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Full Length Research Paper

Identification of some viruses causing mosaic on lettuce and characterization of *Lettuce mosaic virus* from Tehran Province in Iran

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***Lettuce mosaic virus* (LMV), *Cucumber mosaic virus* (CMV) and *Tomato spotted wilt virus* (TSWV) were identified in lettuce fields in Tehran province. In this study, 452 infected lettuce plants having viral infection symptoms including, mosaic, mottling, leaf distortion, stunting defective heading, were collected from the fields throughout Tehran province. Distribution of *Lettuce mosaic virus* (LMV), *Cucumber mosaic virus* (CMV), *Tomato spotted wilt virus* (TSWV) and *Arabis mosaic virus* (ArMV) were determined with DAS-ELISA. LMV, CMV and TSWV were found on lettuce in this region, but no infection by ArMV was found. Percentage of single infection by LMV, CMV or TSWV was 21, 16 and 10% respectively. Also, 16% of samples were co-infected with LMV+CMV, 8% with LMV+TSWV and 8% with CMV+TSWV. 5% of samples were infected to all of these viruses. LMV was found in 49%, CMV in 44% and TSWV in 31% of samples totally. Therefore, LMV is major agent of lettuce mosaic disease in Tehran province. This is the first report of occurrence of TSWV on lettuce in Iran and the first report of CMV and LMV in Tehran province. Three LMV infected samples were collected and their characteristics were determined. After mechanical inoculation, these isolates produced symptoms on *Chenopodium quinoa, Chenopodium amaranticolor, Gomphrena globosa, Nicotiana benthamiana, Lactuca sativa* cv. Mantilia and cv. Terocadero (which contains the *mo1*[1] resistance gene and susceptible respectively), but not cv. Salinas 88 (which contains the *mo1*[2] resistance gene). LMV was purified and LMV polyclonal antiserum was produced in rabbit by a series of intravenous and intramuscular injections, the titre of this antiserum was 1:1024. Gel double diffusion test (GDDT) was performed, and precipitin bands appeared. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting showed the presence of coat protein 29 kDa. In Immunocapture reverse transcription PCR (IC-RT-PCR) with a LMV specific primer pair, an aproximately 1300 bp fragment was amplified.**

Key words: *Lettuce mosaic virus*, *Cucumber mosaic virus*, *Tomato spotted wilt virus*, Immunocapture reverse transcription PCR, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

INTRODUCTION

Lettuce mosaic virus (LMV), *cucumber mosaic virus* (CMV) and *tomato spotted wilt virus* (TSWV) individually cause serious disease in commercial lettuce crops. The reaction of lettuce to LMV (Tomlinson, 1962; Ryder, 1968, 1976; Walkey and Payne, 1990), CMV (Tomlinson et al., 1970; Provvidenti et al., 1980; Walkey et al., 1985b) and TSWV (Moyer, 1999) has been documented. LMV infected plants usually show vein clearing, yellow mottling, and stunted growth. Symptoms are quite variable and depend on the cultivar, the invironmental conditions, and the developmental stage at which the plant became infected (Dinant and Lot, 1992; Candresse et al., 2003). Symptoms in lettuce caused by LMV and CMV are essantially indistinguishable and consist of stunting, chlorosis, mosaic and improper heading of infected plants (Cock, 1968; Bruckart and Lorbeer, 1975).

TSWV infection usually occurs on and affects one side of lettuce plants, then it gradually spreads into the heart leaves. General chlorosis, followed by small brown spot

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and tissue necrosis in older leaves, characterize symptoms in infected lettuce leaves (Sutic et al., 1999). *Arabis mosaic virus* (ArMV) infected plants are chlorotic, dwarfed and fail to form heads (Sutic et al., 1999). LMV belongs to the *Potyvirus* group (Dinant and Lot, 1992; Zerbini et al., 1995), it is aphid-transmitted in a non-persistent manner and is also seed-transmitted (Tomlinson, 1970; Shukla et al., 1994) within the family Potyviridae (Murant et al., 1996). CMV belongs to *Cucumovirus* group, it is aphid-transmitted in a non-persistent manner, but is not seed-transmitted in lettuce (Francki et al., 1979). TSWV belongs to *Tospovirus* group; it is transmitted by thrips in a circulative, propagative manner (Moyer, 1999). ArMV belongs to *Nepovirus* group, and is seed-transmitted in lettuce, also transmitted by soil nematodes (Murant et al., 1996; Mayo and Jones, 1999). Lettuce mosaic virus (LMV) is one of the most destructive viruses in lettuce (*Lactuca sativa* L.) worldwide (Zerbini et al. 1995; Revers et al., 1997; Yang et al., 1998). It has flexous filamentous particles of 750 × 13 nm (Revers et al., 1997, 1999).

