

## Investigation of Mycoviral double-stranded (ds) RNA in *Rhizoctonia solani* Isolates from Cotton in Aydın Province/Turkey

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**Abstract:** Pathogenic forms of *Rhizoctonia solani* are known to attack many species of higher plants from different families. It is one of the causal agent of the damping-off and root rot of cotton. The current damping-off management practices are not satisfactory for preventing yield losses in cotton production. Hypovirulence, caused by particular mycoviruse species, is used in the management of certain plant diseases. The existence of mycoviral dsRNAs in the isolates of *R. solani* has been previously determined in various countries. This study aimed to investigate dsRNA content in the cotton isolates of *R. solani* collected from Aydın Province. In total, 64 *R. solani* isolates collected from cotton seedlings exhibiting light damping-off symptoms were subjected to dsRNA analysis. The dsRNA electrophoretic pattern with the size of 12-18 kb were detected in six *R. solani* isolates. The virulence of these six isolates were compared with a dsRNA-free isolate in apple assay. Two *R. solani* isolates having dsRNA content caused smaller lesions in the inoculation point on apple fruit, which may be the indication of hypovirulence.

**Keywords:** Mycoviruses, Cotton, Damping off, *Rhizoctonia solani*, Hypovirulence

**Aydın İlinde Pamukta *Rhizoctonia solani* İzolatlarında Mikoviral Çift Sarmallı (ds) RNA'nın Araştırılması**

**Öz:** *Rhizoctonia solani*'nin patojenik izolatlarının, farklı familyalardan birçok bitki türünde hastalığa neden olduğu bilinmektedir. Bu patojen, pamukta çökertene ve kök çürüklüğüne neden olan fungal patojenlerin en önemlilerinden biridir. Günümüzde hastalığa karşı kullanılan mücadele yöntemleri hastalık nedeniyle oluşan verim kayıplarının önlenmesinde yeterli olmamaktadır. Geçmişte, farklı ülkelerden *R. solani* izolatları üzerinde yapılan çalışmalarda mycoviral dsRNA'lerin varlığı belirlenmiştir. Bu çalışmanın amacı; Aydın ilinde pamuklardan toplanan *R. solani* izolatlarının mikoviral dsRNA içeriklerinin araştırılmasıdır. Çalışma kapsamında, zayıf çökerten belirtileri gösteren pamuk fidelerinden elde edilmiş toplam 64 adet *R. solani* izolatı dsRNA analizine tabi tutulmuştur. Altı *R. solani* izolatında 12-18 kb büyüklüğünde dsRNA elektroforetik profiller saptanmıştır. dsRNA pozitif 6 izolatın virülensliği dsRNA-negatif bir izolat ile elma testinde karşılaştırılmıştır. İki dsRNA pozitif *R. solani* izolat elma üzerinde inokulasyon noktasında küçük lezyonlar oluşturmuştur ve bu durumun hipovirülslik ile bir ilişkisi olabileceği düşünülmüştür.

**Anahtar Kelimeler:** Mikovirüs, Pamuk, Çökerten, *Rhizoctonia solani*, Hipovirülslik

### INTRODUCTION

Cotton is an important fiber crop being cultivated in many countries around the world, including Turkey (Basal *et al.*, 2019). The damping-off and root rot disease caused by *Rhizoctonia solani* Kühn (telomorph *Thanatephorus cucumeris*), is one of the major factors negatively affecting cotton production throughout the world. In Turkey, *R. solani* is found in the majority of cotton fields and responsible for the death of many cotton seedlings in early season of the cultivation. Management practices for controlling of the disease are limited. Crop rotation is not effective enough since *R. solani* which is a soil-borne fungal pathogen, have a wide host range and can survive in the plant debris and soil one year to another (Sneh *et al.*, 1996 and Agrios, 1997). Certain dsRNA viruses found in fungi were associated with hypovirulence and they were recommended as biological control agents in the management of several plant diseases. The most successful example of plant diseases controlled by the use of mycoviruses is chestnut blight, caused by *Cryphonectria parasitica* (Milgroom and Cortesi, 2004). When *Cryphonectria hypovirus 1* (CHV1) infects *C. parasitica* in

