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Molecular characterization of *Rhizoctonia solani* AG4 using PCR-RFLP of the rDNA-ITS region

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Abstract: *Rhizoctonia solani* isolates obtained from common beans (*Phaseolus vulgaris* L.) were included in an AG4 anastomosis group in accordance with hyphal anastomosis. In the subgrouping of AG4 isolates, PCR-RFLP patterns in the rDNA-ITS were used. After obtaining the genomic DNA belonging to *R. solani* AG4, an approximately 700 bp amplification product of the ITS1-5.8S-ITS2 region was obtained with PCR, using ITS1 and ITS4 universal primers. The PCR products were digested with *Mse*I, *Hinc*II, *Ava*II, and *MfeI* restriction endonucleases, and different PCR-RFLP patterns were obtained for the AG4 HGI and AG4 HGII subgroups. In this study, in addition to the enzymes that were previously used in the AG4 subgrouping, *Ava*II restriction endonuclease was also seen to be effective in the subgrouping. In this way, the isolates that were grouped as *R. solani* AG4 according to anastomosis reactions were separated into 2 subgroups, HGI and HGII. This study indicates that the common beans in the Black Sea coastal region are more commonly infected by *Rhizoctonia solani* AG4 HGI.

Key words: *Rhizoctonia solani,* rDNA-ITS, PCR-RFLP

Rhizoctonia solani AG4 grubu fungusların rDNA-ITS bölgesinin PZR-RFLP ile moleküler karakterizasyonu

Özet: Bu çalışmada, fasulye (*Phaseolus vulgaris* L.) bitkisinden elde edilen *R. solani* izolatları hifal anastomoz reaksiyonlarına göre AG4 anastomoz grubuna dahil edildi. AG4 izolatlarının altgruplarına ayrımında ise rDNA-ITS bölgesinin PZR-RFLP şablonlarından yararlanıldı. *R. solani* AG4 izolatlarına ait genomik DNA elde edildikten sonra, polimeraz zincir reaksiyonuyla (PZR) ITS-1 ve ITS-4 evrensel primerleri kullanılarak ITS1-5.8S-ITS2 bölgesinin yaklaşık 700 bp'lik amplifikasyon ürünü elde edildi. PZR ürünü *Mse*I, *Hinc*II, *Ava*II ve *MfeI* restriksiyon endonükleazları ile reaksiyona tabi tutuldu. Bu enzimatik kesimler sonucunda AG4 HGI ve AG4 HGII gruplarında farklı PZR-RFLP şablonları elde edildi. Bu çalışmada daha önce AG4'ün alt grup ayrımında kullanılan enzimler haricinde *Ava*II restriksiyon endonükleazının da grup ayrımında etkili olduğu belirledi. Böylece anastomoz tiplendirmesiyle *Rhizoctonia solani* AG4 olarak gruplandırılan izolatların HGI ve HGII olarak alt gruplarına ayrılması sağlanmış oldu. Bu çalışma Karadeniz Bölgesi sahil şeridinde fasulye bitkisinin daha yaygın olarak *Rhizoctonia solani* AG4 HGI tarafından enfekte edildiğini göstermektedir.

Anahtar sözcükler: *Rhizoctonia solani*, rDNA-ITS, PZR-RFLP

Introduction

Rhizoctonia solani Kühn (teleomorph: *Thanatephorus cucumeris* (Frank) Donk) is a soil-borne fungal pathogen with a wide host range. It exists in nature as different groups in terms of the number of nuclei in its cells, its cultural features, hosts, and virulence (1). There are 3 main groups of the *Rhizoctonia* form genus in the anamorphic classification: multinucleate *Rhizoctonia* (teleomorphs: *Thanatephorus* and *Waitea*), binucleate *Rhizoctonia* (teleomorphs: *Ceratobasidium* and *Tulasnella*), and uninucleate *Rhizoctonia* (teleomorph: *Ceratobasidium)* (1-2).

Rhizoctonia solani is one of the most common types and belongs to the multinucleate *Rhizoctonia* group. (3). Today *Rhizoctonia solani* isolates are divided into anastomosis groups on the basis of hyphal anastomosis (according to the capability of their hyphae to fuse with members of the designated anastomosis group), cultural morphology, pathogenic features, and DNA homology. Some AGs are also divided into different subgroups according to their various features. So far, 13 AGs have been described: AG1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, and 13 (4-6). AG1, AG2-1, AG2-2, and AG4 are the cause of serious crop losses in several types of plants, especially beans, because of diseases such as root rot, damping off, hypocotyl rot, capsule rot, and fruit rot.

