Melon necrotic spot virus (MNSV), A Newly Reported Virus Diseases for Turkey in Squash species

Gökmen KOÇ* **Hakan FIDAN**** **Saadettin BALOĞLU*****

* Ç.Ü. Pozantı Meslek Yüksekokulu, Pozantı, Adana/Turkey. gkoc@cu.edu.tr
** T.C. Gıda, Tarım ve Hayvancılık Bakanlığı, Biyolojik Mücadele Araştırma İstasyonu. 01321 Adana/Türkiye
*** Ç.Ü. Ziraat Fakültesi Bitki Koruma Bölümü, Adana/Türkiye

Accepted for publication July 7, 2014

ABSTRACT

The study was conducted on summer squash (*Cucurbita pepo.* Linneus, 1753) grown in the plateau of Pozantı which is a county of Adana, Turkey in 2013. During field surveys, systemic chlorotic rings and irregular yellowish dark brown necrotic spots were observed on squash plants. For identification, 49 leaf samples were tested by DAS-ELISA using polyclonal antiserum specific to viruses commonly infecting cucurbits including *Squash mosaic virus* (SqMV), *Cucumber mosaic virus* (CMV), *Zucchini yellow mosaic virus* (ZYMV), *Watermelon mosaic virus 2*(WMV-2), *Watermelon mosaic virus 1*(WMV-1), *Tomato ringspot virus* (ToRSV), *Tobacco ringspot virus*(TRSV) and *Melon necrotic spot virus* (MNSV). Three samples were tested positive for MNSV in DAS-ELISA and the results were confirmed by RT-PCR using total RNA extracted from leaves (100mg). RT-PCR assay was conducted by Sense MNS-C10 and antisense MNS-N40 primer pairs specific to MNSV. As a result of RT-PCR, 485 bp cDNA bands were observed in 2% agarose gel. While occurrence of the ZYMV detected in five samples, remaining viruses were not detected in the tested samples. In Turkey, the occurrence of MNSV was previously reported at melon field samples by serological technique from Thrace region in 2006, also it was detected in cucurbit breeding materials by serologically and molecular techniques in Antalya in 2009. This study is not only first record of MNSV at squash in Turkey but also provide evidence of uncontrolled distribution among both species and regions.

Key words: Squash, MNSV, Pozantı, DAS-ELISA, RT-PCR

INTRODUCTION

Squash (*Cucurbita pepo* Linneus, 1753) is an economically important cucurbit vegetable cultivated in upland, tropical and subtropical climates. There are many factors decreasing the quantity and quality of squash production as in other crops. The most important restrictive factors include plant disease and pests, but plant virus diseases are economically important since yet there are no efficient chemical treatments for protecting plants from virus infection (Ozaslan et al., 2006). Among the important agronomic traits, virus resistance is one of the major breeding objectives, as several diseases caused by viruses have great economic impact on squash production worldwide. For example, include *Cucumber mosaic virus, Squash mosaic virus, Watermelon mosaic virus, Zucchini yellow mosaic virus, Cucumber vein yellowing virus, Papaya ringspot virus* and *Melon necrotic spot virus. Melon necrotic spot virus (MNSV)* is a *Carmovirus* within the family *Tombusviridae* (Hibi and Furuki 1985), which is encountered in cucurbit crops worldwide, and leads to severe yield losses in melon (Kishi, 1966) and cucumber crops (Bos et al., 1984). MNSV can be transmitted mechanically, by the zoospores of the fungus *Olpidium bornovanus* and through seed (Matsuo et al. 1991). MNSV is transmitted through the soil by the fungal vector *O. bornovanus* and/or *O. radicale*, (the taxonomy of these species is apparently still under dispute) via zoospores (Campbell, 1996; Hibi, 1985). Fungal resting spores can survive in the soil for years. The virus (directly or via its vector fungus) can also be spread by seeds (for example at a rate of 10-40% in melon), plant to plant contact, and

mechanical means. Since the virus may be both seed-borne and soil-borne, it may readily be distributed through the cucurbit seed market to become endemic in a district, where it can then be spread locally and persists for a long period in association with its soil-borne fungal vector. Soil fumigation by methyl bromide was used previously to sanitize the soil between cultivations. Since the use of this chemical is now prohibited in many countries for environmental objections. Disease management therefore relies on use of certified virus-free seed and phytosanitary cultural practices (Gu et al. 2008).