active canker the fungus losses ability to destroy tree cambium, that can prevent the tree from dying. CHV1 also alters culture morphology, and cause reduction in pigmentation and sporulation that can be other visible signs of hypovirulence. Approximately 100 viruses from different families have been found in *R. solani* isolates, so far (Abdoulaye *et al.*, 2019). The existence of dsRNA in *R. solani* is very common throughout the world. Forty-nine out of fifty field isolates of *R. solani* had dsRNA in Maine, USA (Zanziger *et al.*, 1984). In another study, the dsRNA is present in 36 of 36 isolates of *R. solani* from different hosts and countries (Bharathan *et al.*, 2005). Hypovirulence in

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dsRNA containing isolates has been frequently reported (Abdoulaye *et al.*, 2019). On the other hand, dsRNA viruses were also detected in virulent strains (Finkler *et al.*, 1985). To date, there has been no report on the virus infected strains of *R. solani* in Turkey. Generally *R. solani* isolates with low virulence are very common in nature (Papavizas *et al.*, 1975), however their association with hypovirulence has not been investigated inadequately. The majority of mycovirus infection do not cause obvious symptoms on the fungal host (Abdoulaye *et al.*, 2019). The viral infection status of the pathogens is needed to be taken into account for better understanding of the pathogenic features of *R. solani* isolates. The objective of this study is to determine mycoviral dsRNA infection status and hypovirulent features of *R. solani* isolates collected from the cotton seedlings exhibiting light root rot symptoms in the field.

## **MATERIALS AND METHODS**

### **Collection of *R. solani* Isolates**

During spring at 2015-2016, cotton seedlings showing light damping-off and root rot symptom were sampled from the cotton production areas of Aydin province in the Aegean Region of Turkey. In total, two hundred samples were collected in three main cotton growing-counties, Koçarlı, Germencik, and Söke. A standart technique developed for the isolation from plant tissue was used in the isolation of the pathogen (Agrios, 1997). Fungal colonies developing around the diseased plant tissue on the surface of the nutrient media in the isolation plates were evaluated for cultural characteristics, such as hyphal branching, colony shape and colony color. A piece of mycelial agar from the colonies being identified as *R. solani* were transferred to new petri plates containing PDA to obtain pure cultures of each isolate.

### **dsRNA Extraction**

Double-stranded RNA extraction from *R. solani* isolates were carried out with a minor modifications of the method described by Balijja *et al.* (2008). All *R. solani* isolates were grown on a sterile cellophane membrane placed on PDA medium at 22°C for 7–10 days. Mycelium (200-300 mg) was harvested using a sterile toothpick and ground into a fine powder in liquid nitrogen using a mortar and pestle. The mycelial powder (200-300 mg) was transferred into 2 ml microcentrifuge tube and treated with 600 µl of EBA extraction buffer. The suspension was centrifuged at 4 °C for 15 min at 16,110 × g and the supernatant was carefully removed and the tubes placed in the micro columns then, centrifuged at 100 × g for 2 min and the eluted liquid was discarded. Columns were centrifuged at 100 × g for 2 minutes after adding 450 µl 1x STE-20% E buffer, which was performed twice. The column was placed into a new 2 ml centrifuge tube. At this stage, to separate dsRNAs, the

column was treated with 400 µl 1x STE buffer and centrifuged at 100 × g for 2 minutes. This stage was repeated twice. Isopropanol was added to an equal volume of the liquid collected in the column then, stirred on the rotator at room temperature for 10 minutes. Then, it was centrifuged at 4 °C at 16 110 × g for 30 min. The dsRNA pellet was washed with 70% ethanol then the pellets were allowed to dry at room temperature. The dried pellets were dissolved in 30 µl of RNase-free water. dsRNA's were separated by gel electrophoresis at 80 w in 0.8% agarose in 1XTBE buffer, then they were visualized under UV light.

