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Partial Capsid Protein Gene Sequence Analysis of *Apple Mosaic Virus* Infecting Apple, Plum and Hazelnut in Turkey

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ABSTRACT

Coat protein (CP) sequences of *Apple mosaic virus* (ApMV) isolates were obtained from apple, plum and hazelnut. These isolates were initially tested by DAS-ELISA. Five out of 38 randomly selected apple, hazelnut and plum trees in Isparta, Düzce and Amasya provinces, respectively were ApMV-infected for determining similarities or differences among Turkish ApMV isolates. The isolates were collected in 2008-2010. Amplification of target regions of selected five isolates was conducted by RT-PCR using coat protein specific primers. PCR products gave bands of 262 bp in gel electrophoresis. Sequence data of 262 bp of the partial coat protein region of the ApMV isolates were obtained using F 5’ AGTAATCCGAAAGGTCCGAATCCGAT 3’ primer. All sequences were compared with ApMV sequences in NCBI and our sequences showed 88-99% similarities with those. Our isolates accession numbers are as follows: HM245753, HM490310, HM490311, HM245751, HM245752. They were located in the same group and it was not seen any differences among them.

Keyword: Apple, Plum, Hazelnut, RT-PCR

INTRODUCTION

Apple mosaic virus (ApMV) (genus *Ilarvirus*, *Bromo viridae*) is an isometric virus with segmented ssRNA genome. Its RNA 3 codes for movement protein and capsid protein (CP). The CP gene of *ilarvirus* is translated from RNA 4, a subgenomic messenger derived from the bicistronic RNA 3. The genomic RNA 1 and RNA 2 encode replication enzymes, the first ORF of RNA 3 encode the movement protein. The CP *ilarviruses* not only forms the shell for the three genome components, but it also plays a major role initiation and propagation of infection (Bol, 1999). ApMV is named after the disease in which causes in apple, the first host in which it was described (Bradford and Joly, 1933). Many woody plants of over 65 species in 19 families (Brunt et al., 1996; Gotlieb and Berbee, 1973; Postman and Cameron, 1987; Sweet and Barbara, 1979; Wong and Horst, 1993) are susceptible hosts of ApMV including rose, hop, birch, raspberry, hazelnut, strawberry, apricot, sweet cherry, almond, red currant, blackberry and raspberry (Crop Protection Compendium 2003) and induces bright yellow patterns on leaves (Nèmeth, 1986). *Prunus domestica* (plum) and *Prunus persica* (peach) are secondary hosts, although there has been no vector reported. ApMV is not pollen-borne except *Rubus* spp. It does not occur in seedling rootstocks and has not been identified in naturally infected weeds. However, its spread through soil in nurseries via root grafting has been observed (Hunter et al., 1958; Dhingra, 1972). The virus is graft-transmissible and it persists in propagative material, which is probably the main source of virus infection.

ApMV has been found in birch, hop, rose and other woody hosts and a number of synonyms of the virus have been assigned, e.g. *European plum line pattern virus, Mountain ash variegation virus, Birch line pattern virus, Birch ringspot virus, Dutch plum line pattern virus, Horsechestnut yellow mosaic virus, Rose mosaic virus, Hop virus A, Hop virus C, Mild apple mosaic virus* and *Severe apple mosaic virus.*

In our country, lots of studies were conducted on ApMV, but majority of them were focuced on detection, damage, spread and dispersal of the virus (Çağlayan et al., 2003; Ulubaş and Ertunç, 2003; Arlı-Sökmen et al., 2004; Akbaş and İlhan, 2005; Arlı-Sökmen, 2005; Yılmaz et al., 2005; Çağlayan et al., 2006; Yardımcı et al., 2006; Dursunoğlu and Ertunç, 2008; Akbas and Değirmenci, 2009; Akbas and Değirmenci, 2010a; Akbas and Değirmenci, 2010b; Değirmenci and Akbas, 2011). However, in the study of Ertunç et al., (2011), molecular characterization of Turkish isolates was reported. In that study, Turkish isolates were compared with Ukranian isolates. The aim of this study was to compare ApMV isolates of apple, hazelnut and plum trees and to determine if a correlation exists between the nucleotide sequence of the CP gene and the natural hosts of the virus. This paper also reports the comparison of partial nucleotide sequences established for five Turkish ApMV isolates and some ApMV isolates selected from NCBI.

