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Seed transmission of *Pepino mosaic virus* in tomato

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Abstract In this manuscript we provide evidence for the seed transmission of *Pepino mosaic virus* (PepMV) in tomato. Fruit was harvested from a tomato crop artificially infected with both European and CH2 genotypes of PepMV and more than 100,000 seeds were extracted and cleaned using an enzymatic treatment without disinfection. Infection assays using indicator plants confirmed the presence of viable virus on the seeds. Seeds were distributed to ten different laboratories in three separate batches, where they were germinated and the young plants tested by ELISA. In total over 87,000 plants were tested and 23 positives detected, indicating an overall transmission rate of 0.026%. However, the observed seed transmission rates varied from 0.005% to 0.057%, depending on the seed batch used. Results clearly showed that PepMV can be transmitted from seeds contaminated with virus to seedlings, highlighting the risk of using seeds from PepMV-infected plants and the potential for seed transmission to contribute to the further spread of PepMV.

Keywords PepMV · Potexvirus · *Solanum lycopersicum* · Viral dissemination

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Introduction

Since first appearing in tomato crops in 1999 *Pepino mosaic virus* (PepMV), a potexvirus originally isolated from pepino (*Solanum muricatum*) in Peru in 1974 (Jones et al. 1980), has become a major disease of greenhouse tomato production worldwide (van der Vlugt et al. 2000; French et al. 2001; Mumford and Metcalfe 2001; Cotillon et al. 2002; Maroon-Lango et al. 2005; Pagan et al. 2006; Hasiów et al. 2008; Hanssen et al. 2008; Ling et al. 2008). The virus causes a wide range of symptoms both on fruits and on the vegetative plant parts, including fruit marbling and flaming, nettle-heads, leaf mosaics, dwarfing, leaf distortions and yellow leaf spots. Although the symptoms are often mild, an increase in symptom severity has been observed and more novel symptoms are now more common, including leaf scorching, sepal necrosis and open fruit (Spence et al. 2006; Hanssen et al. 2008, 2009). Despite the wide range of symptoms, in terms of economic impact, those on fruit are generally regarded as the most damaging as they can lead to fruit being downgraded, reducing the economic value of a crop (Soler et al. 2000; Roggero et al. 2001; Spence et al. 2006).

Originally all PepMV isolates identified in European tomato production shared a high nucleotide sequence homology and differed in biological properties from the orginal pepino isolate (Mumford and Metcalfe 2001; Aguilar et al. 2002; Cotillon et al. 2002; Verhoeven et al. 2003; Lopez et al. 2005; Pagan et al. 2006). For these reasons they were classified as a European tomato genotype (EU). Lopez et al. (2005) isolated a PepMV isolate (LP2001) from *Lycopersicon peruvianum*, which was similar to the original pepino strain in biological properties and shared a high nucleotide sequence identity (96%) with the EU genotype. This isolate is now considered as the type-isolate for the Peruvian genotype of PepMV (LP). Since 2005, three divergent genotypes (US1/CH1, US2 and CH2) have been identified in tomato (Maroon-Lango et al. 2005; Ling 2007). However, as no US2 isolates are available and nucleotide sequence alignment suggests that US2 might be a recombinant of US1 and CH2, we propose to distinguish four PepMV genotype groupings (LP, EU, US1/CH1 and CH2). In several European countries, the CH2 genotype has now become dominant and has largely replaced the EU genotype in commercial tomato production (Davino et al. 2008; Hanssen et al. 2008). However, in the USA, the EU genotype is still predominant (Ling et al. 2008).

