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Additional Information

Incidence and genetic diversity of cucurbit viruses in Spain

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Abstract

Several viral diseases affect cucurbits, with different economic effect depending on the region and the year. However, there are not recent studies on the incidence of specific viruses. During the 2018 summer season, surveys were carried out in open field melon (*Cucumis melo*), squash (*Cucurbita* spp.) and watermelon (*Citrullus lanatus*) crops in most of the main producer areas under commercial (AGL2017-85563-C2 1R and 2R) and organic farming (PROMETEO/2017/078). Samples from plants with virus-like symptoms were analyzed by RT-PCR/PCR with specific primers, hybridization and/or ELISA, to detect *Cucumber mosaic virus* (CMV), *Zucchini yellow mosaic virus* (ZYMV), *Watermelon mosaic virus* (WMV), *Moroccan watermelon mosaic virus* (MWMV), *Cucurbit yellow stunting disorder virus* (CYSDV), *Cucurbit chlorotic yellows virus* (CCYV), *Cucumber green mottle mosaic virus* (CGMMV) and *Tomato leaf curl New Delhi virus* (ToLCNDV). Samples from fields in Murcia (south-eastern Spain), Castilla-La Mancha (central Spain) and Comunidad Valenciana (eastern Spain) were analyzed. At least one of these viruses was detected in approximately 80% of the samples. WMV was the most frequently detected in samples of the three crops. ZYMV, CMV, CYSDV and ToLCNDV were present with lower incidence, mainly identified in mixed infections with WMV.

Keywords: melon, *Cucurbita* spp., watermelon, virus, WMV.

INTRODUCTION

Melon (*Cucumis melo*), squash and pumpkins (*Cucurbita* spp.), and watermelon (*Citrullus lanatus*) are among the most important cucurbits crops in Mediterranean agriculture. Spain is one of the main European producers of these three crops. In 2018, Spain was the first European producing country of pumpkin, squash and gourds (702,278 t), and the second (just preceded by Turkey) of melon (655,677 t) and watermelon (1,113,192 t) (FAOSTAT, 2019).

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Virus-induced diseases represent the most important limiting factor for cucurbits cultivation. At least ten out of the around 60 different viruses reported infecting cucurbits (Lecoq and Desbiez, 2012) have been identified in Spain (Luis-Arteaga et al., 1998; Kassem et al., 2007; Juárez et al., 2013). Among others, *Cucumber mosaic virus* (CMV), *Zucchini yellow mosaic virus* (ZYMV), *Watermelon mosaic virus* (WMV) and *Cucurbit yellow stunting disorder virus* (CYSDV) have for long been widespread in Spanish fields.

Some other viral species are more recent threats in this region. *Cucumber green mottle mosaic virus* (CGMMV) was first described in Spain in cucumber (*Cucumis sativus* L.) in the 1990s (Célix et al., 1996), but it has been notified recently that the incidence of this virus has increased, infecting also watermelon, melon and *Cucurbita* spp. (Crespo et al., 2017). *Tomato leaf curl New Delhi virus* (ToLCNDV) has been reported as the causal agent of a devastating disease affecting cucurbit crops in Spain since 2012 (Juárez et al., 2014). *Moroccan watermelon mosaic virus* (MWMV) has also been reported as a recent threat in this region (Miras et al., 2019). Moreover, *Cucurbit chlorotic yellows virus* (CCYV), not reported in Spain so far, is spreading in the Mediterranean basin (Orfanidou et al., 2014; Al-Saleh et al., 2015).

The most recent study analyzing incidence of cucurbits viruses in Spain gathered data from surveys in 2005 and 2006 (Juarez et al., 2013). Here we present an update of the incidence of some of the most important viral species infecting cucurbits in some of the most important producing areas in Spain.

MATERIALS AND METHODS

Surveys

During the 2018 summer season, surveys were carried out in open field melon, squash and watermelon crops in most of the main producer areas under commercial and organic farming (Figure 1, Table 1).



Figure 1. Locations surveyed in Spain in three different autonomous communities: MU (Murcia), CM (Castilla-La Mancha) and CV (Comunidad Valenciana), in commercial farming fields (solid dots) or organic farming fields (empty dots)

Table 1. Surveys carried out in summer season 2018: crop species surveyed, symptoms observed and viral species detected (see text for viral species full names).

