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Viruses in cucurbit seeds from on-line mail-order providers

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Abstract
Seeds of three important cucurbit species (cucumber, melon and zucchini) were obtained through on-line mail-order providers and tested for selected viruses of biosecurity and agronomic concern. One or more of the targeted viruses were detected in 23 of the 31 seed lots tested. The quarantine pest melon necrotic spot virus was detected by ELISA in melon seeds. Multiple instances of contamination with the quarantine pest cucumber green mottle mosaic virus were also detected, and its presence in seeds of all three plant species was confirmed by inoculation and re-isolation from indicator plants. Cucumber mosaic virus, squash mosaic virus and potyviruses were repeatedly detected, and instances of insect pest and plant trash contamination were also observed. Seeds imported into Australia from some on-line sources have potential to introduce organisms of biosecurity concern.

Keywords Seed-borne virus · Cucurbitaceae · Quarantine pest · Mail-order seeds

Text

On-line commerce ('e-commerce') through global provider platforms has significantly increased the international accessibility of many commodities for consumers. Volumes of global movement of goods have shown associated significant increases; for example, the Australian national postal service provider recently reported a 46% increase of in-bound parcel numbers over the previous corresponding period (Australia Post 2018).

Goods and materials accessible through international e-commerce channels include items whose importation is subject to regulatory control by domestic authorities. Among these are materials of potential biosecurity concern, including unprocessed foodstuffs, live animals and animal products, and plant propagative materials such as plants and seeds. Recently reported instances of global distribution of unsolicited seed consignments (Australian Broadcasting Corporation 2020) have led to a heightened focus on biosecurity-related issues associated with such materials. Amongst various concerns, uncontrolled movement of these materials has potential to spread new agents and strains of disease, and thus to compromise biosecurity controls achieved through more closely controlled pathways.

This study assessed seeds of three agriculturally-important cucurbit species (cucumber, *Cucumis sativus*; melon, *C. melo;* zucchini, *Cucurbita pepo*), sourced through international on-line mail-order providers, for the presence of viruses from several taxa known to include seed-borne members. These included viruses that are quarantine pests for Australia (e.g., cucumber green mottle mosaic virus, melon necrotic spot virus), as well as some previously recorded from Australia (e.g., cucumber mosaic virus). These viruses were also considered to be proxies for the many other viruses that are recognised as being associated with, or transmitted through, seeds of cultivated cucurbit species (DAWE 2020).

Seeds were purchased under permit in lots of 1,500–2,000 seeds through four generic on-line providers over a six month period. Orders were filled by more than 20 supply companies, all self-identified as located in south-east Asia. The specific geographic origins of the supplied seeds were not provided.

None of the accessed seed lots were compliant with Australia's general import requirement for unambiguous identification using the scientific binomial name. Most of the seeds delivered could be matched to those ordered, but

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in some instances shipments contained more than one seed variety without obvious identifications. Consequently, 31 individual seed varieties (16 cucumber, 10 melon and 5 zucchini) were tested for viruses as described, as was one mixed sample of cucumber seeds (CX), and one mixed sample of melon seeds (MX; Tables 1 and 2).

A minimum of 1,000 seeds was initially tested from each seed lot, with serological tests made on 10 batches each of 100 pooled seeds. All seeds were tested by ISTA-accredited enzyme-linked immunosorbent assay (ELISA) procedures using commercially-sourced antisera in accordance with manufacturers' instructions (see Online Resource 1). In some instances samples that returned positive results were further assessed by mechanical inoculation (under permit, and in containment) onto seedlings of herbaceous indicator species (*Nicotiana benthamiana*, *Cucumis sativus* and *C. melo*).

Some seed-derived materials were additionally assessed using reverse transcription polymerase chain reactions (RT-PCR) procedures (see Online Resource 2); the identities of amplified PCR products were confirmed by standard DNA sequencing protocols. For a small number of preparations high-throughput sequencing (HTS) procedures were used to analyse RNA-derived nucleotide sequences (see Online Resource 3).

