Mycologia Iranica 1(2): 99 – 106, 2014 Original Article

New anastomosis Group AG-G of binucleate *Rhizoctonia* sp., the causal agent of root rot disease on miniature roses in Iran

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Abstract: *Rhizoctonia*-like fungi were isolated from the infected roots of miniature rose (*Rosa hybrida* cv. Linda) plant with chlorosis and necrosis symptoms, grown in commercial glasshouse in Rafsanjan, Iran, during the autumn of 2011. All of the isolates were identified as binucleate *Rhizoctonia* sp. on the basis of hyphal characteristics and nuclei number. They were tested for detection of the anastomosis group, optimum growth temperature, rDNA-ITS region traits and pathogenicity on miniature rose *in vivo* and *in vitro*. The analysis of hyphal reaction anastomosis was carried out with the tester isolates of binucleate *Rhizoctonia* AG-A, AG-Ba, AG-G as well as multinucleate *Rhizoctonia* AG2-2IIB and AG4-HGI already detected on miniature rose. The optimum temperature for growth of binucleate *Rhizoctonia* sp. was 35˚C. In *in vivo* test, the symptoms of root rot were observed 35 days after inoculation and mortality happened two weeks later. According to molecular and anastomosis test groups, our results showed that all the isolates have the maximum similarity to AG-G. This is the first report of anastomosis group G (AG-G) of binucleate *Rhizoctonia* sp., the causal agent of root rot disease on miniature roses in Iran.

Key words: miniature rose, pathogenicity, *Rosa hybrida* cv. Linda, ITS-rDNA.

INTRODUTION

The production of ornamental plants is a thriving industry. Rose including the old garden roses (*Rosa gallica* and *Rosa damascena*) and modern roses (miniature roses and hybrid tea roses) are the most important ornamental flowers in the world (Vetricka 1997, Peter Bealis 1990). Miniature roses (*Rosa hybrida* cv. Linda) have become increasingly popular and economically important in the recent years worldwide, including Iran. Similar to the other intensively grown floricultural crops, rose is susceptible to a number of soil-borne phytopathogenic fungi such as *Phytophthora*, *Pythium*, *Fusarium* and *Rhizoctonia*, which cause the plants death (Van der Plaats-Niterink 1981, Hyakumachi et al. 2005). The binucleate *Rhizoctonia* spp. represent diverse groups of saprophytic, pathogenic and mycorrhizal fungi (Adams 1988, Anderson & Rasmussen 1996, Anderson 1982, Honeycutt & Benson 2001, Ogoshi 1987). They cause several diseases, including damping-off, root rot, stem rot, sheath blight, fruit decay and foliar blight on a wide range of important agriculture plants (Parmeter & Whitney 1970, Tanaka et al. 1994). Based on the hyphal anastomosis, binucleate *Rhizoctonia* spp. have been divided into 19 anastomosis groups (AGs), designated as AG-A to AG-S (Ogoshi et al. 1983a, Ogoshi et al. 1983b, Ogoshi et al. 1979, Sneh et al. 1991). The tester strains AG-J and AG-M have been excluded from binucleate *Rhizoctonia* spp., because of having clamp connections. 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. 1991). Differentiation of these three subgroups of AG-B is supported by polymorphism in the ribosomal rRNA gene (Cubeta et al. 1991). Also, AG-D has been subdivided into two subgroups AG-D (I) and AG-D (II), based on the cultural morphology and pathogenicity (Tanaka et al. 1994). The binucleate *Rhizoctonia* sp. AG-G has already been isolated from miniature rose in Japan (Hyamukuchi et al. 2005). The binucleate *Rhizoctonia* spp. and *R. solani* (AG-2-2 IIIB and AG-4 HG-I) were associated with root and stem rot of miniature roses (Priyatmojo et al. 2001). On the other hand, the binucleate-like *Rhizoctonia* spp. (AG-G and AG-T) were isolated from root and stem of cut-flower roses (*Rosa* spp.) (Hyakumachi et al. 2005). These isolates were grouped into two colony morphology types, light brown to brown colony and whitish colony types which belonged to AG-G and AG-T, respectively. The isolates belonging to AG-T did not anastomose with any tester strains of binucleate *Rhizoctonia.* Furthermore, none of the isolates of AG-G and AG-T anastomosed with the tester strains of the previously reported AG-U group collected from miniature roses,

Submitted 2 May 2014, accepted for Publication 25 Nov. 2014
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leaving the identity of this fungal pathogen in doubt, and suggesting the need for employing molecular techniques for precise identification of the causal agent (Hyakumachi et al. 2005).

