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Original article

Screening of some plant pathogenic fungi for the presence of dsRNA mycoviruses

Bazı bitki fungal patojenlerinde dsRNA mikovirüslerinin varlığının taranması

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ABSTRACT

Mycoviruses can infect many fungi and some may cause hypovirulence, which is a common phenomenon used in the biological control of certain fungal diseases of the plants. The most successful example regarding the use of mycoviruses and hypovirulent strains in biological control is the chestnut blight. In this study, the isolates of fungi, namely *Phomopsis viticola* from grapevine, *Verticillium dahliae* from cotton and olive, *Rhizoctonia solani* from cotton and *Leucostoma* spp., from cherry were screened for the presence of dsRNA mycoviruses. Isolation of dsRNAs was performed and dsRNA bands were analyzed by agarose gel electrophoresis. Putative dsRNA bands were detected in eight of 80 *P. viticola* isolates from grapevine, one of 50 *V. dahliae* isolates from olive, three of 50 *V. dahliae* isolates from cotton, six of 50 *R. solani* isolates from cotton and three of 50 *Leucostoma* spp., isolates from cherry. The estimated molecular sizes of the dsRNAs ranged from approximately 12.0 to 20.0 kb.

INTRODUCTION

Mycoviruses are viruses that can cause infection in fungi. They replicate in the fungal cell and are transmitted intracellularly during cell division, sporogenesis and/or hyphal anastomosis. Mycoviruses lack an extracellular stage and are always associated with infection in the hosts. Since the first report of mycoviruses in *Agaricus bisporus* (Son et al. 2015) in 1962, a large number of the mycoviruses belonging to various virus families such as *Hypoviridae*, *Reoviridae*, *Totiviridae*, *Chrysoviridae*, *Partitiviridae*, *Megabirnaviridae*, and *Quadriviridae* have been identified. Some mycoviruses are symptomless; however, some of them can cause significant alterations in the morphology and/or the behaviors of the fungus. As an example, *Hypoviruses* that are members of *Hypoviridae* can cause attenuation in fungal virulence (hypovirulence) along with reduced asexual spore (conidium) production and pigmentation (Son et al. 2015, Xie et al. 2014). Certain dsRNA viruses found in fungi were associated with hypovirulence and they were recommended as biological control agents in the management of several plant fungal diseases. The most

Table 1. Numbers of collected plant samples and obtained fungal isolates from host plants in the Aegean Region of Turkey

Fungal agents	Province	Host plants	Number of samples	Number of obtained isolates
Phomopsis viticola	İzmir, Manisa	grapevine	86	80
Verticillium dahliae	Aydın	olive	80	50
Verticillium dahliae	Aydın	cotton	100	50
Rhizoctonia solani	Aydın	cotton	150	50
Leucostoma spp.	İzmir, Denizli, Manisa, Aydın	cherry	50	50
	Total		466	280

successful example among the disease controlled by the use of hypovirulence is chestnut blight, which is caused by an Ascomycete fungus, *Cryphonectria parasitica*. Hypovirulent strains are known as special forms of *C. parasitica* exhibiting reduced virulence as a result of infection by several dsRNA viruses (CHV-1, CHV-2, CHV-3 and CHV-4). The cankers which are caused by these strains are described non-lethal superficial cracks and sketchy wounds, which is defined as hypovirulent type canker. Hypoviruses can be transmitted cytoplasmically from hypovirulent strains to virulent strains by hyphal anastomosis. This feature of the hypoviruses provides the basis for biological control practices which may result in conversion of the lethal cankers into hypovirulent ones, thus the blighted tree eventually recovers. Mycoviruses have been detected in all of the major phyla of fungi, including the Chytridiomycota, Zygomycota, Ascomycota, Deuteromycota, and Basidiomycota. Some economically important fungal plant pathogens were reported to be the host for mycoviruses, such as *Cryphonectria parasitica* in chestnut, *Phomopsis longicolla* in soybeans, *Phomopsis vexans* in eggplant, *Leucostoma persoonii* in peach, *Diaporthe ambigua* in apple, *Verticillium dahliae* and *Rhizoctonia solani* in cotton, rice, soybeans, and sugarbeets (Bharathan et al. 2005, Canizares et al. 2015, Cao et al. 2011, Koloniuk et al. 2014, Preisig et al. 2000, Zhang et al. 2015). Most mycoviruses were well characterized in terms of their particule morphology and genomic characteristics. According to the most recent reports, more than 250 mycoviruses have been sequenced and registered in the NCBI (National Center for Biotechnology Information) database (Abbas 2016, Xie et al. 2014). Mycoviruses usually have isometric particles, 25–50 nm diameters, and contain segmented or non-segmented double-stranded RNA (dsRNA) genome. However, some possess single-stranded RNA (ssRNA) or double-stranded DNA (dsDNA) genomes (Jiang et al. 2013, Van Regenmortel et al. 2000). In Turkey, mycoviruses have been studied on a limited number of fungal hosts. However, Turkey is a rich country in respect of agricultural crops with great diversity alongside the large number of fungal pathogens. Regarding with the previous reports from elsewhere in the world, *Phomopsis viticola* on grapevine, *Verticillium dahliae* on cotton and olive, *Rhizoctonia solani* on cotton and *Leucostoma* spp. on cherry which are economically important fungal pathogens in Turkey can serve as possible hosts for known or unknown mycoviruses. The objective of this study is to screen some selected fungal pathogens that were found to be economically important in Turkey for the presence of mycoviruses.