All 33 seed lot samples were analysed for the presence of the quarantine pest cucumber green mottle mosaic virus (CGMMV; genus *Tobamovirus*). As indicated in Tables 1 and 2, the presence of CGMMV was detected by ELISA in a total of 13 of the 31 identified varieties (42% incidence), and from each of the three host species. Material from each positive preparation was inoculated onto indicator plants; while none of the indicator plants displayed visually-detectable symptoms of infection, seedlings inoculated with preparations from cucumber, zucchini and melon samples CP, ZD and MC, respectively, returned positive results for infection by PCR analysis.

All cucumber and zucchini seed lots (total n=22) were also tested for the quarantine pest kyuri green mottle mosaic virus (KGMMV) by ELISA; no positive results were recorded. Twelve cucumber (CF-CP, CX) and all five melon seed lots were tested for the quarantine pest zucchini green mottle mosaic virus (ZGMMV), also with no positive results.

Five cucumber seed lots (CA-CE) and all melon seed lots (total n=16) were tested for the quarantine pest melon necrotic spot virus (MNSV; genus *Carmovirus*); a single identified seed lot (MJ) gave a positive result by ELISA, as did the mixed pool seed lot MX.

Table 1 Detections of viruses in seed lots of cucumber sourced from generic on-line suppliers

Host	Sample	Virus[a]				
		CGMMV[a]	CMV	MNSV	SqMV	potyvirus
Cucumber	CA	0[b]	0	0	-[c]	0
	CB	0	0	0	-	0
	CC	0	0	0	-	0
	CD	0	0	0	-	0
	CE	0	0	0	-	0
	CF	0	**4**	-	0	0
	CG	0	**2**	-	0	0
	CH	0	**1**	-	0	0
	CI	0	**3**	-	0	0
	CJ	0	**3**	-	0	0
	CK	**1**	**6**	-	**5**	**2**
	CL	**1**	**6**	-	**1**	0
	CM	**1**	0	-	0	0
	CN	0	**8**	-	**1**	0
	CO	**4**	**10**	-	0	**8**
	CP	**3**	**10**	-	0	**2**
	CX	0	**3**	-	0	**8**

Bold entries are instances of positive virus detections against other reported negative results

[a]CGMMV: Cucumber green mottle mosaic virus; CMV: Cucumber mosaic virus; MNSV: Melon necrotic spot virus; SqMV: Squash mosaic virus; Potyvirus: generic potyvirus test

[b]denotes number of positive 100-seed sub-samples from a total of 10 sub-samples tested

[c]not tested

Table 2 Detections of viruses in seed lots of zucchini and melon sourced from generic on-line suppliers

Host	Sample	Virus[a]				
		CGMMV[a]	CMV	MNSV	SqMV	potyvirus
Zucchini	ZA	0 [b]	0	–[c]	0	**1**[d]
	ZB	**1**	0	–	0	0
	ZC	**2**	0	–	0	**7**
	ZD	**3**	0	–	0	**10**
	ZE	0	0	–	0	**10**
Melon	MA	0	0	0	–	0
	MB	**1**	0	0	–	**10**
	MC	**2**	0	0	–	**6**
	MD	**1**	0	0	–	**6**
	ME	0	0	0	–	0
	MF	0	0	0	–	0
	MG	0	0	0	–	**1**
	MH	0	0	0	–	**1**
	MI	**1**	**4**	0	–	**9**
	MJ	**1**	**2**	**1**	–	**10**[e]
	MX	0	**10**	**9**	–	**1**

Bold entries are instances of positive virus detections against other reported negative results

[a-c]see Table 1 for explanation

[d]PCR product sequence identified as *Watermelon mosaic virus*

[e]PCR product sequence identified as *Zucchini yellow mosaic virus*

Twelve cucumber (CF-CP, CX) and all five melon seed lots were tested for squash mosaic virus (SqMV; genus *Comovirus*); as shown in Tables 1 and 2, three cucumber seed lots returned positive results by ELISA. The same 12 cucumber seed lots, and all other seed lots (total n=28) were tested for the *Nepovirus* arabis mosaic virus, with no positive results observed.

