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PRELIMINARY RESEARCH CONCERNING THE IDENTIFICATION OF REISTANCE GENOTYPES ON CUCURBITACEAE FAMILY IN ARTIFICIAL INFECTION CONDITIONS WITH CMV (CUCUMBER MOSAIC VIRUS)

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Abstract

Cucumber mosaic virus (CMV) was first described in detail in 1916 on cucumber (simultaneously by Doolittle and Jagger) and other cucurbits, but is now known to occur worldwide in both temperate and tropical climates, affecting many agricultural and horticultural crops. Development of genetic resistance to CMV in many vegetables has made a valuable contribution for disease management of this important virus disease. There are some CMV resistant (tolerant) cucumber varieties available that produce a good crop, but most other cucurbits are susceptible to CMV. The screening of the 37 field samples collected from six species of Cucurbitaceae were tested by TAS-ELISA, 13 samples were found to be infected by CMV. Among them. In IC-RT-PCR using the MAbs and specific primers in the region of the coat protein (CP) gene, samples shown that results obtained by TAS-ELISA gave one specific band about 500 nucleotides in length. The validity and reliability of the results of TAS-ELISA and IC-RT-PCR was confirmed by sequencing and phylogenetic analysis of nearly full-length CP genes of the isolates.

Keywords: *Cucumber mosaic virus; Monoclonal antibody; resistance, gene*

INTRODUCTION

Cucumber mosaic virus occurs worldwide and is considered a very important disease in temperate, tropic and subtropic regions of the world.

Crop losses vary from year to year since the amount of disease occurrence depends upon the number of aphids available for virus transmission in the spring or fall when the crops are established as determined by geographical location.

If the spring or fall is cool and wet, aphid numbers are decreased and virus spread is sporadic, with infected plants primarily located in rows bordering the edges of the field.

However, if the spring or fall is warmer with less frequent rains, aphid populations increase rapidly on perennial crops that harbor CMV, and virus spreads rapidly into crops that are young and especially attractive to migrating aphids. In such cases, infection rates may approach 100% and the crop may have to be abandoned.

On average, losses of 10-20% are common, and in some instances the crop may still be harvested, but is of poorer quality and appearance. Historically, *Cucumber mosaic virus* (CMV) was first described in detail in 1916 on cucumber (simultaneously by Doolittle and Jagger) (Fig. 1, 2) and other cucurbits, but is now known to occur worldwide in both temperate and tropical climates, affecting many agricultural and horticultural crops.

Development of genetic resistance to CMV in many vegetables has made a valuable contribution for disease management of this important virus disease.

CMV infects 1200 species in over 100 plant families and can cause significant economic losses in many vegetable and horticultural crops. CMV causes a systemic infection in most host plants, but may remain symptom less in some crops like alfalfa.

Symptoms of cucumber mosaic can vary greatly depending on the crop infected and the age of the plant when infection occurs.

Cucurbits: Almost all cucurbits are susceptible to CMV, with symptoms varying in severity (Fig. 1A-E).

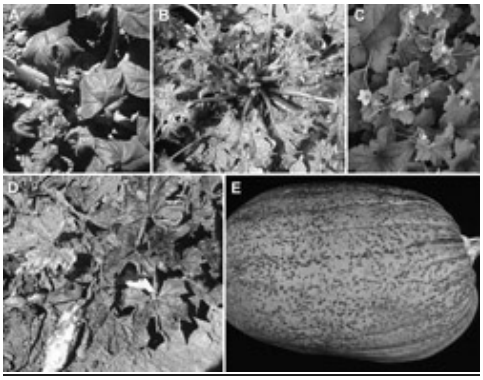

Fig. 1 A-E. CMV infection of cucurbits (www.apsnet.org)

Plants infected early in the season are severely stunted and leaves are malformed, and fruit are unmarketable because of pronounced rugosity (roughness) on the fruit surface, as shown on the infected zucchini plant and fruit in Figure 1B. Infection of vining crops, such as muskmelon, show severely stunted growing tips (Figure 1C), and although fruit may not show symptoms they are of poor quality. If the yellow squash variety grown lacks the precocious gene, color breaking will occur on the fruit, causing the fruit to show green blotchy patterns, but these symptoms are absent in yellow squash varieties with the precocious gene (Fig. 1D).

Color breaking on fruit of varieties without the precocious gene will also occur with *Watermelon mosaic potyvirus* (WMV) infection; however this protection does not hold true for *Papaya ringspot potyvirus* or *Zucchini yellow mosaic potyvirus*, where both foliage and fruit of yellow squash are severely affected. Pumpkin is another cucurbit, that when infected at any early stage, will express severe foliar mosaic and the fruit will show a mosaic pattern and would be unmarketable.