Although *Rhizoctonia* spp. have been reported as a major plant-pathogenic fungus causing a severe economic damage to many species of the ornamental plants, and the binucleate *Rhizoctonia* spp. have been found on miniature rose, to our knowledge, the anastomosis group G has not been reported on miniature rose from Iran so far. The objective of this study was to identify the isolates of a binucleate-like *Rhizoctonia* sp. associated with root and stem rot of miniature roses and its virulence on them *in vitro* and *in vivo*.

MATERIALS AND METHODS

Sampling, fungal isolates and culture maintenance

The infected rose seedlings samples with chlorotic leaves and/or necrotic lesions and dry brown root rot symptoms were collected from the landscapes and glasshouses in Rafsanjan, Iran during the autumn of 2011. Symptomatic roots were washed thoroughly in the running tap water for 30 min to remove the adhered soil particles, air dried, and 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 papers and placed on Petri dishes containing acidified water agar (AWA, pH 4.5) using 10% lactic acid. The cultures were incubated for 2 to 3 days at 25°C in the dark, and then examined microscopically. Fungal colonies with characteristics typical of *Rhizoctonia* like fungi were purified by hyphal tip method 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 sclerotia

To evaluate the cultural appearance of the isolates, a single PDA disk containing mycelium (7-mm diameter) from 2 to 3-day-old cultures of *Rhizoctonia* sp. was placed in the center of 9 cm Petri dishes containing PDA, and incubated at 25°C in the dark or in the refrigerator at 10°C. The cultures were evaluated after 25 days of incubation. The production of sclerotia for each isolate was investigated according to the method used by Oniki et al. (1985). Briefly, fresh cultures of the isolates were grown on the modified Potato Yeast Extract Agar (PYEA) acidified to pH 4.5, using 10% lactic acid. The cultures were incubated at 27°C, and when the hyphae reached the margin of the Petri dish, they were covered to the rim with the air dried soil aggregates. They were then incubated at room temperature with the Petri dish lids removed. Humidity was maintained by watering the soil 1-3 times daily, while the excess moisture was drained. Production of hymenia on the surface of soil was expected within 12-14 days afterwards.

Nuclear conditions and AG determination

The number of nuclei per hyphal cell was determined using the procedure described by Bandoni (1979). A single PDA disk containing mycelium (7-mm diameter) from 2 to 3-day-old cultures of *Rhizoctonia* sp. was placed on a clean sterile microscopic glass slide and incubated for 1-2 days in a moist chamber at 25°C in the dark. Nuclei were stained using a drop of Safranin O and 3% KOH (1:1). Twenty five cells of each isolate were examined at 400× magnification using the bright field microscopy.

To determine the anastomosis group of the isolates, the glass-slide technique was used as described by Kronland & Stanghellini (1988). A single PDA disk containing mycelium (7mm diameter) was cut from the edge of a 2 to 3-day-old culture of *Rhizoctonia* sp. and placed on a clean glass slide. The tester isolates of binucleate *Rhizoctonia* AG-A (C-134), AG-Ba (C-484), AG-G (C-463) and multinucleate *Rhizoctonia* AG2-2IIB and AG4-HGI were placed 3 to 4 cm away from each tested isolate. Slides were put in a moist chamber and incubated at 25°C for 24 to 48 h in the dark. The excess moisture was wiped from the bottom of the slide. Hyphae from the two disks were stained at the meeting zone using safranin O and 3% KOH (1:1), and examined microscopically to determine the anastomosis reaction (Carling 1996, Kronland & Stanghellini 1988).

Hyphal growth rates and diameter determination

A single PDA medium disk containing mycelium (7 mm diameter) from 2 to 3-day-old cultures of *Rhizoctonia* sp. was placed on a clean, sterile, microscopic glass slide covered by 2% WA, and incubated for 1-2 days in a moist chamber at 25 °C in the dark. After 24-48 hours of incubation, the hyphae were stained with Fushin acid and laid under the optical microscope with × 400 magnification. In order to measure the hyphae diameter, at least 50 hyphae were examined, and the average was considered as the hyphal diameter (Sneh et al. 1991).