	Location, autonomous community* (date)	Crop species	Symptoms	Viral species
Commercial farming	El Algar, MU* (August)	Melon	Virus-like symptoms	WMV
	Tomelloso, CM (July)	Melon	Mosaic, leaf deformation	CMV, WMV, ZYMV, ToLCNDV
	Alcázar de San Juan, CM (September)	Melon	Virus-like symptoms	CMV, WMV, ZYMV
	Museros, CV (June)	Melon	Yellowing	WMV, ToLCNDV
		<i>C. moschata</i>	Mosaic, yellowing	WMV
		Watermelon	Yellowing	WMV, CYSDV, ToLCNDV
		Weed	Mosaic, yellowing	WMV
	Museros, CV (July)	<i>Cucurbita</i> spp.	Mosaic, leaf deformation	WMV, CYSDV, ZYMV, ToLCNDV
		Watermelon	Mosaic, leaf deformation	WMV
		Weed	Mosaic, yellowing	ZYMV, ToLCNDV
	Museros, CV (August)	Melon	Mosaic, leaf deformation	WMV, CMV
		<i>Cucurbita</i> spp.	Mosaic, leaf deformation	WMV, CMV, CYSDV, ToLCNDV
Watermelon		Mosaic, leaf deformation	WMV, CMV, ToLCNDV	
Organic farming	Gandía, CV (June)	<i>Cucurbita</i> spp.	Mosaic, yellowing	CMV, WMV
	La Punta, CV (June)	Melon	Mosaic, leaf deformation	WMV, CMV
	Moncada, CV (June)	Melon	Mosaic, leaf deformation	CMV, WMV

* Autonomous community: MU: Murcia; CM: Castilla-La Mancha, CV: Comunidad Valenciana

Virus detection

Samples were analyzed by RT-PCR to determine the presence of CMV, ZYMV, WMV, MWMV, CYSDV, CCYV and CGMMV, and by PCR to detect ToLCNDV, with primers designed based on the coat protein (CP) gene sequence for all of them. Briefly, in the case of RNA viruses, total RNA from leaves was isolated using TRIzol reagent (Thermo Fisher Scientific) followed by chloroform extraction. RNA was precipitated with an equal volume of isopropanol for 20 min. These RNA extracts were used to synthesize cDNA, corresponding to the CP gene of each of the viruses. DNA isolation, to detect ToLCNDV, was carried out as described in Sáez et al. (2017).

In doubtful cases, dot blot molecular hybridization (with digoxigenin-labeled RNA probes) or DAS-ELISA were used to confirm the result.

Partial sequencing of viral isolates

Representative samples infected with the more frequently identified viral species, WMV and CMV, were selected. RT-PCR products were purified from agarose electrophoresis gels by using GeneJET Gel Extraction Kit (Thermo Scientific). The sequencing, using the same primers corresponding to the CP, was carried out in the 'Sequencing Core Service', Instituto de Biología Molecular y Celular de Plantas (IBMCP, CSIC-UPV, Valencia, Spain).

RESULTS AND DISCUSSION

Surveys

1. Commercial farming.

All samples collected in Murcia and Castilla-La Mancha were melon leaf samples of plants that showed symptoms of viral infection, i.e., yellowing, mosaics and leaf deformations. These samples were collected in the final stages of cultivation.

WMV was detected in 67% of the samples from Murcia, with no other viral species identified.

Samples collected in Castilla-La Mancha came from two different localities: Tomelloso and Alcázar de San Juan. Symptoms mainly observed in Tomelloso were mosaic and leaf deformation (Figure 2), with CMV as the suspected causal agent. Apart from this viral species, WMV, ZYMV and ToLCNDV were also identified in these samples. On the other hand, symptoms noticed in Alcázar de San Juan were those typical of ToLCNDV, that is, curled leaves, yellow mosaic and stunted growth. However, samples collected from this area were positive for CMV, WMV and ZYMV, but not for ToLCNDV.



Figure 2. Symptoms in leaves of melon samples collected in Tomelloso (Castilla-La Mancha).

Surveys in Comunidad Valenciana, in the commercial farming field of Museros, were developed sequentially, for three consecutive months. At the beginning of the cycle, most of the plants surveyed of the different species, showed yellowing. In this sampling date, WMV was the most frequent viral species, infecting watermelon (~25%), melon (~50%) and *Cucurbita moschata* (100%). Moreover, the weed samples collected near the *C. moschata* field were also infected with WMV. No other viral species was detected in samples of *C. moschata*. In the case of watermelon, samples infected with WMV were also positive for CYSDV, a different sample was infected with ToLCNDV and the remaining, while showing yellowing, were negative for the viral species analyzed. All melon samples were infected with ToLCNDV.