MATERIALS AND METHODS

Collection of fungal isolates

During autumn, spring and summer in 2015 and 2016, a total of 466 symptomatic plant samples were collected from different hosts including grapevine, olive, cherry and cotton, in the provinces of İzmir, Aydın, Denizli and Manisa in the Aegean Region of Turkey (Table 1). A standart isolation technique was used in the isolation process of the pathogens. Fungal cultures were grown on potato dextrose agar (PDA) or water agar and cultural characteristics and morphology such as color, size and shape of mycelial colonies, hyphal branching, fruiting structures and spores were used in fungal identification. Among the 280 isolates obtained from the isolations, 80 were *Phomopsis viticola*, 50 were *Verticillium dahliae* from olive, 50 were *Verticillium dahliae* from cotton, 50 were *Rhizoctonia solani* and 50 were *Leucostoma* spp.

Extraction of dsRNA

dsRNA extractions were carried out with minor modifications of the method described by Balijja et al. (2008). Isolates were grown on cellophane membranes placed on PDA medium in petri plates at 22 °C for 7–10 days. Mycelia (200-300 mg fresh weight) were harvested using a sterile tooth stick and ground to a fine powder with liquid nitrogen using a pre-cooled mortar and pestle. The powder was stored at –80 °C until use. The powder was transferred into 2 ml microcentrifuge tube and 600 µl of extraction buffer was added. The suspension was centrifuged at 4 °C for 15 min at 16 110 g. After being centrifuged carefully, the collected supernatant was adjusted to a final concentration of 20% ethanol and applied to a micro-column (ultrafree-MC sterile 0.65 µm, Millipore, USA). The micro-column was then centrifuged at 100 g for 2 min and the eluted liquid was discarded. The column was washed twice by adding 450 µl of 1× STE-20% buffer and centrifuged at 100 g for 2 min. The column was placed into a new 2 ml centrifuge tube. The dsRNA was eluted from the column by adding 400 µl of 1× STE buffer twice and centrifuging at 100 g for 2 min. After being collected, the eluate mixed with an equal volume of isopropanol by using a rotator (Rotator 240V, speed 8, Agar Scientific) for 10 min. Then, it was centrifuged at 4 °C for 30 min at 16 110 g. The dsRNA pellet was washed with 70% ethanol, air-dried at room temperature and dissolved in 50 µl of RNase-free water. Size separation of dsRNA elements extracted from individual isolates was conducted by electrophoresis on a 0.8% agarose gel containing ethidium bromide. Electrophoresis was run at 80V (in room temperature) for about 1 h in 1×TBE electrophoresis buffer. Lamda (λ) DNA-HindIII Marker (Thermo Fisher Scientific, USA) was used as the molecular size marker for electrophoresis.

RESULTS AND DISCUSSION

dsRNAs were detected in 8 isolates of *P. viticola* from grapevine, 1 isolate of *V. dahliae* from olive, 3 isolates of *V. dahliae* from cotton, 6 isolates of *R. solani* from cotton and 3 isolates of *Leucostoma* spp. from cherry (Table 2). The estimated molecular size of the dsRNAs ranged approximately from 12.0 to 20.0 kb. The dsRNA's of fungal disease agents have been previously reported in the Diaporthaceae family. For example, mycovirus dsRNAs in *Phomopsis longicolla* isolates from soybean (Koloniuk et al. 2014), in *Phomopsis vexans* isolates from eggplant (Zhang et al. 2015) and *Diaporthe ambigua* isolates from apple (Preisig et al. 2000), and these dsRNAs have been associated with hypovirulence (Koloniuk et al. 2014, Preisig et al. 2000, Zhang et al. 2015). *Phomopsis viticola* (Sacc.) Sacc belonging to the family Diaporthaceae and the causal agents of Phomopsis cane and leaf spot of grapes has not yet been investigated for the presence of dsRNA. In our study, the dsRNA electrophoretic patterns of bands in size of 18-20 kb were detected on agarose gel in eight *P. viticola* isolates (Figure 1). The diagnosis of this new mycoviral dsRNA has not been performed yet and its association with hypovirulence has not been investigated. In the future studies, virulence test on potted grapevine plants by using *P. viticola* isolates possessing dsRNA will be conducted to investigate their hypovirulent characteristics. Moreover, dsRNA of the *P. viticola* will be diagnosed by full genome sequence analysis.

