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Seed transmission of *Melon necrotic spot virus* and efficacy of seed-disinfection treatments

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Rates of seed transmission of *Melon necrotic spot virus* (MNSV) were estimated in seedlings grown from commercial melon (*Cucumis melo*) cv. Galia F_1 seeds. Seedlings at the cotyledon stage and adult plants were assayed for MNSV by DAS-ELISA and RT-PCR. None of the seedling groups tested positive for MNSV by ELISA. The proportion of seedlings infected with MNSV was at least 7 and 8% in seed lots 05 and 06, respectively, as estimated from RT-PCR analysis of grouped seedlings. Fourteen and eight grouped samples (10 seedlings per group), of a total of 200 and 100 seedlings, respectively, grown from infected seeds were MNSV-positive in seed lots 05 and 06, respectively, corresponding to seed-to-seedling transmission rates of 11·3 and 14·8%, respectively. Several seed-disinfection treatments were evaluated for their ability to prevent seed transmission of MNSV. The results suggest that a treatment of 144 h at 70°C can be used to eradicate MNSV in melon seeds without hindering germination.

Keywords: *Carmovirus*, chemical seed treatment, *Cucumis melo*, ELISA, RT-PCR, thermic seed treatment

Introduction

Melon necrotic spot virus (MNSV), a member of the *Carmovirus* genus in the *Tombusviridae* family (Riviere & Rochon, 1990), is a serious pathogen of greenhouse melons (*Cucumis melo*) and cucumbers (*Cucumis sativus*) worldwide, but it also causes problems in fields (Hibi & Furuki, 1985; Jordá *et al*., 2005). MNSV has been reported in the Americas, Europe and Asia, and was recently detected in Tunisia (Yakoubi *et al*., 2008). The symptoms in melon caused by this virus consist of stem necrosis at the crown level and, less frequently, small necrotic spots that sometimes abscise to leave holes on leaves and fruits. Wilting and plant death are also observed (Herrera *et al*., 2006). MNSV is artificially transmissible by mechanical inoculation (Ohshima *et al*., 2000). Additionally, the virus is both seedborne and soilborne, thus it is readily distributed through the seed market and becomes endemic in melon-growing areas, where it persists for a long period in association with its soilborne fungal vector *Olpidium bornovanus* (Kubo *et al*., 2005). MNSV attaches itself to the outer covering of the fungal spores, but is not carried internally (Furuki, 1981). MNSV particles are very stable and can remain viable in the soil for several years. Campbell *et al*. (1996) used the term vector-assisted seed transmission (VAST) because this virus occurs either in or on the seed, irrespective of the presence of the vector, and may also infect at a low rate when the vector is absent. Gosalvez *et al*. (2003) indicated that the highest levels of MNSV were detected in roots at a very early infection stage (4 days post-inoculation) and before the melon plants were transplanted to production areas. At this early development stage, roots seem to act as a reservoir for MNSV, indicating that MNSV replicates in this tissue.

Double antibody sandwich-enzyme linked immunosorbent assay (DAS-ELISA) (Campbell *et al*., 1996) and immunoabsorbent electron microscopy (ISEM) (Tomlinson & Thomas, 1986) have been used to detect MNSV. Molecular hybridization and reverse transcription-polymerase chain reaction (RT-PCR) have also been used to obtain increased sensitivity (Gosalvez *et al*., 2003), as observed in other viral systems (Sánchez-Navarro *et al*., 1998).

Different physical and chemical treatments have been reported to eradicate or significantly reduce the incidence of a number of viruses without affecting seed quality, e.g. *Tobacco mosaic virus* (TMV) (Laterrot & Pécaut, 1968) and *Pepino mosaic virus* (PepMV) (Córdoba-Sellés *et al*., 2007), which like MNSV, are carried on the seed surface. The use of virus-free seeds is an important measure to prevent introduction of the virus into production sites. To date, the only method reported for MNSV-decontamination

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of melon seeds has been acid treatment and drying for 4 days or longer (Dumas de Vaulx, 1970). Although some seed companies currently utilize pretreatments for melon seeds, the details of these seed-treatment protocols are proprietary.

The objectives of this study were (i) to estimate the rate of seed-to-seedling transmission of MNSV by ELISA and RT-PCR, (ii) to test the efficacy of different chemical and physical seed treatments in eradicating MNSV from melon seeds, and (iii) to determine the effect of these treatments on melon seed germination.