In Turkey, some studies have been carried out on the isolation of *Rhizoctonia* fungi from various plants and on the determination of the anastomosis groupings (7-13).

To date, the isolates belonging to different AGs have been obtained from various plants in many regions in Turkey, but these studies have only consisted of isolation and traditional diagnosis. A comparative molecular study on the extremely widespread *Rhizoctonia* in Turkey may reveal the genetic variations of these organisms.

Although morphotaxonomic criteria are currently valid for taxonomically determining *Rhizoctonia* form genus, they may not always provide accurate results. However, some isolates have lost their anastomosis ability and sometimes it is impossible to determine the anastomosis group to which an isolate belongs. On the other hand, isolates of certain AGs can anastomose with more than one AG (e.g., AG2 BI, 3, 6, and 8) (1-3). Furthermore, the determination of AGs by hyphal anastomosis requires meticulous microscopic skill and is a time-consuming procedure. However, several molecular techniques have made isolate characterization easier and more accurate (2).

One of the methods widely used in molecular taxonomy is the characterization of rDNA regions that have been preserved during evolution (14). Fungal nuclear rRNA genes are arranged as tandem repeats with several hundred copies per genome. In each unit, there are 3 rRNA genes: a small rRNA gene (18S), the 5.8S rRNA gene, and a large rRNA gene (28S). Conserved sequences exist in large subunit (LSU) and small subunit (SSU) genes, and these genes have been used in many taxonomic studies. The spacer regions between the subunits are called internal transcribed spacers (ITS), and those between the gene clusters are called intergenic spacers (IGS). These spacer regions are considerably more variable than the subunit sequences and have been widely used in studies on the relationships among species within a single genus or among intraspecific populations (15).

A large number of variations exist within the form genus *Rhizoctonia*. It is possible to determine the AG of an isolate by conventional techniques. However, hyphal anastomosis reactions may not always be sufficient for subgrouping. The molecular studies carried out to date indicate that there are homogeneous AG groups. The purpose of this study was to determine by molecular techniques the AG subgroups that were previously defined by conventional techniques.

Materials and methods

Fungal collection and isolation

Samples of common bean seedlings with root disease symptoms were collected from 4 Turkish provinces located on the coast of the Black Sea during the 2005 vegetation periods. The sampling areas were Giresun [Piraziz (GP), Espiye (GE), Kovanlık (GK), Dereli (GD)], Sinop [Gerze (SG), Dikmen (SD), Ayancık (SA), Erfelek (SE)], Zonguldak [Gökçebey (ZG), Alaplı (ZA), Devrek (ZD), Çaycuma (ZÇ)], and Kırklareli [Demirköy (KDM), Pınarhisar (KP), Kıyıköy (KK), Dereköy (KD)]. Fungal isolation from the samples was carried out using the method proposed by Ogoshi et al. (16).

Hyphal anastomosis reactions and cultural morphology

The determination of AG subgroupings of *Rhizoctonia* isolates obtained from these provinces was done according to the methods used by Kronland and Stanghellini (17), Karaca et al. (18), and Sneh et al. (1). The type of hyphal anastomosis reaction was determined by microscopic observation at 400× magnification. In the process of determining AGs, microscopic investigations were conducted in such a way that isolates could be included in 1 of 4 categories: C0 (no interaction), C1 (contact between hyphae), C2 (death of anastomosed cells), or C3 (no cell death and continuity of cytoplasm) (5).

After the determination of the AGs of *Rhizoctonia* isolates, the activated isolates were inoculated on PDA medium (Oxoid Ltd, Basingstoke, Hampshire, England) and incubated at 25 °C in the dark for 3 weeks, and their cultural features were defined (19).