MATERIALS AND METHODS

Flowering shoots and leaves were collected from apple, hazelnut and plum trees growing in three different locations of Turkey (southern, the Black Sea coast and northern Turkey, respectively). All collecting samples of the trees were production orchards. The samples were collected from symptomatic trees showing ring and line patterns from chloroting to yellowing. These samples were firstly tested for the presence of ApMV by DAS-ELISA. DAS-ELISA was performed with a commercial kit (Agdia, USA) using the manufacture's protocol. Samples from ApMV-symptomatic 22 apple, 35 hazelnut and 32 plum trees were obtained from Isparta province (southern part), Düzce province (the Black Sea coast) and Amasya province (northern part), respectively in 2008-2010 (Fig.1). The high sequence identity of ApMV isolates originating from geographically distant localities points to a hypothesis of a host-conditioned modification and a fixation of nucleotide substitutions in natural hosts compared to each other.

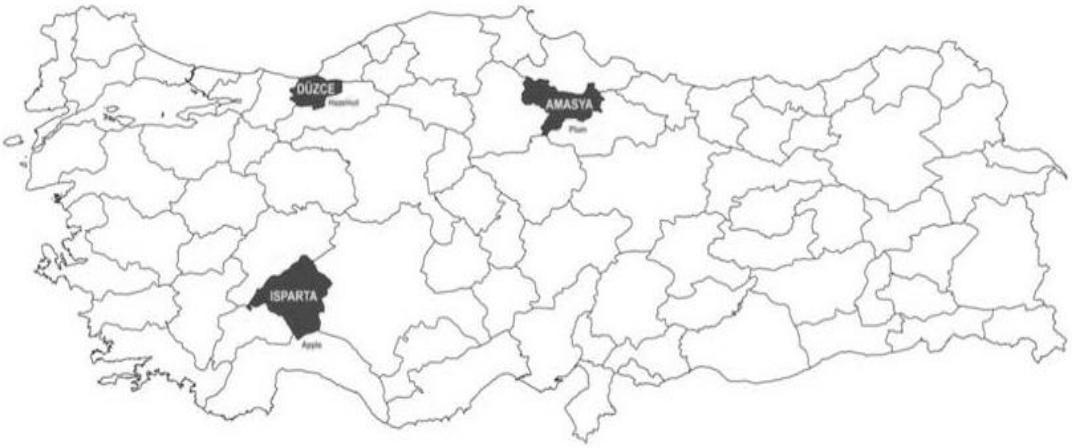

Figure 1. Samples collected provinces

Total RNA was isolated from 100 mg of plant tissue (flowers or leaves) by Foissac et al. (2001) methods. One step RT-PCR was done with the Access RT-PCR kit (Qiagen) and ApMV specific primers: Forward 5′–3′ ATCCGAGTGAACAGTCTATCCTCTAA, reverse 5′–3′ GTAACTCACTCGTTATCACGTACAA were used previously described by Menzel et al. (2002) to amplify of viral sequences specifically a 262 bp product.

The RT-PCR mixture (final volume of 50 µl) contained 5 µl template RNA, 2.5 µl of 5x reaction buffer (Sigma), 1mM dNTP mixture. The mixture also contained 3 mM MgCl2, 2.5 U avian myeloblastosis virus (AMV) reverse transcriptase, 2.5 U *Taq* DNA polymerase, 2 pmol each with reverse and forward primers. In the cycling condition, reverse transcription step was carried out at 37°C for 50 min, activation of the Taq polymerase at 94°C for 2min, followed by 35 cycles of 94°C at 30 s, 52°C at 30 s, 72°C at 1min, and a final extension step at 72°C for 7 min. Amplifications were carried out in a eppendorf mastercycler gradient thermal cycler. PCR products were separated by electrophoresis in 1.5% agarose gels in TAE buffer, stained with ethidium bromide and visualized under UV light.

PCR products were sequenced directly by Iontek Inc. and compared to some ApMV sequences. Sequence analysis were done by using forward primers as unilateral. Phylogenetic tree constructed with partial nucleotide sequences from the CP gene of *Apple mosaic virus* (ApMV) isolates (Düzce-Hocaoğlu-Hazelnut, Düzce-Gölormanı-Hazelnut, Amasya 9-Plum, Amasya 5-Plum, Eğirdir-Apple) from different provinces in Turkey.