In this study, dsRNAs were obtained in one *V. dahliae* isolate from olive and in three *V. dahliae* isolates from cotton. Molecular weights of the dsRNA bands were ranged approximately 18-20 kb (Figure 2a,b). Several researchers have been investigated dsRNA segments in *V. dahliae* isolates collected from cotton and olive (Canizares et al. 2015, Cao et al. 2011, Feng et al. 2013). Four segments of dsRNA element

Table 2. The results of dsRNA analysis of fungal agents

Fungal agents	Number of isolates for dsRNA analysis	Number of dsRNA positive isolates	Number of dsRNA negative isolates
Phomopsis viticola	80	8	72
Verticillium dahliae	50	1	49
Verticillium dahliae	50	3	47
Rhizoctonia solani	50	6	44
Leucostoma spp.	50	5	45

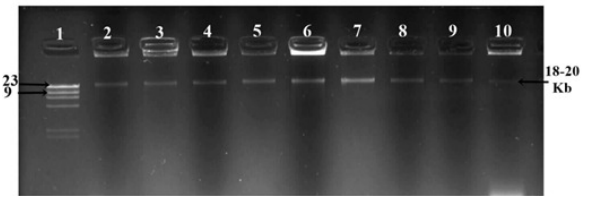

Figure 1. Agarose gel electrophoresis of double-stranded RNA (dsRNA) purified from *Phomopsis viticola* isolates in the Aegean Region of Turkey. The lane 1: Molecular weight marker, ʎDNA/HindIII (23.0 kb), lane 2: Positive control (12.7 kb *Cryphonectria parasitica)*, lanes 3, 4, 6, 7, 8, 9, 10 and 12: Positive isolates of *Phomopsis viticola* (18.0-20.0 kb)

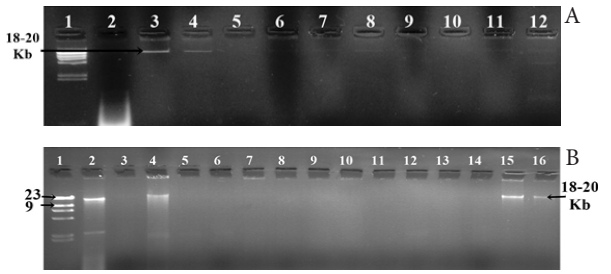

Figure 2. Agarose gel electrophoresis of double-stranded RNA (dsRNA) purified from *Verticillium dahliae* isolates in the Aegean Region of Turkey. The lanes 1 in panels (A and B): Molecular weight marker, ʎDNA/HindIII (23.0 kb), (A) lane 4 and (B) lane 2: Positive control (12.7 kb *Cryphonectria parasitica*), (A) lane 3: Positive isolate of *Verticillium dahliae* (olive) (18.0-20.0 kb), (B) lanes 4, 15 and 16: Positive isolates of *Verticillium dahliae* (cotton) (18.0-20.0 kb)

have been detected in the *V. dahliae* isolates from the cotton. dsRNAs segments which were estimated as molecular sizes ranged from 3.0 to 3.6 kb (Cao et al. 2011). Based on the size of dsRNA's they were termed as dsRNA1, 2, 3 and 4. Canizares et al. (2015) reported that 16 *V. dahliae* isolates from olive in Turkey contained dsRNA viruses.