Pathogenicity

For the agar plate pathogenicity test, 23 isolates were incubated on PDA at 25 °C for 2-3 days, and then agar discs were transferred to a 2% water agar (11 cm diameter petri dish) under sterile conditions and incubated at 25 °C for 2-3 days. Surface sterilization of the bean seeds was done with 1% NaOCl for 3 min and the seeds were washed 5 times with sterile distilled water. Then 4 seeds were placed adjacent to the growing edge of the isolates in each petri dish. After 10 days at 25 °C in a growth chamber with 14 h light and 10 h dark, the roots and hypocotyls of the plants were examined according to a modification of the 1-5 scale (12-20): 1) healthy seedling; 2) very few superficial lesions on roots and hypocotyls; 3) deep and large lesions on roots or hypocotyls; 4) severe root-rot, partially restricted root lengths, or lesions encircling hypocotyls; 5) complete root-rot, collapsed hypocotyls with wilted leaves, or dead seedlings. The pathogenicity scores were analyzed using the Kruskal-Wallis and Mann-Whitney *U* tests.

DNA extraction and PCR of ITS1-5.8S-ITS2 region

The DNA extraction from *Rhizoctonia solani* AG4, selected on the basis of anastomosis group and cultural morphology, was carried out following the procedure of Pascual et al. (21). Two primers [ITS-1 (TCCGTAGGTGAACCTGCGG) (Qiagen) and ITS-4 (TCCTCCGCTTATTGATATGC) (Qiagen)] were used for the amplification of the DNA region encoding ITS-1–5.8S–ITS-2 of the DNA samples (22). The amplification of 50 µL of PCR product was performed using ≤1 µg genomic DNA, 1.5 mM $MgCl_2$, 1 U Taq polymerase (Promega, Go-Taq Flexi DNA Polymerase), 2.5 mM dNTP mix, 1× PCR buffer, and 50 pmol of each primer. For amplification, an MWG Primus thermal cycler was used with the following temperature profile: an initial denaturation at 94 °C for 3 min followed by 30 cycles of 94 °C for 1 min, 49 °C for 2 min, and 72 °C for 3 min, and a final extension at 72 °C for 7 min. The PCR products were electrophoresed on 1% agarose gel (Amresco, Solon, Ohio, USA) in 1× TBE buffer, stained with ethidium bromide, and visualized with the GeneGenius Bio imaging system.

PCR-RFLP and data analysis

Before the RFLP analysis, the PCR products were purified using the QIAquick PCR Purification Kit (Qiagen). Four restriction enzymes [*Mse*I (*Micrococcus* species), *Ava*II (*Anabaena variabilis*), *Hinc*II (*Haemophilus influenzae* Rc.), and *MfeI* (*Mycoplasma fermentas*, isoschizomer of *Mun*I) (New England BioLabs)] were used to distinguish *Rhizoctonia solani* AG4 subgroups with RFLP patterns (23). The purified 4 µL of ITS-PCR product was prepared under conditions recommended by the manufacturer. Five units of enzyme were used in the reaction, with a total volume of 10 µL, and it was incubated overnight at 37 °C. The restriction products were separated with 2% (wt/vol) MetaPhor agarose gel (Lonza Rockland, Rockland, Maine, USA) in 1× TBE buffer. The gels were run at 100 V for 2-3 h, stained with ethidium bromide, and visualized with the GeneGenius Bio imaging system.

Similarities among the isolates were estimated from the proportion of shared restriction fragments, and a dendrogram was constructed from the resulting distance matrix using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) (24). The software package PHYLIP 3.67 was used to perform the distance analysis (25).

Results and discussion

AGs of *Rhizoctonia solani*

The *Rhizoctonia* isolates from the Black Sea coastal region were collected from the roots of common beans and their rhizosphere regions. The 114 *R. solani* isolates obtained were classified into AG4 subgroupings according to anastomosis reactions.

All AG4 isolates anastomosed with both AG4 HGI and AG4 HGII testers at low frequencies. Fusion frequencies among the isolates and AG4 HGI and AG4 HGII tester isolates were determined to be the C2 hyphal type with 30-50% FF, but subgroups of *R. solani* AG4 isolates were not differentiated with anastomosis reactions. Our results also supported the proposition that anastomosis reactions are insufficient for determining AG4 subgroups.

The colony colors were dark brown (HGI) in some *R. solani* AG4 isolates and whitish-brown (HGII) in others on PDA medium.

Virulence of isolates

The virulence of all *R. solani* AG4 isolates was found to be at a high level, corresponding to the fifth level of the pathogenicity scale. There were no differences in mean ranks or median values of AG4 isolates. No significant differences were observed among the isolates (P = 0.05).

