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Van İlinde Yoncada Saptanan Yonca mozaik virüs (AMV) İzolatlarının Kılıf Protein Genomunun Moleküler Karakterizasyonu

Mustafa USTA[1]*, Abdullah GÜLLER[2]

ÖZET: Van ilinde yetiştiriciliği yapılan yonca (*Medicago sativa* L.) bitkilerinde 2019 yılında virüslerin oluşturduğu simptomlara benzer belirtiler görülmüştür. Gözlenen belirtiler arasında cüceleşme, yapraklarda mozaik deseni ve sararma, rozetlenme ve küçük yaprak oluşumu yer almaktadır. Simptom gösteren ve göstermeyen bitkilerden toplanan 19 yonca yaprak örneğine, virüs genomunu tespit edebilmek için uygun primer çiftleri ile RT-PCR testi uygulanmıştır. Yonca yaprağı örneklerinden şiddetli belirtiye sahip altısı beklenen büyüklükte 700 bp DNA band oluşturmuştur. Bunlar arasından rastgele seçilen ikisi bir plazmid vektörüne klonlanmıştır. Elde edilen rekombinant plazmidler her iki yönde dizilenmiştir. Dizi analizi sonuçlarına göre infekteli yoncalardaki virüsün Yonca mozaik virüs olduğu açığa çıkarılmıştır. Dizi bilgileri MT210179 ve MT210178 erişim numaraları ile gen bankasına yüklenmiştir ve sırasıyla Alakoy Y9 ve Alakoy Y1 olarak isimlendirilmiştir. Her iki dizi için gen bankasına kayıtlı 16 AMV dizisi ile oluşturulan filogenetik ağaca göre, her iki izolat da nükleotit düzeyinde ABD, Brezilya ve Puglia izolatları ile en yüksek benzerlik ve Güney Kore izolatı ile en düşük benzerlik oranı göstermiştir. Ayrıca her iki dizinin birbirleri arasında, 7 nükleotit değişikliği ile %98.45 oranında nükleotit benzerliği gösterdiği ortaya çıkarılmıştır. Gerçekleştirilen litaratür tarama çalışmalarına göre, bu çalışma Türkiye'nin Van ilinde yetiştirilen yonca bitkilerindeki Yonca mozaik virüsü (AMV)' nün ilk raporu ve moleküler analizidir.

Anahtar Kelimeler: Dizileme, filogenetik analiz, RT-PCR, karakterizasyon, yonca,

Molecular Characterization of the Coat Protein Genome of *Alfalfa Mosaic Virus* (AMV) Isolates from Alfalfa in Van Province

ABSTRACT: Virus-like symptoms were observed in alfalfa plants (*Medicago sativa* L.) grown in Van province of Turkey in 2019. The symptoms observed were dwarfing, mosaic pattern and yellowing of leaves, rosetting, and decreasing in leaf sizes. Genomic RNA of 19 symptomatic and non-symptomatic alfalfa leaves were extracted for the detection of virus RNA using virus-specific primer pair by RT-PCR. Of the 19 alfalfa leaf specimens, the six yielded the expected 700 bp DNA band in severely symptomatic alfalfa specimens. Two of them randomly selected were inserted into a plasmid vector. Obtained recombinant plasmids were sequenced in both directions. According to the results of the sequence analysis, it was revealed that the virus in infected alfalfa was the *Alfalfa mosaic virus*. The sequence data were recorded into the GenBank with access numbers MT210179 and MT210178 and denominated as Alakoy Y9 and Alakoy Y1 isolates, respectively. According to the phylogenetic tree created with 16 AMV sequences registered in the GeneBank for both sequences, the AMV CP gene sequence indicated the highest similarity with USA (JN256023), Brazil (FJ858265), and Puglia (Y09110) isolate and the lowest with Korea isolate (KY348844), at the nucleotide level. It was also revealed that both sequences show 98.45% nucleotide similarity with 7 nucleotide exchanges. According to the literature research results, this is the first report and molecular analysis of *Alfalfa mosaic virus* (AMV) in *Medicago sativa* L. in Van province of Turkey.