Materials and methods

The melon seeds used in this study were from cv. Galia F_1 produced in Spain and known to be highly susceptible to MNSV (Naval-Merino, 2005). Two seed batches were used for comparison, lot 05 and lot 06, from 2005 and 2006, respectively.

Seed-to-seedling transmission of MNSV

To determine transmission rates from melon seeds to seedlings, 355 and 150 seeds from seed lots 05 and 06, respectively, were germinated in small sterile plastic trays containing paper towels and covered with transparent polyethylene film. After 15 days, 0·1-g root samples were taken from each seedling, which were then transferred individually into the wells of 24-well trays containing sterilized substrate (2 : 1 peat: sand) and placed in a climatic chamber with 60% relative humidity, a 12-h photoperiod, day and night temperatures of 26°C and 18°C, respectively, and irrigation with distilled water. Stringent sanitary measures were used to prevent any spurious virus spread. A total of 200 and 100 seedlings at the cotyledon stage from lots 05 and 06, respectively, were randomly selected and transferred to the trays. Forty-five days later, the plants, now considered to be adults, were taken individually from the tray with a shovel so as not to damage the root system. Roots were washed with distilled water to remove the substrate and 0·1-g root samples were again taken from each plant.

The root samples from every 10 plants were mixed and grouped into plastic bags. From every composite sample of about 1 g, samples of 0·5 g and 0·1 g were taken to be analysed in parallel by DAS-ELISA and RT-PCR, respectively, to detect MNSV infection in melon plants at both developmental stages. All the plants were replanted for subsequent analysis.

DAS-ELISA analysis

The presence of MNSV was determined by DAS-ELISA with virus-specific antiserum (BIO-RAD) as recommended by the manufacturer. The 0·5-g samples were homogenized in a plastic bag in extraction buffer (2% PVP in PBS-Tween, 1 : 20, w/v). The homogenates were filtered through muslin and duplicate 100-μL aliquots detected by DAS-ELISA with MNSV-specific antiserum. Healthy and virus-infected melon root samples were included as negative and positive controls, respectively. Absorbance values (A_{405} nm) were measured in a Titertek Multiskan immunoplate reader. Only samples with values more than twice those of healthy root-extract controls were recorded as infected (DAS-ELISA-positive).

RNA extraction and RT-PCR analysis

The 0·1-g root samples were ground in liquid nitrogen, and total RNAs extracted using the RNAwiz™ kit (Ambion) following the manufacturer's instructions. RT-PCR was carried out in a single step using the SuperScript™ II RT with Platinum® Taq kit (Invitrogen Life Technologies) and MNSV-specific primers, MNSV1 (5′-GGAGGCAACATTTCGTACA-3′) and MNSV2 (5′-AGAGACCAAGCGATCAAAC-3′), designed to amplify a 651-bp viral genome fragment corresponding to nucleotides 172–822 of the MNSV coat protein gene (p42). Total RNAs were denatured at 65°C for 5 min and cDNA synthesized at 50°C for 30 min in a reaction mix that included 0·4 μM primers, 1 × reaction buffer (containing 0·4 mM dNTPs and 2·4 mM $MgSO_4$) and 0·25 μL of the enzyme mix, in a final reaction volume of 12·5 μL. The PCR conditions were: 94°C for 2 min, followed by 35 cycles of 1 min at 94°C, 1 min at 50°C and 1 min at 72°C, with a final elongation step of 10 min at 72°C, using a Mastercycler personal 5332 thermocycler (Eppendorf). RT-PCR-amplified products were separated by electrophoresis on 1·2% agarose gel in 1 × TAE buffer (40 mM Tris-acetate and 1 mM EDTA at pH 8·0) and stained with ethidium bromide. Fragment sizes were determined by comparison with a 100 bp DNA Ladder Plus (MBI Fermentas).

Percentage viral incidence from the grouped samples was estimated using the formula of Gibbs & Gower (1960): $P = 1 - (1 - y/n)^{1/k}$, where P = probability of transmission by a single MNSV-infected seedling, y = number of RT-PCR-positive samples, n = total number of samples assayed and k = number of seedlings per sample ($k = 10$).