With the sudden appearance and rapid establishment of the different PepMV genotypes across large geographical areas, the question of how PepMV is spread over long distances remains unanswered. Along with the movement of germplasm and trade in infected fruit, the potential role of contaminated seed has also been suggested. Seed transmission has been reported for approximately 20% of plant viruses and can, even with low transmission rates, be an important means of viral dissemination (Yang et al. 1997). Although potexviruses are generally not considered to be seed-transmitted, the highly infectious nature of PepMV combined with an extremely rapid cross-continental spread has raised concerns with respect to seed transmission. However, despite the fact that it has previously been shown that seeds from PepMV-infected tomato plants contain high viral loads (Krinkels 2001; Córdoba-Sellés et al. 2007; Ling 2008), and that viral particles can be found on the seed coat, but not in the embryo (Ling 2008), the ability of PepMV to be transmitted via seed is still unclear. Previous studies have been ambiguous, as

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seed transmission rates obtained varied from zero to 1.84% (Krinkels 2001; Salomone and Roggero 2002; Córdoba-Sellés et al. 2007; Ling 2008). In most previous studies the numbers of seeds tested were low; in others the seeds were not cleaned to 'industry-standards'. Therefore, the work presented here was designed to obtain a statistically-sound estimation of the PepMV seed transmission rate in tomato.

Materials and methods

Seed harvest and cleaning

Seeds used in this study were harvested from tomatoes artificially infected with PepMV (cv. Tricia; De Ruiter Seeds, Bergschenhoek, The Netherlands) and grown in plastic tunnels in Belgium during the 2007 growing season (Hanssen et al. 2009). Plants were inoculated with a mixture of the EU and CH2 genotypes, and fruit was harvested at 8, 12 and 15 weeks post-inoculation (WPI). Enzyme-linked immunosorbent assay (ELISA) analyses on leaf samples confirmed similarly high viral concentrations in the mother plants at the three different harvesting points (Hanssen et al. 2009).

In each harvest, all ripe tomatoes (500 to 800) were collected and cleaned to 'industry standards'. Seeds were manually separated from the tomato pulp and collected in containers to which an equal volume of tap water was added. Subsequently, citric acid pH 4 (6.7% *v*/*v*) and pectinase (Pectinex® Ultra SP-L, Novozymes A/S, Denmark; 0.25% *v*/*v*) were added and the pulp incubated for 3 h at 28°C, stirring every 30 min. Next, seeds were retrieved by sieving, thoroughly rinsed with tap water and dried for 24 h in an oven at 26°C until the water content was below 6%.

Determination of viral presence and infectivity on seeds

PepMV contamination of the seeds was assessed using a commercially available ELISA assay according to the suppliers' instructions (Prime Diagnostics, Wageningen, The Netherlands). Twenty seeds were tested per seed batch, each sample containing one seed from the infected batch spiked into 250 healthy seeds according to a PepMV-specific seed testing protocol designed by the International Seed Health Initiative - section Vegetables (ISHI-Veg; Krinkels 2001). All samples were tested in duplicate and were rated positive if the mean optical density at 405 nm (OD) of the sample was at least twice the mean OD of two wells containing extract from healthy tomato seeds. The PepMV genotypes present on the seed batches were determined by RT-PCR-RFLP (Hanssen et al. 2008).

Part of the seed batch harvested at 15 WPI was used for seed infectivity assays on *Nicotiana occidentalis* P1. Seeds were divided into subsamples, crushed in a mixture of sand and water and inoculated on-to two plants using carborundum powder and cotton wool. Plant symptoms were evaluated two weeks after inoculation.

Seed transmission grow-out trials

The tomato seeds harvested from PepMV-infected plants were distributed to ten different partner laboratories, in batches of 4,000–5,000 seeds per laboratory. These were sown in stonewool blocks (10 cm^2) within six weeks of the seed being harvested. Seedlings were irrigated individually with nutrient solution according to local practices and grown for at least 4 weeks in a glasshouse. Strict hygiene measures were taken to prevent external contamination of seeds and seedlings. Leaf samples were then taken from each plant and pooled together and tested in groups of ten. In total, 8,778 pooled samples were obtained from 87,780 seedlings, across all ten laboratories. All pooled samples were analysed for the presence of PepMV by the ELISA assay as described above. Positive ELISA results were confirmed by an additional ELISA analysis or by RT-PCR.