Radial growth rates of the isolates were determined at 5, 15, 20, 25, 30 and 40°C. A single agar disk (7 mm diameter) containing mycelium was transferred from the margin of a 2 to 3-day-old colony to the center of a 9 cm Petri dish of PDA. Measurements were performed 12h afterwards to allow the diffusion of temperature 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 the experiment was repeated twice.

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

In vitro pathogenicity tests of the isolates were performed on detached miniature rose branches

according to the method described by Afek et al. (1990), with some modifications. Two-year-old dormant shoots, 3 cm in diameter and 25 cm in length were collected. A 5 mm bark disk removed from the detached shoot by a cork borer was replaced by a 5-mm mycelial plug of a 4-day-old binucleate *Rhizoctonia* sp. culture on PDA. Five replicates of each isolate were used. Controls were represented by shoots inoculated with a sterile PDA plug (three replicates). The exposed surface of the wounding site was wrapped by Parafilm (Pechiney, Japan). After inoculation, the shoots were incubated in a chamber for one week at 25°C and relative humidity of >90%, and the length of the bark necrosis was measured on each shoot.

In vivo pathogenicity test was carried out on 2-year-old miniature roses applying the methods described by Yang & Verma (1992). The inoculum was prepared by growing each *Rhizoctonia* isolate in a 500 mL Erlenmeyer flask containing 100 g of barley grain and 100 mL of distilled water. The flasks were sterilized at 121°C for 20 min, inoculated with three 7-mm diameter mycelial disks of the isolates cut from the edges of 3-day-old *Rhizoctonia* sp. cultures on PDA, incubated at 25°C for 10 days in the dark and shaken regularly to aid the uniform colonization. The infested barley grain was air dried for 1 week and stored at 4°C until the usage time. On the other hand, the gravel containing soil was partially sterilized for two consecutive days at 121°C for 30 min, and then infested with 2% (w/w) barley grain colonized with *Rhizoctonia*. The healthy uniform seedlings were carefully transferred into 1000g of *Rhizoctonia*-infested soil in 15 cm-diameter plastic pots. The soils inoculated with sterile barley grain served as negative controls. The pots were covered with black vinyl sheets, incubated at room temperature for 24 h to stimulate the growth of *Rhizoctonia* sp., and then transferred to the greenhouse. Disease severity was determined 2-4 weeks after inoculation.

Genotype analysis

Mycelium extraction was carried out by filtering the mycelial mats of the 1-week-old cultures on liquid potato dextrose medium kept at 25±1°C with shaking (150 rpm), washing three times with sterile distilled water and powdering with liquid nitrogen using a mortar and pestle. The extracted mycelium was kept at -80°C. Genomic DNA was extracted using a modified procedure of Cetyl Trimethylammonium Bromide (CTAB), described by Alaei *et al.*, (2009). Briefly, the extracted mycelium was subjected to 450 µl of an extraction buffer containing 0.7 M NaCl (Merck, Germany), 50 mM Tris-HCl (pH 8.0) (Sigma, St. Louis, USA), 0.01 M Na_2-EDTA (Merck), 1% (v/v) β-mercaptoethanol (Sigma) and 1% CTAB (Sigma). The mixture was briefly vortexed and incubated at 65°C in a water bath for 30 min. Then, an equal volume of chloroform: isoamyl alcohol (24:1 (v/v)) was added to the sample. The mixture was vortexed and subsequently centrifuged at 16,000 g for 15 min. The clear supernatant was transferred to a new tube, precipitated with isopropyl alcohol and centrifuged at 16,000 g for 5 min. The pellet was washed in 70% EtOH, re-centrifuged, dried at room temperature, re-suspended in 50µl of sterilized milliQ water and stored at -20°C. DNA concentrations were determined using Nanodrop (Thermo Scientific, USA). PCR amplification of the rDNA ITS1-5.8S-ITS2 region was carried out in a GeneAmp PCR System C-1000 (Bio Rad, USA) thermocycler, using the primer pair ITS1-F (5'-CTT-GGT-CAT-TTA-GAG-GAA-GTA-A-3') (Gardes & Bruns, 1993) and ITS4 (5'-TCC-TCC-GCT-TAT-TGA-TAT-GC-3') (White et al. 1990). The PCR reaction mixture (25µL) contained PCR Buffer (10 mM Tris-HCl, 50 mM KCl; pH 8.8) (Sinaclon, Iran), 2.5mM $MgCl_2$ (Sinaclon), 0.2 mM of dNTP (Roche, Mannheim Germany), 0.2 µM of each primer, 1.5 units of Ampli*Taq* polymerase (Sinaclon) and 5µL of DNA. Amplification was performed using the following conditions: an initial denaturation at 94°C for 5 min, followed by 40 cycles of denaturation at 94°C for 1 min, 1 min of annealing at 45°C, 1 min of extension at 72°C and a final extension at 72°C for 10 min. Five µl of PCR product was used for electrophoresis on 1.5% agarose gel.

