potato, AG-3, lane5: Pepper, AG-4.

Table 1 Estimated length of digestion fragments of the amplified rDNA-ITS region of binucleate *Rhizoctonia* AG-F using primer set ITS1f-ITS4 in compare to other AGs.

Rhizoctonia isolates (AG)	Host	Fragment lengths (bp)	
		Tru9I	*Bstf5I*
AG-F	Pistachio	80, 190, 220	150, 600
AG-4	Pepper	100, 120, 220, 260	120, 210, 520
AG-3	Potato	80, 210, 220	150, 500
AG-2-2	Sugar beet	100, 150, 250, 290	-

Discussion

Soil borne plant pathogenic fungi that cause root and stems rots and yield reduction in most pistachio growing regions of the world as well as in Iran are *Rhizoctonia*, *Phytophtora*, *Pythium*, *Verticillium, Fusarium* and *Armillaria* spp. (Holtz *et al.*, 1996; Banihashemi, 1995). Isolates of *R. solani* AG-4 cause seed rot, post-emergence damping off and root rots of pistachio have already been detected and identified in the US (Holtz *et al.*, 1996) and Iran (Ashkan and Abusaidi, 1995; Ilkhan *et al.*, 2011) but to our knowledge according to available literatures there is no reported data for the BNR on Pistachio. In current study we obtained ten BNR isolates from symptomatic roots and stems rot on pistachio seedlings from nursery in Rafsanjan. The isolates were in morphology of light colony type having binucleate vegetative hyphal cells and were identified as AG-F based on the results of anastomosis tests that confirmed the C2 anastomosis reaction type as well as, by rDNA-ITS sequence analysis. Therefore this is the first report of binucleate *Rhizoctonia* sp. AG-F as inducer of stem and root rot of pistachio in Iran and the world. Mature cultures of AG-F collected from pistachio were similar in appearance to those of AG-F tester strains on PDA. Some conventional (anastomosis tests) and molecular analysis results from several studies (Martin, 2000; Sharon *et al.*, 2008) have suggesting that the BNR species do not form a

homogenous species and are mostly considered as non-pathogenic mycorrhizal fungi (Adams, 1988; Andersen and Rasmussen, 1996; Anderson, 1982; Burpee and Goulty, 1984) but in this study *in vitro* and *in vivo* pathogenicity tests of isolates on pistachio seedling showed that they are pathogenic. These data are supported by other researchers who indicated that isolates belonging to anastomosis group F can cause diseases on pea, radish, onion, lettuce, tomato, and cotton (Sneh *et al.*, 1998). In addition, although our results provide an evidence for homogeneity of species in this anastomosis group (AG-F) and which is supported by other researchers based on anastomosis (Eken and Demirci, 2004) or molecular (Fang *et al.*, 2013) studies, our results were not in accordance with data reported by Gurkanli and Ozkoc (2011) who have introduced the subsets of AG-Fa and AG-Fb from Turkey. Also, it still needs further studies using more AG-F samples from a wide variety of pistachios and different locations. The PCR amplification of the rDNA ITS region of binucleate *Rhizoctonia* sp. isolates using the ITS1F-ITS4 primer pairs gave 719 bp fragment as supported by (Gurkanli and Ozkoc, 2011). Identification and separation of binucleate *Rhizoctonia* sp. AG-F from other anastomosis groups including *R. solani* AG-4, AG-3 as well as AG-2-2 by PCR-RFLP analysis using two restriction endonucleases *Tru9I* and *Bstf5I* was successful (Fig. 3) and showed a different pattern from that of the others, in which was supported by other researchers (Cubeta *et al.*, 1991; El-Bakali and Martín Esteban, 2000; Falahati-Rastegar *et al.*, 2010; Mahmoudi *et al.*, 2005; Tajik-Ghanbari *et al.*, 2005) who used this technique to identify different anastomosis groups of *Rhizoctonia* species. Sequencing analysis of rDNA-ITS showed high similarity to reference data of anastomosis group F in Genbank. None of the tested isolates in our study could produce the teleomorphic stage under cold condition but further research in this issue is necessary. Pathogenicity test revealed that the isolates of AG-F cause stem and root rot only on six months old pistachio seedlings with severe damage on roots but not on one year-old seedlings. The evidence suggested that much attention should be paid during this stage in propagation of seedlings in nursery. *In vitro* pathogenicity tests on different crops revealed that the binucleate *Rhizoctonia* sp. AG-F could have a high potential to cause disease on many crops such as watermelon. This finding is supported by other researchers (Nischwitz *et al.*, 2013; Sharon *et al.*, 2007). Due to reports on pathogenicity of binucleate *Rhizoctonia* sp. AG-F on other hosts including watermelon, cucumber, soybean, sweet potato, pea and strawberry (Nischwitz *et al.*, 2013; Sharon *et al.*, 2007) also with regard to the optimum growth of pathogen at 35 °C and its disease severity, it is a potentially important pathogen in Southern Iran where different susceptible crops including onion, lettuce, tomato and cotton are grown in nursery and field condition and would require greater attention to provide better disease control strategy.