The symptoms induced by MNSV in melon include local necrotic spots or large necrotic lesions on leaves, and necrosis on stems and petioles (Matsuo et al. 1991) and occasional plant death(Gu et al. 2008) The MNSV host range is restricted to members of the Cucurbitaceae family, where severity of the symptoms depends on the growing conditions, and the species and cultivar used. The MNSV genome is a single-stranded positive sense RNA molecule of 4.3 kb with five open reading frames (Diaz et al. 2004; Riviere and Rochon 1990). The spread of MNSV in cultivated cucurbits can be controlled through the use of virus-free seeds, control of the fungal vector and production materials; and the use of resistant cultivars (Morales et al., 2005; Coudriet et al. 1981).

In this study surveys (2013) were conducted to determine the presence and distribution of common cucurbit viruses, including MNSV in Pozantı upland (having high potential for summer production) where existence of cucurbit viruses has not been explored. Additionally, other viruses infecting cucurbits, including *Squash mosaic virus* (SqMV), *Cucumber mosaic virus* (CMV), *Zucchini yellow mosaic virus* (ZYMV), *Watermelon mosaic virus 2* (WMV-2), *Watermelon mosaic virus 1* (WMV-1), *Tomato ringspot virus* (ToRSV) and *Tobacco ringspot virus* (TRSV), were analyzed in 2013 due to symptomatic similarities. Furthermore, MNSV detected by ELISA was confirmed by molecular tests.

MATERIALS AND METHODS

The research was conducted in Pozantı Upland of the Çukurova Region in 2013 (Fig 1). A total of 49 samples were collected from suspected plants in cucurbit fields at different locations in the Upland.

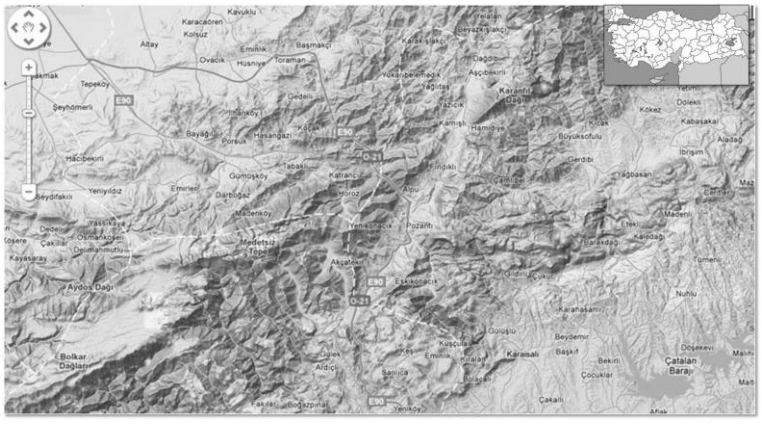

Fig.1. The map of Pozantı Upland

Serological Tests

The study was conducted on summer squash grown in plateau in Pozantı county of Adana, Turkey in 2013. During the surveys, systemic chlorotic rings and irregular yellowish dark brown necrotic spots were observed on squash plants. Surveys were conducted from May to August of the growing season and only symptomatic samples were collected randomly throughout the area in the county. The samples, were labeled and placed in separate plastic bags, brought to the Virology Laboratory by placing in ice bucket and kept at 4°C. All samples were processed within 24 hours.