Analysis of the amplification product of PCR-RFLP

Twenty-three isolates representing different sampling areas were selected from the AG4 isolates, which had been determined on the basis of hyphal anastomosis and cultural morphology. Then the ITS-1–5.8S–ITS-2 rDNA region was amplified by PCR, the obtained PCR product was digested with 4 restriction endonuclease enzymes (*Mse*I, *Ava*II, *Hin*cII, and *MfeI*), and the RFLP patterns were obtained.

As seen in Figure 1, the ITS-1–5.8S–ITS-2 amplification product of AG4 HGI isolates was

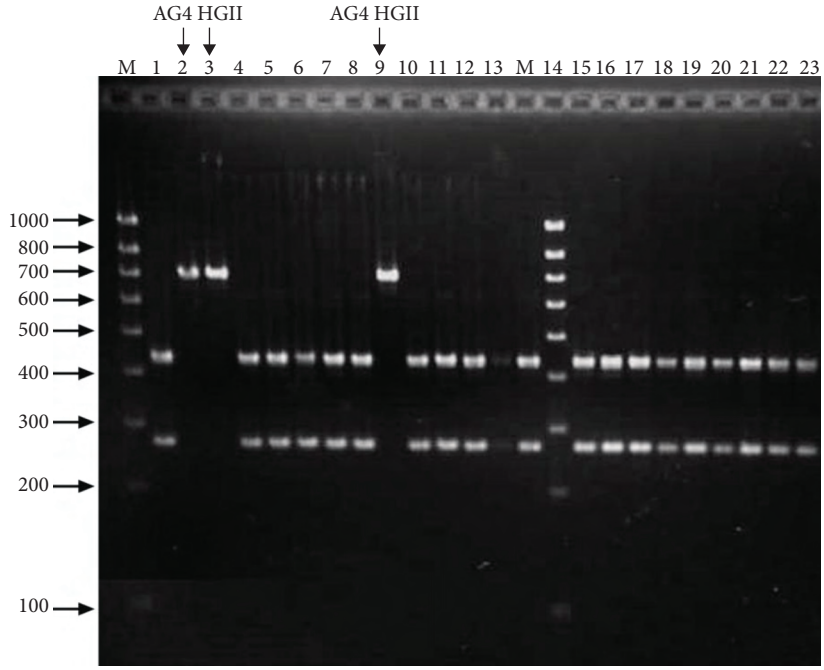

Figure 1. RFLP pattern of ITS1-5.8S-ITS2 with *Hin*cII restriction endonuclease on 2% MetaPhor agarose gel. M: DNA marker, 1: ZA (AG4 HGI), 2: ZD (AG4 HGII), 3: KP, 4: KDM, 5: GD, 6: ZG, 7: KD, 8: KDM, 9: SA, 10: ZG, 11: ZÇ, 12: ZG, 13: ZA, 14: ZÇ, 15: ZG, 16: GP, 17: GD, 18: GK, 19: GP, 20: GK, 21: GD, 22: GE, 23: GE.

digested by the *Hinc*II restriction endonuclease enzyme at one recognition site. As a result of the RFLP of the PCR product of AG4 HGI isolates, approximately 425 bp and 266 bp bands were evident. However, the PCR amplification product of AG4 HGII isolates was not digested by the *Hinc*II restriction endonuclease enzyme.

As in Figure 2, the ITS-1–5.8S–ITS-2 amplification products of AG4 HGI and AG4 HGII isolates were digested by the *MfeI* restriction endonuclease enzyme at one recognition site. The restriction fragments corresponding to a digestion by *MfeI* were 435 bp and 256 bp, approximately.

When the ITS-1–5.8S–ITS-2 amplification product of AG4 HGI isolates was digested by the *Ava*II restriction endonuclease enzyme, 550 bp and 110 bp bands, approximately, were obtained. The restriction fragments for the AG4 HGII isolates that were digested by the same enzyme at one recognition site were 550 bp and 150 bp, approximately (Figure 3).