PCR products were purified using the AxyPrep® PCR Clean-up Kit (Roche Molecular Biochemicals) and sequenced in both directions by the DNA sequencing laboratory of MilleGen (Labege, France).

Sequence data were analyzed using Chromas 1.45 (copyright ©1996-1998, Conor McCarty), verified manually, aligned using ClustalX 1.81 (Thompson et al. 1997), edited manually and subjected to similarity search against NCBI GenBank (http://www.ncbi.nlm.nih.gov/BLAST/) DNA sequence data obtained in this study have been deposited in GenBank.

RESULTS

Sampling and morphological identification of the isolates

A total number of five infected miniature rose samples were collected. Twelve isolates were obtained from the infected roots and stems, including seven isolates of *Fusarium* spp. and five isolates of typical *Rhizoctonia*-like hyphal criteria. The vegetative mycelia of *Rhizoctonia* isolates on PDA had a shade of white to a light color when young and turned to light brown when aged. The aerial mycelia were produced in all of the isolates after 7 days, but sclerotia were not produced neither on PDA at room temperature nor in the refrigerator temperature. All the cultures produced alternating brown and light concentric rings in the dark (Fig. 1b).

Nuclear condition and AG determinations

Rhizoctonia isolates recovered from the infected roots and stems of miniature rose had binucleate vegetative hyphal cells (Fig.1d). The binucleate

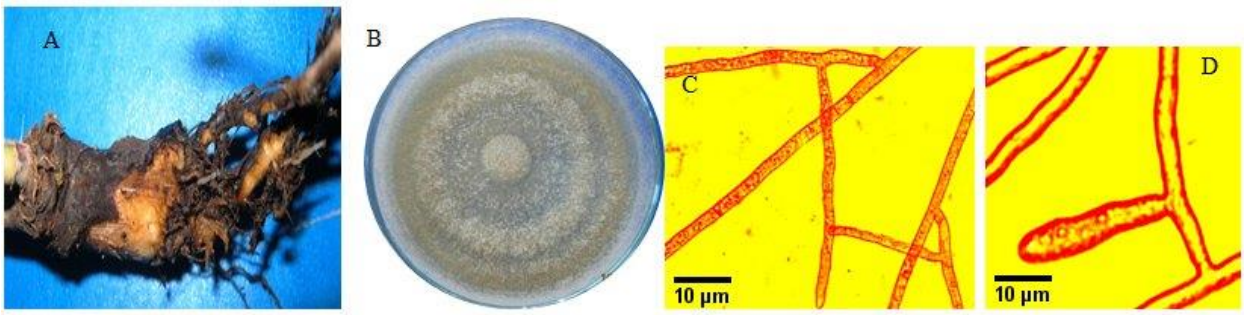

Fig. 1. *Rhizoctonia* sp. AG-G. **(A)** Symptom of root and stems rot on rose seedling, **(B)** Morphology of colony on PDA after 25 days at 25˚C, **(C)** Hyphal reaction anastomosis between the isolates and the tester, **(D)** Binucleate cell.

Rhizoctonia isolates failed to anastomose either with the binucleate tester isolates AG-A and AG-Ba or with the multinucleate isolates AG2-2IIB and AG4-HGI, but they had the anastomosis reaction of C3 with the AG-G tester. (Fig. 1C).