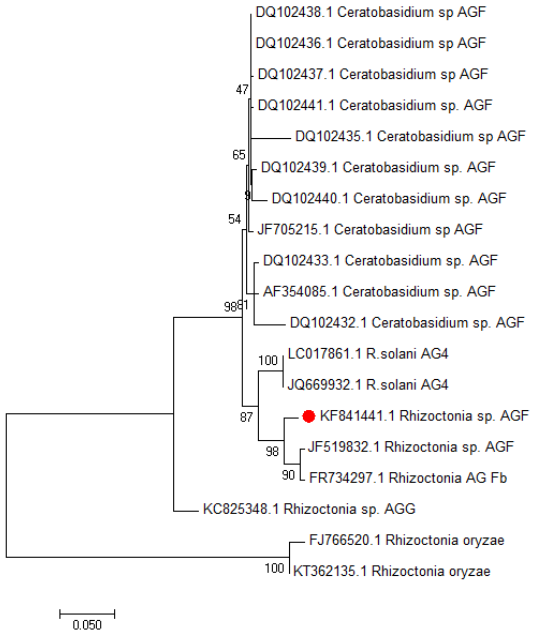

Figure 5 Phylogenetic tree of representative isolates of *Rhizoctonia* spp. AG-F, inferred by Maximum Likelihood analysis of ITS1, 5.8s and ITS2 sequences in MEGA 7.0. The bootstrap support from 1000 replication is indicated on the branches.

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گروه آناستوموزی جدید ریزوکتونیایی دوهسته‌ای AG-F عامل پوسیدگی ریشه و ساقه پسته

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چکیده: تعداد ده جدایه از قارچ‌های شبیه به *Rhizoctonia* از ریشه و ساقه‌های آلوده پسته (*Pistacia vera*) با علائم زردی و نکروز از خزانه‌های تجاری شهرستان رفسنجان، ایران، در پاییز ۱۳۹۰ جداسازی شد. گیاهچه‌های آلوده علائم زردی و نکروزه داشتند. براساس خصوصیات هیف و تعداد هسته، تمام جدایه‌ها به‌عنوان ریزوکتونیای دوهسته‌ای شناسایی شدند. جدایه‌های نماینده برای تعیین گروه آناستوموزی، دمای بهینه رشد، و ویژگی نواحی ITS-rDNA و بیماریزایی آنها روی پسته در شرایط گلخانه و آزمایشگاهی مورد بررسی قرار گرفتند. بررسی پیوند هیفی با جدایه‌های شاخص از گروه‌های آناستوموزی دوهسته‌ای AG-A، AG-Ba، AG-G و AG-F و هم‌چنین جدایه‌های چندهسته‌ای AG-4 که قبلاً از روی پسته جداسازی شده بودند انجام شد. دمای بهینه رشد برای تمام ریزوکتونیاهای دوهسته‌ای ۳۵ درجه سلسیوس بود. در شرایط گلخانه، علائم پوسیدگی ریشه ۳۰ روز پس از آلودگی مشاهده شد و مرگ کامل گیاه دو هفته بعد صورت گرفت. طبق داده‌های مولکولی و تعیین گروه‌های آناستوموزی نتایج نشان داد که تمام جدایه‌ها بیش‌ترین شباهت را به گروه آناستوموزی F دارند. این اولین گزارش گروه آناستوموزی F (AG-F) از ریزوکتونیاهای دوهسته‌ای، عامل پوسیدگی ریشه و ساقه پسته در ایران و جهان است.

واژگان کلیدی: نهال پسته، *Rhizoctonia* sp.، واکنش زنجیره‌ای پلیمراز، استخراج دی.ان.ای، AG-F