Because of its wide host range, numerous weeds can serve as reservoirs for CMV and contribute to virus spread to crops at the beginning of the season. Perennial, biennial, and winter annual weeds harboring CMV in roots, tubers and underground organs throughout the winter include common milkweed (*Asclepias syriaca*), yellow rocket (*Barbarea vulgaris*), marsh yellowcress (*Rorippa islandica)*, and yellow toadflax (also called butter-and-eggs, *Linaria vulgaris*). These were shown to be important sources for infection of lettuce in upstate New York. Seven additional weeds, including common chickweed (*Stellaria media*) were shown to be important overwintering sources in Britain for lettuce (Tomlinson, J.A., and A.L. Carter. 1970). It is important to note that infected weeds are often symptomless.

Development of genetic resistance is the simplest and most effective method of controlling virus diseases and is especially appropriate for CMV.

Success has been noted, especially for cucumber and spinach, but is variable among most other crops. The history of CMV resistance in cucumber (*Cucumis sativus*) dates back to 1927 when the oriental varieties 'Chinese Long' and 'Tokyo Long Green' were introduced to the US. After extensive study, it was concluded that homozygosity of three partially dominant genes was needed to convey a high level of resistance for cucumber.

This resistance formed the background for all modern day slicing and pickling cucumber varieties and has been effective for many decades, perhaps because resistance is based upon several genes.

Genetic resistance for CMV in melon (*Cucumis melo*) is derived from oriental melons, and depending on the source, resistance is controlled by two or three complementary recessive genes.

Some of these factors for resistance are strain and/or temperature dependent, with plants developing symptoms at temperatures below 20°C. No commercial muskmelon varieties with CMV resistance are available. Fortunately, most varieties of watermelon (*Citrullus lanatus*) are resistant to the most prevalent strains of CMV, with the exception of a specific strain that can infect plants systemically.

Eradication of weed hosts is often a difficult task because of the extensive host range of this virus.

However, elimination of several of the key perennial or biennial weeds located near the crop may reduce severe virus pressure, and has successfully been used to control CMV.

MATERIAL AND METHOD

The objectives for this work are the behavior of different genotypes of cucumbers, melons, loofah, that hybrids and varieties, the CMV infection; the implementation of the main methods for detection of viruses in plants; and the implementation of Markers assisted selection (MAS) for detecting CMV-resistant genotypes.

Virus sources

CMV virus is of French origin from INRA Bordeaux.

Plant material

The plant material consists in different species of cucurbitaceous and several varieties:

Varieties and hybrids of the species *Citrullus lanatus* :

1.Dulce de dabuleni
2.1AS1WM001
3.CATHERINE F1
4.SUGAR BABY
5.PEACE F1
6.CRIMSON SWEET
7.OLTENIA
8.1AS1WM011

Varieties and hybrids of the species *Cucumis sativus*:

1.Crisan
2.Cornison de Paris
3.Merengue F1
4.Kybria F1
5.Maresa F1
6.Mirabele F1

Varieties and hybrids of the species *Cucumis melo*:

1. Galben necacios
2. Machidimon F1
3. Citirex F1
4. Bulgaresti
5. Raymond F1

The species

1.*Cucurbita pepo var Maxima:*
2.Cucurbita moscata
3. Luffa cylindrica
4. Lagenaraia siceraria
5. Kivano (castravetele tepos)

Methods

ELISA. Triple antibody sandwich (TAS)-ELISA (Zhou et al., 1997) and antigen-coated plate (ACP)-ELISA (Jiang et al., 2003) were used for detection and characterization of MAbs against CMV with an alkaline phosphatase-conjugated goat anti-mouse IgG antibody (Sigma). Healthy tobacco plants and buffer alone were used as controls. The positive threshold was fixed at twice the average of the optical density obtained with the healthy controls. All tests were duplicated.

Immunocapture RT-PCR (IC-RT-PCR). Three CMV specific primers were designed based on the cDNA sequences of the CMV CP gene deposited in the GenBank database.

The forward primers CMV I-F (5′-CGACTTAATAAGACGTTAGCAGC-3′, corresponding to nucleotides 121–143 of CP gene of CMV S-I isolates) and CMV II-F (5′-TCCCAATGCTAGTAGAACCTCC-3′, corresponding to nucleotides 18–39 of CP gene of CMV S-II isolates) located at the upstream end of CP gene were specific for CMV S-I and CMV S-II isolates, respectively.