Seed-disinfection treatments

To determine the efficacy of seed-disinfection treatments in eradicating MNSV from melon seeds, the two seed batches were compared using seven treatments. The effect of high temperature on eradication of MNSV was evaluated by heating seeds in single layers in an incubator at 70°C for (i) 72 h (T72), (ii) 96 h (T96), (iii) 120 h (T120) or (iv) 144 h (T144). Two disinfectant solutions, proven effective with other viruses, were tested. Seeds were submerged in either (v) 10% trisodium phosphate solution for 3 h (TTP) or (vi) 0·1 N HCl for 30 min (THC). For each chemical treatment, a mesh tea strainer containing the seeds was immersed in 100 mL disinfectant solution in a 250-mL glass beaker, covered and placed on an orbital shaker at 220 r.p.m. for the specified duration at room temperature. A combined treatment (thermic and chemical) was also assayed in which seeds were (vii) heated for 120 h at 70°C and then submerged in 10%

Table 1 Effect of disinfection treatments on melon seed germination rates

	Lot 05			Lot 06		
Treatment[a]	Number of seeds[b]	Germination rate (%)[c]	Variation rate[d]	Number of seeds[b]	Germination rate (%)[c]	Variation rate[d]
NT	355	86	–	150	83	–
T72	175	79	–7	150	82	–1
T96	175	76	–10	175	83	0
T120	175	80	–6	175	82	–1
T144	350	80	–6	175	84	1
TTC	175	77	–9	175	85	2
TTP	175	75	–11	175	84	1
THC	175	79	–7	175	82	–1

[a]Disinfection treatments used: NT, no treatment; T72, T96, T120, T144, heat treatment at 70°C for 72, 96, 120 and 144 h, respectively; TTC, heat treatment at 70°C for 120 h followed by immersion in 10% trisodium phosphate for 3 h; TTP, immersion in 10% trisodium phosphate for 3 h; THC, immersion in 0·1 N HCl for 30 min.

[b]Total number of seeds put in the germination trays.

[c]Germination rates recorded 15 days after seeds germinating.

[d]Difference in germination rate between untreated and treated seeds.

trisodium phosphate for 3 h (TTC). Following all treatments, seeds were triple-rinsed in sterile deionized water, dried on sterile paper towels, germinated in trays with paper towels and transferred to trays with substrate as described earlier. For all treatments, the germination rate was calculated by counting seedlings 15 days after depositing the seeds in trays and the effect of the treatments determined by comparing with the germination values obtained for untreated melon seeds. The numbers of seeds used for each batch/treatment are presented in Table 1. After counting, the germinated seedlings were selected and tested for MNSV by ELISA and RT-PCR at the cotyledon and adult stages, as previously described.

Statistical analysis

In all experiments, ELISA absorbance data and the results of each RT-PCR assay were recorded and analysed using the multifactorial ANOVA procedure in STATGRAPHICS PLUS for Windows (version 5·1; Manugistics Inc). The 'growth stage at the time of analysis' in the untreated seeds and 'seed lot assayed' were considered fixed effects in the MNSV infection rate detected by ELISA or RT-PCR. Fisher's protected least significance difference (LSD; $P < 0{\cdot}05$) procedure was used to determine which means significantly differed from the others. The same statistical procedure was used with the germination data obtained. In this case, 'seed treatment' and 'seed lot assayed' were considered fixed effects.

Sequencing and analysis

To confirm the identity of the virus detected in infected seedlings grown from the commercial melon seeds, the RT-PCR product was purified using a High Pure PCR Product Purification Kit (Roche Diagnostics) and directly sequenced with MNSV1 primer using a Big Dye Terminator Version 3·1 Cycle Sequencing Kit in a 3100 Genetic Analyzer sequencer (Applied Biosystems). The nucleotide sequence was compared by BLAST (basic local alignments search tool) (Altschul *et al*., 1997) with MNSV sequences available from the National Centre of Biotechnology Information (NCBI).

Results

Seed-to-seedling transmission of MNSV

DAS-ELISA analysis

None of the composite samples tested, corresponding to 200 and 100 plants from lots 05 and 06, respectively, were positive for MNSV by DAS-ELISA at either the cotyledon or adult stage. Absorbance values for duplicate samples of 12 negative controls ranged from –0·078 to 0·162 (mean 0·022), and for duplicate samples of 12 positive controls from 0·195 to 2·368 (mean 1·452). The mean absorbances of the cotyledon and adult stages were $0{\cdot}039 \pm 0{\cdot}004$ and $0{\cdot}003 \pm 0{\cdot}008$, respectively, in seed lot 05, and $0{\cdot}038 \pm 0{\cdot}004$ and $0{\cdot}007 \pm 0{\cdot}015$, respectively, in seed lot 06. The plant growth stage at the time of the DAS-ELISA analysis significantly affected the absorbance values obtained in the ELISA test of the untreated melon lots ($P = 0{\cdot}0001$). However, there were no significant differences in absorbance between the two seed lots ($P = 0{\cdot}6593$).