Statistical analyses

To determine the number of seeds required for the grow-out trial, a binomial distribution of the probability of seed transmission (P) was assumed (Table 1). The seed transmission rate was calculated from grouped sample test results using the equation $P^* = 1 - (1 - R/N)^{1/i}$, where P^* is the maximum likelihood estimate of the seed transmission rate (0 to 1), N is the number of grouped samples, i is batch size, and R is the number of infected seedlings (adapted from Gibbs and Gower 1960). Transmission rates of seeds, harvested at 8, 12 and 15 WPI, were compared using an analysis of deviance (Generalised Linear Model with a binomial distribution error term

Table 1 Estimation of the number of PepMV-infected seeds to be included in PepMV grow-out trials under different assumptions of transmission rates

		X Value (Number of positive groups)					
		P=0.0001		P=0.001		P=0.01	
N=10000		X	CI	X	CI	X	CI
	K=50	1	0–0.0185	10	0–0.02	79	0.0012–0.0357
	K=25	1	0–0.0093	10	1.8E-7–0.011	89	0.0027–0.0252
	K=10	1	0–0.0039	10	2.5E-5–0.0055	96	0.0048–0.0182
N=50000							
	K=50	5	0–0.0035	49	3.5E-5–0.0066	395	0.0039–0.0191
	K=25	5	0–0.0019	49	0.0001–0.0035	444	0.0069–0.0146
	K=10	5	0–0.0009	50	0.0003–0.0023	478	0.0074–0.0131
N=100000							
	K=50	10	0–0.0020	98	0.0001–0.0036	790	0.0061–0.0154
	K=25	10	1.8E-8–0.0011	99	0.0003–0.0026	889	0.0072–0.0136
	K=10	10	2.5E-6–0.0006	100	0.0005–0.0018	960	0.0082–0.0121

N=Total number of seeds. K=Number of seeds per group. P=Probability of seed transmission. X=Number of positive groups. CI= Confidence interval. The confidence intervals were calculated using the binomial distribution $f(X) = [N!/(X!(N-X)!] P^X (1-P)^{N-X}$. If N seeds are analysed in batches of K, the frequency of positive batches, in which transmission has occurred, will be i and thus 1-i is the frequency of batches in which no seed transmission has occurred. Therefore, applying the above binomial probability distribution, $f(0) = 1-i = [K!/(0!(K-O)!] P^0 (1-P)^K = (1-P)^K$. Therefore, $1-i = (1-P)^K$ and $p=1 - (1-i)^{1/K}$

and logit-link function); 95% lower and upper confidence intervals were calculated to indicate the errors associated with the transmission rates (Table 2). Generalised Linear Model analysis was carried out using Genstat Release 10.2 (Lawes Agricultural Trust). Confidence intervals were calculated using Seedcalc version 8.1 (International Seed Testing Association).

Results

A total of > 100,000 seeds was obtained by enzymatic treatment from fruit harvested from tomato plants artificially infected with PepMV. Three batches of seeds were produced at 8, 12 and 15 WPI. Seeds were tested for the presence and viability of PepMV particles and used in grow-out trials to determine the

Table 2 Number of ELISA-positive plots out of the total number of plots[a] tested in grow-out trials

Partner	8 WPI harvest	12 WPI harvest	15 WPI harvest	Total
Bulgaria	0/382	0/395	0/338	0/1115
Denmark	0/240	0/360	2/320	2/920
Greece	0/270	0/366	2/416	2/1052
Italy	1/249	1/393	1/410	3/1052
Norway	0/346	0/350	2/411	2/1107
Slovenia	0/71	0/153	2/240	2/464
UK	0/329	0/365	2/348	2/1042
Poland		0/495	5/520	5/1015
Portugal		0/350	3/350	3/700
Belgium		2/311		2/311
Total	1/1887	3/3538	19/3353	23/8778
Transmission rate (%)[b]	0.0053a	0.0085a	0.0567b	0.026
Confidence interval (P=0.05)	0.0002–0.0295	0.0021–0.0248	0.0345–0.0885	0.0166–0.0395

[a] 8.760 plots consisting of 10 tomato seedlings each grown from infected seeds

[b] Different letter labels indicate significant differences (P=0.05)

seed transmission rate. In total ten partner laboratories from different European countries participated in the grow-out trials.