When the ITS-1–5.8S–ITS-2 amplification product of AG4 HGI isolates (such as lane 4) was digested by the *Mse*I restriction endonuclease enzyme, 138 bp, 152 bp, and 205 bp bands, approximately, were obtained. Unlike the other AG4 HGI isolates, M46 (lane 1) and M67 (lane 13) produced approximately 152 bp, 171 bp, and 205 bp bands. However, when AG4 HGII isolates (lane 2) were digested by the same enzyme, the bands obtained were 114 bp, 142 bp, 152 bp, and 205 bp, approximately (Figure 4).

Each band formed by the digestion by the restriction endonuclease enzyme in RFLP analysis was considered to be a character, and a data matrix was created in accordance with whether they exist in the tested groups. As a consequence of these data, the UPGMA similarity tree was formed (Figure 5).

Of the subdivided isolates to undergo RFLP analysis, 107 isolates (93.8%) were defined as AG4 HGI and 7 isolates (6.2%) were defined as AG4 HGII. Meinhardt et al. (26) found that all isolates obtained from beans in Brazil were AG4 HGI. The majority of the isolates obtained in this study were found to be AG4 HGI, indicating that the beans in the Black Sea coastal region are primarily infected by this subgroup. In this study, all *R. solani* AG4 isolates had high virulence on beans. The results of pathogenicity studies clearly show that the isolates of *R. solani* AG4

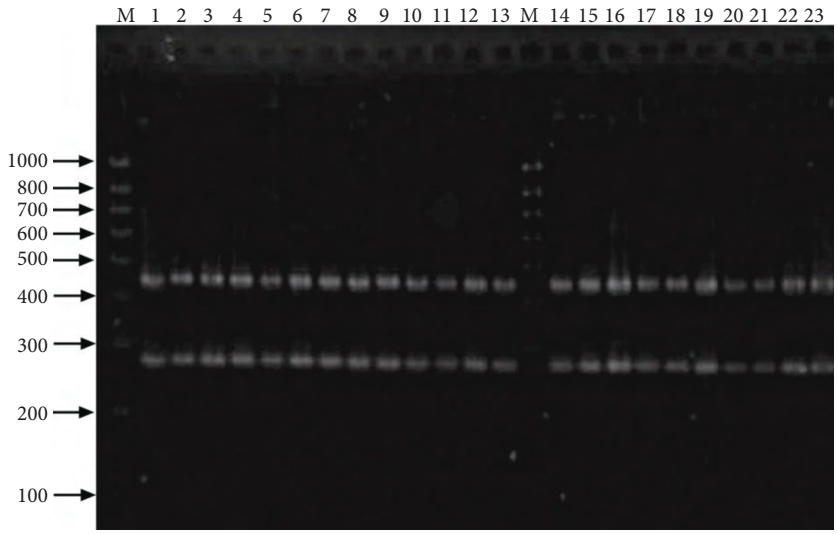

Figure 2. RFLP pattern of ITS1-5.8S-ITS2 with *MfeI* restriction endonuclease on 2% MetaPhor agarose gel. M: DNA marker, 1: ZA (AG4 HGI), 2: ZD (AG4 HGII), 3: KP, 4: KDM, 5: GD, 6: ZG, 7: KD, 8: KDM, 9: SA, 10: ZG, 11: ZÇ, 12: ZG, 13: ZA, 14: ZÇ, 15: ZG, 16: GP, 17: GD, 18: GK, 19: GP, 20: GK, 21: GD, 22: GE, 23: GE.