RT-PCR analysis

Fourteen and eight of the composite samples tested at the cotyledon stage from lots 05 and 06, respectively, were positive for MNSV by RT-PCR (Table 2). The expected DNA fragment of 651 bp was amplified from extracts of infected seedlings, but not from healthy root extracts (Fig. 1). The data confirmed that MNSV was seed-transmitted in these seed lots. The probability of infection was 11·3 and 14·8% in seed lots 05 and 06, respectively, as indicated in Table 2. None of the RT-PCR positive melon seedlings showed obvious symptoms of MNSV

Table 2 Efficacy of seven disinfection treatments for eradication of *Melon necrotic spot virus* (MNSV) from infected melon seed, infection rates obtained after each seed treatment, and probability of seedling infection, using reverse transcription-polymerase chain reaction (RT-PCR)

		Lot 05				Lot 06			
		Seedlings				Seedlings			
Growth stage[a]	Treatment[b]	Tested[c]	Positive samples[d]	Range of seedling infection (%)	Probability of infection[e]	Tested[c]	Positive samples[d]	Range of seedling infection (%)	Probability of infection[e]
Cotyledon	NT	200	14	7–70	11·3	100	8	8–80	14·8
Adult		200	0	0	0·0	100	0	0	0·0
Cotyledon	T72	100	7	7–70	11·3	100	7	7–70	11·3
	T96	100	4	4–40	4·9	100	4	4–40	4·9
	T120	100	4	4–40	4·9	100	0	0	0·0
	T144	200	0	0	0·0	100	0	0	0·0
	TTC	100	1	1–10	1·0	100	0	0	0·0
	TTP	100	6	6–60	8·7	100	6	6–60	8·7
	THC	70	4	6–57	8·1	70	4	6–57	8·1

[a]Growth stage at time of RT-PCR test: cotyledon or adult stage (45 days after transplanting). Only roots were tested.
[b]Disinfection treatments used: NT, no treatment; T72, T96, T120, T144, heat treatment at 70°C for 72, 96, 120 and 144 h, respectively; TTC, heat treatment at 70°C for 120 h followed by immersion in 10% trisodium phosphate for 3 h; TTP, immersion in 10% trisodium phosphate for 3 h; THC, immersion in 0·1 N HCl for 30 min.
[c]Number of seedlings tested by RT-PCR after each disinfection treatment.
[d]Number of MNSV-positive grouped root samples (each containing roots from 10 melon seedlings).
[e]Probability of infected seedlings grown from infected seeds (i.e. rate of seed-to-seedling MNSV transmission) calculated using the formula of Gibbs & Gower (1960) to estimate proportions from grouped samples.

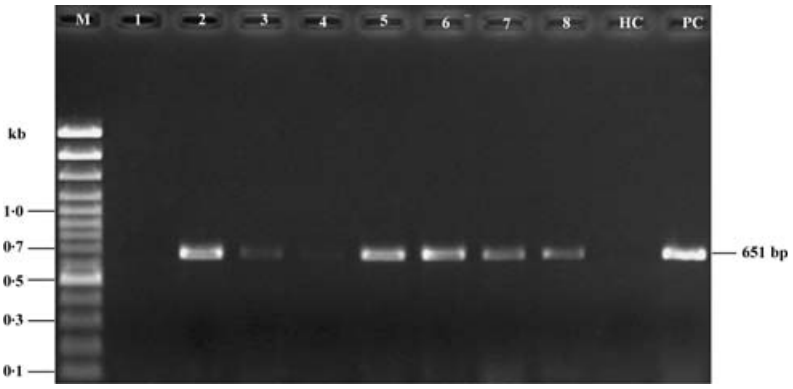

Figure 1 RT-PCR amplification of a 651-bp fragment representing the partial sequence of the *Melon necrotic spot virus* (MNSV) coat protein gene (p42). Lanes 1–8, composite root samples from 10 seedlings at the cotyledon stage germinated from untreated seeds; HC, healthy control; PC, MNSV-positive control (MNSV-PAN2); M, 100-bp DNA Ladder Plus (MBI Fermentas).

infection. At the adult stage, no composite sample was MNSV-positive by RT-PCR (Table 2). As with the ELISA results, significant differences ($P = 0·0000$) in the RT-PCR results were noted between the analyses performed at either the cotyledon or adult stage. However, there were no significant differences ($P = 0·7548$) between seed lots.