Determination of viral presence and infectivity on the seeds

Before distribution of the seeds to the ten partners, the presence of PepMV on the seeds was determined by ELISA analyses on 20 samples per harvest, following the spiking method described above. The ELISA results revealed a high concentration of PepMV on or in seeds, as all samples were positive with mean OD values of 3.39 (S.D. (±) 0.29), 3.35 (± 0.37) and 3.12 (± 0.57) obtained for the three subsequent harvests; mean OD values of 2.92 (± 0.60) and 0.07 (± 0.006) were obtained for the positive and negative control wells, respectively. Genotype analyses by RT-PCR-RFLP revealed that, like the mother plants, seeds from all three harvests were infected by both the EU and CH2 PepMV genotype.

Infectivity of PepMV particles present in seeds from the third harvest was determined by inoculation on *N. occidentalis*. To this end, 15 samples of 10 seeds each and 10 samples of 50 seeds each were inoculated on two *N. occidentalis* seedlings per sample. Five out of 30 plants inoculated with the 10 seed samples developed PepMV symptoms, while 11 out of 20 plants inoculated with the 50 seed samples developed PepMV symptoms, indicating that these seed batches contained infectious PepMV.

PepMV seed transmission

In order to determine the PepMV seed transmission rate in tomato, an extensive grow-out trial was performed. Based on previous studies, a seed transmission rate between 0 and 1% was anticipated (Krinkels 2001; Salomone and Roggero 2002; Córdoba-Sellés et al. 2007). Assuming that the probability of seed transmission is binomially distributed and that the rate is not < 0.01%, probability calculations resulted in the estimation that a total number of 100,000 seedlings in blocks of ten would be sufficient to obtain a reliable estimate (Table 1). The germination and grow-out of this many seedlings, arranged to avoid physical contact between the blocks, would require a huge amount of greenhouse space. Therefore the seed transmission grow-out trials were run in parallel in the greenhouse facilities of plant pathology laboratories of ten partners in Belgium, Bulgaria, Denmark, Greece, Italy, Norway, Poland, Portugal, Slovenia and the UK. To assess the influence of the time span between infection of the mother crop and seed harvest on the transmission rate, seeds harvested at 8, 12 and 15 WPI were analysed in three subsequent grow-out trials. Since the seeds were harvested from a commercial tomato hybrid, a heterogeneous germination and a suboptimal germination rate of 70–80% were expected. Therefore, seeds were sown in blocks of 16 to obtain at least ten homogenous seedlings per block to pool into one sample for ELISA analysis.

In total, 8,776 blocks consisting of 10 seedlings each were sampled four weeks after sowing, 23 of which tested positive for PepMV by ELISA (Table 2). Positive ELISA results were generally clearly above the threshold value (twice the negative control) (Table 3) and were in all 23 cases confirmed by additional ELISA analyses or by RT-PCR. In addition, typical PepMV symptoms such as nettlehead and leaf deformation were seen in infected plots. Thus, at least 23 out of 87,760 seeds resulted in an infected seedling, leading to a minimum PepMV transmission rate of 0.026% (confidence interval 0.0166 to 0.0359; Table 2). Interestingly, while only one and three infected blocks were obtained from the first and second harvest, respectively, the third harvest gave rise to 19 infected blocks, resulting in a significantly higher ($P<0.05$) transmission rate (0.0567%) compared to the first and second harvest (0.0053% and 0.0085%, respectively). Positive samples obtained in Denmark, Italy, Norway Slovenia and the UK were analysed by a genotype-specific TaqMan qRT-PCR (unpublished) or by RT-PCR-RFLP (Hanssen et al. 2008). Out of the 11 positive blocks obtained in those four countries, seven were infected only with the EU genotype, three with the CH2 genotype, and one with both the EU and CH2 genotype.