In our study, electrophoretic bands of a large dsRNA (12-18 kb) were detected on agarose gel in six *R. solani* isolates and this dsRNAs were similar in molecular size of the 21 *R. solani* isolates found by Bharathan et al. (2005) (Figure 3). Many studies have been carried out on the presence of dsRNA in different hosts of *R. solani* isolates (Bharathan et al. 2005, Das et al. 2014, Kousik et al. 1993). The dsRNA fragments were obtained ranging between 0.6 and 23 kb for *R. solani* isolates in the North America and Japan (Bharathan and Tavantzis 1990, Bharathan and Tavantzis 1991, Hyakumachi et al. 1985, Kousik et al. 1994, Zanzinger et al. 1984). Bharathan et al. (2005) dsRNAs were detected in 36 isolates belonging to nine anastomosis groups (AGs) and molecular sizes of the dsRNA

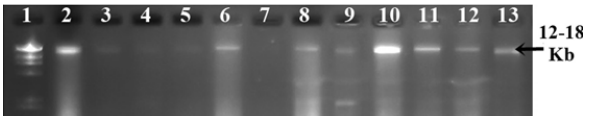

Figure 3. Agarose gel electrophoresis of double-stranded RNA (dsRNA) purified from *Rhizoctoni solani* isolates in the Aegean Region of Turkey. The lane 1: Molecular weight marker, ʎDNA/HindIII (23.0 kb) and lane 2: Positive control (12.7 kb *Cryphonectria parasitica*), lanes 6, 8, 10, 11, 12 and 13: Positive isolates of *Rhizoctoni solani* (12.0-18.0 kb)

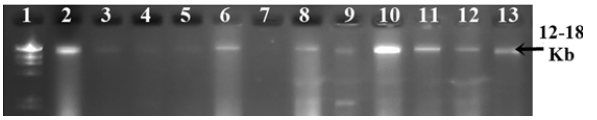

Figure 4. Agarose gel electrophoresis of double-stranded RNA (dsRNA) purified from *Leucostoma* spp. isolates in the Aegean Region of Turkey. The lane 1: Molecular weight marker, ʎDNA/HindIII (23.0 kb) and lane 2: Positive control (12.7 kb *Cryphonectria parasitica*), lanes 8, 9 and 11: Positive isolates of *Leucostoma* spp. (18.0-20.0 kb)

ranged between 0·74 and 23·0 kb.

In this study, dsRNA profile with 18-20 kb molecular weight was obtained in 3 isolates (Figure 4). Jensen et al. (1995) in the North Carolina, USA, found that isometric virus-like particles (VLP) were visible in hyphae of a hypovirulent isolate of *Leucostoma persoonii*, and this VLP contained at least six segments of dsRNA. This dsRNA's molecular weights were 7.9, 3.0, 2.8, 2.6, 2.3 and 0.7 kb. The presence of dsRNA from *Leucostoma* spp. isolates was investigated on the limited number of isolates; and dsRNA bands were found in six isolates for the first experiment in Turkey (Tonguslu and Acikgoz 2015).

In summary, this study is the first to investigate mycoviral dsRNAs in the important fungal pathogens of the main crops grown in the Aegean Region of Turkey. The dsRNAs having molecular sizes ranged from 12-20 kb were detected in the eight isolates of *P. viticola* from grapevine, one and three of *V. dahliae* from olive and cotton, respectively, six of *R. solani* from cotton and three of *Leucostoma* spp. from cherry. As in the case of *C. parasitica*, a model fungus for studying mycoviruses, the findings in this study provide a fundamental knowledge base for further understanding of mycoviruses and their influences on fungi. This knowledge will be valuable for the future development of biological control strategy for economically important fungal diseases

in Turkey by the use of hypovirulence-associated isolates.

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ÖZET

Mikovirüsler birçok fungal hastalık etmenini enfekte edebilir ve bunların bazıları hipovirülensliğe neden olabilmektedir. Bu mikovirüsler bitkilerin biyolojik mücadelesinde yaygın olarak kullanılmaktadırlar. Mikovirüslerin kullanılması ile gerçekleştirilen biyolojik mücadeleye en başarılı örnek, Kestane kanseri hastalığıdır. Bu çalışmada, asmadan elde edilen *Phomopsis viticola*, pamuktan ve zeytinden *Verticillium dahliae*, pamuktan *Rhizoctonia solani* ve kirazdan *Leucostoma* spp. izolatlarındaki mikovirüslerin varlığı dsRNA metodu ile araştırılmıştır. dsRNA ekstraksiyonu yapılmış ve agaroz jel elektroforez yöntemi ile analiz edilmiştir. Asmada 80 *P. viticola* izolatının sekizinde, zeytinde 50 *V. dahlia* izolatının birinde, pamukta 50 *V. dahliae* izolatının üçünde, pamukta 50 *R. solani* izolatının altısında ve kirazda 50 *Leucostoma* spp. izolatının üçünde dsRNA bantları elde edilmiştir. Elde edilen dsRNA bantlarının tahmini moleküler ağırlıkları 12-20 kb arasında bulunmuştur.

Anahtar kelimeler: mikovirüs, *Phomopsis viticola*, *Rhizoctonia solani*, *Verticillium dahliae*, *Leucostoma* spp.

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