Keywords: alfalfa, characterisation, phylogenetic analysis, sequencing, RT-PCR,

[1]Mustafa USTA (**Orcid ID:** 0000-0002-3940-2774), Department of Plant Protection, Faculty of Agriculture, Van YuzuncuYil University, Van, TURKEY
[2]Abdullah GÜLLER (**Orcid ID:** 0000-0003-3887-4208), Department of Plant Protection, Faculty of Agriculture, Bingol University, Bingöl, TURKEY

*Sorumlu Yazar / Corresponding Author:Mustafa USTA, e-mail: mustafausta@yyu.edu.tr

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INTRODUCTION

Alfalfa crops, known botanically as *Medicago sativa* L., is a perennial legume fodder plant and is mostly cultivated in more than 80 countries due to its importance. Based on archaeological records, it has been cultured in southwest Asia about 4000 years ago and has been cultivated to date (Radovic et al., 2009; Dale et al., 2012). Due to its high protein value, high digestibility, rich nutrition content, perfect forage quality, and improve soil texture, it is preferred more than almost all other forage crops. Alfalfa roots and leaves can also be used in industry as raw materials (i.e. ethanol, biodegradable plastic), bioenergy production and its flowers can serve as a source of pollen and nectar for honey bees (Soya et al., 2004; Geren et al., 2009; Monteros and Bouton, 2009;Taha, 2015). It can survive ranging from -50 °C to 60 °C temperatures and can adapt highly to different climatic conditions and altitudes (Moreira and Fageria, 2010; Kavut and Avcıoglu, 2015). Although this is so and its cultivation area increases, alfalfa production is not at the desired level. In Turkey, a total of 2609878 hectares has been allocated for planting forage crops, but approximately 2.49 % of it has been used for alfalfa (Anonymous, 2017). Turkey's alfalfa crop yield (2675 kg da^{-1} is well above the world average (729 kg da^{-1}) (Anonymous, 2014). The principal regions for alfalfa production in Turkey include Van, Muş, Iğdır, Sivas, Erzurum, Konya, and Aksaray (TUIK, 2017). Van province, eastern Turkey located, ranks first in terms of the planting area (1042504 da) and the crop quantity of alfalfa (2050522 tons), according to TUIK, (2017a). The vast majority of communities living in rural areas are occupied in agriculture and animal husbandry for a livelihood. The feed requirement of livestock is usually met by natural meadow and pasture areas. However, roughage production is limited due to the short vegetation period (Turan et al., 2017).

Besides physiological imbalances and pests, many fungal ve bacterial diseases also attack to the alfalfa plant, including rust, common leaf spot, anthracnose, fusarium and bacterial wilt, bacterial leaf spot, dwarf, and crown gal (Palumbo et al., 1998; Samac et al., 1998; Sisterson et al., 2010; Ali et al., 2011; Vasić et al., 2011; Qin et al. 2016; Peterson et al., 2018).

Alfalfa health can be affected by about thirty-one viruses (Erwin et al., 1990), including AMV (Alfalfa mosaic), provisionally *Alfalfa virus S* (AVS), Alfalfa enation (AEV), Bean yellow mosaic (BYMV), Cucumber mosaic (CMV), Bean leaf roll (BLRV), Tobacco streak (TSV), Bean common mosaic (BCMV), Clover vein yellow (CVYV), Pea enation mosaic (PEMV), Clover yellow vein (CLYVV), and Peanut stunt (PSV) (Paliwal, 1982; Cook and Wilton, 1984; Rahman et al., 1993;Alan et al., 1996; Shah et al., 2006; Massumi et al., 2012; Al-Saleh and Amer, 2013; Trucco et al., 2016; Al-Shahwan et al., 2017; Nemchinov et al., 2017).