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

The results of *in vitro* pathogenicity test showed that all the isolates of *Rhoizoctonia* sp. could infect the detached branches of miniature rose (Fig. 2b). The fungus penetrated the tissue and caused rotting inside the branches. The infected branches color turned to brown to black after one week. The *Rhizoctonia* sp. was re-isolated from infected tissues.

All the isolates of *Rhizoctonia* originally obtained from rose caused the disease symptoms after five weeks. In the other words, the symptoms began to appear about 30 to 34 days after inoculation. The first symptoms included chlorosis and necrosis on the upper part of the plants which later caused defoliation and dry root rot. All the lateral roots were destroyed and the main root showed the dry rot symptoms (Fig. 2a). Mortality occurred as roots were not formed and plants declined. *Rhizoctonia* sp. was re-isolated from the inoculated tissues in order to perform Koch's postulation. No symptoms of disease were observed on the control treatments.

Hyphal growth rates and diameter determination

Hyphal growth rates of all the binucleate isolates of *Rhizoctonia* were similar at different temperature. All of the isolates grew at a temperature range of 10 to 40˚C, but none of them could grow at 5˚C. Their optimum growth temperature was 35˚C. The diameter of hyphal cells varied from 3 to 5 µm, which is less than the diameter reported for *R. solani*.

Amplification and characterization of the rDNA ITS sequences

PCR amplification of the rDNA ITS region of *Rhizoctonia* isolates using the ITS1F-ITS4 primer pair gave the PCR products of 695 bp. (Fig. 3), containing 72 bp of the 3' end of 18S rDNA, 177 bp of ITS1, 162 bp of 5.8S rDNA, 254 bp of ITS2 and 30 bp of the 5' end of 28S rDNA (Fig. 4). The complete genomic DNA sequence of the amplified region between the primers ITS1Fand ITS4 was obtained for the binuclate *Rhizoctonia* isolate VRU-R3. The rDNA ITS nucleotide sequences of the isolates showed high sequence homology (100% identity) and no significant intraspecific variation was observed. The sequence is available in GeneBank with Accession No. KC825348.1.

Fig. 2. Pathogenicity tests of *Rhizoctonia* sp. AG-G. on rose seedlings, **(a)** *in vivo* test, **(b)** *in vitro* test, **A:** control **B:** inoculated by *Rhizoctonia* sp. AG-G.

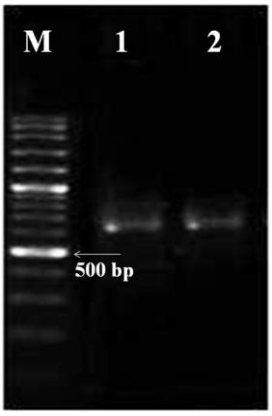

Fig. 3: Typical amplification products of genomic DNA of *Rhizoctonia* sp. AG-G isolate VRU-R3 in conventional PCR with the primer pair ITS1F/ITS4. M=100-bp DNA ladder (Invitrogen). Lanes 1 and 2 results from PCR reaction with pure genomic DNA.