All seed lots were tested for the presence of cucumber mosaic virus (CMV; genus *Cucumovirus*), with 11 cucumber and three melon samples providing positive results.

All seed lots were tested for the presence of members of the *Potyvirus* genus using broadly-reactive antiserum. The presence of potyviruses was detected in four cucumber, 8 melon and four zucchini seed lots, respectively. Products amplified and sequenced from seeds lots MJ and ZD were identified as being derived from watermelon mosaic virus (WMV) and zucchini yellow mosaic virus (ZYMV), respectively.

The inter-sample variations in virus incidence and prevalence suggest that most, if not all seed lots originated from different sources of seeds. Given the relatively small sizes of the samples tested from each seed lot, these results indicate high levels of overall contamination with seed-borne viral pathogens. High-throughput sequencing of selected samples gave a further indication of the likely health status of the plants from which these seeds were derived. In addition to further detections of WMV (e.g., seed lots CC and MX) and ZYMV (seed lot MX), the cucurbit aphid-borne yellows virus (CABYV; genus *Luteovirus*) was detected in several cucumber seed lots (e.g., CC and CP); while known to be seed-associated this virus is not considered to be seed transmissible, but its presence is nevertheless indicative of parental plant health status.

In some instances (Tables 1 and 2) more than one pool of 100 seeds was contaminated by a targeted virus. Thus, for example, cucumber seed lot CP, in which the presence of CGMMV was verified by mechanical transmission and PCR detection, returned 3 CGMMV positives among the 10 sample pools tested, as well as 10 positives for CMV. *Prima facie*, this result suggests a likely contamination rate of approximately 0.3% with CGMMV, accepting however that the small sample sizes tested preclude robust statistical assessment of likely prevalences of contaminating viruses.

The observation of multiple seed lot detections of CGMMV and MNSV even with the small number of samples tested suggests that, in a number of instances at least, the levels of virus prevalence are in excess of those tolerated in seeds entering Australia through other pathways (DAWE 2020). Australia's requirements for entry of large seed lots of these same cucurbit species include testing of 9,400 seeds, such that non-detection of quarantine pest viruses indicates with 99% confidence that the level of contamination is less than 0.05%.

Although several of the viruses detected on multiple occasions in this study (e.g., CMV, SqMV) are not quarantine pests for Australia, the risks associated with importation of new virus strains with different pathogenic characteristics, and the potential for recombination between these and virus strains already present, suggest that it is highly desirable to limit their further entry wherever possible.

In addition to detections of the viral pathogens that were the focus of this study, multiple instances of seed lot contamination with plant trash, other contaminating materials and arthropod pests were observed. Specimens of the latter identified by morphological or molecular techniques comprised booklice (*Liposcelis entomophila*; Psocoptera, Liposcelididae), grain beetles (*Oryzaephilus surinamensis* [Col., Silvanidae] and *Cryptolestes* sp. [Col., Laemophloeidae]), and a lepidopteran (*Plodia interpunctella*; Lep., Pyralidae). While none of these identified species are quarantine pests for Australia, other high-risk arthropods such as Khapra beetle (*Trogoderma granarium*) are also associated with seeds and other stored products (Athanassiou et al. 2019).

Any inspection of the Worldwide Web will show that there are reputable companies that offer seeds through on-line mail-order services. This study indicates, however, that it is important for consumers to select on-line suppliers with caution, given that it is also apparent that obtaining seeds from some sources has potential to introduce organisms of biosecurity concern.

In late 2020 the Australian Government implemented emergency measures to address the risk associated with Khapra beetle, banning entry into Australia of a number of high-risk plant products including seeds of the genera *Cucurbita*, *Cucumis* and *Citrullus*, through several identified pathways, including international mail (World Trade Organization 2020).

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