Discussion

The results presented in this extensive study, based upon a grow-out trial using almost 90,000 tomato seedlings, clearly demonstrated that PepMV can be transmitted to the next generation via contaiminated seed and provided a statistically

Table 3 ELISA optical density (OD) values of controls and positive samples obtained from the grow-out trials by the different partner laboratories

Samples	Belgium	Denmark	Greece	Italy	Norway	Poland	Portugal	Slovenia	UK
Healthy controls[a]	0.111± 0.004	0.090± 0.008	−0.024± 0.026	0.117± 0.011	0.016± 0.013	0.024± 0.005	0.020± 0.008	0.082± 0.015	0.064± 0.003
Positive controls[a]	3.450± 0.120	1.682± 0.120	1.940± 0.034	3.136± 0.056	1.034± 0.128	1.944± 0.140	3.355± 0.135	1.321± 0.177	0.819± 0.145
Positive sample 1	0.340	0.480	1.983	0.689	1.593	0.575	0.124	1.631	0.357
Positive sample 2	0.280	1.303	1.217	0.307	0.695	0.571	0.120	0.964	1.045
Positive sample 3	–	–	–	2.930	–	0.585	0.840	–	–
Positive sample 4	–	–	–	–	–	0.202	–	–	–
Positive sample 5	–	–	–	–	–	0.734	–	–	–
Negative samples[b]	0.116± 0.006	0.087± 0.006	0.014± 0.020	0.102± 0.022	0.020± 0.007	0.029± 0.006	0.018± 0.005	0.076± 0.003	0.066± 0.008

[a] Mean OD value ± standard deviation obtained from all control wells

[b] Mean OD value ± standard deviation obtained from all negative sample wells

sound estimation of 0.026% as the PepMV seed transmission rate in tomato.

In the past, the seed transmission of PepMV has proved a controversial subject, with previous studies on the subject giving conflicting results. For example, Salomone and Roggero (2002) did not find any seed transmission by testing 52 seedlings. Likewise, in a more recent study, none of 10,000 grow-out seedlings from an infected commercial seed lot was infected by PepMV as determined by symptom expression, ELISA tests or infectivity assays, although mechanical transmission demonstrated the virus on the seed was still viable (Ling 2008). In contrast, other studies have found seed transmission, including one conducted in collaboration with the seed industry, which revealed seed transmission rates between 0.06 and 0.03% for seeds that were cleaned by natural fermentation and dried (Krinkels 2001). In another grow-out trial with 168 seedlings a seed transmission rate of 1.84% was found for PepMV-infected tomato seeds that were rinsed without fermentation or enzymatic treatment (Córdoba-Selles et al. 2007). The contrasting conditions used in these previous studies and the study presented in this paper does make direct comparison of results difficult. The fact that different PepMV genotypes, seed ages and seed treatments were used will potentially have influenced the final results obtained. Moreover, in contrast to our latest study, the numbers of seeds used in most of the previous studies were too low for a statistically sound estimation of the seed transmission rate. However it is interesting that the results found in our study are in line with those from Krinkels (2001), who obtained similar transmission rates and applied a comparable seed treatment. In contrast, the much higher transmission rate reported by Córdoba-Selles et al. (2007) can be explained by the fact that seeds were not cleaned to 'industry-standards'. A high viral titre on seeds harvested from PepMV-infected tomato plants was previously reported (Córdoba-Selles et al. 2007; Ling 2008). Taking into account the highly efficient mechanical transmission of the virus, it is strongly suspected that seed transmission of PepMV occurs as a result of contact between the germinating seedling and the virus-contaminated seed coat. For this reason, seed cleaning and treatment will have a large influence on the transmission rate. This was demonstrated by further seed treatment and disinfestation studies performed by Córdoba-Selles et al. (2007), who were able to significantly reduce transmission from their uncleaned seed using various chemical treatments.