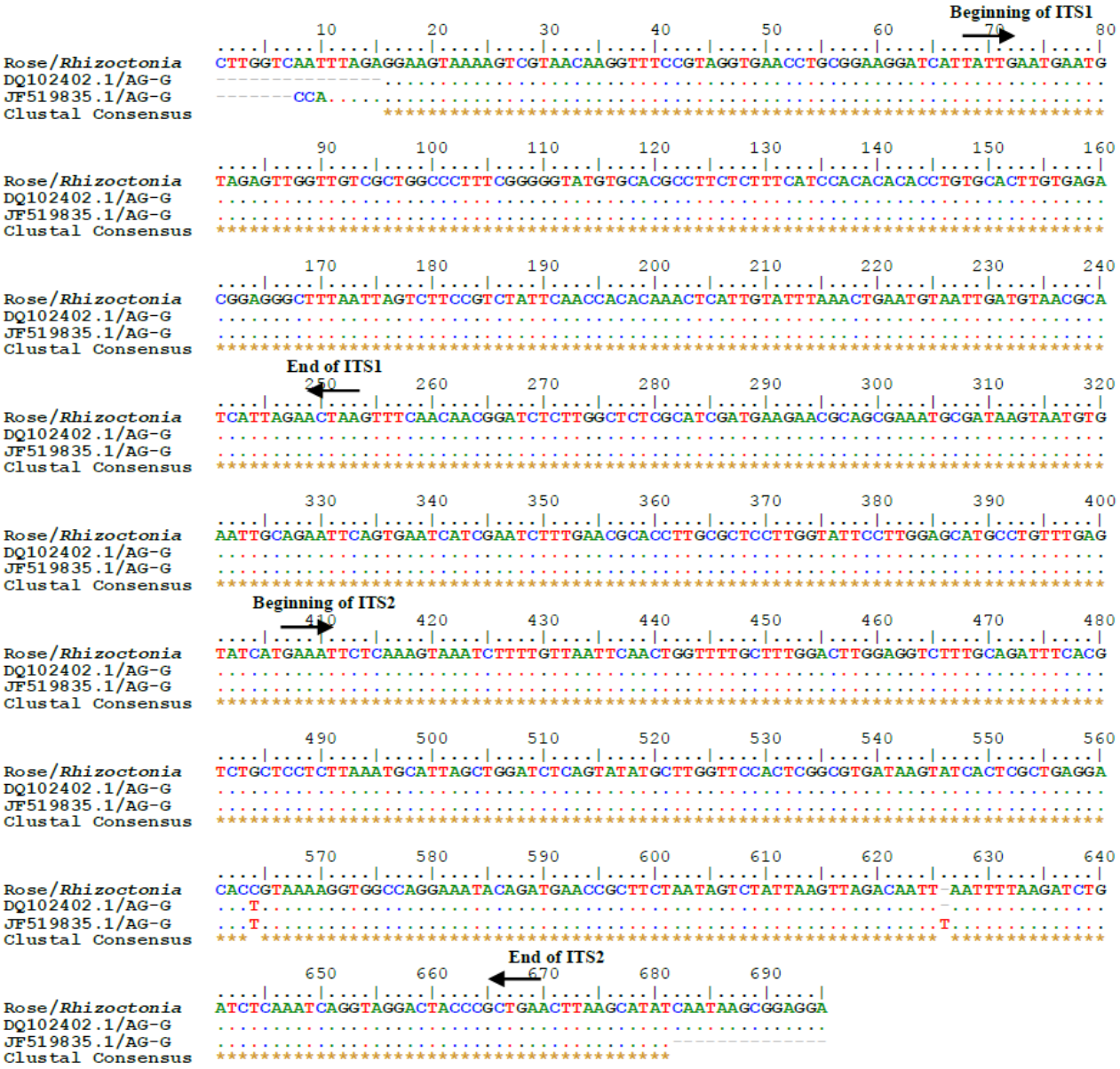

Fig. 4. Alignment of the complete nucleotide sequence of the internal transcribed spacer (ITS1 and ITS2) region and the 5.8S subunit of the nuclear ribosomal RNA genes of *Rhizoctonia* sp. AG-G isolate VRU-R3 isolated from miniature rose. The sequences are written 5′ to 3′. Identical nucleotides are indicated by dots. The ITS1 and ITS2 regions are marked with arrows. The sequence was conserved in ITS1 but differed in ITS2 at position 564 (T instead of C) and at position 626 (addition of T).

The BLAST similarity search revealed the binucleate *Rhizoctonia* sp. AG-G as the most similar sequence (>99% identity) with GenBank entries DQ10402.1 and JF519835.1, which are partial and complete sequences of ribosomal RNA regions of strawberry (*Fragaria* x *ananassa*) and apple rootstock M9, respectively (Sharon et al. 2007). The average nucleotide composition of the ITS1-5.8S-ITS2 sequences were as follows: 19.88% C, 30.40% T, 27.81% A and 21.90% G.