Amongst 31 viral agents, AMV is a prevalent dangerous pathogen of alfalfa. In addition to the pathogenicity of AMV, the synergistic effect of two or more viruses can adversely affect the yield, durability, and quality of alfalfa. AMV is a typical member of the genus *Alfamovirus*, which belongs to the Bromoviridae family. AMV can infect a wide-host range, mostly belonging to the Solanaceae, Compositae, Fabaceae, and Umbelliferae families, containing about 600 plant species (Jaspars and Bos, 1980; Brunt et al, 1990). The virus is phloem-limited and can rapidly spread from plant to plant in a non-persistent manner by a minimum of fourteen aphid species fed with stylets, especially *Myzus persicae* (Sulzer) (Šutic et al., 1999; Ragsdale et al., 2001). The virus has also been reported to be transmitted by seeds, pollen, and *Cuscuta* spp (Frosheiser, 1974; Hemmati and McLean, 1977). AMV posses a single strand positive sense genome (+ssRNA) with 4 segments consisting of 3 genomics, and 1 subgenome. RNA1 and RNA2 are responsible for viral replicase proteins. RNA3 and subgenomic RNA4 (sgRNA4) are responsible for direct translation of movement protein (MP), and coat protein (CP) synthesis, which is necessary for infection, respectively (Tenllado and Bol, 2000; Bol, 2003).

Although AMV on *M. sativa* or the other plants has been declared elsewhere in Turkey, information associated with the prevalence of disease and its characterization has poorly reported. This current study depicts the symptoms and molecular characterization of AMV as the causal agent of mosaic disease on alfalfa in Van province.

MATERIAL AND METHOD

Plant specimens

In 2019, alfalfa-growing fields, where are the Alakoy district of Van province, were visually investigated for the occurrence of plants showing symptoms typical of AMV infection as defined below. A total of 20 samples were collected and brought to the virology laboratory in an icebox for further extraction and detection of the virus. Besides the conspicuous symptomatic plants, non-symptomatic specimens were also collected due to the possibility of latent viral infection.

Total RNA extraction and first strand complementary DNA (cDNA)

The extraction of RNA of the test virus was made from the mature leaves of alfalfa with the silica-capture method, with minor modifications (Foissac et al., 2001). Obtained RNAs were stored at -20 °C until the cDNA for subsequent use.

Total RNAs were utilized as a template for reverse transcription reaction by using the CP-specific antisense primer. The first strand complementary DNA synthesis was performed in two steps. In the first step, a volume of 12 µl mixture containing 5 µl of RNA, dNTP mix of 1 µl, reverse primer of 1 µl (available below), and RNase free water of 5 µl was incubated at 65 °C for 5 minutes, then directly kept in ice for 5 minutes. The mixture obtained in the first step was completed to 20 µl using the 5X RT buffer (4 µl), 0.1M DTT (2 µl), RNase-free water (instead of inhibitor) (1 µl), and RT enzyme (1 µl) and incubated at 42 °C for 50 minutes, followed by held at 70 °C for 15 minutes to terminate the reaction. Obtained cDNAs were kept in the deep-freezer to avoid RNA degradation until later use.

AMV detection by PCR

The synthesized cDNA was used as the basis for the PCR reaction. Two AMV-specific oligonucleotide primers and PCR schedule to amplify a portion of the AMV CP gene were used as specified by Martinez et al. (2004). PCR assay was carried out in an automated thermal cycler (Eppendorf, USA) in a final volume of 25 µl containing 2.5 µl of 10× Taq Buffer, 1.5 µl of $MgCl_2$, 1 µl of total cDNA, 0.5 µl each dNTP (10 mM) mix and, primer pairs with 0.2 µl of Dream Taq DNA polymerase (Fermentas, USA), 18.3 µl of Nuclease-free H_2O. To control the presence and size of the PCR yields, fifteen µl of cycled DNAs and DNA ladder (Fermentas, 1000 bp) were loaded into agarose gel containing Ethidium bromide (EtBr) in Tris Acetic EDTA (1×TAE) buffer and run using electric current (60 minutes at 80V). Separated DNA fragments were visualized and captured using UV illumination and gel documentation device. The cDNA from healthy alfalfa extract was used as the negative control. The AMV isolate, which was recently characterized by sequence analysis for this study, was used as a positive control.