### **Virulence Assay**

Virulence of the *R. solani* isolates was determined by using the apple assay according to Erincik *et al.* (2008). Six dsRNA containing isolates and one dsRNA-free isolate were used in the apple assay. 'Granny Smith' apples were surface sterilized with 70% ethanol. Two wells with 6-mm diameter, each of which was evenly positioned at opposite site, were created by removing tissue plugs using a sterile cork borer. A plug of fungal mycelia of each isolate was placed into well for inoculation. During this process, the plug was placed mycelium side down using a sterile spatula to ensure that the mycelia fully touch the bottom of the well. The inoculation point was sealed with a small piece of parafilm. Four apples were inoculated per isolate and each apple was accepted as one replication. Each well on an apple was accepted as subsample. Negative control apples were only inoculated with sterile-agar plug. All apples were incubated in a plastic box covered by a plastic bag at 22 ° C in the growth chamber. The lesions formed on the apples at the 15th day of incubation were evaluated by measuring their size. The data were analyzed statistically using analysis of variance (ANOVA).

## **RESULTS AND DISCUSSION**

### **Sampling and Isolation of *R. solani***

From the isolations conducted on the 200 diseased-plant samples, 64 *R. solani* isolates were obtained. During growing on PDA, *R. solani* isolates showed a light-brown or cream color colonies with full growth and developed hyphae branching at right angle. Only 32 percent of the samples gave *R. solani*, which is the indication of low frequency rate. This can be explained by our selective sampling strategy to obtain hypovirulent *R. solani* isolates. During the sampling, we chose the seedlings exhibiting light damping-off and root rot symptoms and purposely avoided to pick the heavily diseased ones.

### **dsRNA Analysis**

The result of dsRNA electrophoresis showed that six *R. solani* isolates gave dsRNA profiles, which were illustrated in Figure-1. The estimated molecular size of the dsRNAs 12-18 kb. According to the previous reports, most of the

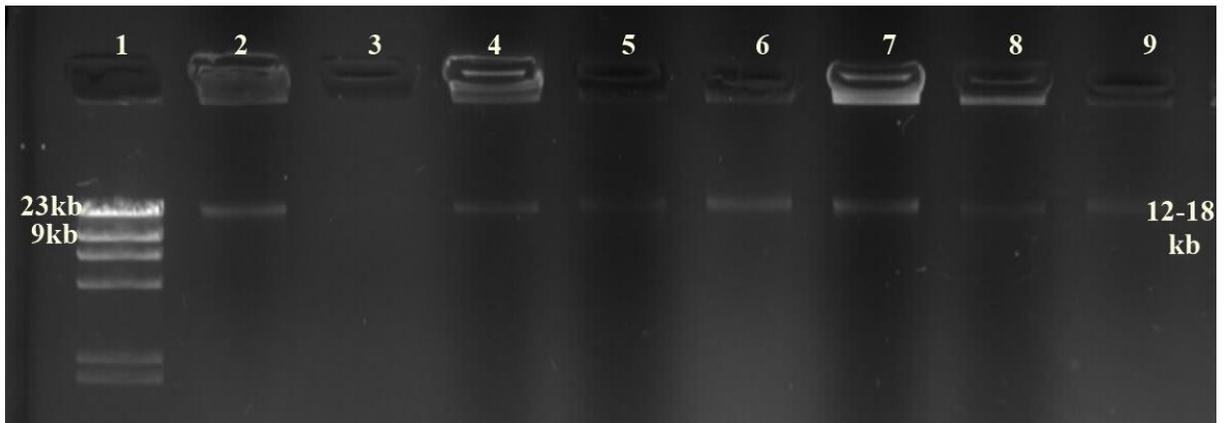


Figure 1. Electrophoresis in agarose gel of double-stranded RNA (dsRNA) purified from *Rhizoctonia solani* collected from cotton seedlings in the Aydın province. The lanes 1 molecular marker  $\lambda$ DNA/HindIII (23.0 kb), lane 2 positive control isolate (12.7kb *Cryphonectria parasitica*), lanes 4, 5, 6, 7, 8 and 9 (18-12 kb) dsRNA positive *Rhizoctonia solani* isolates

