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Identification and characterization of lettuce big vein disease (LBVD) in lettuce (*Lactuca sativa*) crops in Adana and Mersin provinces in Turkey

Adana ve Mersin illerinde (Türkiye) yetiştirilen marullarda (*Lactuca sativa*) marul iri damar hastalığının tanılanması ve karakterizasyonu

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ABSTRACT

This study was carried out for the purpose of determination of Lettuce big vein disease (LBVD), identification of the virus or virus complex (MiLBVV and/or LBVaV) causing disease and characterization of causal agent in the fields where the lettuce cultivation was made extensively in Adana and Mersin provinces during autumn and winter months between 2015 and 2017. A total of 160 samples were collected from lettuce (*Lactuca sativa* L.) plants suspected to be infected with LBVD simptomatologically in surveys. In the result of the ELISA tests, 52 samples were found to be infected with MiLBVV. RT-PCR studies were performed to identify the causative agents of LBVD by using specific primer pairs for MiLBVV (MiLBV-F; MiLBVV-R) and LBVaV (VP-248; VP-249) showed that LBVD is caused by MiLBVV or mixed infection of MiLBVV+LBVaV. LBVaV infection was not detected alone in lettuce plants with LBVD symptoms. Sequence analyses showed that Adana and Mersin isolates of MiLBVV formed in separate groups on phylogenetic tree. While Yakapınar (2-MiLBVV 1 E10), Yumurtalık (6-MiLBVV 1 G10) and Yüreğir (4-MiLBVV 1 F10) isolates from Adana province clustered with Argentina and Iran isolates, Yenice 1 (11-MiLBVV 2 H10) and Yenice 2 (12-MiLBVV 2 E11) isolates from Mersin province were in the same group with Netherlands and Egypt isolates in another group. In addition, the Yüreğir isolate of LBVaV (4-VP 248 A12) clustered with isolates from Saudi Arabia, United States, United Kingdom, Australia and Netherlands.

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ÖZ

Bu çalışma, Marul iri damar hastalığı (Lettuce big-vein disease, LBVD)'nın saptanması, bu hastalığa neden olan virüs veya virüs kompleksinin (MiLBVV ve/veya LBVaV) belirlenmesi ve bu etmenlerin karakterizasyonu amacıyla, 2015-2017 yılları arasında sonbahar ve kış aylarında, Adana ve Mersin illerinde yaygın olarak marul yetiştiriciliğinin yapıldığı alanlarda, yürütülmüştür. Arazi çıkışlarında, simptomatolojik olarak LBVD ile enfekteli olduğundan şüphelenilen toplam 160 adet marul bitkisinden örnekleme yapılmıştır. ELISA testleri sonucunda, 52 marul örneği MiLBVV ile enfekteli bulunmuştur. LBVD'ye sebep olan etmenlerin belirlenmesi amacıyla, MiLBVV (MiLBVV-F; MiLBVV-R) ve LBVaV (VP-248;VP-249)'ye spesifik primer çiftleri kullanılarak yapılan RT-PCR çalışmaları sonucunda, MiLBVV veya MiLBVV+LBVaV karışık enfeksiyonunun varlığı ortaya konmuştur. LBVD simptomu gösteren marul bitkilerinde tek başına LBVaV enfeksiyonu saptanmamıştır. Sekans analizlerinde, MiLBVV'nin Adana ve Mersin izolatları filogenetik ağaç üzerinde kendi aralarında ayrı birer grup oluşturmuşlar ve Adana ilinden, Yakapınar (2-MilBVV 1 E10), Yumurtalık (6-MiLBVV 1 G10) ve Yüreğir (4-MiLBVV 1 F10) izolatları, Arjantin ve İran izolatları ile birlikte yer alırken, Mersin ilinden Yenice 1 (11-MiLBVV 2 H10) ve Yenice 2 (12-MiLBVV 2 E11) izolatları, Hollanda ve Mısır izolatları ile aynı grupta yer almışlardır. Bunun yanında, LBVaV'nın Yüreğir izolatı (4-VP 248 A12), Suudi Arabistan, ABD, Birleşik Krallık, Avustralya ve Hollanda izolatları ile grup oluşturmuştur.