Phylogenetic analyses were done by Mega 4.0 package with selected some ApMV sequences from NCBI. Topology of trees was inferred by means of MEGA 4.0 program were performed to check the statistical significance. Additional data of previously sequenced ApMV isolates were used for the phylogenetic analysis for acquaint with our isolates source and relationships between the other isolates. The sequences were searched for by computing the phylogenetic profiles with the program MEGA 4.0 (Tamura et al., 2007).

RESULTS AND DISCUSSION

Detection of ApMV isolates in orchards

Thirty eight out of testing of 89 samples (22 apples from Isparta, 35 hazelnuts from Düzce and 32 plums from Amasya) showed the presence of ApMV infections. Thirty eight samples detected by ELISA were confirmed by one step RT-PCR. Nine apple, 13 plum and 16 hazelnut samples were found to be infected according to test results. RT-PCR amplification of theApMV isolates gave a product of approximately 262 bp with the ApMV primer set corresponding to the CP of ApMV (Fig.2).

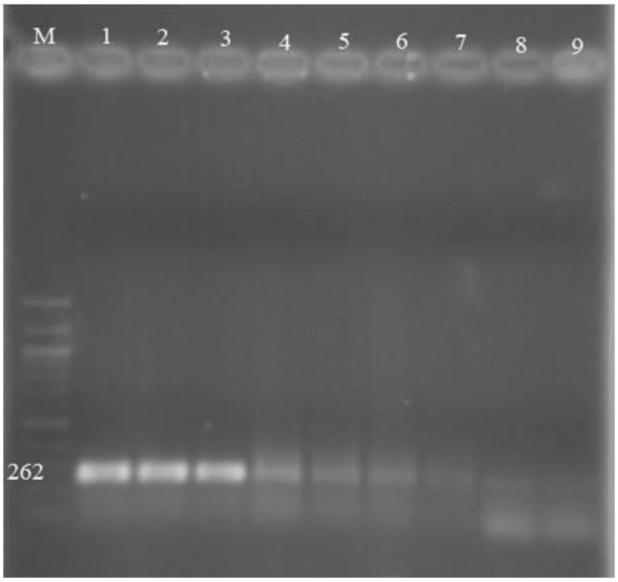

Figure 2. Gel electrophoresis of RT-PCR products of *Apple mosaic virus* (ApMV) with specific primers. Lane 1-3, ApMV apple isolates (from Isparta); lane 4–6 ApMV plum isolates (from Amasya); lane 7–9 ApMV hazelnut isolates (from Düzce)

Sequence data and phylogenetic analysis

Five ApMV *CP* gene PCR products were subsequently sequenced. The alignment of nucleotide sequences of five ApMV-Turkish isolates, named Eğirdir (Acc. No. HM245753-apple isolate), Amasya-9 (Acc. No.HM490310-plum isolate), Amasya-5 (Acc. No. HM490311-plum isolate), Düzce-Hocaoğlu (Acc. No. HM245751-hazelnut isolate) and Düzce-Gölormanı (Acc. No. HM245752-hazelnut isolate), with the corresponding gene sequence of the type member in GenBank (Acc. no: AF473588-hop isolate), showed a level of nucleotide variation ranging from 2 to 9 %.

By using Australian, Korean, Indian, Brazil and Czech Republic ApMV isolates (AF 473593,AF 548367, FN 564150, GQ 131805 and AY 542542) in NCBI, phylogenetic tree of these five ApMV isolates were constructed. Accordingly, the phylogenetic tree placed the Turkish isolates in two most parsimony trees. The apple isolate (Eğirdir) was closely related to both plum isolates in same sub-clade, whereas it was not related hazelnut isolates in another sub-clade. Two hazelnuts were appeared in same sub-clade with Indian apple isolate (FN564150), whereas our other isolates were placed in other sub-clade. The other Turkish ApMV isolates from apple and hazelnut hosts in GenBank were not related with our ApMV isolates surprisingly. The other plum isolate (Amasya-9) was not taken part in any subclades, however it was closely related with two subclades containing Australian and Indian ApMV isolates, but far from ApMV Turkish hazelnut isolates (Fig.3).