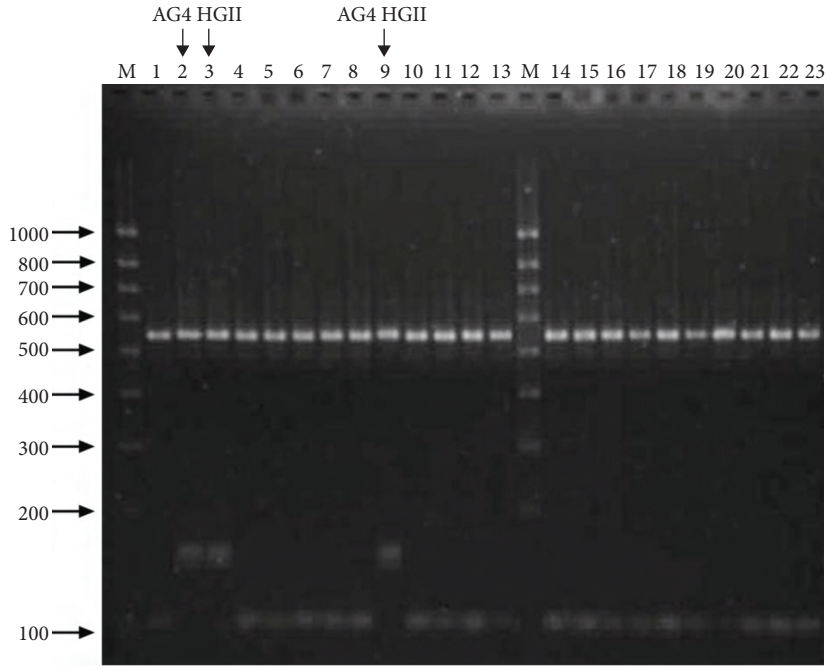

Figure 3. RFLP pattern of ITS1-5.8S-ITS2 with *Ava*II restriction endonuclease on 2% MetaPhor agarose gel. M: DNA marker, 1: ZA (AG4 HGI), 2: ZD (AG4 HGII), 3: KP, 4: KDM, 5: GD, 6: ZG, 7: KD, 8: KDM, 9: SA, 10: ZG, 11: ZÇ, 12: ZG, 13: ZA, 14: ZÇ, 15: ZG, 16: GP, 17: GD, 18: GK, 19: GP, 20: GK, 21: GD, 22: GE, 23: GE.

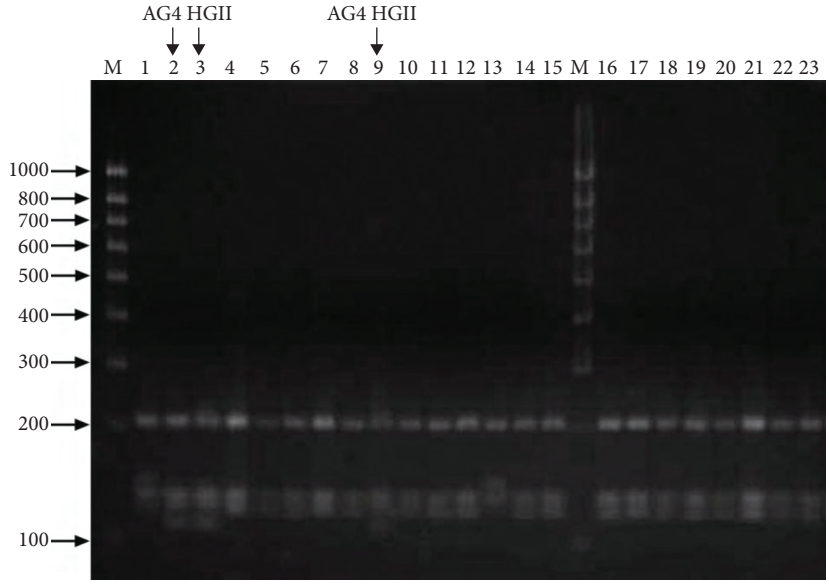

Figure 4. RFLP pattern of ITS1-5.8S-ITS2 with *Mse*I restriction endonuclease on 2% MetaPhor agarose gel. M: DNA marker, 1: ZA (AG4 HGI), 2: ZD (AG4 HGII), 3: KP, 4: KDM, 5: GD, 6: ZG, 7: KD, 8: KDM, 9: SA, 10: ZG, 11: ZÇ, 12: ZG, 13: ZA, 14: ZÇ, 15: ZG, 16: GP, 17: GD, 18: GK, 19: GP, 20: GK, 21: GD, 22: GE, 23: GE.