Cloning of amplified products and Sequencing of DNA

Among the positive reaction specimens, two were randomly selected and purified from agarose gel using the GeneJET Gel Extraction Kit (Thermo Scientific™), according to the supplier's specifications. Purified DNA fragments were directly cloned into the pGEM T-Easy vector (Promega). Recombinant DNA products were transformed into *E. coli* (JM 109 strain) by electroporation. The recombinant plasmid containing cDNA inserts were purified using the GeneJET Plasmid Miniprep Kit

(Thermo Scientific™), and sent bi-directionally for sequencing (Sentebiolab, Ankara, Turkey). Both resulting sequences were recorded in the GeneBank (NCBI, National Center for Biotechnology Information).

Phylogenetic relationship

The phylogenetic dendrogram was created with two Van AMV sequences and other AMV sequences derived from the GeneBank. The evolutionary relationship was calculated with the neighbor-joining method (NJM) of the CLC Main Workbench 6.7.1.The robustness of the trees was identified by bootstrap using 100 resamplings to support for the branches. *Barley yellow dwarf virüs*-PAV (BYDV-PAV) CP gene (KC900900) was defined as outsource to root the tree. Sixteen isolates were selected to reveal phylogenetic relationships.

RESULTS AND DISCUSSION

Symptoms of infected alfalfa

During the visual assessment, symptomatic plants exhibiting mosaic and chlorosis in alfalfa leaves were observed as shown in Fig 1.



Figure 1. Symptoms of *Alfalfa mosaic virus* on alfalfa leaves, including lateral yellowish streaks to leaf midribs and yellow-green mottling

Viral detection of AMV in field-infected alfalfa

Leaf extracts from the infected and symptomless alfalfa specimens planted under natural conditions in Van province were screened by RT-PCR aganist the AMV occurrence. PCR- amplified products revealed a typical bands of 700 bp size in agarose gel, corresponding to the viral CP genom. As shown in Fig 2, amongst 19 specimens of alfalfa tested, 6 of 12 symptomatic specimens reacted with AMV-specific primers, but remaining others (13) were negative for AMV.

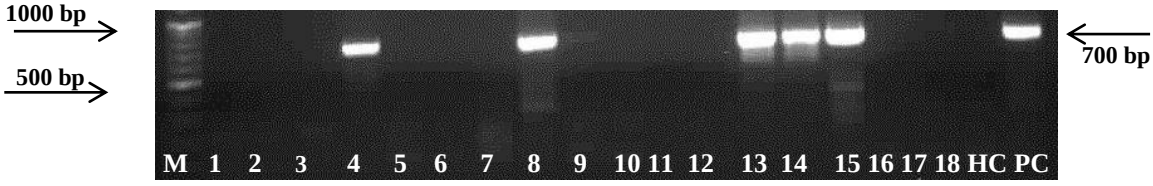


Figure 2. Electropherogram resulted from the PCR tests applied to the alfalfa AMV infected specimens taken from the Van province. M: 1000bp standart DNA ladder; 4, 8, 13, 14, 15: Infected alfalfa specimens from Van; HC: Healthy alfalfa control; PC: Positive control;

Sequence identity and Phylogenetic relationship

Van- AMV sequences obtained from alfalfa were submitted to the GenBank database under accession number MT210179 and MT210178 and denominated as Alakoy Y9 and Alakoy Y1, respectively. Both sequences were nearly similar (98.45%) when analyzed between them, indicating that

low genetic variability within the Van isolates. Further characterization showed that 7 bases exchange was found between the two CP sequences at different positions. Namely, the Alakoy Y9 (MT210179) isolate has Adenine (A) instead of Guanin (G) at 71 positions, Cytosine (C) instead of Timin (T) at 194 positions, Timin (T) instead of Cytosine (C) at 217 positions, Cytosine (C) instead of Timin (T) at 348 positions, Adenine (A) instead of Guanin (G) at 357 positions, Timin (T) instead of Adenine (A) at 524 positions, and Cytosine (C) instead of Timin (T) at 348 positions at 631 positions compared to Alakoy Y1 (MT210178) isolate. That finding provides a genetic basis for future manipulation of the AMV reverse historical evolution, since its existence (Fig. 3).