1. Introduction

Vegetables adorn our tables, with their beautiful appearance, unique tastes, and aromas, take an important place in human nutrition (Abak ve ark. 2010). Turkey plays a role as one of the major producers of a large number of vegetable species, ranking in the top five countries in the world. It has been reported that the highest yield in Turkey is taken from the Mediterranean region where greenhouses are able to thrive in the advantageous climate (TOBB 2013). According to FAO in 2017, China ranks first with the estimated annual production of lettuce approximately 15 million tons, while, Turkey seventh places in the world with production of 490423 tons (FAO 2019). According to the data of Turkish Statistical Institute in 2016, 53603 tons of lettuce was produced in 18215 da in Adana, while 56164 tons of lettuce was produced in 22847 da in Mersin province of Turkey (TÜİK 2019a, 2019b).

Lettuce is taking important place among vegetables in many countries including Turkey, and numerous viral agents have been reported in lettuce varieties including *Alfalfa mosaic virus*, *Beet western yellows virus*, *Broad bean wilt virus*, *Mirafiori lettuce big vein virus*, *Lettuce big vein associated varicosavirus*, *Lettuce mosaic virus*, *Lettuce necrotic yellows virus*, *Tomato spotted wilt virus* and *Turnip mosaic virus* (Moreno and Fereres 2012; Sertkaya 2015). Among these, lettuce big-vein disease (LBVD) is a major disease of lettuce worldwide. Due to the disease, chlorotic opening of the leaf veins in infected lettuce plants causes symptoms such as leaf deformation, delay in maturation, and decrease in head size or no head formation. The overall quality and yield of the product are affected (Araya et al. 2011). *Lettuce big-vein associated virus* (LBVaV, genus *Varicosavirus*) is a rod-shaped virus transmitted by *Olpidium brassicae* was thought to be causal agent of LBVD, historically, but recently the *Mirafiori lettuce big-vein virus* (MiLBVV) was detected to be causal agent of the big vein symptom in lettuce plants. MiLBVV or mixed infection by both MiLBVV and LBVV has been reported in several European countries, USA, and Japan (Roggero et al. 2003). In our country, the presence of MiLBVV and/or LBVaV has been detected in a limited number of studies conducted on LBVD (Alan and Kamberoğlu 2015; Sertkaya 2015; Zelyüt Randa 2016), but the effects of these viral agents on disease development and symptom expression alone or mixed infections and their molecular characterizations has not been studied.

This study was conducted with the aims of detection of LBVD in the lettuce cultivation areas in Adana and Mersin provinces and to reveal the virus or virus complex causing LBVD and molecular characterization of the virus isolates. For this purpose, collected leaf samples were tested by serological (DAS-ELISA) and molecular (PCR) methods and molecular characterization were performed on isolated viruses that were obtained.

2. Materials and Methods

2.1. Sample collection and storage

The areas of lettuce cultivation in Adana (Ceyhan, Çukurova, Karaisalı, Merkez, Sarıçam, Seyhan, Yumurtalık and Yüreğir districts, and the village of Yakapınar) and Mersin (Akdeniz and Tarsus districts, and the village of Yenice) provinces were surveyed during the autumn and winter periods of 2015-2017.

Leaf samples were taken from lettuce plants suspected to be infected with LBVD as a result of simptomatological observations. The plants showing at least one of the symptoms such as general regression in growth, stunting, deterioration of head development and mosaic, chlorotic areas around the vein tissue, vein enlargement, vein clearing, deformation, blistering, chlorosis and sometimes necrosis in leaves were subjected to the serologic and molecular studies. The collected samples were photographed, numbered, and placed in nylon bags and brought to the laboratory in an ice box. The samples were stored at 4ºC and analyzed within 1 week.

2.2. Serological tests

The presence of MiLBVV in collected samples was tested with commercially available MiLBVV-specific DAS-ELISA kit. The double antibody sandwich enzyme linked immunosorbent assay (DAS-ELISA) method was applied according to the antisera manufacturer's procedure (BIOREBA AG, Reinach, Switzerland) with some modifications.