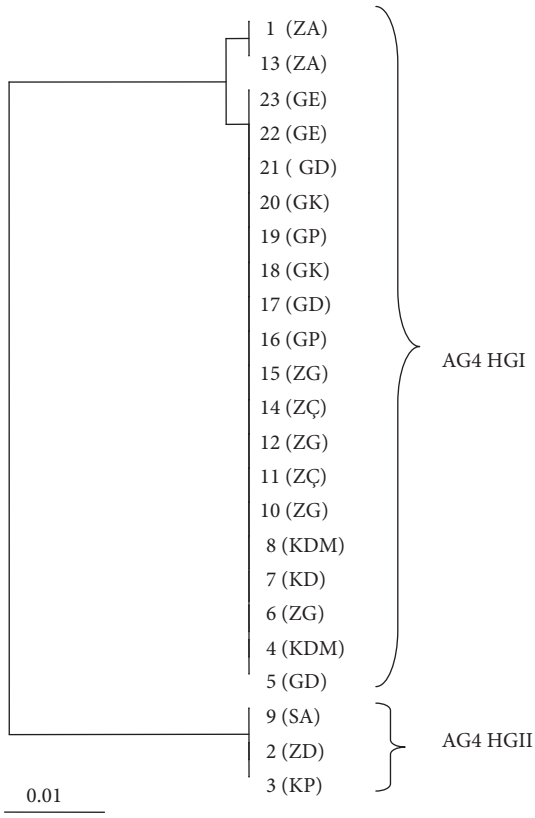

Figure 5. Dendrogram generated by UPGMA showing the genetic similarity between *R. solani* AG4 isolates from the Black Sea coastal region based on rDNA ITS-RFLP analysis.

from different plants caused diseases and varied in their virulence according to plant types (12-18-26-27-28-29-30-31).

The DNA fingerprints obtained in the RFLP analysis have been widely used in the differentiation of fungi. This method, based on the restriction analysis of rDNA amplified with PCR, has been important because it allows *R. solani* populations to be rapidly characterized at the AG level.

Using RS1 and RS4 primers, Guillemaut et al. (23) amplified the ITS sequences of 219 *R. solani* isolates belonging to anastomosis groups AG1 to AG13, which vary in terms of their locations and hosts, and then compared the restriction patterns of the ITS sequences (475-550 bp) to each other. They determined 40 RFLP patterns of the ITS sequences of the isolates with 4 distinguishing enzymes (*Mse*I, *Ava*II, *Hinc*II, and *MfeI*). It was evident from their study that these 4 enzymes facilitated the subdivision of AG1, AG2, AG3, and AG4. Guillemaut et al. (23) found that *Hinc*II alone could be used to differentiate some AG4 isolates into subgroups. Nevertheless, in our study, it was determined that *Mse*I and *Ava*II enzymes, in addition to *Hinc*II, were effective in the differentiation of subgroups. A longer amplification product (approximately 700 bp) was obtained because the primers that were used (ITS-1 and ITS-4) were different. In a study carried out in Brazil in 2002, Meinhardt et al. (26) used the RFLP method for the characterization of *R. solani* AG4 isolates obtained from beans. When the PCR products obtained from the ITS-1 and ITS-4 primers were digested by the Taq, *Hha*I, *Hae*III, and *Mse*I enzymes, the researchers found that the *Mse*I enzymes divided the isolates into AG4 HGI and AG4 HGII groups. Pannecoucque et al. (32) isolated various *R. solani* isolates and determined that the AG4 isolates only belonged to the AG4 HGII subgroup. Therefore, in the PCR-RFLP study, they examined the RFLP patterns of the AG4 HGII isolates, using primers ITS-4 and ITS-5 and restriction endonucleases *Mse*I *Ava*II, *Hin*cII, and *Mun*I (isoschizomer of *MfeI*). They found different patterns than those found in the current study because they used different primers. On the other hand, in the current study, it was found that in addition to these 2 enzymes (*Hinc*II and *Mse*I), the *Ava*II restriction endonuclease was effective in the subdivision of AG4.

In this study, it was seen that although AG4 HGI and AG4 HGII isolates were distinguished with different RFLP patterns, some AG4 HGI isolates, unlike the others, produced a 171 bp band instead of a 138 bp band when ITS-1–5.8S–ITS-2 amplification products were digested by the *Mse*I enzyme. These data indicate that there may be a genetic variation in the organisms of the AG4 HGI group. Therefore, it will be useful to determine the advanced genetic diversity of these organisms by means of a sequence analysis.

In conclusion, it was found that the *Hinc*II, *Mse*I, and *Ava*II enzymes could be used for the subdivision (HGI and HGII) of the organisms of the AG4 group. However, the *MfeI* restriction endonuclease was ineffective in distinguishing subdivisions of this group. It is possible to safely determine the subgroups

of *R. solani* AG4, an important plant pathogen, using the 3 enzymes mentioned above. Consequently, it is recommended that sequence analysis be carried out to determine whether there is further genetic diversity.

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