Double Antibody Sandwich Enzyme Linked Immunosorbent Assay (DAS-ELISA)

For identification viruses infecting summer squash, leaf samples were tested by DAS-ELISA using polyclonal antiserum specific *to Squash mosaic virus* (SqMV), *Cucumber mosaic virus* (CMV), *Zucchini yellow mosaic virus* (ZYMV), *Watermelon mosaic virus 2* (WMV-2), *Watermelon mosaic virus 1* (WMV-1), *Tomato ringspot virus* (ToRSV), *Tobacco ringspot virus* (TRSV) and *Melon necrotic spot virus* (MNSV) supplied by Agdia (USA)

Tests involved detection of cucurbit viruses in infected young leaves and carried out according to Clark and Adams, (1977). Polystyrene microtiter plates were coated with 1:200 dilution of gamma globulin . The leaves of infected young plants were grounded in the extraction buffer (phosphate buffered saline, PBS, pH 7.0). Plant samples were applied at a dilution of l/5 (Wff) in PBS (pH7.0), containing 0.05 volume Tween-20, 0.2% polyvinyl pyrrolidone (PVP-40) and 2% bovine serum albumin (BSA). IgG-conjugate was applied at the concentration of 1 ul/200 ul. Alkaline phosphatase conjugate was used at a 1/1000 dilution.

Results were acquired spectrophotometrically at 405 nm Medispec ESR 200 ELISA microplate reader. Also, negative controls (healthy samples), twice the mean value of healthy specimen were considered positive. Positive controls of all viruses were supplied in lyophilized form with the kits and were resuspended in the sampled buffer as recommended by the manufacturers (Agdia, Inc.).

Total RNA isolation and Reverse-Transcription Polymerase Chain Reaction (RT-PCR)

Total RNA extracts (Astruc et al., 1996) of MNSV infected squash plants were used in a reverse-transcription polymerase chain reaction (RT-PCR) with sense (MNS-C10 (-) CTCCATAAGCGCCAAGCAACC and antisense primer MNS-N40 (+) AGCGGGGGAAAACAGAAGAA primers ,designed based on an nucleotide sequences alignment of CP genes of diverse MNSV isolates(Jung et.al., 2005). First and second strand cDNAs were synthesized from MNSV RNA using murine leukemia virus reverse transcriptase at 42 ºC (45 min) in a termocycler. cDNA's were amplified from the first strand cDNA of MNSV RNA using Taq DNA polymerase and the specific primers. The template cDNA's and a set of primers were mixed in a reaction solution containing 10mM tris-HCl (pH 8.9), 50mM KCl, 1.5 mM $MgCl_2$, 0.01% (w/v) gelatin, 0.24 mM of each dNTP and 5 units of Taq DNA polymerase. Samples were overlaid with one drop of mineral oil and amplification proceeded through a cycle of denaturation at 95 ºC (45 sec), annealing at 50 ºC (1 min) and extension at 72 ºC (1 min) for a total of 35 cycles in Techne Genius thermal cycler.

Analysis of RT-PCR products

Ten microlitres of the PCR reaction mixture was combined with gel loading buffer and analyzed on a 2% agarose gel containing 0.5 mg/ml of ethidium bromide and photographed (Sambrook et al., 1989). One kb DNA ladder (Gibco, BRL, MD) or Bio Marker™ Low (Bio Ventures, Inc.) were used on each gel to determine the length of the amplified product.

RESULTS

Occurrence and detection of viruses by ELISA.

Two (MNSV and ZYMV) out of eight viruses were detected in samples collected from Pozantı county, while none of the samples were positive with *Squash mosaic virus* (SqMV), *Cucumber mosaic virus* (CMV), *Watermelon mosaic virus 2* (WMV-2), *Watermelon mosaic virus 1* (WMV-1), *Tomato ringspot virus* (ToRSV), *Tobacco ringspot virus* (TRSV) which is a severe virus of Squash and other cucurbits. MNSV and ZYMV have been previously reported on cucurbits in Turkey (Yılmaz et. al.1992; Köklü and Yılmaz, 2006; Fidan et al., 2009). MNSV was the most prevalent virus, being detected in 3 samples collected from different squash plants in the Pozantı county. Disease incidence of MNSV from collected samples was 6% and ZYMV was 10% by DAS-ELISA. No results confirmed the mixed infections of MNSV and ZYMV in collected plants.