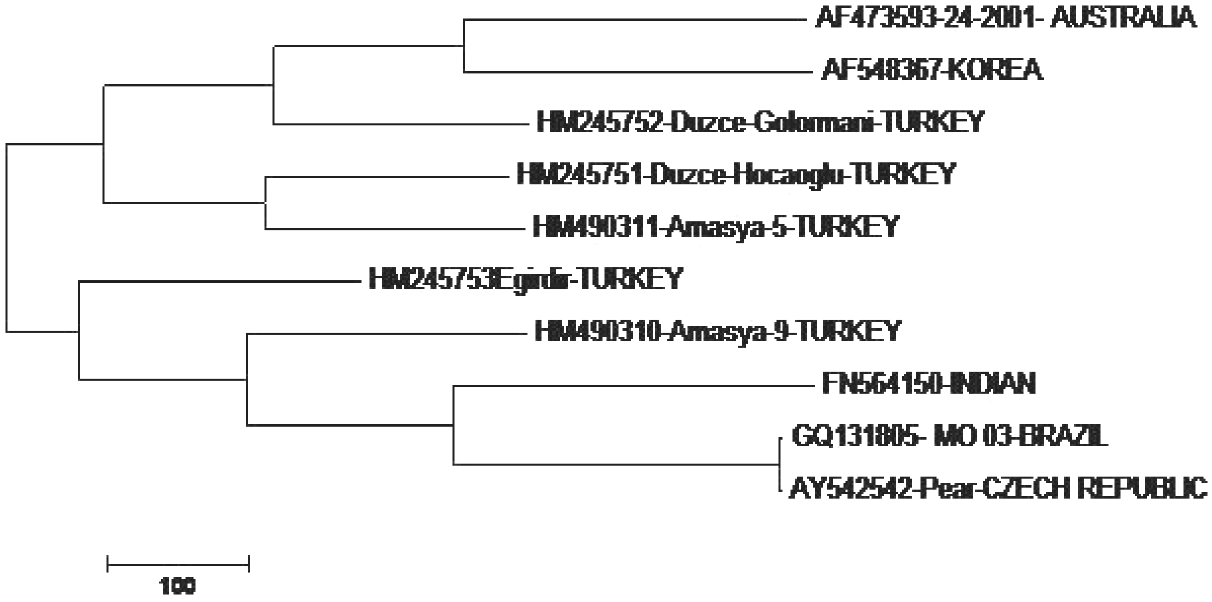

Figure 3. Phylogenetic trees inferred by parsimony analysis of the putative coat protein

The evolutionary history was inferred using the Maximum Parsimony method. The most parsimonious tree with length = 2653 is shown. The consistency index is 0.613268 (0.611364), the retention index is 0.410684 (0.410684), and the composite index is 0.251859 (0.251077) for all sites and parsimony-informative sites (in parentheses). The MP tree was obtained using the Close-Neighbor-Interchange algorithm (Nei and Kumar, 2000) with search level 1 [0, 1] in which the initial trees were obtained with the random addition of sequences (10 replicates). The tree is drawn to scale, with branch lengths calculated using the average pathway method and are in the units of the number of changes over the whole sequence. The analysis involved 10 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 619 positions in the final dataset. Evolutionary analyses were conducted in MEGA4 (Tamura et al., 2007).

This is the first report of comparison of different ApMV isolates, taken from different host (apple, hazelnut, plum) in Turkey. Our study extends knowledge on virus sequence analysis in those crops in Turkey for which only scanty information was previously available. These partial sequences of ApMV isolates were compared with different ApMV isolates from different site of the world in NCBI. One apple isolate was similar to 80-89% the other Turkish isolates in NCBI, 83-95% with the other ApMV isolates in NCBI.

The hazelnut isolates were similar to 90-100% the other Turkish ApMV isolates in NCBI, 91-98% the other isolates in NCBI. The plum isolates 86-95%, 83-94% with the other isolates in NCBI. This could explain that our ApMV isolates show the high similarities with the other ApMV isolates in NCBI.

Analysis of aligned CPnt sequences revealed five ApMV isolate clusters with each other closely related (Fig. 2). Within the ApMV isolates, one group contained two hazelnut isolates. The remaining isolates formed two or three close but distinct clusters.

Based on these results, the correlation was found among the isolates although isolates were taken from different host and geographical regions. The cause of observed usual high sequence identity in the apple, hazelnut and plum isolates remains certain. It is suggested that the structure for *Ilarviruses* is stable. *Ilarviruses* have a common spatial organization and some conserved sequences. These results showed similarities with Ertunç et al. (2011)'s study.