The genome of LMV is a single, positive-sense 10080 nt RNA molecule polyadenylated at its 3´-end, and containing a large open reading frame encoding a polyprotein of 3255 amino acids (Redondo et al., 2001; Krause-Sakate et al., 2002). LMV isolates have been classified into four pathotypes, according to their virulence on lettuce varieties carrying the three resistance or tolerance genes *mo1*^{1}, *mo1*^{2} and *Mo*2, which are identified in *L. sativa* cultivars (Pink et al., 1992; Bos et al., 1994). The *mo1*^{1} gene was initially identified iv cv. Gallega de Invierno (Bannerot et al., 1969). It was mostly used by European breeders and has now been introduced into all types of lettuce, including butterhead, looseleaf, crisphead, and cos lettuce (Pink et al., 1992; Candresse et al., 2002). The *mo1*^{2} gene identified in PI 251245, an accession of *L. sativa* from Egypt and initially named mo, has mostly been used by North American breeders (Pink et al., 1992). The *mo1*^{1} and *mo1*^{2} genes are recessive and are believed to be either closely linked or allelic (Dinant and Lot, 1992; German-Retana et al., 2000). They are deployed worldwide and allow a reasonably effective control of the disease (German-Retana et al., 2000; Candresse et al., 2002) although resistance-breaking isolates have been reported (Pink et al., 1992). LMV is controlled using virus-free seeds (Walkey and Dance, 1979) and deployment of genetic resistance (Ryder, 1970; Walkey et al., 1985; Ryder et al., 2003). The aim of this study is identification of viruses causing mosaic on lettuce in Tehran province and then the determination of some molecular and biological properties of lettuce mosaic virus isolates from Iran.

MATERIALS AND METHODS

Samples collection and virus identification

In addition, 452 infected lettuce plants having viral infection symptoms including: mosaic, mottling, leaf distortion, stunting and defective heading were collected from 23 fields throughout Tehran province in two growing seasons. Each plant sample was placed separately in a plastic bag and kept at 4°C until analyzed by LMV, CMV, TSWV and ArMV polyclonal antibodies, and DAS-ELISA was performed as described by Clarck and Adams (1977). Samples were considered positive if the absorbance at OD_{405nm} were twice the average value of healthy controls.

Biological tests

Three isolates of LMV were collected and then were ground 1:2 (wt/vol) in 0.05 M potassium phosphate buffer, pH 7.0, containing 0.1% Na_2SO_3, and carborundum was added before rub-inoculating. Lettuce plants, cv. Mantilia and Salinas 88, which contain the *mo1*^{1} and *mo1*^{2} resistance genes respectively, and susceptible lettuce cv. Terocadero, *Chenopodium quinoa*, *Chenopodium amaranticolor*, *Nicotiana benthamiana* and *Gomphrena globosa*, at the three to five leaf stage, were rub-inoculated with sap and maintained in an insect-free greenhouse at 20 to 25°C. Seeds of the differential lettuce cultivars were provided by T. Candresse (INRA, France). The presence of the virus in the plant apex was assessed based on the appearance of symptoms 3 weeks after inoculation and testing each plant by DAS-ELISA.

Antiserum production

One of the virus isolates was propagated in *C. quinoa* and was purified from infected plants. Antiserum to LMV was produced in rabbit by a series of intravenous and intramuscular injections. Rabbit was bled 6 weeks after the initial injection. Antiserum was crossabsorbed with healthy *C. quinoa*. Leaf tissue (0.4 g) was homogenized and diluted with 20 ml of TBST buffer (10 mM Tris, pH 8.0, 150 mM NaCl, 0.05% Tween 20). This solution was mixed with the antiserum.

Serological analysis

ELISA was performed as antigen coated indirect assay, essentially as described previously by Creamer and Falk (1989). Samples were extracted in coating buffer (0.05 M sodium carbonate, pH 9.6). Plant extracts, LMV antiserum and goat anti-rabbit IgG alkaline phosphatase conjugate (Sigma, St. Louis, MO) were incubated for approximately 2 h, and *p*-nitrophenyl phosphate substrate was incubated for 30 min at 37°C on microtitre plates. Absorbance values were determined photometrically at 405 nm. Samples were considered positive if the absorbance was twice average value of healthy controls. Gel double diffusion test (GDDT) was performed, using 0.8 g of agarose in 100 ml potassium phosphate buffer containing 0.85 g of NaCl, 0.5 g of SDS and 2% of sodium azide.