Type of symptoms

Infected leaves in the fields were reduced in size and weight (Fig.2-3). Also, Leaf deformation, caused by ZYMV.

Fig.2. Chlorotic spots on squash leaf induced by MNSV

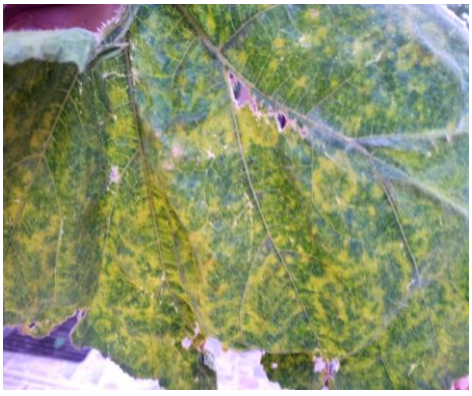

Fig.3. Leaf distortion, mosaic, necrosis spots on squash induced by MNSV

The RT-PCR amplification

In order to verify the infection, RT-PCR amplified MNSV RNA yielding an expected product of 485 bp (Fig.4) on agarose gel (2%) in all tree samples of squash were tested. Consequently, RT-PCR results confirmed the infections of MNSV in DAS-ELISA for positive plants.

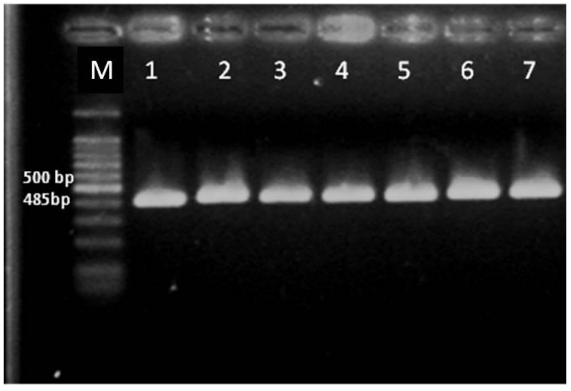

Fig.4. RT-PCR product of 485 bp of MNSV CP on agarose gel (2%); First lane MDNA ladder, Lane 1-3 samples positive of squash in Pozantı; Lane 4 Positive control of melon; Lane 5-7 MNSV cucurbit isolates provided by Dr. Hakan Fidan

DISCUSSION

This study revealed the natural distribution and occurrence of cucurbit and squash viruses in areas located in Pozantı county of Adana. The previous study by Yılmaz et. al. (1992) reported the incidence of viruses in Turkey. Our results pointed out the sanitary conditions for the eight viruses in cucurbit growing fields in Pozantı. ZYMV was the most widespread virus infecting squash crops in Pozantı, belonging to the genus Potyvirus and is transmitted in a nonpersistent manner by aphids, management of which are difficult.

The study was focused on MNSV due to first occurrence on squash which has a less incidence than ZYMV. Also the ZYMV is not a new finding (Özaslan et al., 2006). Similar infections were observed in summer squash and other cucurbits in Turkey (Yılmaz et.al.1989; Özaslan et al., 2006). Another important point is that, MNSV may survive in various weed species not belonging to the family *Cucurbitaceae* serving as a reservoir in the early season

for fungus vectors. This allows MNSV to be spread more progressively and to occur with high incidence, after primary infection by infected seedling or seed inoculums sources (Yılmaz et.al.1989; Özaslan et al., 2006).

The previous study by Köklü and Yilmaz, (2006) on melon and Fidan et al. (2009) on cucurbits had been done. In Turkey, the occurrence of MNSV was reported at melon field samples by serological techniques from Thrace region in 2006 (Köklü and Yılmaz, 2006), also at cucurbit breeding materials by serological and molecular techniques in Antalya in 2009 (Fidan et al., 2009).