Seventy of the seedlings that gave positive results as grouped samples were individually analysed by DAS-ELISA and RT-PCR. None of the plants were MNSV-positive by DAS-ELISA. However, 12 individual seedlings were MNSV-positive by RT-PCR, but only at the cotyledon stage.

Efficacy of seed-disinfection treatments

Effects of seed treatments on melon seed germination

For all treatments (but not controls), the germination rate of seed lot 06 was significantly higher ($P > 0·0002$) than that of lot 05 (Table 1). Disinfection treatment did not have a significant effect ($P > 0·7636$) on the germination rate of either seed lot. Treatment T96 did not affect the germination of seed lot 06 compared with that of untreated seeds. TTP had the greatest affect on germination rate in seed lot 05, reducing it to 75%, compared with 86% for untreated seeds, but did not have this effect in lot 06. Reduction in germination of seeds from lot 06 relative to untreated seeds was seen in treatments T72, T120 and THC, while treatments T144, TTC and TTP increased the germination rate; TTC had the most favorable effect on seed germination, an increase of 2% compared to untreated seeds (Table 1).

Detection of MNSV in seedlings by DAS-ELISA

No MNSV infection was detected by ELISA in melon plants at either the cotyledon or adult stages after various seed-disinfection treatments in both seed lots. In all treatments, however, a significant reduction ($P = 0·0000$) in background ELISA absorbance values was detected compared with the values for untreated seeds.

Detection of MNSV in seedlings by RT-PCR

Results for RT-PCR detection of MNSV infection in seedlings after various seed-disinfection treatments, as well as the transmission rates of MNSV from treated and untreated seeds to seedlings, are provided in Table 2. Only treatment T144 totally eradicated MNSV infection in the seedling assay in both seed lots. With other treatments, the incidence of infection ranged from 1 to 70% in seed lot 05 and from 4 to 70% in seed lot 06, compared to 70 and 80% in untreated seeds of lots 05 and 06, respectively. Reduced incidence of MNSV compared to untreated seeds was evident in seedlings following all seed treatments, except for T72, which remained in the same range as that of seedlings from untreated seeds in seed lot 05. Seed treatments T96 and T120 reduced seedling infection by 42·9% in seed lot 05, and by 50 and 100%, respectively, in seed lot 06. Treatment TTC reduced seedling infection by 85·7 and 100% in seed lots 05 and 06, respectively, while TTP and THC reduced infection by only 14·3 and 18·6%, respectively, in seed lot 05, and 25 and 28·8%, respectively, in seed lot 06, compared with seedlings from untreated seeds. Treatments T120 and TTC in seed lot 06 and T144 in both seed lots presented a probability of infection of 0·0 at the cotyledon stage (Table 2); otherwise, the probability of detecting an infected seedling after treating seeds ranged from 1·0 to 11·3 in seed lot 05 and from 4·9 to 11·3 in seed lot 06, compared with 11·3 and 14·8 in seed lots 05 and 06, respectively, for seedlings from untreated seeds. Disinfection treatments of seeds significantly affected ($P = 0{\cdot}0000$) the incidence of MNSV infection of seedlings, as detected by RT-PCR. However, there were no significant differences ($P = 0{\cdot}6439$) between seed lots.

All plants from seeds of treatments T72, TTP and THC (those with the highest probabilities of seedling infection) were tested at the adult stage for MNSV by DAS-ELISA and RT-PCR, but no positive results were obtained.

Sequence analysis of the MNSV isolate

The sequence of the MNSV isolate (MNSV-SEED1) (GenBank Accession No. DQ443545) detected in infected seedlings grown from commercial melon seeds had 100% sequence homology in the genome region studied with several MNSV isolates obtained from different geographic regions (Herrera-Vásquez *et al.*, 2007), including the MNSV-PAN2 isolate from Panama (DQ443547) used as an MNSV-positive control in this study.