The reverse primer CMV-R (5′-TGCTCRAYGTCRACATGAAG-3′, complementary to nucleotides 601–620 of CP gene of CMV S-I and S-II isolates) was degenerate and based on the conserved sequence of CMV CP genes in both subgroups. The immunocapture was carried out as described (Jiang and Zhou, 2002).

RESULTS AND DISCUSSIONS

Table 1 Results (TAS)-ELISA

Species	Varieties	ELISA	PCR
CITRULUS LANATUS	Dulce de dabuleni	+	+
	1AS1WM001	-	+
	Katherine F1	+	+
	Sugar baby	-	-
	Peace F1	-	-
	Crimson sweet	+	+
	Oltenia	-	-

	1AS1WMO11	-	-
CUCUMIS SATIVUS	Castravetele tepos	+	+
	Cornison de Paris	+	+
	Crisan	+	+
CUCUMIS MELO	Galben necacios	-	-
	Makdimon	-	-
	Citirex	-	-
	Raimond	-	-
	Bulgaresti	-	-
CUCURBITA MAXIMA	Dovleacul de placinta	-	-
	Dovlecelul	-	-
TIGVA	-	+	+
LUFA	-	+	+
CASTRAVETE AMAR	-	-	-

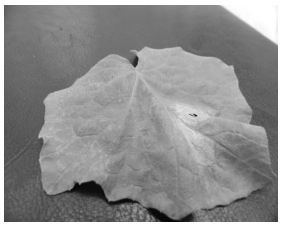

Fig. 2 and 3: CMV symptoms on the cucumber genotypes.

Aneling temperature of primers of 46 ° C for five cycles, followed by recovery at 50 ° C for 30 repeated cycles gave clear bands in agarose gel electrophoresis. Subsequently, these parameters were used in IC-RT-PCR.

Based on the optimization procedure, primers CMV IF / CMV-R (specific for isolated SI) gave a single band of approximately 500 nucleotides of CMV-Fny, CMV II-F/CMV-R while primers (specific for S -II isolates) produced a band of approximately 600 nucleotides of CMV-G2 (Fig. 2A). There was no band in terms of control samples, using primer pairs specific S-II.

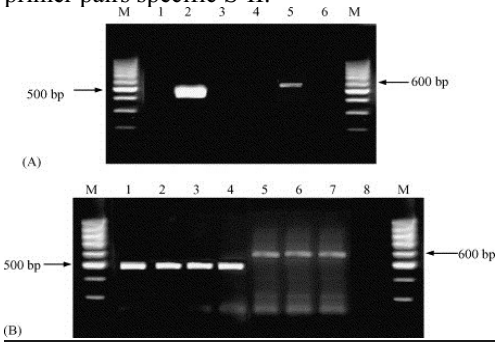

Fig. A and B: Electrophoresis gel 1.5% to revaluated a CMV infection.

Development of IC-RT-PCR for various genotypes cucurbitaceae in terms of CMV infection virus isolated subset I and II. (A), the amplified bands were obtained in terms of some genotypes II of CMV and CMV-R, amplified with CMV IF / CMV-R. (B), IC-RT-PCR from melon probs: 1.Dulce of Dabuleni, 2. Catherine 1, 3. 1AS1Wmoo1; 4.Peace F1: 5. Crimson Sweet and 6. Oltenia. Results demonstrated that the 6 genotypes studied only 2, namely Catherine and Crimson Sweet were revealed to be positive (presence elecroforeza band in agarose gel) electrophoresis A.

Using primers Begomo1 (CCGTGCTGCTGCCCCCATTGTCCGCGTC AC) and Begomo2 (CTGCCACAACCATGGATTCACG CACAGGG) for pumpkin and certain genotypes 1.LUFA. 2. Gourds, 3. Pumpkin pie, for cucumbers of varieties 4. Cornison de Paris, 5. Cucumber spiky, 6. Crisan, 7.melon Crimson sweet and 8. Negative control sample of tobacco showed a band of each PCR product of 500 bp and 600 bp respectively in 1.5% agarose gel. Electrophoresis B.

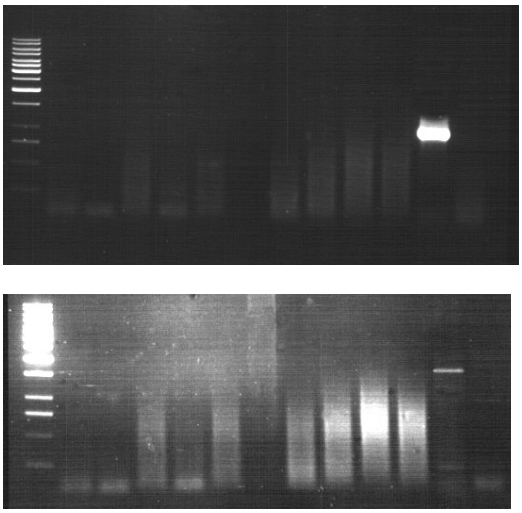

Fig 2 a and b: PCR detection of CMV in different melon genotypes.