Figure 3. Alignment showing the nucleotide consensus between two related sequences (MT210178 and MT210179) created by CLC Main Workbench 6.7.1

Although the host range of the 15 AMV isolates characterized in other countries was variable, the molecular properties of their on different hosts were slightly different. As tabulated in Table 1, the nucleotide sequence similarity among all isolates ranged from 93.95-98.60%. Alakoy Y9 sequence (MT210179) had the highest identity to sequences of Brazil (FJ858265, alfalfa isolate), Puglia (Y09110, tomato isolate) and USA (JN256023, soybean isolate) by 98.29%, while Alakoy Y1 sequence (MT210178) was with Brazil (FJ858265) and Puglia sequences by 89.60%. The lowest similarity of both of them (94.26%) was found between one isolate from *Trifolium repens* in South Korea (KY348844) when compared with all isolates. Based on the similarity incidence amongst different AMV sequences, we can say that its sequence similarity is not dependent on either the host or the region. As in Fig 4, the consensus tree of AMV isolates constructed from the 700 nt size CP gene supported these results. Turkish Van isolates of AMV are highlighted by underlining.

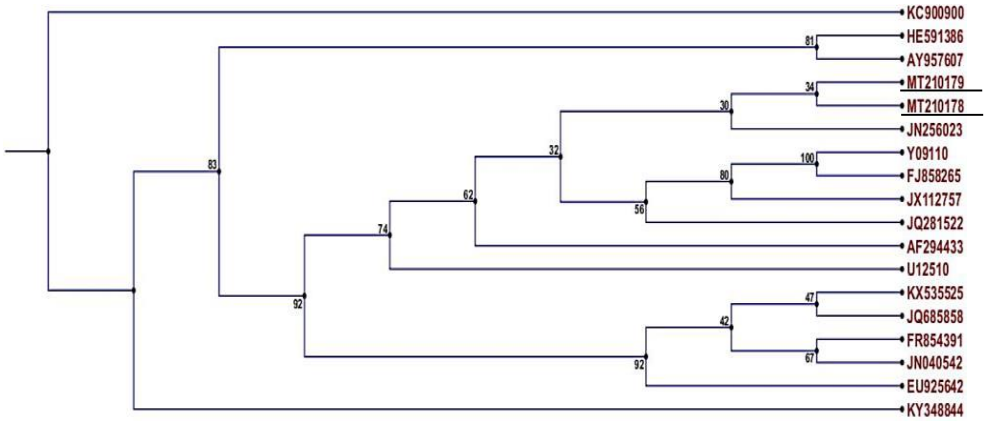


Figure 4. The phylogenetic dendrogram, created by the (NJM), showing the phylogenetic relationship of *Alfalfa mosaic virus* from alfalfa of Van/Turkey and those available in GenBank, based on CP gene

Table 1. Nucleotide similarity incidence of CP gene sequences among AMV Van/Turkey isolates along with those of AMV isolates originating in various locations downloaded from NCBI