Plant extracts in extraction buffer were added to plates coated with MiLBVV specific polyclonal antisera in carbonate buffer and incubated at 4ºC overnight. After washing, enzyme conjugated antibody in conjugate buffer was added and plates were incubated for 3 h at 35ºC. MiLBVV infection was detected by addition of substrate after incubating at room temperature for 60-120 min. Absorbance values were measured at 405 nm using an ELISA reader (MEDISPEC ESR-200). The samples with absorbance value two to three times greater than that of negative control were considered to be infected (Wang and Gonsalves 1990). All samples were tested in duplicate and their average results were taken.

2.3. Total nucleic acid (TNA) extraction

TNAs were obtained from leaf of infected lettuce plants according to Astruc et al. (1996). Leaf tissue was extracted in 2 volumes of TE buffer (100 mM Tris-HCl pH 8.0, 50 mM EDTA, 500 mM sodium chloride and 0.1% 2-mercapthoethanol) and centrifuged at 4 000 rpm for 5 min. After adding of 50 µl of 20% SDS, the tubes were kept at 65ºC for 15 min. Then, 250 µl of 6 M potassium acetate (pH 6.5) were added and the extracts were chilled on ice for 20 min. After precipitation in 500 µl ethanol, nucleic acids were resuspended in 50 µl RNAse-free sterile water.

2.4. RT-PCR

RT-PCR studies were performed in a two step procedure as described by Araya et al. (2011) and Navarro et al. (2004) using MiLBVV specific primers (MiLBVV-F- 5'-CAG CAC TTT TTG GAT TTT GTC C-3 ' and MiLBVV-R- 5'-AGA GAA GCC TGT TCC TGC AA-3'), which yielded a 233 nucleotide (nt) fragment overlapping with a region of coat protein (CP) located on viral RNA3 and LBVaV specific primers (VP-248-5'-CGC CAG GAT CTT TGA TCC ATC TG-3' and VP-249-5'-TTG CGA CAT GTT CCT CCT CAT CG-3'), which yielded a 296 nucleotide (nt) fragment overlapping with a region of coat protein (CP) located on viral RNA2. Selected isolates of MiLBVV, Yakapınar (2-MiLBVV 1 E10), Yüreğir (4-MiLBVV 1 F10), Yumurtalık (6-MiLBVV 1 G10), Yenice 1 (11-MiLBVV 2 H10) and Yenice 2 (12-MiLBVV 2 E11) and isolate of LBVaV, Yüreğir (4-VP 248 A12) were used in molecular studies. PCR reactions included an initial denaturation of 2 min at 94ºC, 35 cycles of 30 sec at 94ºC, 30 sec at 55ºC and 2 min at

72ºC, and a final extension for 10 min at 72ºC. PCR products were separated on a 1.5% agarose gel and illuminated under UV light after staining with ethidum bromide (Gallitelli and Minafra 1994).

Sequencing was carried out procuring commercial services. Sequence analyzes were performed using NCBI, BLAST and MEGA 7 programs and the similarity rates of MiLBVV and LBVaV isolates obtained from this study were compared with MiLBVV and LBVaV isolates reported from various countries in the world by phylogenetic trees.

3. Results

3.1. Sample collection and serological tests

During the surveys, 32 fields were visited in 9 districts in Adana province and 14 fields in 3 districts in Mersin province. A total number of 160 samples were collected in Adana (11 samples from Ceyhan, 12 from Çukurova, 21 from Karaisalı, 13 from the Center, 9 from Sarıçam, 21 from Seyhan, 7 from Yakapınar, 15 from Yumurtalık and from 2 Yüreğir districts) and Mersin (2 from Akdeniz, 34 from Tarsus and 13 from Yenice districts) provinces (Table 1).

Various symptoms such as regression in growth and lack of head formation of the plant, yellowing, mosaic, friability, blistering, necrosis, expansion of veins, vein coloration, vein clearing and deformation of the leaves were observed in lettuce plants (Figure 1 and 2).

As a result of ELISA tests, out of the 160 lettuce samples tested, 52 (32.5%) were found to be infected with MiLBVV, 37 of which were from Adana and 15 from Mersin provinces (Table 1).

Table 1. Provinces and districts, number of surveyed fields, tested plants and MiLBVV infected plants.