It should be noted that as the seeds used in this study were harvested from heavily infected plants, where all the fruit was infected, and where no post-cleaning disinfection treatment of the seeds was applied prior to sowing, the transmission rate obtained

does represent a potential worst case scenario. In practice, procedures (both statutory and internal quality ones) are in place that should virtually eliminate the risk from commercial seed. For example, within the EU, PepMV has had quarantine status on seeds since 2001 and regulations are in force to prevent the introduction and further spread of PepMV through infected tomato seeds (Commission Decision 2001/536/EC and 2004/200/EC). These include the seed producer having to provide proof of absence of PepMV, either in the production area, in the mother crop by monitoring, or through seed testing. In addition, acid extraction of tomato seeds is mandatory. In general, seed production companies combine the different criteria. Established seed production methods do exist and protocols such as the widely-used ISHI-Veg approved ELISA-based testing procedure (http://www.worldseed.org/en-us/international_seed/ishi_vegetable.html) can provide reliable detection of PepMV in the contaminated seed lots, as shown in this study. However, this study does provide conclusive proof that PepMV can be transmitted from tomato seeds produced from infected fruit and for that reason the risk must be taken seriously.

The continued imposition of strict regulations for seed harvest and reliable PepMV seed testing methods are necessary to prevent spread of PepMV by tomato seeds. While the efficiency of transmission is obviously low, the highly infectious nature of PepMV in tomato crops means that even one infected plant derived from contaminated seed is sufficient to start an outbreak in a commercial crop that might have tens if not hundreds of thousands of plants. So ultimately in risk matrix terms, while the risk of infection from seed is low, the probability of it causing an outbreak is high. For example, transmission via seed provides the most likely explanation of how PepMV was able to spread so widely and so rapidly throughout worldwide greenhouse tomato production in the late 1990s and early 2000s, prior to the introduction of the current regulations.

While the occurrence of seed transmission is clear, the results do include some interesting observations. Firstly, they show that infected seedlings obtained from tomato seeds infected by two different genotypes of PepMV are not necessarily infected by both genotypes. Only one out of 11 infected plots from which the genotypes were determined was infected by both genotypes. These results strongly suggest that only a low number of viral particles initiated the infection and represent strong evidence of a population bottleneck during PepMV seed transmission. Population bottlenecks during horizontal transmission of plant viruses from host to host have thus far only been reported during aphid transmission (Ali et al. 2006). Very small numbers (between one and two) of effective founders have been reported for *Cucumber mosaic virus* (CMV) transmitted from one plant to another by the aphid vector *Aphis gossyppi* (Betancourt et al. 2008). Also the number of *Potato virus Y* (PVY) particles transmitted by the aphid vector *Myzus persicae* was found to be very limited, from 0.5 to 3.2 (Moury et al. 2007). However, to our knowledge, this is the first indication of a population bottleneck during seed transmission.

The second interesting observation made during this study was the apparent increase in seed transmission risk seen as the interval between infection of the mother crop and seed harvest was extended. A ten-times higher transmission rate was observed for seeds harvested at 15 WPI, as compared to seeds harvested at 8 and 12 WPI. The observed difference could not be explained by a higher viral titre in the mother plants at the time of harvest, or by a higher concentration of virus in or on the seed batches. It has previously been reported that the time interval between infection of the mother plants and fruit harvest influences seed transmission rates and it was suggested that infection before initiation of florescence is required to obtain seed transmission (Blaszczak 1964). Taking into account a period of 2 weeks between inoculation and overall systemic spread of the virus (Hanssen et al. 2009), and a period of nine to ten weeks between initial fruit set and tomato harvest, fruit set of tomatoes harvested at 15 WPI was initiated after systemic spread of the virus in the mother plants, in contrast to the 8 WPI harvest. If systemic spread of the virus at the time of fruit set is required for seed transmission to occur, this might explain both the absence of seed transmission in the 8 WPI harvest and the relatively high rate obtained from the 15 WPI harvest. Fruits harvested at 12 WPI were either set just before or just after systemic spread of the virus, which might account for the intermediate rate. While it is tempting to suggest a physiological explanation, further studies would undoubtedly be required to clarify the possible mechanisms behind this observation.

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