Western blotting was done as described previously by Creamer (1992). Samples electrophoresed through a 5% stacking gel and 12% resolving sodium dodecyl sulfate polyacrylamide gel (SDS)-PAGE at 100 V for 1 h in a mini-protean II system. The fractionated proteins were electro-blotted onto nitrocellulose membrane, blocked with PBST plus 2% no fat dry milk, and probed with LMV polyclonal IgG (2 h) and then goat anti-rabbit conjugated to alkaline phosphatase (2 h). After rinsing, the membrane was placed in 10 ml phosphatase buffer (0.1 M Tris-HCl, 0.1 M NaCl, 50 mM $MgCl_2$, pH 9.5) containing 0.4 mM of nitroblue tetrazolium chloride and 0.45 mM of 5-bromo-4-chloro-3-indolyl phosphate. After 10 to 15 min the reaction was stopped by extensive rinsing in water. Color development was evident for the infected samples.

IC-RT-RCR

Infected leaves were ground 1:3 (wt/vol) in PBST buffer containing

Table 1. Symptoms of LMV infection on test plants.

Test plant	Symptoms
Chenopodium quinoa	vc, sl, ld, chll
Chenopodium amaranticolor	vc, sl, chll
Lactuca sativa var. terocadero	vc, s, m, ld , dh
Lactuca sativa var. mantilia	vc, m, d,dh, lds
Lactuca sativa var. salinas 88	-
Nicotiana benthamiana	m
Gomphrena globosa	nll

vc: vein clearing	chll: chlorotic local lesion	s: stunting
sl: systemic lesion	m: mosaic	d: dwarfing
ld: leaf destortion	dh: defective heading	nll: necrotic local lesion

Figure 1. Systemic symptoms of *lettuce mosaic virus* on *Lactuca sativa* cv. Terocadero.

2% polyvinil pyrolidon (mol/Wt 40,000). The plants extracts were incubated overnight at 4°C in 0.2 ml tubes precoated with anti-LMV immunoglobulins. After washing the tube 3 times with PBST buffer, RT-PCR reaction was performed by primer pair described previously by Zerbini et al. (1995). 100 µl of RT-PCR reaction mix (250 µM each of dNTPs, 100 pM each of the two primers, 0.25 units of M-MuLV reverse transcriptase, and 1 unit of *Taq* DNA polymerase). The tubes were incubated 40 min at 42°C for reverse transcription, were incubated 5 min at 95°C for denaturation, the PCR profile was 35 cycle with the following parameters:

Denaturing for 1 min at 94°C, annealing for 2 min at 42°C, and extension for 2 min at 72°C, followed by an extension step at 72°C for 5 min. RT-PCR products were separated by electrophoresis in 1% agarose gel at 80 V and detected by ethidium bromide staining.

RESULTS

Virus distribution

The results of this study were including: single infection to LMV, CMV and TSWV was determined in 93, 72 and 45 samples respectively. No infection to ArMV was detected in any of the samples. Mixed infection to LMV+CMV, LMV+TSWV and CMV+TSWV was detected in 69, 37 and 35 samples respectively. 25 samples were infected to all of them. Therefore, percentage of single infection to LMV, CMV and TSWV were 21, 16 and 10% respectively. Percentage of mixed infection of LMV+CMV, LMV+TSWV and CMV+TSWV was 15, 8 and 8% respectively. 5% of tested plants were infected to all of these three viruses. LMV was found in 49%, CMV in 44% and TSWV in 31% of samples totally.

Biological tests

The symptoms of three isolates of LMV on test plants are summarized in Table 1. LMV produced symptoms including: mosaic, leaf distortion, vein clearing and defective heading on susceptible lettuce cv. Terocadero, but did not produce symptoms on cv. Salinas 88, which contains the *mo1*^{2} resistance gene. Lettuce plants cv. Mantilia (containing the *mo1*^{1} resistance gene) also showed dwarfing, leaf detortion, mosaic and defective heading symptoms. In cv. Mantilia, the level of virus detected by ELISA was less than the corresponding level found in the susceptible cv. Terocadero. *C. quinoa* and *C. amaranticolor* plants developed chlorotic local lesions on inoculated leaves 3 to 5 days after inoculation, and systemic symptoms that included chlorotic lesion and leaf distortion 2 weeks after inoculation, *N. benthamiana* developed systemic symptoms, *G. globosa* developed necrotic local lesions 2 to 3 weeks after inoculation, and did not show systemic symptoms (Figures 1, 2, and 3).