Our isolates and the other isolates in NCBI consisted of two mother clades in the phylogenetic analysis. Accordingly, the phylogenetic tree placed our isolates in two subclades. The hazelnut isolates were placed in one subclade, as to our other apple and plum isolates placed in other clade. The hazelnut isolates and an Indian apple isolates placed in same clade. Our isolates were closely related to another clade containing Brazil-apple, Czech-pear, Shimla-India-apple, Ukraine-apple, but far from isolates from Australian hop isolates.

ApMV is the most widespread virus in various fruit crops in Turkey. For all that, there have not been enough studies regarding with the virus isolates up to now. This study depicts detailed information on ApMV Turkish isolates. The realization of the virus full genome analysis gives the real comparisons among isolates. However, this preliminary result gives idea about ApMV Turkish isolates. Additional research will be required to determine the differences of the other ApMV isolates.

ÖZET

TÜRKİYE'DE ELMA, ERİK VE FINDIKTA ENFEKSİYON YAPAN *APPLE MOSAIC VİRUS'*UN KISMİ KILIF PROTEİN GEN SEKANS ANALİZİ

Apple mosaic virus (ApMV) izolatları elma, erik ve fındıklardan elde edilmiştir Bu izolatlar öncelikli olarak DAS-ELISA ile test edilmiştir. Üç farklı ilden (Amasya, Düzce ve Isparta) ApMV ile enfekteli bitkilerden alınmış 38 elma, erik ve fındık izolatından 5 tanesi Türk izolatları arasındaki farklılığı belirlemek için tesadüfi olarak seçilmiştir. Seçilen 5 izolata ApMV'nin kılıf protein gen bölgesine spesifik primerler kullanılarak RT-PCR işlemi gerçekleştirilmiştir. PCR ürünleri 262 bp büyüklüğünde bantlar vermiştir. PCR ürünlerinin forward primer (F 5' AGTAATCCGAAAGGTCCGAATCCGAT 3') kullanılarak sekans dizileri çıkartılmıştır. Bütün sekanslar GenBank ApMV sekansları ile karşılaştırılmıştır. Bizim sekanslarımız GenBank sekansları ile % 88-99 oranında benzerlik göstermiştir. İzolatlarımıza ait sekanslar GenBank da HM245753, HM490310, HM490311, HM245751, HM245752 erişim numaraları ile depolanmıştır. Bu izolatların filogenetik analizlerinin sonucu ürünlerinin aynı grupta yer aldığı göstermiştir.

Anahtar Kelimeler: Elma, Erik, Fındık, RT-PCR

LITERATURE CITED

Akbas, B. and Ilhan, D. 2005. Widespread distribution of Apple mosaic virus on apple in Turkey. Plant Disease 89 (9): 1010.

Akbas, B. and Değirmenci, K. 2009. Incidence and natural spread of ApMV on hazelnut in west Black Sea coast of Turkey and its effects on yield. Journal of Plant Pathology 91 (3):767-771

Akbas B. and Değirmenci, K. 2010a. Simultaneous detection of Apple mosaic virus in cultivated hazelnuts by one-tube RT-PCR. African Journal of Biotechnology 9 (12):1753-1757

Akbas B. and K. Değirmenci, 2010b. First report from Turkey of European plum line pattern caused by *apple mosaic virus*. Plant Disease, 94 (5) 641

Arlı-Sökmen, M. Şevik, M.A. Yılmaz, N.D.K. 2004. Samsun'da fındık (Corylus avellana L.) alanlarının elma mozayik virüsü ile bulaşıklık durumunun belirlenmesi. Türkiye I. Bitki Koruma Kongresi Bildirileri 8-10 Eylül 2004. Samsun s.173.

Arlı-Sökmen, M. 2005. A preliminary study on molecular differantiation of apple and hazelnut isolates of ApMV. Journal of Turkish Phytopathology, 32 (3): 115-123.

Bol, J.F. 1999. Alfalfa mosaic virus and ilarviruses: involvement of coat protein in multiple steps of the replicationcycle. Journal of General Virology 80: 1089-102.

Bradford, F.C. and Joly, L. 1933.Infectious variegation in the apple. Journal of Agricultural Research 46: 901–908.