DISCUSSION

Binucleate-like *Rhizoctonia* sp. isolates were isolated predominantly from symptomatic roots and stems of cut-flower roses in Rafsanjan, Iran. They all had the light brown to brown colonies. Mature cultures of AG-G collected from miniature roses were similar to the AG-G tester strains on PDA. This similarity was confirmed by the C3 anastomosis reaction between AG-G collected from miniature roses and the AG-G tester strains. These isolates belonged to the anastomosis group G. Isolates of AG-G infect sugar beet, bean, melon, peanut, sunflower and strawberry (Martin 2000, Martin 1988, Oniki & Araki 1982, Sneh et al. 1991). The occurrence of root and stem rot of miniature roses caused by AG-G has been reported previously from Japan (Hyakumachi et al. 2005). In addition to AG-G, the binucleate *Rhizoctonia* AG-T and AG-U as well as *R. solani* AG-2-2 IIIB and AG-4 HG-I isolates have been also associated with root and stem rot in miniature roses (Priyatmojo et al. 2001; Hyakumachi et al. 2005). This study is the first report of AG-G on miniature rose in Iran. Diversity in the composition of *Rhizoctonia* populations on miniature rose can be influenced by growth stage of plants, plant organs, sampling season and geographical location of crop production areas. The infected rose seedling with these AGs was introduced from the certain nursery sites to the other areas with different climatic conditions for rose production. In addition to the binucleate *Rhizoctonia* sp., *Fusarium* spp. were also isolated from miniature rose. Symptoms on rose roots caused by *Rhizoctonia* and *Fusarium* are similar. However, infection by the binucleate *Rhizoctonia* sp. is commonly characterized by a darker reddish brown necrosis. In this study, only *Rhizoctonia* sp. was isolated from miniature rose and no isolates of *Fusarium* spp. were isolated from the infected stems (Hyakumachi et al. 2005). Actually, the binucleate *Rhizoctonia* sp. was consistently isolated from the rotting stems. Pathogenicity tests revealed that all the three isolates of AG-G were pathogenic on roots and stems of miniature roses. None of the isolates tested in our study could be induced to produce the teleomorph stage under the mentioned conditions. The length of the rDNA-ITS region amplified by ITS1F -ITS4 primers was about 680-700 bp as reported by Toda et al. (1999). Sequencing analysis showed 99% similarity to the anastomosis group G reference isolate and AG-G isolates on the other hosts. The results showed that the optimum growth of all the isolates of binucleate *Rhizoctonia* occurred at the temperature of 30−40˚C. Regarding to the warm climatic conditions in the southern Iran, especially Kerman, Fars and Isfahan provinces, this fungus can be an important pathogen on some of the other crops including strawberry, sunflower, melon and sugar beet that could be grown in different parts of this area.

ACKNOWLEDGEMENTS

The authors wish to thank N. Kondo (Faculty of Agriculture, Hokkaido University Japan) and Parisa Taheri (Faculty of Agriculture, Mashhad University, Iran) who supplied the tester strain of *Rhizoctonia* spp.

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گروه آناستوموزی جدید AG-G برای *Rhizoctonia* sp. دو هسته‌ای، عامل پوسیدگی ریشه رز مینیاتوری در ایران

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چکیده: قارچ‌های شبیه به *Rhizoctonia* از ریشه های آلوده گیاهان رز مینیاتوری (*Rosa hybrida* cv. Linda) با علائم زردی و نکروز از گلخانه‌های شهرستان رفسنجان، ایران، در پاییز ۱۳۹۰ جداسازی شد. بر اساس خصوصیات هیف و تعداد هسته، تمام جدایه‌ها به عنوان ریزوکتونیای دو هسته ای شناسایی شدند. این جدایه ها برای تعیین گروه آناستوموزی، دمای بهینه رشد، و ویژگی نواحی ITS-rDNA و بیماریزایی آنها روی رز مینیاتوری در شرایط گلخانه و آزمایشگاهی مورد بررسی قرار گرفتند. بررسی پیوند هیفی با جدایه های شاخص از گروه‌های آناستوموزی دو هسته‌ای AG-A، AG-Ba و AG-G و همچنین جدایه‌های چند هسته‌ای AG-4HGI و AG2-2IIIB که قبلا از روی رز مینیاتوری جداسازی شده بودند انجام شد. دمای بهینه رشد برای تمام ریزوکتونیا های دو هسته ای ۳۵ درجه سلسیوس بود. در شرایط گلخانه، علائم پوسیدگی ریشه ۳۵ 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 G دارند. این اولین گزارش گروه آناستوموزی G (AG-G) از ریزوکتونیایی دو هسته‌ای، عامل پوسیدگی ریشه رز مینیاتوری در ایران است.

کلمات کلیدی: رز مینیاتوری، بیماریزایی، *Rosa hybrida* cv. Linda، ITS-rDNA.

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