Serological analysis

The LMV antiserum reacted positively with purified LMV and LMV infected *C. quinoa*, and did not react with healthy *C. quinoa* in ELISA. Pre-immune serum did not

Figure 2. Systemic symptoms of *lettuce mosaic virus* on *Chenopodium quinoa.*

Figure 3. Necrotic local lesion of *lettuce mosaic virus* on *Gomphrena globosa.*

react to purified LMV or LMV infected plants. The titre of this antiserum was 1:1024. In GDDT a precipitin band appeared between antibody and infected plant sap and purified LMV but not between antibody and healthy plant sap (Figure 4). In Western blot, purified LMV and greenhouse-infected *C. quinoa* tissue reacted positively to LMV antiserum. A disease-specific protein of approximately 29 kDa was observed in SDS-PAGE and blot using LMV antiserum (Figure 5).

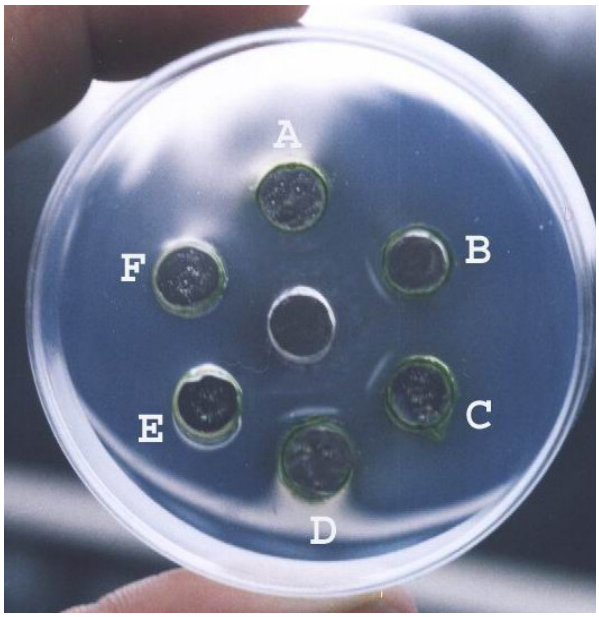

Figure 4. Gel double diffusion test of *lettuce mosaic virus* using LMV polyclonal antiserum B,C: LMV infected *C.quinoa* D: Purified LMV A,E,F: Healthy *C.quinoa.*

IC-RT-PCR

An approximately 1300 bp DNA fragment was amplified from LMV-infected plants by IC-RT-PCR with a LMV specific primer pair. No DNA fragment amplified from uninfected plants (Figure 6).

DISCUSSION

The two closely linked or allelic genes, *mo1*[1] (Von der Pahlen and Crnko, 1965; Pink et al., 1992) and *mo1*[2] (Ryder, 1970; Pink et al., 1992) are currently used worldwide to protect lettuce crops against the detrimental effects of LMV infection. As is frequently observed for resistance to potyviruses (Fraser, 1990; Grumet et al., 2000), these genes are recessive and do not provide immunity to viral infection by common LMV strains. Levels of resistance conferred by *mo1*[1] and *mo1*[2] genes can either be low (systemic virus accumulation but no symptoms, sometimes referred to as tolerance) or high (no virus multiplication), and this may depend on the virus

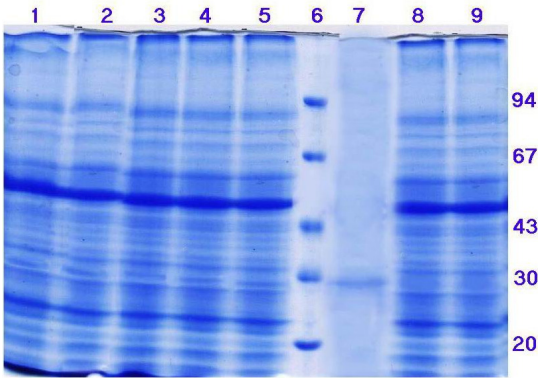

Figure 5. SDS-PAGE of LMV infected *C.quinoa* and purified LMV, Lane 1 to 5**:** LMV infected *Chenopodium quinoa*. Lane 6: Protein molecular weight standards Lane 7: Purified LMV Lane 8 to 9: Healthy *C.quinoa*.