Accession Number	Country	Host	Isolate/Strain	Nucleotide similarity (%)	
				MT210179	MT210178
JQ685858	Iran	*Medicago sativa*	Ke.Sh.Al	96.59	97.36
FR854391	Italy	*Teucrium fruticans*	Tef-1	96.74	97.52
JN040542	Chile	*Viburnum tinus*	-	97.05	98.14
FJ858265	Brazil	*Medicago sativa*	-	**98.29**	**98.60**
Y09110	Puglia	*Solanum lycopersicum*	Danza	**98.29**	**98.60**
JX112757	Australia	*Medicago sativa*	EW	97.67	97.98
JQ281522	China	*Medicago sativa*	-	97.05	97.36
JN256023	USA	*Glycine max* (soybean)	SturdyII	**98.29**	97.98
EU925642	Serbia	*Syringa vulgaris* (lilac)	100-08	96.12	96.90
AY957607	Mexico	*Leonotis nepetaefolia*	-	95.19	95.19
HE591386	Spain	*Hibiscus rosa-sinensis*	TM2	94.88	95.50
KX535525	Iran	*Plantago sp.*	Kh.Sa.Pl	96.28	97.05
AF294433	Korea	*Solanum tuberosum*	KR2	97.36	97.36
U12510	New Zealand	*Medicago sativa*	NZ2	97.05	97.52
KY348844	South Korea	*Trifolium repens*	Gimcheon	93.95	94.26
MT210179	**Turkey**	***Medicago sativa***	**Alakoy Y9**	**98.45**	**98.45**
MT210178	**Turkey**	***Medicago sativa***	**Alakoy Y1**	**98.45**	**98.45**

AMV is a prevalent virus that causes infection in economic crops. AMV was first described in 1931 and then its presence from different geographical region was reported formerly (Weimer, 1931), including in South Australia from lucerne (Hajimorad and Francki, 1988), in USA from the snap bean, chickpea, and tumble pigweed (Shah *et al*., 2006), in Saudi Arabia from cowpea, potato, eggplant and weeds (Al-Shahwan et al., 2017; Abdalla et al., 2019), in Spain from the ornamental plant lavandula, *Tecoma capensis*, pepper, tomato and common borage (Mallor et al., 2002; Martínez-Priego et al., 2004; Parrella et al., 2011), in Canada from potato (Xu and Nie, 2006), in Poland from yellow lupin (Frencel and Pospieszny, 1979), in North Cyprus from alfalfa, pepper, and potato (Fidan et al., 2012), in Iran from alfalfa, potato, pepper, cowpea, and mung bean (Mangeli et al., 2019), in Lithuania from tomato (Zitikaitė and Samuitienė, 2008), in Czech Republic, Serbia, Hungary, and Slovenia from pepper (Petrovic et al., 2010; El-Helaly et al., 2012; Svoboda and Svobodová-Leišová, 2012), in *Viburnum tinus* in Chile (Peña et al., 2011), in *Teucrium fruticans* in Italy (Parrella et al., 2012), in *Carica papaya* in Brazil (Moreira et al., 2010).

AMV infections of alfalfa naturally have been reported in Australia (Garran and Gibbs, 1982), Canada (Paliwal, 1982), New Zealand (Forster et al., 1985), western North America (Rahman and Peaden, 1993), Lithuania (Staniulis, 1994), Jordan (Sawalha and Mansour, 1996b), Spain (Mallor et al., 2002), Oman (Mughal et al., 2003), USA (Shah et al., 2006), Iran (Massumi et al., 2012), Italy (Parrella et al., 2012), and Saudi Arabia (Al-Saleh and Amer, 2013).

In this study, we attempted to identify the causal agent in the alfalfa specimens in Van, 2019. The diagnosis of AMV isolates can broadly be detected by serological methods such as ELISA assay and dot blot hybridization (Al-Shahwan et al., 2017; Alhudaib, 2019) and molecular methods such as PCR-based techniques (Parrella et al., 2012). In the current study, the RT-PCR method is used for AMV-detection. In six out of 19 alfalfa specimens, AMV was detected, but not detected in 13. Six specimens showing virus-like symptoms were negative for AMV. This situation may be caused by physiological disorders, abiotic factors or the presence of another pathogen, such as a virus or phytoplasma.

