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First report of cucumber mosaic virus subgroups I and II on soybean, pea, and eggplant in Iran

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Summary. – Cucumber mosaic virus (CMV) has the widest host range (> 1000 plant species) of any known plant virus, a large number of vector species, and a wide geographical distribution. A survey was conducted to determine the incidence of CMV of subgroups I and II on selected host crops in northern Iran. A total of 935 leaf samples from 10 host crops (tomato, pea, tobacco, soybean, watermelon, broad bean, squash, cucumber, eggplant, and lettuce) showing virus disease-like symptoms were collected in 12 cities of Golestan and Mazandaran provinces (northern Iran) during 2009 and 2010. Among the field samples tested by double-antibody sandwich ELISA (DAS-ELISA), 275 samples were found to be infected by CMV. These were subsequently evaluated by compound ELISA with monoclonal antibodies. We found that 198 samples were infected by subgroup I, 98 samples by subgroup II and 45 samples by both virus subgroups. Twenty-four samples showed no reaction in compound ELISA. In presented paper, CMV subgroups I and II (CMV-I and CMV-II) have been reported for the first time on soybean, pea and eggplant in Iran, with subgroup I being dominant in the north of the country.

Keywords: CMV; subgroups I and II; compound ELISA; monoclonal antibody

Introduction

CMV, a positive-sense RNA plant virus with a tripartite genome, is the type member of the genus *Cucumovirus*. CMV has a worldwide distribution and exists as a variety of isolates that differ in host range and pathogenicity (Cui *et al*., 2005). CMV causes great losses in vegetables, ornamentals and fruits, and is destructive due to its rapid spread by more than 75 aphid species in the field (Akhtar *et al*., 2010).

Many CMV isolates have been described and they can be divided into two main subgroups, CMV-I and CMV-II based on serological relationships, peptide mapping of the coat protein (CP), nucleic acid hybridization and nucleotide sequence identity (Thompson and Tepfer, 2009; Yordanova *et al*., 2002). More recently, a further division of subgroup I into IA and IB has been proposed based on the nucleotide sequences of the 5′ non-translated region (NTRs) and CP gene of RNA3 (Moury, 2004). The host range of the two subgroups is quite similar with only a few exceptions reported in the literature. CMV-I usually causes severe symptoms in dicotyledonous plants, while CMV-II can show reduced virulence and leads to milder symptoms (Carrere *et al*., 1999). Subgroups IA and II appear worldwide, but subgroup IB appears predominantly in East Asia (Du *et al*., 2007). CMV-I is predominant in the tropics and subtropics, while CMV-II is prevalent in temperate regions (Berniak *et al*., 2009).

Due to its economic importance, several serological methods have been developed for detection and differentiation of CMV isolates. Because the two subgroups are serologically closely related and usually cannot be distinguished by polyclonal antibodies, monoclonal antibodies (MAbs) against CMV-I and CMV-II isolates have been raised and used to differentiate the two subgroups (Porta *et al*., 1989).

In Iran, CMV isolates from over 73 species in 26 families of host plants have been obtained (Ahoonmanesh *et al*.,

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Abbreviations: CMV = cucumber mosaic virus; CMV-I = cucumber mosaic virus subgroup I; CMV-II = cucumber mosaic virus subgroup II; CP = coat protein; DAS-ELISA = double-antibody sandwich ELISA; MAbs = monoclonal antibodies

1997; Farhangi *et al*., 2004; Golnaraghi *et al*., 2004; Rahimian and Izadpanah, 1978; Rasoulpour and Izadpanah, 2008; Sokhandan Bashir *et al*., 2006; Soleimani *et al*., 2004). Little data, however, is available about the subgrouping of CMV isolates that appear in Iran. In order to better understand the epidemiology of CMV in Golestan and Mazandaran provinces (north of Iran), MAbs against the two CMV subgroups were used in compound ELISA for efficient identification and subgrouping of CMV in the present study.

Materials and Methods

Samples. A total of 935 leaf samples including cucumber, squash and watermelon (from *Cucurbitaceae*), tobacco, tomato and eggplant (from *Solanaceae*), soybean, pea and broad bean (from *Fabaceae*) and lettuce (from *Asteraceae*), which showed symptom(s) similar to those caused by CMV were collected from several locations in Azad-Shahr, Agh-Ghala, Bandar Torkaman, Bandar Gaz, Ramian, Ali-Abad, Kordkuy, Kalaleh, Gorgan, Gonbad Kavoos, Minoodasht (in Golestan province) and Behshahr (in Mazandaran province) during 2009–2010. In general, no more than 10 samples per crop were collected at each site (Hord *et al*., 2001). Samples were immediately placed in plastic bags, transported in cold boxes, and were transferred to the laboratory in order to detect the CMV. They were stored for short-term at 4ºC, until tested by ELISA.