Provinces	Districts	Number of Fields Sampled	Number of plants tested	Number of plants MiLBVV infected
	Ceyhan	2	11	-
	Çukurova	1	12	9
	Karaisalı	5	21	13
ADANA	Merkez	2	13	1
	Sarıçam	3	9	1
	Seyhan	5	21	7
	Yakapınar	4	7	3
	Yumurtalık	8	15	2
	Yüreğir	2	2	1
		32	111	37
	Akdeniz	2	2	-
MERSİN	Tarsus	8	34	6
	Yenice	4	13	9
		14	49	15
TOTAL		46	160	52

Figure 1. General view of a lettuce plant infected with LBVD.

Figure 2. MiLBVV (a, b) and MiLBVV+LBVaV (c, d) detected plants showing yellowing, necrosis, deformation, blistering, big vein and vein clearing symptoms.

3.2. RT-PCR studies and sequence analysis

RT-PCR studies were carried out in order to molecular detection of MiLBVV and also LBVaV which could not be tested serologically. MiLBVV was detected in 21 samples while mixed infection of MiLBVV+LBVaV was determined in 16 samples by PCR. LBVaV infection was not detected alone in lettuce plants showing LBVD symptoms.

A band of approximately 233 bp was obtained by RT-PCR using MiLBVV specific primers, MiLBVV-F and MiLBVV-R and sequenced. Sequence comparisons showed that selected isolates of MiLBVV (Yakapınar (2-MilBVV 1 E10), Yüreğir (4-MiLBVV 1 F10), Yumurtalık (6-MiLBVV 1 G10), Yenice 1 (11-MiLBVV 2 H10) and Yenice 2 (12-MiLBVV 2 E11) have 95 to 97% nucleotide sequence identity with MiLBVV isolates reported from different part of the world (Table 2).

A phylogenetic tree was constructed using sequences of the CP genes of 15 selected MiLBVV isolates together with Turkish virus isolates, 2-MilBVV 1 E10, 6-MiLBVV 1 G10, 4-MiLBVV 1 F10, 11-MiLBVV 2 H10 and 12-MiLBVV2 E11. Sequence analyses showed that Adana and Mersin isolates of MiLBVV formed in separate groups on phylogenetic tree. While Yakapınar (2-MilBVV 1 E10), Yumurtalık (6-MiLBVV 1 G10) and Yüreğir (4-MiLBVV 1 F10) isolates from Adana province clustered with isolates from Argentina (FJ864680.1, GU295451.1) and Iran (JN576418.1), Yenice 1 (11-MiLBVV 2 H10) and Yenice 2 (12-MiLBVV2 E11) isolates from Mersin province were in the same group with Netherlands (AF525935.1) and Egypt (LT721898.1) isolates in another group (Figure 3).

On the other hand, a fragment with the size of 296 bp was amplified in the PCR by using LBVaV specific primers, VP-248 and VP-249 and sequenced. After the sequence comparison, Turkish isolate of LBVaV (4-VP 248 A12) from Yüreğir in Adana province shared 92-96% sequence identity with the virus isolates reported from other countries of the world (Table 3). The highest similarity rate (96%) was found with an isolate from Saudi Arabia (KU586443.1) while the similarity rate was 92% with an isolate from Japan (AB114138.1).

As a result of sequence analysis of PCR products, the LBVaV found in the Yüreğir isolate (4-VP 248 A12) belongs to the same group on the phylogenetic tree with isolate from Saudi Arabia (KU586443.1, KJ701037.1), the USA (AY496053.1), the United Kingdom (AY496054.1), Australia (AY496055.1) and the Netherlands (AY496056.1) (Figure 4).

Table 2. Origin and genbank accession number of MiLBVV isolates from different parts of the world used for phylogenetic comparison.