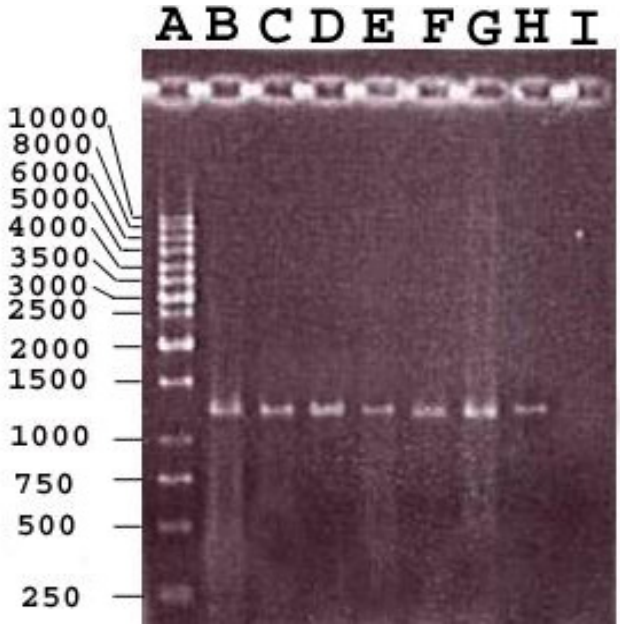

Figure 6. Detection of *lettuce mosaic virus* (LMV) by IC-RT-PCR. A 1300 bp fragment was amplified from LMV infected *C Chenopodium quinoa*. Lane A: Ladder B to H: LMV infected *C. quinoa* **I**: Healthy *C. quinoa*.

isolate (Pink et al., 1992; Revers et al., 1997). These gene restrict the long-distance movement of the virus or affect symptoms expression and/or viral accumulation (Bos et al., 1994; Dinant and Lot, 1992; Revers et al., 1997). The dominant gene *Mo*2 affords immunity to the virus but is overcome by most LMV isolates (Revers et al., 1997; Krause-Sakate et al., 2002). New strains of the virus appear at intervals. Most seem to be restricted to certain areas or may disappear quickly.

However, in recent years, several strains have persisted for a longer period of time, and at least two can overcome the resistance conferred by one or the other or both alleles (Ryder et al., 2003). Based on biological tests LMV isolates from Iran overcame the *mo1*^{1} resistance gene and produced symptoms on cv. Mantilia, but did not produce symptoms on cv. Salinas 88, which contains the *mo1*^{2} resistance gene. LMV antiserum reliably detected LMV in plants such as *C. quinoa* and *L. sativa* by ELISA and GDDT. Using RT-PCR would allow a more specific virus identification. Recently, efforts have been made to develop more sensitive techniques for the detection of LMV based on the PCR (Revers et al., 1999; Peypelut et al., 2004; Candresse et al., 2007; Moreno et al., 2007). LMV has been reported on lettuce by Manouchehri-Kashani from Khuzestan province (1968), CMV has been reported on lettuce by Izadpanah from Fars province (1983), but TSWV has not been reported on lettuce previously in Iran. This is the first report of TSWV on lettuce from Iran and first report of LMV and CMV from Tehran province. Infection to LMV, CMV and TSWV was determined in 93, 72 and 45 samples respectively. Therefore, LMV is the major agent of lettuce mosaic disease in Tehran province.

Conclusion

In this survey, LMV was found in 49%, CMV in 44% and TSWV in 31% of infected samples. These results indicate widespread occurrence of TSWV and CMV alongside the dominant LMV infection. These viruses directly affected the quality of leaves and preventing sale of affected plants (Pavan et al., 2008). According to the high percentage of virus infection in lettuce fields, detection of best methods for control of these viruses is very important. As a result of difficulties in controlling TSWV vectors (Manoussoloulos et al., 1999), and the transmission of LMV and CMV by aphids in a non-persistent manner, these viruses can be responsible for the loss of up to 100% of lettuce crops (Dinant and Lot, 1992). Thereupon the control of LMV, relied on prophylactic measures such as the use of virus free-seeds, vector aphids control in order that keep insect population low in the fields, elimination of alternative hosts (Pavan et al., 2008) and on genetic resistance (Dinant and Lot, 1992; Ryder, 1970).

LMV isolates capable of overcoming the resistance afforded by *mo1*^{1} and *mo1*^{2} have been described in various parts of the world (Dinant and Lot, 1992; Pink et al., 1992). Lettuce cultivar 'Cuesta' introduced in 2007 with that carries multiple resistance genes to tospoviruses, *lettuce mottle virus* and most strain of LMV-Common (controlled by the genes *mo1*^{1} and/or *mo1*^{2}) and LMV. Most strains (overcoming *mo1*^{1} and *mo1*^{2}) (Pavan et al., 2008). For control of TSWV, breeding cultivars for high resistance level seems to be the best control strategy, since thrips control has not been

efficient. Eradication of weeds and volunteer growth close to the lettuce fields, in association with other cultural practice, could minimize and prevent infection by tospoviruses. There is no information of resistance cultivars to CMV and the disease can be prevented by controlling the vector sources (Pavan et al., 2008).

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