Brunt, A.A, Crabtree, K.Dallwitz, M.J. Gibbs, A.J. Watson, L. Zurcher, E.J. (1996) *Apple mosaic ilarvirus*. Plant Viruses Online: Description and Lists from the VIDE Database. http://biology.anu.edu/Groups/MES/vide.

Çağlayan, K. Ulubaş, Ç. Gazel, M. and Jelkmann, W. 2003. First report on molecular identification of apple viruses in Turkey. Journal of Turkish Phytopathology 32 (2): 57-61

Çağlayan, K. Gazel, M. Ulubaş, Ç. Jelkmann, W. 2006. "Detection of four Apple Viruses by ELISA and RT-PCR Assay in Turkey", Turkish Journal of Agriculture and. Forestry, 30, 241-246.

Değirmenci, K. And Akbaş, B. 2011. Amasya ve Tokat illerinde yetiştirilen eriklerde Elma mozaik virüs (*Apple mosaic virus*, ApMV)'ü enfeksiyonu ve yaygınlığı. Bitki Koruma Bülteni, Cilt 51, Sayı 2.

Dhingra, K.L. 1972.Transmission of Apple mosaic by natural root grafting. Indian Journal of Horticulture 29: 348-350.

Dursunoğlu, S. and Ertunc, F. 2008. Distribution of apple mosaic ilarvirus in Turkey. Acta Horticulturae 781:131-135.

Ertunç, F. Canik, D. Gospodaryk, A. Budzanıvska, I.G. Polıschuk, V.P. 2011. Elma mozaik ilarvirüsü Türkiye ve Ukrayna izolatlarının moleküler karakterizasyonu. Tarım Bilimleri Dergisi, 17, 95-104.

Foissac, X, Svanella-Dumas, L. Dulucq, M.J. Candresse, T. Gentit, P. 2001. Polyvalent detection of fruit tree tricho, capillo and foveaviruses by nested RT-PCR using degenerated and inosine containing primers (PDO RT-PCR). ActaHortic. 550: 37-43.

Gotlieb, A.R, Berbee, J.G. 1973. Line pattern of birch caused by Apple mosaic virus. Phytopathology, 63: 1470-1477.

Hunter, J.A, Chamberlain, E.E. Atkinson, J.D. 1958. Note on the transmission of Apple mosaic by natural root grafting. New Zealand Journal of Agricultural Research 1: 80-82.

Menzel, W, Jelkmann, W. Maiss, E. 2002. Detection of four apple viruses by multiplex RT-PCR assays with coamplification of plant mRNA as internal control. J. Virol. Methods 99: 81-92.

Nemeth, M. 1986.Viruses, mycoplasma and ricketsia diseases of fruit trees. Boston, USA: Kluwer Academic Publishers.

Nei M and Kumar S. 2000. Molecular Evolution and Phylogenetics. Oxford University Press, New York.

Postman, J.D.Cameron, H.R. 1987.Apple mosaic virus in U.S. filbert germplasm. Plant Disease 71: 944-945.

Sweet, J.B.Barbara, D.J. 1979.A yellow mosaic diseases of horse chestnut caused by apple mosaic virus. Annu.Appl. Biol. 92: 335- 341.

Tamura, K.Dudley, J. Nei, M. and Kumar, S. 2007. MEGA4: Molecular evolutionary genetics analysis (MEGA) software version 4.0. Molecular Biology and Evolution 24:1596-1599.

Ulubaş, Ç. and Ertunç, F. 2003. The Use of RT-PCR for specific detection of Apple mosaic virus (ApMV) in apple. Journal of Turkish Phytopathology 32 (2): 91-97.

Wong, S.M.Horst, R.K. 1993. Purification and characterisation of an isolate of Apple mosaic virus from rose in the USA. J. Phytopathology 139: 33-47.

Yardımcı, N. Çevik, B. and Eryiğit, H. 2006. Detection of apple mosaic virus on apple cultivars in south-west Turkey by ELISA and RT-PCR methods. Proceedinngs, 20th Int. Symposium On Virus And Virus-Like Diseases of Temperate Fruit Crops, 22-26 Mayıs 2006, Antalya.

Yılmaz, N. D. K. Yanar, Y. Kadıoğlu, I. and Yanar, D. 2005. Study on distribution of Apple mosaic virus in apple orchards in Tokat province. 19 Mayıs Universitesi Ziraat Fakültesi Dergisi 20 (3): 12-15.