This is the first report of MNSV infected Squash for Adana and Turkey. It is also infers uncontrolled distribution according to both species and regions, from the North West to the southern side of the country.

Further studies are planned for screening of seed sources, identifying respective vectors and unknown epidemiological acts and characteristics of the virus, possible new viruses perpetuating in weeds and adjacent crop species at the county. Novel management strategies for diseases will be devised upon these studies.

ÖZET

Melon necrotic spot virus (MNSV), Kabak Türlerinde Türkiye için Yeni Bir Virüs Hastalığı

Çalışma 2013 yılında Türkiye'nin Adana ili *Pozantı* ilçesinde yayla koşullarında yetiştirilen yazlık kabak (*Cucurbita pepo* Linneus, 1753) bitkilerinde gerçekleştirilmiştir. Gözlemler sırasında kabak bitkilerinin yapraklarında, sistemik klorotik halka ve düzensiz sarımsı koyu kahverengi nekrotik lekeler gözlenmiştir. Tanılanma için 49 yaprak örneği Agdia (USA)'dan temin edilen spesifik poliklonal antiserum ile DAS-ELISA tekniğine göre *Squash mosaic virus* (SqMV), *Cucumber mosaic virus* (CMV), *Zucchini yellow mosaic virus* (ZYMV), *Watermelon mosaic virus 2* (WMV-2), *Watermelon mosaic virus 1* (WMV-1), *Tomato ringspot virus* (ToRSV), *Tobacco ringspot virus* (TRSV) ve *Melon necrotic spot virus* (MNSV) gibi bilinen kabakgil virüslerine karşı testlenmiştir. Üç örnekteki MNSV pozitif DAS-ELISA sonuçları, 100 mg yapraktan ekstrakte edilen toplam RNA'ların RT-PCR'da kullanılmasıyla doğrulanmıştır. RT-PCR, Sense ***MNS***-C10(-) ve antisense primerleri ***MNS***-N40 (+) kullanılarak yapılmıştır. RT-PCR sonucu kılıf proteinine ait 485 bp büyüklüğünde DNA bantları %2'lik agaroz jelde gözlenmiştir. Beş örnekte ise ZYMV varlığı saptanmıştır. Hastalık 2006 yılında, Türkiye'nin Trakya bölgesi kavun bitkilerinde serolojik tekniklerle, 2009 yılında ise Antalya ilindeki hıyar bitkilerine ait ıslah materyallerinde hem serolojik hem moleküler olarak saptanmıştır. Bu çalışma yalnızca MNSV'nin Türkiye'de kabak bitkisindeki ilk kaydı olmayıp aynı zamanda etmenin türler ve bölgeler arasında kontrolsüz yayılımının göstermektedir.

Anahtar Kelimeler: Kabak, MNSV, Pozantı, DAS-ELISA, RT-PCR

Acknowledgments

This work was supported partly by Virology Labs of Biological Research Station of Adana and Plant Protection Dept. at Çukurova University.

LITERATURE CITED

Bos, L., Van Dost, H. J. M., Huttigna, H. And Maat, D. Z. 1984. Further characterization of melon necrotic spot virus causing severe diseases in glasshouse cucumbers in the Netherlands and its control. Neth. J. Plant Pathol. 90:55-69.

Compbell, R. N. 1996. Fungal transmision of plant viruses. Annu.Rev. Phytopathol. 34:87-108.

Clark, M.F., And Adams, A.N., 1977. Charecteristic of Microplate method of Enzyme- linked Immunosorbent Assay for the Detection of Plant Viruses. J. Gen. Virol., 342 475-483.