Fig 2. PCR for detection of begomovirus using these primers for these genotypes of melon (Red Star, Raymond, Romanza, Nostalgia, Sorento, black spot, Macapadimon, Citirex, Colorado, Galben Necacios, Bulgarian). Fig. 2a. PCR with universal primers begomoviruses to produce a PCR product of approximately 1.1 kb. The Marker 1 Kb class is a scale used in most cases. Lanes 2-11 are samples from melon where no PCR product was not amplified. Lane 12 positive control in this case constitutes a sample of tomato and the tape 13 is negative control, where distilled water was added to the reaction mixture.

Method of IC-RT-PCR was then used to test the presence of CMV in samples of Melon, cucumber and pumpkin. A 500-nucleotide band could be amplified with primers specific pair and the evidence were revealed by TAS-ELISA negative. Similarly, samples containing S-II produced a band of 600 nucleotides (Fig. 2B). S I and S II pairs of primers have amplified specific1 melon genotypes, but have grown loofah, gourds, pie pumpkins, cucumbers (Paris Gherkins, Cucumber spiky and Crisan).

For detection of CMV's on cucurbitaceae (watermelon, cucumber, squash) serological ELISA methods are widely used. However, PCR has proved more reliable for detecting CMV isolates (Porta et al, 1989 and Hsu et al., 2000). In this study, we continued with the identification of new genotypes from Cucurbitaceae in terms of resistance to CMV may be proposed improvement works continue in these species.

Also in this paper has tried PCR's simple, and then IC-RT-PCR method. Differences were relevant. First, RNA extraction is difficult and costly, and RNA molecules are also slightly degraded due to the presence of RNase almost everywhere. Second, potential contamination of RT-PCR analysis was more difficult. However, the method of IC-RT-PCR Total viral RNA extraction avoid replacing the total DNA of plants, and was easily achieved in a single tube. These procedures have been developed to detect several viruses in plants, including Apple stem pitting virus, Cherry leaf roll virus, Grapevine leafroll-Associated Virus 1, Pepino mosaic virus, Plum pox virus and Raspberry Bushy Dwarf Virus (Wetzel et al , 1992, Kokko et al, 1996, James et al, 1997, KEIM-Konrad and Jelkmann, 1997, Werner et al., 1997, Sefc et al, 2000 and Mansilla et al., 2003)

CONCLUSIONS

In this paper, we developed a technique for IC-RT-PCR to detect CMV isolates from different genotypes cucurbitacea. Testing the samples in artificial conditions of infection using TAS-ELISA and IC-RT-PCR showed that this method of IC-RT-PCR is the most sensitive eficenta. Further analyzes aimed at identifying other genotypes of interest in terms of resistance to CMV. Of the 22 local and foreign genotypes of melons and cucumbers two genotypes: Yellow and black spot necacios their interest in terms of resistance to CMV. The IC-RT-PCR technique, we found that a single strong band of approximately 500 nucleotides that could be easily amplified by plants containing viral particles of insulation and while the band of 600 nucleotides in plants containing viral particles CMVizolatul S-II was relatively weak. These results coincide with those obtained by TAS-ELISA, suggesting that isolated S-II concentration in plants is lower than isolated. Although it is a natural infection and mixed with isolated CMV S-II in the samples tested, IC-RT-PCR procedure should be improved to detect possible mixed infections TAS-ELISA techniques and IC-RT-PCR developed in this study to detect CMV isolates

were also relevante. De use of molecular markers may be useful for screening a large number of genotypes to identify those of interest in terms resistance to CMV. Among the 33 samples tested in this study, only six samples (6.9%) were found to be infected with CMV isolate S-II. The reason for this is probably that the samples tested in this study were collected from the green house conditions in south of the country. In addition, host species could be an important factor for the incidence of CMV infection different (Hristova et al., 2002). To determine the prevalence of such infections in the south of Romania, are still necessary in other studies on multiple samples from different cultures, locations and years. RCP is actually more sensitive than serological methods ([Bousalem et al, 2000.] And [Jacobi et al, 1998.]). This may have some advantage in detecting the different CMV isolates that have low concentrations in plants . Moreover, PCR is more suitable for obtaining information on the viral genome. In contrast, TAS-ELISA facilitates rapid processing of large numbers of samples. A combination of the two above methods may be most suitable for epidemiological analysis and to study the genomic variations of the CMV strains.

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