Many records are available in the literature, regarding the determination of AMV by RT-PCR test from symptomatic plants. Data presented in Fig. 2 show that RT-PCR- amplified Van AMV RNAs are resulted in an amplification product size of approx. 700 bp in agarose gel. The size of these sequences supported the specificity of the primers in our study, which also used by various researchers (Al-Saleh

and Amer, 2013; Abdalla et al., 2019), but not that of described by Xu and Nie (2006), Al- Abrahim (2014) (351 bp), Massumi et al., (2012) (780 bp), and Zitikaitė and Samuitienė, (2008) (620 bp). This difference arises from different primers designed specifically for the CP gene used by the researchers.

The most typical symptoms produced by AMV-Van isolates in alfalfa were the yellow patch areas parallel to leaf veins on the leaf, little leaf, and vein banding that consistent with the symptoms in many reports described by various researchers (Bailiss and Ollennu, 1986; Al-Saleh and Amer, 2013;Al-Shahwan et al., 2017).

Although AMV has been present in Turkey for about 40 years (Çıtır, 1982), little data is available about the prevelance and molecular characteristics of its. In addition, studies on the AMV virus of alfalfa are scanted. The first study on Alfalfa AMV was performed by Erdiller and Laseman, (1985) in Ankara, followed by Çalı, (1990) in central Anatolia and, in Erzurum (Yardımcı and Açıkgöz, 1997) using mechanical inoculation, serological immunoelectron microscopy tests.

AMV infection has a destructive effect on alfalfa worldwide, due to reduces the feed protein and yield, adversely affects seed germination, reduces nodulation and nitrogen binding ability via bacteria living its roots and, causes yellowing by degrading chloroplasts (Bailiss and Oilennu, 1986; McLaughlin and Boykin, 1988; Balasubramaniam et al., 2014). Accordingly, it causes significant crop loss at varying rates in cultivated plants such as tomatoes, peppers, potatoes and alfalfa from nationwide and worldwide. Natural AMV disease in Turkey have been detected in the potato (*Solanum tuberosum*) in Hatay, Erzurum, Balıkesir province (Özdemir et al., 2011;Sertkaya et al., 2017), and in the Jimson Weed (*Datura stramonium* L.), in the eggplant in Manisa (Ozdemir et al., 2011), in the bean and its seeds in Burdur and Samsun (Güzel and Arlı-Sökmen, 2003;Çulal Kılıç and Yardımcı, 2015), and husk tomato in Hatay (Sertkaya et al., 2013; Sertkaya et al., 2017), pepper in Samsun (Arlı-Sökmen et al., 2005; Özdemir and Erilmez, 2007), red pepper in Kahramanmaraş, Şanlıurfa and Gaziantep (Demir, 2005; Buzkan et al., 2006), pepper and alfalfa in Adana and Mersin (Çetinkıran and Baloğlu, 2011).

AMV has an increased risk from year to year, depending on natural inoculum sources and effective vectors. Spreading from the alfalfa to the surrounding susceptible economic crops, which are the hosts of AMV in adjacent fields through aphids is common. Informing farmers about viral transmission and following the proper management strategy about this disease is important for both preventing the viral spread and minimizing future crop losses.

Also, it will be useful to follow the situation of AMV symptoms and other viral agents in the cultivated production fields. Viral infections cannot be cured with any drug in chemical-manner. Hence, to limit crop losses of alfalfa and to refrain the epidemic of AMV, it is recommended to focus on its vectors and the destruction of weeds that have natural epidemiological reservoirs during the off-season times. Plus, the cultivating of resistant ones among 87 varieties of alfalfa will also reduce the risk of viral infections (Hiruki and Miczynski, 1990; Small, 2011).

CONCLUCION

Despite AMV importance, information on the variance and genome structure of its natives is limited and has not been investigated in detail before. According to the available literature, this article is the first etiology record of alfalfa mosaic virus that naturally infects alfalfa plant (Medicago sativa) in Van province of Turkey, confirmed with the sequence analysis of the causal virus, at the molecular level.

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