Coudriet Dl, Kishaba An, Bohn Gw 1981. Inheritance of resistance to muskmelon necrotic spot virus in a melon aphidresistant breeding line of muskmelon. J Am Soc Hort Sci 106:789–791

Di´Az Ja, Nieto C, Moriones E, Truniger V, Aranda Ma (2004) Molecular characterization of a Melon necrotic spot virus strain that overcomes the resistance in melon and non-host plants. Mol Plant Microbe Interact 6:668–675

Fidan, H., Unlu, A., Koc, G., Unlu, M. 2010 Turkiye'de Hıyar Bitkisinde Yeni Bir Virus Hastalıgı. Melon Necrotic Spot Virus (MNSV) VIII. Sebze Tarymy Sempozyumu 23 Haziran-26 Haziran 2010 - VAN / TURKYEVan S:490-494

Gu, Q.-S., Bao W.-H.,, Tian, Y.-P., Prins, M., Yang, H.-X., Lu, J., Liu, L.-F, and Penga, B.2008, Melon necrotic spot virüs newly reported in China, Plant Pathology (2008) 57, 765, Doi: 10.1111/j.1365-3059.2008.01847.x

Hibi T, And Furuki, I., (1985) Melon necrotic spot virus. In: (eds) CMI/ AAB Descriptions of plant viruses. Association of applied biologists, Warwick, UK

Jung, J. A., Kim, S. M., Kim, H. R., Lee, S. H., Choi, H. S., Park, J. W. And Kim, T. W. 2005. Diagnosis of Melon necrotic spot carmovirus by using RT-PCR and efficiency comparison with other diagnostic methods Plant Pathol. J. 21:434 (abstract).

Kishi, K. 1966. Necrotic spot of melon, a new virüs disease. *Ann.Phytopath. Soc. Japan* 32:138-144.

Koklu, G., And Yilmaz, O., 2006 Occurrence of cucurbit viruses on field-grown melon and watermelon in the Thrace region of Turkey Phytoprotection, vol. 87, n° 3, 2006, p. 123- 130.

Matsuo K, Kameya-Iwaki M, Ota T 1991. Two new strains of Melon necrotic spot virus. Ann Phytopatol Soc Jpn 57:558–567

Morales, M., Orjeda, G., Nieto, C., Van Leeuwen, H., Monfor, A., Charpentier, M., Caboche, M., Arús, P., Puigdomènech, P., Aranda, M.A., Dogimont, C., Bendahmane, A., And Garcia-Mas, J., 2005. A physical map covering the nsv locus that confers resistance to Melon necrotic spot virus in melon (Cucumis melo L.). Theor Appl Genet (2005), DOI 10.1007/s00122-005-0019-y

N. Astruc, J.F. Marcos, G. Macquaire, T. Candresse, V. Pallás, 1996. Studies on the diagnosis of hop stunt viroid in fruit trees: identification of new hosts and application of a nucleic acid extraction procedure based on non-organic solvents Eur. J. Plant Pathol., 102 (1996), pp. 837–846

Ozaslan, M., Aytekin, T., Bas, B., Kilic, H.I., Afacan D., And Dag,D.S. 2006. Virus Diseases of Cucurbits in Gaziantep-Turkey. *Plant Pathology Journal, 5: 24-27.*

Riviere Cj, Rochon Dm 1990. Nucleotide sequence and genomic organization of Melon necrotic spot virus. J Gen Virol 71:1887– 1896

Sambrook, J., Fritsch, E.F. And Maniatis, T. 1989. Molecular Cloning: A Laboratory Manual, 2nd edn. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Yilmaz, M.A., Ozaslan, M., Ozaslan, D., 1989. Cucumber Vein Yellowing Virus in Cucurbiteceae in Turkey. Plant DiseaseT3 (7)z 610 (Abst).

Yilmaz,M.A., Lecoq, H., Abak, K., Baloglu, S., Ve Sari, N., 1992. Ttirkiye'de Kabakgil Sebze TiirlerindeZarar Yapan Virusler. Türkiye I. Ulusal Bahge Bitkileri Kongresi, Ege Universitesi Ziraat Fakiiltesi, Bornova, izmir, Cilt I Sebze, (439- 442) 13-16 Ekim, 1992.