Isolates	Origin	Accession Number	% Identity
MiLBVV-ITA1	Italy	AY581699.1	97
MiLBVV-GER3	Germany	AY581698.1	97
MiLV-GAL1	Spain	AY366416.1	97
MLBVV-LP2	Argentina	FJ864680.1	97
CP-5	Argentina	GU295451.1	97
MLBVV-LP1	Argentina	FJ864681.1	97
MiLBVV-DEN1	Denmark	AY581692.1	96
Bauru-58	Brazil	DQ530358.1	95
CP-gene	Egypt	LT721898.1	95
CP-gene	Egypt	LT721900.1	95
LS301-0	Holland	AF525935.1	95
MiLBVV-SH	Iran	JN576418.1	95
MiLV-ALM2	Spain	AY366418.1	95
MiLBVV-HOL2	Holland	AY581693.1	95
MiLBVV-GER1	Germany	AY581695.1	95

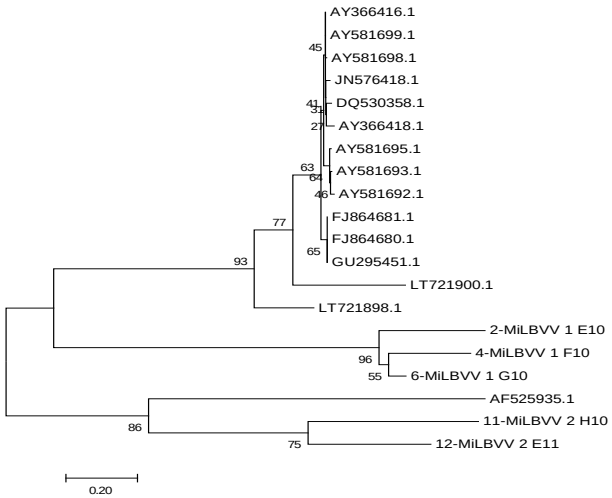

Figure 3. A phylogenetic tree was constructed with nucleotide sequences of 5 isolates of MiLBVV from Turkey and 15 isolates of MiLBVV from different parts of the world (Bootstrap 1000 replicates).

Table 3. Origin and genbank accession number of LBVaV isolates from different parts of the world used for phylogenetic comparison.

Isolate	Origin	Accession Number	% Identity
LBVaV-4U	Saudi Arabia	KU586443.1	96
Ls302	Holland	JN710441.1	95
LBVaV-UK2	United Kingdom	AY581690.1	95
LBVaV-GRA1	Spain	AY581689.1	94
LBVV-ALM1	Spain	AY366413.1	94
AUSB2	Australia	GU220725.1	94
LBVV-GAL1	Spain	AY366412.1	94
LBVV-SA-41	Saudi Arabia	KJ701037.1	94
LBVaV-AUS	Australia	AY496055.1	94
LBVV-NL	Holland	AY496056.1	94
LBVV-UK	United Kingdom	AY496054.1	94
LBVV-USA	ABD	AY496053.1	94
LBVaV-SON5	Spain	AY839622.1	93
LBVV-RNA2	Japan	AB114138.1	92
Isolate:Wa	Japan	AB190528.1	92
Isolate:Hy	Japan	AB190527.1	92
Isolate:A	Japan	AB190526.1	92
LBVV-CP	Japan	AB050272.1	92

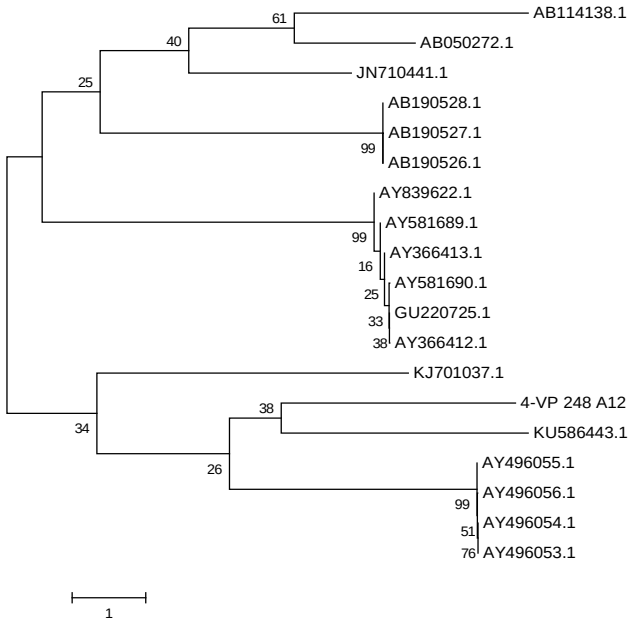

Figure 4. A phylogenetic tree, constructed with nucleotide sequences of a LBVaV isolate (4-VP 248 A12) from Turkey and 18 isolates of the virus from different parts of the world (Bootstrap 1000 replicates).