DAS-ELISA. ELISA kits with polyclonal antibodies against CMV were from DSMZ (Germany). Positive and negative controls were provided by Tirtash Research and Education Center (Behshahr, Iran) and greenhouses of Gorgan University, respectively. The DAS-ELISA procedure was performed according to the DSMZ protocol for CMV using the Clark-Adams method (Clark and Adams, 1977). Samples infected with CMV were further tested by compound ELISA for differentiation of CMV-I and CMV-II.

Compound ELISA reagent set for CMV-I and CMV-II with specific monoclonal antibodies and positive controls for each subgroup were obtained from Agdia (USA) by the Biofords (France). Compound ELISA and alkaline phosphatase label were performed according to the Agdia protocol for CMV subgroups. Microtiter plates (Agdia, USA) were coated with 100 µl of 1:100 concentrated capture antibody (CAB 44700 for CMV-I and CAB 44800 for CMV-II) in carbonate coating buffer (CCB) and incubated in a humid box for 4 hrs at room temperature. Wells were washed three times with washing buffer (PBST), 100 µl of plant extract was dispensed to each well, the plate was placed inside a humid box and incubated for 2 hrs at room temperature. Wells were washed 8 times with washing buffer, 100 µl of alkaline phosphatase enzyme conjugate and detection antibody in ECI buffer were added and incubated in a humid box for 2 hrs at room temperature. Wells were washed again 8 times, 100 µl of PNP substrate was added and plates were incubated in a humid box for 60 min. The absorbance was determined at 405 nm by an ELISA-reader (BioTek-ELx800).

Results and Discussion

During 2009–2010, 935 symptomatic leaf samples were collected from 60 fields in Golestan and Mazandaran provinces, which included 153 samples from tobacco (*Nicotiana tabacum*), 127 from pea (*Pisum sativum*), 83 from soybean (*Glycine max*), 122 from broad-bean (*Vicia faba*), 79 from eggplant (*Solanum melongena*), 24 from lettuce (*Lactuca sativa*), 91 from cucumber (*Cucumis sativus*), 62 from squash (*Cucurbita pepo*), 90 from watermelon (*Citrullus lanatus*) and 104 from tomato (*Lycopersicon esculentum*). These samples were evaluated by DAS-ELISA method using polyclonal antibody. Results of this survey indicated that among the total of 935 samples, 275 samples (29.4%) were infected by CMV and showed positive reaction in DAS-ELISA test. Between the hosts tested, the highest and the lowest rate of CMV infection was associated to watermelon (62.44%) and lettuce (0%), respectively.

CMV-infected samples were further tested by compound ELISA using monoclonal antibodies for the detection of CMV subgroup I and subgroup II. Of the 275 infected samples, 198 samples were infected by subgroup I, 98 samples by subgroup II and 45 samples by both subgroups (Table 1). Twenty-four samples showed no infection by these subgroups. The highest CMV-I incidence among the surveyed hosts was reported on watermelon (85.36%), followed by squash (58.33%), eggplant (54.45%), tobacco (53.17%), tomato (38.67%), broad bean (35%), soybean (25%), cucumber (20%), pea (16.67%) and lettuce (0%). Incidence of CMV-II in decreasing order was on tomato (59.46%), eggplant (44.07%), cucumber (42.86%), tobacco (24.93%), watermelon (21.34%), squash (13.88%), soybean (12.5%), broad bean (10%), pea (8.33%) and lettuce (0%). The most and the least infection by both subgroups was observed on tomato (17.62%) and soybean and lettuce (0%), respectively. Among the studied locations, the most and the least infection by CMV-I was observed in Behshahr (100%) and Ramian (19.78%), by CMV-II in Gonbad Kavoos (58.13%) and Kordkuy (0%) and by both subgroups in Behshahr (20.5%) and Bandar Torkaman and Kordkuy (0%), respectively.

According to the research records on the hosts, it was found that between the selected hosts only three hosts (cucumber, tobacco and soybean) were among previously reported CMV hosts in Golestan province and six other hosts (broad bean, watermelon, pea, squash, eggplant, and tomato) were determined as new CMV hosts in Golestan province. In addition, CMV subgroups from 9 hosts in Golestan province were tested for the first time. Across Iran, CMV was reported on all of the studied hosts, but CMV subgroups were isolated from cucumber, tobacco, watermelon, tomato, squash and broad bean. In this work, CMV-I and CMV-II were reported for the first time in Iran on soybean, pea and eggplant. This confirms the correctitude of the host selection for this research.