In the middle stage of the growing cycle, symptoms were more severe in all the crop species, with mosaics, leaf deformation and chlorosis. WMV was the only viral species infecting watermelon. All *Cucurbita* ssp. samples were again infected with WMV, some of them in mixed infection with CYSDV, but also with ZYMV and ToLCNDV. A weed sample collected near the melon field was positive for ZYMV and ToLCNDV.

In the third sampling date, symptoms were even more severe, with symptoms in fruits of the three crop species (Figure 3).



Figure 3. Symptoms in watermelon (left), melon (center) and *Cucurbita moschata* (right) fruits collected in Museros (Comunidad Valenciana). Watermelon and melon plants were infected with WMV and ZYMV, and *C. moschata*, with WMV.

All watermelon samples showed clear symptoms of WMV in leaves. All of them were infected with WMV, with some of them also positive for CMV and ToLCNDV or ZYMV. WMV and CMV were detected in melon samples. In the case of *Cucurbita* ssp. all the samples were infected with at least either WMV or CMV, and in 44% of the samples with both of them. CYSDV and ToLCNDV were also detected in some samples.

2. Organic farming

Three fields, located in Gandía, La Punta and Moncada, conducted with organic agricultural practices, were surveyed. The viral species identified in the three fields were WMV and CMV, with different relative importance depending on the locality. Aphids were consistently detected in these fields, although treatments with diatomaceous earth were carried out.

All samples in Gandía corresponded to *Cucurbita* spp, which showed yellowing and leaf deformation (Figure 4). In some cases, leaves were asymptomatic, while symptoms appeared in fruits. CMV and WMV were detected in 5% and 41% of the

samples analyzed, respectively. No mixed infections were identified. Samples infected with CMV were collected at early stages.



Figure 4. Symptoms in *Cucurbita* spp. plants in Gandía (Comunidad Valenciana). Samples were positive for CMV (left) and WMV (right).

In La Punta, where melon was cultivated, soil-borne fungal diseases were the most important limiting factor. Samples from plants not affected by fungi and with symptoms of viral infection were collected. A total of 84% of the samples were positive for WMV, and 47% for CMV, in most cases in mixed infection (Figure 5).



Figure 5. Symptoms in melon plants in La Punta (top) and Moncada (bottom) (Comunidad Valenciana). Samples were positive for CMV and WMV.

Contrarily, in Moncada, also cultivated with melon, CMV was the prevalent virus (91%), while WMV was only detected in 15% of the samples, later in the growing cycle,

and frequently in mixed infections (Figure 5).

WMV was the viral species most frequently identified in this survey. Previous studies revealed the importance of WMV in different Spanish cucurbits producing areas (Luis-Arteaga et al., 1998; Moreno et al., 2004; Juárez et al., 2013). The high percentage of mixed infections was also highlighted in preceding surveys (Kassem et al., 2007; Juárez et al., 2013), as well as the consequences in terms of symptom enhancement of these multiple infections (Gómez et al., 2010). Moreover, the presence of the different viruses in weeds should be further explored, since the importance of alternative hosts to understand population biology of multi-host pathogens has been demonstrated (Kassem et al., 2013).

Sequencing analysis

The CP gene PCR product of samples infected with WMV from different origins, representative of the surveys and the hosts, were sequenced. Nucleotide identity between sequences varied between 92% and near 100%. However, no specific association was found between CP gene sequences and host or location. This lack of association has previously been reported for other cucurbit viruses (Kassem et al., 2007; 2013). Sequences obtained showed the highest similarity with either the Spanish isolate WMV-Vera (GenBank accession number MH469650) or with Asian isolates.

The same analysis was carried out with CMV-infected samples from Moncada (Comunidad Valenciana), where this virus was detected in 91% of the samples. All the sequences obtained showed high similarity among them, and high similarity with European isolates belonging to the subgroup IA (Aramburu et al., 2007).

CONCLUSIONS

- WMV was the viral species most frequently detected in melon, watermelon and *Cucurbita* spp. samples. ZYMV, CMV, CYSDV and ToLCNDV were present with lower incidence, mainly identified in mixed infections with WMV.
- CMV sequences showed high identity among them and with European isolates.
- WMV sequences were more variable, with nucleotide identity between 92 and near 100%. These sequences showed the highest similarity with either Spanish or Asian isolates.
- Future work will include the surveying of other producer areas in Spain and other weeds present in cucurbits crops.

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