4. Discussion

In this study, lettuce plants showing the symptoms of vein formation and deformation in their leaves were sampled. Factors causing LBVD (Lettuce big-vein disease) and LBVaV (Lettuce big-vein associated virus) were also researched. The virus isolates obtained via testing by ELISA and RT-PCR were molecularly characterized.

This study showed that MiLBVV by itself or as a mixed infection with LBVaV caused LBVD. Single infection by LBVaV was not found alone in lettuce plants showing LBVD symptoms. Similarly, Hayes et al. (2006) and Araya et al. (2011) reported that in MiLBVV or MiLBVV+LBVaV mixed infections could caused by LBVD symptoms, LBVaV alone didn't have any role in causing LBVD symptoms. In contrast, Roggero et al. (2003), in their serological studies in Italy, determined that MiLBVV was negative and LBVaV was positive in plants showing big-vein symptoms. MiLBVV was accepted as the main factor in this disease, as it was able to develop big vein symptom alone, with this finding backed by studies performed in Italy, France, Japan and Argentina. (Roggero et al. 2003; Lot et al. 2002; Sasaya et al. 2005; Barcala Tabarozzi et al. 2010). However, Navarro et al. (2004) in Spain, Fletcher et al. (2005) in Australia, Pavan et al. (2008) in Brazil, and Alemzadeh and Izadpanah (2012) in Iran in their conducted studies, determined that samples containing both viruses MiLBVV and LBVaV have been reported to cause LBVD. In addition, Al-Saleh et al. (2015) and Heidari et al. (2010) reported that MiLBVV and LBVaV alone or their mixed infections caused LBVD.

In the PCR studies using specific primer pairs for the molecular diagnosis of MiLBVV and LBVaV, bands with a size of 233 bp were observed in the samples infected with MiLBVV and bands with a size of 296 bp were observed in LBVaV infected lettuce samples. The results obtained were similar to the results reported by Navarro et al. (2004) and Araya et al. (2011). The same investigators reported that they observed

bands with 233 and 296 sized bp in samples infected with MiLBVV and LBVaV, respectively.

As a result of the molecular characterization studies, Yakapınar (2-MiLBVV 1 E10), Yumurtalık (6-MiLBVV 1 G10) and Yüreğir (4-MiLBVV 1 F10) isolates from the selected MiLBVV isolates in Adana province were in the same group with Argentina and Iran, and Yenice 1 (11-MiLBVV 2 H10) and Yenice 2 (12-MiLBVV 2 E11) isolates from Mersin province were in the same group with the Netherlands and Egypt isolates. In addition, the Yüreğir isolate of LBVaV (4-VP 248 A12) was similar to Saudi Arabia, Australia, the Netherlands, the United Kingdom and the United States. The isolates of the MiLBVV Adana and Mersin isolates used in this study were included in the same group within themselves on the phylogenetic tree but they were also divided into two groups by forming separate groups among themselves. Maccarone (2013) reported that the presence of MiLBVV and LBVaV isolates in different groups on the phylogenetic tree indicated a second inoculum entry. In addition, Umar et al. (2017) reported that in their phylogenetic studies with MiLBVV and LBVaV, six Saudi Arabia isolates were present in separate groups, but there was a low difference between them, which could possibly be due to multiple inoculums of MiLBVV and LBVaV. They also reported as another possibility that after the introduction of a general inoculum into the country, these viruses could spread from this source or be able to develop variations due to adaptation.

5. Conclusions

This study showed that MiLBVV alone or in combination with LBVaV caused LBVD and MiLBVV produced big vein symptom without depending on the presence or absence of LBVaV. In this study, viral factors causing LBVD were determined and characterized using serological and molecular methods.

It will now be useful to conduct a study to determine the effective control of LBVD and to take necessary measures to determine the sources of inoculum, to determine the virus vector relationships, and to develop and use the resistant and tolerant varieties found.

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