mycoviruses belong to the virus families having dsRNA genome (Abdoulaye *et al.*, 2019) and their molecular sizes varied in a broad range. Bharathan *et al.* (2005) had detected dsRNA fragments with ranging sizes from 0.74 to 23 kb in 36 *R. solani* isolates from different host and countries. Supyani and Hardjono (2014) reported that the sizes of RNA genomes found in *R. solani* ranged from 1 to 12.7 kb. In the present study, a large dsRNA of 12-18 kb were detected on agarose gel in six *R. solani* isolates and this dsRNA's are similar in weigh to the two isolates of *R. solani*, which was reported by Bharathan *et al.* (2005).

#### Virulence Assay

The results of virulence assay showed that 6 *R. solani* isolates having dsRNA content produced varying levels of virulence. At the 15<sup>th</sup> day of the incubation, the high level of virulences were found in SKP 9/4, GP 2/3 and GP 3/3 isolates, which produced lesions with mean of 46.2, 43.3 and 42.3 mm in diameter, respectively. The lowest virulence was obtained in UP 2/2 isolate which produced lesions with the mean of 19.2 mm in diameter. The other two isolates, OP 1/3 and GP 5/5, produced smaller lesions with mean of 38.3 and 23.3 mm diameters, respectively. UP 2/2 caused 60% and GP 5/5 51% reduction in virulence comparing to the dsRNA negative isolates. These two isolates can be considered as relatively low virulent ones (Figure 2 and Table 1).

One of the most important signs of hypovirulence is the reduction in virulence of the fungal isolates after infection by viral agent (Ghabrial, 2001). In this study, the two dsRNA positive isolates (UP2/2 and GP5/5) showing low level virulence in apple assay have possibility of being hypovirulent strains. Eventhough apple is not a host, its tissue has been commonly used in the screening of hypovirulent strains of *C. parasitica*, the causal agent of



Figure 2. Lesions developed on the 'Granny Smith' apple fruit inoculated with *Rhizoctonia solani* isolates at the 15th day of inoculation. BP1/5 (dsRNA-free) isolate, GP3/3, GP2/3, SKP9/4, OP1/3, UP2/2 ve GP5/5 (dsRNA containing) isolates

Table 1. Lesion development on the 'Granny Smith' apple fruit inoculated with *Rhizoctonia solani* isolates at the 15th day of incubation

Isolates	Lesion Diameter <sup>2</sup> (mm)	Reduction in virulence %
UP 2/2 <sup>(+)</sup>	19.2 c	60
GP 5/5 <sup>(+)</sup>	23.3 c	51
OP 1/3 <sup>(+)</sup>	38.3 b	20
GP 3/3 <sup>(+)</sup>	42.3 ab	11
GP 2/3 <sup>(+)</sup>	43.3 ab	9.05
SKP 9/4 <sup>(+)</sup>	46.2 a	2.5
BP 1/5 <sup>(-)</sup>	47.5 a	-

<sup>2</sup>different letters in the same column indicated that means are significantly different from each other according to Duncan Multiple Range Test (P $\leq$ 0.05)

chestnut blight (Erincik *et al.*, 2008). It was demonstrated that, hypovirulent strains of *C. parasitica* developed small lesions on apple fruit. The apple assay was the first time used for the virulence determination of *R. solani* isolates; therefore, virulence results obtained in this study needs to be confirmed by another virulence test which will be

conducted on the real host. This study revealed that there are dsRNA viruses in *R. solani* populations in Turkey. In future studies, hypovirulence tests should be conducted on the main host to verify the results obtained in this study. The full genome analysis of these dsRNAs is also necessary for identification of virus and for establishing its taxonomy. All the findings in this study will be helpful for changing our aspect to manage damping-off and root rot in cotton. There are many good examples of hypovirulence that can lead the way for us in the development of novel biocontrol strategies for the management of *R. solani* related diseases on cotton, and other hosts as well.

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