Discussion

Previous studies indicated that MNSV is seed-transmitted in commercial melons (Gonzalez-Garza *et al.*, 1979), while others found limited evidence of infection in the absence of the vector, *O. bornovanus* (Campbell *et al.*, 1996). In the present study, ELISA did not detect MNSV in any seedling groups grown from commercial seeds, yet MNSV was detected in at least 14 composite samples by RT-PCR. This was most likely a result of the low sensitivity of ELISA for detection of MNSV in symptomless plants (Gómez *et al.*, 2005). In the future, a larger number of seedlings should be tested to confirm the absence of transmission, as should an amplified ELISA, different sources of antibodies and decreasing the dilution of root material in the extraction buffer.

RT-PCR was previously used to detect MNSV (Gosalvez *et al.*, 2003), and is used for large-scale routine testing for the presence of other viruses in seeds, e.g. *Cucumber mosaic virus* (CMV) in lupins (Wylie *et al.*, 1993). In this study, MNSV was detected by RT-PCR in seedlings at the cotyledon stage, but not at the adult stage, which may indicate potential resistance of the melon cultivar used to the virus. Although the virus may initially be present in the roots, its movement may be restricted as a result of plant resistance and it may not be able to multiply. This reduced concentration was also observed in the case of PepMV (Córdoba-Sellés *et al.*, 2007). Therefore, there is a risk that the virus could be acquired from infected seedlings by the fungal vector once they have been transplanted into the field, and subsequently transmitted to adult plants.

With MNSV infection, which largely depends on environmental conditions, the subsequent behaviour of a seedling from an infected seed is not predictable. A high incidence of MNSV was observed by RT-PCR in the small sample analysed, higher than that obtained by Campbell *et al.* (1996) using MNSV-infected fruits and a higher sample size, but with a less sensitive method, ELISA. However, more testing of melon seed lots from MNSV-infected melon fruits by RT-PCR is required before a general recommendation regarding the routine use of this technique can be made.

MNSV could be controlled by preventing the causal agent from entering the field, either by the resistance to the disease found in some muskmelon cultivars (Mallor *et al.*, 2004), *O. bornovanus* control by applying Agral surfactant, which affects the zoospores of this fungal vector (Tomlinson & Thomas, 1986), or the use of virus-free seeds (Dumas de Vaulx, 1970). As the testing of all seedlings is impractical, legislation should regulate treatments to ensure that melon seeds are MNSV-free. Thermic treatment was used to eliminate TMV (Laterrot & Pécaut, 1968), *Cucumber green mottle mosaic virus* (CGMMV; Sang-Min *et al.*, 2003) and (PepMV; Córdoba-Sellés *et al.*, 2007) by heating seeds at least 82°C for 72 h, without affecting the germination of seeds. The results of the present study suggest that treatment for 144 h at 70°C (T144) is effective enough to eliminate MNSV and to increase the germination rate of treated seeds relative to untreated seeds. However, this was the most prolonged of all the treatments assayed, and should only be applied by qualified laboratory personnel.

Seedling transmission rates were progressively reduced with treatments T72, T96 and T120 in relation to untreated seeds. The high stability of MNSV may be the reason why T72 was so ineffective. Trisodium phosphate treatment was used to eliminate other viruses located in the seed coat, without affecting seed germination rates (Green *et al.*, 1987). In the present study, treatment TTP

reduced but did not eliminate MNSV infection, because the virus was not only in the seed coat. Inactivation of contaminating MNSV, either located in the paper layer or enclosed in the seed coat (Campbell *et al*., 1996), was previously demonstrated by hydrochloric acid treatment (Dumas de Vaulx, 1970), although the results were probably based on symptomatology. In this work, THC treatment did not effectively eradicate MNSV from seeds. Thermic treatment at 80°C for 24 h combined with immersion in a 10% trisodium phosphate solution for 3 h was previously successful in deactivating viruses located in the same part of the seed as MNSV (Dinant & Lot, 1992; Córdoba-Sellés *et al*., 2007). TTC treatment strongly reduced but did not completely eradicate the incidence of the MNSV in seedlings in this study, and a low presence of the virus can pose a danger to production as the fungal vector spreads.

The sequence of the MNSV isolate obtained in this work is not fully disseminated in countries where the seeds are obtained, e.g. Spain. However, in Latin American countries, where seeds are mostly imported, several MNSV isolates with 100% nucleotide sequence identity with this sequence have been reported (Herrera-Vásquez *et al*., 2007). The dissemination of MNSV with this sequence into these countries through commercial seeds is possible.

A legislated protocol for seed treatment and testing is desirable. More research needs to be undertaken to optimize seed-testing protocols against MNSV infection, with a view to controlling and eradicating the disease.

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