Table 1. List of CMV hosts from north of Iran and number of samples infected with CMV-I, CMV-II and both subgroups (CMV-I & II)

Crops	No. of samples collected	[a]CMV-I (%)	[b]CMV-II (%)	[c]CMV-I & II (%)
Broad Bean	122	9 (35.00)	6 (10.00)	3 (5.00)
Cucumber	91	3 (20.00)	8 (42.86)	2 (5.71)
Eggplant	79	27 (54.45)	12 (44.07)	5 (13.43)
Lettuce	24	0	0	0
Pea	127	4 (16.66)	3 (8.33)	1 (2.78)
Soybean	83	9 (25.00)	1 (12.50)	0
Squash	62	8 (58.33)	3 (13.88)	1 (5.55)
Tobacco	153	56 (53.16)	15 (24.93)	14 (16.59)
Tomato	104	35 (38.66)	39 (59.46)	16 (17.62)
Watermelon	90	47 (85.36)	11 (21.34)	3 (6.70)
Total	935	198	98	45

[a,b,c]Number of samples infected with CMV-I, CMV-II, CMV-I and II, respectively (percentage of infection).

Several strains of CMV induce different symptoms on various hosts (Tamashiro *et al*., 2004). While collecting the samples, it was attempted to take leaves with typical viral symptoms. The results of the ELISA performed on collected samples are in agreement with the severity of the virus symptoms on them. Samples collected from soybean had no viral symptoms and initially were thought to be virus-free, but 14 out of 83 samples were infected, which suggests latent infection (Carrere *et al*., 1999). On the other hand, cucumber, as one of the main hosts of CMV and with typical symptoms, did not exhibit infection and only 9 out of 91 collected samples gave positive reaction in ELISA. The negative reaction of samples in ELISA despite typical viral symptoms could be due to the infection of plants with other viruses or may be related to non-biotic factors, such as cold weather or application of pesticides and fertilizers (Bos, 1999; Sokhandan Bashir *et al*., 2006).

CMV-I is predominant in the tropics and subtropics and usually causes severe symptoms in those regions. In contrast, CMV-II is prevalent in temperate regions. Among 275 CMV-infected samples tested in this work, 198 samples (72%) showed infection with CMV-I. Thus, the subgroup I was more common than the subgroup II. This was expected, as the climate in Golestan province is subtropical. Our results are consistent with those obtained in Australia and Costa Rica (Rizos *et al*., 1992; Hord *et al*., 2001). In addition, several groups studying CMV in northwestern Iran, Turkey and Poland showed that CMV-II was more common in regions with moderate climate (Sokhandan Bashir *et al*., 2006; Balci, 2005; Berniak *et al*., 2009).

Between samples infected with cucumber mosaic virus, 45 samples (16.4%) were infected with both subgroups I and II. From the hosts studied in this work, mixed infection was not observed only in lettuce and soybean. Mixed infections with different CMV strains in one plant are well known for a long time (Price, 1934). Single infections by defined strains of specific subgroups occur in the field only rarely, as surveys in various parts of the world revealed the presence of a great variety of CMV strains in many locations (Varveri and Boutsika, 1999). Therefore, mixed infections with strains of different subgroups or mixed infections of CMV with other viruses can be considered a common event in the field, which was confirmed by Fraile *et al*. (1997) in Spain and Kumar *et al*. (2009) in India. But mixed infections with both subgroups in a single plant are not very common and only few reports exist about that, e. g. mixed infections of tobacco with CMV subgroup I and II in Germany (Hellwald *et al*., 2001).

In this work, 24 samples positive in DAS-ELISA did not show positive reaction with any of the monoclonal antibodies used in compound ELISA. Failure of the CMV-I and II MAbs to detect the virus may have two reasons. First, the concentration of CMV-I and CMV-II may be below the ELISA detection limit (Yu *et al*., 2005). Second, although MAbs are usually more reliable than polyclonal antibodies, their high specificity may be a problematic, since they react with only one epitope (Sokhandan Bashir *et al*., 2006).

The host species might be an important factor for the incidence of the different subgroups of CMV. To establish the prevalence of the two subgroups in Iran, continuous studies on more samples of different crops, locations and years are required.

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